METHODS IN MOLECULAR MEDICINE™

Celiac Disease

Methods and Protocols

Edited by

Michael N. Marsh, MD, DSc, FRCP

Humana Press

Celiac Disease

METHODS IN MOLECULAR MEDICINE™

John M. Walker, SERIES EDITOR

52. **Atherosclerosis Methods and Protocols,** edited by *Angela F. Drew*, 2001
51. **Angiotensin Protocols**, edited by *Donna H. Wang*, 2001
50. **Colorectal Cancer: *Methods and Protocols***, edited by *Steven M. Powell*, 2001
49. **Molecular Pathology Protocols**, edited by *Anthony A. Killeen*, 2000
48. **Antibiotic Resistance Methods and Protocols,** edited by *Stephen H. Gillespie*, 2000
47. **Ocular Molecular Biology Protocols,** edited by *P. Elizabeth Rakoczy*, 2000
46. **Angiogenesis: *Reviews and Protocols,*** edited by *J. Clifford Murray*, 2000
45. **Hepatocellular Carcinoma: *Methods and Protocols*, edited by** *Nagy A. Habib*, 2000
44. **Asthma: *Mechanisms and Protocols*, edited by** *K. Fan Chung and Ian Adcock*, 2000
43. **Muscular Dystrophy: *Methods and Protocols,* edited by** *Katherine B. Bushby and Louise Anderson*, 2000
42. **Vaccine Adjuvants: *Preparation Methods and Research Protocols***, edited by *Derek T. O'Hagan*, 2000
41. **Celiac Disease: *Methods and Protocols***, edited by *Michael N. Marsh*, 2000
40. **Diagnostic and Therapeutic Antibodies,** edited by *Andrew J. T. George and Catherine E. Urch*, 2000
39. **Ovarian Cancer: *Methods and Protocols***, edited by *John M. S. Bartlett*, 2000
38. **Aging Methods and Protocols,** edited by *Yvonne A. Barnett and Christopher P. Barnett*, 2000
37. **Electrochemotherapy, Electrogenetherapy, and Transdermal Drug Delivery:** *Electrically Mediated Delivery of Molecules to Cells*, edited by *Mark J. Jaroszeski, Richard Heller, and Richard Gilbert*, 2000
36. **Septic Shock Methods and Protocols**, edited by *Thomas J. Evans*, 2000
35. **Gene Therapy of Cancer: *Methods and Protocols***, edited by *Wolfgang Walther and Ulrike Stein*, 2000
34. **Rotaviruses: *Methods and Protocols***, edited by *James Gray and Ulrich Desselberger*, 2000
33. **Cytomegalovirus Protocols,** edited by *John Sinclair*, 2000
32. **Alzheimer's Disease: *Methods and Protocols,*** edited by *Nigel M. Hooper*, 2000
31. **Hemostasis and Thrombosis Protocols,** edited by *David J. Perry and K. John Pasi*, 1999
30. **Vascular Disease: *Molecular Biology and Gene Therapy Protocols,*** edited by *Andrew H. Baker*, 1999
29. **DNA Vaccines: *Methods and Protocols,*** edited by *Douglas B. Lowrie and Robert G. Whalen*, 2000
28. **Cytotoxic Drug Resistance Mechanisms,** edited by *Robert Brown and Uta Böger-Brown*, 1999

METHODS IN MOLECULAR MEDICINE™

Celiac Disease

Methods and Protocols

Edited by

Michael N. Marsh, MD, DSc, FRCP

Department of Medicine,
University of Manchester, Salford, UK

Humana Press  **Totowa, New Jersey**

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

This publication is printed on acid-free paper. ♾
ANSI Z39.48-1984 (American Standards Institute) Permanence of Paper for Printed Library Materials.

Cover design by Patricia F. Cleary

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel.: 973-256-1699; Fax: 973-256-8341; E-mail: humana@humanapr.com

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging in Publication Data

TO COME FROM

Main entry under title:

LIB. OF CONGRESS

Methods in molecular medicine™.

Celiac disease: methods and protocols / edited by Michael N. Marsh.
p. cm. -- (Methods in molecular medicine™)
Includes index.
ISBN 0-89603-650-2 (alk. paper)
1. XXXXXXX--Laboratory manuals. I. Marsh, Michael N. II. Series.
[DNLM: 1. XXXXXX. 2. XXXXX. QXXXXX 2000]
QXXXXX 2000
DNLM/DLC 00-4625
for Library of Congress CIP

Preface

Research into gluten sensitivity has never been more popular nor more exciting. Thus a call for a new book, *Celiac Disease: Methods and Protocols*, devoted entirely to techniques and technology seemed a most appropriate undertaking. I am therefore grateful to Professor J. M. Walker for inviting me to complete this task for Humana Press. To do this would have been impossible without the contributions of friends and colleagues from around the world who have devoted so much interest to the project. It has also been necessary for them to master the unique chapter-writing skills required of every manuscript published in this series of laboratory monographs.

With regard to gluten sensitivity we are in a period of great change, occasioned by the introduction of reproducible methods for cloning lymphocytes, the application of physical methods to identify gluten sequences as T-cell antigens, the study of peptide responses in vitro and in vivo by either jejunal or rectal challenge, elucidating the locations of other genes concerned in pathogenesis, or the use of elegant immunohistocytochemical and mRNA probing techniques for analyzing the finer points of the mucosal inflammatory response to gluten. Never was a detailed laboratory handbook, of the quality assembled here through its contributing experts, so necessary as at this time!

It has been a privilege to put together *Celiac Disease: Methods and Protocols* and it is offered in the hope that its pages will contain the necessary information for any newcomer to this field to get himself or herself organized quickly with the least technical hindrance.

Michael N. Marsh, MD, DSc, FRCP

Dedication

This book is dedicated to the memory of Professor Anne Ferguson, whose untimely death has deprived gastrointestinal science of one of its most renowned and prolific investigators. Although our professional lives had almost run concurrently, she and I met only rarely, usually transiently at various international meetings or gatherings.

It was her earlier work with Delphine Parrott in Glasgow, and subsequently with Thomas MacDonald and Allan Mowat in Edinburgh, that provided the initial evidence that mucosal T lymphocytes influence mucosal architecture, and hence may play a fundamental role in the immunopathogenesis of celiac disease. Indeed, the most recent studies with cloned mucosal T lymphocytes have confirmed such pioneering work and affirmed that lymphocytic sensitization to gluten, and subsequent antigen-induced activation, does result in the spectrum of mucosal changes that has now been identified across the ever-widening perception of what, today, should be known as gluten sensitivity, rather than by its restrictive 1970s clinical designation as "celiac disease." Anne's later contributions to the measurement, and diagnostic use, of inflammatory/immunologic mediators in intestinal lavage fluid are further novel applications of her approach to the understanding of gastrointestinal disease: these studies have been brought together by her colleagues for publication in this volume.

Anne's name will go down in the history of gluten sensitivity along with those of Dicke, Shiner, Rubin, and Crosby.

May she forever rest in peace.

Michael N. Marsh

Contents

Contributors

Reinout Amons • *Department of Immunohaematology and Bloodbank, University of Leiden, Leiden, The Netherlands*

Ian D. R. Arnott • *Department of Gastroenterology, Western General Hospital, Edinburgh, UK*

Conrad G. Beckett • *Department of Gastroenterology, Bradford Royal Informary, Bradford, West Yorkshire, UK*

Stephen Bevan • *Institute of Cancer Research, Sutton, Surrey, UK*

Per Brandtzaeg • *Laboratory for Immunohistochemistry and Immunopathology (LIIPAT), Institute of Pathology, The National Hospital, Oslo, Norway*

Paul J. Ciclitira • *Department of Gastroenterology, The Rayne Institute, St. Thomas' Hospital, London, UK*

Peter T. Crowe • *University Department of Medicine, Hope Hospital, Salford, UK*

Anna Dahele • *Department of Gastroenterology, Western General Hospital, Edinburgh, UK*

Walburga Dieterich • *Medical Clinic I, University of Erlangen, Erlangen, Germany*

Jan W. Drijfhout • *Department of Immunohaematology and Bloodbank, University of Leiden, Leiden, The Netherlands*

Hazel E. Drummond • *Department of Gastroenterology, Western General Hospital, Edinburgh, UK*

Arzu Ensari • *Department of Pathology, University of Ankara Medical School, Ankara, Turkey*

Roger J. Fido • *Department of Agricultural Sciences, IACR-Long Ashton Research Station, Long Ashton, Bristol, UK*

Subrata Ghosh • *Department of Gastroenterology, Western General Hospital, Edinburgh, UK*

Simon M. Gilbert • *Department of Agricultural Sciences, IACR-Long Ashton Research Station, Long Ashton, Bristol, UK*

Hoebert S. Hiemstra • *Department of Immunohaematology and Bloodbank, University of Leiden, Leiden, The Netherlands*

Syed S. Hoque • *Department of Gastroenterology, Western General Hospital, Edinburgh, UK*

Richard S. Houlston • *Institute of Cancer Research, Haddow Laboratory, Sutton, Surrey, UK*

Kenneth Humphreys • *Department of Gastroenterology, Western General Hospital, Edinburgh, UK*

Frode L. Jahnsen • *Laboratory for Immunohistochemistry and Immunopathology (LIIPAT), Institute of Pathology, The National Hospital, Oslo, Norway*

Finn-Eirik Johansen • *Laboratory for Immunohistochemistry and Immunopathology (LIIPAT), Institute of Pathology, The National Hospital, Oslo, Norway*

Frits Koning • *Department of Immunohaematology and Bloodbank, University of Leiden, Leiden, The Netherlands*

Knut E. A. Lundin • *Institute of Immunology, Rikshospitalet, University of Oslo and Department of Medicine, Ullevaal sykehus, Oslo, Norway*

Maria Maglio • *Department of Pediatrics, University Federico II, Naples, Italy*

Michael N. Marsh • *Department of Medicine, University of Manchester, Hope Hospital, Salford, UK*

Guiseppe Mazzarella • *Istituto di Scienze dell'Alimentazione CNR, Avellino, Italy*

Stephen N. McAdam • *Institute of Immunology, Rikshospitalet, University of Oslo, Oslo, Norway*

Øyvind Molberg • *Institute of Immunology, Rikshospitalet, University of Oslo, Oslo, Norway*

Kieran J. Moriarty • *Department of Medicine (Gastroenterology), Bolton General Hospital, Bolton, UK*

Ellen M. Nilsen • *Laboratory for Immunohistochemistry and Immunopathology (LIIPAT), Institute of Pathology, The National Hospital, Oslo, Norway*

Franco Paparo • *Department of Pediatrics, University Federico II, Naples, Italy*

Ernst Otto Riecken • *University Clinic Benjamin Franklin, Free University of Berlin, Berlin, Germany*

Detlef Schuppan • *Medical Clinic I, University of Erlangen, Erlangen, Germany*

PETER R. SHEWRY • *Department of Agricultural Sciences, IACR-Long Ashton Research Station, Long Ashton, Bristol, UK*

LUDVIG M. SOLLID • *Institute of Transplantation Immunology, Rikshospitalet, University of Oslo, Oslo, Norway*

ARTHUR S. TATHAM • *Department of Agricultural Sciences, IACR-Long Ashton Research Station, Long Ashton, Bristol, UK*

RICCARDO TRONCONE • *Department of Pediatrics, University Federico II, Naples, Italy*

DAVID J. UNSWORTH • *Department of Immunology, Southmead Hospital, Bristol, UK*

YVONNE VAN DE WAL • *Department of Immunohaematology and Bloodbank, University of Leiden, Leiden, The Netherlands*

PETER A. VAN VEELEN • *Department of Immunohaematology and Bloodbank, University of Leiden, Leiden, The Netherlands*

DEBBIE WILLIAMSON • *Department of Medicine, Hope Hospital, Salford, UK*

1

Celiac Disease

A Brief Overview

Debbie Williamson and Michael N. Marsh

1. Introduction

Historically, the term *celiac disease* evolved within pediatric practice during the nineteenth century, defining children with severe wasting and putrid stools ***(1)***. In the earlier twentieth century, similar complaints in adults were categorized as "intestinal insufficiency" or "idiopathic steatorrhea." It was also realized at that time that, for many of these adult patients, celiac-like features had been present since early childhood.

The pathological link followed the introduction of the peroral jejunal biopsy technique that now revealed that in both conditions, the proximal jejunal mucosa was highly abnormal. Thus, "celiac disease" (juvenile) and "idiopathic steatorrhea" (adult) came to be seen as facets of a lifelong disorder. Celiac disease (ca. 1960–1970) assumed a compact diagnostic format based on a previous (long) history of severe, fatty diarrhea, weight loss and inanition; the presence of villus-effacing mucosal damage of upper jejunum; and a response to a gluten-free diet. This latter advance, based on the discovery that wheat protein (gluten) is the dietary cause of this condition, was pioneered by the Dutch pediatrician Willem Dicke and his collaborators Jan van de Kamer and Dolf Weijers ***(2)*** toward the end of World War II.

This "clinical-descriptive" definition of overt celiac disease served reasonably well, although in retrospect it clearly failed to encompass patients with (gluten-driven) dermatitis herpetiformis, whose jejunal mucosa was often found to display minimal architectural changes and somewhat uninterpretable lymphocytic infiltrations of the villous epithelium ***(3)***. It also failed to account satisfactorily for the death of unresponsive patients from a form of end-stage

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

intestinal failure invariably due to progressive lymphoma. Both categories, despite evidence for the gluten sensitivity, fell outside the limited scope of this early definition. A third exception to the definition came with studies in which jejunal morphology in approx 10–15% of first-degree family members also revealed a severe, flat-destructive proximal lesion of the jejunum ***(4)*** of whom at least 50% were asymptomatic. Indeed, many such individuals would never have considered themselves to be ill had the surveillance operation not identified their status ***(5)***.

The realization that a patient may be asymptomatic despite having a severe lesion of the proximal lesion seemed a curious anomaly. However, the rational answer to this paradox was provided by MacDonald et al. ***(6)***, in Seattle, Washington, who revealed that the development of symptoms depends not on the appearance of the proximal lesion but on the length of bowel involved with lesion pathology. We still have no means of determining this clinically.

Pathophysiologically, it is more helpful to consider the compensatory action of the residual distal bowel and colon, which, in overcoming any malabsorptive defect of the upper intestine ***(7)***, prevents diarrhea and renders the patient asymptomatic. In recent years, the concept of compensated-latent disease has evolved, and with it the necessary realization that the clinical-descriptive term *celiac disease* is no longer an appropriate designation ***(8)***; a better alternative is *gluten sensitivity* (*see* **Subheading 1.1.**).

The period of compensated latency may be relatively short, accounting for the peak in early childhood (**Fig. 1**); the minimum "induction" period between weaning (i.e., introduction of dietary gluten) and symptomatic presentation is 3 mo ***(9,10)***. Here the male:female ratio over this 5-yr period is equal. The second peak begins around the second decade, and broadly extends into the geriatric age group; in this adult group, note the preponderance and earlier presentation of females. From these data it is evident that many children escape diagnosis during childhood, the teenage-adolescent period is specifically associated with a continuing latent-compensated phase, and the number of compensated-latent individuals in later decades is unknown. Indeed, it is evident that the classical symptomatic triad with which celiac disease invariably presented during the earlier part of the twentieth century has decreased dramatically over the last few decades (**Fig. 1**). Thus, it follows that other "patients" will get through life without ever knowing that they were gluten sensitized. Neither do we know how many *de novo* presentations of malignancy (e.g., esophagus, stomach, jejunum, intestinal lymphoma) are due to an underlying gluten sensitivity. It should be evident, therefore, from an understanding of the applied physiopathology, that gluten sensitivity is more than likely to exist in a compensated-latent mode, unless unmasked by specific environmental factors at any time point throughout life (**Fig. 2**).

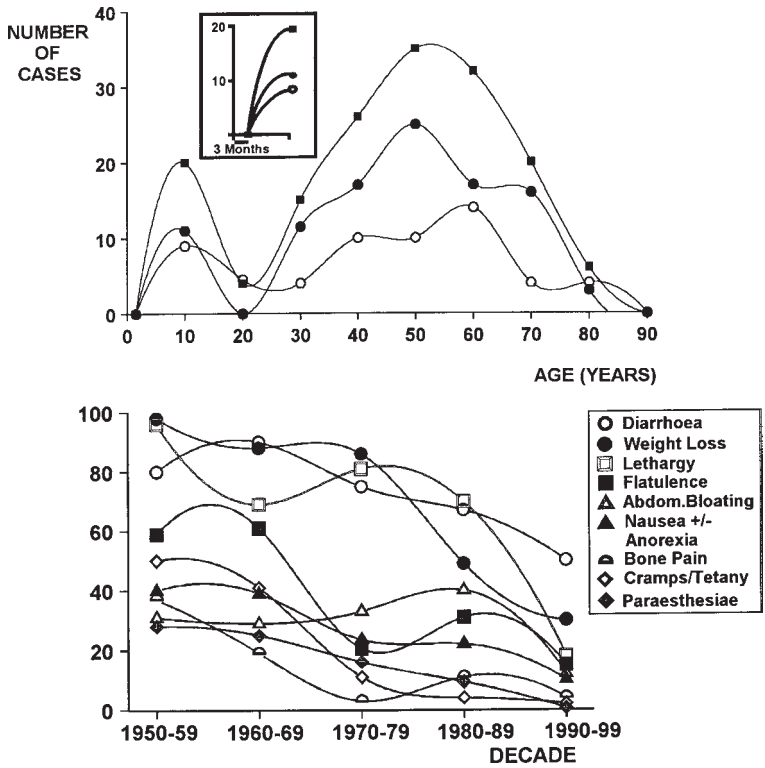


Fig. 1. Epidemiological data from the Celiac Clinic at Hope Hospital (**left**), which mirrors national trends. The early childhood peak (inset: note minimum 3-mo induction period) has equal numbers of boys and girls and probably reflects an "infective" and hence diarrheal form of presentation. The adult peak extends over seven decades with females presenting earlier than males. Here the more likely symptom complex will be caused by to anemia (especially iron deficiency), dermatitis herpetiformis, other atypical forms of presentation, or diarrhea acquired through foreign travel. If we evaluate presenting features of celiac disease (**right**), as detailed in various studies since 1960 onward ***(27)***, we can see to what extent the classic presenting features of diarrhea, weight loss, and weakness have fallen up to the present era.

1.1. Definition and Rationale of the Book

Gluten sensitivity is a more useful term that encompasses patients with classical malabsorption disease, dermatitis herpetiformis, other nongastrointestinal manifestations of the condition, and those with compensated-latent disease ***(5)*** (**Fig. 3**).

Gluten sensitivity may be defined ***(11)*** as a state of heightened cell-mediated (T-lymphocyte) and humoral (B-lymphocyte) reactivity to prolamin pep-

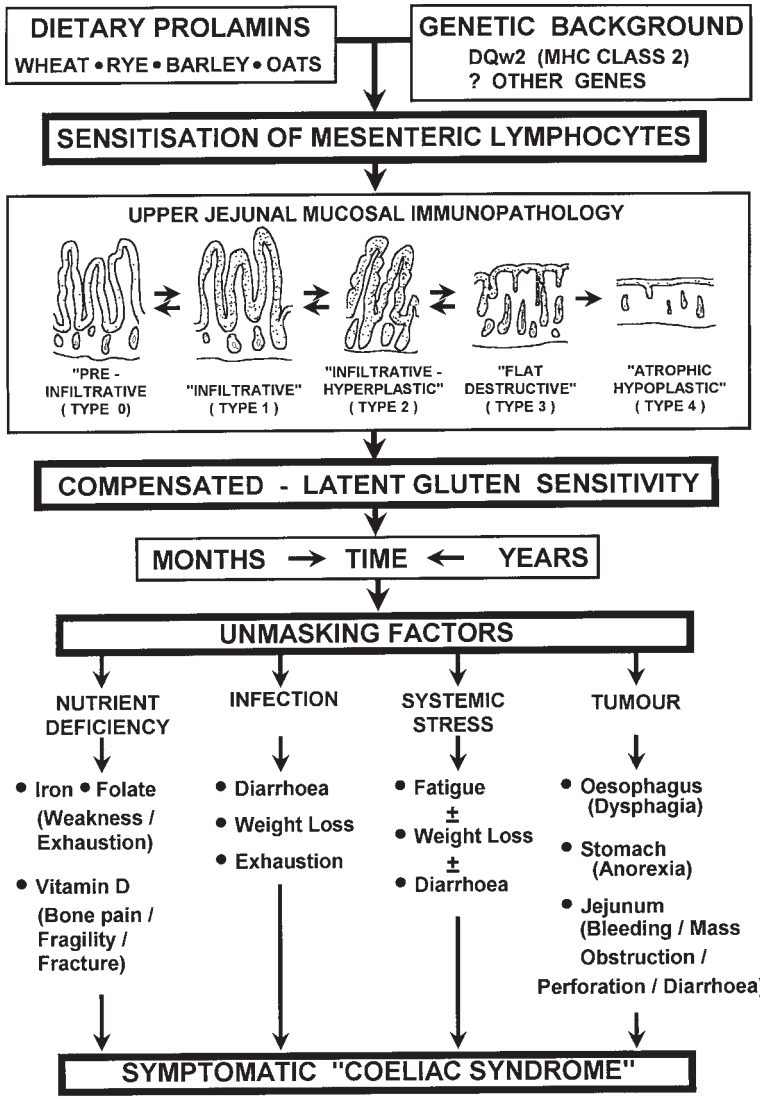


Fig. 2. Pathogenesis of gluten sensitivity and the compensated-latent state, with factors precipitating a symptomatic "celiac" syndrome. The view proposed is that the proximal gluten-induced lesion (a T-cell-mediated, host-mediated response by activated mesenteric lymphocytes to "foreign" gluten protein in the upper intestinal wall) results in a compensated-latent state, irrespective of the degree of severity of this proximally located lesion. If that were not so, everyone so predisposed would develop symptoms and be diagnosed within 6–12 mo of age, which clearly does not happen. The environmental triggers that unmask the compensated-latent stage, in whatever decade of life (**Fig. 1**) can be usefully classified into four groups, of which infection and nutrient deficiency (separate or combined) account for the most common modes of clinical presentation.

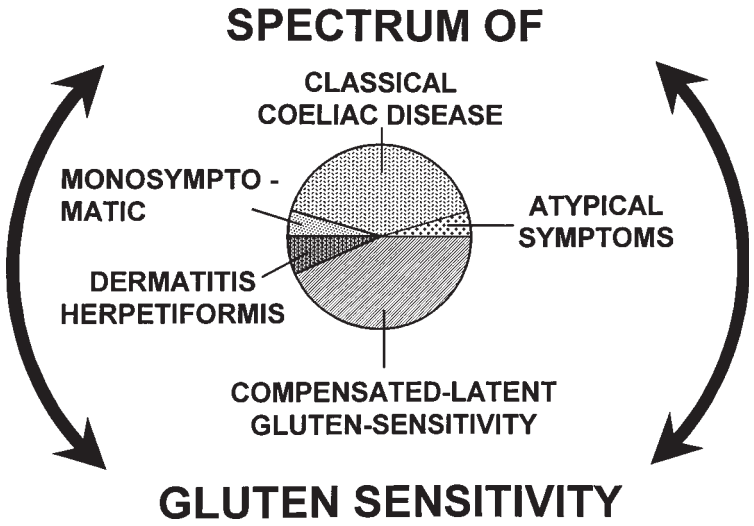


Fig. 3. The clinical spectrum of gluten sensitivity. This includes patients of all ages presenting with "classical" features ("celiac disease"). Other groups of individuals fall outside that restrictive definition, including individuals with atypical or monosymptomatic presentations (which may not always immediately suggest a gastrointestinal basis), and dermatitis herpetiformis. Others comprise a seemingly important group that remains in a compensated-latent phase of this hypersensitivity reaction to gluten protein.

tides in genetically predisposed (DQw2) individuals, resulting in variable degrees of mucosal change and injury. The sensitization is to various groups of prolamin peptides: glutens (wheat), hordeins (barley), and secalins (rye); avenins (oats) do not appear to be disease-activating proteins.

In the last two decades some formidable laboratory techniques have been applied to the study of gluten sensitivity. This book elucidates those techniques and their detailed practice. However, as more research is carried out, the complexity of the immunopathology of this condition becomes ever more apparent. We are therefore still a long way from resolving the puzzle. Nevertheless, newer insights are likely to appear rapidly with the application of (lymphocyte) cloning techniques, and the investigation of the involved proteins by powerful physical techniques (mass spectrometry).

1.2. Prolamin Separation and Peptide Elucidation

The prolamins of wheat, barley, and rye are not easy proteins to work with, and to separate them in highly purified form is still a quite difficult task, but essential for determining which species of these numerous proteins is relevant

to disease activity. However, the amino acid sequences of many such proteins have been adduced *(12)*, and such knowledge permits the synthesis of highly purified oligopeptides that are amenable to study by in vivo or in vitro techniques.

Further attempts at evaluating peptide activity require identification of material contained with the antigen-presenting groove of the class II major histocompatibility complex molecular (DQ2) thought to be central to pathogenesis. This highly technical approach and its allied techniques will clearly provide further information.

1.3. Genetic Background

Although 95% of gluten-sensitized individuals are $DQ2^+$ *(13)*, the molecular structure of this heterodimer is identical to that in $DQ2^+$-nonceliac individuals. Therefore, other genes must clearly be involved, and this can be examined through automated linkage analysis, genotyping, and positional cloning strategies. It seems odd that the quest for alternative genetic components has not definitively identified the other genes that must clearly be involved in pathogenesis *(14)*.

1.4. Cloned Mucosal T-Lymphocytes

The recent development of techniques for isolating and cloning T-lymphocytes from celiac mucosa has been a major advance in furthering our understanding of gene (DQ2-transfected Epstein-Barr virus–transformed B-lymphocytes), peptide, and lymphocyte interactions *(15)*. Such techniques bring gluten sensitivity into the test tube, and provide the opportunity for rapid appraisal of gene mutations (at key binding sites in the groove) and for residue substitutions in known active oligopeptides. The signal observation that mucosal transglutaminase has a high affinity for gliadin peptides residues (thereby creating possible new epitopes that may have disease-activating or mucosal-damaging propensities) is a very recent, but exciting observation *(16,17)* whose biological significance still requires elucidation.

Clinically, the formation of "antiendomysial" antibodies to tissue transglutaminase enzyme *(18)* (or gluten-transglutaminase neoepitopes) has revolutionized the clinical approach to diagnosis, especially in recognizing patients in the compensated-latent phase *(19)*, and even with nongastrointestinal manifestations. These are aspects of the clinical manifestations of gluten sensitization that still need detailed evaluation *(20,21)*.

1.5. Mucosal Immunopathology

Ultimately, the intestinal mucosa is the site of T-lymphocyte-DQw2 interactions, and gluten *(22–25)*. On current dogma, it must be presumed that at weaning in a genetically predisposed individual, naive T-cells are sensitized

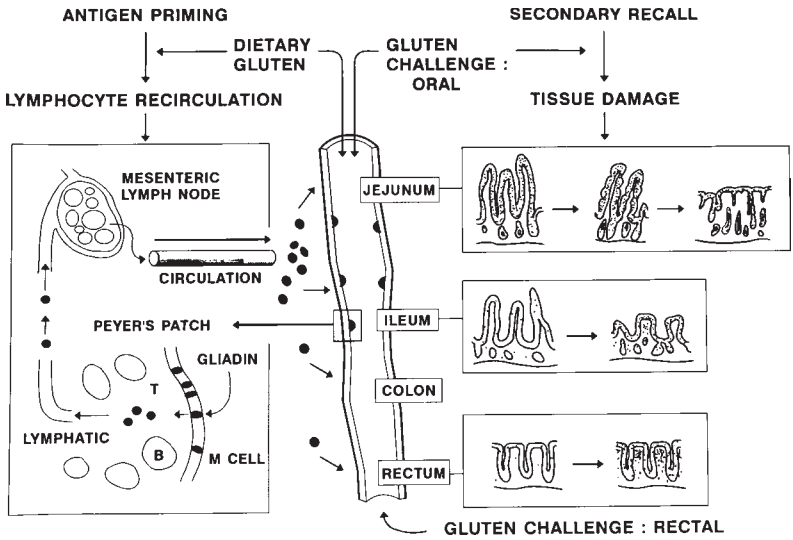


Fig. 4. Mechanism of gluten sensitization of mesenteric lymphocytes. Initial priming occurs in Peyer's patches (**left**) from which primed T- and B-lymphocytes emigrate via lymphatics and mesenteric lymph nodes. After recirculating in the blood, the lymphocytes randomly home to the epithelium and mucosa (lamina propria) throughout the intestinal tract (via $\alpha_4\beta_7$ and $\alpha_4\beta_E$ integrins). Secondary (recall) challenge (**right**) leads to the lymphocytes' reactivation and hence the promotion of an immune/inflammatory response with nonspecific secondary recruitment of many other cell types to the locus where antigen is present. In conformity with previous animal experiments, secondary gluten-induced pathology (1) can be evoked at places remote from the site of initial priming, e.g., distal ileum and rectum, as well as the upper jejunum; and (2) the reaction remains restricted to the site to which antigen is applied.

within Peyer's patches, from which such cells ultimately migrate into the recirculation, and then return to the intestinal lamina propria and epithelium.

It is these primed lymphocytes within the mucosa that evoke secondary responses in the presence of gluten that cause each phase of injury (**Fig. 4**). In the absence of gluten (a gluten-free diet), the mucosa returns to normal, implying that there is no intrinsic fault with the mucosa itself. This also explains why it is possible to bring about identical responses on rectal mucosal challenge, simply because sensitized T-lymphocytes recirculate there (as presumably to all other mucosal sites) (**Fig. 4**).

Although we have a good idea of the descriptive features of mucosal pathology in gluten sensitivity ***(26)***, how such changes come about is far less certain. Issues concerning the role of the microvasculature and of connective tissue reorganization (within the lamina propria), the interplay between enterocytes

and lamina propria or between other lymphocytes, and the curiously elevated numbers of $\gamma\delta^+$ T-cell receptor lymphocytes within the epithelium are being explored by computerized image analysis, highly sophisticated immunohistochemical and immunocytochemical techniques, and the application of molecular biological approaches in identifying key cytokines involved in these events. Nevertheless, the mucosal reaction, as it evolves in gluten sensitivity, is immensely complicated, and despite analysis of in vivo and in vitro mucosal tissues, a clear answer to the immunopathology of gluten sensitivity, other than its basic T-cell modulating basis, still needs to be elucidated.

2. Conclusion

The investigation of the biomolecular aspects of celiac disease is not for the fainthearted. But for those who wish to immerse their feet, or even plunge into this complex pool of intrigue, this book should provide good introductory exposure.

References

1. Gee, S. J. (1888) On the coeliac affection. *St. Bart Hosp. Rep.* **24,** 17–20.
2. Dicke, W. K., Weijers, H. A., and van de Kamer, J. H. (1953) Coeliac disease. 2—The presence in wheat of a factor having a deleterious effect in cases of coeliac disease. *Acta. Paediatr. Scand.* **42,** 34–42.
3. Fry, L., Seah, P., Hoffbrand, A. V., and McMinn, R. (1972) Lymphocytic infiltration of epithelium in diagnosis of gluten-sensitive enteropathy. *Br. Med. J.* **3,** 371–374.
4. Marsh, M. N. (1989) Lymphocyte-mediated intestinal damage—human studies, in *The Cell Biology of Inflammation of the Gastrointestinal Tract*, Peters, T. J., ed., Corner's Publications, Hull, East Riding, UK, pp. 203–229.
5. Marsh, M. N. (1995) The natural history of gluten sensitivity: defining, refining and re-defining. *Q. J. Med.* **85,** 9–13.
6. MacDonald, W. C., Brandborg, L. L., Flick, A. L., Trier, J. S., and Rubin, C. E. (1964) Studies of celiac sprue. IV—The response of the whole length of the small bowel to a gluten-free diet. *Gastroenterology* **47,** 573–589.
7. Marsh, M. N. (1993) Mechanisms of diarrhoea and malabsorption in gluten-sensitive enteropathy. *Eur. J. Gastroenterol. Hepatol.* **5,** 784–795.
8. Marsh, M. N. (1992) Gluten sensitivity and latency: the histological background, in *Dynamic Nutrition Research, Vol. 2: Common Food Intolerances: 1. Epidemiology of Coeliac Disease*, Auricchio, S. and Visakorpi, J. M., eds., Karger, Basel, Switzerland, pp. 142–150.
9. Young, W. F. and Pringle, E. M. (1971) 110 children with coeliac disease, 1950–1969. *Arch. Dis. Child.* **46,** 421–436.
10. McNeish, A. S. and Anderson, C. M. (1974) The disorder in childhood. *Clin. Gastroenterol.* **3,** 127–144.
11. Marsh, M. N. (1992) Gluten, major histocompatibility complex, and the small intestine: a molecular and immunobiologic approach to the spectrum of gluten-sensitivity ('celiac sprue'). *Gastroenterology* **102,** 330–354.

12. Shewry, P. R., Tatham, A. S., and Kasarda, D. D. (1992) Cereal proteins and coeliac disease, in *Coeliac Disease*, Marsh, M. N., ed., Blackwell Scientific, Oxford, UK, pp. 305–348.
13. Lundin, K. E. A., Scott, H., Hansen, T., Paulsen, G., Halstensen, T., Fausa, O., Thorsby, E., and Sollid, L. (1993) Gliadin specific, HLA-DQ(α1*0501, β1*0201) restricted T cells isolated from the small intestinal mucosa of coeliac disease patients. *J. Exp. Med.* **178,** 187–196.
14. Houlston, R., Tomlinson, I., Ford, D., Seal, S., and Marsh, M. N. (1997) Linkage analysis of candidate regions for coeliac disease genes. *Hum. Mol. Genetics* **6,** 1335–1339.
15. Nilsen, E. M., Lundin, K., Krajci, P., Scott, H., Sollid, L., and Brandtzaeg, P. (1995) Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon-γ. *Gut* **37,** 766–776.
16. Molberg, Ø., McAdam, S., Körner, R., Quarsten, H., Scott, H., Noren, D., et al. (1998) Tissue transglutaminase selectively modifies gliadin peptides that are recognised by gut derived T cells in celiac disease. *Nature Med.* **4,** 713.
17. van de Wal, Y., Kooy, Y., van Veelen, P., Pena, S., Mearin, L., and Koning, F. (1998) Selective diamidation by tissue transglutaminase strongly enhances gliadin-selective T cell reactivity. *J. Immunol.* **161,** 1185.
18. Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, V., and Riecken, E. O. (1997) Identification of tissue transglutaminase as the auto-antigen of celiac disease. *Nature Med.* **3,** 797–801.
19. Unsworth, D. J. and Brown, D. L. (1994) Serological screening suggests that adult coeliac disease is under-diagnosed in the UK and increases the incidence by up to 12%. *Gut* **35,** 61–64.
20. Marsh, M. N. (1997) Transglutaminase, gluten and celiac disease: food for thought. *Nature Med.* **3,** 725–726.
21. Mulder, C. J. J., Rostami, K., and Marsh, M. N. (1998) When is a coeliac a coeliac? *Gut* **42,** 594.
22. Ferguson, A. (1987) Models of immunologically driven small intestinal damage, in *The Immunopathology of the Small Intestine*, Marsh, M. N., ed., Wiley, Chichester, pp. 225–252.
23. Mowat, AMcI and Ferguson, A. (1982) Intraepithelial lymphocyte count and crypt hyperplasia measure the mucosal component of the graft-versus-host reaction in mouse small intestine. *Gastroenterology* **83,** 417–423.
24. MacDonald, T. T. (1992) T cell-mediated intestinal injury, in *Coeliac Disease*, Marsh, M. N., ed., Blackwell Scientific, Oxford, UK, pp. 283–304.
25. Marsh, M. N. and Cummins, A. (1993) The interaction role of mucosal T lymphocytes in intestinal growth, development and enteropathy. *J. Gastroenterol. Hepatol.* **8,** 270–278.
26. Marsh, M. N. (1992) Mucosal pathology in gluten sensitivity, in *Coeliac Disease*, Marsh, M. N., ed., Blackwell Scientific, Oxford, UK, pp. 136–191.
27. Howdle, P. D. and Losowsky, M. S. (1992) Celiac disease in adults, in *Coeliac Disease*, Marsh, M. N., ed., Blackwell Scientific, Oxford, UK, pp. 49–80.

2

Genotyping Methodologies

Stephen Bevan and Richard S. Houlston

1. Introduction

This chapter details DNA extraction through polymerase chain reaction (PCR) amplification, gel running, allele assignment, and data management so that the genotyping data produced is suitable for use in linkage analysis programs.

1.1. DNA Extraction

The most convenient source of genomic DNA is via EDTA blood samples, which after collection can be frozen and stored at –70°C for long periods. Since only white blood cells (WBCs) contain DNA, the first process in the extraction protocol is to separate the red blood cells (RBCs) and WBCs either by centrifugation or by lysis of the RBCs in a hypotonic solution.

1.2. Considerations Before PCR

A genomewide search is typically based on between 250 and 400 markers to give 10–20 cm separation across the genome. Before embarking on a genomewide search, several factors need to be considered. These include detection of PCR products, what label should be used, and, given the large number of results that will be generated, which system will maximize throughput.

1.3. Detection of PCR Products

Fluorescent labeling and radioactive labeling are the two main methods of detecting PCR products with the resolution required for allele calling. Both methods have advantages and disadvantages, primarily in terms of cost and the laboratory equipment needed to detect them.

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

The simplest way of labeling a PCR product is to label the primer before the PCR begins. In the case of fluorescence, the labeled primer is usually acquired from a commercial source, whereas with radioactivity, the primer can be labeled with ^{32}P on the bench. The two main advantages of using fluorescent primers are that they are nonhazardous and that they can be multiplexed to speed up analysis. The disadvantages of this approach are the expense and the requirement for specialized equipment such as an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA). Detection of fluorescently labeled PCR products works by electrophoresing the products through denaturing polyacrylamide gels along with a labeled size marker. The products migrate through the path of an argon laser beam and emit fluorescence, which is then detected. Four colors can be detected, allowing multiple samples to be loaded into a single lane. Furthermore, products of different sizes migrate at different rates, so more than one sample can be loaded with the same colored marker. This allows up to nine markers to be simultaneously loaded in a single lane. For radioactive markers, it is only possible to load a single marker in any one lane of the gel because there is only one type of output signal and the resolution is not as high as that for fluorescent markers. The main advantages of radioactively labeled markers over fluorescent ones is that they are relatively cheap, easy to generate and detect, and no expensive detection equipment is required. However, the allele numbers have to be scored manually, which can be time-consuming. Use of fluorescent primers and an ABI 377 means that the data are stored digitally and can be analyzed by computer programs such as genotyper or Genetic Analysis System (GAS) software.

1.4. Design of Fluorescent Marker Panels

Fluorescent markers are available either individually or in panels. The panels consist of a range of markers designed to be run together in a single gel lane to give maximal throughput. The main disadvantage of these panels is that they have been designed with a genomewide search in mind, and, as such, markers from a single chromosome are randomly distributed through the panels depending on the size of product they produce. Microsatellite markers, the size of the product they amplify, and their position in the genome can be found at several Internet sites, which are given in **Table 1**. Note, however, that these sites tend to use their own maps, and the distances quoted will vary from site to site. Thus, markers should be chosen from only one map rather than several. Also provided for each marker is a heterogeneity score ranging from 0 to 1. This is a measure of how informative the marker is for linkage; the higher the number the more informative the marker.

Table 1
Contact Addresses for Information on Fluorescent Primers and Related Technical Data

Marshfield Centre for Medical Genetics
http://www.marshmed.org/genetics/
Genethon
http://www.genethon.fr/genethon-en.html
Cooperative Human Linkage Centre
http://www.chlc.org/
Genetic Location Database
http://cedar.genetics.soton.ac.uk/public_html/
Genome Database
http://www.hgmp.mrc.ac.uk/gdb/gdbtop.html
CEPH Genotyping Database
http://www.cephb.fr/cephdb/
National Centre for Biotechnology Information
http://www.ncbi.nlm.nih.gov/
Perkin-Elmer Applied Biosystems
http://www.perkin-elmer.com/ab
Helena Bioscience
e-mail: info@helena.co.uk

1.5. Radioactive Primers

The best alternative to fluorescent primers is radioactively labeled primers. The two main methods of radioactively labeling are either to label the primer before performing PCR or to use a radioactively labeled dNTP for incorporation during PCR. However, the latter method provides a lower level of resolution, making radioactively labeled primers the method of choice. Endlabeling of primers relies on the use of T4 polynucleotide kinase to catalyze the transfer and exchange of phosphate from adenosine triphosphate to the 5' hydroxyl terminus of polynucleotides.

1.6. PCR

Standard PCR protocols can be used for both fluorescent and radioactive primers, and the commercial suppliers of fluorescently labeled primers will usually provide the PCR conditions suitable for their primers.

1.7. Allele Calling from Fluorescent Primers

Multiple PCR reactions can be mixed before genescan gel loading to enhance sample throughput. Ideally, approx 5–10 ng of DNA of each sample should be

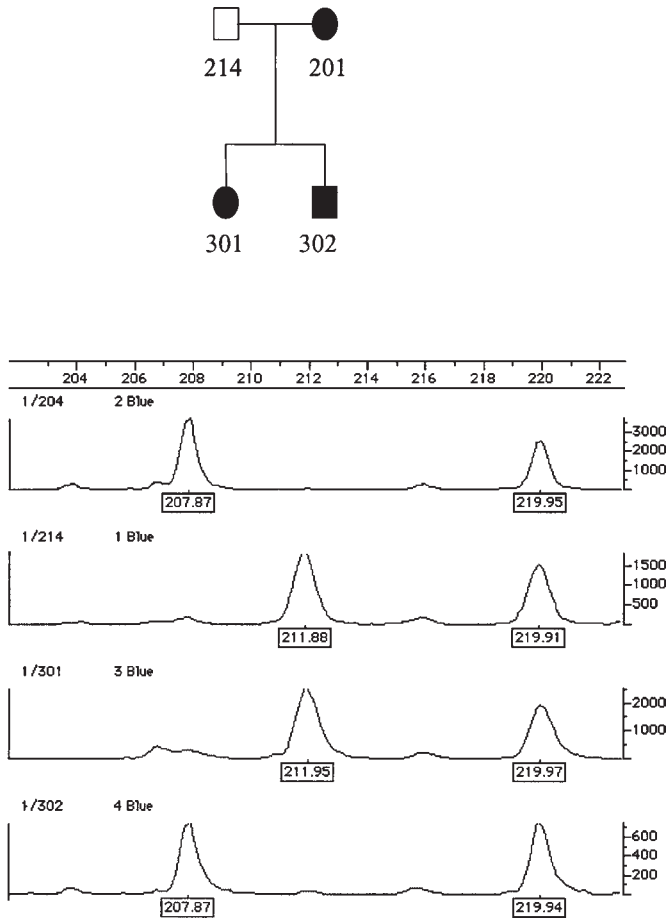


Fig. 1. Pedigree and corresponding Genotyper output for a single marker D10S1677.

loaded onto each lane of the gel. During electrophoresis the fluorescent data are collected and stored using the GeneScan Collection Software (Perkin-Elmer), and analyzed by the GeneScan Analysis Software at the end of the run. These programs come with the ABI 377 DNA sequencer. The gel file can then be downloaded to a computer (typically an Apple Macintosh) and alleles scored using the Genotyper software. This software produces a plot of size in base pairs against fluorescence intensity. A PCR product will produce a peak in fluorescence corresponding to its size. The allele number can then be scored either manually or automatically. **Figure 1** shows the pedigree of a small family and the corresponding Genotyper output for marker D10S677. This marker is a tetranucleotide repeat and has a predicted size range of between 197 and 225 bp.

From the Genotyper output in **Fig. 1** it can be seen that alleles should be labeled at 207, 211, 215, 219 bp, and so on. With a larger number of families, the number of different alleles will increase and accurate allele frequencies can be calculated. Because the expected size of D10S677 is 197–225 bp, 199 bp should be labeled allele 1, 203 bp allele 2, and so on. If an allele does not occur, then it can be given a frequency of 0 in subsequent linkage analysis. The family in **Fig. 1** would be scored as 3 6, 4 6, 4 6, 3 6 for person 1/201, 1/214, 1/301, and 1/302 respectively.

1.8. Detection of Radioactive PCR Products

Radioactive PCR products are run on urea denaturing gels, and in our laboratory, they are set up on a standard vertical gel electrophoresis apparatus (Model S2, Gibco BRL, Paisley, UK). The gels are 30 × 40 cm and can accommodate SO samples at any one time.

On radioactive gels, alleles are generally scored from top to bottom, assigning the highest band as allele 1, the next as allele 2, and so on. If two gels are being run with the same marker, it is essential to run a duplicate sample on both gels to ensure uniformity in calling alleles. This is important when calculating frequencies of alleles for linkage analysis.

1.9. Data Management

Following the assignment of alleles to individual DNA samples, the inheritance of these alleles should be examined for Mendelian transmission as a prelude to linkage analysis. This can be done by eye from a sheet of paper, but the process can become quite complex in families with many markers. A suitable program for displaying pedigrees and markers is the commercial software package Cyrillic (Cherwell Scientific, Oxford, UK).

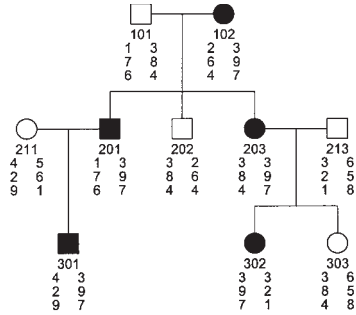
1.10. Cyrillic

In Cyrillic each pedigree can be drawn, along with the relevant individual's phenotype and marker alleles. The benefit of this is that a family can be associated with more than one disease and data for each disease kept separate. Cyrillic will also haplotype families automatically. Cyrillic has an export function, enabling marker data to be transferred out to analysis packages such as MLINK and FASTLINK. However, the program is inflexible since pedigrees cannot be automatically drawn by importing allele data from other programs.

1.11. Data Format

For linkage analysis, data must be written into or imported/read into the analysis software in a specific format. This is usually set out as follows:

Family ID, PID, FID, MID, Sex, Affection Status, Marker Typings



1	101	0	0	1	1	1	3	7	8	6	4
1	102	0	0	2	2	2	3	6	9	4	7
1	201	101	102	1	2	1	3	7	9	6	7
1	202	101	102	1	1	3	2	8	6	4	4
1	203	101	102	2	2	3	3	8	9	4	7
1	211	0	0	2	1	4	5	2	6	9	1
1	213	0	0	1	1	3	6	2	5	1	8
1	301	201	211	1	2	4	3	2	9	9	7
1	302	213	203	2	2	3	3	9	2	7	1
1	303	213	203	2	1	3	6	8	5	4	8

Fig. 2. A family pedigree typed with three markers and the output file for the pedigree ready for analysis by linkage analysis software.

where Family ID is the family number; PID is the person number; FID is the number of the person's father (0 = unknown); MID is the number of the person's mother (0 = unknown); Sex is 1 for male and 2 for female; Affection Status is 0 for unknown, 1 for unaffected, and 2 for affected; and the Marker Typings are the alleles produced from the genescan analysis or radioactive gel runnings. **Figure 2** shows a family (given the family ID of 1) typed for three markers and the format in which this family's data would be arranged for linkage analysis.

Cyrillic can be used to create and export output files from pedigrees, but these have to be drawn first, which is time-consuming. We have found it easier to create a database using Microsoft Access, which allows the marker alleles to be typed into a table. By setting up a table containing the standard family pedigree information (columns 1–6 in **Fig. 1**), marker alleles can be merged for analysis. No graphic representation is required, and as few or as many markers as required can be merged for analysis at any one time. The major advantage of Access is that tables can be linked together, so that inputting data into one table automatically adds the same data to other tables. This has allowed us to input data into tables on a panel-by-panel basis, automatically allocating them to

chromosome-specific tables. The chromosomal table is then merged with the pedigree table ready for linkage analysis.

Although Access does have an export function, exporting changes the spacing of the fields. A simple way to overcome this problem is to cut the table data you want to analyze (by highlighting the text and using the Edit, Cut command), opening Microsoft Word and using the Edit, Paste Special command. This gives two options: to paste either as formatted text (rich text format [RTF]) or as unformatted text. The data must be pasted as unformatted text and then saved as a text only file (*.txt). This maintains the spacing of the fields, and the text file can be read directly by linkage analysis software. Transfer between PC and a UNIX machine running linkage software can be conveniently performed by a file transfer protocol (ftp).

Genotyping data can also be managed by a suite of programs collectively called GAS. This program has the advantage that it will automatically put raw data into a format suitable for linkage analysis, and it also has analysis software. The GAS program, manual, and example files are available from ftp.well.ox.ac.uk by anonymous ftp and are available for IBM-PC, Vax UMS, DEC Ultrix, DEC Alpha, Sun solaris and Sun os. When logging in, your user name should be anonymous and your password your e-mail address.

2. Materials

1. Sucrose lysis mix: 218 g of sucrose, 20 mL of 1 *M* Tris (pH 7.5), 2 g of $MgCl_2$, 20 mL of Triton X-100. Make up to 2 L with dH_2O to provide enough solution for forty 10-mL blood samples.
2. Resuspension buffer: 2.6 mL of 5 *M* NaCl, 0.84 mL of 0.5 *M* EDTA (pH 8.0), 15 mL of 10% sodium dodecyl sulfate. Make up to 175 mL with dH_2O to provide enough solution for forty 10-mL blood samples.
3. GTB buffer (20X): 432 g of Tris, 144 g of taurine, 8 g of EDTA. Make up to 2 L with dH_2O and stir until dissolved.

3. Methods

3.1. Sucrose Lysis DNA Extraction

1. Decant 10 mL of blood into a 50-mL Falcon tube and add 40 mL of ice-cold dH_2O.
2. Invert the tube five times to mix the solutions gently, and then centrifuge at 500*g* for 20 min at 4°C. This lyses the RBCs and pellets the remaining WBCs.
3. Remove the supernatant and keep the pellet on ice. Add 25 mL of ice-cold sucrose lysis solution to the pellet and resuspend by moderate manual shaking to lyse the WBCs.
4. Centrifuge at 500*g* for 20 min at 4°C to pellet the released genomic DNA.
5. Discard the supernatant and resuspend the pellet in 3.5 mL of resuspension buffer supplemented with 20 mg/mL of proteinase K (0.5 mL of 20 mg/mL of proteinase K should be added to 175 mL of resuspension buffer immediately prior to use).

6. Following gentle resuspension, incubate overnight at 37°C or for 3 h at 60°C to allow protein digestion.
7. Add 1.2 mL of 5 *M* NaCl to the tube and shake vigorously for 20 s to precipitate digested protein. Centrifuge at room temperature for 30 min at 3000*g* to pellet the protein.
8. Transfer the supernatant to a 15-mL tube and add 2 vol of 100% ethanol. Then invert gently to precipitate the DNA. If necessary the sample can be left at –20°C for 30 min to enhance precipitation. If the DNA is visible, it can be removed with a pipet to a separate tube and dried before resuspending in Tris-EDTA (TE). If it is not visible, centrifuge at 2800*g* for 30 min to pellet the DNA, remove the supernatant, dry the pellet, and then resuspend in TE (200–500 µL depending on the size of the pellet).

3.2. Endlabeling of Primers with ^{32}P

1. Add to a 1.5-mL microfuge tube 20 µL of primer (at 5 outer diameter [OD] conc.), 2.5 µL of 10X kinase buffer, 1 µL of T4 polynucleotide kinase, 1 µL of ^{32}P, and make up to 25 µL with dH_2O.
2. Incubate at 37°C for 40 min to allow addition of the ^{32}P to the primer. Then add to the PCR stock mix ready for PCR.

3.3. PCR Protocol

All of the volumes in this protocol are applicable to a 96-well plate used on a Biomek 1000 robot (Beckman Coulter, Fullerton, CA), i.e., for one hundred 15-µL PCR reactions.

1. In a 1.5-mL microfuge tube, mix 530 µL of dH_2O, 150 µL of reaction buffer, 150 µL of dNTP mix (10 m*M*), 90 µL of 25 m*M* $MgCl_2$ (1.5 m*M* final), 15 µL of 10 mg/mL of bovine serum albumin, 30 µL of each primer (40 pmol), and 6 µL of *Taq* (5 U/µL).
2. Add 10 µL of the stock mix to 5 µL of DNA (at 2.5 ng/µL) and cover with 20 µL of mineral oil in a 96-well plate by the robot. (The PCR machine can be set to hot lid if required, dispensing with the need for oil, but the presence of oil helps reduce evaporation and condensation after PCR when the samples are stored at 4°C.)
3. Then run the PCR at conditions specific to the primer pair in question. For example, a primer pair with an annealing temperature of 55°C would have 30 cycles of 55°C for 1 min, 72°C for 2 min, and 94°C for 1 min before a final cycle of 55°C for 1 min followed by 72°C for 7 min to ensure full extension and maximization of double-stranded PCR product.

3.4. Electrophoresis of Radioactive Markers

1. Clean both gel plates with soapy water and then with 100% EtOH, and coat the small plate in a silane solution such as sigmacote (Sigma Aldrich, St. Louis, MO) to prevent the gel from sticking to it.
2. Once dry, add the spacers and tape the plates ready for gel pouring.

3. Make the gel by mixing 40 g of urea, 4 mL of 20X GTB buffer and 31 mL of dH_2O. Swirl gently until most of the mix has dissolved, and then heat for 20 s on full power in a microwave. Swirl gently until completely dissolved.
4. Add 12 mL of 40% acrylamide (6% final), 300 µL of adenosine 5'-phosphosulfate (APS), and 24 µL of TEMED; mix gently; and pour. Polymerization should occur within 30 min to 1 h.
5. To your 15-µL PCR reaction add 20 µL of running dye (200 µg of bromophenol blue, 200 µg of xylene cyanol in 100 mL of formamide) and immediately before loading heat to 94°C for 5 min. Then place on ice and load. This procedure is done to denature all double-stranded molecules and anneal them slowly to reduce to a minimum the amount of nonspecific binding, which leads to false bands on the gel.
6. Run at 80 W for as long as necessary to electrophorese the product into the bottom third of the plate (the longer the better since the further the products travel the better the separation).
7. Once the gel has run, remove the plates from the gel apparatus, separate the plates, and transfer the gel to filter paper (Whatman 3MM paper or similar) by laying the paper onto the gel and applying gentle pressure before peeling up from one corner, being careful to mark the orientation of the gel. Place a piece of Saran wrap over the gel and dry under vacuum at 80°C on a gel dryer for 40–60 min.
8. Check the activity of the gel with a Geiger counter and then expose to X-ray film in an autoradiography cassette for as long as required. Develop the gel in the usual manner and score the alleles.

4. Notes

1. Several commercial kits are available for the extraction of DNA from blood and solid tissues, but these are generally quite expensive—particularly when a large number of samples are to be extracted. For this reason, most laboratories have adopted the sucrose lysis method of genomic DNA extraction from whole blood. This method uses water to lyse the RBCs and a sucrose solution to burst the WBCs, allowing the genomic DNA to be precipitated following incubation with proteinase K to remove any contaminating protein.
2. Ten milliliters of fresh whole blood should yield between 200 and 1000 µg of genomic DNA. Following resuspension in TE, the DNA concentration can be determined by calculating A_{26O}, with an OD of 1.0 corresponding to 50 µg of DNA. The $A_{260/280}$ ratio can also be calculated to determine the protein level in the sample. Clean DNA should have a ratio of approx 1.6; a higher ratio implies contaminating protein and a lower ratio implies contaminating RNA. If contaminating protein is present, the sample can be reincubated with proteinase K, and contaminating RNA can be removed by incubation with RNase. Once the DNA purity is satisfactory, a 50 ng/µL working stock should be made ready for PCR.
3. Fluorescent markers can be purchased from a commercial supplier such as Genset (distributed by Helena Bioscience, Sunderland, UK) or Perkin-Elmer (Warrington, Cheshire, UK).

4. This is equivalent to taking 2.5 μL of an average PCR reaction into a final volume of 50 μL; i.e., for a genescan mix of 5 markers, take 2.5 μL each and add to 37.5 μL of dH_2O. Genescan mixes should ideally be made the day before the genescan is to be run, to allow adequate mixing of the samples. Mixes can be made just before running, but in our experience the quality of the genescan output is inferior.
5. The end-labeling protocol provides enough labeled primer for approx one hundred 15-μL PCR reactions—enough for one 96-well plate if automation is used.
6. If a large number of PCR reactions are to be performed, as is the case in a genomewide search, it is highly advantageous to consider some form of automation, either by simple multichannel pipetting or full automation on a robot such as the Biomek 1000. Both allow 96-well plates to be used, giving a total PCR reaction volume of 15 μL plus oil (oil is not necessary with heated-lid PCR machines, further speeding up sample preparation time). With automation only one stock mix per plate is required, incorporating everything except DNA. This is provided from a separate 96-deep-well tray, which means that every 96-well plate has the same template, thus decreasing the chance of the pipetting errors associated with manual handling.
7. Following PCR the samples are ready for gel electrophoresis. For radioactive PCR reactions, simply add the labeled primer in place of the fluorescent primer in the PCR protocol detailed in **Subheading 3.3.**
8. Following PCR, samples should be checked by agarose gel electrophoresis (run 5 μL of the 15-μL reaction on a 2% agarose gel) to estimate DNA concentration. A 15-μL PCR reaction will typically yield 25–200 ng/μL of DNA. These estimates are helpful since loading too much DNA onto a genescan invariably leads to "bleeding" of one colored dye into another, thus restricting the number of samples that can be analyzed at any one time.
9. The genescan mixes are run on denaturing urea gels, and plate cleaning, gel casting, and sample running should be carried out in accordance with the user's manual. However, we have found that the following gel recipe appears to give slightly better results than that detailed in the user's manual:
   a. Add 18 g of urea, 18 mL of dH_2O, and 5.7 mL of 40% (29:1) acrylamide/bisacrylamide solution to a clean glass beaker and stir until dissolved.
   b. Make up to 50 mL with dH_2O and add 250 μL of 10% APS and 35 μL of TEMED to polymerize the gel. Mix gently and pour.

Acknowledgment

We thank the Coeliac Society for granting a fellowship.

3

From Linkage to Genes

Positional Cloning

Stephen Bevan and Richard S. Houlston

1. Introduction

Linkage analysis in families containing affected individuals can be used to identify the location of disease susceptibility genes. The aim of this chapter is to provide an overview of the molecular methods employed to clone these susceptibility genes on the basis of linkage data.

The minimum distance across which there is strong linkage should first be refined by examination of additional markers within the region. This can rarely be refined to less than 1 cm (approx 1035 kb). If this is the case, identification of a gene from linkage alone is unlikely unless a strong candidate gene lies within this region. If a candidate gene exists, mutation detection should be performed by a technique such as single-stranded conformation polymorphism (SSCP) or conformation-sensitive gel electrophoresis (CSGE) followed by sequencing of any possible mutations. However, if there is no candidate gene, then a positional cloning strategy should be undertaken to identify and isolate genes from the linked area that may be responsible for the disease under study.

Positional cloning relies on the isolation of large fragments of genomic DNA followed by isolation of expressed sequences from within the linked region. Yeast artificial chromosomes (YACs) are generally used for the replication and isolation of large human genomic fragments, whereas bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PACs) are suitable for smaller genomic fragments.

1.1. Yeast Artificial Chromosomes

Three types of *cis*-acting DNA sequence elements are necessary for yeast chromosome function: the telomeres, an origin of replication, and a centromere.

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

These three elements have been extensively analyzed, and each can be isolated on a fragment of approx 1 kb ***(1,2)***. Since yeast chromosomes range in size from 250 to 2000 kb, removal of nonessential yeast sequence and replacement with human genomic DNA fragments allows the large-scale isolation and replication of human DNA as molecular clones ***(3)***. YACs spanning the entire human genome are commercially available.

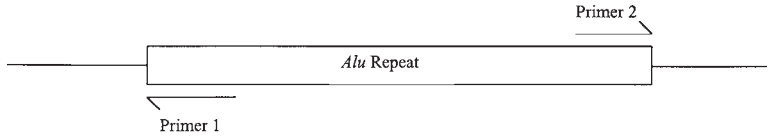
The first step in YAC screening is to plate the YACs in a regular pattern or grid so that any regions of interest can be easily identified. This "ridding can be done either manually or using a robot such as the Biomek 1000 (Beckman Coulter, Fullerton, CA) ***(4,5)***. It is first necessary to screen YACs to determine their relationship with each other to see whether they overlay and, if so, by how much. YACs are generally screened in one of two: by identifying unique sequence-tagged sites (STSs) by polymerase chain reaction (PCR) analysis ***(6,7)***; or by Southern analysis of individual YACs using repetitive sequence probes. This is sometimes called *Alu* fingerprinting ***(8)***.

1.2. YAC Analysis

Initial screening concentrates on how YACs are related to each other in terms of overlap and sequence identity. One technique for determining this is partial restriction digest analysis to determine an approximate restriction map of a YAC insert. Each YAC is partially digested in duplicate with a range of restriction enzyme concentrations, and the products are separated by pulsed-field gel electrophoresis (PFGE). Following blotting to a membrane, one set is hybridized to a probe that detects the left arm of the YAC (yeast sequence) and the other a right-arm probe. This allows the approximate distance of each restriction site to either end of the YAC to be determined.

Analysis of YAC clones can also be performed using PCR directly on individual colonies, which is useful for mapping STSs or for *Alu* PCR. A single colony is touched with a sterile loop and added to a previously prepared reaction mix including appropriate primers. *Alu* PCR uses primers that are specific to the human repeat sequence *Alu*, which on average occurs once every 3 to 4 kb in human genomic DNA. If they are close enough together, a PCR product results from between two *Alu* repeats, leading to a characteristic fingerprint for any particular region of human genomic DNA (*see* **Fig. 1**). The fingerprints from two or more YACs can therefore be compared in order to find overlapping regions ***(9,10)***.

A similar technique, known as *Alu*-vector PCR, can be used to recover the end of a YAC clone. In this case, an *Alu* primer is used in conjunction with a vector-specific primer. However, the technique relies on the close association of an *Alu* repeat with the end of the clone. An alternative method is known as vectorette PCR, which is a complex protocol that relies on successive rounds of restriction digestion and ligation (for further information *see* **ref. *11***).



Primer 1	5'	GCCTCCCAAAGTGCTGGGATTACAG	3'	25mer
Primer 2	5'	CCA$^{T}/_{C}$TGCACTCCAGCCTGGG	3'	20mer

Fig. 1. Position and sequence of the *Alu* repeat primers used for *Alu* PCR fingerprinting. The primers read away from each other in an attempt to bridge the intervening genomic DNA between adjacent *Alu* repeats.

The use of large genomic fragments in YACs is not always appropriate, and once areas of interest have been identified, these large fragments may even be a hindrance. Several other smaller-scale cloning systems are available based on a bacterial host that may be more suitable.

1.3. Bacterial Cloning Systems

Bacterial cloning systems have several advantages over YACs, including a higher transformation efficiency when generating libraries and easier isolation of inserted DNA. However, these bacterial systems do have a smaller cloning capacity than YACs.

The first bacterial cloning system was based on bacteriophage P1 and has a cloning capacity of approx 100 kb. Both cloned DNA and vector DNA are packaged into phage particles in a linear form. They are then injected into *Escherichia coli* by the phage's natural activity, where the DNA is circularized using P1 loxP recombination sites and the host-expressed enzyme P1 Cre recombinase. Vectors have a kanamycin resistance gene for selection, and both human and mouse genomic libraries are commercially available (*see* **ref. *12*** for further details).

A second cloning system based on *E. coli* utilizes an F-factor vector, and is called a BAC. This system has an advantage over the P1-based cloning system in that the insert size can be as large as 300 kb, and electroporation can be used to transform the bacterial host, thus avoiding the use of vector packaging sequences. The most recent cloning system is a combination of both the P1 cloning system and the F-factor cloning system, called the PAC ***(13)***. Again, this system uses electroporation to transform the bacterial host and allows insert sizes of 100–300 kb to be examined. Both BACs and PACs are commercially available.

1.4. Identification of Coding Sequence from Genomic DNA

Once a YAC, BAC, or PAC library has been generated and ordered, the search for candidate genes can begin. The first step is to look for any cross species–conserved sequences in the linkage region that would indicate some functionality ***(14)***. If this search is unsuccessful, specific sequences that are associated with genes can be searched for. A good example is the CpG island. The only methylated base so far identified in vertebrate DNA is 5-methylcytosine, and in mammalian cells more than 90% of this methylated nucleotide occurs in the dinucleotide sequence 5'-CpG-3'. There is an inverse relationship between the extent of methylation in the vicinity of a promoter and the rate of transcription of the corresponding gene: only weak transcription occurs from methylated DNA, whereas unmethylated DNA is strongly transcribed. Thus, searching for CpG islands is a good method of identifying possible genes. This can be done by using a pair of isoschizomeric restriction endonucleases such as *Hpa*II and *Msp*I, which recognize sites containing the 5'-CG-3' sequence and compare the digest pattern produced. The analysis is based on the fact that *Hpa*II will cleave only unmethylated CCGG sequences, whereas *Msp*I cleaves both methylated and unmethylated CCGG sequences ***(15)***. If this method is unsuccessful, a more direct approach may be required, such as direct screening of cDNA libraries.

1.5. Direct Screening of cDNA Libraries

The simplest, and one of the most powerful, techniques for locating expressed DNA in cloned genomic DNA is to hybridize the cloned genomic DNA from a YAC, PAC, or BAC onto a cDNA library. The hybridized cDNA can then be isolated and mapped back to the relevant genomic insert to find the corresponding gene ***(16,17)***.

1.6. cDNA Selection

cDNA selection was designed to allow the quick and efficient isolation of cDNAs from cloned genomic DNA ***(17a,18)***. In this system, cloned genomic DNA from a YAC, BAC, or PAC is biotinylated and then hybridized to cDNA. The hybridized cDNA and genomic target are then purified by binding to streptavidin-coated beads. The cDNA is eluted and then cloned and/or PCR amplified before sequencing. If necessary, further rounds of purification can be performed to enhance the specificity of the procedure. The result will be a copy of all exonic material encoded by the genomic insert.

1.7. Exon Trapping

The two previously described procedures of direct screening and cDNA selection will only result in isolation of cDNAs being expressed in the cell line

from which the cDNA library was constructed. If a gene has a tissue-specific distribution or a developmental distribution, it may not be detected. In this case, the genomic sequence itself must be used for identification of coding sequences by a procedure known as exon trapping. This method uses splice donor and acceptor sites in the vector to screen for acceptor and donor sites in the genomic DNA fragments inserted into the vector ***(19–22)***. A YAC, BAC, or PAC insert is restriction digested into 4 to 5 kb fragments that are then ligated into the exon-trapping vector encoding a splice donor and acceptor site on either side of the ligation point. Following ligation, the vector is transformed into a host cell, allowed to express then total RNA isolated and reverse transcriptase-PCR performed for the presence of trapped exons.

The first step in exon trapping is to produce a partial digest of PAC DNA. This is a standard partial restriction digest with lowering dilutions of the enzyme. The fragments can then be gel purified prior to cloning.

The DNA is now ready for ligation into the exon-trapping vector, for which a standard ligation protocol can be used. The ligation should run for 5 h at room temperature or 24 h at 4°C. Then it is transformed into competent cells for plasmid amplification. Following transformation and incubation overnight, a pooled liquid culture should be generated, and after 24 h of growth the plasmid DNA is isolated by a standard alkaline lysis "miniprep" protocol. The plasmid DNA should then be electroporated into the host cell (*see* **Note 6**).

The cells should then be incubated for 48 h at 37°C in a 5% CO_2 tissue culture incubator prior to total RNA isolation and cDNA synthesis. There are a number of commercially available kits for this procedure, such as TRIZOL™ and the Superscript Preamplification Kit (Gibco-BRL, Gaithersburg, MD). Following this procedure, vector-specific primers can be used to amplify any trapped exons prior to direct sequencing of the PCR product. Once direct sequencing information has been obtained from one or more of the previously described methods, a computer-based analysis and homology search should be performed. There are a multitude of DNA and protein sequence databases, and more detailed information on the most useful of these can be found in the annual database issue of *Nucleic Acids Research*.

2. Materials

1. SCM broth: 1.7 g of yeast nitrogen base without amino acids and without $(NH_4)_2SO_4$, 5 g of $(NH_4)_2SO_4$. 560 mg of amino acid mix (minus uracil and tryptophan). Make up to 1 L with dH_2O and adjust pH 5.8. Autoclave and then add 50 mL of filter sterilized 40% glucose.
2. Guanidinium chloride (GuHCl) solution: 4.5 *M* GuHCl, 0.1 *M* EDTA, 0.15 *M* NaCl, 0.05% sarkosyl (pH 8.0).

3. YPD agar: 10 g of yeast extract, 20 g of peptone. Add 1 L of dH_2O and adjust pH to 5.8. Add 20 g of Bacto-agar and autoclave. Add 50 mL of filter-sterilized 40% glucose solution before the agar sets, and pour.
4. YAC isolation solution 1: 1 M sorbitol, 50 mM EDTA, 1 mg/mL of zymolase, 28 mM b-mercaptoethanol (b-ME).
5. YAC isolation solution 2: 1 M sorbitol, 50 mM EDTA, 1 mg/mL of zymolase.
6. YAC isolation solution 3: 100 mM EDTA, 10 mM Tris, 1% v/v sarkosyl.
7. Alu PCR mix (for 10 reactions): 15 μL of dNTPs (5 mM), 15 μL of 10X PCR buffer, 5 μL of bovine serum albumin (10 mg/mL), 2 μL (1:20 with Tris-EDTA [TE]) of 1:20 b-ME, 2 μL of AmpliTaq, 31 μL of TE, 15 μL (1 ng/μL) of primer 1, 15 μL (1 ng/μL) of primer 2.
8. Biotinylation mix: 2 μg of genomic DNA, 2 μL of 10X biotinylation buffer, 3 μL of dGTP, dATP, and dCTP mix (0.4 mM each dNTP), 1 μL of dTTP mix (2 μL of dTTP [0.4 mM], 0.5 μL of biotin-16-dUTP diluted to 0.1 mM), 0.5 μL ^{32}P (as a tracer), 2 μL enzyme mix.
9. 2X Hybridization solution: 1.5 M NaCl, 40 mM sodium phosphate (pH 7.2), 10 mM EDTA (pH 8.0), 10X Denhardt's solution, 0.2% v/v sodium dodecyl sulfate (SDS).

3. Methods

3.1. Culture of Yeast Cells and YAC Isolation

1. Inoculate a 10-mL culture of SCM broth with a loop of yeast cells and incubate overnight at 30°C with gentle shaking.
2. Inoculate 100 mL of SCM broth with 0.2 mL of overnight culture and incubate for 24 h at 30°C with vigorous shaking.
3. Pellet the cells at 3000*g* for 5 min at room temperature, discard the supernatant, and resuspend in 5 mL of 0.9 *M* sorbitol, 20 m*M* EDTA, 4 m*M* 2-β-mercaptoethanol, pH 7.5.
4. Add 20 μL of 10 mg/mL of zymolyase and incubate for 1 h at 37°C with gentle shaking. Spin at 2000*g* for 10 min and discard the supernatant.
5. Resuspend in 5 mL of GuHCl solution and heat at 65°C for 10 min to break open the cells and release the nucleic acids.
6. Allow to cool to room temperature, and then add an equal volume of 100% EtOH and pellet the DNA at 2000*g* for 10 min.
7. Discard the supernatant and resuspend in 2 mL of TE at pH 7.4. Once resuspended, add 200 μg of RNase and incubate at 37°C for 30 min to allow digestion of RNA.
8. Add 400 μg of proteinase K and incubate at 65°C for 1 h to allow digestion of any protein.
9. Perform a phenol chloroform extraction twice, then a chloroform extraction followed by ethanol precipitation to purify the DNA, and resuspend in 200–300 μL of TE at pH 7.4 ready for PCR.

3.2. Isolation of Specific YACs

1. Streak out yeast into patches approx 1 cm^2 on YPD agar and incubate overnight at 30°C.
2. Scrape off the yeast cells with a sterile scraper into a 1.5-mL microcentrifuge tube and resuspend in 125 μL of isolation solution 1 by gently vortexing.
3. Incubate at 37°C for 1 h with gentle shaking.
4. Add 125 μL of 1% LMP agarose (made up in solution 2) at 45°C and mix gently by pipetting. Then add to pulse-field gel electrophoresis plug molds on ice to set.
5. After 10 min on ice, transfer the plugs to 3 mL of isolation solution 3 and shake gently at 37°C for 1 h. The plugs are now ready for use in PFGE. PFGE should be performed in accordance with the user's manual until an adequate chromosomal spread is achieved.
6. Cut the recombinant chromosome from the gel using a sterile scalpel blade, trim away as much excess agarose as possible, and transfer the excised band to a sterile 1.5-mL microcentrifuge tube.
7. Wash the excised band in 500 μL of Agarase buffer (New England Biolabs, UK) for 30 min. Remove the buffer and repeat the wash three times. Then after removing the final wash, melt the agarose at 65°C for 10 min.
8. Add 1 U of Agarase for every 200 μL of agarose and incubate at 42°C for 2 h to digest the agarose.
9. Add 5 μL of dextran T40 as a carrier, ammonium acetate to 2.5 *M* final concentration, and 2.5 vol of 100% EtOH. Then store at –70°C for a minimum of 30 min. Spin in a microcentrifuge at 12,000*g* for 20 min, wash three times in 70% EtOH, dry, and resuspend in 15 μL of TE.

3.3. Alu *PCR*

1. Streak out the clones onto YPD agar and incubate for 24 h at 30°C to obtain single colonies.
2. Pick the single colonies with a sterile toothpick and resuspend in 100 μL of TE in a sterile microcentrifuge tube.
3. Prepare the *Alu* PCR mix (*see* **Subheading 2.**, **item 7**), aliquot 10 μL into each PCR tube, add 5 μL of resuspended colony, and cover with mineral oil. The following PCR conditions should be used: 94°C for 5 min, 30 cycles of 65°C for 1 min, 72°C for 5 min, 93°C for 1 min, and then a final cycle of 65°C for 1 min and 72°C for 10 min to ensure completion of all double-stranded molecules.
4. After PCR compare the samples on a 2% Nusieve agarose gel.

3.4. cDNA Selection

1. Prepare the biotinylation mix (*see* **Subheading 2.**, **item 8**) and incubate at 15°C for 90 min to allow labeling.
2. Stop the reaction by incubating at 65°C for 10 min, allow to cool to room temperature, and make up to 100 μL with TE.

3. Remove the unincorporated dNTPs by adding the mix to a Sephadex G-50 spin column and centrifuging for 5 min at 2000*g*. (Commercially available biotinylating kits are available and can be used if preferred.)
4. Precipitate the genomic DNA by adding 20 μL of 3 *M* sodium acetate (pH 5.4) and 300 μL of 100% EtOH, and leave at –70°C for a minimum of 20 min.
5. Pellet the DNA, remove the supernatant, and wash the pellet with 70% EtOH. Then dry and resuspend to an approximate concentration of 20 ng/μL, ready for hybridization selection.
6. For hybridization, denature 5 μL of biotinylated genomic DNA by overlaying with mineral oil and heating at 95°C for 5 min.
7. Add 2 μg of cDNA and 5 μL of 2X hybridization solution, and incubate at 65°C for 54 h with gentle shaking for hybridization to occur.
8. Wash 2 mg of Dynabeads three times in binding buffer (10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, pH 8.0, 1 *M* NaCl), and then resuspend at 10 mg/mL in binding buffer.
9. Add the hybridized DNA and incubate at room temperature for 15 min with occasional shaking.
10. Remove the beads with a magnet and wash with 1 mL of wash solution 1 (1X standard saline citrate [SSC], 0.1% SDS) for 15 min at room temperature, then three times at 65°C with wash solution 2 (0.1X SSC, 0.1% SDS).
11. Elute the cDNA from the genomic DNA by incubating the beads in 50 μL of 100 m*M* NaOH for 10 min at room temperature. Then neutralize by adding 50 μL of 1 *M* Tris-HCl (pH 7.5) and desalt by running through a Sephadex G-50 column as before. The cDNA is now ready for cloning and/or PCR and sequencing.

3.5. Purification of DNA from Agarose

1. After excising the band, trim as much excess agarose as possible and transfer the band to a 1.5-mL microcentrifuge tube.
2. Add 5 vol of 20 m*M* Tris-HCl and 1 m*M* EDTA (pH 8.0), and incubate at 65°C for 5 min to melt the slice of gel.
3. Cool to room temperature and add an equal volume of phenol. Vortex for 20 s and recover the aqueous phase by centrifuging at 12,000*g* for 7 min (the white substance at the interface is powdered agarose). Remove the aqueous phase to a new tube.
4. Add 250 μL of phenol and 250 μL of chloroform, vortex for 20 s, and spin as before. Remove the aqueous phase to a new tube and repeat the extraction with 500 μL of chloroform.
5. Add 0.2 vol of 10 *M* ammonium acetate and 2 vol of 100% EtOH and leave at –70°C for a minimum of 30 min.
6. Pellet the DNA at 12,000*g* for 5 min, remove the supernatant, and wash with 70% EtOH. Then resuspend in 30 μL of 10 m*M* Tris-HCl, pH 8.5.

3.6. Transformation of Competent Cells

1. Take an aliquot of competent cells from –70°C and place on ice to thaw.
2. Once thawed, add the whole ligation reaction and mix gently by stirring with a pipet tip. Do not pipet up and down.
3. Leave on ice for 15 min, heat shock at 42°C for 2 min, and then transfer back to ice for 5 min.
4. Add 1 mL of prewarmed L-broth and incubate at 37°C for 45 min. Then plate out one-tenth on L-agar and leave at 37°C overnight.

4. Notes

1. As in all work involving human DNA, it is essential to ensure sterility of solutions and reagents. It is particularly important to wear gloves at all times given the high abundance of *Alu* repeat sequences in human genomic DNA.
2. **Subheading 3.1.** detailing YAC DNA isolation typically results in DNA fragments ranging from 50 to 200 kb. However, some protocols require intact chromosomes, and **Subheading 3.2.** is a modification of the rapid method detailed by Coulson *(23)*.
3. In **Subheading 3.2.**, the agarose plugs containing the isolated yeast DNA and YACs only need to undergo PFGE if it is essential to isolate the specific recombinant chromosome. If this is not essential, the agarose plugs can be stored in isolation solution 3 or in 0.5 *M* EDTA at 4°C until required.
4. When performing *Alu* PCR, it is important to use a negative control of TE to ensure that there is no contamination of stock solutions owing to the high abundance of *Alu* sequences in the human genome. It is also advisable to use an empty host yeast cell as a further control to check for any nonspecific binding of primers.
5. The large volumes of primer used in the *Alu* PCR protocol are required because of the high number of *Alu* sites in human genomic DNA. Using less primer would lead to the production of suboptimal levels of product, and a subsequent failure to detect them by agarose gel electrophoresis.
6. Electroporation should be performed according to the manufacturer's instructions. As an approximate guide, the sample should be pulsed at 0.25 kV and 960 μF, with an average time constant of 17–25 ms. However, this will vary depending on the host strain used. An ideal host for electroporating DNA into would be COS-7 cells.
7. In direct screening techniques, it is important that the genomic DNA be free of vector sequences because these will generate prohibitively high background levels and render any results meaningless.

Acknowledgment

We thank the Coeliac Society for providing a fellowship.

References

1. Stinchcomb, D. T., Strahl, K., and Davis, R. W. (1979) Isolation and characterisation of a yeast chromosomal replicator. *Nature* **282,** 39–43.
2. Szostak, J. W. and Blackburn, E. H. (1982) Cloning yeast telomeres on yeast plasmid vectors. *Cell* **29,** 245–255.
3. Burke, D. T., Carle, G. F., and Olson, M. V. (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* **236,** 806–812.
4. Bently, D. R., Todd, C., Collins, J., Holland, J., Dunham, I., Hassock, S., Bankier, A., and Giannelli, F. (1992) The development and application of automated gridding for efficient screening of yeast and bacterial ordered libraries. *Genomics* **12,** 534–541.
5. Ross, M. T., Hoheisel, J. D., Monaco, A. P., Larin, Z., Zehetner, G., and Lehrach, H. (1992) High density gridded YAC filters: their potential as genome mapping tools, in *Techniques for the Analysis of Complex Genomes* (Anand, R., ed.), Academic, London, pp. 137–154.
6. Silverman, G. A., Jockel, J. I., Dower, P. H., Mohr, R. M., Tallion-Miller, P., and Korsmeyer, S. J. (1991) Yeast artificial chromosome cloning of a 2-megabase size contig with chromosomal band 18q21 establishes physical linkage between BCL2 and plasminogen inhibitor type-2. *Genomics* **9,** 219–228.
7. Green, E. D. and Green, P. (1991) Sequence tagged sites (STS) content mapping of human chromosomes: theoretical considerations and early experiences. *PCR Methods Appl.* **1,** 77–90.
8. Wada, M., Little, R. D., Abidi, F., Porta, G., Labella, T., Cooper, T., Della-Valle, G., D'Urso, M., and Schlessinger, D. (1990) Human Xq24-Xq28: approaches to mapping with yeast artificial chromosomes. *Am. J. Hum. Genet.* **46,** 95–106.
9. Nelson, D. L., Ledbetter, S. A., Corbo, L., Victoria, M. F., Ramirez-Solis, R., Webster, T. D., Ledbetter, D. H., and Caskey, C. T. (1989) *Alu* polymerase chain reaction: a method for rapid isolation of human specific sequences from complex DNA sources. *Proc. Natl. Acad. Sci. USA* **86,** 6686–6690.
10. Coffey, A. J., Roberts, R. G., Green, E. D., Cole, C. G., Butler, R., Anand, R., Giannelli, F., and Bently, D. R. (1992) Construction of a 2.6 Mb contig in yeast art)ficial chromosomes spanning the human dystrophin gene using an STS based approach. *Genomics* **12,** 474–484.
11. Riley, J., Butler, R., Ogilvie, D., Finniear, R., Jenner, D., Powell, S., Anand, R., Smith, J. C., and Markham, A. F. (1990) A novel, rapid method for the isolation of terminal sequences from yeast artificial chromosome (YAC) clones. *Nucleic Acids Res.* **18,** 2887–2890.
12. Sternberg, N. (1990) Bacteriophage P1 cloning system for the isolation, amplification and recovery of DNA fragments as large as 100 kb. *Proc. Natl. Acad. Sci. USA* **87,** 103–107.
13. Ioannou, P. A., Amemiya, C. T., Games, J., Kroisel, P. M., Shizuya, H., Chen, C., Batzer, M. A., and deJong, P. J. (1994) A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. *Nat. Genet.* **6,** 84–89.

14. Monaco, A. P., Neve, R. L., Colletti-Feener, C., Bertelson, C. J., Kurnit, D. M., and Kunkel, L. M. (1986) Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. *Nature* **323,** 646–650.
15. Bird, A. P. and Southern, E. M. (1978) Use of restriction enzymes to study eukaryotic DNA methylation: the methylation pattern in ribosomal DNA from *Xenopus laevis. J. Mol. Biol.* **118,** 27–47.
16. Kendall, E., Sargent, C. A., and Campbell, R. D. (1990) Human major histocompatibility complex contains a new cluster of genes between the HLA-D and complement loci. *Nucleic Acids Res.* **18,** 7251–7257.
17. Elvin, P., Slynn, G., Black, D., Graham, A., Butler, R., Riley, J., Anand, R., and Markham, A. F. (1990) Isolation of cDNA clones using yeast artificial chromosome probes. *Nucleic Acids Res.* **18,** 3913–3917.
17a. Parimos, S., Patanjali, S., Shukla, H., Chaplain, D., and Weissman, S. (1991) cDNA Selection: an efficient PcR approach for the selection of cDNAs encoded in large chromosomal DNA fragments. *Proc. Natl. Acad. Sci. USA* **88,** 9623–9627.
18. Lovett, M., Kere, J., and Hinton, L. M. (1991) Direct selection: a method for the isolation of cDNAs encoded by large genomic regions. *Proc. Natl. Acad. Sci. USA* **88,** 9628–9632.
19. Auch, D. and Reth, M. (1990) Exon trap cloning: using PCR to rapidly detect and clone exons from genomic DNA fragments. *Nucleic Acids Res.* **18,** 6743, 6744.
20. Buckler, A., Chang, D. D., Graw, S. L., Brook, D., Haber, D. A., Sharp, P. A., and Housman, D. E. (1991) Exon amplification: a strategy to isolate mammalian genes based on RNA splicing. *Proc. Natl. Acad. Sci. USA* **88,** 4005–4009.
21. Church, D., Stotler, C., Rutter, J., Murrell, J., Trofatter, J., and Buckler, A. (1994) Isolation of genes from complex sources of mammalian genomic DNA using exon amplification. *Nat. Genet.* **6,** 98–105.
22. Harnaguchi, M., Sakamoto, H., Tsuruta, H., Sasaki, H., Muto, T., Sugimura, T., and Terada, M. (1992) Establishment of a highly sensitive and specific exon trapping system. *Proc. Natl. Acad. Sci. USA* **89,** 9779–9783.
23. Coulson, A. R., Waterston, R., Sulston, J., and Kohara, Y. (1988) Genome linking with yeast artificial chromosomes. *Nature* **335,** 184–186.

4

Linkage and the Transmission Disequilibrium Test in Complex Traits

Celiac Disease as a Case Study

Stephen Bevan and Richard S. Houlston

1. Introduction

Many disorders such as celiac disease do not conform to a simple Mendelian model of inheritance and display a complex pattern of inheritance indicative of the interaction of a number of distinct susceptibility genes. Susceptibility to celiac disease is genetically determined by possession of specific HLA DQ alleles, acting in concert with one or more non-HLA-linked genes. Haplotype-sharing probabilities across the HLA region in affected sibling pairs suggest that genes within the major histocompatibility complex (MHC) contribute no more than 30% of the sibling familial risk of celiac disease, making the non-HLA-linked gene (or genes) the stronger determinant of celiac disease susceptibility ***(1)***. Locating these non-HLA-linked genes can be undertaken by either linkage or association. The relative merits of these two approaches depend critically on the frequency and genotypic risks associated with susceptibility genes.

2. Linkage

The essential prerequisite for utilizing the classical linkage approach to detect disease genes is that the model of inheritance of the disease can be specified with some degree of certainty. To circumvent the requirement for a specified model of inheritance, several nonparametric methods have been developed. These are based on determining which regions of the genome are identical by descent (IBD) in affected relatives.

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

2.1. Affected Sibling-Pairs

The most common paradigm of this approach utilizes affected sibling-pairs (ASPs) and is based on comparing the IBD allele sharing at a given marker with the expectation under the null hypothesis that no deleterious gene is present. A marker close to a susceptibility gene would be expected to display an excess in IBD sharing with ASPs sharing both alleles IBD more frequently than sharing neither allele. The allele-sharing probabilities of ASPs depend on the contribution any gene makes to the genetic variation of the trait. This is generally measured in terms of the risk to relatives of affected probands compared with the population risk (i.e., the relative risk denoted by λ). $\lambda_s = K_s/K$ and $\lambda_{po} = K_o/K$, where K_s and K_o are the sibling and offspring recurrence risks, respectively, and K is the population risk.

The 0, 1, and 2 allele-sharing probabilities of ASPs are given by ***(2)***:

$$Z_0 = \alpha_0 \cdot 1/\lambda_s$$
$$Z_1 = \alpha_1 \cdot \lambda_o/\lambda_s$$
$$Z_2 = \alpha_2 \cdot \lambda_{mz}/\lambda_S$$

where $Z_i = P$(sibs share i marker alleles IBD|ASP) and $\alpha_i = P$(sibs share marker alleles IBD).

For $i = 0, 1, 2$, α_i equals 1/4, 1/2, and 1/4, respectively. When a marker is unlinked, $Z_i = \alpha_i$. These formulae hold true irrespective of the mode of inheritance at the disease locus, the number of alleles and their frequencies, penetrance, and population prevalence ***(2)***. The only requirement is that recombination (θ) be negligible. Incorporating θ, the ASP probabilities are given by ***(2)***:

$$Z_0 = \alpha_0 - \alpha_0(2\psi - 1) \cdot 1/\lambda_s \cdot (\lambda_s - 1)$$
$$Z_1 = \alpha_1 + \alpha_1(2\psi - 1) \cdot 1/\lambda_s \cdot (\lambda_s - \lambda_o)$$
$$Z_2 = \alpha_2 + \alpha_2(2\psi - 1) \cdot 1/\lambda_s \cdot (\lambda_m - \lambda_s)$$

where the parameter $\psi = \theta^2 + (1 - \theta)^2$.

For a given λ_s, an increase in the value of θ leads to a reduced deviation of marker sharing from its null expectation (**Fig. 1**). This has the consequence that the number of ASPs required to demonstrate linkage is increased as θ increases.

Given a set of observed sharing results, the maximum likelihood sharing (MLS) is defined as the 0-1-2 ratio for which the likelihood of producing the observed data is highest. This can be expressed as follows:

$$\text{MLS}(Z_0, Z_1, Z_2) = \max \log_{10}[L(Z)/L(0.25, 0.5, 0.25)]$$

where the null hypothesis H_0 $(Z_0, Z_1, Z_2) = (0.25, 0.5, 0.25)$, $L(Z)$ = likelihood of data given Z (dependent on family structure and genotypes) and Z is the value of Z maximizing $L(Z)$.

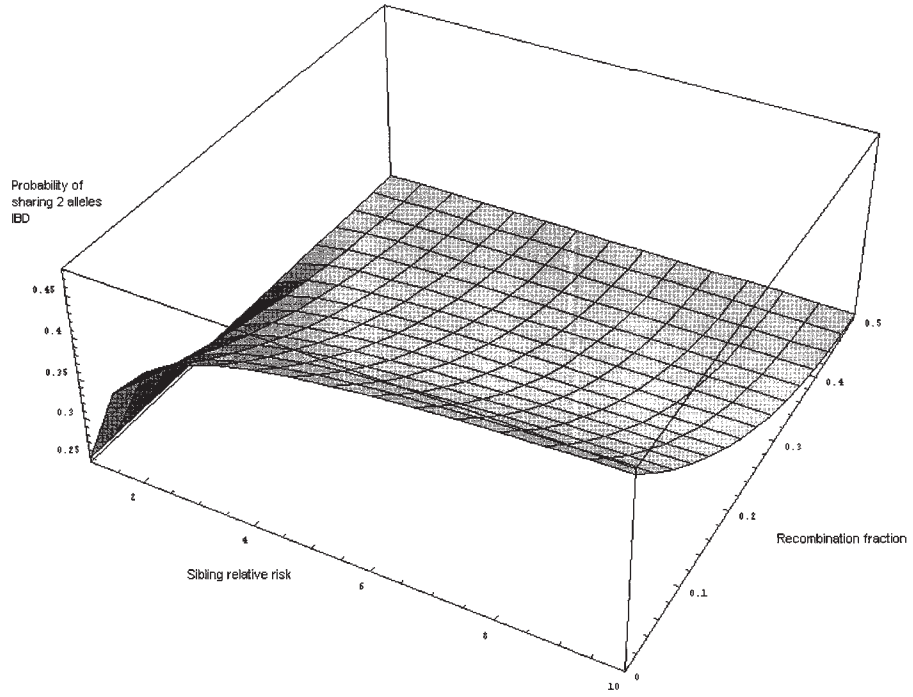


Fig. 1. The expected IBD 2-sharing in ASPs as a function of λ_s and θ.

A single affected sib pair sharing 2 alleles IBD will generate a log of the odds (LOD) of 0.3. This approach to mapping disease predisposition is therefore more conservative than a parametric method, but is highly robust.

When searching for the MLE, not all 2-1-0 ratios correspond to realistic genetic models. These restrictions are referred to as the "possible triangle" ***(3)*** defined as follows:

$$Z_i > 0$$

$$Z_0 + Z_1 + Z_2 = 1$$

$$Z_2 + Z_0 > Z_1$$

$$Z_1 > Z_2$$

The MLE is therefore defined as the corresponding ratio that maximizes the LOD score while simultaneously satisfying the foregoing restrictions. The further assumption of no "dominance variance" is equivalent to the constraint $Z_1 = 1/2$. In this case, the allele-sharing probabilities are described by the single parameter Z_2.

Sibships with more than two affected siblings tend to have a disproportionate effect on the results obtained. A weighting parameter can partially compensate for this. Families with more than two affected children provide more information than $n - 1$ independent pairs from families with single affected siblings but less information than $1/2 \cdot n \cdot (n - 1)$ pairs. The Hodge weighting parameter ***(3)*** is as follows:

$$4[(2n - 3(1/2)n - 1)/(n(n - 1))]/3$$

The statistical method of demonstrating linkage described above is only one of a number of approaches using ASPs; however, it is widely used and is implemented in the MAPMAKER-SIBS program ***(4)***, detailed under **Subheading 5.1.**

2.2. Nonparametric Linkage in Extended Families

The principle of detecting excess sharing of alleles between affected family members can be extended to families containing affected relative pairs other than ASPs, such as uncle-nephew or cousin pairs. A number of methods have been introduced to make use of these other types of multiple-case families. Here we describe the nonparametric linkage (NPL) statistic for assessing linkage, implemented in the GENEHUNTER program ***(5)***. The NPL score is based on the score defined by:

$$S_{\text{all}}(\boldsymbol{V}) = 2^{-a} \Sigma [\pi b_i(h)!]$$

where $\boldsymbol{V}$ = given inheritance vector; and h = collection of alleles, one from each affected $b_i(h)$ = number of times ith founder allele appears in h for $i = 1,\ldots,$ $2f$(f = number of founders).

For full details of the methodology, *see* **ref. *6***. However, the following example should serve to illustrate how the NPL statistic is derived. Consider an ASP whose parents have genotypes 16 and 24. If the genotypes of the affected sibs are 12 and 46, i.e., share 0 alleles, the possible vectors of inheritance are as given in **Table 1**. That is, the number of ways to choose h multiplied by the number of permutations that preserve the vector h equates to the average number of ways to permute alleles to preserve the inheritance vector. However, if the genotypes of the affected sibs are 12 and 26, i.e., share 1 allele, the possible vectors of inheritance are as given in **Table 2**. Whereas if the genotypes of the affected sibs are 12 and 12, i.e., share 2 alleles, the possible vectors of inheritance are as given in **Table 3**.

The mean value of S is $\mu = E[S(V)] = P(\text{share } 0) + P(\text{share } 1) + P(\text{share } 2)$. In this example, $\mu = 1/4 + 5/8 + 3/8 = 1.25$. The standard deviation σ of S is given by $\sqrt{[E((S(V))^2 - (E(S(V)))^2]}$. In this example, $\sigma = 0.177$. The normalized NPL scores for an ASP given by $Z(V) = [S(V) - \mu]/\sigma$ are therefore:

Table 1
ASP Sharing 0 Alleles IBD

	Founder alleles			
$b_i(h)$	**1**	**2**	**6**	**4**
16	1	0	1	0
14	1	0	0	1
26	0	1	1	0
24	0	1	0	1

$S_{all}(V) = 2^{-a} \Sigma [\pi b_i(h)!] = \frac{1}{4}(1 + 1 + 1 + 1) = 1.$
Parental genotypes are 16 and 24.
Sibling genotypes are 12 and 64.

Table 2
ASP Sharing 1 Allele IBD

	Founder alleles			
$b_i(h)$	**1**	**2**	**6**	**4**
16	1	0	1	0
12	1	1	0	0
26	0	1	1	0
22	0	2	0	1

$S_{all}(V) = 2^{-a} \Sigma [\pi b_i(h)!] = \frac{1}{4}(1 + 1 + 1 + 2) = \frac{5}{4}.$
Parental genotypes are 16 and 24.
Sibling genotypes are 12 and 26.

Table 3
ASP Sharing 2 Alleles IBD

	Founder alleles			
$b_i(h)$	**1**	**2**	**6**	**4**
16	2	0	0	0
14	1	1	0	0
26	1	1	0	0
24	0	2	0	0

$S_{all}(V) = 2^{-a} \Sigma [\pi b_i(h)!] = \frac{1}{4}(2 + 1 + 1 + 2) = \frac{3}{2}.$
Parental genotypes are 16 and 24.
Sibling genotypes are 12 and 12.

Sharing 2 alleles: 1.414, i.e., (1.5 – 1.25)/(0.177)

Sharing 1 allele: 0.0, i.e., (1.25 – 1.25)/(0.177)

Sharing 0 alleles: –1.414, i.e., (1.0–1.25)/(0.177)

To combine scores across a number of families ($i = 1,\ldots, m$), the overall score is normalized to have a mean of 0, and variance of 1.0 according to:

$$Z = \Sigma\, \gamma\, Z_i; \text{ where } \gamma = 1/\sqrt{m}$$

2.3. Statistical Thresholds

The conventional critical threshold in classical linkage studies for genomic searches is a maximum LOD score of 3.0. This corresponds approximately to a *p* value of about 0.0001. The justification for imposing such a 1ow significance level is that the prior probability of linkage to any given marker is low. In a typical genome search based on several hundred markers, almost all will be unlinked. An LOD of 3.0 actually corresponds to a probability of about 9% that any marker tested on a given set of families will be positive by chance. The appropriate threshold for a 5% genomewide false-positive rate is an LOD score of 3.3 *(7)*. Many linkage studies in complex diseases make use of multiple comparisons and demand tougher levels of significance. **Table 4** details the thresholds for suggestive and significant linkage according to pedigree structure in complex traits *(7)*, and **Table 5** shows the NPL scores and the corresponding equivalent LOD scores for significance levels of 0.1–0.0001.

2.4. Multipoint Analysis

A serious limitation of using a single marker is that not all pairs in a pedigree will be informative; that is, the number of alleles shared by pedigree members cannot be determined unambiguously. Multilocus methods utilize the information from adjacent markers to estimate the sharing in these ambiguous cases. Using several highly polymorphic markers, which are closely spaced, theoretically makes almost all sib-pairs in a family fully informative.

3. Experimental Design in Linkage Studies

Several factors influence the power of a study to demonstrate linkage. These include the informativity of markers, the distance of the marker from the disease locus, clinical and laboratory errors, and the magnitude of the genetic trait.

3.1. Informativity of Markers

If intervening relatives are genotyped, the proportion of achievable power is proportional to the polymorphism information content (PIC) *(6)*. (For a detailed definition of PIC refer to Appendix C.) The power of a given set of families

Table 4
Thresholds for Mapping Loci Underlying Complex Traits

	Suggestive linkage p value (LOD)	Significant linkage p value (LOD)
LOD score analysis	1.7×10^{-3} (1.9)	4.9×10^{-5} (3.3)
Allele-sharing methods		
Sibs and half-sibs	7.4×10^{-4} (2.2)	2.2×10^{-5} (3.6)
Grandparent-grandchild	1.7×10^{-3} (1.9)	4.9×10^{-5} (3.3)
Uncle-nephew	5.6×10^{-4} (2.3)	1.8×10^{-5} (3.7)
First cousin	5.2×10^{-4} (2.3)	1.6×10^{-5} (3.7)
First cousin, one removed	4.8×10^{-4} (2.4)	1.5×10^{-5} (3.8)
Second cousin	4.2×10^{-4} (2.4)	1.3×10^{-5} (3.8)

Table 5
LOD and Corresponding NPL Statistic and Significance Values

LOD[a]	NPL[b]	Asymptotic p
0.59	1.65	0.05
1.17	2.33	0.01
2.07	3.09	0.001
3.00	3.72	0.0001
3.95	4.27	0.00001

[a]Assumes one-half of p value.
[b]Uses one-sided normal p value.

cannot, however, be increased indefinitely by refining marker density. The only way of increasing power is by increasing the number of families analyzed.

3.2. Clinical and Laboratory Errors

Errors in the diagnosing of family members, genotyping, or data transcription can all contribute to the erosion of the power of a set of families to demonstrate linkage, and the power of any set of families to demonstrate linkage is greatly reduced if markers are too widely spaced.

3.3. Magnitude of the Genetic Trait

If a disease is caused by a single gene, the power required to detect linkage is simply a function of λ_s. However, if disease susceptibility is the result of more than one gene, the power to detect linkage depends on the contribution to the overall familial risk made by each locus and how the different loci interact,

Table 6
Number of ASPs Required to Give an Expected LOD Score of 3.0 Under a Range of Genetic Models

	One gene				Two genes[a]			
	$\lambda_s = 3$	$\lambda_s = 3$	$\lambda_s = 2$	$\lambda_s = 2$	$\lambda_s = 3$	$\lambda_s = 3$	$\lambda_s = 2$	$\lambda_s = 2$
Dominant gene								
p	0.001	0.05	0.001	0.05	0.001	0.05	0.001	0.1
γ	50	21	35	10	35	10	24	6
ASPs	57	56	106	104	244	239	439	239
Recessive gene								
p	0.01	0.2	0.01	0.2	0.01	0.2	0.01	0.2
γ	289	26	203	15	203	15	143	9
ASPs	13	26	23	45	51	100	88	175

[a]Based on an additive model, but assuming that both genes have identical frequency and risk ratios (γ).

e.g., whether they act additively or multiplicatively. **Table 6** shows that the numbers of ASPs required to demonstrate linkage can be significantly different under a range of modes of inheritance. The power of an affected sibling-pair test to detect linkage can be easily determined using simple formulae ***(2)***. For more complex family structures, the power to detect linkage can be estimated by simulation under a range of possible genetic models, using programs such as SLINK ***(8)*** or SIMLINK ***(9)***. Calculations of power derived from such computations are often expressed in terms of the expected LOD score (or ELOD), which will be generated given a set of families, under a range of alternative models and types of marker.

3.4. Can the Power of a Study Be Increased by Collecting Specific Types of Family?

Subcategorization of a disease can be used to obtain larger familial relative risks and hence increase power. An example is in the field of cancer genetics, where for most common cancers familial risks show considerable age dependency. Therefore, collecting blood samples from siblings affected at a young age offers an opportunity to increase the probability of enriching for genetic cases.

For a disease characterized by a large λ and in which θ is small, distant relatives are better for linkage. For small values of λ, affected siblings will provide a more optimal sampling frame.

3.5. Who Should Be Genotyped?

If a marker is sufficiently polymorphic, the chance that a pair of alleles in a sibship is identical by state will be close to that of them being IBD. Theoreti-

Table 7
Contingency Table of Transmitted and Nontransmitted Parental Alleles[a]

Transmitted allele	Nontransmitted allele	
	M	N
M	*a*	*b*
N	*c*	*d*

[a]TDT = $(b - c)^2/(b + c)$.

cally, in those circumstances, it may be more profitable in terms of genotyping to type only children in a larger number of families rather than to type a smaller number of complete nuclear families. However, most markers have several common alleles requiring the weighting of expected sharing probabilities derived from genotypes. This inevitability leads to a significant reduction of power in any given data set. It is therefore always advantageous to genotype intervening relatives since the inheritance of alleles can be unambiguously determined, rather than to rely on the likely patterns of inheritance given the frequency of marker alleles. If parents are not genotyped, any statistic derived from the analysis of ASPs will depend on the frequency of marker alleles. Furthermore, it is difficult to discover genotyping errors in nuclear families if the parents are not genotyped.

4. The Transmission Disequilibrium Test

An alternative strategy for identifying the location of a gene conferring susceptibility to celiac disease is by allelic association, based on demonstrating overrepresentation of a specific allele in affected individuals. The simplest means of undertaking this is to compare the frequency in affected individuals (cases) with the frequency in the general population (controls). Marker alleles that are positively associated with the disease are analogous to risk factors in epidemiology. A major problem inherent in this approach is that spurious associations can arise as a result of population stratification. One method of overcoming the problem of hidden population stratification is to use family-based controls. The most common approach, introduced by Spielman et al. ***(10)***, is the transmission disequilibrium test (TDT), which is based on the McNemar test for matched-pair data. It considers only parents whose transmitted and nontransmitted alleles are different (i.e., heterozygous parents) and assesses the evidence for preferential transmission of one allele over the other (**Table 7**). One additional attractive feature of the TDT is that it is also a test of linkage and not merely of linkage disequilibrium, since only linkage disequi-

librium can distort the distribution of marker genotypes among parents of affecteds.

4.1. Dealing with Multialleles

One drawback of the TDT as originally formulated is the assumption of a biallelic marker locus. Generalizing the TDT to handle polymorphic markers is not straightforward unless there is a candidate-associated allele, in which case all the other alleles can be combined so that the marker system is effectively biallelic. This technique can also be applied when there is no candidate association.

A natural extension to the two-allele TDT statistic is

$$[(k-1)/k]\ \Sigma_i(n_{i.}-n_{.i})2/(n_{i.}+n_{.i}-2n_{ii})$$

where summation is over all k alleles ***(11)***. The statistic has nearly a χ^2 distribution with $k-1$ degrees of freedom (df) when the null hypothesis of no linkage is true. Another approach to dealing with multiple alleles includes logistic-regression ***(11)***. Unfortunately, the number of dfs can still be large, and a multiple testing problem arises when any attempt is made to assess the significance of the maximal TDT score. Terwilliger ***(12)*** proposed a test of association having 1 df, that is appropriate if only one (unknown marker allele) might be positively associated with the disease. Alternatively, to overcome the multiple-testing problem, Morris et al. ***(13)*** developed a randomization procedure.

4.2. Experimental Design in TDTs

Probably one of the major concerns in carrying out TDTs is the presence of incomplete genotype data. Sham and Curtis ***(14)*** pointed out that when genotype data are unavailable for one parent, bias in the TDT may arise.

5. Computing Requirements for Running Linkage and TDT Programs

In recent years there has been an almost universal adoption of the UNIX operating system as a platform for analytical software in genetics. Appendix E provides a list of the essential UNIX commands necessary for file management and the running of most of the linkage programs. The following sections detail two of the most widely used programs for undertaking nonparametric linkage analyses: MAPMAKER-SIBS and GENEHUNTER.

5.1. MAPMAKER-SIBS Program

MAPMAKER-SIBS provides a method of performing exclusion mapping of qualitative traits, maximum likelihood mapping of qualitative traits, and

analysis of quantitative traits. Only nuclear families are suitable for analyses using this program (i.e., parents and children).

Reference: Kruglyak, L., Lander, E. S. (1995) *Am. J. Hum. Genet.* **57,** 439–454.
Source: http://www-genome.wi.mit.edu/ftp/distribution/software/mapmaker

To extract required files from sibs-2.1.tar.Z, use the following commands:

uncompress sibs-2.1.tar.Z
tar xvpf sib-2.1.tar.Z

The following files will be created:

sibs.sun	MAPMAKER executable for Sun Sparc stations
sibs.alpha	MAPMAKER executable for DEC Alpha machines
sibs.help	on-line help
sibs.ps	Postscript version of help
makefile	Unix Makefile for compilation on other systems
linkloci.dat	Sample marker datafile
linkped.pre	Sample pedigree file

src and **anslib** contain the source codes.

If MAPMAKER-SIBS is to be run on a SUN Sparc station or a Dec Alpha machine the executables '**gh.sun**' or '**gh.alpha**' can be used without compilation.

Input format

The data set is divided into a pair of files: the parameter file and pedigree file.

The pedigree file required should be in the standard LINKAGE format: (Family ID), (Individual ID), (Father's ID), (Mother's ID), (Sex; 1 = M, 2 = F), (Affection status; 1 = Unaffected, 2 = Affected, 0 = Unknown), (Liability class if used), (Marker genotypes; $m_1 \ldots m_i$). **Figures 2** and **3** show example pedigree and parameter files.

A typical set of commands for performing an analysis using the MAPMAKER-SIBS program is as follows:

sibs.sun *to run program*
prepare pedigrees fam.dat
load markers mk5.dat
photo sample.out
postscript on
pairs used
3
estimate
quit

1	101	0	0	1	0	1 2	6 7	2 3	1 5
1	102	0	0	2	0	3 4	8 9	2 4	2 6
1	103	101	102	1	2	1 3	6 8	2 2	1 2
1	104	101	102	1	2	1 3	6 8	2 2	1 2
2	101	0	0	1	1	2 3	7 8	3 4	2 6
2	102	0	0	2	1	4 5	9 10	3 5	3 7
2	103	101	102	1	2	2 4	7 9	3 3	2 3
2	104	101	102	2	2	2 4	7 9	3 3	2 3
3	101	0	0	1	1	1 2	2 4	4 6	1 2
3	102	0	0	2	1	3 4	3 5	2 4	3 4
3	103	101	102	2	2	1 3	3 4	2 4	1 3
3	104	101	102	2	2	1 3	3 4	2 4	1 3
3	105	101	102	1	2	1 4	3 4	2 4	13

Fig. 2. Example of a pedigree file of three families.

```
5 0 0 5 << NO. OF LOCI, RISK LOCUS, SEXLINKED (IF 1) PROGRAM
O 0.0 0.0 0 << MUT LOCUS, MUT RATE, HAPLOTYPE FREQUENCIES (IF 1)
1 2 3 4 5<< ORDER OF LOCI
1 2 << AFFECTATION, NO. OF ALLELES
0.8 0.2 << GENE FREQUENCIES
1 << NO. OF LIABILITY CLASSES
0.0001 0.0001 0.5 << PENETRANCES (RECESSIVE MODEL SHONN)
3 8 #marker 1 (locus 2)
0.125 0.125 0.125 0.125 0.125 0.125 0.125 0.125 << ALLELE
FREQUENCIES
3 5 #marker 2 (locus 3)
0.5 0.1 0.1 0.1 0.1 0.1 .
3 5 #marker 3 (locus 4)
0.5 0.1 0.1 0.1 0.1 0.1
3 5 #marker 4 (locus 5)
0.5 0.1 0.1 0.1 0.1 0.1.
5 7 8 << DISTANCES (cM) BETWEEN MARKERS
1 0.1 0.45 << REC VARIED, INCREMENT, FINISHING VALUE
```

Fig. 3. Example of a parameter file.

5.2. GENEHUNTER Program

The GENEHUNTER program allows nonparametric linkage based on the NPL score to be undertaken. In addition, it allows reconstruction of marker haplotypes in pedigrees by maximum likelihood, and exact computation of multipoint LOD score under a defined model of inheritance.

Reference: Kruglyak, L., Daly, M. J., Reeve-Daly, M. P., Lander, E. S. (1996) *Am. J. Hum. Genet.* **58,** 1347–1363.
Source: http://www-genome.wi.mit.edu/ftp/distribution/software/genehunter

To extract required files from gh.tar.Z, use the following commands:

uncompress gh.tar.Z
tar xvpf gh.tar

The following files will be created:

gh.sun	GENEHUNTER executable for Sun Sparc stations
gh.alpha	GENEHUNTER executable for DEC Alpha machines
gh.help	On-line help
gh.ps	Postscript version of help
Makefile	Unix Makefile for compilation on other systems
linkloci.dat	Sample marker datafile
linkped.pre	Sample pedigree file

src and anslib contain the source codes.

If GENEHUNTER can be run on a SUN Sparc station or a Dec Alpha machine, the executables '**gh.sun**' or '**gh.alpha**' can be used without compilation.

The GENEHUNTER program requires a pair of files: the parameter file and the pedigree file. The pedigree file required should be in the standard LINKAGE format. The parameter file is identical to that required by MAPMAKER-SIBS except that the first locus (i.e., the disease locus) should not be included in the line ordering of loci.

A typical set of commands for performing an analysis using the GENEHUNTER program are as follows:

gh.sun *to run program*
load markers mk.dat
photo sample.out
Postscript on
skip off
scan fam.dat
total stat net
Quit

5.3. Other Programs for Nonparametric Linkage Analysis

Several other programs are available, some of which use different statistical methods of detecting linkage. Appendix D provides a list of other linkage programs and how to obtain them.

6. Programs for Performing TDTs

Many programs are available for carrying out TDTs. All of these can deal with multiple alleles.

ETDT: Performs the TDT on markers with more than two alleles using a logistic regression analysis.
Reference: Sham, P. C. and Curtis, D. (1995) *Ann. Hum. Genet.* **59,** 323–336.
Source: http://www.gene.ucl.ac.uk/~dcurtis/software.html

GASSOC: computes general score tests for disease and genetic marker associations using cases and their parents. These methods include an extension of the TDT for multiple marker alleles, as well as additional general tests sensitive to associations that depend on dominant or recessive genetic mechanisms.
Reference: Rowland, C. and Schaid, D. (1996) *Am. J. Hum. Genet.* **(Suppl.) 59,** A234; Schaid (1996) *Genet. Epidemiol.* **13,** 423–449.
Source: http://www.mayo.edu/statgen

DISEQ: Based on a likelihood-based approach to test linkage disequilibrium for multiple loci based on a test with 1 df.
Reference: Terwilliger, J. D. (1995) *Am. J. Hum. Genet.* **56,** 777–787.
Source: ftp://linkage.cpmc.columbia.edu/software/diseq

TRANSMIT: Allows tests for association between genetic marker and disease by examining the transmission of markers from parents to affected offspring. The main features that differ from other similar programs are that it can deal with transmission of multilocus haplotypes, even if phase is unknown; and that parental genotypes may be unknown.
Reference: Clayton, D. (1998) Transmit (version 2.3); MRC Biostatistics Unit, Cambridge, UK.
Source: ftp://ftp.mrc-bsu.cam.ac.uk/methodology/genetics/

TDTMAX: randomization test based on the TDT max statistic and the sequential version of the randomization test are implemented in this program and the ancillary program PATTERN.
Reference: Morris, A. P., Curnow, R. N., Whittaker, J. C. (1997) *Ann. Hum. Genet.* **61,** 49–60.
Source: http://www.Rdg.ac.uk/AcaDepts/sn/wsn1/dept.html

On-line documentation is available for all of these programs. All programs can be supported by the UNIX operating system. After downloading programs from the Internet, the required files for most of these programs can be extracted using these commands:

uncompress program.tar.Z
tar -xvpf program.tar.Z

Input format. Most of the programs require family information to be in the standard LINKAGE format for pedigrees.

7. Comparing the Power of Linkage and TDT Methods to Detect Non-HLA-Linked Genes in Celiac Disease

To compare the sample sizes required to detect a predisposition gene using linkage and association methods requires consideration of the level of acceptable type I (false-positive) and Type II (false-negative) error rates (denoted by α and β, respectively). The probability (power) that a test will correctly detect a deleterious locus is given 1-β. Most linkage searches are based on approx 250–400 markers at a density of 10–20 cm throughout the genome. Kruglyak and Lander ***(4)*** have proposed that an LOD score of 3.4 be used to define as the appropriate level of significance (equating to α of 2.2×10^{-5}) for genomewide searches using ASPs. Association studies can be based on a candidate gene approach or can be genomewide. Risch and Merikangas ***(15)*** proposed that 5×10^{-8} be adopted as a critical value for α in genomewide association tests. This is based on a Bonferroni correction to account for testing of five biallelic markers in 100,000 genes ***(15,16)***. Contingent on the probable impact of the non-HLA-linked genes to the development of celiac disease, a comparison of the relative power of linkage using ASPs and association tests using the TDT approach was made using the formulas derived by Camp ***(17)***.

The sample size required to detect any disease gene depends on its frequency and associated risk ratio ψ. Both these parameters are unknown for the non-HLA-linked component of celiac disease. However, the sibling relative risk can be derived from estimated overall and HLA-linked sibling relative risks. Estimates of the prevalence of celiac disease based on the disease presenting symptomatically vary considerably in different areas of Europe and the United States. However, population studies based on antibody screening have all shown that the prevalence of celiac disease is of the order of 1 in 300 ***(1)***. Using this population prevalence rate and a 10% recurrence risk, the sibling relative risk is 30. The sibling relative risk of celiac disease associated with the HLA-linked genes (derived from the allele-sharing probabilities of markers across the MHC region, whereby $\gamma_s = 0.25 \cdot Z_0$) is 3.3 (95% confidence interval;

Table 8
Sample Sizes Necessary to Gain 80% Power in ASP Linkage Analysis and TDTs[a]

γ	*p*	λ_s	ASP	TDT
16.0	0.01	3.4	59	34
	0.1	6.9	31	14
	0.2	4.5	43	18
	0.5	1.9	161	49
8.0	0.01	1.5	417	119
	0.1	3.1	66	29
	0.2	2.8	76	30
	0.5	1.7	235	62
4.0	0.01	1.1	7912	523
	0.1	1.5	342	86
	0.2	1.6	264	72
	0.5	1.4	549	106
2.0	0.01	1.01	550,000	4154
	0.1	1.1	9995	533
	0.2	1.1	4630	360
	0.5	1.1	4630	348
1.5	0.1	1.002	86,000,000	16,369
	0.01	1.02	130,000	1952
	0.2	1.03	48,532	1197
	0.5	1.04	33,374	971

[a]Values are based on a multiplicatively acting non-HLA-linked gene.

2.9–3.5) ***(17)***. Hence, the non-HLA-linked gene or genes will be the stronger determinant of celiac disease compared to those linked to HLA. The actual relative risk will depend upon the mode of interaction between the HLA-linked and -unlinked genes. These genes could interact either additively (i.e., the penetrance of the disease is represented by the sum of the penetrances contributed by two or more loci) or multiplicatively (i.e., the penetrance of the disease is the product of the penetrances contributed by two or more loci). The familial risks seen in siblings and monozygotic twins are most parsimonious with a multiplicative model. Based on this model, the relative risk associated with the non-HLA-linked genes will be approx 9.0, accounting for approx 70% of the familial risk of celiac disease ***(17)***.

Table 8 gives the sample sizes necessary to gain 80% power in ASP linkage analysis and TDT approaches. Estimates are based on gene frequencies of 0.01, 0.1, 0.2, and 0.5, and risk ratios commensurate with the sibling relative risks that can be attributable to the HLA-unlinked loci in celiac disease. Although

the number of TDT trios is smaller in all cases than that necessary for linkage analysis using ASPs, **Table 2** shows that relatively small numbers of ASPs are required to detect a predisposition gene when it is less common and the sibling relative risk is high. Under this scenario, linkage using ASPs will offer the most efficient strategy for detecting a disease gene, particularly given the limited amount of genotyping required compared to undertaking an association study. As the sibling relative risk (λ_s) becomes smaller, the deviation of allelic sharing in ASPs from the null expectation becomes smaller. As a consequence, the numbers of siblings required to detect linkage increases dramatically. Once the disease allele is relatively common and its risk ratio is small, the number of ASPs required will be prohibitively large ***(16)***.

The sibling relative risk associated with the non-HLA-linked genes in celiac disease is comparatively large; however, the relative power of linkage and association detection methods will depend on whether this risk can be ascribed to a single locus or a number of different genes acting in concert. If the sibling risk is conferred by a single locus, a linkage search will clearly be the best strategy. However, if susceptibility to celiac disease is controlled by a number of non-HLA-linked genes, each of small effect, an association-based strategy should prove more effective.

Acknowledgments

We thank the Coeliac Society for providing a fellowship to S. Bevan.

Appendix A

Suggested Further Reading

Ott, J. (1991) *Analysis of Human Genetic Linkage*, rev. ed., The Johns Hopkins University Press, Baltimore, MD.

Risch, N. (1990) Linkage strategies for genetically complex traits. I. Multilocus models. *Am. J. Hum. Genet.* **46,** 222–228.

Risch, N. (1990) Linkage strategies for genetically complex traits. II. The power of affected relative pairs. *Am. J. Hum. Genet.* **46,** 242–253.

Risch, N. (1990) Linkage strategies for genetically complex traits. III. The effect of marker polymorphism on analysis of affected relative pairs. *Am. J. Hum. Genet.* **46,** 242–253.

Terwilliger, J. D. and Ott, J. (1994) *Handbook of Human Genetic Linkage*, The Johns Hopkins University Press, Baltimore, MD.

Appendix B

Mapping Functions

A mapping function is a formula that relates recombination fractions to genetic distances. If no interference is assumed, then the Haldane mapping function can be used:

$$x = -1/2 \cdot \log_e (1 - 2\theta)$$
$$\theta = 1/2(1 - e^{-2x})$$

where x is the genetic distance in Morgans and θ is the recombination fraction.

Depending on the assumed mechanism by which interference occurs, a number of map functions have been derived. One of the most commonly used to incorporate interference is the Kosambi mapping function:

$$x = 1/2 \tanh -1(2q) = 1/4 \ln[(1 + 2\theta)/(1 - 2\theta)]$$
$$\theta = 1/2 \tanh(2x) = 1/2[(\exp(4x) - 1)/(\exp(4x) + 1)]$$

Appendix C

Polymorphism Information Content

At a locus with n alleles of frequencies $p_1, p_2, \ldots, p_n$, the PIC is defined as the proportion of matings in which a parent is expected to be informative:

$$1 - \sum_{i=1}^{n} p_i^2 - \sum_{i=1}^{n} \sum_{j \neq i} p_i^2, p_j^2$$

Appendix D

Linkage Programs and How to Obtain Them

The following list is not exhaustive but provides an overview of some of the most commonly used programs for human genetic linkage analysis. Most programs have on-line documentation.

BETA
Description: Nonparametric linkage analysis using allele sharing in sib pairs.
Authors: Collins, A. (arc@soton.ac.uk), Morton, N. E.
ftp: http://cedar.genetics.soton.ac.uk/pub/PROGRAMS/BETA
Operating systems: UNIX(SUN.)
References: Morton, N., Lio, P., and Collins, A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3471–3476; Collins, A. and Morton, N. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 9177–9181; Lio, P. and Morton, N. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 5344–5348.

FASTLINK
Description: Faster version of LINKAGE.
Authors: Cottingham, R. W., Gupta, S., Idury, R. M., and Schaffer, A. A. (schaffer@helix.nih.gov), Shriram web: http://www.cs.rice.edu/~schaffer/fastlink.html
ftp: ftp://fastlink.nih.gov/pub/fastlink or ftp://softlib.cs.rice.edu/softlib/fastlink
Operating systems: UNIX, VMS, PC(DOS/..)
References: (1993) *Am. J. Hum. Genet.* **53,** 252–263; Schaffer, A. A., Gupta, S. K., Shriram, K., and Cottingham, R. W. (1994) *Hum. Hered.* **44,** 225–237.

GAS (Genetic Analysis System)
Description: Suite of programs for statistical analysis of genetic linkage data, sib-pair analysis, association studies.
Author: Young, A. (ayoung@vax.ox.ac.uk)
Web: http://users.ox.ac.uk/~ayoung/gas.html
ftp: ftp://ftp.ox.ac.uk/pub/users/ayoung
Operating systems: PC(DOS), UNIX(SunOS/Solaris/AIX/OSF1/Ultrix/SGI-IRIX), VMS

GENE HUNTER-PLUS
Description: GENEHUNTER-PLUS is a modification of the GENEHUNTER software package, which produces output files containing the null and conditional distributions of the test statistic (in nullprobs.dat and probs.dat, respectively). These files can then be used as input to the KAC program, which incorporates the allele sharing modeling for LOD scores and likelihood ratio tests as developed by Kong and Cox (1997).
Authors: Augustine Kong (kong@galton.uchicago.edu), Mike Friggs
ftp: ftp://galton.uchicago.edu/pub/kong
Reference: Kong, Cox (1997) *Am. J. Hum. Genet.* **61,** 1179–1188.

LINKAGE—general pedigrees
Authors: Young, A., Weeks, D., Lathrop, M.
ftp: ftp://linkage.rockefeller.edu/software/linkage
Operating systems: PC(DOS/OS2/..), UNIX, VMS
References: Lathrop, Lalouel, Julier, Ott (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3443–3446; Lathrop, Lalouel (1984) *Am. J. Hum. Gene.* **36,** 460–465; Lathrop, Lalouel, White (1986) *Genet. Epidemiol.* **3,** 39–52; American Society Human Genetics annual meeting 1995 (Young, Weeks, Lathrop [1995] *Am. J. Hum. Genet.* **[Suppl.] 57(4),** A206).

SIB-PAIR
Description: A program for simple nonparametric genetic analysis. Includes IBD- and IBS-based APM, Haseman-Elston sib pair, TDT, and association analyses.
Authors: Duffy, D. (Queensland Institute of Medical Research, Australia; davidd@qimr.edu.au)
Web: http://www.qimr.edu.au/davidD/davidd.html
Reference: American Society of Human Genetics annual meeting 1997 (Duffy [1997] *Am. J. Hum. Genet.* **[Suppl.] 61,** A197.

SIMLINK
Description: A computer program to estimate the probability ("power") of detecting linkage given family history information on a set of identified pedigrees.
Authors: Boehnke, M. (boehnke@sph.umich.edu)

Web: http://www.sph.umich.edu/group/statgen/software
Operating systems: PC (DOS/..), UNIX(SunOS/..), VMS
References: Boehnke (1986) *Am. J. Hum. Genet.* **39,** 513–527; Ploughman, Boehnke (1989) *Am. J. Hum. Genet.* **44,** 543–551.

SLINK
Authors: Weeks, D. E., Ott, J.
ftp: ftp://linkage.rockefeller.edu/software/slink
Reference: Ott (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4175–4178; American Society of Human Genetics annual meeting 1990 (Weeks, D., Ott, J., and Lathrop, M. [1990] *Am. J. Hum. Genet.* **[Suppl.] 47,** A204).

SPLINK
Description: Affected sib pairs linkage analysis.
Author: Clayton, D. (MRC Biostatistics Unit, Cambridge, UK)
ftp: ftp.mrc-bsu.cam.ac.uk/pub/methodology/genetics
Web: http://www.mrc-bsu.cam.ac.uk/pub/methodology/genetics/
Reference: Holmans (1993) *Am. J. Hum. Genet.* **52,** 362–374.

VITESSE
Description: Calculates likelihood on pedigrees.
Author: O'Connell, J. (jeff@sherlock.hgen.pitt.edu)
ftp: ftp://watson.hgen.pitt.edu/pub/vitesse/
Operating systems: UNIX, VMS, PC (DOS)
References: American Society of Human Genetics annual meeting 1995 (O'Connell, J. and Weeks, D. [1995] *Am. J. Hum. Genet.* **[Suppl.] 57,** A199); O'Connell, Weeks [1995] *Nat. Genet.* **11,** 402–408.

Appendix E

Basic Unix Commands and Information

Basic information

Login and password procedures are standard.
When you log in, the working directory is your home directory.
The symbols $ or % are prompts.
To back up and correct typos press Backspace or, Delete or #(Shift 3)
When you have typed a command, press Enter (or return).
To log out, type exit.
To find out where you are, type pwd (present working directory).

Pathnames

The root directory of the disk is called /.
If a pathname starts with a slash (/), it originates at the root directory.
A pathname consists of directory names separated by slashes (/).

Filenames

Capital and small letters are different in filenames.
Filenames can contain letters, numbers, and dots. Avoid using punctuation and slashes because these have special meaning.
Filenames should not have spaces.
The * wildcard stands for a bunch of characters.
The ? wildcard stands for a single character in a filename.

Basic Commands

Copy a file	cp oldfile newfile
Copy a file to another directory	cp oldfile dirname
Copy a bunch of files to another directory	cp file* dirname
Erase a file	rm filename
Rename a file	mv oldname newname
Move a file to another directory	mv oldfile dirname
Listfile names	1s
Listfile names with sizes and dates	1s – 1
View a test file	more filename
Compare two files	diff file 1 file 2
Printing	1pr textfile

References

1. Houlston, R. S. and Ford, D. (1996) Genetics of coeliac disease. *QJM* **89,** 737–743.
2. Risch, N. (1990) Linkage strategies for genetically complex traits. II. The power of affected relative pairs. *Am. J. Hum. Genet.* **46,** 229–241.
3. Holmans, P. (1993) Asyptoric properties of affected-sib-pair analysis. *Am. J. Hum. Genet.* **52(2),** 362–374.
4. Holmans, P. (1993) Asyptoric properties of affected-sib-pair linkage analysis. *Am. J. Hum. Genet.* **52,** 362–374.
5. Kruglyak, L. and Lander, E. S. (1995) Complete multipoint sib-pair analysis of qualitative and quantitative traits. *Am. J. Hum. Genet.* **57,** 439–454.
6. Kruglyak, L., Daly, M. J., Reeve-Daly, M. P., and Lander, E. S. (1996) Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am. J. Hum. Genet.* **58,** 1347–1363.
7. Lander, E. and Kruglyak, L. (1995) Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat. Genet.* **11,** 241–247.
8. Hodge, S. E. (1984) The information contained in multiple sibling pairs. *Genet. Epidemiol.* **1,** 109–122.
9. Risch, N. (1990) Linkage strategies for genetically complex traits. III. The effect of marker polymorphism on analysis of affected relative pairs. *Am. J. Hum. Genet.* **46,** 242–253.
10. Weeks, D. E., Ott, J., and Lathrop, G. M. (1989) SLINK: a general simulation program for linkage analysis. *Am. J. Hum. Genet.* **47,** A204.

11. Spielman, R. S. and Ewens, W. J. (1996) The TDT and other family-based tests for linkage disequilibrium and association. *Am. J. Hum. Genet.* **59,** 983–989.
12. Ploughman, L. M. and Boehnke, M. (1989) Estimating the power of a proposed linkage study for a complex trait. *Am. J. Hum. Genet.* **44,** 543–551.
13. Terwilliger, J. D. (1995) A powerful likelihood method for the analysis of linkage disequilibrium between trait loci and one or more polymorphic marker loci. *Am. J. Hum. Genet.* **56,** 777–787.
14. Morris, A. P., Curnow, R. N., and Whittaker, J. C. (1997) Randomisation tests of disease-marker associations. *Ann. Hum. Genet.* **61,** 49–60.
15. Sham, P. C. and Curtis, D. (1995) An extended transmission/disequilibrium test (TDT) for multi-allele marker loci. *Ann. Hum. Genet.* **59,** 323–336.
16. Risch, N. and Merikangas, K. (1996) The future of genetic studies of complex human disease. *Science* **273,** 1516, 1517.
17. Curtis, D. and Sham, P. C. (1995) A note on the application of the transmission disequilibrium test when a parent is missing. *Am. J. Hum. Genet.* **56,** 811, 812.
18. Camp, N. J. (1997) Genomewide transmission/disequilibrium testing—consideration of the genotypic relative risks at disease loci. *Am. J. Hum. Genet.* **61,** 1424–1430.
19. Bevan, S., Popat, S., Ferguson, A., et al. Contribution of the MHC region to the familial risk of coeliac disease. *J. Med. Genet.* **36(9),** 687–690.

5

Extraction, Separation, and Purification of Wheat Gluten Proteins and Related Proteins of Barley, Rye, and Oats

Arthur S. Tatham, Simon M. Gilbert, Roger J. Fido, and Peter R. Shewry

1. Introduction

1.1. Wheat Gluten Proteins

The wheat proteins, which are active in celiac disease and other gluten-related conditions, are defined as prolamins, in that they are soluble as individual subunits in alcohol-water mixtures, such as 50% (v/v) aqueous propan-1-ol or 60–70% (v/v) aqueous ethanol. However, in wheat grain and flour, about half of these subunits are present in polymers that are not soluble in alcohol-water mixtures unless disulfide bonds between the component subunits are reduced using an agent such as 2-mercaptoethanol (2-ME) or dithiothreitol (DTT). These alcohol-insoluble polymers are traditionally called glutenins and the related alcohol-soluble monomers are called gliadins; the two groups of proteins together form the major part of the gluten fraction. Gluten can be readily prepared from wheat by washing dough to remove the bulk of the starch, cell-wall material, and soluble components. It is a cohesive viscoelastic mass that contains, in addition to the gluten proteins, small amounts of other proteins, residual starch (about 25% dry wt), and lipid.

The two major wheat species used for food production are bread wheat (*Triticum aestivum*), which is a hexaploid with three genomes termed A, B, and D, and durum (pasta) wheat (*T. turgidum* var *durum*), which contains only the A and B genomes. However, related hexaploid, tetraploid, and A genome diploid species were cultivated and consumed historically and are still marketed to a limited extent today. They include spelt (*T. aestivum* var *spelta*, AA,

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

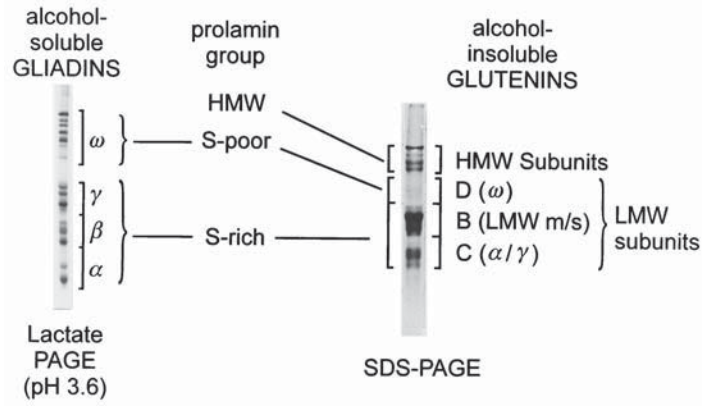


Fig. 1. Classification of the prolamins of wheat. Separation of nonreduced alcohol-soluble gliadins by lactate polyacrylamide gel electrophoresis (PAGE), and reduced alcohol-insoluble reduced glutenins by sodium dodecyl sulfate (SDS)-PAGE.

BB, DD), emmer (*T. turgidum* var *dicoccum*, AA, BB), and einkorn (*T. monococcum* var *monococcum*, AA). The A, B, and D genomes of these cultivated wheats are derived from related wild grass species of the genera *Triticum* and *Aegilops* and therefore encode related proteins. Consequently, it is not valid to expect any cultivated or wild wheat species to be nontoxic to those suffering from celiac disease despite claims to the contrary.

Gluten is a complex mixture of proteins, with more than 50 different components present in hexaploid bread wheat. These can be divided into subgroups on the basis of their structures and properties; the gliadins are divided into α-, γ-, and ω-type on the basis of their amino acid sequences and mobility on electrophoresis at low pH, and the reduced glutenin subunits are divided into high molecular weight and low molecular weight groups (**Fig. 1**) ***(1)***.

Although the gliadin/glutenin classification provides a valuable operational framework, the availability of amino acid sequences of individual proteins has allowed the introduction of a new classification based on molecular relationships. This classification divides the gluten proteins into three groups called sulfur-rich (S-rich), S-poor, and high molecular weight prolamins. These groups comprise the α-type gliadins, γ-type gliadins, and low molecular weight subunits; the ω-type gliadins; and the high molecular weight subunits, respectively. The various groups and names of gluten proteins can be confusing to the nonexpert and therefore we have summarized than in **Fig. 1** and **Table 1** ***(2)***.

Because of the complexity of the gluten protein fraction, and the high degree of relatedness of many components, it has proved difficult, and in many cases impossible, to purify single homogeneous components in sufficient amounts

Table 1
Groups of Prolamins of the Triticeae and Their Presence as Monomers or Polymers[a]

	Wheat	Barley	Rye	Polymeric or monomeric
S-rich prolamins				
γ-type	γ-type gliadins	γ-type hordeins	M_r 40,000 γ-secalins	Monomer
	—	—	M_r 75,000 γ-secalins	Polymer
α-type	α-type gliadins	—	—	Monomer
Aggregated type	LMW subunits of glutenin	B hordeins	—	Polymer
S-poor prolamins	ω-gliadins	C hordeins	ω-secalins	Monomer
HMW prolamins	HMW subunits of glutenin	D hordeins	HMW secalins	Polymer

[a]HMW, high molecular weight; LMW, low molecular weight.

for detailed characterization, or clinical challenges. It is therefore usual to work with fractions or subfractions comprising mixtures of related components such as total γ-type gliadins and "slow" ω-gliadins. An alternative approach is to express individual proteins in *Escherichia coli* or another heterologous host, using cloned cDNAs or genes, as reported recently by Tanabe et al. ***(3)*** and Maruyama et al. ***(4)*** in a study of dietary allergy of wheat. However, this approach requires the availability of a cloned cDNA or gene and presupposes that the expressed protein is correctly folded and processed.

The complexity of the gluten fraction within a single homozygous cultivar or line of wheat is one aspect of protein polymorphism. A second aspect is that considerable variation occurs in the number and electrophoretic properties of the individual components present in different cultivars or lines. It is therefore important to define the line used for protein purification and to avoid changing between lines with different protein patterns.

1.2. Barley and Rye Prolamins

Barley (*Hordeum vulgare*) and rye (*Secale cereale*) are closely related to wheat and are classified in the same botanical Tribe, the Triticeae. Although it is not possible to make gluten from these species, both contain groups of prolamins, called hordeins in barley and secalins in rye, which are related to the gliadin and glutenin components of wheat (**Fig. 2**, **Table 1**). However, there are some subtle differences: rye lacks proteins related to the low molecular

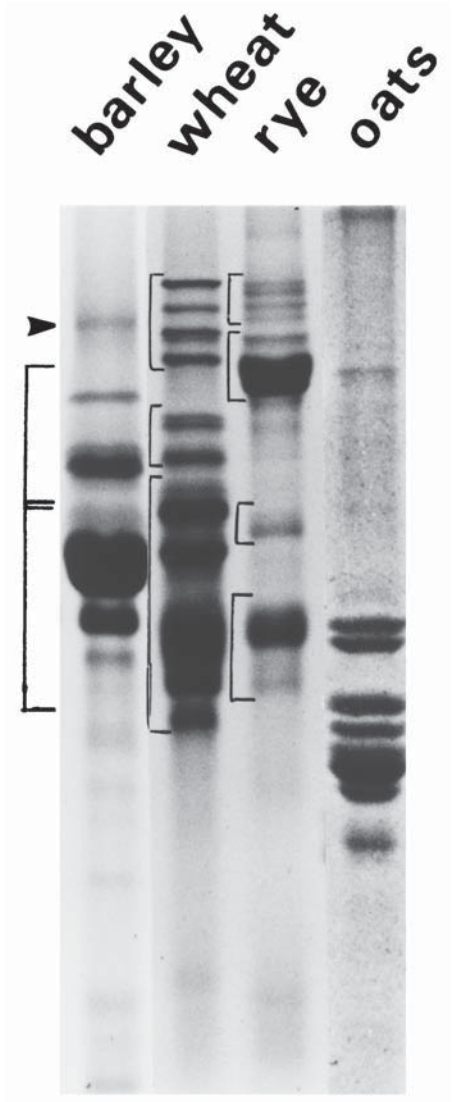


Fig. 2. SDS-PAGE of total prolamin fractions from barley (hordeins), wheat (gliadins and glutenins), rye (secalins), and oats (avenins). The arrow in the barley track indicates D hordein and the brackets indicate, in order of increasing mobility downward, C hordeins and B + γ-hordeins. The brackets in the wheat track indicate, in order of increasing mobility downward, the high molecular weight subunits of glutenin, the S-poor ω-gliadins, and the S-rich α-type gliadins + γ-type gliadins + low molecular weight subunits of glutenin. The brackets in the rye track indicate, in order of increasing mobility, high molecular weight secalins, M_r 75,000 γ-secalins, ω-secalins, and M_r 40,000 γ-secalins.

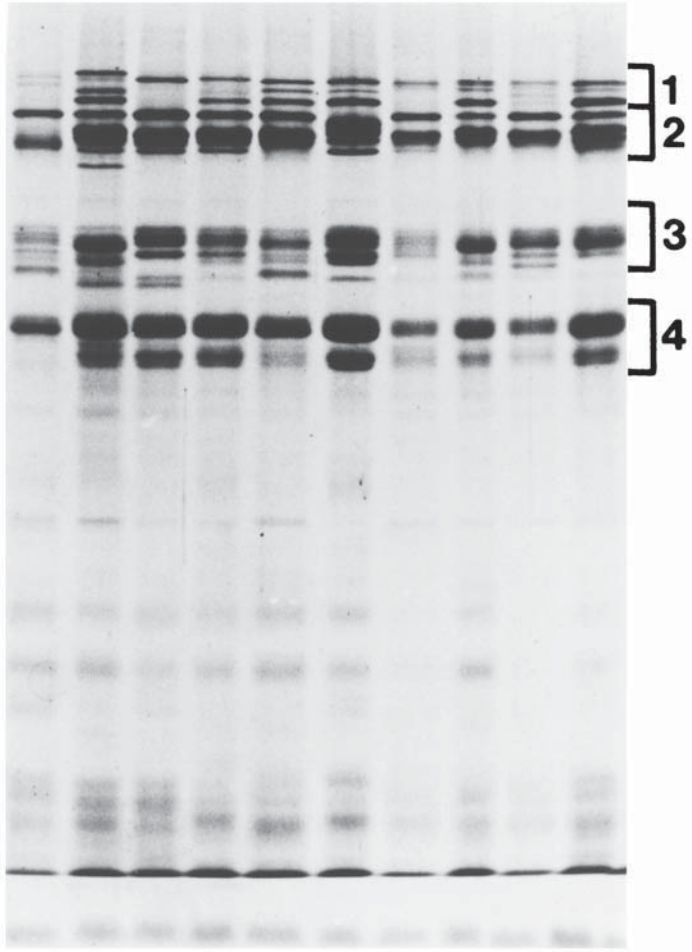


Fig. 3. Polymorphism in secalins extracted from single seeds of rye cultivar "Gazelle," and separated by SDS-PAGE. 1, high molecular weight secalins; 2, M_r 75,000 γ-secalins; 3, ω-secalins; and 4, M_r 40,000 γ-secalins.

weight subunits and B hordeins but contains a polymeric type of γ-secalin, whereas both rye and barley lack close homologs of the α-type gliadins of wheat.

Both barley and rye are diploid species, and the level of polymorphism in homozygous lines is therefore generally lower than in tetraploid and hexaploid wheats. However, whereas barley and wheat are inbreeding species, rye is outbreeding. Consequently, rye cultivars are complex mixtures of genotypes with individual plants being heterozygous. This means that individual seeds of cultivars show highly complex and variable patterns of secalin subunits (**Fig. 3**),

and it is virtually impossible to purify homogeneous single components. Although inbred rye lines with homogeneous secalin compositions are available, these lack vigor and seeds are difficult to obtain in large amounts.

1.3. Oat Prolamins

Oats are classified in the botanical Tribe Aveneae, and oat prolamins account for only about 10–15% of the total grain protein, in contrast to about 50% in members of the Triticeae. Cultivated oats (*Avena sativa*) are hexaploid and contain about 10–12 major prolamin (avenin) components (**Fig. 2**). All appear to be related to each other and to the S-rich prolamins of the Triticeae, but the existence of discrete subgroups is still uncertain.

1.4. Prolamin Purification

It is clearly impossible to provide detailed methods for the purification of all the individual prolamin components from wheat, barley, rye, and oats. Instead, we focus on presenting methods that we have found to have wide applicability, using examples largely from our own work.

2. Materials

2.1. Protein Extraction and Sample Preparation: Extraction of Seed Tissues

1. Total protein extraction buffer: 0.0625 *M* Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-ME or 1.5% DTT, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue.

2.2. Electrophoresis Systems

2.2.1. Laemmli System

1. Solution A: 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide.
2. Solution B: 1.5 *M* Tris-HCl, pH 8.8.
3. Solution C: 0.5 *M* Tris-HCl, pH 6.8.
4. Solution D: 10% (w/v) SDS.
5. Solution E: 10% (w/v) ammonium persulfate (freshly prepared).
6. Solution F: 0.004% (w/v) riboflavin.
7. Running buffer: 0.025 *M* Tris, 0.192 *M* glycine, pH 8.3, 0.1% (w/v) SDS.
8. Sample buffer: Same as total protein extraction buffer (*see* **Subheading 2.1.1.**).

2.2.2. Tris-Borate System

1. Separating gel buffer: 1.25 *M* Tris-borate, with 1% (w/v) SDS prepared by dissolving 378 g of Tris, 25 g of SDS, and 95 g of boric acid to a volume of 2.5 L. The pH should be 8.9 with no adjustment required.
2. Stacking gel buffer: 1.0 *M* Tris-HCl, pH 6.8, 10% (w/v) SDS.
3. Ammonium persulfate (prepared fresh), 10% (w/v).

4. Sample buffer: Mix 6.55 mL stacking gel buffer (pH 6.8), 3.3% (w/v) SDS, 10 mL (v/v) glycerol, and 1.54% (w/v) DTT (100 m*M* final concentration). Make up to 100 mL with water.
5. Running buffer: Use separating gel buffer diluted 10X. This may be reused by mixing the buffer from the upper and lower chambers.
6. Acrylamide (40% w/v) and *NN′*-methylenebisacrylamide (2% w/v) solutions are purchased from BDH (Poole, Dorset, UK).

2.2.3. Lactate-PAGE System

1. Solution A: 32% (w/v) acrylamide, 1% (w/v) bisacrylamide.
2. Solution B: 0.032% (w/v) ferrous sulfate.
3. Upper reservoir buffer (1 L): 0.1% (w/v) aluminum lactate, 0.16% (v/v) lactic acid (85%); pH should be 3.1.
4. Lower reservoir buffer (4 L): 0.4% (v/v) lactic acid (85%); adjust pH to 2.6 with further lactic acid.
5. Sample buffer: 35% (v/v) aqueous ethanol, 10% (v/v) glycerol, 3 *M* urea, with one crystal of basic fuchsin.

3. Methods

3.1. Preparation of Gliadin and Glutenin Fractions

The following methods are recommended to prepare total gliadin, glutenin, and gliadin + glutenin fractions, as summarized in **Fig. 4**.

3.1.1. Extraction of Separate Gliadin and Glutenin Fractions

1. Stir 100 g of flour continuously with 1 L of water-saturated butan-1-ol for 1 h at 20°C. Centrifuge (5000*g*, 10 min, 20°C) and discard the supernatant (*see* **Note 1**).
2. Stir the pellet with 1 L of 0.5 *M* NaCl for 1 h at 20°C. Centrifuge (5000*g*, 10 min, 20°C) and discard the supernatant. Repeat one time.
3. Stir the pellet for 10 min with 1 L of distilled water. Centrifuge (5000*g*, 10 min, 20°C) and discard the supernatant.
4. Stir the pellet for 1 h at 4°C with 1 L of 70% (v/v) aqueous ethanol. Centrifuge (5000*g*, 10 min, 20°C) and remove the supernatant. Repeat one time and bulk the supernatants that contain gliadins and some alcohol-soluble glutenin polymers.
5. Stir the pellet with 1 L of 50% (v/v) aqueous propan-1-ol + 2% (v/v) 2-ME + 1% (v/v) acetic acid for 1 h at 20°C. Centrifuge (5000*g*, 10 min, 20°C) and remove the supernatant. Repeat one time and bulk the supernatants that contain reduced subunits of alcohol-insoluble glutenins.

3.1.2. Extraction of a Combined Gliadin + Glutenin Fraction

1. Follow **steps 1–3** in **Subheading 3.1.1.** (*see* **Note 2**).
2. Stir the pellet for 1 h at 20°C with 1 L of 50% (v/v) aqueous propan-1-ol containing 2% (v/v) 2-ME and 1% (v/v) acetic acid. Centrifuge (5000*g*, 10 min, 20°C)

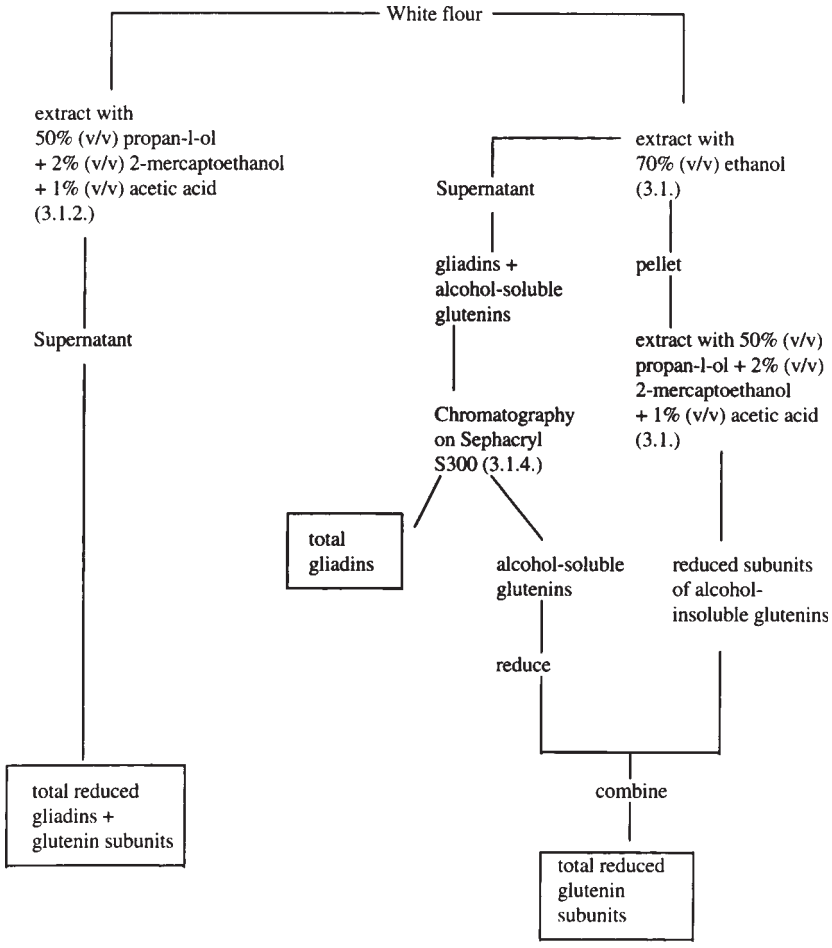


Fig. 4. Flowchart for preparation of gliadin and glutenin fractions.

and remove the supernatant. Repeat two times and bulk the supernatants that contain total reduced gliadins and glutenin subunits (*see* **Notes 3** and **4**).

3.1.3. Salt Precipitation

1. Mix the bulked supernatants with 2 vol of 1.5 *M* NaCl and let stand overnight at 4°C.
2. Centrifuge (5000*g*, 10 min, 4°C) and discard the supernatants.
3. Redissolve the pellets in about 50 mL of 0.1 *M* acetic acid. Dialyze extensively against 0.1 *M* acetic acid (at least four changes of 5 L) for 48 h at 4°C.
4. Lyophilize the dialyzed proteins.

3.1.4. Separation of Gliadins from Alcohol-Soluble Glutenins by Gel Filtration on Sephacryl S300

The gliadins prepared by this procedure can be used without reduction or after reduction of intrachain disulfide bonds. The glutenins can be reduced and combined with the fraction from **Subheading 3.1.1.** to give total reduced glutenin subunits.

1. Pack and equilibrate a 90 × 4.4 cm column of Sephacryl S300 in 6 *M* urea and 1% (v/v) acetic acid.
2. Dissolve 1 g of unreduced gliadins and alcohol-soluble glutenins in 25 mL of the same buffer.
3. Centrifuge the protein solution (5000*g*, 5 min, 20°C) and apply to the column.
4. Elute with the same buffer, and monitor fractions by OD_{280} and SDS-PAGE.
5. Bulk the fractions of interest, dialyze against 0.1 *M* acetic acid for 60 h at 4°C, and lyophilize.

3.2. Preparation of Prolamin Fractions from Barley and Rye

Total prolamin fractions from barley and rye are prepared as described under **Subheadings 3.1.2.** and **3.1.3.** (*see* **Note 5**).

3.3. Alkylation of Prolamin Fractions (5)

It is sometimes advantageous to reduce and *S*-pyridylethylate the cysteine residues present in the prolamin fractions of barley and rye. This may facilitate handling and purification and also give better resolution on electrophoresis (*see* **Note 6**).

1. Dissolve 1 g of prolamin in 50 mL of 50 m*M* Tris-HCl, pH 7.5, containing 8 *M* urea and 1% (v/v) 2-ME. Stir for 1 h at 20°C.
2. Add 1.8 mL of fresh 4-vinylpyridine and stir for 20 min at 20°C in the dark. (**Warning:** *see* **Note 7**.)
3. Stop the reaction by transferring the mixture to dialysis tubing and dialyze against ice-cold water. After initial dialysis, dialyze against water for 48 h at 4°C and lyophilize.

3.4. Purification of Gliadinsof Wheat, Hordeins of Barley, and Secalins of Rye

The following combination of procedures is recommended (**Fig. 5**).

3.4.1. Ion-Exchange Chromatography on CM Cellulose at pH 4.6

Ion-exchange chromatography at pH 4.6 separates on the content of basic amino acids (lysine, arginine, histidine, and the *S*-pyridylethyl derivative of cysteine) that are positively charged at this pH (*see* **Note 8**).

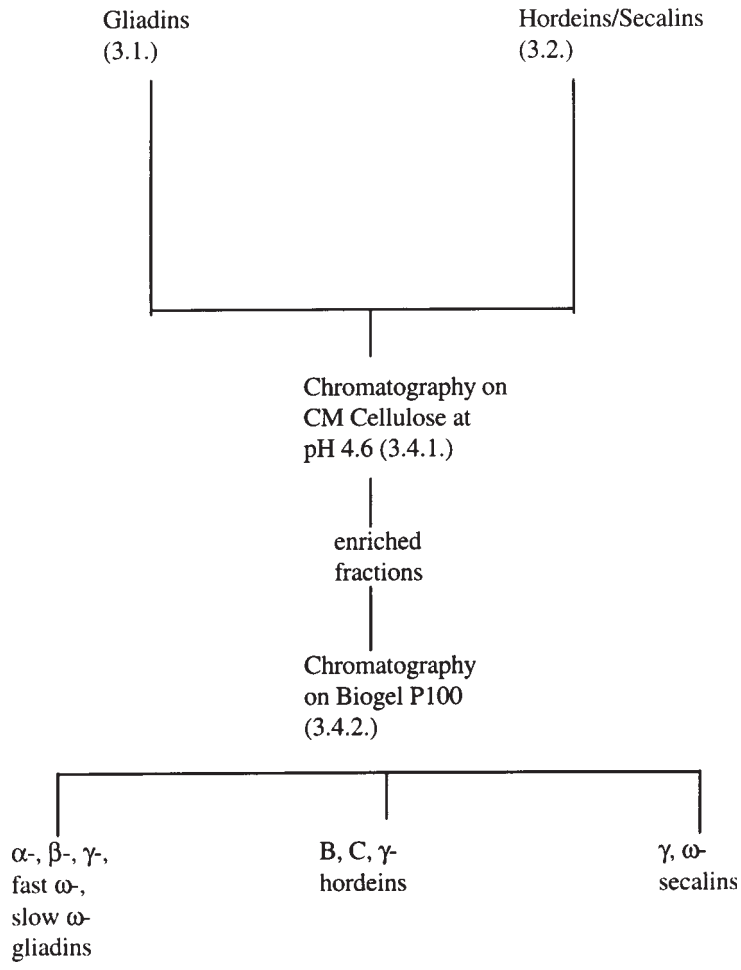


Fig. 5. Flowchart for recommended procedure for the separation of groups of gliadins, hordeins, and secalins.

1. Prepare a 4.4 × 30 cm column with a bed volume of approx 150 mL of CM cellulose equilibrated with 10 m*M* glycine/acetic acid buffer, pH 4.6, containing 3 *M* urea.
2. Dissolve 500 mg to 1 g of protein in the same buffer and dialyze overnight at 4°C against column buffer.
3. Load the sample, wash the column with one bed volume of buffer, and elute with a linear gradient of 0–0.5 *M* NaCl in the same buffer over 500 mL.
4. Monitor OD_{280} and separate selected samples by lactate-PAGE for gliadins (*see* **Subheading 3.7.3.**) or SDS-PAGE using either the Laemmli system

(*see* **Subheading 3.7.1.**) or Tris-borate system (*see* **Subheading 3.7.2.**) for other prolamins (*see* **Notes 9** and **10**).
5. Bulk the fractions of interest, dialyze against 0.1 *M* acetic acid at 4°C, and lyophilize.

3.4.2. Gel Filtration Chromatography on Biogel P100

1. Pack and equilibrate a 90 × 4.4 cm column of Biogel P100 in 0.1 *M* acetic acid.
2. Dissolve 1 g of gliadins in 0.1 *M* acetic acid (50 mL) and apply to the column (*see* **Note 11**).
3. Elute with 0.1 *M* acetic acid and monitor the fractions by OD_{280} and lactate-PAGE (*see* **Subheading 3.7.3.**).
4. Lyophilize the bulked fractions containing components of interest.

3.5. Purification of High Molecular Weight and Low Molecular Weight Subunits of Wheat Glutenin

The following method is specifically designed to purify individual high molecular weight subunits and groups of low molecular weight subunits (**Fig. 6**).

3.5.1. Extraction

This extraction method for the isolation of prolamins is based on Shewry et al. ***(6)*** (*see* **Notes 12** and **13**).

1. Stir 100 g of flour continuously with 800 mL of chloroform for 2 h at 20°C. Filter and then discard filtrate. Repeat one time. Air-dry the flour.
2. Stir the flour with 800 mL of 70% (v/v) aqueous ethanol for 2 h at 20°C. Centrifuge (5000*g*, 10 min, 20°C) and then discard the supernatant.
3. Disperse the pellet in 600 mL of 70% (v/v) aqueous ethanol and 1% (v/v) 2-ME, and stir for 2 h at 4°C. Centrifuge (5000*g*, 10 min, 4°C) and then discard the supernatant.
4. Disperse the pellet in 500 mL of 50% (v/v) aqueous propan-1-ol, 1% (v/v) acetic acid, and 2% (v/v) 2-ME, and stir for 18 h at 20°C. Centrifuge (5000*g*, 10 min, 20°C) and then discard the pellet.
5. Neutralize the supernatant with 50% (w/v) NaOH.
6. To the supernatant add 2 vol of 1.5 *M* NaCl and let stand overnight at 4°C.
7. Centrifuge (10,000*g*, 10 min, 4°C).
8. Dialyze the pellet against water for 48 h and lyophilize.

3.5.2. Preparation of Enriched High Molecular Weight and Low Molecular Weight Subunit Preparations by Selective Precipitation

This procedure is adapted from a method ***(7)*** developed for the purification of low molecular weight subunits.

1. Dissolve 1 g of prolamin from **Subheading 3.5.1.** in 400 mL of 80 m*M* Tris-HCl, pH 8.0, containing 2% (w/v) DTT and 50% (v/v) aqueous propan-2-ol at 60°C for 30 min. Centrifuge (12,000*g*, 5 min, 20°C) and discard the pellet.

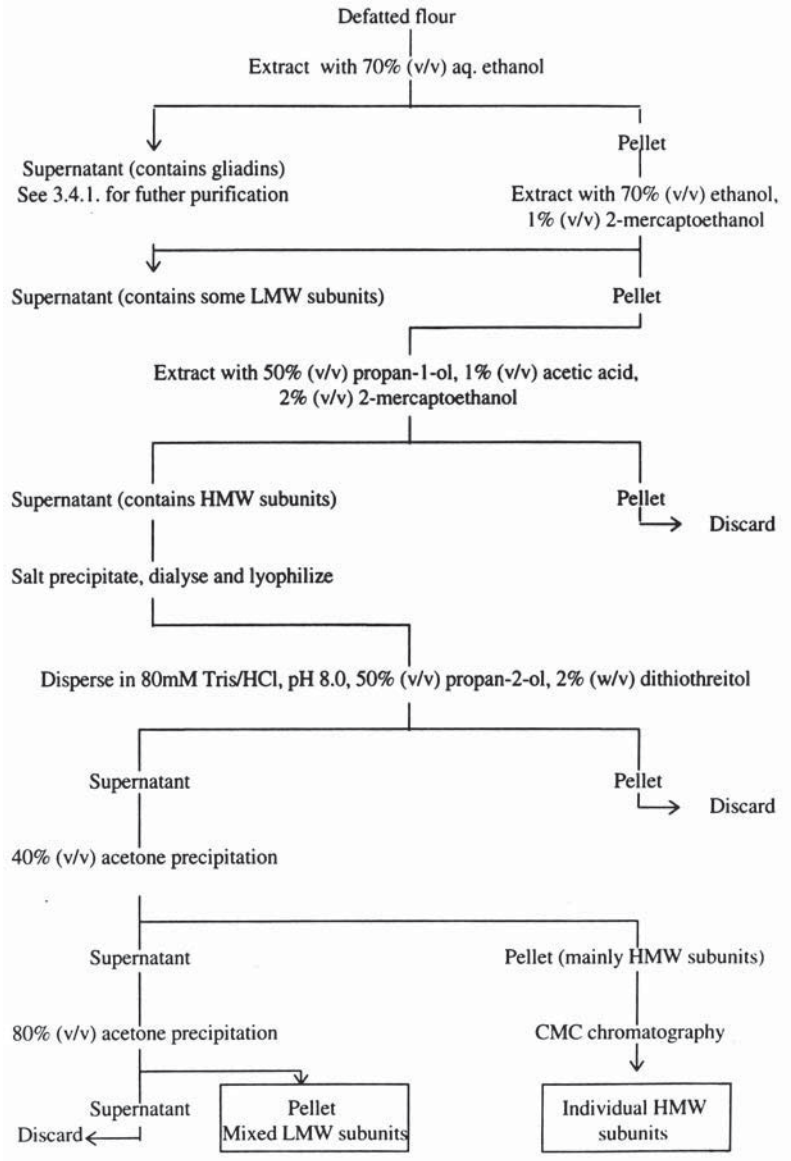


Fig. 6. Flowchart for the preparation of high molecular weight subunits of glutenin. LMW, low molecular weight; HMW, high molecular weight.

2. Add acetone to the supernatant to a concentration of 40% (v/v) and let stand for 10 min at 20°C. Centrifuge (12,000*g*, 10 min, 20°C) and then retain the pellet and supernatant.

3. Wash the pellet with water before lyophilizing to obtain a fraction enriched in high molecular weight subunits.
4. To the supernatant increase acetone concentration to 80% (v/v) and centrifuge (12,000*g*, 10 min, 20°C). Discard the supernatant.
5. Wash the pellet with water before lyophilizing to obtain a fraction enriched in low molecular weight subunits.

3.5.3. Ion-Exchange Chromatography on CM Cellulose at pH 4.6

1. Prepare the CM cellulose per the manufacturer's recommendations. Pour into a 25 × 4.4 cm column to give a bed volume of approx 150 mL.
2. Equilibrate the column in 50 m*M* glycine/acetate buffer, pH 4.6, containing 3 *M* urea and 2% (v/v) 2-ME.
3. Dissolve 1 g of prolamins from **Subheading 3.5.2.** in 50 mL of column buffer and apply to the column.
4. Elute with 3 column volumes of buffer and then elute with a gradient of 0–150 m*M* NaCl over 500 mL in the same buffer.
5. Monitor the fractions by SDS-PAGE minigels using the Tris-borate system described under **Subheading 3.7.2.**
6. Pool the fractions, dialyze against 1% (v/v) acetic acid and then water for 48 h at 4°C, and lyophilize.

3.6. Purification of Avenins of Oats

Oats contain relatively high contents of lipid- and salt-soluble albumins and globulins. The following method is designed to give total avenins with little lipid and albumin/globulin contamination.

1. Stir 100 g of flour continuously with 1 L of butan-1-ol for 2 h at 20°C. Centrifuge (7500*g*, 15 min, 20°C) and discard the supernatant. Repeat three times. Air-dry the pellet.
2. Stir the pellet with 1 L of 50% (v/v) aqueous ethanol for 1 h at 20°C. Centrifuge (7500*g*, 15 min, 20°C) and save the supernatant. Repeat one time. Bulk the supernatants.
3. Add 2 vol of 1.5 *M* NaCl to the bulked supernatants and let stand overnight at 4°C.
4. Centrifuge (7500*g*, 15 min, 4°C) and discard the supernatant that contains albumins and globulins.
5. Dissolve the pellet in 3 *M* urea and 0.1 *M* acetic acid and dialyze against 0.1 *M* acetic acid for 60 h.

3.7. Electrophoresis Systems

It is essential to use high-resolution electrophoresis systems to monitor the purification and final purity of proteins. The Laemmli system *(8)* is widely used and is recommended for general use. The Tris-borate system can be used to give high resolution of the high molecular weight subunits of glutenin.

Table 2
Volumes Required for Casting Two Laemmli *(8)* Separating Gels[a]

	Acrylamide (%)				
	5	7.5	10	12.5	15
A (mL)	1.66	2.5	3.32	4.16	5.0
B (mL)	2.5	2.5	2.5	2.5	2.5
D (μL)	100.0	100.0	100.0	100.0	100.0
H_2O (mL)	5.7	4.8	4.0	3.2	2.3
E (μL)	50.0	50.0	50.0	50.0	50.0
TEMED (μL)	5.0	5.0	5.0	5.0	5.0

[a]Using the Bio-Rad Mini Protein II gel apparatus.

The lactate-PAGE system should be used to separate gliadins into α, β, γ, and ω types (*see* **Fig. 1**).

3.7.1. The Laemmli System

Since its introduction, the Laemmli *(8)* system has been widely used for proteins of animal, plant, and microbial origin. We have provided recipes for a range of acrylamide concentrations with volumes calculated for two widely used types of apparatus: the Hoefer SE600 (San Francisco, CA) (which is identical to the Bio-Rad [Richmond, CA] Protean I) with 14 cm × 16 cm × 1.5 mm gels, and the Bio-Rad Mini Protean II with 8 cm × 7.3 cm × 0.75 mm gels (*see* **Tables 2** and **3**). Whereas the Mini Protean II apparatus is invaluable for rapid routine analyses, the larger gel size of the Hoefer SE600 can give better resolution of complex mixtures.

1. Cast separating gels according to the manufacturer's instructions, using the volumes given in **Tables 2** and **3**. The solutions are mixed before the addition of solution E and TEMED but not degassed. The gel is carefully overlaid with water or water-saturated butan-1-ol and allowed to set for about 1 h.
2. Pour off water-saturated butan-1-ol and gently wash the gel surface with distilled water. Dry the gel surface and the inside of the cassette by inserting a folded no. 1 filter paper to just above the surface of the gel.
3. Place combs into the gel cassettes and pipet the stacking gel to form wells. The stacking gel is prepared as follows: 1.3 mL of solution A, 2.5 mL of solution C, 100 μL of solution D, 50 μL of solution E, and 4.7 mL of water. The solution is well mixed before the addition of 1.3 mL of solution F and 10 μL of TEMED.
4. After about 30 min, remove the combs and wash the sample wells with running buffer to remove nonpolymerized acrylamide (*see* **Note 14**).
5. Load about 10–20 μL of sample per well for the Hoefer SE600 (20 wells/gel) or 10 μL/well for the Bio-Rad Mini Protean II (10 wells/gel).

Table 3
Volumes Required for Casting Two Laemmli *(8)* Separating Gels[a]

	Acrylamide (%)				
	5	7.5	10	12.5	15
A (mL)	10	15	20	25	30
B (mL)	15	15	15	15	15
D (µL)	600	600	600	600	600
H_2O (mL)	34	29	24	19	14
E (µL)	300	300	300	300	300
TEMED (µL)	30	30	30	30	30

[a]Using the Hoefer SE600 gel apparatus.

Table 4
Volumes REquired for Casting Two Tris-Borate Gels[a]

	Separating gel, 10%	Stacking gel, 3%
Acrylamide (mL)	15.00	1.50
Bisacrylamide (mL)	3.12	0.80
Separating gel buffer (mL)	6.00	—
Stacking gel buffer (mL)	—	2.48
10% SDS (w/v)	—	0.20
Water (mL)	35.30	14.80
Ammonium persulfate 10% (w/w)	0.56	0.20
TEMED (µL)	25	20

[a]Using the Hoefer SE600 gel apparatus.

6. Run the gels at 20°C with water cooling for the Hoefer SE600 (*see* **Notes 15–17**). The Hoefer SE600 has an initial power setting of 20 mA/gel (for 30 min) followed by 30 mA/gel for about 3 h. The Bio-Rad Mini Protean II is run at constant 200 V (approx 70 mA at the start and 35 mA at the finish) for 50 min.

3.7.2. The Tris-Borate System

The Tris-borate system, based on Koenig et al. ***(9)***, is a useful alternative to the Laemmli system, providing better separation of the high molecular weight subunits of wheat glutenin.

1. **Table 4** gives the volumes for two Hoefer SE600 Gels (60 mL total volume).
2. Follow the method given for the Laemmli system (*see* **Subheading 3.7.1.**).
3. Run 1.5-mm thick gels at 30 mA/gel for about 3 h (*see* **Note 15**).

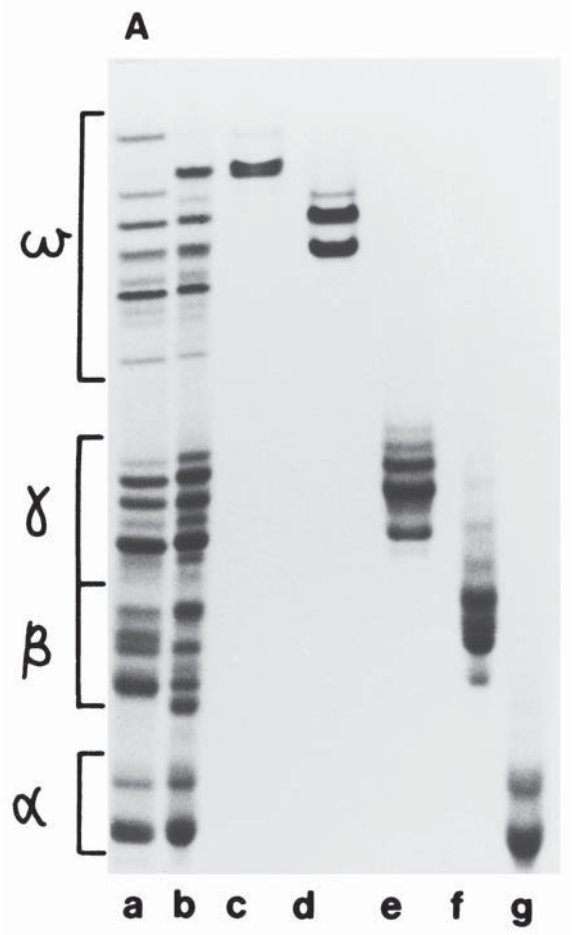


Fig. 7. Lactate-PAGE separation of gliadins. Tracks **a** and **b** are total gliadins, track **c**, slow ω-gliadins; track **d**, fast ω-gliadins; track **e**, γ-gliadins; track **f**, β-gliadins; and track **g**, α-gliadins.

3.7.3. The Lactate-PAGE System

The gliadins of wheat are classified into groups on the basis of their mobility at low pH. A number of low pH electrophoretic systems have been used, initially with starch gels but more recently using polyacrylamide gels. Most are based on lactate/lactic acid buffer systems, with special catalysts to allow polymerization at low pH (3.0–3.2). The system described here is based on Clements ***(10)*** and is used in our laboratory with a dedicated Bio-Rad Protean II gel apparatus. A typical separation of gliadin fractions is shown in **Fig. 7**.

1. Precool buffers to 4°C.
2. Volumes are given for the Bio-Rad Protean II System. Mix the following (sufficient for two gels): 25 mL of solution A, 10 mL of solution B, 45 mL of water, 0.4 mL of lactic acid (85%), and 80 mg of ascorbic acid.
3. Precool at –20°C until ice crystals start to appear. Add 0.2 mL of 0.6% (v/v) hydrogen peroxide and pour rapidly at room temperature. The mixture will polymerize in 2 to 3 min (*see* **Note 18**).
4. Dissolve the protein samples in sample buffer at about 1 mg/mL, centrifuge, and load up to 100 μL.
5. Run with reverse polarity (compared to SDS-PAGE) at 40–45 mA/gel, either at 4°C in a cold room or using a cooling system (*see* **Note 19**). Run for 30 min after the tracking dye has reached the front (a total of 2.0–2.5 h).
6. Carefully remove the gels and place into 350 mL of 10% (w/v) trichloroacetic acid for 30 min to fix the proteins. Then add 5 mL of 0.5% (w/v) aqueous Coomassie Brilliant Blue R250 and leave overnight. No destaining is required.

4. Notes

1. It is best to use white flour from a single variety of wheat. This is most readily produced by laboratory scale milling using a Brabender Quadrumat Junior (Brabender OHG, Duisberg, Germany) mill. Whole-meal flours can be used if white flour is not available, produced on a standard laboratory mill (e.g., Cyclone, Retsch Gmbh Co., KG, Haan, Germany) with a fine (0.5 mm or below) sieve.
2. A less pure total gliadin + glutenin fraction can be prepared by "direct extraction," as described under **Subheading 3.1.2.** but omitting **step 1**.
3. All manipulations involving 2-ME should be carried out in a fume cupboard. 2-ME can be replaced by DTT at the same concentration. This is safer but can be expensive if large volumes of solvent are used.
4. Protein preparations that contain reducing agent (2-ME or DTT) should be dialyzed separately from those that do not, to prevent diffusion of the reducing agent into the unreduced samples.
5. This procedure gives total reduced protein subunits. Fractions corresponding broadly to gliadins and glutenins can also be prepared by following procedures under **Subheadings 3.1.1.**, **3.1.3.**, and **3.1.4.**
6. *S*-pyridylethylation adds positively charged pyridylethyl groups to the proteins, improving the separation by chromatography at low pH and preventing the reformation of disulfide bonds. It does not usually affect the antigenic status of the proteins, but this could occur.
7. 4-Vinylpyridine is highly toxic by inhalation. Carry out the initial dialysis in a fume hood until most of the reagent is gone. Fresh reagent should always be used because it degrades rapidly after opening.
8. The basic method can be modified by increasing or reducing the concentration of urea or by including a reducing agent (2-ME or DTT) for reduced protein preparations.

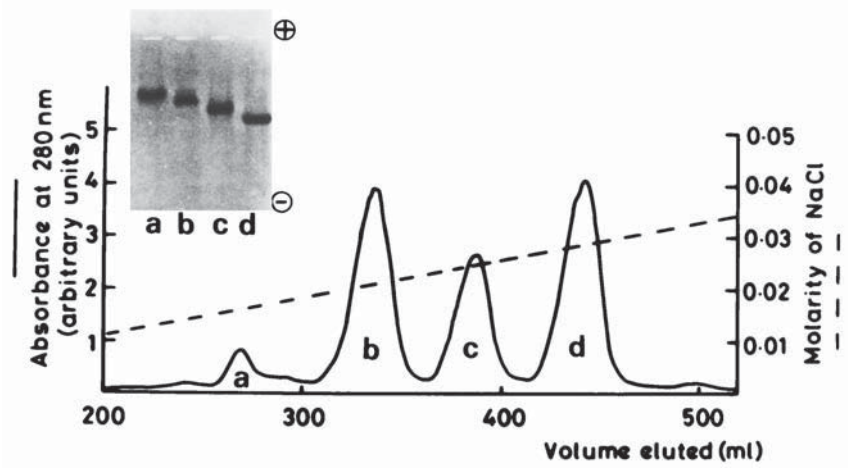


Fig. 8. C hordein fractions separated by ion-exchange chromatography. Inset lactate-PAGE of C hordein components ***(14)***.

9. The basic method gives broad fractions containing α-, β-, γ-, and ω-gliadins of wheat; the latter is eluted in groups of "fast" and "slow" components. Similarly, it will separate C hordein from B and γ-hordeins of barley and the ω- and γ-secalins of rye.
10. Optimization of the salt gradient can give very fine resolution of components within groups. **Figure 8** shows the resolution of C hordein components achieved with a linear gradient of 0–0.02 *M* NaCl.
11. It is necessary to incorporate 3 *M* urea into the 0.1 *M* acetic acid to ensure solubility of some hordeins and secalins.
12. Several related methods for the preparation of high molecular weight and low molecular weight subunits based on selective precipitation have been reported ***(11,12)***.
13. The purification procedure works well with unalkylated protein samples, which can then be alkylated if required.
14. The inclusion of up to 4 *M* urea in the separating and stacking gels can result in improved resolution, but also leads to decreased mobility and, in some cases, changes in the relative mobilities of the different components of a mixture ***(13)***.
15. It is often convenient to run large gels overnight with a constant setting of 50 V. This is adjusted to 30 mA/gel for the final hour.
16. The choice of gel size can have a great effect on the separation.
17. The Laemmli system gives good resolution of proteins over a wide range of M_r.
18. The apparatus and especially the plates must be scrupulously clean and free of SDS.
19. Efficient cooling is essential because the gel is run at a high voltage.

References

1. Shewry, P. R. and Tatham, A. S. (1990) The prolamin storage proteins of cereal seeds: structure and evolution. *Biochem. J.* **267,** 1–12.
2. Shewry, P. R., Tatham, A. S., Forde, J., Kreis, M., and Miflin, B. J. (1986) The classification and nomenclature of wheat gluten proteins: a reassessment. *J. Cereal Sci.* **4,** 97–106.
3. Tanabe, S., Arai, S., Yanagihara, Y., Mita, H., Takahashi, K., and Watanabe, M. (1996) A major wheat allergen has a Gln-Gln-Gln-Pro-Pro motif identified as an IgE-binding epitope. *Biochem. Biophys. Res. Commun.* **219,** 290–293.
4. Maruyama, N., Ichise, K., Katsube, T., Kishimoto, T., Kawase, S., Matsumura, Y., Takeuchi, Y., Sawade, T., and Utsumi, S. (1998) Identification of major wheat allergens by means of the *Escherichia coli* expression system. *Eur. J. Biochem.* **255,** 739–745.
5. Friedman, M., Krull, L. H., and Cavins, J. F. (1970) The chromatographic determination of cystine and cysteine residues in proteins as *S*-β-(4-pyridylethyl)-cysteine. *J. Biol. Chem.* **245,** 3868–3871.
6. Shewry, P. R., Field, J. M., Faulks, A. J., Parmar, S., Miflin, B. J., Dietler, M. D., Lew, E. J.-L., and Kasarda, D. D. (1984) The purification and N-terminal amino acid sequence analysis of the high molecular weight glutenin polypeptides of wheat. *Biochim. Biophys. Acta* **788,** 23–34.
7. Melas, V., Morel, M.-H., Autran, J.-C., and Feillet, P. (1994) Simple and rapid method for purifying low molecular weight subunits of glutenin from wheat. *Cereal Chem.* **71,** 234–237.
8. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
9. Koenig, R., Stegemann, H., Francksen, H., and Paul, H. L. (1970) Protein subunits in the potato virus N group: determination of the molecular weights by polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta* **207,** 184–189.
10. Clements, R. L. (1987) A study of gliadins of soft wheats from the Eastern United States using a modified polyacrylamide gel electrophoresis procedure. *Cereal Chem.* **64,** 442–448.
11. Verbruggen, I. M., Veraverbeke, W. S., Vandamme, A., and Delcour, J. A. (1998) Simultaneous isolation of wheat high molecular weight and low molecular weight glutenin subunits. *J. Cereal Sci.* **28,** 25–32.
12. Marchylo, B. A., Kruger, J. E., and Hatcher, D. W. (1989) Quantitative reversed-phase high-performance liquid chromatographic analysis of wheat storage proteins as a potential quality prediction tool. *J. Cereal Sci.* **9,** 113–130.
13. Bunce, N. A. C., White, R. P., and Shewry, P. R. (1985) Variation in estimates of molecular weights of cereal prolamins by SDS-PAGE. *J. Cereal Sci.* **3,** 131–142.
14. Shewry, P. R., Lew, E. J.-L., and Kasarda, D. D. (1981) Structural homology of storage proteins coded by *Hor-1* locus of barley (*Hordeum vulgare* L.). *Planta* **153,** 246–253.

6

Structure Elucidation of Gluten-Derived Peptides by Tandem Mass Spectrometry

Peter A. van Veelen, Jan W. Drijfhout, and Frits Koning

1. Introduction

1.1. General

In recent years there have been major developments in the field of mass spectrometry (MS) that permit the analysis and characterization of peptides and proteins at the femtomolar level ***(1–8)***. This chapter deals with the use of MS for the elucidation of peptide sequences of gliadin- and/or glutenin-derived fragments. It is meant to give an overview of what modern mass spectrometric methods can mean for your research in the biomolecular field rather than an in-depth step-by-step practical protocol. Important aspects are covered to give the reader an idea of what is needed to perform, and what can be expected from, this mass spectrometric application.

1.2. Mass Spectrometry

In essence, MS (*see* **Table 1**) is a technique for determining the mass of individual molecules. More important, it allows the analysis of individual components in complex mixtures, and it is highly sensitive. In principal, the following steps comprise mass spectrometric analysis:

1. Inject the solubilized sample and ionize the components to be analyzed.
2. Separate the formed ions on the basis of their mass and charge.
3. Detect the ions.

More advanced mass spectrometric analysis allows the elucidation of the structure of compounds, which can be selected from mixtures. Here we briefly describe the principles of MS and describe its use for the elucidation of the amino acid sequence of a gliadin peptide. The mass spectrometric procedures

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

Table 1
Interesting Internet Sites

General information about MS and many other links
http://pangea.ija.csic.es/icpms/msres.html
http://www.embl-heidelberg.de
http://thompson.mbt.washington.edu
Biotechnology information and databases
http://www.ncbi.nlm.nih.gov
http://www.expasy.ch
http://www.sanger.ac.uk
http://www.tigr.org
http://www.ebi.ac.uk
Major MS companies
http://www.finnigan.com
http://www.micromass.co.uk
http://www.perkin-elmer.com

described start with partially purified high-performance liquid chromatography (HPLC) fractions from a pepsin/trypsin digest of gluten.

For analysis of the samples, a tandem mass spectrometer equipped with an electrospray ionization source is used (a Q-TOF). **Figure 1** shows a schematic representation of this instrument. In the electrospray source, a fluid is nebulized by the action of an electrical field at atmospheric pressure. As a result of this process, gas-phase ions are formed that are subsequently transported to the first mass spectrometer, a quadrupole mass spectrometer. This quadrupole mass spectrometer acts as a mass filter and thus allows the selection of a narrow mass window of ions to pass through the device, while all other ions are deflected. The second mass spectrometer, a time-of-flight (TOF) mass spectrometer, is used to determine the mass-to-charge ratio of the ions. Between these two mass spectrometers, a collision cell is placed that can be used to generate fragments of the ions selected in the quadrupole. The masses of these fragments hold the clue to the amino acid sequence of the peptide analyzed and are recorded by the TOF MS. **Figure 2** illustrates the power of this arrangement. **Figure 2A** shows the total number of ions that were present in an enzymatic digest of gluten. For this analysis, all ions generated in the electrospray source were allowed to pass through the quadrupole MS and detected by the TOF-MS. Next, the quadrupole MS was used to select ions in a particular mass range, indicated by the arrow. **Figure 2A** shows the result of this selection. Only ions with a mass-to-charge ratio of 662, a minor component of the total sample, are now detected by the TOF-MS. All other ions are deflected. Subse-

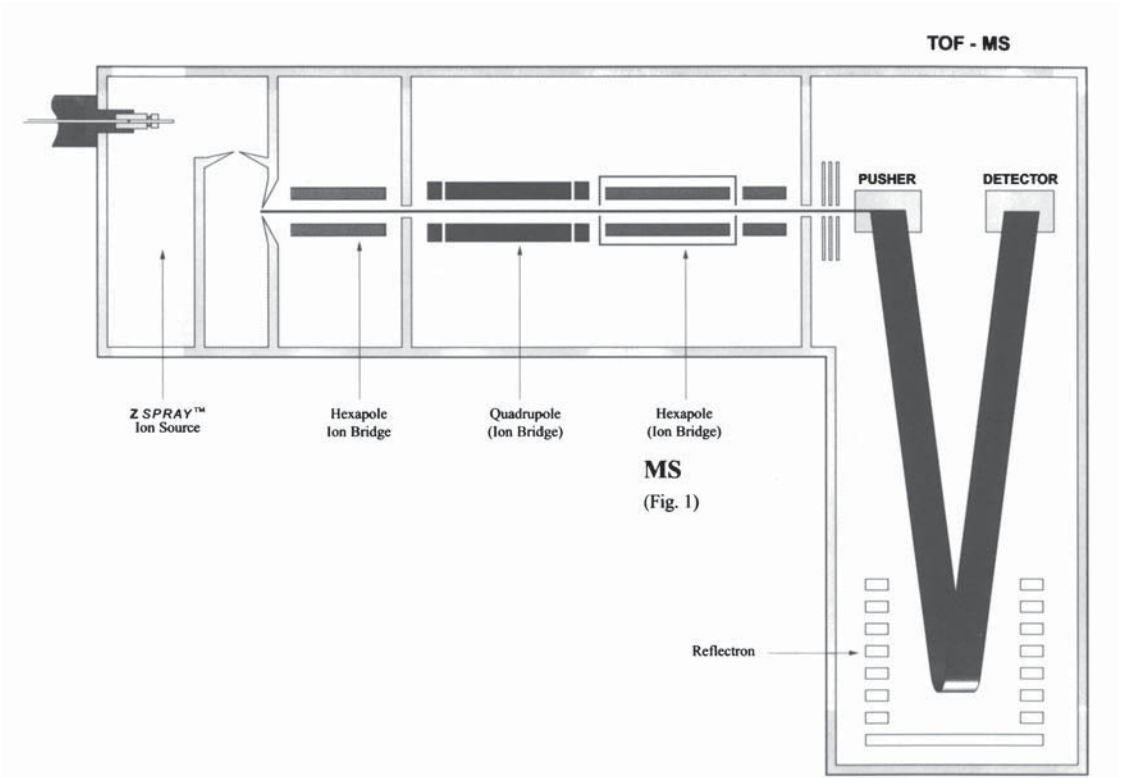
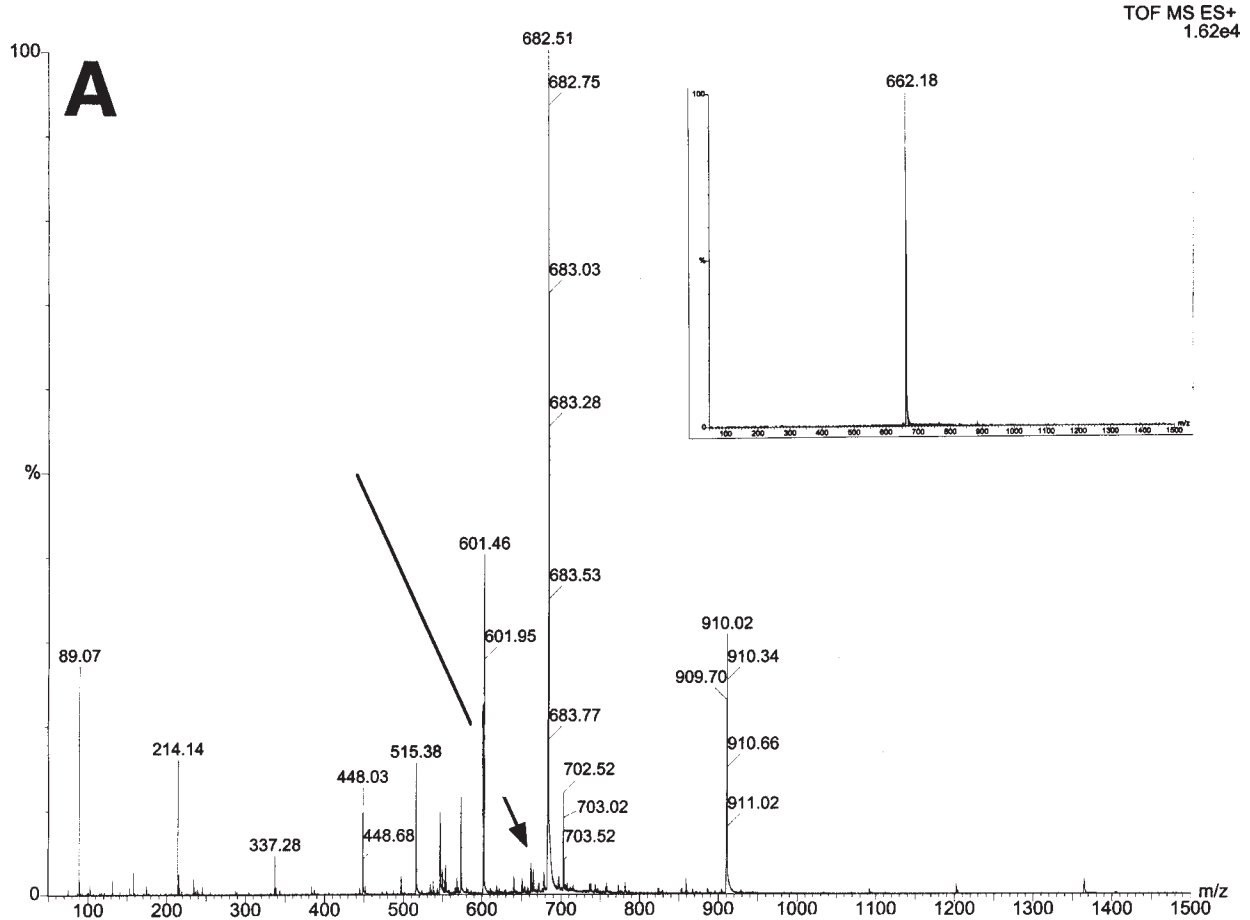


Fig. 1. Schematic of a modern tandem mass spectrometer, termed a Q-TOF. It comprises two mass spectrometers, a quadrupole MS, and a TOF-MS, with a collision cell (hexapole) in between. The solution containing the analytes is introduced into the MS source and nebulized by an electrical field at atmospheric pressure. The ions formed in this process are guided to the TOF-MS, in which their mass-to-charge ratio is determined on the basis of time the ions need to traverse the flight tube, the smallest ions arriving first. Now that the "normal" mass spectrum is known, individual components can be selected to undergo collisions with argon atoms in the collision cell, using the quadrupole, which acts as a mass filter, allowing only a small mass window of ions to exit the device. The mass of the fragments, formed as a result of the collisions, are measured by the TOF-MS.


A
TOF MS ES+
1.62e4
100
%
0
682.51
682.75
683.03
683.28
683.53
683.77
662.18
601.46
601.95
910.02
910.34
909.70
910.66
911.02
89.07
214.14
515.38
448.03
448.68
337.28
702.52
703.02
703.52
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z

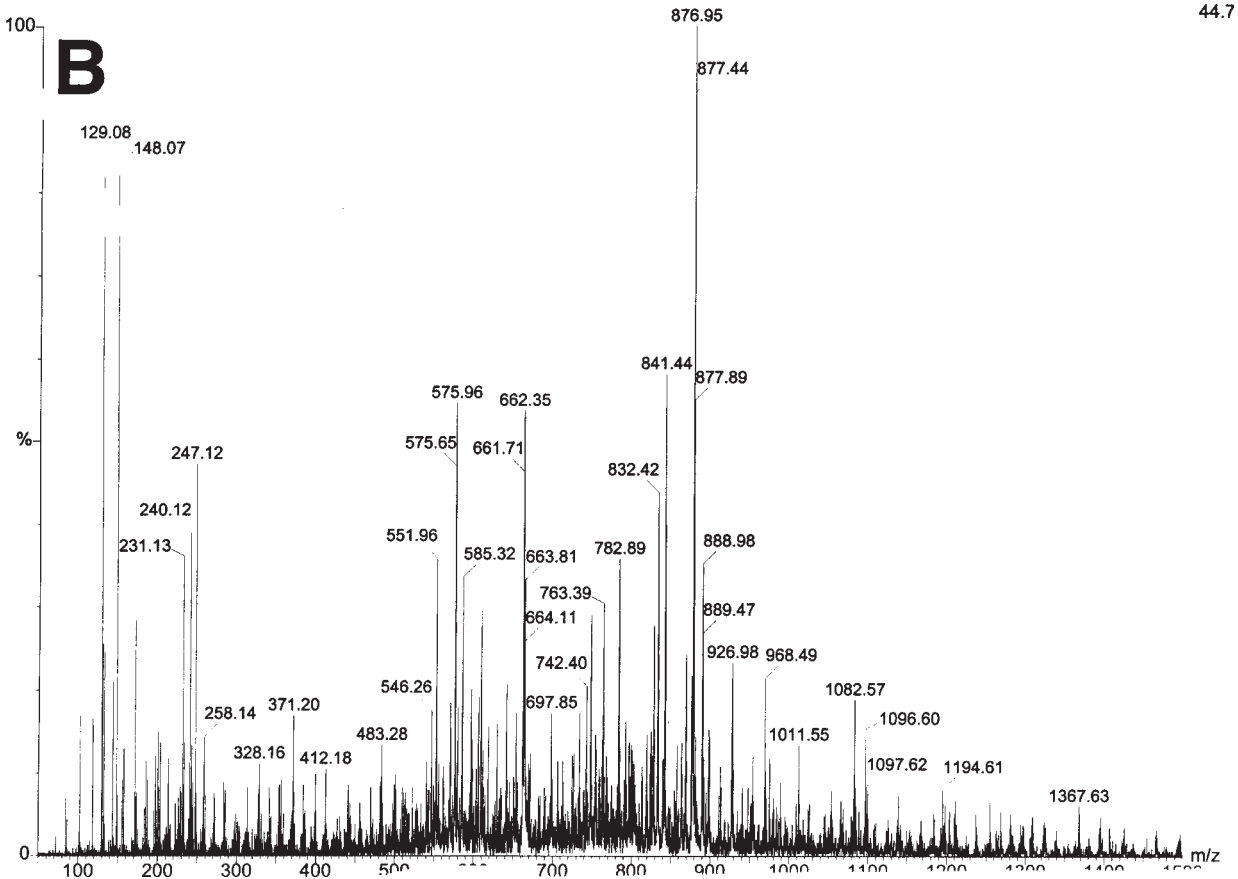


Fig. 2. Mass spectrum of a peptide mixture. **(A)** The inset shows the ions that were allowed to pass the first quadrupole, which acts as a mass filter. **(B)** Tandem mass spectrum of the ions with mass/charge 662 (3+) illustrating the capacity of the technique to sequence individual components in complex mixtures.

quently, these ions with a mass-to-charge ratio of 662 are fragmented in the collision cell by impacting with an inert gas, e.g., argon, and the resulting ions are recorded, which yields a so-called tandem mass spectrum (MS/MS spectrum) (*see* **Fig. 2B**).

Figure 3 illustrates how such an MS/MS spectrum can be used to elucidate the amino acid sequence of the peptide. An arbitrary sequence is displayed at the top of the figure. Fragmentations in the collision cell at the low collision energies used primarily occur on the peptide bonds, leading to so-called b- and y-ions. The b-ions contain the N-terminus whereas the y-ions contain the C-terminus of the peptide. Since these cleavages occur at (almost) any peptide bond, many different fragments are formed. **Figure 3** displays a number of them: b5–b10 and y5–y10. The formation of these ions gives rise to the (partial) MS/MS spectrum (*see* lower part of **Fig. 3**). It can easily be seen that the mass difference between b7 and b8 is the mass of a glutamine (Q) whereas the difference between b8 and b9 represents that of a valine (V) and between b9 and b10 a leucine (L). The proper alignment of these ions thus reveals the sequence QVL. Identification of all ions that belong together in the same series can therefore lead to the elucidation of the complete amino acid sequence of the peptide.

A major advantage of this type of instrumentation, therefore, is the possibility of analyzing individual components in complex mixtures. In principle, all components present in the digest could be sequenced individually using the procedure just described.

A high-quality MS/MS spectrum can thus lead to the elucidation of the primary amino acid sequence of peptides. However, in practice, such spectra do not always contain sufficient information. This can be owing, e.g., to a low precursor ion intensity or a large precursor ion.

In cases in which only limited sequence information is obtained from the MS/MS spectrum, the use of intelligent database searching approaches becomes indispensable. Naturally, these approaches will become more valu-

---

Fig. 3. *(opposite page)* Simplified representation of how a peptide falls apart in a tandem MS experiment, resulting in the spectrum at the bottom, and how this leads to elucidation of the (partial) amino acid sequence of a peptide. (Top) An arbitrary peptide in one-letter code is given. When this peptide is subjected to collisions with argon atoms, cleavages occur that preferentially take place at peptide bonds. This leads to the formation of so-called b- and y-ions, a few of which are depicted in the spectrum at the bottom. B-ions include the N-terminus and y-ions contain the C-terminus. When the peptide breaks between the two leucines (L), the ions b10 and y5 are formed. In analogous fashion, the other fragments are also formed. In the mass spectrum, this leads to a series of b-ions and a series of y-ions. It can easily be seen that the difference between the b9 and b10 ion is a leucine, and the difference between the b8 and b9 is a valine (V). From the b5-b10 series, the sequence L-M-Q-V-L can be deduced.

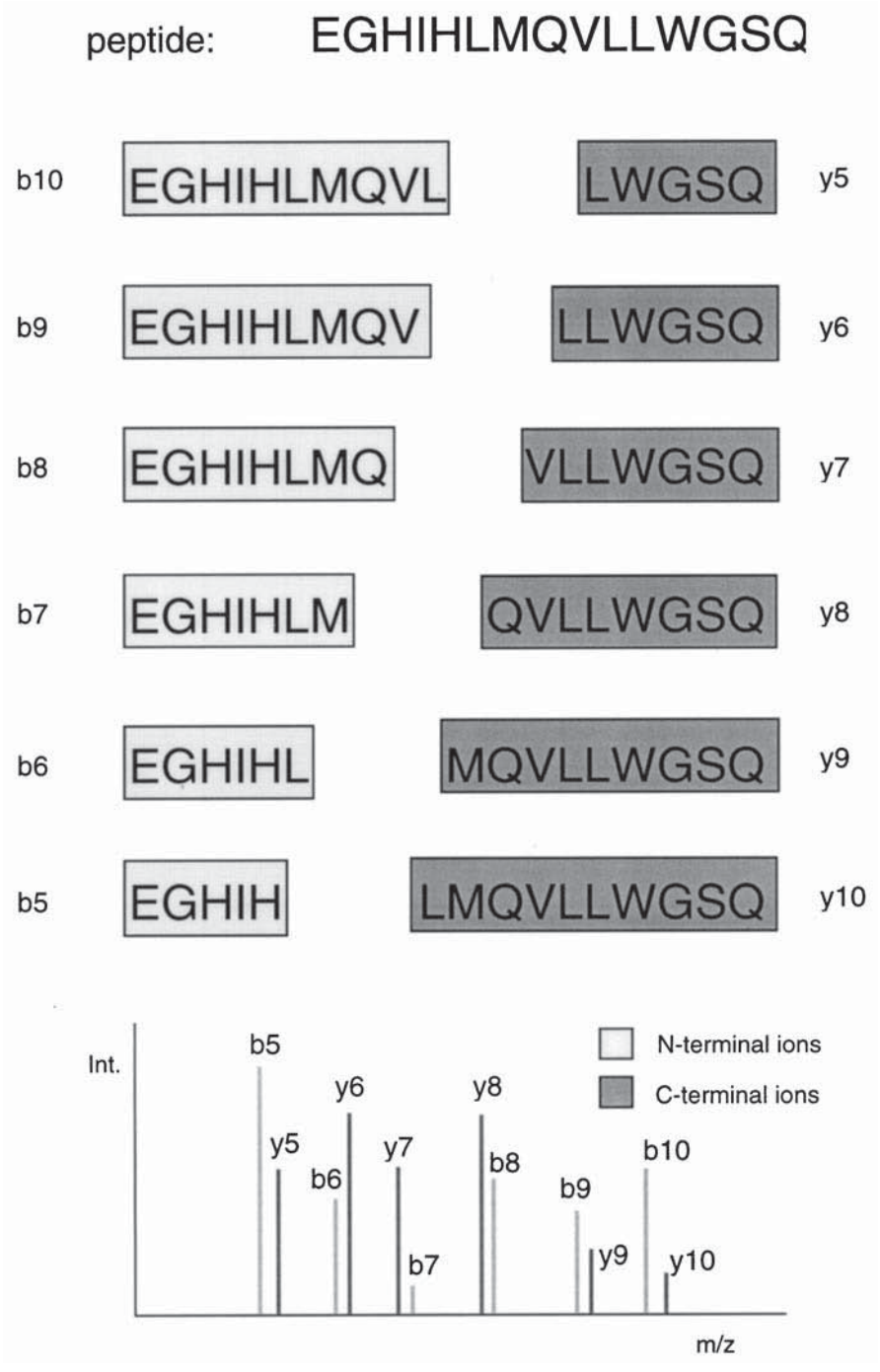

peptide: EGHIHLMQVLLWGSQ
b10
EGHIHLMQVL
LWGSQ
y5
b9
EGHIHLMQV
LLWGSQ
y6
b8
EGHIHLMQ
VLLWGSQ
y7
b7
EGHIHLM
QVLLWGSQ
y8
b6
EGHIHL
MQVLLWGSQ
y9
b5
EGHIH
LMQVLLWGSQ
y10
Int.
b5
y5
y6
b6
y7
b7
y8
b8
b9
y9
b10
y10
N-terminal ions
C-terminal ions
m/z

Fig. 3.

able the more complete the databases become (*see* **Note 1**). This implies that, even though only a partial sequence is used for a database search, one can expect to find the correct complete peptide sequence in the database, and thereby also the corresponding protein and gene.

Next we discuss the structural determination of a 22-mer peptide encountered in the course of our work in the elucidation of gluten-derived T-cell epitopes.

2. Materials

1. Perform on-line flow injection analysis electrospray ionization-mass spectrometer (ESI-MS) on a hybrid quadrupole TOF mass spectrometer, a Q-TOF (Micromass, Manchester, UK).
2. Use an on-line nano-electrospray interface (capillary tip with a 20 μm id and 90 μm od) with an approximate flow rate of 250 nL/min (*see* **Note 2**).
3. Obtain this flow by splitting the 0.4 mL/min flow of a conventional high-pressure gradient system with an Acurate flow splitter (LC Packings, Amsterdam, The Netherlands).
4. Dissolve HPLC fractions in water:methanol:acetic acid 50:50:1 (v/v/v).
5. Inject with a dedicated micro/nano HPLC autosampler, the FAMOS (LC Packings, Amsterdam, The Netherlands (*see* **Note 3**).
6. Record mass spectra from mass 50–2000 Dalton every second with a resolution of 5000 full width half maximum (*see* **Note 4**).
7. Expand the system for on-line HPLC-MS with a PEPMAP C-18 nano-HPLC column (LC Packings).

3. Methods

3.1. Sequence Determination of a Gluten-Derived Peptide

1. HLA-DQ2- and DQ8-restricted, gluten-specific T-cells are present in small intestinal biopsies of celiac patients. The gluten preparations that are used to stimulate such T-cells are usually pepsin/trypsin digests of gluten that contain a large number of different peptides. MS can be used to characterize the amino acid sequence of individual gluten peptides in such preparations by following these steps: Fractionate the gluten preparation by reversed-phase HPLC.
2. Take these individual fractions of varying complexity as a starting point for analysis *(9)* (*see* **Note 5**). **Figure 4A** shows the mass spectrum of one of these rpHPLC fractions analyzed. Several components are present, all different gluten-derived peptides (*see* **Note 6**).
3. Select a particular ion, in this example the ions with mass-to-charge ratio 827 (triply charged) with the first quadrupole, and subject it to MS/MS; that is, generate the fragment ion spectrum (**Fig. 4B**).
4. Determine the sequence from the spectrum (not shown here). This sequence is based on the identification of a series of b-ions in the spectrum. The mass of

these identified b-ions is indicated in bold (*see* **Fig. 5**) in the list of theoretical fragments of this peptide. In addition, a few y-ions are found that confirm this sequence. As can be seen in **Fig. 5**, the presence of ions from both series gives a reading from both termini, which together cover the total sequence.

3.2. Database Searching with SEQUEST and PepSearch

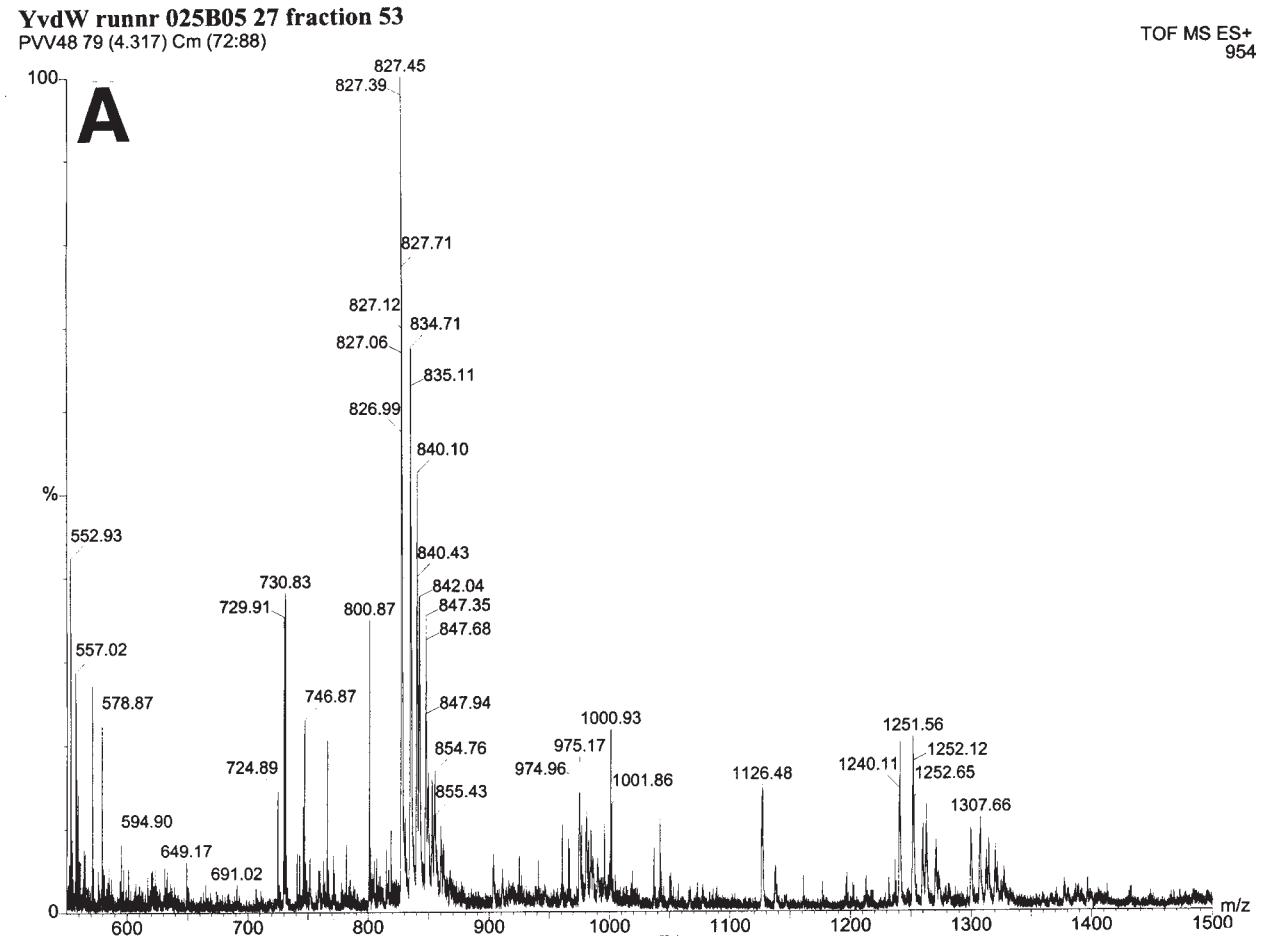
Here we discuss two database searching approaches, both of which give a good impression of the enormous value of mass spectrometric information, using SEQUEST ***(10)*** and PepSearch ***(11)***, which are both commercially available.

SEQUEST does not rely on any human interpretation. The tandem MS spectrum, together with the mass of the precursor ion, is the input for the program. The program compares all appropriate theoretical mass spectra within the database (based on a number of conditions, such as the precursor ion mass) with the MS/MS spectrum of the unknown peptide, and gives a matching score. This comparison results in a list of best scores, with the best ones at the top of the list. As an example, the MS/MS spectrum of **Fig. 4B** was fed into the program, using a *Triticum aestivum* subset of the combined SwissProt, PIR, and TREMBL databases. **Figure 6** presents the result of this search. The correct peptide can be found with a high score as the second and third hit, and the first hit is a quite similar peptide with one amino acid substitution.

PepSearch requires a (very limited) manual spectral interpretation, resulting in the elucidation of a short amino acid stretch in the unknown peptide, e.g., PQN interpreted from spectrum 4b. Obviously, the start and end mass of this stretch are known and together. This information is used to form a sequence tag such as (762.4)PQN(1102.6). In addition, the mass of the precursor is known—in this case 2478 Dalton. This information is quite specific. Application to our problem using the *T. aestivum* subset resulted in the list presented in **Fig. 7**. All hits satisfy the boundary conditions.

Although in this particular case the sequence tag was sufficient to find the right peptide, it is not always enough for a unique hit. Additional information can then be used, such as when the peptide is known or expected to be, e.g., a trypsin fragment. This is an optional and very stringent condition in the search. Moreover, molecular mass and p*I* data about the original protein, from which the peptide stems, can be used as extra limiting conditions.

The best hits in both approaches are easily checked by comparison of the predicted fragments with the actual spectrum. Both programs can also be helpful, even if the exact correct sequence is not found. Thus, by working interactively, spectrum interpretation is facilitated. Because of all the checks in the overall procedure, mistakes are not easily made. As a final check, predicted peptides can be synthesized and their fragmentation patterns compared with those of the native peptide.


YvdW runnr 025B05 27 fraction 53
PVV48 79 (4.317) Cm (72:88)
TOF MS ES+
954
A
100
%
0
827.45
827.39
827.71
827.12
834.71
827.06
835.11
826.99
840.10
552.93
840.43
730.83
842.04
729.91
800.87
847.35
847.68
557.02
578.87
746.87
847.94
1000.93
975.17
854.76
724.89
974.96
1001.86
1126.48
1251.56
1252.12
1240.11
1252.65
855.43
1307.66
594.90
649.17
691.02
600
700
800
900
1000
1100
1200
1300
1400
1500
m/z

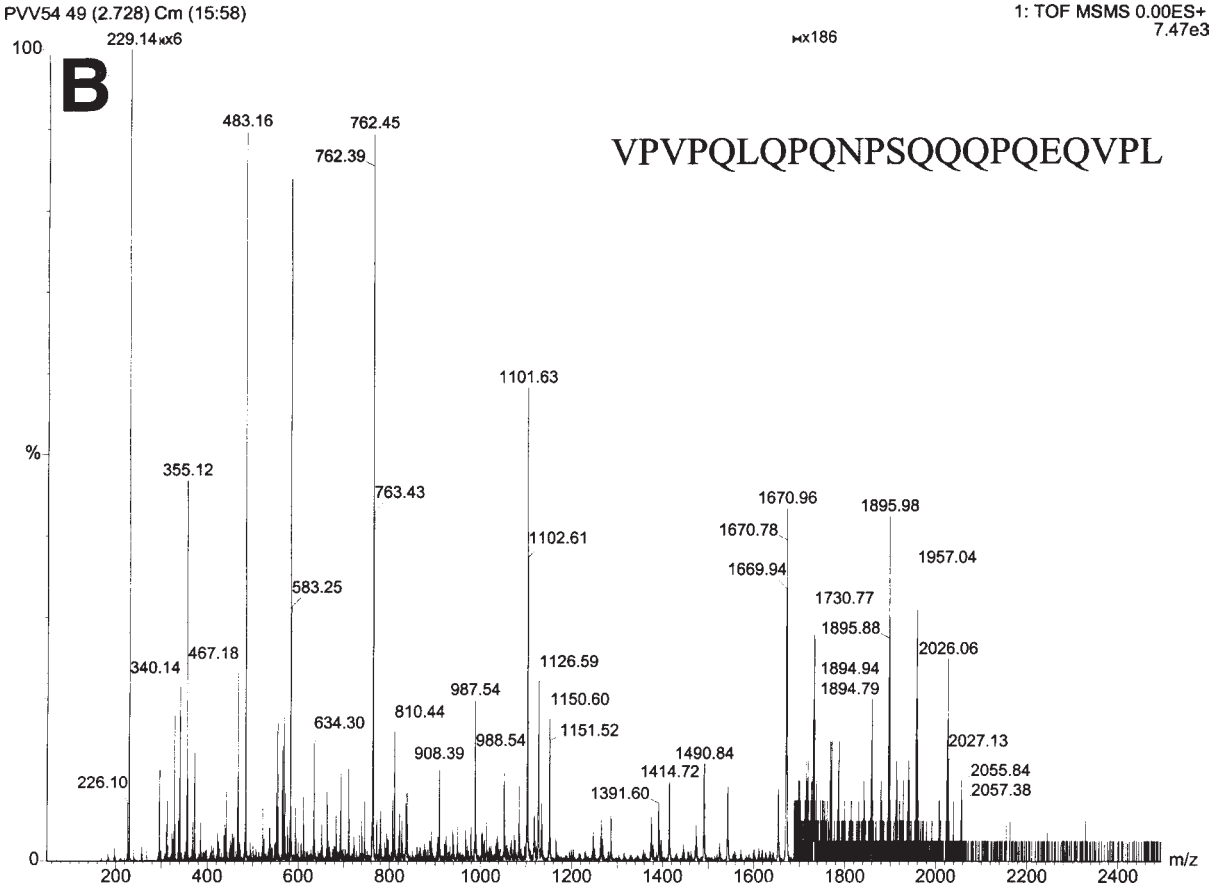


Fig. 4. Mass spectrum of an HPLC purified fraction after work-up of a pepsin/trypsin digestion of gluten **(A)**. The spectrum displays several components, of which 827 (3+) was selected for MS/MS. **(B)** the tandem MS spectrum from which the indicated sequence was deduced.

b-ions	100.1	**197.1**	**296.2**	393.3	**521.3**	**634.4**	**762.5**	**859.5**	**987.6**	**1101.6**	**1198.7**
Sequence (1-11)	Val	Pro	Val	Pro	Gln	Leu	Gln	Pro	Gln	Asn	Pro
y-ions	#	2380.2	2283.2	2184.1	2087.1	1959.0	1846.0	1717.9	1620.8	1492.7	1378.7
b-ions	**1285.7**	**1413.8**	**1541.8**	**1669.9**	**1766.9**	**1895.0**	**2024.0**	2152.2	2251.2	2348.2	#
Sequence (12-22)	Ser	Gln	Gln	Gln	Pro	Gln	Glu	Gln	Val	Pro	Leu
y-ions	1281.6	1194.6	1066.6	**938.5**	**810.4**	713.4	585.3	456.3	**328.2**	**229.2**	132.1

Fig. 5. Sequence of the gliadin peptide (*see* **Fig. 4**) and the theoretical mass spectrometric fragment ions (b- and y-ions). The actual fragments found in the tandem MS spectrum are displayed in bold.

1. GDA4_WHEAT +1 **VPVPQLQPQNPSQQQPQKQVPL**
   D22364 alpha/beta-gliadin precursor (clone A735) - wheat
2. GDA7_WHEAT +3 **VPVPQLQPKNPSQQQPQEQVPL**
   Q41546 Q41546 triticum aestivum (wheat). alpha/beta-glia
   Q41529 Q41529 triticum aestivum (wheat). alpha-gliadin s
   S07924 alpha/beta-gliadin precursor - wheat
3. GDA1_WHEAT +9 **VPVPQLQPQNPSQQQPQEQVPL**
   GDA3_WHEAT P04723 triticum aestivum (wheat). alpha/beta-
   GDA5_WHEAT P04725 triticum aestivum (wheat). alpha/beta-
   GDA9_WHEAT P18573 triticum aestivum (wheat). alpha/beta-
   Q41545 Q41545 triticum aestivum (wheat). (t. aestivum) a
   Q41509 Q41509 triticum aestivum (wheat). alpha-gliadin.
   Q41533 Q41533 triticum aestivum (wheat). alpha-gliadin m
   S10015 alpha/beta-gliadin precursor (clone MM1) - wheat
   A22364 alpha/beta-gliadin precursor (clone A42) - wheat
   E22364 alpha/beta-gliadin precursor (clone A1235) - whea
4. CYF_WHEAT **VLANGKKGGLNVGAVLILPEGFELA**
5. GDA4_WHEAT +6 **QGSFQPSQQNPQAQGSVQPQQLP**
   GDA7_WHEAT P04727 triticum aestivum (wheat). alpha/beta-
   GDA9_WHEAT P18573 triticum aestivum (wheat). alpha/beta-
   Q41546 Q41546 triticum aestivum (wheat). alpha/beta-glia
   S10015 alpha/beta-gliadin precursor (clone MM1) - wheat
   S07924 alpha/beta-gliadin precursor - wheat
   D22364 alpha/beta-gliadin precursor (clone A735) - wheat
6. GDA7_WHEAT +3 **LPQLPYPQPQSFPPQQPYPQQ**
   Q41546 Q41546 triticum aestivum (wheat). alpha/beta-glia
   Q41529 Q41529 triticum aestivum (wheat). alpha-gliadin s
   S07924 alpha/beta-gliadin precursor - wheat
7. ATPB_WHEAT **VTFPPGKLPYIYNALVVQSRDT**

Fig. 6. Output of the database search program SEQUEST after input of the tandem MS spectrum (**Fig. 4**), showing the potential of the approach. A *T. aestivum* subset of a nonredundant database was searched. Hits 2 and 3 are the correct ones. Hit 1 is identical except for one (E → K18) residue.

Protein	Mol. Mass	Description line	Sequence found
GDA_1	30403	P04721 triticum aestivum	VPVPQLQPQNNPSQQQPQEQVPL
GDA_3	32236	P04723 triticum aestivum	VPVPQLQPQNNPSQQQPQEQVPL
GDA_4	34238	P04724 triticum aestivum	VPVPQLQPQNNPSQQQPQKQVPL
GDA_5	36666	P04725 triticum aestivum	VPVPQLQPQNNPSQQQPQEQVPL
GDA_7	36117	P04727 triticum aestivum	VPVPQLQPQNNPSQQQPQEQVPL
GDA_9	35397	P18573 triticum aestivum	VPVPQLQPQNNPSQQQPQEQVPL
Q41545	36538	Q41545 triticum aestivum	VPVPQLQPQNNPSQQQPQEQVPL
Q41546	36120	Q41546 triticum aestivum	VPVPQLQPQNNPSQQQPQEQVPL
Q41509	33046	Q41509 triticum aestivum	VPVPQLQPQNNPSQQQPQEQVPL
Q41529	36144	Q41529 triticum aestivum	VPVPQLQPQNNPSQQQPQEQVPL
Q41533	29995	Q41533 triticum aestivum	VPVPQLQPQNNPSQQQPQEQVPL
S10015	35397	Alpha/beta-gliadin precursor	VPVPQLQPQNNPSQQQPQEQVPL
S07924	36120	Alpha/beta-gliadin precursor	VPVPQLQPQNNPSQQQPQEQVPL
D22364	37783	Alpha/beta-gliadin precursor	VPVPQLQPQNNPSQQQPQKQVPL
A22364	36666	Alpha/beta-gliadin precursor	VPVPQLQPQNNPSQQQPQEQVPL
E22364	37016	Alpha/beta-gliadin precursor	VPVPQLQPQNNPSQQQPQEQVPL

Fig. 7. Output of the database search program PepSearch after input of the peptide sequence tag (762.4)PQN(1102.6), total mass 2478 Dalton, which arose from manual interpretation. A *T. aestivum* subset of a nonredundant database was searched. Within the applied boundary conditions, a variant peptide with a lysine (K) instead of a glutamic acid (E) was found.

4. Notes

1. The major DNA sequencing efforts being carried out today, such as the human genome project, but also in the field of various agrohorticultures, will continue to demonstrate the ever-expanding potential of these search routines.
2. Alternatively, use borosilicate needles of type A (Micromass) for off-line nanoelectrospray studies. Load the needles with 1.5 μL of solutions of the analyte in water:methanol:acetic acid 50:50:1 (v/v/v). The needles spray for 45–60 min in favorable cases.
3. Since these samples are often extremely precious, it is important not to use more of them than is actually used in the analysis. The injection system should be capable of doing so, but many standard HPLC injectors are not optimal. Miniaturization is a key element in an analytical procedure, when only small absolute sample amounts are available. In practice, this means that sample handling is of utmost importance, because quantitative transfer of minute samples, e.g., is not a trivial matter, as a result, among others, of aspecific interactions with chromatographic materials or vial walls. Be sure not to introduce dead volumes when making connections. Miniaturization in the systems described here means, e.g., working with 25-μm id transfer capillaries or, in the case of off-line nanoelectrospray, working with internal needle diameters of a few micrometers. Therefore, filtering of solvents, preferably through 0.5-μm filters is strongly recommended.
4. This resolution allows direct determination of the charge state and the monoisotopic mass. Select ions in MS/MS mode with a window of 2 Daltons with the

first quadrupole, and collect the fragments after collision with high efficiency with the orthogonal TOF mass spectrometer. Apply argon as the collision gas (4×10^{-5} mbar), and adjust the collision voltage around 30 V.

5. These fractions only contain peptides with a mass below 10 kDa. Because of the reversed-phase fractionation, they are free of salts and, therefore, can be directly analyzed by electrospray MS.
6. An important characteristic of electrospray ionization is that ions with different charge states are formed from the same component (generally one charge on every 8–15 amino acids). In addition, salt adducts of such components can be formed, which can complicate the spectra.

References

1. Dongre, A. R., Eng, J. K., and Yates, J. R. III (1997) Emerging tandem-mass-spectrometry techniques for the rapid identification of proteins. *Trends Biotechnol.* **15(10),** 418–425.
2. Yates, J. R. III (1996) Protein analysis by mass spectrometry. *Methods Enzymol.* **271,** 351–377.
3. Gillece-Castro, B. L. and Stults, J. (1996) Peptide characterization by mass spectrometry. *Methods Enzymol.* **271,** 403–427.
4. Chait, B. T. and Kent, S. B. H. (1992) Weighing naked proteins: practical, high-accuracy mass measurement of peptides and proteins. *Science* **257,** 1885–1893.
5. Hillenkamp, F., Karas, M., Beavis, R. C., and Chait, B. T. (1991) Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal. Chem.* **63,** 1193A–1203A.
6. Fenn, J. B., Mann, M., Kai Meng, C., Fu Wong, S., and Whitehouse, C. M. (1989) Electrospray ionization for mass spectrometry of large biomolecules. *Science* **246,** 64–71.
7. Neubauer, G., King, A., Rappsilber, J., Calvio, C., Watson, M., Ajuh, P., Sleeman, J., Lamond, A., and Mann, M. (1998) Mass spectrometry and EST-database searching allows characterization of the multi-protein spliceosome complex. *Nat. Genet.* **20(1),** 46–50.
8. Den Haan, J. M., Sherman, N. E., Blokland, E., Huczko, E., Koning, F., Drijfhout, J. W., Skipper, J., Shabanowitz, J., Hunt, D. F., Engelhard, V. H., and Goulmy, E. (1995) Identification of a graft versus host disease-associated human minor histocompatibility antigen. *Science* **268,** 1476–1480.
9. Van de Wal, Y., Kooy, Y., van Veelen, P., Pena, S., Mearin, L., Molberg, O., Lundin, K. E. A., Sollid, L. M., Mutis, T., Benckhuijsen, W., Drijfhout, J. W., and Koning, F. (1998) Small intestinal T cells of celiac disease patients recognize a natural pepsin fragment of gliadin. *Proc. Natl. Acad. Sci. USA* **95,** 10,050–10,058.
10. Eng, J., McCormack, A., and Yates, J. III (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in protein databases. *J. Am. Soc. Mass Spectrom.* **5,** 976–989.
11. Mann, M. and Wilm, M. (1994) Error-tolerant identification of peptides in sequence databases by peptide sequence tags. *Anal. Chem.* **66,** 4390–4399.

7

Synthetic Peptide Libraries for T-Cell Epitope Identification

Hoebert S. Hiemstra, Jan W. Drijfhout, and Frits Koning

1. Introduction

This chapter describes a methodology for elucidating immunogenic epitopes stimulatory for $CD4^+$ T-cell clones (**Fig. 1**). The methodology makes use of synthetic peptide libraries and must be regarded as an alternative to other approaches, such as peptide elution or the application of genetic libraries. The methodology only requires knowledge about the restriction element of the T-cell clone. The restriction element determines which major histocompatibility complex (MHC)–binding anchor motif must be built into the library peptides. A synthetic peptide library is prepared comprising approx 8 million peptides. The synthesis proceeds via a mix-and-split protocol using a solid-phase approach on a hybrid resin ***(1,2)***. On a hybrid resin, most of the peptide material (84%) is attached via an acid-labile linker whereas the remaining part of the peptide material is acid-stable attached ***(3)***. During synthesis, resin-bound peptides comprising 14 amino acid residues are produced, with each resin bead containing one unique peptide ***(4,5)***. The beads are split into 384 pools, with each pool containing 20,000 beads. From each pool, about 28% of the peptide material is cleaved from every bead. Subsequently, in the first screening round, the 384 pools, each containing 20,000 solubilized peptides, are tested in a proliferation assay with the T-cell clone.

Stimulating pools are selected for screening in the second round. Beads from these pools, still containing 72% of the peptide material, are split into 288 pools, each containing about 70 beads. Again 28% of the peptide material is cleaved off and tested for its proliferative activity. Active pools are selected for the third screening round in which solubilized peptides (28%) are tested in 0/1 bead/well 96 wells. The remaining 16% of the peptide material on the

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

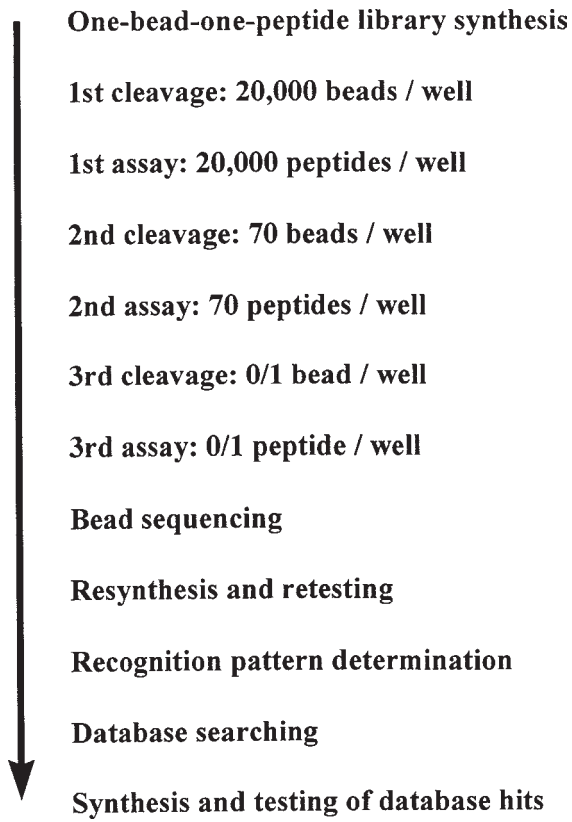


Fig. 1. Flow diagram of the complete procedure for the identification of T-cell epitopes using synthetic peptide libraries ***(1)***.

bead, corresponding to the active peptide, is sequenced by Edman degradation, thus providing the sequence of the stimulatory peptide (the mimicry epitope). These mimicry epitopes can be used, e.g., in the design of antagonists for immune intervention ***(6)***. The mimicry epitope sequence may also be used as lead sequence for epitope definition. In that case, the importance of each amino acid in the sequence for T-cell recognition is determined by amino acid omission and substitution analysis, using synthetic peptides ***(2)***. This yields a recognition pattern, i.e., a pattern that reflects for each amino acid position in the sequence those amino acids necessary for recognition ***(2)***. This recognition pattern is used for database searching with the program PeptideSearch. The database to be searched can contain all information about protein sequences known to date, but it can also be limited to a relevant subset database. For a celiac disease–related T-cell clone, a database comprising only gliadin and

glutenin proteins can be searched ***(7–11)***. Peptides from the database that fit the search pattern are synthesized and tested for recognition, thus yielding the natural epitope.

2. Materials

All solvents are synthesis grade or pro analysi.

1. Tentagel-S-AM peptide synthesis resin, loading about 0.2–0.25 mmol/g, particle size 90 μm, no. S30022 (Rapp Polymere, Tübingen, Germany).
2. Tentagel-H-AM library synthesis resin, loading about 0.2–0.25 mmol/g, particle size 90 μm, 84% Fmoc-linker, 16% Fmoc-norleucine, no. BH30902.1922 (Rapp Polymere).
3. Fmoc-amino acids ***(12)***; side chain–protecting groups as follows:
   a. tBu (*tert* butyl) for aspartic acid, glutamic acid, serine, threonine and tyrosine.
   b. Trityl for asparagine, glutamine, histidine, and cysteine.
   c. *Tert* butyloxycarbonyl for lysine and tryptophan.
   d. 2,2,5,7,8-Pentamethylchroman-6-sulfonyl for arginine.

3. Methods

3.1. Peptide Library Synthesis

In the following protocol all natural amino acids are used, except cysteine (cysteine complicates synthesis and handling). All procedures are performed at room temperature.

1. Weigh 4 g of Tentagel-H-AM resin (containing about 8 million beads) into a polypropylene 175-mL flask (no. 2110-0006, Nalgene, Hereford UK). On each bead, about 90–100 pmol of peptide can be synthesized.
2. Swell the beads for 30 min with 40 mL of *N*-methylpyrrolidon (NMP).
3. Divide the slurry into equal portions by transferring to 19 individual 5-mL reactors (no. V050PE000, Multisyntech, Bochum, Germany).
4. Remove the solvent on a vacuum box and wash each reactor three times with 2 mL of NMP.
5. Remove the Fmoc-protection with 20% piperidine in NMP. Do this three times, each for 5 min. Use 1.5 mL of piperidine solution per reactor.
6. Wash each reactor six times with 2 mL of NMP.
7. Next add to each reactor in the following order freshly prepared solutions of 65 mg (200 μmol) of Fmoc-γ-aminobutyric acid (Fmoc-Gaba-OH) in 400 μL of NMP; 104 mg (200 μmol) of benzotriazole-1-yl-oxy-*tris*-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) in 400 μL of NMP; and 100 μL of a solution of *N*-methyl morpholin (NMM)/NMP 1:1 (v/v). Stir to homogenize.
8. Allow mixture to react for 1 h.
9. Repeat as in **step 6**.
10. Repeat as in **step 5**.
11. Repeat as in **step 6**.

3.2. Commencement of Coupling Cycle

1. Couple in each reactor a different natural amino acid (except cysteine) by adding, to the reactor, freshly prepared solutions of (*see* **Note 1**) 200 μmol of Fmoc-amino acid-OH in 400 μL of NMP; 200 μmol of PyBOP in 400 μL of NMP; and 100 μL of a solution of NMM/NMP 1:1 (v/v). Stir to homogenize.
2. Allow mixture to react for 1 h.
3. Repeat as in **Subheading 3.1.**, **step 6**.
4. Remove the resin from each reactor by:
   a. Suspending the resin from each reactor in 2 mL of 1,2-dichloroethane (DCE)/acetonitrile (ACN) 82:18 (v/v) and transferring to a 175-mL polypropylene flask.
   b. Suspending the remaining resin in each reactor in 2 mL of DCE/ACN 82:18 (v/v) and transferring to the same 175-mL polypropylene flask.
5. Agitate the resin suspension for at least 1 min on a vortex in order to homogenize the beads completely.
6. Divide the slurry into equal portions by transferring to the 19 individual 5-mL reactors.
7. Repeat as in **Subheading 3.1.**, **step 6**.
8. Repeat as in **Subheading 3.1.**, **step 5**.
9. Repeat as in **Subheading 3.1.**, **step 6**.

3.3. Completion of the Coupling Cycle

1. Repeat **steps 1–9** in **Subheading 3.2.** until peptides are of the desired length (e.g., 14-mers for class II binding peptides).
2. After the last Fmoc-deprotection, wash each reactor with 10 mL of dichloromethane (DCM), 10 mL of ether/DCM 1:1 (v/v), and 10 mL of diethyl ether (ether), successively.

3.4. Peptide Library Screening

1. Suspend the resin from each reactor in 1 mL of DCE/ACN 82:18 (v/v) and transfer to a 175-mL polypropylene flask.
2. Suspend the remaining resin in each reactor in 1 mL of DCE/ACN 82:18 (v/v) and transfer to the same 175-mL polypropylene flask.
3. Divide the suspended resin equally over 4 × 96 well solvent-resistant multiscreen-PF1 filter plates (no. MAR1N1010, Millipore, Molsheim, France), and remove the solvent by suction on the multiscreen assay system (Millipore).
4. Wash each well with 3X 200 μL of ACN and 3X 200 μL of ether, respectively.
5. Air-dry overnight.
6. Protocol for one plate: Place one filtration plate on the multiscreen assay system and add to each well 150 μL of trifluoroacetic acid (TFA)/water/ACN 10:1:9 (v/v/v). Make sure the wells are not leaking.
7. After exactly 90 min, apply vacuum and collect the filtrate in a serocluster polypropylene U-bottomed plate (no. 3794, Costar, Cambridge, MA).
8. Directly wash each well with 75 μL of TFA/water/ACN 10:1:9 (v/v/v) and collect in the same U-bottomed plate.

9. Remove the U-bottomed plate and directly wash the beads with 0.5 *M* Tris-HCl (pH 7.5)/ACN 1:1 (v/v), 4X 200 µL per well, and incubate in the same buffer for 1 h (*see* **Note 2**).
10. Wash the beads successively with water/ACN 1:1 (v/v), ACN, DCM, and ether, 2X 200 µL per well and dry.
11. Store the beads containing plate at –20°C.
12. Dry the plate from **step 8** with the filtrates containing about 28% of the peptides under vacuum overnight or in a system that provides (for a 96-well format) multiple nitrogen streams for approx 30 min.
13. Remove the protecting groups by adding to each well 100 µL of TFA/water/ethanethiol 18:1:1 (v/v) and allow to react for 3 h.
14. Dry the plate as described in **step 12**.
15. Dissolve the peptides by adding 5 µL of dimethylsulfoxide (DMSO) to each well.
16. Dilute and neutralize by adding 200 µL of 30 m*M* sodium phosphate (pH 7.5) to each well. This solution is used in a proliferation assay.
17. For the second screening, divide beads from active pools containing about 20,000 beads into three 96-well filtration plates, and perform **steps 6–16** using a reaction time of 180 min at **step 7**.
18. For the third screening, divide beads from active pools containing about 70 beads in one 96-well filtration plate, and perform **steps 6–16** using a 30-min reaction time at **step 7**, now using TFA/water 19:1 (v/v).
19. Apply beads from active wells in the third screening to gas-phase sequencing using Edman degradation to determine the amino acid sequence of the peptide on the bead (*see* **Note 3**). This provides the sequence of the mimicry epitope (*see* **Note 4**).
20. Resynthesize and test this peptide to confirm the activity.

3.5. Proliferation Assay

1. Perform assay in flat-bottomed 96-well plates (no. 655160, Greiner, Frickenhausen, Germany) in complete Iscove's modified Dulbecco's medium (no. 041-90898, Gibco-BRL, Paisley, Scotland), containing 10% pooled human serum.
2. For $CD4^+$ T-cell proliferation assays for testing library pools and synthetic peptides, use 1×10^4 T-cells and 5×10^4 irradiated HLA-matched peripheral blood mononuclear cells per well (*see* **ref. *8*** for cell preparation).
3. Test library pools in a quantity of 7 µL of library solution per well giving final test concentrations of 5 n*M* for each individual peptide and 0.1% DMSO (v/v) (*see* **Notes 5–7**).
4. Add ^3H labeled thymidine after 72 h, harvest cells, and count activity of the T-cell DNA after a further 18 h.

3.6. Determination of the Recognition Pattern

1. Based on the amino acid sequence of the mimicry epitope, determine a recognition pattern. A recognition pattern comprises particular amino acids that are allowed at each position of the peptide. In most cases, a recognition pattern is restricted to the

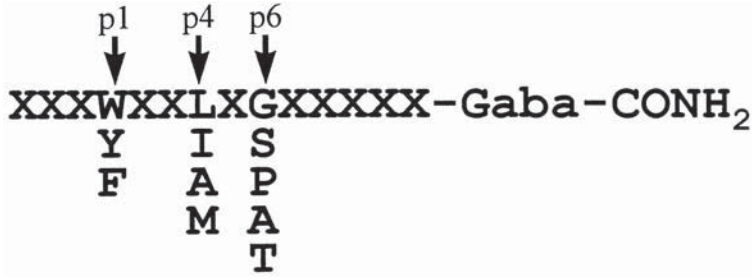


Fig. 2. Design of an HLA-DR1-dedicated library ***(1)***. Indicated are the anchor amino acids of relative positions 1, 4, and 6. X represents a random position. All 19 amino acids are present ***(1)***.

amino acid stretch located in the MHC-peptide binding groove. As such, a stretch of nine amino acids is sufficient. Normally the library is constructed such that these nine amino acids are in the middle part of the library peptides, i.e., the part that also contains the anchor residues for MHC binding (**Fig. 2**).

2. Various ways for determining the recognition pattern are possible. For example, synthesize 180 peptides comprising all possible substitutions with natural amino acids at all nine positions and test. Their activity: The results immediately yield the recognition pattern ***(2)***.
3. Alternatively, use partial substitution analysis and omission analysis. Synthesize 9 peptide mixtures, each mixture having 19 amino acids at each specific position, except the one that is defined in the mimicry epitope ***(2)***.

3.7. Searching the Databases with the Recognition Pattern

1. To search with PeptideSearch, databases or subsets of databases must be in FASTA-format. The recognition pattern is used as the input for the program PeptideSearch. All peptide hits from a database completely match the pattern and therefore have no ranking, in contrast to the output of a homology search.
2. Synthesize 14-mer database peptides (corresponding to the length of the initial library-derived 14-mer mimicry epitope) ***(3)*** and test for proliferation induction. Instead of searching in a particular subset database, selection of hits can also be applied after searching a more general database ***(13)***.

4. Notes

1. If anchor positions are synthesized, only the anchor residues can be incorporated in that particular cycle.
2. After first- and second-round cleavage, attention must be paid to the washing of the beads before storage. The morphology of the beads is such that liquid transport into and out of the bead is slow. This implies that washings must be performed extensively to prevent the content of the bead remaining acidic (which would cleave the peptide material on storage).

3. During Edman degradation, beads should be washed and extracted at least 50% longer than normal to cope with the slow liquid transport in the bead.
4. After the third screening round, peptide identification might be performed by tandem mass spectrometric sequencing of the solubilized peptide instead of by Edman sequencing of the bead.
5. In all screening rounds, stimulating peptides are normally present in suboptimal concentration (5 n*M*). This implies that only sensitively recognized mimicry epitopes are identified.
6. Normally, pools that show (in duplicate) counts that are fivefold higher than background are interpreted as positive. Counts are normally higher in the second and third screenings, compared with those in the first.
7. The concentration of DMSO in T-cell proliferation tests should normally not exceed 0.1%.

References

1. Hiemstra, H. S., Duinkerken, G., Benckhuijsen, W. E., Amons, R., de Vries, R. R. P., Roep, B. O., and Drijfhout, J. W. (1997) The identification of CD4+ T cell epitopes with dedicated synthetic peptide libraries. *Proc. Natl. Acad. Sci. USA* **94,** 10,313–10,318.
2. Hiemstra, H. S., van Veelen, P. A., Schloot, N. C., Geluk, A., van Meijgaarden, K. E., Willemen, S. J. M., Leunissen, J. A. M., Benckhuijsen, W. E., Amons, R., de Vries, R. R. P., Roep, B. O., Ottenhoff, T. H. M., and Drijfhout, J. W. (1998) Definition of natural T cell antigens with mimicry epitopes obtained from dedicated synthetic peptide libraries. *J. Immunol.* **161,** 4078–4082.
3. Hiemstra, H. S., Benckhuijsen, W. E., Amons, R., Rapp, W., and Drijfhout, J. W. (1998) A new hybrid resin for stepwise screening of peptide libraries combined with single bead Edman sequencing. *J. Peptide Sci.* **4,** 282–288.
4. Lam, K. S., Salmon, S. E., Hersh, E. M., Hruby, V. J., Kazmierski, W. M., and Knapp, R. J. (1991) A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* **354,** 82–86.
5. Houghten, R. A., Pinilla, C., Blondelle, S., Appel, J. R., Dooley, C. T., and Cuervo, J. H. (1991) Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery. *Nature* **354,** 84–86.
6. De Koster, H. S., Vermeulen, C. J., Hiemstra, H. S., Amons, R., Drijfhout, J. W., and Koning F. (1999) Definition of agonists and design of antagonists for alloreactive T cell clones using peptide libraries. *Int. Immunol.* **11,** 585–591.
7. Van de Wal, Y., Kooy, Y. M. C. Drijfhout, J. W., Amons, R., Papadopoulos, G. K., and Koning, F. (1997) Unique peptide-binding characteristics of the disease-associated DQ(α1*0501, β1*0201) vs the non-disease-associated DQ(α1*0201, β1*0202) molecule. *Immunogenetics* **46,** 484–492.
8. Van de Wal, Y., Kooy, Y. M. C., van Veelen, P. A., Peña, S. A., Mearin, L. M., Molberg, O., Lundin, K. E., Sollid, L. M., Mutis, T., Benckhuijsen, W. E., Drijfhout, J. W., and Koning, F. (1998) Small intestinal T cells of celiac disease patients recognize a natural pepsin fragment of gliadin. *Proc. Natl. Acad. Sci. USA* **95,** 10,050–10,054.

9. Van de Wal, Y., Kooy, Y. M. C., Drijfhout, J. W., Amons, R., and Koning, F. (1996) Peptide-binding characteristics of the Coeliac disease associated DQ(α1*0501, β1*0201) molecule. *Immunogenetics* **44,** 246–253.
10. Van de Wal, Y., Kooy, Y. M. C., van Veelen, P., Vader, W., August, S. A., Drijfhout, J. W., Peña, S. A., and Koning, F. (1999) Glutenin is involved in the gluten-driven mucosal T cell response. *Eur. J. Immuol.* **29,** 3133–3139.
11. Van de Wal, Y., Kooy, Y., van Veelen, P., Pena, S., Mearin, L., Papadopoulos, G., and Koning, F. (1998) Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. *J. Immunol.* **161,** 1585–1588.
12. Fields, G. B. and Noble, R. L. (1990) Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Peptide Protein Res.* **35,** 161–214.
13. Mann, M. and Wilm, M. S. (1994) Error-tolerant identification of peptides in sequence databases by peptide sequencing. *Anal. Chem.* **66,** 4390–4399.

8

Characterization of HLA-DQ-Specific Peptide-Binding Motifs

Yvonne van de Wal, Reinout Amons, and Frits Koning

1. Introduction

Several immunological disorders display a striking association with particular HLA alleles. Although the basis for these HLA-disease associations is not completely understood, it is likely that peptides bound to the disease-associated molecules play a role in pathogenesis. The function of HLA molecules is to bind and present peptide antigens to T-cells. Because of polymorphisms in the peptide-binding site, different HLA molecules bind different sets of peptides since the polymorphic residues within the binding pockets of the HLA molecule determine which amino acid side chains can be bound. Such preferences for certain amino acids (anchor residues) at particular sites in the HLA-bound peptide determine the so-called allele-dependent peptide-binding motif (for a review *see* **ref. *1***). Such a motif can be used to predict whether a particular peptide will bind to a given HLA-molecule. The characterization of the peptide-binding motifs of disease-associated class II molecules, therefore, can be a useful tool to identify potential disease-inducing peptides and antigens. Peptide-binding motifs can be defined using different approaches:

1. Characterization of naturally processed HLA-bound peptides ***(2–8)***
2. Analysis of large peptide pools from M13 bacteriophage peptide display libraries ***(9)*** or synthetic peptide libraries ***(10–12)***
3. Analysis of peptide-binding requirements in assays using peptide analogs with single amino acid substitutions ***(13–15)***.

Interestingly, for many immunological disorders, HLA-DQ rather than HLA-DR alleles are implicated as the primary genetic susceptibility factor. Genetic susceptibility to celiac disease, e.g., is strongly associated with the

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

expression of the HLA-DQ2 (α1*0501,β1*0201) molecule. In this chapter, we describe a method for the identification of HLA-DQ-specific peptide-binding motifs. This method includes the purification of HLA-DQ-specific monoclonal antibodies (mAb) from culture supernatants, the immobilization of these antibodies to Sepharose beads, the purification of HLA molecules from detergent-solubilized cells, the elution of peptides from the purified HLA molecules, the separation of the peptides by reverse-phase high-performance liquid chromatography (HPLC), and the characterization of their sequences by Edman degradation. Using the identified peptide sequences, cell-free peptide-binding assays can be established, which then allows analysis of the contribution that each individual amino acid residue makes to peptide binding ***(16,17)***. Finally, by performing extensive amino acid substitution analyses, the overall peptide-binding characteristics of the HLA-DQ molecule can be determined ***(16,17)***.

2. Materials

2.1. Purification of mAbs

1. Protein Free Hybridoma Medium (Gibco, Grand Island, NY).
2. Hemoflow F-40-S capillary dialyzer (Fresenius, Bad Homburg, Germany).
3. IgG binding buffer (Pierce, Rockford, IL).
4. Immunopure IgG elution buffer (Pierce).
5. Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden).
6. Bicinchominic acid (BCA) protein determination assay (Pierce).

2.2. Immobilization of Antibodies

1. Centriprep-30 filters (Amicon, Beverly, MA).
2. CNBR-activated Sepharose 4B beads (Pharmacia).
3. Coupling buffer: 0.1 *M* $NaHCO_3$, 0.5 *M* NaCl, pH 8.3.
4. Blocking buffer: 0.1 *M* Tris-HCl, pH 8.0.
5. Acetate buffer: 0.1 *M* NaAc, 0.5 *M* NaCl, pH 4.0.
6. Tris-HCl buffer: 0.1 *M* Tris, 0.5 *M* NaCl, pH 8.0.

2.3. Purification of HLA Molecules

1. 0.5% NP-40 lysis buffer: 50 m*M* Tris; 150 m*M* NaCl; 5 m*M* EDTA; 0.5% NP-40; 10 m*M* iodoacetamide; 0.1 m*M* AEBSF; and 1 μg/mL of the protease inhibitors leupeptin, chymostatin, pepstatin A, and antipain (all from Sigma, St. Louis, MO), pH 8.0.
2. Sepharose CL-4B beads (Pharmacia).
3. CNBR-activated Sepharose beads (Pharmacia).
4. Tris-NaCl buffer: 50 m*M* Tris, 150 m*M* NaCl, pH 8.0.
5. Centriprep-10 filters (Amicon).
6. *n*-Octylglucopyranoside (Sigma).

7. Elution buffer: 50 m*M* diethylamine (Fluka, Buchs, Switzerland), 150 m*M* NaCl, 1% *n*-octylglucopyranoside (fresh preparation).
8. Glycine buffer: 2 *M* glycine, pH 2.5.
9. SMART microHPLC system (Pharmacia).

2.4. Cell-Free Peptide-Binding Assay

1. Citrate-phosphate buffer: 42 m*M* citric acid, 90 m*M* Na_2HPO_4, pH 5.0.
2. Protease inhibitor mix: 10 m*M* AEBSF (Calbiochem, Ja Jolla, CA); 50 m*M* EDTA; and 10 μg/mL each of leupeptin, chymostatin, pepstatin A, and antipain (all from Sigma).
3. Sephadex G-50 (Pharmacia).

3. Methods

3.1. Purification of HLA-Specific mAbs

1. Use supernatant of hybridomas producing HLA-DQ-specific antibodies as a source of antibodies.
2. Culture the hybridoma cell lines in protein-free hybridoma medium. Depending on the total amount of antibodies required, 2–5 L of supernatant are collected.
3. Concentrate the supernatant using Hemoflow F-40-S-mediated capillary dialysis according to the manufacturer's specifications.
4. Purify the DQ-specific monoclonal antibodies (mAbs) from the concentrated culture supernatant using protein A Sepharose CL-4B beads, packed into a column as follows:
   a. Equilibrate the column with three bed volumes of binding buffer.
   b. Mix the concentrated culture supernatant 1:1 (v/v) with Immunopure IgG binding buffer and pass over the protein A-column.
   c. Wash the column with 5 bed volumes of binding buffer.
   d. Elute the mAbs with 3 bed volumes of Immunopure IgG elution buffer in fractions of one-half the bed volume.
   e. Directly adjust the pH of the fractions to pH 7.0 by adding binding buffer (usually one-tenth of the elution volume suffices).
   f. Determine the protein concentration with the BCA protein determination assay according to the manufacturer's specifications.

3.2. Immobilization of HLA-Specific Antibodies

1. Covalently couple the purified antibodies to Sepharose beads as follows:
   a. Concentrate the protein A–purified mAbs using 30-kDa filter systems.
   b. Wash CNBr-activated Sepharose 4B beads with 1 m*M* HCl (100 mL/g of beads; 1 g of beads can bind 5 mg of mAbs) on a glass filter.
   c. Equilibrate the beads with three bed volumes of coupling buffer.
   d. Mix the beads with the mAbs in a 1:1 ratio (v/v) and incubate for 2 h at room temperature while rotating.

e. Remove the supernatant and block the remaining active sites on the beads by incubating them in blocking buffer for 2 h at room temperature while rotating.
f. Wash the beads in three cycles, changing the washing buffers from acetate (pH = 4.0) to Tris (pH = 8.0).
g. Check the activity/specificity of the sample by immunoprecipitation.
h. Store the beads at 4°C.

3.3. Purification of HLA Molecules from Detergent-Solubilized Cells

1. Use bulk cultures of Epstein-Barr virus–transformed B-cell lines as a source of HLA molecules. Cell pellets are collected and can be stored at –70°C until use.
2. Solubilize about 30×10^9 cells in 150 mL of 0.5% NP-40 lysis buffer (*see* **Note 1**).
3. Remove insoluble material by centrifugation at 13,000*g* for 60 min.
4. Preclear the lysate by incubating the lysate with a bed volume of 10–20 mL of Sepharose CL-4B beads for 2 h at room temperature while rotating gently.
5. Remove the beads via a centrifugation step at 13,000*g* for 5 min.
6. Mix the supernatant with the CNBR-Sepharose-coupled mAbs and incubate overnight at 4°C while rotating gently.
7. Wash the beads on a glass filter with 5 bed volumes 0.005% NP-40 lysis buffer (without protease inhibitors), followed by 10 bed volumes of Tris-NaCl buffer (*see* **Note 2**).
8. Pack the beads in a column and elute the HLA-peptide complexes with 10% Hac. This results in both the release of the HLA-peptide complexes from the antibody column and the release of the peptide from the HLA molecule.
9. Separate the peptides from the HLA class II α- and β-chains by 10-kDa filtration.
10. Concentrate the <10-kDa fraction to approx 0.3 mL by lyophilization.
11. Fractionate the peptide material by reverse phase HPLC.
12. Sequence the dominant peptides in the fractions by (1) Edman degradation (*see* **Subheading 3.5.**) or (2) tandem mass spectrometry.

Use a different protocol for the purification of DQ molecules for cell-free peptide-binding assays:

1. Repeat **steps 1–6**, as previously detailed.
2. Wash the beads on a glass filter with five bed volumes of 0.5% NP-40 buffer, followed by five bed volumes of 0.005% NP-40 buffer, and five bed volumes of a Tris-NaCl buffer containing 1% *n*-octylglucopyranoside (all buffers without protease inhibitors) (*see* **Note 3**).
3. Pack the beads into a column and elute the HLA-peptide complexes with five bed volumes of elution buffer.
4. Neutralize the eluate to pH 7.0 by adding glycine buffer (*see* **Note 4**).
5. Concentrate the HLA-peptide complexes by 10-kDa filtration.
6. Determine the protein concentration with the BCA protein determination assay.
7. Check the purity of the HLA preparations on sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by staining with Coomassie blue (*see* **Note 5**).

3.4. Cell-Free Peptide-Binding Assay

1. Select a peptide on the basis of the eluted peptide sequences to establish a cell-free peptide-binding assay.
2. Synthesize this peptide with an additional N-terminal tyrosine residue to allow iodination with ^{125}I.
3. Establish peptide binding by mixing 1 µL of purified DQ (±17 nmol/mL); 1 µL of ^{125}I-radiolabeled peptide (±0.5 nmol/mL, giving rise to ±40,000 cpm); 6 µL of citrate-phosphate buffer; 2 mL of protease inhibitor mix, dissolved in a 0.5% NP-40 solution; and 5 mL of 15% dimethyl sulfoxide containing 0.02% NaN_3. (In the case of competition experiments, 5 µL of competitor peptide [ranging in concentration from 1–10 nmol/mL] is added simultaneously with the ^{125}I-labeled wild-type peptide.)
4. Incubate for 48 h at 37°C in the dark.
5. Separate the DQ-peptide complexes from unbound peptide by gel filtration on a 10-mL Sephadex G-50 column using phosphate-buffered saline containing 0.5% NP-40 as elution buffer.
6. Collect individual fractions and measure in a γ-counter.
7. Calculate the percentage of specific peptide binding as the ratio of counts per minute in the void volume (= HLA-bound peptide) to the total counts per minute recovered.

3.5. Edman Sequencing

1. Load the peptide samples on pretreated sequencing biphasic columns of the Hewlett-Packard protein sequencing system according to the company's instructions (*see* **Note 6**).
2. Sequence the sample on the Hewlett-Packard G1006A (Palo Alto, CA) protein sequencer system, which is equipped with a 2.1 × 250 mm narrow-bore phenylthio-hydentoin (PTH) amino acid column, and a Model 1100 liquid chromatograph with diode array detection. Use the 3.0 or 3.1 sequencing programs.

Alternatively, especially when a rather hydrophobic C-terminal end of the peptide is suspected, the following protocol is used to covalently couple the peptides to aminoaryl polyvinylidene difluoride (PVDF) (Millipore, Etten-Leure, NL):

1. Spot the peptide material present in a volatile solvent, e.g., 0.2% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile (ACN), repetitively in small increments (e.g., 1 µL) to one quarter of a disk of aminoaryl PVDF. Use a hair dryer if larger volumes are applied.
2. Dry the disks thoroughly by lyophilization.
3. Couple the peptide to the disk by applying 5 µL of a solution of 1 mg/mL of 1-(3 dimethylaminopropyl)-3-ethyl carbodiimide in 50 m*M* *N*-morpholino ethanesulfonic acid, pH 5.0, in 50% (v/v) ACN.
4. Let the moist disk segment air-dry at room temperature (20–30 min).
5. Repeat the coupling procedure once.

6. Wash the air-dried disk segment in distilled water.
7. Cut the disk segment into small strips.
8. Load the strips onto an empty sequencing column.
9. Sequence the sample as in **steps 1** and **2** of the first procedure under **Subheading 3.5.** (*see* **Note 7**).

4. Notes

1. Solubilization of cell pellets in 0.5% NP-40 lysis buffer should occur *slowly* and is best done overnight on a stirrer at 4°C.
2. For the elution of peptides from HLA molecules, freshly prepared buffers should be used (preferably autoclaved).
3. *n*-Octylglucopyranoside-containing preparations cannot be stored since precipitation occurs with time. A fresh preparation must be made for each experiment.
4. Adjustment of pH (±11.0–7.0) of diethylamine-eluted DQ molecules should be done cautiously.
5. HLA preparations of certain DQ alleles (in particular HLA-DQ8) tend to be relatively unstable. If possible, plan to do the experiments short term once the DQ preparation has been purified.
6. Pretreatment uses either the 3.0 program provided by the company or a cleaning program utilizing acetic anhydride to block any residual, sequenceable material in advance. Our experience (unpublished) suggests that with the latter program, in particular, a consistently lower initial background is obtained.
7. In our experience, all peptides coupled in this way could be sequenced completely. Although the acidic amino acids D and E are partially bound to the aminoaryl PVDF via their side chain carboxyl groups, they always remain clearly visible on sequencing, albeit in reduced amounts.

References

1. Rammensee, H. G., Friede, T., and Stevanovic, S. (1995) MHC ligands and peptide motifs: first listing. *Immunogenetics* **41,** 178–228 (review).
2. Rudensky, A. Y., Preston-Hurlburt, P., Hong, S. C., Barlow, A., and Janeway, C. A., Jr. (1991). Sequence analysis of peptides bound to MHC class II molecules. *Nature* **353,** 622–627.
3. Chicz, R. M., Urban, R. G., Gorga, J. C., Vignali, D. A., Lane, W. S., and Strominger, J. L. (1993) Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J. Exp. Med.* **178,** 27–47.
4. Kropshofer, H., Max, H., Muller, C. A., Hesse, F., Stevanovic, S., Jung, G., and Kalbacher, H. (1992) Self-peptide released from class II HLA-DR1 exhibits a hydrophobic two-residue contact motif. *J. Exp. Med.* **175,** 1799–1803.
5. Chicz, R. M., Urban, R. G., Lane, W. S., Gorga, J. C., Stern, L. J., Vignali, D. A. A., and Strominger, J. L. (1992) Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature* **358,** 764–768.

6. Chicz, R. M., Lane, W. S., Robinson, R. A., Trucco, M., Strominger, J. L., and Gorga, J. C. (1994) Self-peptides bound to the type I diabetes associated class II MHC molecules HLA-DQ1 and HLA-DQ8. *Int. Immunol.* **6,** 1639–1649.
7. Verreck, F. A. W., Van de Poel, A., Termijtelen, A., Amons, R., Drijfhout, J. W., and Koning, F. (1994) Identification of an HLA-DQ2 peptide binding motif and HLA-DPw3-bound self-peptide by pool sequencing. *Eur. J. Immunol.* **24,** 375–379.
8. Verreck, F. A. W., Van de Poel, A., Drijfhout, J. W., Amons, R., Coligan, J. E., and Koning, F. (1996) Natural peptides isolated from Gly86/Val86-containing variants of HLA-DR1, -DR11, -DR13, and -DR52. *Immunogenetics* **43,** 392–397.
9. Hammer, J., Takacs, B., and Sinigaglia, F. (1992) Identification of a motif for HLA-DR1 binding peptides using M13 display libraries. *J. Exp. Med.* **176,** 1007–1013.
10. Fleckenstein, B., Kalbacher, H., Muller, C. P., Stoll, D., Halder, T., Jung, G., and Wiesmüller, K. H. (1996) New ligands binding to the human leukocyte antigen class II molecule DRB1*0101 based on the activity pattern of an undecapeptide library. *Eur. J. Biochem.* **240,** 71–77.
11. Hammer, J., Valsasnini, P., Tolba, K., Bolin, D., Higelin, J., Takacs, B., and Sinigaglia, F. (1993) Promiscuous and allele-specific anchors in HLA-DR-binding peptides. *Cell* **74,** 197–203.
12. Hammer, J., Belunis, C., Bolin, D., Papadopoulos, J., Walsky, R., Higelin, J., Danho, W., Sinigaglia, F., and Nagy, Z. A. (1994) High-affinity binding of short peptides to major histocompatibility complex class II molecules by anchor combinations. *Proc. Natl. Acad. Sci. USA* **91,** 4456–4460.
13. Sidney, J., Oseroff, C., Southwood, S., Wall, M., Ishioka, G., Koning, F., and Sette, A. (1992) DRB1*0301 molecules recognize a structural motif distinct from the one recognized by most DR beta 1 alleles. *J. Immunol.* **149,** 2634–2640.
14. Geluk, A., van Meijgaarden, K. E., Janson, A. A., Drijfhout, J. W., Meloen, R. H., de Vries, R. R., and Ottenhoff, T. H. (1992) Functional analysis of DR17(DR3)-restricted mycobacterial T cell epitopes reveals DR17-binding motif and enables the design of allele-specific competitor peptides. *J. Immunol.* **149,** 2864–2871.
15. O'Sullivan, D., Sidney, J., del Guercio, M. F., Colon, S. M., and Sette, A. (1991) Truncation analysis of several DR binding epitopes. *J. Immunol.* **146,** 1240–1246.
16. Wal, Y. van de, Kooy, Y. M. C., Drijfhout, J. W., Amons, R., and Koning, F. (1996) Peptide-binding characteristics of the Coeliac disease associated DQ(α1*0501, β1*0201) molecule. *Immunogenetics* **44,** 246–253.
17. Wal, Y. van de, Kooy, Y. M. C., Drijfhout, J. W., Amons, R., Papadopoulos, G. K., and Koning, F. (1997) Unique peptide-binding characteristics of the disease-associated DQ(α1*0501, β1*0201) versus the non-disease associated DQ(α1*0201, β1*0202) molecule. *Immunogenetics* **46,** 484–492.

9

Studies of Gliadin-Specific T-Cells in Celiac Disease

Øyvind Molberg, Stephen N. McAdam, Knut E. A. Lundin, and Ludvig M. Sollid

1. Introduction

Celiac disease is an immune-mediated disorder that primarily affects the small intestinal mucosa. It is one of the few human disorders of which it is possible, and ethically acceptable, to obtain samples from the disease-affected tissue. This chapter describes how small intestinal biopsy specimens are utilized for studies of cell-mediated immune responses in celiac disease. The focus is mainly on practical procedures for isolation, growth under sterile conditions, and subsequent analyses of gliadin-specific T-cells derived from the small biopsy specimens. This chapter also provides guidelines for the preparation of different gliadin antigens suitable for T-cell analysis. Note that most of the T-cell assays described necessitate serological and/or genomic HLA typing of the celiac disease patients from whom the T-cells are derived.

2. Materials

2.1. Preparation of Antigen

1. Wheat flour (from different cultivars, *see* **Note 1**) and complex gliadin (G-3375, Sigma, St. Louis, MO).
2. Enzymes for gliadin digestion: pepsin (P-7012, Sigma), trypsin (T-0134, Sigma), and chymotrypsin (C-4129, Sigma).
3. Tissue transglutaminase (T-4398, Sigma): Reconstitute in phosphate-buffered saline (PBS), aliquot, and store at –70°C. Activate with 0.8 m*M* $CaCl_2$ in PBS.
4. Synthetic gliadin peptides: Dissolve purchased peptides, quality controlled by high-performance liquid chromatography (>80% purity) and mass spectrometry, in distilled H_2O, aliquot in vials, dry in a SpeedVac, store at –70°C, and resuspend in PBS before use.

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

5. Preparation of recombinant gliadins: pET 17xb expression system (Novagen, Madison, WI), carbenicillin (Novagen), isopropylthiogalactoside (IPTG) (I-5502, Sigma).

2.2. Isolation and Culturing of T-Cells

1. T-cell growth media: Complete medium (CM), consisting of RPMI-1640 (Gibco, Paisley, Scotland) supplemented with penicillin/streptomycin; and 0.01 *M* 2-mercaptoethanol (2-ME) (M-6250, Sigma). Store at 4°C. In T-cell medium, CM is supplemented with 15% human serum (HS-CM). The human serum is a pool of sera from several blood donors (*see* **Note 2**). Inactivate quality-tested, pooled serum for 30 min at 56°C and store at –70°C.
2. T-cell growth factors: Recombinant human interleukin-2 (IL-2) (ARM1050, Amersham Pharmacia Biotech AB, Uppsala, Sweden), and recombinant IL-15 (247-IL, R+D Systems, Abingdon, UK). Dissolve in a 1% human albumin/RPMI-1640 solution. Store stock solutions of 50,000 IU/mL for IL-2 and 100 ng/mL for IL-15 at –20°C. Dissolve phytohemagglutinin (PHA) (HA16, Murex, Dartford, UK) in PBS and store at 4°C.
3. Isolation of T-cells: Collagenase A (Boehringer Mannheim, Mannheim, Germany). Rat antimouse (RAM) IgG1 Dynabeads M-450 (Dynal, Oslo, Norway) coated with IgG1 anti-CD25 monoclonal antibody (MAb) (2C8, Eurogenetics, Tessenderloo, Belgium).
4. Freezing of T-cells: Dimethyl sulfoxide (DMSO) (Sigma D-5890), 1.8-mL Nunc cryo tube vials (363401, Nunc, Roskilde, Denmark). Inactivate fetal calf serum (FCS) (Gibco) for 30 min at 56°C and store at –70°C.
5. Mycoplasma detection in cell cultures is performed using a polymerase chain reaction (PCR) mycoplasma detection kit (90-1001K, American Type Culture Collection [ATCC], Manassas, VA) and/or by *in situ* detection of mycoplasma infection with an indicator cell line (Vero 76, CRL-1587, ATCC) with fluorescent Hoechst 33258 staining ***(1)***.
6. Laminar flow sterile bench.
7. Water-jacketed incubator (Forma Scientific, Marietta, OH).
8. Tissue culture plates (Corning Costar, Cambridge, MA): 3799, 96-well U-bottomed; 3595, 96-well flat-bottomed; 3548, 48-well; 3524, 24-well.
9. Terasaki plates for T-cell cloning (163118, Nunc).

2.3. Preparation and Culturing of Other Cells

1. Preparation of peripheral blood mononuclear cells (PBMCs): Sterile 100-mL glass flasks containing glass beads in 0.9% NaCl. Lymphoprep (Nycomed Pharma, Oslo, Norway). Hank's balanced salt solution (Gibco) or sterile PBS supplemented with 11 mg/mL of glucose ("endothelial buffer").
2. Depletion of T-cells from PBMCs: Pan T (CD2) Dynabeads M-450 (Dynal), RAM IgG1 Dynabeads M-450 coated with IgG1 anti-CD3 MAb (T10B9,

kindly provided by J. S. Thompson, University of Kentucky Medical Center, Lexington, KY).
3. Epstein-Barr virus (EBV) transformation of PBMCs: Supernatant from the B95-8 cell line (no. CRL-1612, ATCC).
4. Growth media for EBV-transformed B-lymphoblastoid cell lines (B-LCLs): RPMI-1640 (without antibiotics) supplemented with 10% FCS. B-LCL is grown in 25 (Falcon, Becton Dickinson, Franklin Lakes, NJ) and 75-cm^3 (Falcon) tissue culture flasks.

2.4. Reagents for T-Cell Assays

1. Thymidine incorporation: (Methyl-^{3}H) thymidine (Amersham Pharmacia Biotech). Stock solution of 50 μCi/mL stored at 4°C. Harvest 96-well plates with a Skatron Micro cell harvester (Skatron, Lier, Norway). Count β-scintillation with a 1205 Betaplate liquid scintillation counter (LKB Wallac, Turkee, Turku, Finland).
2. Proliferation inhibition assays: Purified anti-HLA MAb: B8.11 anti-DR monomorphic (hybridoma a gift from B. Malissen, Centre d'Immunologie, Marseille, France), SPV-L3 anti-DQ monomorphic (hybridoma a gift from H. Spits, Netherlands Cancer Institute, Amsterdam), W6/32 anti-HLA class I monomorphic (HB-95, ATCC). Store stock solutions of the MAbs (0.5–2 mg/mL) at –70°C. Other anti-HLA MAbs used infrequently: L243 anti-DR monomorphic (HB-55, ATCC), B7/21 anti-DP monomorphic (hybridoma a gift from I. Trowbridge, The Salk Institute, La Jolla, CA), 2.12.E.11 anti-DQ2 (a gift from H. Viken, Institute of Immunology, University of Oslo, Norway).

3. Methods

3.1. Preparation of Antigens

3.1.1. Preparation of Gliadin from Wheat Flour

Gliadin is the major storage protein in wheat. Wheat flour has a total protein content of about 10% and approx 10% of these proteins are made up of alcohol-soluble gliadin proteins. Extraction of gliadin from wheat flour is a multistep process.

1. Wash wheat flour repeatedly in water-saturated *n*-butanol.
2. Centrifuge the solution at 200*g* for 5 min between washings and discard the supernatant (consisting of carbohydrates, lipids, and water-soluble proteins).
3. Dissolve the resulting wheat gluten fraction in 70% ethanol and put on a magnetic stirrer overnight at room temperature.
4. Centrifuge this solution (400*g* for 5 min) to remove the alcohol-insoluble glutenin fraction.
5. Recover the gliadin proteins by "salting out," that is, precipitate with a 1.5% w/v NaCl solution.

6. Dissolve the precipitate in 0.01 *M* CH_3COOH and store at 4°C until subjecting it to enzymatic digestion.

3.1.2. Production of Recombinant Gliadins *in* Escherichia Coli

Multiple α- and γ-type gliadin alleles can be cloned from individual wheat cultivars using PCR of either cDNA or genomic DNA and using oligonucleotide primers designed to anneal to conserved 3' and 5' sites ***(2)***.

1. We chose to express mature proteins lacking the leader peptide sequence. The following sets of primers were successfully used: α-gliadins (5' primer) 5'-GCT ATG GAT CCA TAT GGT TAG AGT TCC AGT GCC-3' and (3' primer) 5'-GCA TCA AGC TTC ATC GAT AGT TAG TAC CGA AGA TGC C-3'. γ-gliadins (5' primer) 5'-GCT ATG GAT CCA TAT GAA TAT CCA GGT CGA CCC-3' and (3' primer) 5'-GCA TCA AGC TTC ATC GAT ATT GGC CAC CAA TGC CCG C 3'. PCR products from PCRs using these primers can be positionally cloned into the *Nde*I and *Hind*III sites of the vector pET17xb according to the manufacturer's instructions (Novagen).
2. Identify plasmids containing full gliadin genes by sequencing, transform into the bacterial expression host BL21 (DE3) pLysS (Novagen), and select on Luria Bertani (LB) plates containing 100 μg/mL of ampicillin and 34 μg/mL of chloramphenicol.
3. Grow freshly transformed colonies in LB media containing 100 μg/mL of carbenicillin and 34 μg/mL of chloramphenicol at 37°C until the OD_{600} reaches 0.6–1.
4. Induce gliadin expression by the addition of 0.4 m*M* IPTG and leave the cultures shaking at 37°C for a further 16 h. Harvest cultures by centrifuging at 5000*g* for 30 min.
5. Resuspend the bacterial cell pellet in 70% ethanol, preheated to 50°C; sonicate; and incubate for 2 h at 50°C.
6. Remove the bacterial debris by centrifugation at 10,000*g* for 30 min and add 2 vol of 1.5 *M* NaCl.
7. Leave the solution at 4°C overnight to allow the gliadin to precipitate completely.
8. Rinse the preciptate briefly in H_2O before dissolving it in 5 *M* urea and assessing for quantity and purity. Typically, a yield of between 5 and 30 mg of gliadin per liter of culture is obtained that stains as a single band with Coomassie blue after separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

3.1.3. Pepsin and Trypsin Digestion of Gliadins

1. Dissolve gliadin in 0.01 *M* CH_3COOH.
2. Adjust the pH to 1.8 with 1 *M* HCl for pepsin digestion.
3. Add pepsin at a 1:100 ratio (w/w) and stir the solution for 4 h at 37°C.
4. Terminate the pepsin digestion by bringing the pH to 7.8 with 1 *M* NaOH.
5. For trypsin digestion, add trypsin at a 1:100 ratio (w/w) to the solution and stir for 4 h at 37°C.
6. Deactivate trypsin by heating the solution to 85°C for 45 min.

7. Cool the peptic-tryptic-digest (PT-gliadin) to 20°C, adjust the pH to 4.5 with 1 *M* HCl, and dialyze extensively against 0.01 *M* NH_4HCO_3.
8. For storage, lyophilize the gliadin solution. The freeze-dried gliadin is stored in a dry container at room temperature.
9. Reconstitute the PT-digest in PBS (pH 7.4), run through a 45-μm filter, and use directly in T-cell assays.
10. It is essential to perform a quality test of all new batches of PT-gliadin for their ability to stimulate PT-gliadin-specific T-cells (*see* **Note 3**).

3.1.4. Chymotrypsin Digestion of Gliadin

1. Dissolve gliadin in 0.01 *M* NH_4HCO_3 with 2 *M* urea, stir for 2 h at 37°C, and add chymotrypsin at a 1:200 ratio (w/w) ***(3)***.
2. Run the enzymatic digestion overnight and terminate it by heat inactivation of the enzyme (98°C for 5 min).
3. Dialyze the chymotrypsin-digested gliadin extensively against 0.01 *M* NH_4HCO_3, lyophilize, and store at room temperature. Reconstitute in PBS when needed.
4. Chymotrypsin-digested gliadins are often subjected to acid/heat treatment prior to use in T-cell assays (*see* **Subheading 3.12.**). Dissolve the chymotrypsin-digested proteins in 0.01 *M* CH_3COOH at a pH of 1.8 (adjust with 1 *M* HCl) and heat treat at 98°C for 60 min.
5. Dry the acid/heat-treated gliadin preparations in a SpeedVac and store at –70°C (*see* **Note 4**).

3.1.5. Tissue Transglutaminase Treatment of Gliadin Proteins and Peptides

1. Dissolve guinea pig tissue transglutaminase (tTG) in PBS, and adjust the concentration of the enzyme to 10–100 μg/mL.
2. Activate tTG with 0.8 m*M* $CaCl_2$ and incubate with gliadin for 2 h at 37°C before addition to antigen-presenting cells (APCs) (*see* **Subheading 3.12.2.**) (*see* **Note 5**).

3.2. Preparation of "Feeder Cells" for T-Cell Cultures

T-cells require support from other cells to proliferate in vitro. We routinely use allogeneic PBMCs (from healthy donors) as the source of cells for T-cell support. The supportive cells are termed *feeder cells*.

1. Prepare the PBMCs from heparin- or citrate-treated whole blood, or as we prefer, prepare them from defibrinated blood.
2. Remove the fibrin from the blood by gently shaking it for 7 min in sterile flasks containing glass beads.
3. Isolate the PBMCs by density-gradient separation, wash two to three times in HBSS or endothelial buffer, and suspend in HS-CM preheated to 37°C.
4. Adjust the PBMCs to 1×10^6 cells/mL in HS-CM and γ-irradiate (with an energy of 25 gy) before addition to T-cell cultures (*see* **Note 6**).

3.3. In Vitro Antigen Challenge of Small Intestinal Biopsy Specimens

1. Obtain duodenal biopsy specimens via a gastroduodenoscope using biopsy forceps.
2. Transfer the specimens immediately to HS-CM at 4°C and keep on ice.
3. Within 1 to 2 h orient (with the epithelial surface facing upward) the specimens on a metal grid in one well on a 24-well plate, and immerse in 3 mL of HS-CM culture medium with or without antigen.
4. Perform antigen challenge of the biopsy specimens for 20 h at 37°C in an organ culture chamber with 95% O_2, 5% CO_2, and at 1 bar pressure ***(4)***.
5. The gliadin preparations routinely used for challenge consist of 5 mg/mL of PT-digested gliadin in HS-CM or 0.5 mg/mL of chymotrypsin-digested gliadin in HS-CM.

3.4. Isolation of Cells from In Vitro–Challenged Biopsy Specimens

1. Immediately after the biopsy specimens are taken out of the organ culture chamber, centrifuge the remaining culture medium to recover the cells that migrate out of the biopsies during antigenic challenge. This population of cells is later referred to as emigrant cells (*see* **Note 7**).
2. Remove the biopsy specimens from the wells, chop finely with a scalpel, and subject to enzymatic digestion for 45 min at 37°C in 1 mg/mL of collagenase A in HS-CM solution.
3. Pass the digested biopsy material through a 70-μm sterile filter to remove nondegradable material and epithelial cells (*see* **Notes 8** and **9**).

3.5. In Vitro Handling of Biopsy-Derived T-Cells

The conditions for the primary culture (i.e., the first 7–10 d in vitro culturing) of biopsy-derived cells have evolved through trial and error, and it is likely that they can be further optimized (*see* **Note 10**).

1. Obtain blood (used for preparation of autologous feeder cells) from the celiac disease patients at the time of endoscopy. Store the autologous PBMCs in HS-CM at 4°C overnight, and irradiate prior to use.
2. Wash all biopsy-derived cell fractions, resuspend in HS-CM, count, and then keep them on ice. In our original protocol, the resident cells were at this stage subjected to positive selection of CD25-expressing cells using RAM IgG1 Dynabeads M-450 coated with antihuman CD25 MAb ***(5–7)***. However, recent experiments have demonstrated that this selection step is unnecessary (*see* **Note 11**).
3. Evaluate the number and viability of emigrant and resident cells using phase-contrast microscopy and seed dilutions of each population into wells on tissue culture plates. These wells contain autologous, irradiated PBMCs as feeder cells and recombinant human IL-2 as a T-cell growth factor.

3.6. Restimulation and Expansion of T-Cells

*3.6.1. Restimulation and Expansion of T-Cell Lines (*see ***Note 12****)*

1. Expand the biopsy-derived cultures by cyclic restimulation each seventh day. After 14–17 d of growth in vitro, the culture contains mainly $CD4^+$ T-cells; that is, it can be referred to as a polyclonal T-cell line.
2. Seven days after the first restimulation (on d 14–17), transfer the T-cell line to a tube, centrifuge (350*g* for 7 min), resuspend in HS-CM, and adjust to 1×10^6 viable cells/mL.
3. Perform further restimulation of T-cell lines according to a standardized scheme.
4. Seed T-cells ($0.75–1 \times 10^6$) into a 24-well plate containing 1 mL of a "feeder mix" (thereby giving a total volume of 1.75–2 mL of CM in the well).
5. Use a feeder mix for restimulation consisting of allogeneic, irradiated PBMCs (1×10^6 cells/mL), PHA (1 μg/mL), and IL-2 (10 IU/mL).
6. Split the T-cell lines and add fresh CM supplemented with 5–10 IU/mL of IL-2 only on d 3, 4, and 5 in each cycle.

3.6.2. Expansion of T-Cell Clones

Antigen-specific T-cell clones (*see* **Subheading 3.8.**) can be expanded using the same protocol as the T-cell lines, but if large numbers of cells are required an alternative procedure is preferable. The principle of this procedure is to induce optimal proliferation of the T-cell clone by combining two efficient activation stimuli: allogeneic HLA-molecules and a mitogen.

1. Thaw the T-cell clone, and, depending on its previously delineated growth properties, adjust to $5–50 \times 10^5$ cells/mL in HS-CM supplemented with a mixture of allogeneic, irradiated PBMCs (1×10^6 cells/mL) from at least three different donors, and 1 μg/mL of PHA.
2. Plate out the mixture of T-cell clone/PBMCs and PHA in a volume of 100 μL/well ($5–50 \times 10^4$ T-cells/well) on U-bottomed 96-well plates.
3. On d 4 supplement the wells with 5 IU/mL of IL-2 in CM (50 μL/well), and on d 7 transfer the cells, at a 1:1 ratio, to 24-well plates and supplement with 1 mL of HS-CM containing 1×10^6 PBMCs and 1 μg/mL of PHA.
4. On d 10 or 11 supplement each well with 5 IU/mL of IL-2 in CM (500 μL/well).
5. Test the T-cell clones for sustained antigen-specific proliferative responses and freeze down aliquots of them on d 14.
6. Maintenance of T-cells in culture for long periods of time can be both difficult and tedious. In addition, for reasons that remain unclear, T-cell lines and clones often lose antigen specificity during prolonged culture. To circumvent these problems, we chose to produce a cell line expressing the T-cell receptor (TCR) of a gliadin-specific T-cell clone by transfecting it into a mouse T-cell line that expresses no endogenous TCR. This transfectant displays the same antigen specificity as the original clone but is considerably easier to keep in culture (*see* **Note 13**).

3.6.3. Use of IL-15 to Maintain Quiescent T-Cells in Culture

Recently IL-15 has been shown to protect activated $CD4^+$ T-cells from apoptosis and maintain them in a quiescent state *(8)*. The addition of IL-15 to the media of T-cell clones following restimulation allows resting T-cells to be kept in culture for many weeks, if not months. These resting T-cells can then be used whenever they are needed. By contrast, T-cells that undergo periodic restimulation can only be used on d 6 or 7 following restimulation. IL-15 should be added to a final concentration of 1 ng/mL to thawed T-cell clones or on d 5 or 6 following restimulation. CM containing 1 ng/mL of IL-15 should then be used to feed clones when necessary. Although we have primarily used IL-15 for the culturing of T-cell clones, it is likely that it can also be used for similar purposes with T-cell lines.

3.7. Isolation of Gliadin-Specific T-Cells from PBMCs (see Note 14)

1. Primary cultures are made by stimulating 10×10^6 PBMCs (at a concentration of 2×10^6 cells/mL) from celiac disease patients or healthy controls with 5 mg/mL of PT-digest of gliadin in 25-cm^3 tissue culture flasks.
2. After 5 d wash the gliadin-stimulated PBMCs in CM, count viable cells, and perform a positive selection of activated cells, i.e., cells expressing the IL-2 receptor α-chain (CD25).
3. Perform the selection step by incubating the PBMCs with Dynabeads-M450 RAM IgG1 (coated with an IgG1 antihuman CD25 mAb) at 4°C for 45 min, using a bead:cell ratio of 5 beads per large lymphocyte-like cell.
4. Incubate positively selected cell:bead complexes overnight in CM to detach the beads.
5. After removing the beads with a magnet, wash the positively selected cells, resuspend in HS-CM, and count.
6. A fraction of the cells are cloned directly *(see* **Subheading 3.8.***)* and the remaining are restimulated with feeder mix and cultured for 7 d before being screened for recognition of the PT-gliadin used for primary stimulation.

3.8. Cloning of T-Cells

T-cell clones are established from gliadin-specific biopsy-derived T-cell lines or positively selected T-cells from gliadin-stimulated PBMCs. The T-cell lines are mostly cloned in connection with restimulation, i.e., on d 7 after PHA stimulation, but it is also feasible to thaw a T-cell line and clone it directly.

1. Dilute the T-cells to 200–600 cells/mL in a standard feeder mix (*see* **Subheading 3.6.**).
2. Immediately seed 20-μL aliquots of the T-cell/feeder mixture into separate wells in Terasaki plates and incubate for 7 d (*see* **Note 15**).
3. Transfer the growing T-cell clones from the Terasaki wells using a pipet.

4. Transfer each clone to a well in a 48-well plate containing 400 μL of feeder mix.
5. Culture the clones in the 48-well plates for 7 d (with HS-CM/IL-2 supplementation on d 3–5 if necessary) (*see* **Note 10**).
6. Screen for antigen-specific proliferative responses on d 14 after cloning.
7. On d 14 the T-cell clones can be either frozen down or restimulated once more with feeder mix and expanded for another week.

3.9. Phenotyping and Genotyping of T-Cells

Qualitative and quantitative phenotyping of T-cells, as well as genotyping of T-cell clones, can be performed with several different methods. A detailed description of such methods is not within the scope of this chapter, but brief descriptions, including appropriate references are included (*see* **Note 16**).

3.10. Freezing and Thawing of T-Cells

3.10.1. Freezing of T-Cells (see **Note 17**)

Because of the limited life-span in vitro, it is essential to freeze down sufficient numbers of viable T-cells during the expansion period. As a prerequisite for maintaining the growth potential of the T-cells following freezing and subsequent thawing, it is critical that the viability of the T-cells is excellent when cryopreserving them.

1. Prior to freezing the T-cells, label 1.8-mL cryovials and keep at –20°C.
2. Centrifuge the T-cells that are to be frozen in a cool centrifuge (400*g* for 7 min), resuspend in ice-cold CM supplemented with 50% FCS, and keep on ice. Subsequently add a solution of ice-cold 20% DMSO in 20% FCS CM to the T-cell suspension at a 1:1 ratio. Add this DMSO mixture in a dropwise fashion while gently mixing the cells.
3. After adding DMSO, immediately aliquot the cells into the prechilled cryovials (1 mL in each vial) and put in a –70°C freezer.
4. After 24 h at –70°C, transfer the cryovials to a liquid nitrogen tank for longtime storage at –180°C.

3.10.2. Thawing of T-Cells

Thawing of the T-cells is a two-step process:

1. Gently immerse the cryovial in water at 37°C until the suspension melts.
2. Immediately after the last of the ice in the vial has melted, transfer the cell suspension to a larger tube and add 8 mL of ice-cold 20% FCS-CM with a pipet. The T-cells are very vulnerable at this stage and it is critical that the FCS-CM, in particular the first 2 mL, is added dropwise, and with a speed that does not exceed 1 mL/min. Following addition of FCS-CM, immediately centrifuge the thawed T-cells and resuspend in CM. The thawed T-cells must be restimulated within a few minutes.

3.11. Screening Cell Cultures for Mycoplasma and Other Infections

Mycoplasma infections are devastating for in vitro culturing of human T-cells. We therefore strongly recommend that all laboratories working with T-cells establish good routines for mycoplasma detection. We currently use two complementary methods: detection of mycoplasma in cultures by PCR and detection of mycoplasma by Hoechst DNA staining (*see* **Note 18**). As a general rule, all T-cell lines established should be screened for mycoplasma infection, with either PCR or DNA staining, at least once. If mycoplasma is detected, all infected T-cells should be discarded, because there is currently no established protocol for decontamination. Moreover, all reagents used during culturing of the affected cells must be discarded.

Cell cultures can also be subject to bacterial and fungal infections, but this poses less of a problem since these infections are readily detectable and cells can be discarded before the infection spreads. We have chosen to monitor maintenance of appropriate sterile culture conditions by culturing B-LCLs in medium without antibiotics.

3.12. T-Cell Assays: T-Cell Proliferation Assay

3.12.1. Detection of Gliadin-Specific T-Cells in PBMCs (see **Note 19**)

1. Incubate freshly made PBMCs with a complex gliadin antigen for 6 to 7 d and test in a proliferative assay.
2. Seed triplicates of 1×10^5 PBMCs (in a volume of 150 µL of HS-CM) into 96-well U-bottomed plates, add PT-gliadin (dilutions from 0.1 to 10 mg/mL), and incubate for 6 d at 37°C.
3. After 6 d, add 20 µL of a 50 µCi/mL (Methyl-^{3}H) thymidine solution to each well, and culture the plate for a further 8–24 h before harvesting.
4. Harvest the plates using an automated microcell harvester, and measure ^{3}H-thymidine incorporation in each well with a liquid scintillation counter.

3.12.2. Preparation of APCs

The proliferative T-cell assays are routinely performed with B-LCLs as APCs. The B-LCLs are derived from different sources: autologous B-LCL, allogeneic B-LCL derived from Norwegian celiac disease-patients, and also the well-characterized panel of HLA homozygous B-LCL established during the 10th and 11th International Histocompatibility Workshops ***(9)***.

1. Culture the B-LCL in tissue culture flasks using RPMI-1640 with 10% FCS as growth medium. The culturing of B-LCL is done in medium without antibiotics (*see* **Subheading 3.11.**).
2. Before incubating with antigen, subject the B-LCL to gamma irradiation (75 gy), wash, resuspend in HS-CM, and adjust to 1×10^6 cells/mL.

3. PBMCs are also used frequently as APCs. In some instances, the PBMCs are depleted of T-cells prior to usage. Perform the T-cell depletion by incubating PBMCs with Dynabeads M-450 coated with anti-CD2 mAb (bead:cell ratio 3:1) and Dynabeads M-450 coated with anti-CD3 MAb (bead:cell ratio 3:1) at 4°C for 45 min.
4. Following negative selection, wash the (T-cell depleted) PBMCs once in HS-CM, gamma irradiate (25 gy), and adjust to 1×10^6 cells/mL.

3.12.3. Incubation of APCs with Antigen

1. Incubate gliadin antigens with APCs in 96-well U-bottomed plates.
2. Dissolve gliadin antigens (*see* **Subheading 3.1.**) and dilute in PBS (pH 7.4) prior to use in T-cell assays. This also includes tTG-modified gliadin and gliadin peptides.
3. Incubation with antigen is typically done in a volume of 100 µL. Each well contains 5×10^4 APCs in 80 µL of HS-CM and 20 µL of antigen solution.
4. Test each dilution of antigen in duplicates or triplicates.
5. The incubation time is dependent on the nature of the gliadin antigen. Complex gliadin antigens are incubated with APCs overnight at 37°C before addition of T-cells, whereas it is sufficient to incubate gliadin peptides with APCs for only a few hours before addition of T-cells.

3.12.4. Addition of T-Cells to APCs Incubated with Antigen

Test T-cells directly after thawing, 7 d after restimulation, or after a period of culture in IL-15.

1. Transfer the T-cells that are to be tested to tubes, wash, resuspend in fresh HS-CM at 37°C, adjust to 1×10^6 cells/mL, and then add to the 96-well plates containing APCs preincubated with gliadin antigens.
2. Typically, 5×10^4 T-cells in a volume of 50 µL of HS-CM are added to each well.
3. Following addition of T-cells, incubate the plates for 48 h.
4. Pulse each well with 20 µL of a 50 µCi/mL (Methyl-^{3}H) thymidine solution, and culture for a further 8–24 h before harvesting as described above (*see* **Subheading 3.12.1.**).

3.12.5. Determination of the HLA Restriction of Gliadin-Specific T-Cells

Determine the HLA restriction of T-cell lines and clones by either one of two complementary assays: an APC panel assay, or a proliferation inhibition assay employing anti-HLA mAb. Perform the APC panel assay with well-characterized HLA homozygous B-LCLs. Incubate different B-LCLs with gliadin in a standard proliferative T-cell assay.

The principle of the proliferation inhibition assay is that proliferation of T-cells can be inhibited by isotype-specific anti-HLA mAb. Three different, purified mAbs are routinely used in this assay: anti-DR (B8.11), anti-DQ (SPV-

L3), and anti-HLA class I (W6/32). The ability to inhibit proliferation has been tested in assays with T-cells with known HLA restriction. Based on data from these assays, we use all three mAbs at a final concentration of 10 μg/mL. The mAbs are added in a volume of 30 μL, to antigen-pulsed APCs, 30 min prior to addition of T-cells. The rest of the inhibition assay is performed as other T-cell proliferative assays. Some assays have also been performed with mAb against HLA-DP (B7/21). Finally, the same type of proliferation inhibition assays can also be performed with mAb that block receptors on the T-cells. We have performed such assays with anti-CD3, anti-CD4, and anti-CD8 mAb.

3.13. Discussion of the Applied Methods

3.13.1. Preparation of the Antigen

In their native form, gliadin proteins are insoluble in physiological solutions. However, they can be solubilized by limited, enzymatic proteolysis, thereby becoming suitable as antigens in T-cell assays. In research related to celiac disease, pepsin and trypsin have traditionally been the enzymes applied for proteolytic digestion of gliadin. The historical reason for this is that it was demonstrated in a seminal study, performed 40 yr ago, that digestion with pepsin and trypsin does not abolish the in vivo activity of gliadins ***(10)***. PT-gliadins have been instrumental in identifying gliadin-reactive small intestinal T-cells (as a corollary, we would probably not have identified any gliadin-specific T-cells if we had performed biopsy-challenge and in vitro testing of the T-cells with a chymotrypsin-digest of gliadin). Moreover, pepsin and/or trypsin digestion of gliadin is still widely used in studies of the immunopathogenesis of celiac disease ***(11–17)***.

Owing to the broad specificity of both enzymes, combined pepsin and trypsin proteolysis catalyzes the formation of quite complex mixtures of protein fragments. Indeed, analysis of PT-digested gliadin by two-dimensional electrophoresis revealed thousands of different protein fragments of varying size ***(18,19)***. The complexity is further increased by the introduction of negative charges into the gliadin fragments through spontaneous and random deamidation of glutamine residues in the gliadin proteins. This deamidation spontaneously occurs at the low pH (<2.0) used during pepsin digestion ***(20)***.

As a T-cell antigen, PT-gliadin has both advantages and disadvantages. The advantages are as follows: First, PT-gliadin challenge induces activation markers on lamina propria $CD4^+$ T-cells in the majority of celiac lesions ***(11,21)***. More important, when these $CD4^+$ T-cells are tested in vitro, they reproducibly recognize the challenging antigen. Second, T-cell clones specific for PT-gliadin made from one wheat strain are, as a rule, crossreactive with PT-gliadin made from other wheat strains, indicating the presence of a large number of

T-cell epitopes within each digest. Third, it is a very stable antigen, and it is relatively easy to produce a large batch that can be used throughout a whole series of T-cell experiments.

The disadvantages of PT-gliadin are mostly related to its complex nature. First, it is difficult to estimate the fraction of gliadin protein fragments within the mixture that are immunologically relevant. Second, identification and purification of protein fragments that contain T-cell epitopes is demanding. Third, mapping of T-cell epitopes within natural gliadins is technically challenging ***(3,17)***. This is partly because the closely related structures and amino acid sequences of the different protein fragments, as well as their inherent physiochemical properties, causes problems in isolation and purification of the epitope. An additional problem is that the microheterogeneity of the proteins ***(19)***, combined with chemical modifications, makes it extremely difficult to deduce epitopes from partial protein sequences and/or published genomic gliadin sequences.

The limitations caused by the complexity of natural gliadins have compelled us to clone, sequence, and express several recombinant gliadins. Initially this technology required considerable effort to set up, but once established, it became relatively easy to produce milligram quantities of individual gliadin proteins. A great advantage of recombinant antigen is that the sequence of each individual antigen can be deduced from the DNA sequence. This has proved quite valuable for the mapping of T-cell epitopes ***(2,22)***.

Because the low pH during pepsin digestion leads to deamidation, PT-gliadin is not suitable for analyzing the general importance of deamidation for T-cell recognition of gliadin. To address this, a water-soluble, nondeamidated gliadin would be optimal. In practice, this is not possible, but gliadin, enzymatically digested with chymotrypsin, is a close approximation ***(3,23)***. Indeed, limited chymotrypsin proteolysis of gliadin efficiently increases its solubility in water, and since the proteolysis is performed at neutral pH, the spontaneous deamidation of glutamine residues remains minimal.

The effect of active tTG on the proliferation of gliadin-specific, gut-derived, DQ-restricted T-cells can be evaluated with very simple assays ***(16,22,23)***. However, note that no human tTG was available when these assays were established, and guinea pig tTG was chosen because it had been reported to have the same substrate specificity as human tTG ***(24)***.

3.13.2. Isolation and Growth of the Biopsy-Derived T-Cells

During organ culture, the antigen used for ex vivo challenge interacts with mucosal APCs to form peptide-HLA complexes capable of activating resident T-cells. This *in situ* activation of the T-cells is, in our hands, a prerequisite for

obtaining growing T-cells recognizing the challenging antigen. Other groups have shown that lamina propria lymphocytes isolated from nonstimulated biopsies are highly susceptible to apoptosis ***(25)*** and that addition of exogenous IL-2 can rescue activated T-cells from apoptosis ***(26)***. Taken together, these observations could indicate that the combination of *in situ* activation and immediate supply of IL-2 in vitro selectively enhances the survival of the antigen-specific mucosal T-cells.

The growth of the biopsy-derived cells is dependent on HS-CM. Among the most critical components are probably the essential nutrients contained within the human serum. (Another advantage of human serum is that it contains few foreign proteins, and thus it limits cross-priming of the T-cells to a minimum.) Finally, inclusion of the reducing agent 2-ME in CM presumably reduces the growth-inhibiting effect of oxidants released by adherent macrophages in the wells ***(27)***.

3.13.3. The T-Cell Assays

T-cell recognition of antigens has mostly been evaluated using a ^{3}H thymidine incorporation assay. The same robust assay has also been used for all screenings of antigen-specific T-cell clones, thereby selecting exclusively for cells that proliferated on TCR ligation. However, because many studies of $CD4^{+}$ memory T-cells have found a close correlation between proliferation and other effector functions ***(28)***, this criterion of selection was regarded as relevant and sufficient. Moreover, the analyses of cytokines secreted from our T-cells on stimulation by antigen (using the same reagents as in the ^{3}H thymidine incorporation assays) directly supported this notion ***(29)***.

4. Notes

1. We have used wheat flour from many different wheat strains (including Kadett, Cheyenne, Scout 66), but also commercial blended wheat obtained from our local grocery store. Overall, we have not observed gross differences in T-cell reactivity among PT digests from the different cultivars, nor among different brands of blended wheat. Presently, we use batches made from the defined cultivar Kadett and batches made from commercial blended wheat. A presumed advantage of the latter is that it contains gliadins from wheat strains to which our patient population is currently exposed.
2. The serum from each individual donor is tested for its ability to sustain growth of PBMCs stimulated with anti-CD3 mAb (T10/B9 coated Dynabeads M-450) and 10 IU of IL-2. A 15% HS-CM solution is made from each individual serum. PBMCs (1×10^{5}/well) seeded in each of the HS-CM samples are stimulated with 3.5×10^{5} anti-CD3 mAb coated beads plus 5 IU/mL of IL-2. The proliferation of PBMCs grown in each HS-CM sample is evaluated by ^{3}H-thymidine incorporation from 48 to 72 h after stimulation. The sera that sustain growth are chosen and pooled.

3. It is important to be aware of the large variability in the T-cell stimulatory capacity of different batches of PT-digested gliadins. We have observed that batches of PT-gliadin made from the same gliadin preparation and with similar reagents can differ in their T-cell stimulatory capacity. The reason for this variation among batches is not well understood, but it is likely related to differences in both the efficacy of enzymatic digestion and the degree of spontaneous nonenzymatic deamidation of glutamine residues occurring during treatment at low pH. Each new batch should be tested for recognition by PT-gliadin-specific T-cells. If such T-cells are not available, test the new batch for its ability to induce proliferation of PBMCs from treated celiac disease patients (*see* **Subheading 3.12.1.**). It is particularly useful to include old batches of PT-gliadin that are known to efficiently stimulate gliadin-specific T-cells as internal controls.
4. A similar acid/heat-treatment has also been applied on purified (chymotrypsin-digested) gliadin preparations ***(30)***, and is also used to modify synthetic gliadin-peptides ***(3)***. As was described for PT-digested gliadins, all new batches of acid/heat-treated chymotrypsin digested gliadins must be tested against an old reference batch before addition into T-cell assays.
5. tTG has been used to modify PT-digested gliadin (incubated with tTG in dilutions from 1 μg/mL to 10 mg/mL), chymotrypsin-digested gliadin (in dilutions of 0.1 μg/mL to 5 mg/mL), recombinant gliadins, and various synthetic gliadin peptides ***(22,23)***.
6. Nonirradiated PBMCs can be frozen (*see* **Subheading 3.10.**), γ-irradiated on thawing, and thereafter used as a source of feeder cells.
7. The term *emigrant cells* is adapted from Pope et al. ***(31)***, who characterized cells migrating out of skin biopsies during in vitro culture and defined these as "emigrant cells."
8. The adhesive properties of the mucosal epithelial cells have a tendency to induce formation of large clusters of cells. Many of these cell clusters are removed by filtering the cell suspension through a 70-μm filter. Because the epithelial cells die within a few days in vitro, it is advantageous to remove them prior to culturing the lymphoid cells. We cannot exclude that a fraction of the lamina propria lymphocytes is trapped in the clusters and lost during filtering. However, we feel that this is less of a problem than the increased contamination with epithelial cells observed when using larger (200-μm) filters.
9. The single-cell suspension obtained after filtering is referred to as resident cells (i.e., cells still resident in the biopsy specimens after antigen challenge).
10. To date, the highest success rate with primary cultures has been obtained with plating biopsy-derived cells in 96-well plates (particularly U-bottomed, but also flat-bottomed have worked well in our hands). We coculture approx $1–2 \times 10^4$ biopsy-derived cells and 1×10^5 autologous PBMCs in a volume of 150 μL of CM supplemented with 10 IU/mL of IL-2. These cultures are supplemented with fresh HS-CM (with 10 IU/mL of IL-2) on d 3, 4, and/or 5 (depending on the growth). In some cases, particularly when the biopsy-derived cells are proliferating slowly, activation of macrophages in the wells may cause problems. In these

cases, it has been beneficial to transfer the T-cell cultures into new wells on d 5 and add fresh HS-CM/IL-2. The cultures are restimulated for the first time 7–10 d after being plated out. At this stage, they have optimally proliferated to approx 2×10^5 cells in each well. On d 7–10, the cultures are resuspended in their growth medium and transferred to new wells and stimulated with an additional 500 μL of HS-CM containing autologous, irradiated PBMCs (1×10^6 cells/mL), 10 IU/mL of IL-2, and 1 μg/mL of PHA. The most efficient expansion has been observed when each culture is transferred from 1 well in the 96-well plate to 1 well in a 48-well plate.

11. Positive selection of cells using anti-CD25 MAbs was omitted as a result of control experiments demonstrating that T-cells cultured directly from digested biopsy specimens (without any selection step) displayed gliadin-specific responses and HLA restriction patterns similar to parallel T-cells that had been subjected to bead selection. *See* **ref. *32*** for further details.
12. This scheme allows resting of the T-cells for at least 48 h at the end of each growth cycle. The main advantage of this scheme is that it drives the T-cells into a cycle in which they are in a nonproliferating ("resting") state on d 7. This optimizes the conditions for performing proliferative T-cell assays (**Subheading 3.12.**), cloning of T-cell lines (**Subheading 3.8.**), and freezing of T-cells (**Subheading 3.10.**).
13. Many groups have developed alternatives to culturing normal T-cells, such as transformation of T-cells with Herpes virus ***(33)***. However, we have chosen to produce a TCR transfectant expressing the Vα and Vβ gene segments from a gliadin-specific gut-derived T-cell clone ***(5)***. These were amplified from genomic DNA and cloned into the TCR expression vectors, pTαcass and pTβcass, respectively ***(34)***. These vectors allow for functional expression of murine or human variable region TCR spliced to murine-constant regions (Madsen, L., Svejgaard, A., and Fugger, L., unpublished observations). Transfection of these two plasmids together with CD4:pBs and a Neo selection marker into the TCR-deficient mouse T-cell hybridoma BW 58α-/β- ***(35)*** allowed for the selection and cloning of a stable transfectant (60.6) expressing human CD4 and chimeric TCR. Ligation of this TCR induces the mouse cell line to produce IL-2. Comparisons of the TCR transfectant with the original T-cell clone using a panel of synthetic peptide variants has confirmed that the specificity of the chimeric TCR is maintained.
14. When this method was applied on PBMCs from treated celiac disease patients, a high frequency of gliadin-specific T-cell clones was found ***(36)***.
15. By cloning at limiting dilution, the likelihood of clonality can be assessed using Poisson distribution ***(37)***.
16. For phenotyping of T-cells, we have often used a rosette-forming assay ***(38)*** that has the advantage of being rapid and easy to perform. This assay is, however, mainly qualitative. Thus, if quantitative data are necessary, other assays, such as flow cytometry, are more suitable. The TCR genes of some of the gliadin-specific T-cell clones ***(5)*** have been characterized, and the methods we used are described elsewhere ***(39)***. In brief, the TCRα genes were amplified, cloned, and

sequenced by a slight modification of the rapid amplification of the cDNA ends (RACE) technique ***(40)***. The TCRβ genes were characterized by cloning and sequencing of PCR products that were obtained either with a degenerate primer specific for the leader sequences in conjunction with a Cβ primer or by the RACE technique.

17. We have no indications that the cells should be frozen down at any particular concentration. Excellent viability can be obtained with $1–50 \times 10^6$ cells/cryovial.
18. Detection of mycoplasma by PCR or Hoechst staining is performed according to protocols supplied by the manufacturer (*see* **Subheading 2.2.**). The PCR method requires 5×10^5 cells, whereas the Hoechst staining can be performed using supernatants from growing T-cells (i.e., it does not consume cells). However, an advantage of the PCR method is that it can be performed on cells immediately after they are thawed.
19. We have regarded a stimulation index (SI) >10 as a positive gliadin-specific PBMC response. (The SI is defined as mean of counts per minute [cpm] in triplicates of PBMCs stimulated with antigen divided by mean cpm in triplicates of PBMCs stimulated with medium alone.)
20. As a general rule, we have regarded a positive T-cell response as SI > 3 (mean cpm in wells with [T-cells + APCs + gliadin] divided by mean cpm in wells with [T-cells + APCs without gliadin] and Δcpm > 1000. Δcpm = mean cpm of [T-cells + APCs + gliadin] – mean cpm of [T-cells + APCs without gliadin]).

Acknowledgments

We wish to thank Kjell Kett and the nursing staff at the Gastroenterological Unit, Rikshospitalet, for help with obtaining duodenal biopsies. Helge Scott at the Institute of Pathology, Rikshospitalet was invaluable as a collaborator in the human T-cell studies; he performed the in vitro antigen challenge of the biopsy specimens. Ove Norén and Hans Sjöström at the Department of Medical Biochemistry and Genetics, The Panum Institute, University of Copenhagen, Denmark, were important collaborators on gliadin antigen characterization. We also acknowledge Lars Fugger at the Department of Clinical Immunology, Aarhus University Hospital, Skejby, Aarhus, Denmark, and Lars Siim Madsen, Department of Clinical Immunology, Rikshospitalet, University of Copenhagen, Denmark, for producing the TCR transfectant; and Dorit Anthonsen, Eli Brundtland, Tore Jensen, Christel Kristiansen, and Liv Mangschau for their excellent technical assistance. Finally, we wish to thank Erik Thorsby, head of the Institute of Transplantation Immunology, for providing excellent working facilities and invaluable expert advice.

References

1. Chen, T. R. (1977) In situ detection of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell Res.* **104,** 255–262.

2. Arentz-Hansen, E. H., McAdam, S., Molberg, Ø., Kristiansen, C., and Sollid, L. M. (2000) Production of a panel of recombinant gliadins for the characterisation of T cell reactivity in coeliac disease. *Gut* **46,** 46–51.
3. Sjöström, H., Lundin, K. E. A., Molberg, Ø., Körner, R., Mcadam, S. N., Anthonsen, D., Quarsten, H., Norěn, O., Roepstorff, P., Thorsby, E., and Sollid, L. M. (1998) Identification of a gliadin T-cell epitope in coeliac disease: general importance of gliadin deamidation for intestinal T-cell recognition. *Scand. J. Immunol.* **48,** 111–115.
4. Browning, T. H. and Trier, J. S. (1969) Organ culture of mucosal biopsies of human small intestine. *J. Clin. Invest.* **48,** 1423–1432.
5. Lundin, K. E. A., Scott, H., Hansen, T., Paulsen, G., Halstensen, T. S., Fausa, O., Thorsby, E., and Sollid, L. M. (1993) Gliadin-specific, HLA-DQ(α1*0501, β1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J. Exp. Med.* **178,** 187–196.
6. Lundin, K. E. A., Scott, H., Fausa, O., Thorsby, E., and Sollid, L. M. (1994) T cells from the small intestinal mucosa of a DR4, DQ7/ DQ8 celiac disease patient preferentially recognize gliadin when presented by DQ8. *Hum. Immunol.* **41,** 285–291.
7. Molberg, Ø., Kett, K., Scott, H., Thorsby, E., Sollid, L. M., and Lundin, K. E. A. (1997) Gliadin-specific HLA-DQ2 restricted T cells are commonly found in small intestinal biopsies from coeliac disease patients, but not from controls. *Scand. J. Immunol.* **46,** 103–109.
8. Dooms, H., Desmedt, M., Vancaeneghem, S., Rottiers, P., Goossens, V., Fiers, W., and Grooten, J. (1998) Quiescence-inducing and antiapoptotic activities of IL-15 enhance secondary CD4+ T cell responsiveness to antigen. *J. Immunol.* **161,** 2141–2150.
9. Yang, S. Y., Milford, E., Hammerling, U., and Dupont, B. (1987) The B-cell panel designed for the 10th International Histocompatibility Workshop, in *Immunobiology of HLA* (Dupont, B., ed.), Springer-Verlag, New York, pp. 11–19.
10. Frazer, A. C. (1959) Gluten-induced enteropathy, the effect of partially digested gluten. *Lancet* **ii,** 252–255.
11. Maiuri, L., Picarelli, A., Boirivant, M., Coletta, S., Mazilli, M. C., De Vincenzi, M., Londei, M., and Auricchio, S. (1996) Definition of the initial immunologic modifications upon in vitro gliadin challenge in the small intestine of celiac patients. *Gastroenterology* **110,** 1368–1378.
12. Marsh, M. N. and Crowe, P. T. (1995) Morphology of the mucosal lesion in gluten sensitivity. *Baillieres. Clin. Gastroenterol.* **9,** 273–293.
13. Maiuri, L., Auricchio, S., Coletta, S., De Marco, G., Picarelli, A., Di Tola, M., Quaratino, S., and Londei, M. (1998) Blockage of T-cell costimulation inhibits T-cell action in celiac disease. *Gastroenterology* **115,** 564–572.
14. Nilsen, E. M., Jahnsen, F. L., Lundin, K. E. A., Johansen, F. E., Fausa, O., Sollid, L. M., Jahnsen, J., Scott, H., and Brandtzaeg, P. (1998) Gluten induces an intestinal cytokine response strongly dominated by interferon-γ in patients with celiac disease. *Gastroenterology* **115,** 551–563.

15. Troncone, R., Gianfrani, C., Mazzarella, G., Greco, L., Guardiola, J., Auricchio, S., and De Berardinis, P. (1998) Majority of gliadin-specific T-cell clones from celiac small intestinal mucosa produce interferon-γ and interleukin-4. *Dig. Dis. Sci.* **43,** 156–161.
16. van de Wal, Y., Kooy, Y., van Veelen, P., Pena, S., Mearin, L., Papadopoulos, G., and Koning, F. (1998) Selective deamidation by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. *J. Immunol.* **161,** 1585–1588.
17. van de Wal, Y., Kooy, Y. C., van Veelen, P. A., Pena, S. A., Mearin, L. M., Molberg, Ø., Lundin, K. E. A., Sollid, L. M., Mutis, T., Benckhuijsen, W. E., Drijfhout, J. W., and Koning, F. (1998) Small intestinal T cells of celiac disease patients recognize a natural pepsin fragment of gliadin. *Proc. Natl. Acad. Sci. USA* **95,** 10,050–10,054.
18. Shewry, P. R., Tatham, A. S., and Kasarda, D. D. (1992) Cereal proteins and coeliac disease, in *Coeliac Disease* (Marsh, M. N., ed.), Blackwell Scientific, Oxford, UK, pp. 305–348.
19. Wieser, H. (1995) The precipitating factor in coeliac disease, in *Coeliac Disease* (Howdle, P. D., ed.), Bailliere Tindall, London, pp. 191–208.
20. Hamada, J. S. (1994) Deamidation of food proteins to improve functionality. *Crit. Rev. Food Sci. Nutr.* **34,** 283–292.
21. Halstensen, T. S., Scott, H., Fausa, O., and Brandtzaeg, P. (1993) Gluten stimulation of coeliac mucosa in vitro induces activation (CD25) of lamina propria CD4+ T cells and macrophages but no crypt-cell hyperplasia. *Scand. J. Immunol.* **38,** 581–590.
22. Arentz-Hansen, E. H., Körner, R., Molberg, Ø., Quarsten, H., Vader, W., Kooy, Y., Lundin, K. E. A., Koning, F., Roepstorff, P., Sollid, L. M., and McAdam., S. (2000) The intestinal T cell response to α-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *J. Exp. Med.*, in press.
23. Molberg, Ø., Mcadam, S. N., Körner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Norĕn, O., Roepstorff, P., Lundin, K. E. A., Sjöström, H., and Sollid, L. M. (1998) Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat. Med.* **4,** 713–717.
24. Aeschlimann, D. and Paulsson, M. (1994) Transglutaminases: protein cross-linking enzymes in tissues and body fluids. *Thromb. Haemost.* **71,** 402–415.
25. Boirivant, M., Pica, R., DeMaria, R., Testi, R., Pallone, F., and Strober, W. (1996) Stimulated human lamina propria T cells manifest enhanced Fas- mediated apoptosis. *J. Clin. Invest.* **98,** 2616–2622.
26. Mueller, D. L., Seiffert, S., Fang, W., and Behrens, T. W. (1996) Differential regulation of bcl-2 and bcl-x by CD3, CD28, and the IL-2 receptor in cloned CD4+ helper T cells: a model for the long-term survival of memory cells. *J. Immunol.* **156,** 1764–1771.
27. Gordon, S., Clarke, S., Greaves, D., and Doyle, A. (1995) Molecular immunobiology of macrophages: recent progress. *Curr. Opin. Immunol.* **7,** 24–33.

28. Lundin, K. E. A., Sollid, L. M., and Thorsby, E. (1996) Class II restricted T cell function, in *HLA and MHC: Genes, Molecules and Function* (Browning, M. and McMichael, A., eds.), BIOS Scientific, Oxford, pp. 329–352.
29. Nilsen, E. M., Lundin, K. E., Krajci, P., Scott, H., Sollid, L. M., and Brandtzaeg, P. (1995) Gluten specific HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon-γ. *Gut* **37,** 766–776.
30. Lundin, K. E. A., Sollid, L. M., Anthonsen, D., Noreˇn, O., Molberg, Ø., Thorsby, E., and Sjöström, H. (1997) Heterogeneous reactivity patterns of HLA-DQ-restricted, small intestinal T-cell clones from patients with celiac disease. *Gastroenterology* **112,** 752–759.
31. Pope, M., Betjes, M. G., Hirmand, H., Hoffman, L., and Steinman, R. M. (1995) Both dendritic cells and memory T lymphocytes emigrate from organ cultures of human skin and form distinctive dendritic-T-cell conjugates. *J. Invest. Dermatol.* **104,** 11–17.
32. Molberg, Ø., Lundin, K. E. A., Nilsen, E. M., Scott, H., Kett, K., Brandtzaeg, P., Thorsby, E., and Sollid, L. M. (1998) HLA restriction patterns of gliadin- and astrovirus-specific CD4+ T cells isolated in parallel from the small intestine of celiac disease patients. *Tissue Antigens* **52,** 407–415.
33. Meinl, E., Hohlfeld, R., Wekerle, H., and Fleckenstein, B. (1995) Immortalization of human T cells by Herpesvirus saimiri. *Immunol. Today* **16,** 55–58.
34. Kouskoff, V., Signorelli, K., Benoist, C., and Mathis, D. (1995) Cassette vectors directing expression of T cell receptor genes in transgenic mice. *J. Immunol. Methods* **180,** 273–280.
35. Letourneur, F. and Malissen, B. (1989) Derivation of a T cell hybridoma variant deprived of functional T cell receptor α and β chain transcripts reveals a nonfunctional alpha-mRNA of BW5147 origin. *Eur. J. Immunol.* **19,** 2269–2274.
36. Gjertsen, H. A., Sollid, L. M., Ek, J., Thorsby, E., and Lundin, K. E. A. (1994) T cells from the peripheral blood of coeliac disease patients recognize gluten antigens when presented by HLA-DR, -DQ, or -DP molecules. *Scand. J. Immunol.* **39,** 567–574.
37. Taswell, C. (1981) Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* **126,** 1614–1619.
38. Gaudernack, G. and Lundin, K. E. A. (1989) Rapid immunomagnetic phenotyping of cells. *J. Immunogenet.* **16,** 169–175.
39. Hansen, T., Lundin, K. E. A., Markussen, G., and Thorsby, E. (1992) T cell receptor usage by HLA-DQw8-specific T cell clones. *Int. Immunol.* **4,** 931–934.
40. Frohmann, M. A. (1990) RACE: rapid amplification of cDNA ends, in *PCR Protocols* (Innis, M. A., Gelfand, D. H., and Sninsky, J. J., eds.), Academic, San Diego, pp. 28–32.

10

Morphometric Analysis of Intestinal Mucosa

The Measurement of Volume Compartments and Cell Volumes in Human Intestinal Mucosa

Michael N. Marsh, Peter T. Crowe, Kieran J. Moriarty, and Arzu Ensari

1. Introduction

The widespread use of peroral (capsule) and, more recently, endoscopically obtained mucosal biopsies from jejunum and duodenum provides an easy source of material for diagnostic (clinical) and investigative scientific study. The basis of our understanding of small intestinal diseases has stemmed directly from these sources since, in addition to the purely morphological (and pathological/immunopathological) domain, these biopsies have helped to elucidate other types of mucosal disease (e.g., the "disaccharidase" deficiencies, defects in water and electrolyte transfer, and amino acid transport and lipid metabolism).

Gluten sensitivity (celiac sprue disease) is essentially a disease in which bystander injury to the upper intestinal mucosa occurs in genetically predisposed individuals ***(1,2)*** as a result of wheat prolamin, barley hordein, rye secalin, and oats (?) avenin protein ingestion ***(3,4)***. Since the highest concentrations of dietary prolamin occur in the duodenum and upper small intestine, mucosal damage is most marked in these regions, becoming progressively less severe toward the midjejunum and beyond. Thus, in vivo biopsy techniques tend to sample areas of greatest mucosal damage. It is also important to stress that mucosal biopsies are superficial and, at best, penetrate no farther than the muscularis mucosae (a point frequently ignored). Therefore, the material observed comprises only the mucous membrane, which comprises surface epithelium, lamina propria, and muscularis mucosa. The submucosa is also

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

inflamed in celiac sprue, but this region of the mucosa has never been studied quantitatively.

Despite much descriptive work in the literature, only a few attempts have been made to apply strict morphometric rules to the analysis of ex vivo biopsy material. However, despite the widespread availability of computerized image-analysis packages, the same semiquantitative, or descriptive, approaches (with all their intrinsic errors) continue to be slavishly adhered to: few new or original approaches have been evolved to take full advantage of the computerized facilities that are ripe for development. This chapter details approaches made in Manchester to evaluate quantitatively the tissue changes associated with celiac sprue disease, in comparison with disease-control tissues ***(5,6)***.

At the outset, it should be appreciated that the intestinal mucosa is partially anisotropic; that is, epithelium and muscularis mucosae have an orientation with respect to the plane of sectioning, whereas lamina propria does not. By convention, therefore, mucosal biopsies are sectioned "vertically," i.e., at right angles to the mucosal (luminal) surface. Good sectioning orientation is indicated by crypts that are cut precisely along their long axes.

How can valid, comparative measurements be effected between a control (villus-bearing) and a classical "flat" celiac mucosa with almost complete effacement of villi and massive hypertrophy of the crypts? In developing our techniques, we figured that one way of ensuring direct quantitative comparability would be to reconstruct the three-dimensional (3D) characteristics of each specimen, in relation to a standardized invariant third-party reference area ***(7)***. This comprises a 100 × 100 μm^2 of muscularis mucosa. The volumes of crypt epithelium, surface epithelium, and lamina propria per specimen were calculated with respect to that invariant reference, thereby ensuring the interspecimen comparability desired (**Fig. 1**). Furthermore, the complex 3D shape of each tissue compartment may be easily envisioned as a cube of equivalent volume, thereby permitting quantitative diagrammatic illustration of the results thus obtained ***(8–10)***.

Our system was put together with components built around the original Kontron-Mop Videoplan (Mark 1) system. The latter comprised a computer, drawing (digitizing) tablet, and stylus. We incorporated an Olympus (Olympus Optical, Southall, Middlesex, UK) research microscope equipped with ×4, ×10, ×20, ×40, ×60, and ×100 (oil planapochromat) objectives. A small, high-quality color TV camera was attached to the microscope tube in series with a variable zoom magnifier. The camera was used to give visual signals to a large, floor-mounted TV color monitor (screen size 24 × 24 in.) with which the operator could work with greatly magnified images. All components were assembled in and around a specially built work console.

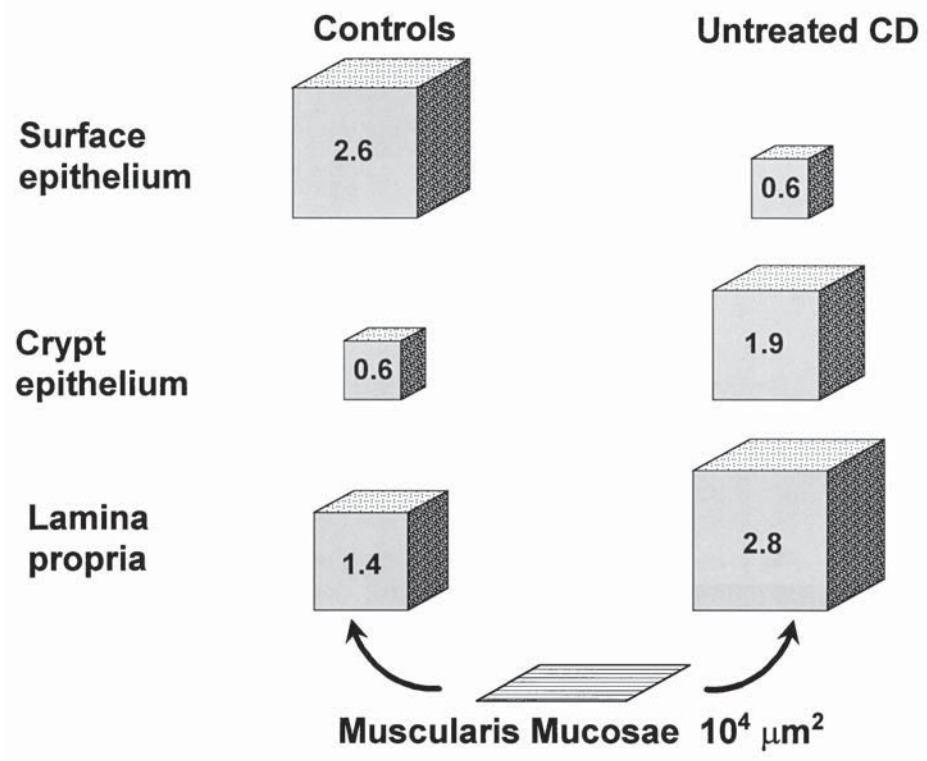


Fig. 1. The image-analysis technique described here is based on an arbitrarily chosen test square of muscularis mucosae, of $100 \times 100 = 10^4$ μm^2. All comparative measurements of either mucosal volume compartments, as illustrated here for surface and crypt epithelium and lamina propria, or of absolute cell populations contained therein, are related to that test area. Volumes are expressed semiquantitatively as cubes-of-equivalent volume.

2. Materials

2.1. Small Bowel Biopsy

Most of our work was performed with jejunal mucosae, obtained from the duodeno-jejunal flexure with a capsule, located under radiographic control.

2.2. Histological Technique

When performing biopsies, either from jejunum (Crosby Capsule) or duodenum (gastroduodenoscopic), retrieve the specimen(s) rapidly and orientate on dental wax (Agar, Stanstead, Essex, UK). Use a small hand lens (×10 magnification) to obtain optimal spreading of tissue on to the wax substrate. Proceed to tissue processing as described below under **Subheading 2.4.**

2.3. Fixation and Embedding Media

1. 0.1 *M* Sodium cacodylate buffer: Dissolve 10.7 g of sodium cacodylate (BDH, Poole, Dorset, UK) in 500 mL of deionized, distilled water. Add 0.17 g of $CaCl_2$. Adjust pH to 7.4 with 0.1 *N* HCl. Store at 4°C (*see* **Note 1**).
2. 2.5% Glutaraldehyde fixing solution: Use 25% ultrapure glutaraldehyde (TAAB, Emmer Green, Reading, Berks, UK). Dilute 1 mL of concentrate with 9 mL of 0.1 *M* cacodylate buffer (*see* **item 1**). Store at 4°C.
3. 1% Osmium tetroxide (OsO_4) (Agar): Dissolve 1 g in 100 mL of distilled, deionized water. Store at 4°C in a brown glass bottle, and cover in aluminum foil (*see* **Note 1**).
4. Epoxy resin embedding/infiltration mixtures: Araldite® (Ciba Geigy Agrochemicals AG, Basel, Switzerland) (obtained from Agar). Take 10 g of Araldite CY212, 10 g of dodecyenyl succinic anhydride (DDSA), 0.4 g of N-benzyl-N,N-dimethylamine (BDMA), and 1 g of dibutyl phthalate. Mix thoroughly and vacuum extract to remove all air bubbles. Store in deep freeze (at –70°C) in a 50-mL airtight plastic syringe. For infiltration, dilute this mixture 50:50 (v:v) with acetone. For use, remove from freezer up to 1 h before needed (*see* **Note 2**).
5. Toluidine blue stain (Charles Lamb, Wembley, Middlesex, UK): Dissolve 2.5 g of powder in 250 mL of distilled water. Store stock solution at 4°C. For daily use, draw up 20 mL into a plastic syringe. Fit a 0.22-µm Millipore filter to nozzle of the syringe to filter out debris and bacteria before flooding a slide. (Millipore, Watford, Herts, UK) (*see* **Note 3**).

3. Methods

3.1. Preparation of Tissues

1. Flood the fresh, well-orientated mucosal specimen with 2.5% cacodylate-cacodylate-buffered glutaraldehyde at room temperature (*see* **Note 4**) while still on wax.
2. After about 15 min, when the tissue is firm, cut into thin strips (1 to 2 mm wide by 5 to 6 mm long) with a chloroform-cleaned sharp blade on dental wax.
3. Reimmerse the mucosal strips in fresh glutaraldehyde in labeled glass pots (Agar).
4. After a further 3 h of fixation, decant off the glutaraldehyde from the pot.
5. Wash four times in sodium cacodylate buffer, 2 h each change.
6. Immerse tissue in 1% OsO_4 for 1 h (*see* **Note 4**).
7. Use a pipet to remove the OsO_4 and wash tissue four times in 0.1 *M* cacodylate buffer (with calcium, *see* **Subheading 2.3.**, **item 1**) and store overnight at 4°C. If further blackening of buffer solution occurs, replace with fresh buffer.
8. The next day decant off the buffer and dehydrate in acetone (two times).
9. Flood the tissue with 50:50 (v:v) Araldite:acetone solution and allow to infiltrate for 6 h at room temperature.
10. Replace 50:50 (v:v) dilute epon with embedding mixture, and allow the tissue to infiltrate for 24 h at room temperature.
11. Reembed each strip in fresh Araldite, in individual wells of a silicon block (Agar), and incubate for 24 h in an oven at 60°C (*see* **Note 5**).

12. Mount trimmed epon blocks on a microtome chuck. Section the block face, maintaining vertical orientation with check sections, at 1 μm thickness with a glass knife (*see* **Note 6**).
13. Stain-dry sections with buffered (pH 7.2) toluidine blue; rinse in 50, 70, and 100% (v:v) alcohol:distilled water; and mount in DPX (Charles Lamb).

3.2. Calibrating the Image-Analysis System

The image-analysis system must be calibrated for each new series of observations, so that distances measured with the stylus are related to real dimensions.

1. Use a calibrated micrometer in the microscope stage and obtain its image on the TV monitor. If tissue volumes are to be measured, use a ×10 or ×20 objective, or for cellular measurements, select the ×40 or ×100 objective. Use zoom magnification to obtain an optimal image on the monitor. Use the stylus to mark off an appropriate length i.e., 100 and 1 μm, respectively, from the TV-displayed micrometer, using two contacts with the digitizing board. Incorporate the actual number of micrometers, to which this calibration distance corresponds, into the program that has to be set up for each category of measurement. For convenience and consistency, once all these steps have been carried out, the procedure can be simplified. Given the chosen microscope objective and zoom magnification settings, the calibration distance can be transferred to a plastic ruler in which holes, capable of admitting the tip of the stylus, are drilled at the appropriate distance. The rule must also be inscribed with the category of measurement (such as volume determination or cell diameter, the objective required, and zoom setting).
2. Programming the computer: Each program is a "circular" program (follow manufacturer's directions) comprising a series of logical stages. The following is a representative example:
   a. Outline the lamina propria with the stylus (**IIIB**, **Fig. 2**).
   b. Outline crypt profiles: Program the computer to subtract this value from stage A.
   c. Mark off the corresponding length of the muscularis mucosae (**IIIC**).
   d. Store the data.
   e. Repeat stages **A–C**, or exit (**Fig. 2**). In this way, a series of observations can be made and accumulated at one sitting. The statistical manipulation of the data acquired depends on individual requirements, but can also be programmed into the computer.
3. Decide on your criteria for defining the junction between surface (villous) epithelium and crypt epithelium (**Fig. 3**).

3.3. Determining Mucosal Volumes

1. Calibrate the image analyzer for volume measurement, using predetermined criteria (*see* **Subheading 3.2.**, **item 1**).
2. Insert slide, bearing stained sections, on the microscope stage and obtain an image on the TV monitor. Use the stylus to draw around the tissue structure of interest, such as surface epithelium, crypt epithelium, or lamina propria (*see* **Note 7**). Once

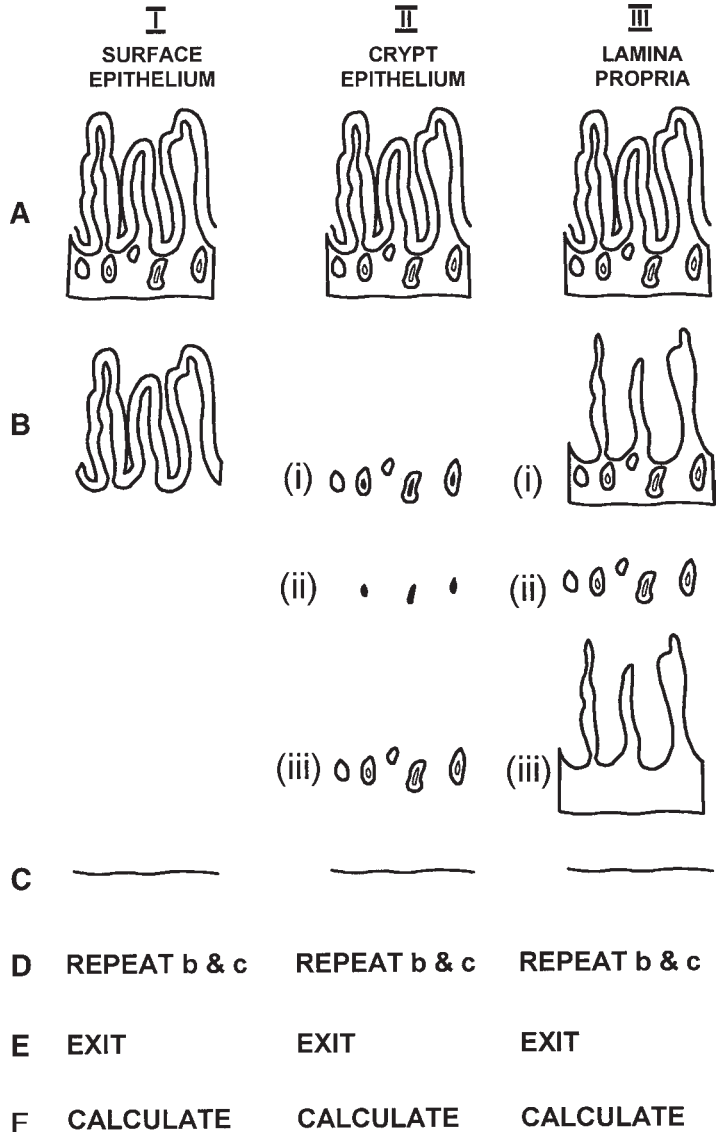


Fig. 2. The procedure used to measure mucosal compartment volumes of surface epithelium **(I)**, crypt epithelium **(II)**, and lamina propria **(III)**. A representative mucosal profile is shown in **(a)**. In **(b)** the component relevant to each compartment column is outlined with the stylus. In **II (b) (i)** and **III (b) (ii)**, it must be remembered that the crypt lumen **(II [b] [ii])**, or all crypt profiles **(III [b] [ii])**, must be subtracted, thus leaving the actual tissue profile required: **II (b) (iii)** and **III (b) (iii)**, respectively. Such manipulations can be built into each measuring program. The length of muscularis mucosae underlying the tissue area must then be marked off. Further repeats of **(b) + (c)** are made and successively stored, followed ultimately by exit **(e)** from the cycle, once sufficient data have provided a satisfactory mean volume, as calculated **(f)** by the computer.

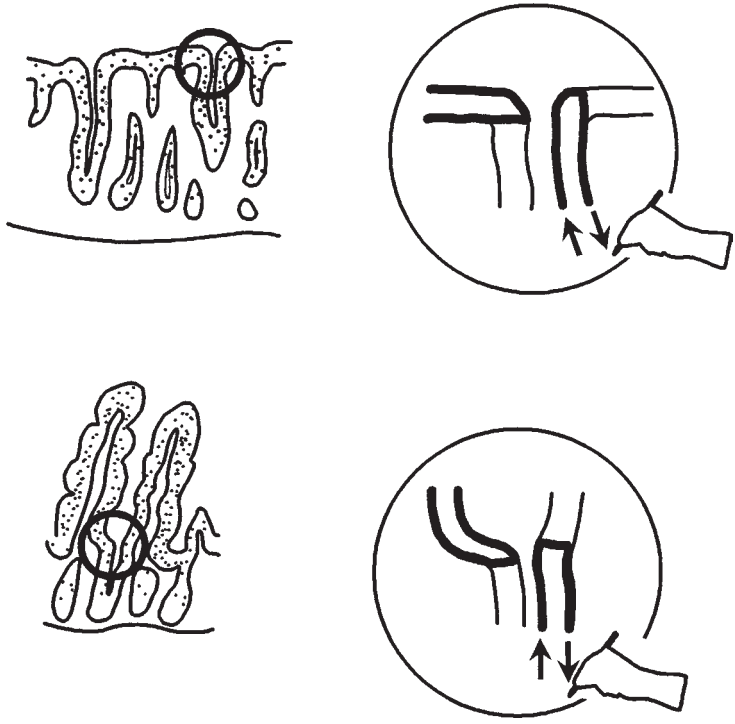


Fig. 3. It is necessary to make arbitrary decisions concerning the "cutoff" points between crypt and surface epithelium (flat mucosa) **(top)** and the crypt-villus function (normal mucosa) **(bottom)**. Our preference was for each solution in the circled enlargements.

the stylus has traced all the profile outlines, and unwanted structures where appropriate have been excluded, proceed to the next step in the program; that is, mark off the length of the muscularis mucosae underlying the profiles previously drawn. Next, enter the data in the computer, store, and return to the start of the program. Using another section, repeat the cycle of measurements and store the data. Repeat (**Fig. 2**) with sufficient measurements to give a mean value that is constant and satisfies the desired coefficient of variation (CV).

3.4. Determining Absolute Populations of Cells within a Defined Tissue Volume (per 10^4 μm^2 muscularis mucosae)

3.4.1. Background

The purpose here is to express any desired cell type as an absolute population, rather than as a profile-density count, which still remains the standard (if erroneous) approach in the literature. For cell population measurements,

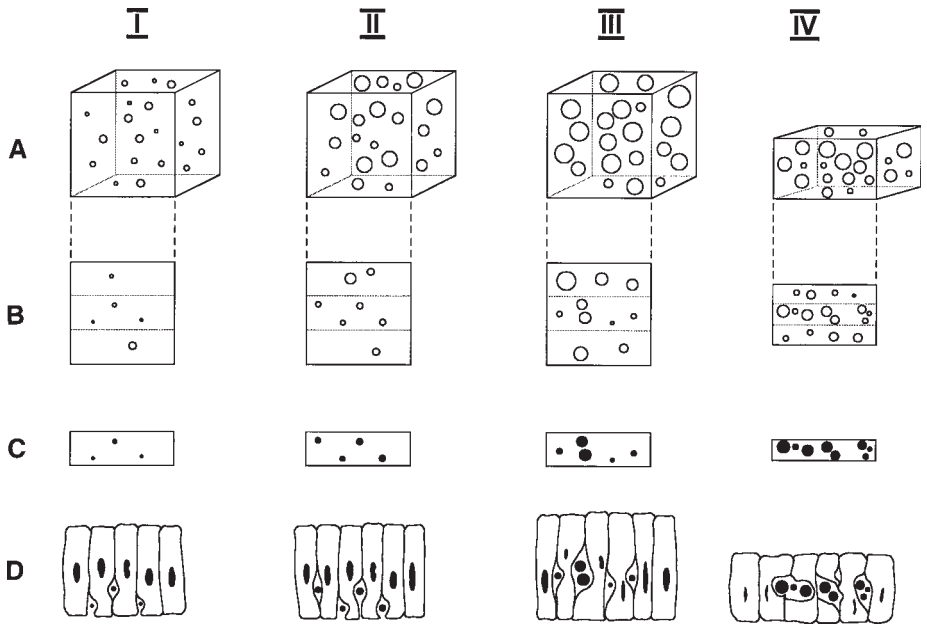


Fig. 4. The effect of (1) cell size and (2) volume of distribution in determining "profile densities" in any random tissue section. In each box **(A I–IV)**, there are 20 cells, whose mean size is small **(I)**, normal **(II)**, and large **(III, IV)**. Take an imaginary section **(B)** from each volume compartment (*see* **Fig. 1**) and at **(C)** a representative strip from **(B)**. At **(D)** an imaginary epithelium has been drawn around the profiles present in each strip **(C)**. Now, if these "epithelia" were read blind in the microscope, it might be concluded that "infiltration" progressively increased from **D I–D IV**. But this would be a fatal conclusion because, as the initial premise stated, the actual number of cells in each box **(A)** is identical.

it is not necessary to measure the actual volume of the mucosal compartment in which they are contained, since the cells are still counted relative to a 10^4 μm^2 test area of muscularis mucosae ***(9,11,12)***.

Cells are counted by their nuclei. Cytoplasmic boundaries can never be defined reliably at light microscope level (even if the cell of interest is only identifiable with immunohistochemical decoration of a marker specific to its plasma membrane).

In the two-dimensional (2D) realm of a tissue section, cells (nuclei) are represented as profile discs; a count of profile discs is a measure of cell density (number per volume) and is *not* a direct measure of the absolute number of cells present (**Fig. 4**). The profile discs seen reflect section thickness (assumed to be constant), cell (nuclear) size, and volume of distribution. Indeed, any

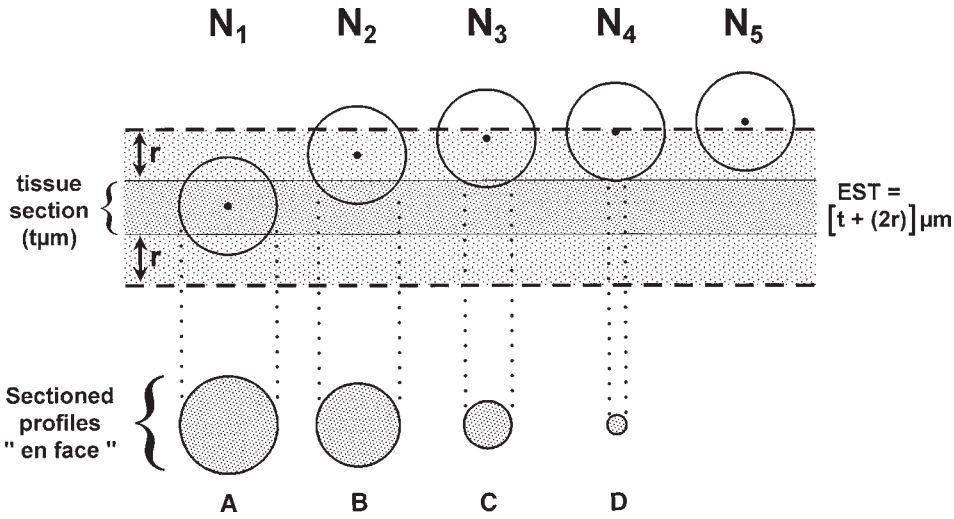


Fig. 5. **(Top)** A tissue section (of thickness *t* μm), widened on either side by the radius (*r*) of the nuclei (N1, 2…5) that occupies different positions relative to the section, *t*. As the nucleus (N_1, N_2, N_3, etc.) is progressively moved away from the microtome section (positions N2, 3, 4), its profile, as seen *en face* **(B, C, D)** gradually decreases in size. Once the center of the nucleus lies outside its radius (*r*), it will not have a profile in section *t* (N5). This point defines what is meant by EST, which numerically is $t + 2r = (t + D)$ μm. Thus, to make proper counts of cells from tissue sections, the (nuclear) diameter of such cells must be previously determined.

rough count of cell densities must be divided by effective section thickness (EST), which is section thickness (*t* μm) added to mean nuclear diameter (*D*) of the cell type being counted (**Fig. 5**). Thus, if $t = 1$ μm and $D = 5$ μm, then a count density of, say, 600 profiles is equivalent to $600 \div (5 + 1) = 100$ actual cells. The procedure is explained descriptively in the following sections.

3.4.2. Measurement of Nuclear Diameter

1. Calibrate the image analyzer, using a ×100 oil-immersion objective if working with 1-μm toluidine blue–stained plastic sections.
2. With the stylus, draw around the perimeters of 100 cell nuclei (e.g., intra-epithelial lymphocytes [IEL]) (*see* **Note 8**).
3. Store the data from each measurement and program the computer to convert circumferences into corresponding diameters. (*See* the manufacturer's instructions for establishing a computer program required for these manipulations.)
4. Draw a rough frequency diagram for the nuclei thus measured and determine the mode value for this distribution, and from its half-value, project a line back to the zero point (**Fig. 6**).

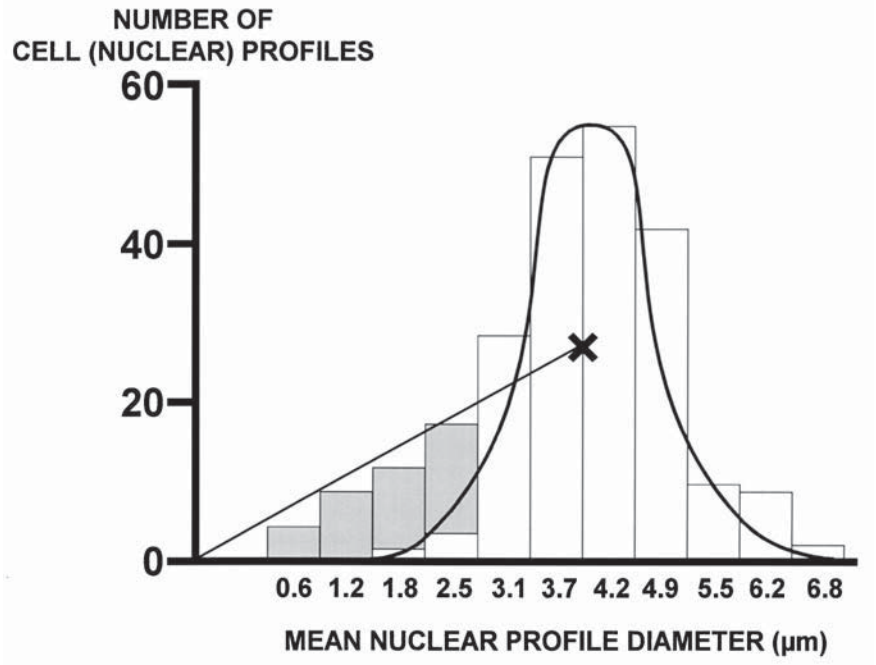


Fig. 6. A frequency distribution of profile diameters for a selection of 100 IEL. The circumference of each nucleus selected (using ×100 oil-objective optics) is drawn with the stylus; the computer then calculates the corresponding diameter. The halfway point on the mode **(X)** of this distribution is joined to zero, and the number of "lost profiles" at each size interval is determined (LH axis). The total number of lost profiles for each size interval is added to the original 100 observations; the computer recalculates the new diameter (D1) of the completed sample. Because this sample of profiles represents a frequency distribution of nuclear diameters (since not all nuclear profiles in any section will be sagittal), the recalculated diameter (D1) is multiplied by $4/\pi$ to correct for such nonsagittal sectioning. The result is the true mean diameter of the sample measured.

5. Add deficient "lost profiles" (owing to difficulty in recognizing very small tangential nuclear cuts as nuclear, as opposed to other adjacent cellular material in the section), as illustrated (**Fig. 6**).
6. Determine, by inspection, the number of lost profiles in each size class.
7. Use the keyboard to add manually lost-profile data to the original data set, and recalculate new mean D_1.
8. Another correction is now required, to compensate for the fact that all the profiles measured are not true diameters (i.e., equatorial cuts across all the nuclei sampled). Indeed, the series of measurements made represents a frequency distribution of those variables (**Fig. 5**). The correction is simply done by multiplying

D_1 by 4/π. The resulting value is the true nuclear diameter, from which the EST may then be calculated ***(7,8)***.

9. Now return to the original tissue slide. Count all nuclear profiles of cells appearing in the specific tissue component (epithelium, crypts, or lamina propria).
10. Input data with the keyboard.
11. Proceed to the next step in the program; that is, mark off the corresponding length of muscularis mucosae. Store the data and repeat this procedure until a constant density count is obtained. Scale up the count, appropriate to the ratio between the total length of muscularis mucosae actually counted and 10,000 μm of muscularis (≡100 × 100 = 10^4 μm^2 of test area).
12. Divide by the EST, as previously calculated. This gives the absolute number of cells overlying 10^4 μm^2 muscularis mucosae, distributed within that tissue component (i.e., surface epithelium, crypts, or lamina propria) (*see* **Note 9**).
13. **Figure 7** illustrates the graphical representation of data relevant to the spectrum of mucosal responses in gluten sensitivity (*see* **Note 10**) and their related immunopathological stages (types 1–3).

3.5. Measuring Epithelial Cell Volumes

In severely damaged mucosae, the surface epithelial cells often appear shortened and cuboidal (or square shaped) in sectioned profile. This phenomenon is related to the process of villus flattening, rather than to any particular disease process, because it is seen in a variety of conditions (tropical sprue, gluten sensitivity, giardiasis, etc.). Are such cuboidal epithelial cells smaller in volume, in comparison with normal columnar epithelial cells?

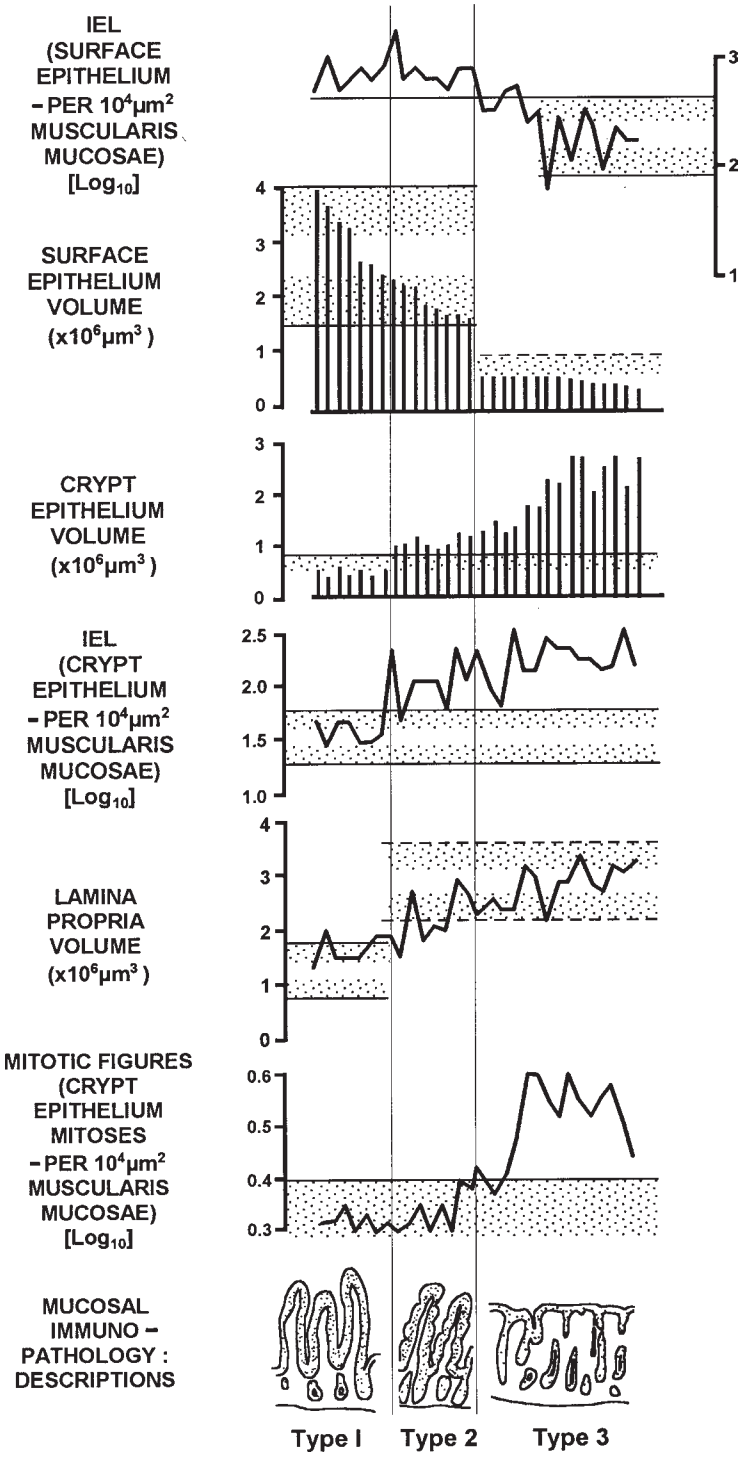
The factors that determine and maintain cell shape are varied; therefore, it is surprising that much analysis of cellular dimensions is confined to the 2D realm (i.e., widths and heights). Yet, it is clearly evident from studies of wound healing, e.g., that alterations in cell shape and volume occur with great rapidity, thus influencing the appearance of sectioned cells involved directly or indirectly in such processes.

3.5.1. The Geometry of Cell Volume Measurement

Two methods were devised to quantitate actual cell volumes in control, and disease, mucosae (*see* **Appendix**).

The first method assumes that an epithelial cell is a cylinder. This is the simplest model; its volume is described by the rotation of a planar rectangle vertically through 360° (**Fig. 8A**). The sides of the cylinder are parallel and both ends are assumed to be circular. The length of the cylinder is the vertical, sectioned, profile height of the epithelial cell (h) and its volume V is therefore $\pi r^2.h$, where r is one-half the mean profile cell width. The latter is the average, based on the width of the cell at its base (w_1) and along its luminal surface (w_2):

$$r = [(w_1 + w_2)/4]$$


IEL (SURFACE EPITHELIUM – PER $10^4 \mu m^2$ MUSCULARIS MUCOSAE) [Log_{10}]
3
2
1
SURFACE EPITHELIUM VOLUME ($x10^6 \mu m^3$)
4
3
2
1
0
CRYPT EPITHELIUM VOLUME ($x10^6 \mu m^3$)
3
2
1
0
IEL (CRYPT EPITHELIUM – PER $10^4 \mu m^2$ MUSCULARIS MUCOSAE) [Log_{10}]
2.5
2.0
1.5
1.0
LAMINA PROPRIA VOLUME ($x10^6 \mu m^3$)
4
3
2
1
0
MITOTIC FIGURES (CRYPT EPITHELIUM MITOSES – PER $10^4 \mu m^2$ MUSCULARIS MUCOSAE) [Log_{10}]
0.6
0.5
0.4
0.3
MUCOSAL IMMUNO – PATHOLOGY : DESCRIPTIONS
Type I
Type 2
Type 3

The second method assumes the cell can be described volumetrically as an inverted, truncated conoid. Its volume is described by the rotation of a truncated right-angled triangle through 360° (**Fig. 8B**). Volume, V, is given by the following expression:

$$V = \pi(h/3)[r_1^2 + (r_1 \times r_2) + r_2^2]$$

The vertical height of the cell is h, r_1 is the half-width (i.e., radius) of the cell at its apical pole, and r_2 is the corresponding half-width measured at its basal pole ***(13)***.

Information necessary for either equation is obtainable from the 2D tissue section. Epon sections, 1 μm thick, of glutaraldehyde-fixed material and stained with toluidine blue are examined under oil-immersion optics with a ×100 objective. This approach is less easily undertaken with thicker, wax-embedded material.

3.5.2. Image Analysis Procedure for Cell Volumes

1. Calibrate the digitizing tablet. Use an oil-immersion ×100 objective.
2. For the purposes of measuring h and w, select individual epithelial cells only where it is judged that sectioning is maximally "vertical" through each individual cell. For each cell selected, measure its width, both at the base and lumen, and height with the stylus. Program the computer to calculate volumes, according to each formula.
3. Sufficient measurements are made to achieve the desired CV. In our hands, it was necessary to measure 60 individual (surface) epithelial cells to achieve a steady mean, CV < 10%. Log-transform data to achieve a normal distribution. *See* **Table 1** for representative values for normal (control) jejunal mucosae and severely damaged ("flat") celiac mucosae (gluten sensitivity).

Fig. 7. *(opposite page)* Dermatitis herpetriformis provides the means of examining the entire immunopathological spectrum of the mucosal lesions (type 1–3) seen in gluten sensitivity. In the type 1 (infiltrated) lesion, the volume of villous epithelium is within the control reference range, but the absolute number of IEL is greater than the reference range. All other indices are normal. In the type 2 (infiltrative-hyperplastic) lesion, villous epithelium still remains normal and infiltrated with lymphocytes, but crypt epithelial volume is now increased above the control reference range; crypt epithelium is also infiltrated with lymphocytes. Note that lamina propria volume is also beginning to increase into the type 3 (flat) celiac range. With the classical type 3 (flat-destructive) lesion, villous epithelium is reduced, as is the complement of surface epithelial IEL, whereas hypertrophy and lymphocytic infiltration of crypt epithelium remain elevated. Lamina propria volumes are increased to at least twice those of control mucosae (and contain increased numbers of mast cells, basophils, and neutrophils, which are absent in type 1 lesions). At this stage, there is a marked rise in the mitotic activity of crypt epithelium. (Adapted from **ref. *17***.)

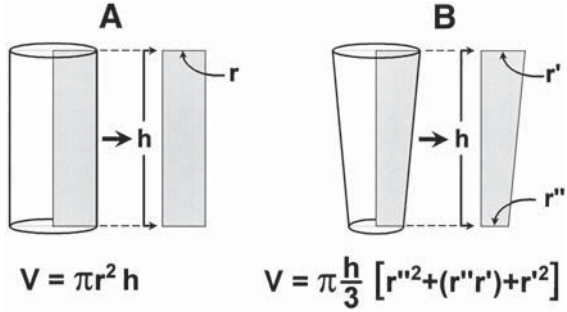


Fig. 8. Measuring cell volumes as **(A)** cylinders or **(B)** truncated conoids. The volume of a cylinder is described by the rotation of a rectangle, of height *h*, and width (= radius, *r*) through 360°. The volume of a truncated conoid is described by the rotation of a truncated, right-angled triangle of height *h*, base width r_1, and upper truncated width r_2 through 360°. Data for *h*, r_1, and r_2 can be obtained from the planar two-dimensional aspects of any sectioned cell. The relevant formulae for calculating each respective volume are shown and can be programmed into a computer.

Table 1
Morphometric Data for Small Intestine Mucosa[a]

Quantity	Disease-control mucosae	Untreated gluten sensitivity
Volume of epithelium, V_{SE} (per 10^4 μm² muscularis mucosae)	2.3×10^6 μm³	0.4×10^6 μm³
Mean volume of enterocyte	800 μm³	610 μm³
Number of enterocytes in V_{SE}	3000	600
Number of IEL in V_{SE}	350	190
Absolute ratio of IEL/100 enterocytes (morphometric)	12 (5–35)[b]	32 (16–65)[b]
Ratio IEL:enterocyte (profile density count) (%) ***(14)***	24 (11–53)	61 (31–122)
Mean height of enterocyte	37 (30–43) μm	33 (27–39)
Mean width of enterocyte	5.1 μm	4.7 μm
Mean width of enterocyte nucleus (i.e., diameter of cylinder)	3.3 μm	3.5 μm
Mean nuclear diameter (IEL)	4.9 μm	5.2 μm
EST (1-μm section)		
Enterocytes	4.3 μm	4.5 μm
IEL	5.9 μm	6.2 μm

[a]IEL have virtually circular (spherical) nuclei. Widths of enterocyte nuclei are equivalent to "diameter" and have been determined by direct measurement. Data are obtained from precisely oriented 1-μm epon sections; the sections have a very accurate thickness since they are cut with a high-precision EM-type ultramicrotome.

[b]Log-transformed mean and range.

4. Notes

1. Glutaraldehyde, sodium cacodylate (containing arsenic), and OsO_4 are unpleasant, toxic compounds. They should be handled with gloved hands in a proper fume cupboard. Avoid inhaling the vapor from either glutaraldehyde or OsO_4, which results in sore eyes, a blocked nose, and a sensation of a blocked nose that lasts for a day or two after exposure. Consider wearing protective spectacles and a face mask. OsO_4 takes a long time to dissolve, and it is best if the glass vial containing the substance is cut open and then immersed in the water. Dissolution of 1 g will take several days, and therefore the container needs to be put in to a dark environment while this takes place. It is also important to remember that the vapor escaping from the OsO_4 solution will blacken objects adjacent to it, so the bottle itself must be encased in another screw-top container large enough to hold it and then covered in aluminum foil. Preserved in this way, the OsO_4 stock solution will last for several months when stored at 4°C. If the solution should turn black or purple, then it must be discarded.
2. Araldite is unpleasant to work with. Its components are possibly carcinogenic in the unpolymerized state. Make up in bulk and store in a sterile, airtight container (i.e., 50-mL syringe) at –70°C. Before the Araldite is required, take the syringe out of the freezer and allow thawing: it takes about 1 h. After thawing, the appropriate volume required can be extruded from the syringe. Once that has been done, the syringe is replaced in the freezer. We have experienced no loss of activity by repeated freeze-thawing when required.
3. Bulk toluidine blue stain is susceptible to bacterial contamination. The bacteria seem to adhere to tissue sections despite the alcohol wash. We use, for current needs, a small volume of the prepared dye in a small syringe to which is fitted a Millipore filter containing a 0.22-μm membrane (type GS). This prevents debris and other bacteria from contaminating the slide.
4. Do not dispose of glutaraldehyde, OsO_4, or alcohol via a sink. Use properly designated toxic-disposal waste units. These should be available, and part of the routine safety procedures, in any laboratory.
5. Embed one strip per well in the block, with the long axis of the tissue strip set transversely across one end of the rectangular wall. In this way, optimal tissue sections can be obtained.
6. Repeated sectioning of a block may lead to suboptimal orientation. Random sections must always be stained and observed in the microscope to guard against this problem, particularly when a large series of slides for measurement is being cut off for staining and mounting.
7. It is best to examine the lamina propria separately in terms of its volume and the cells contained, i.e., mast cells, plasma cells, eosinophils, or neutrophils. In toluidine blue sections, mast cell granules appear magenta purple, eosinophils have bright green cytoplasmic granules, neutrophils appear as dark speckled greenish-gray cytoplasm. Hence, 1-μm plastic sections should be used because the resolution is excellent under ×1000 oil-immersion optics and the staining permits recognition of the majority of cell types encountered in intestinal mucosa. How-

ever, the distinction between plasma cells and lymphocytes is difficult, and immunocytochemical image analysis is necessary for good results, as described by Ensari (*see* **Chapter 11**).

8. In our experience, a population of 100 cells provides a good sample, including CVs <10%.
9. In linear terms, the test area (100 × 100) μm^2 is equivalent to a total length of 10,000 μm of muscularis mucosa. When volumes are being measured, approx 2000 μm of linear muscularis mucosae must be accumulated for one completed set of measurements. The total accumulated volumes are then multiplied by 5, and to scale up to 10,000 linear micrometers of muscularis and for convenience, they are expressed per 10^6 μm^3. For nuclei, EST must be taken into account. For example, if EST is equivalent to ($t + D$) or (1 + 5), the calculation will not depend on 10,000 μm (100 × 100 μm^2) muscularis mucosae, but [(100 ÷ 6) ×100]μm. The data are then scaled up appropriately to that revised linear length of muscularis mucosae (i.e., 1670 μm).
10. The description of these procedures seems immensely complicated. On the contrary, it is quite easy. Once everything is up and running, it takes about 30 min to obtain a real diameter (D) of a cell (nuclear population). It takes another 30 min to obtain a constant value of volumes of surface and crypt epithelium (and cells contained as absolute populations).

Appendix

We used ***(13)*** a third method for determining individual cell volumes, V_{ENT}, by using IEL/enterocyte ratios as determined by the conventional method of Ferguson and Murray ***(14)***. **Figure 9** illustrates the procedure. However, values for V_{ENT} were approx 1600 μm^3, values that were almost twice those obtained by the geometric methods (about 800 μm^3/enterocyte).

Furthermore, we found a disparity between the IEL/enterocyte ratios calculated by the Ferguson and Murray ***(14)*** method and our results, based on morphometry. Thus, for control mucosae, there are approx 350 IEL and 3000 enterocytes within a standardized V_{SE} (2.6 × 10^6 μm^3), giving a true, absolute ratio of 12 IEL (10–16) per 100 enterocytes (**Fig. 10**). In our hands (counting 1000 enterocyte nuclei in 1-μm toluidine blue–stained epon sections), the Ferguson-Murray ratio yielded data of 24 IEL (11–53) per 100 enterocytes. Clearly there are problems with this latter technique! We therefore investigated why the Ferguson-Murray technique overestimated, by a factor of 2, the actual number of IEL present within epithelium ***(15)***.

First, an EST must be calculated for IEL nuclei, as well as for enterocyte nuclei (**Fig. 11**) assuming (here) the use of 1-μm toluidine blue–stained plastic sections. For enterocyte nuclei, EST is 3.3 + 1 = 4.3 μm whereas for IEL, EST is 4.8 + 1 = 5.8 μm, for control mucosae. Corrections must therefore be applied separately for the count of enterocyte nuclei, and for IEL nuclei. Thus, for

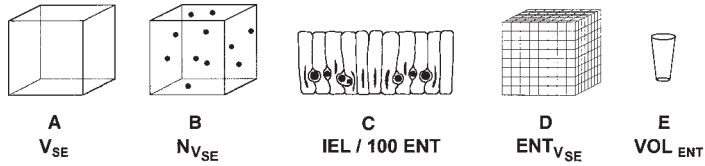


Fig. 9. Another method of calculating cell volumes was based on a 3-deductive approach, derived from image-analysis data. As described in this chapter, a volume of epithelium (V_{SE}) (**Fig. 1**) relative to a constant test area of muscularis mucosae can be defined **(A)**, and its content of lymphocytes calculated in absolute terms **(B)**. Using the Ferguson-Murray technique, the relative ratio of lymphocytes to epithelial cells is determined **(C)**, so that from **(A)** and **(B)**, the total number of enterocytes contained within V_{SE} **(A)** can be deduced, from which the average volume (V_{ENT}) of an enterocyte may be determined **(E)**. The errors intrinsic to this method, deriving solely from the inaccuracies of the Ferguson-Murray technique, are pointed out (*see* **Appendix**) and illustrated (**Table 1**).

100 enterocyte nuclei, only 100 ÷ 4.3 or 23 *actual* enterocytes would have been counted, whereas for IEL, representative figures would be 23 ÷ 5.8 = 4 actual IEL. Scaling up, this actual ratio (4 IEL/23 enterocytes) becomes 16 IEL/100 enterocytes.

Second, a further problem that results from counting "enterocyte nuclei" must be addressed. This problem occurs because enterocytes (and their nuclei) are not arranged in parallel rows, but in short-range hexagonal array, as has been demonstrated both by transmission electron microscopy (TEM) and scanning electron microscopy (SEM), and as a result, several nuclei will be "lost" in any routine count of enterocyte nuclei. In **Fig. 12**, enterocytes, and their nuclei, are drawn *en face*, but also to scale based on other morphometric data ***(13,15,16)***. The lines represent random planes of section through this "epithelial layer," and the ratios the number of nuclear and enterocyte (cytoplasmic) hits along each "section" plane. Representative epithelial profiles visualized from sections A, B, and C are demonstrated underneath, illustrating the extent of cell losses incurred when only enterocyte *nuclei* are counted. Unfortunately, cell boundaries are quite difficult to visualize, even with 1-μm sections observed under oil-immersion optics. But, as these data reveal, in an average count of 100 enterocytes, approx 50 enterocytes will be lost. This is another important reason that the Ferguson-Murray "counting" technique yields grossly inflated values for IEL.

Thus, taking the previously described example, our raw count is no longer 4 IEL/23 enterocytes, but 4 IEL/34 enterocytes (i.e., 23 + 11) or approx 12 IEL/

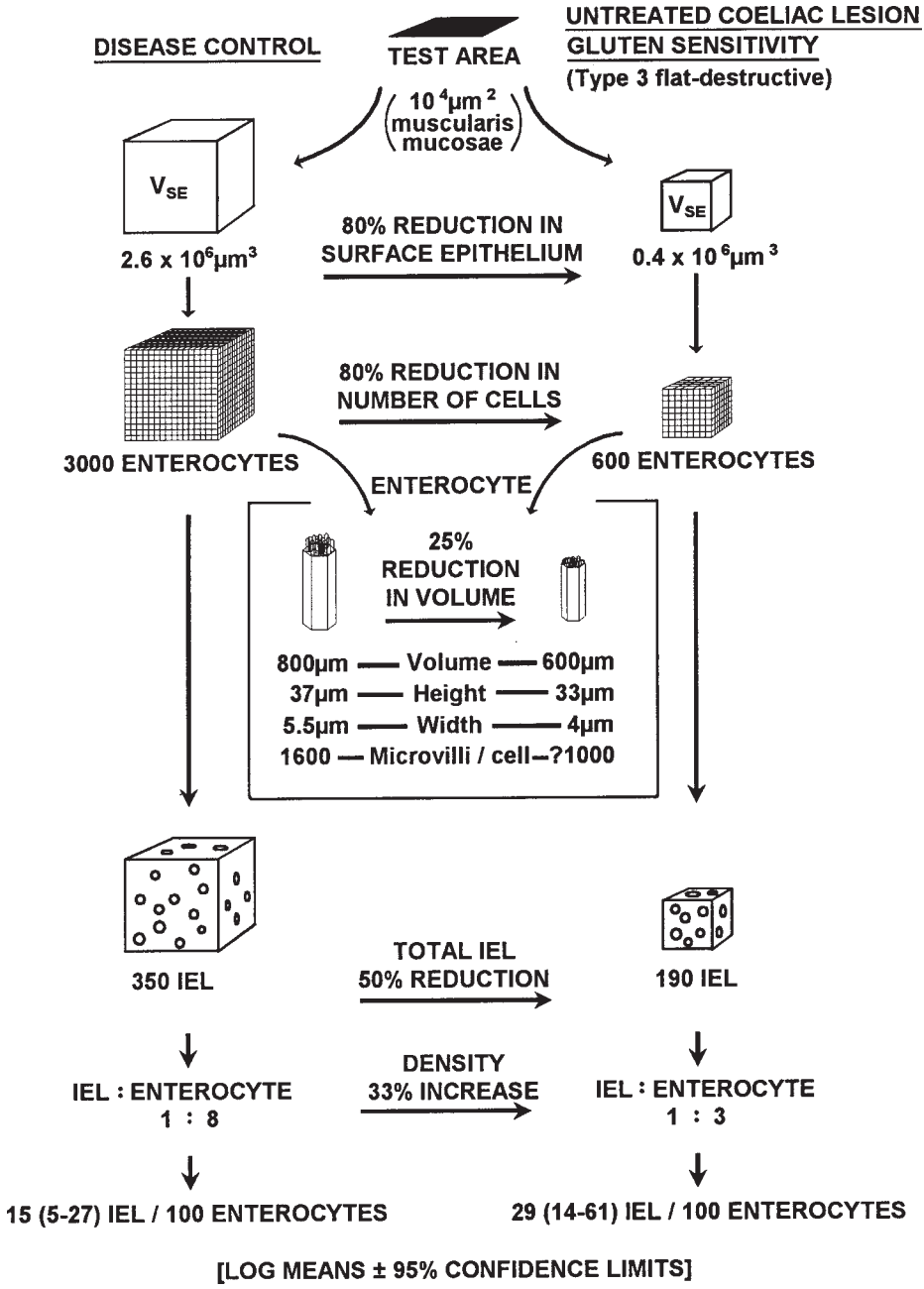


Fig. 10. Semivisual display of morphometric data for disease control and untreated celiac mucosae obtained by image analysis. Note the correct figures for IEL: enterocyte ratios, following logarithmic transformation.

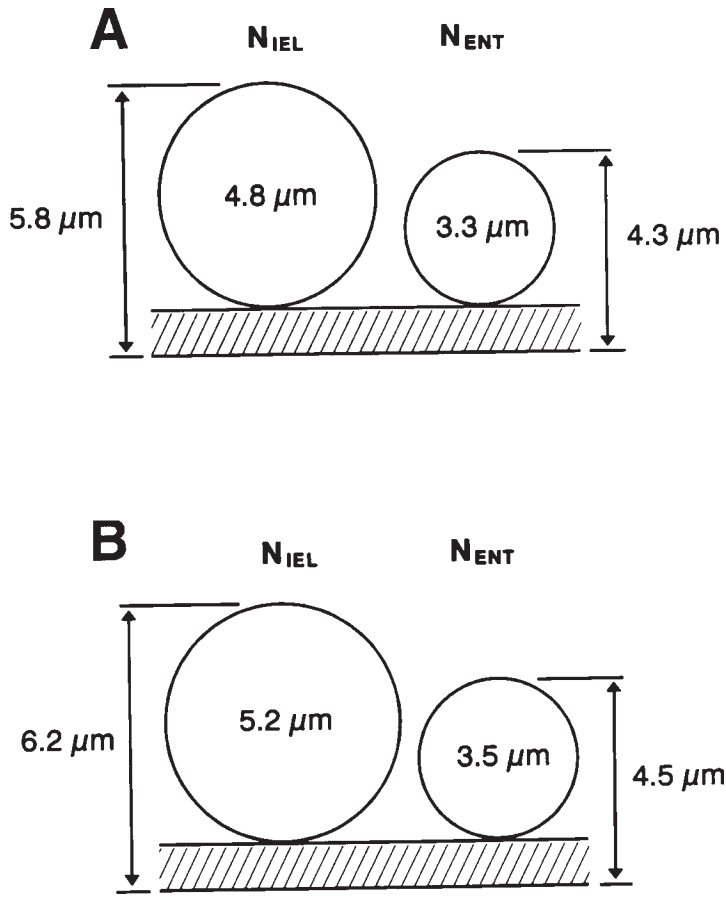


Fig. 11. Based on the principle explored in **Fig. 5**, this figure gives values for EST for enterocyte nuclei (N_{ENT}) and IEL nuclei (N_{IEL}) in **(A)** disease-control and **(B)** untreated gluten-sensitive mucosae. Note that although enterocyte nuclei are prolate spheroids, their "diameter" corresponds to their cross-sectional (radial) dimensions, so that EST is easily calculable.

100 enterocytes, a figure that now agrees closely with the computerized morphometric data. This affirms how crudely inaccurate the Ferguson-Murray technique is, in overestimating the ratio of IEL to enterocytes, it accounts for the inordinately high values for V_{ENT}, obtained when these data were employed in its calculation.

Finally, because the IEL data are not normally distributed (despite the erroneous claims of Ferguson and Murray *[14]*), it is necessary to log-transform the information. Sensible figures from our observations are given in **Table 1**.

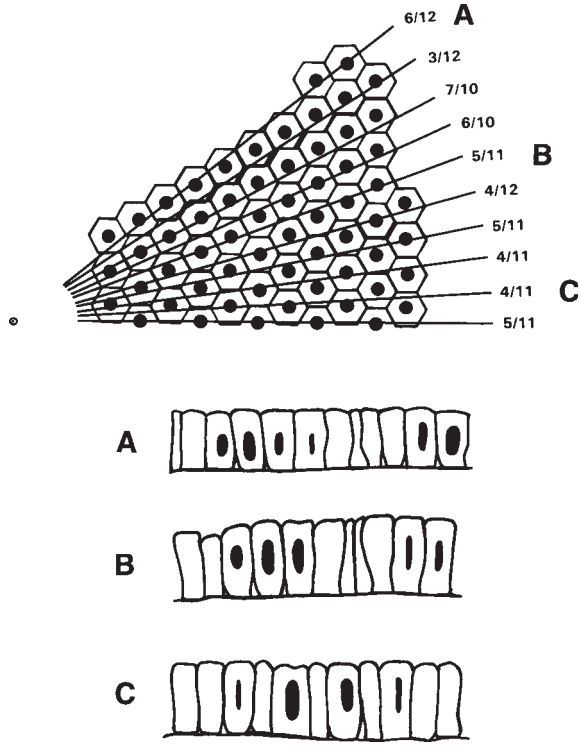


Fig. 12. **(Top)** An "epithelium" viewed *en face*: it is drawn to scale, based on previously known data. The lines represent random sectioning planes through this epithelium. Note that, on average, there are only approx 50% nuclear hits, as opposed to cytoplasmic hits, per cell sectioned. **(Bottom)** A, B, and C are drawn imaginatively, as they might appear in sectioned profile. Note, therefore, how many cells are "lost" when cell counts are solely dependent on scored nuclei.

References

1. Marsh, M. N. (1983) Immunocytes, enterocytes and the lamina propria: an immunopathologic framework of coeliac disease. *J. R. Coll. Phys. (Lond.)* **17,** 205–212.
2. Marsh, M. N. (1987) Coeliac disease, in *Immunopathology of the Small Intestine* (Marsh, M. N., ed.), John Wiley & Sons, Chichester, UK, pp. 371–399.
3. Marsh, M. N. (1992) Gluten, major histocompatibility complex, and the small intestine: a molecular and immunobiologic approach to the spectrum of gluten sensitivity ("celiac sprue"). *Gastroenterology* **102,** 330–354.
4. Marsh, M. N. (1993) Mechanisms of diarrhoea and malabsorption in gluten-sensitive enteropathy. *Eur. J. Gastroenterol. Hepatol.* **5,** 784–795.
5. Marsh, M. N. (1992) Mucosal pathology in gluten sensitivity, in *Coeliac Disease* (Marsh, M. N., ed.), Blackwell Scientific, Oxford, UK, pp. 136–191.

6. Marsh, M. N. (1988) Studies of intestinal lymphoid tissue. XI—The immunopathology of cell-mediated reactions in gluten sensitivity and other enteropathies. *Scanning Microsc.* **2,** 1663–1684.
7. Weibel, E. R. (1979) *Stereological Methods*, Academic, New York.
8. Niazi, N. M., Leigh, R., Crowe, P., and Marsh, M. N. (1984) Morphometric analysis of small intestinal mucosa. I. Methodology, epithelial volume compartments and enumeration of inter-epithelial space lymphocytes. *Virchows Arch. (Pathol. Anat.)* **409,** 11–22.
9. Dhesi, I., Marsh, M. N., Kelly, C., and Crowe, P. (1984) Morphometric analysis of small intestinal mucosa. II. Determination of lamina propria volumes: plasma cell and neutrophil populations within control and coeliac disease mucosae. *Virchows Arch. (Pathol. Anat.)* **403,** 173–180.
10. Marsh, M. N. and Hinde, J. (1986) Morphometric analysis of small intestinal mucosa. III. The quantitation of crypt epithelial volumes and lymphoid cell infiltrates, with reference to celiac sprue mucosae. *Virchows Arch. (Pathol. Anat.)* **409,** 11–22.
11. Marsh, M. N., Mathan, M., and Mathan, V. I. (1983) Studies of intestinal lymphoid tissue. VII—The secondary nature of lymphoid. *Am. J. Pathol.* **112,** 301–312.
12. Marsh, M. N. and Hinde, J. (1985) Inflammatory component of celiac sprue mucosa. I—Mast cells, basophils and eosinophils. *Gastroenterology* **89,** 92–101.
13. Crowe, P. T. and Marsh, M. N. (1993) Morphometric analysis of small intestinal mucosa. IV. Determining cell volumes. *Virchows Arch. (Pathol. Anat.)* **422,** 459–466.
14 Ferguson, A. and Murray, D. (1971) Quantitation of intra-epithelial lymphocytes in human jejunum. *Gut* **12,** 988–994.
15. Crowe, P. T. and Marsh, M. N. (1993) Morphometric analysis of intestinal mucosa. VI—Principles in enumerating intra-epithelial lymphocytes. *Virchows Arch. (Pathol. Anat.)* **422,** 459–466.
16. Marsh, M. N. (1972) The application of scanning electron microscopy to the study of control and diseased human intestinal mucosa, in *Recent Advances in Gastroenterology* (Badenock, J. and Brooke, B. N., eds.), Churchill Livingstone, London, pp. 49–72.
17. Marsh, M. N. (1989) Studies of intestinal lymphoid tissue. XV—Histopathological features suggestive of cell-mediated reactivity in jejunal mucosae of patients with dermatitis herpetiformis. *Virchows Arch. A. Pathol. Anat.* **416,** 125–132.

11

Morphometry of Rectal Mucosa

**Arzu Ensari, Michael N. Marsh,
Peter T. Crowe, and Kieran Moriarty**

1. Introduction

1.1. Background

The diagnosis of many gastrointestinal disorders is made by assessing lesions in an endoscopic mucosal biopsy subjectively, or by objective parameters and morphometric techniques ***(1–4)***. Morphometry was first introduced into pathology 80 yr ago. It arose from doubts about qualitative observations and the need to correlate changes in morphology with function ***(5,6)***. The quality of the results of morphometry is mainly affected by the preparation of the specimens. Since biological structures such as mucosae are highly organized structurally and, in mathematical terms, are extremely anisotropic, a proper allowance for the orientation of the measured structures must be made. Thus, in comparative morphometric studies, it is essential to ensure that only sections obtained in proper and consistent orientation are used ***(2,7,8)***.

1.2. Basis of Quantitative Analysis

The fundamental need is specific visualization of the structure(s) to be measured, or enumerated. Immunohistochemistry can contribute to the precise demonstration of either specific (morphological) features or cell populations, so that the combination of immunohistochemistry with computer-assisted image-analysis systems ("immunemorphometry") permits objective, reproducible quantification of the stained structures in relation to total tissue architecture ***(9,10)***.

In the last few years, however, several morphometric techniques have been developed such as simple measurements using linear scales or grids inserted into the ocular ***(11,12)***, point counting ***(13)***, image analysis on a television

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

monitor ***(14)***, measurements of surface-to-volume ratios ***(15)***, as well as sophisticated methods such as "computer analysis projections" ***(4,16–19)***. In quantitative histological studies, all measurements and counts have to be referred to other baseline or reference measurements ***(14)***. A confusing variety of quantitative approaches have been used, with heterogeneity in the methods of cell counting yielding results that were also not uniform ***(20)***. In some studies, the density of cells was calculated for a length of muscularis mucosae ***(8,21)*** whereas in others an area of muscularis mucosae ***(15,22–24)*** was used as a third-party reference; these are both philosophically valid.

1.3. The Three-Dimensional Factor

The rectal mucosa is a three-dimensional (3D) structure; hence, observations must not be based solely on the two-dimensional appearances in tissue sections ***(24)***. When sections are quantitated by microscopy, all structures observed are profiles of their actual 3D reality. The number of cellular profiles actually observed depends on three variables:

1. Section thickness (which for quantitative work should be constant and hence irrelevant)
2. The actual number of cells present in the tissue (to be determined more reliably when immunohistochemical staining is performed)
3. More important, the size (i.e., diameter) of those cells ***(25,26)***

The size (diameter) of cells influences the number of profile discs present in any unselected tissue section when viewed under the microscope; thus, larger cells create larger profiles and hence appear more frequently in a given section compared with smaller cells, whose profiles will appear less often. Therefore, in quantitating unknown tissues, it is essential to know mean cell size (diameter). Nuclei, in general, are easier to work with because they stain well and lend themselves more effectively to accurate quantitation because their profiles are more regular in outline. All cell profiles, as far as rectal morphometry is concerned, are deemed to be nuclear cellular profiles ***(26)***.

The first systematic, prospective approach to formal morphometry of rectal mucosa was carried out by Loft et al. ***(27)*** with computerized image analysis to permit objective assessment of mucosal structure and cellular content. He used toluidine blue–stained 1-μm thick epon sections and morphometric methods that had been validated previously for jejunal mucosa in gluten sensitivity ***(22)***. Loft's method ***(27)*** was based on the use of a constant test square of muscularis mucosae, of side 100 μm, so that all comparative measurements were related to the same test area (10^4 μm^2). This methodological approach has been used with consistent results in a wide variety of studies from the same laboratory ***(15,27–29)***. Our methodology, as described in this chapter, is adapted from Loft's ***(30)***. It is

modified by the additional use of immunohistochemistry ***(31–33)***, hence called immunomorphometry.

In contrast to the purely histological analysis performed by Loft ***(30)*** with ultrathin epon sections, the immunohistochemical techniques used in our studies proved superior in quantitating inflammatory cells. The discrepancy between Loft et al.'s results ***(27)*** and those presented in later studies ***(31–34)*** arises from the difficulty of deciding, purely on morphological criteria, whether a cell is a lymphocyte, macrophage, or neutrophil. The use of specific monoclonal antibodies (mAbs) with immunohistochemical techniques allows precise distinction, via cell-surface antigens expressed specifically on particular cell types, without dependence on the morphological features that can mislead the observer, resulting usually in an underestimation of the number of cells actually present. This is evident from the fact that, although identical morphometric procedures were employed in both studies (cells were enumerated with respect to a standard test area of 10^4 μm^2 of muscularis mucosae), the actual number of lymphocytes in Loft's analysis ***(27,30)*** was much smaller than that revealed in our studies, hence proving the power of morphometry when used with immunohistochemistry (immunemorphometry) in the diagnosis of inflammatory diseases of the gastrointestinal (GI) tract.

2. Materials

2.1. Frozen Tissue Preparation

1. Dental wax.
2. Thin strip of carrot (20 × 4 × 2 mm).
3. Aluminum foil boat (*see* **Fig. 1**).
4. OCT embedding compound (BDH, Poole, UK) (store at room temperature).
5. Liquid nitrogen-cooled isopentane (–160°C).
6. Cardice (solid carbon dioxide) (Distillers MG, Reigate, Surrey, UK).
7. Dichlorodifluoromethane spray (store at room temperature).

2.2. Immunohistochemical Techniques

2.2.1. Antibodies

All antibodies should be stored at 4°C. All dilutions are made with Tris-buffered saline (TBS).

1. DAKO-CD3: Antihuman T-cell, CD3 UCHT1 IgG1, kappa, dilution 1:50 (Dako, High Wycombe, UK).
2. Identi-T TCRδ1: Murine mAb to δ chain of human γδ T-cell antigen receptor, 5A6.E9, IgG1, dilution 1:15 (T-Cell Diagnostics, Endogen Inc., Woburn, MA).
3. DAKO-M1: Monoclonal mouse antihuman granulocyte-associated antigen (CD15), C3D-1, IgM, kappa, dilution 1:50 (Dako).

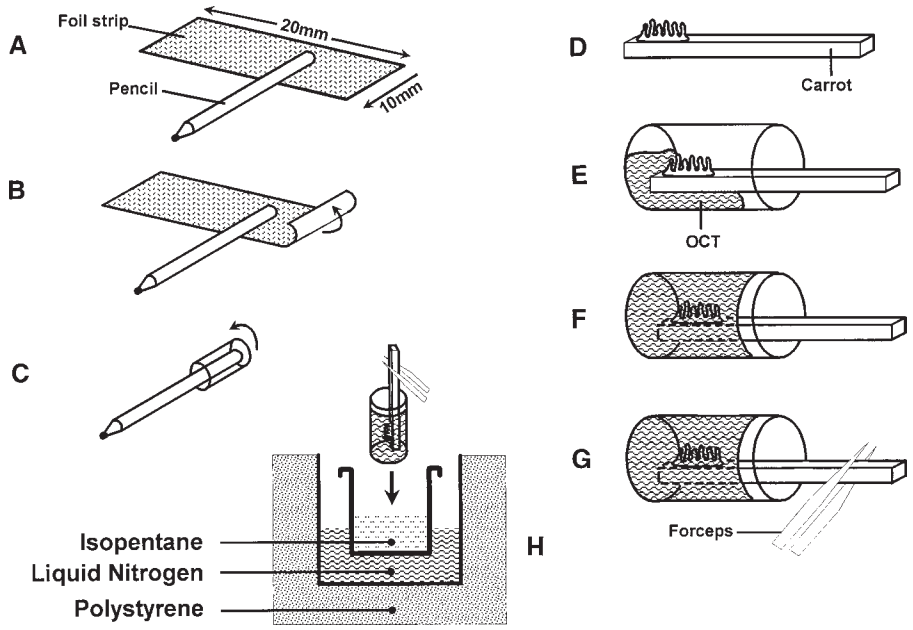


Fig. 1. How rectal mucosal biopsies are handled. Take a smooth piece of aluminum foil **(A)** and wrap it around a pencil **(B)** until a cylindrical cap has been made **(C)**. Next mount mucosal biopsy on a small strip of carrot, 30 mm long, 2 to 3 mm wide and deep **(D)**. Introduce some OCT compound into the base of the aluminum cap and gently place the carrot strip (with biopsy) on to the OCT **(E)**. Gradually fill the remainder of the aluminum cap without creating an air bubble **(F)**. Then, using forceps, plunge the entire assembly **(G)** into liquid isopentane surrounded by a container of liquid nitrogen **(H)**. Place frozen material into a small plastic bag, fasten, label, and store in a liquid nitrogen Dewar.

4. DAKO-CD25: Monoclonal mouse antihuman interleukin-2-receptor (CD25), ACT-1, IgG1, kappa, dilution 1:50 (Dako).
5. DAKO-CD68: Monoclonal mouse antihuman macrophage (CD68), KP1, IgG1, kappa, dilution 1:50 (Dako).
6. DAKO-HLA-DR: Monoclonal mouse antihuman HLA-DR, DK22, IgG2a, kappa, dilution 1:50 (Dako).
7. Rabbit antimouse (RAM) immunoglobulins: dilution 1:50 (Dako).
8. Peroxidase-conjugated rabbit antimouse (PxRAM) immunoglobulins: dilution 1:25 (Dako).
9. Alkaline phosphatase-antialkaline phosphatase (APAAP) mouse monoclonal: AP7/6/7, IgG1, kappa, dilution 1:50 (Dako).
10. One hundred microliters of antibody solution (AS) is needed for each section (n) for immunohistochemistry. After calculating the total antibody solution (TAS = $n \cdot$ AS) for the slides to be stained, the amount of antibody for the recommended dilution is calculated as TAS/dilution ratio. The volume of antibody is then sub-

tracted from TAS to indicate how much buffer solution is necessary to make the appropriate dilution for *n* number of sections.

2.2.2. Buffers, Solutions, and Substrates

1. 0.5 *M* Tris-HCl, pH 7.6 (10×).
2. 1.5 *M* NaCl (10×).
3. TBS.
4. 3,3'-Diaminobenzidine tetrahydrochloride (DAB) (Dako): Dissolve one DAB tablet in 10-mL of TBS. Immediately before applying to sections, add 7.5 µL of 3% hydrogen peroxide per 1 mL of DAB solution (toxic and light sensitive).
5. Fast Red solution: Dissolve 2 mg of Naphthol AS-MX phosphate (Sigma [St. Louis, MO]) in 200 µL of dimethylformamide (Sigma) (toxic and light sensitive) in a glass tube. Dilute to 10 mL with TBS (pH 8.2) and add 30 µL of levamisole (Sigma) to block endogenous alkaline phosphatase activity. Immediately before staining, add 10 mg of Fast Red salt (Sigma) to solution.

3. Methods

3.1. Rectal Biopsies

1. Take rectal biopsies approx 5–10 cm from the anal margin through an illuminated rigid sigmoidoscope. At each time interval (if appropriate) obtain four pieces, orientate and spread on dental wax, and handle as described below. Use Brock biopsy 4-mm angled-up cup forceps (cat. no. 19.4031, Seward Medical, London, UK) (**Fig. 2**) (*see* **Note 1**).

3.2. Cryostat Sections

1. Mount the biopsy specimen carefully on a thin strip of carrot (20 × 4 × 2 mm), place in an aluminum foil boat filled with OCT embedding compound (BDH), plunge immediately into liquid nitrogen–cooled isopentane (–160°C) (*see* **Fig. 1**), and subsequently store in a liquid nitrogen Dewar (*see* **Note 2**).
2. Immediately before sectioning, remove the aluminum foil around the tissue block on Cardice (solid carbon dioxide) (Distillers MG). Mount the block on a microtome chuck with OCT embedding compound, and freeze with dichlorodifluoromethane spray (*see* **Note 3**).
3. Cut two consecutive 6-µm sections on a Bright cryostat (*see* **Note 4**), place on glass slides, and air-dry.
4. Wrap sections in aluminum foil and keep in a –70°C freezer until needed further (*see* **Note 5**).

3.3. Immunohistochemical Staining Techniques

3.3.1. Preparation of Buffer Solution

1. Prepare 0.5 *M* Tris-HCl pH 7.6 (10×) by dissolving 60.55 g of Trizma base in slightly less than 1 L of distilled water. Adjust pH to 7.6 with concentrated HCl, and make up to 1 L with distilled water (*see* **Note 6**).

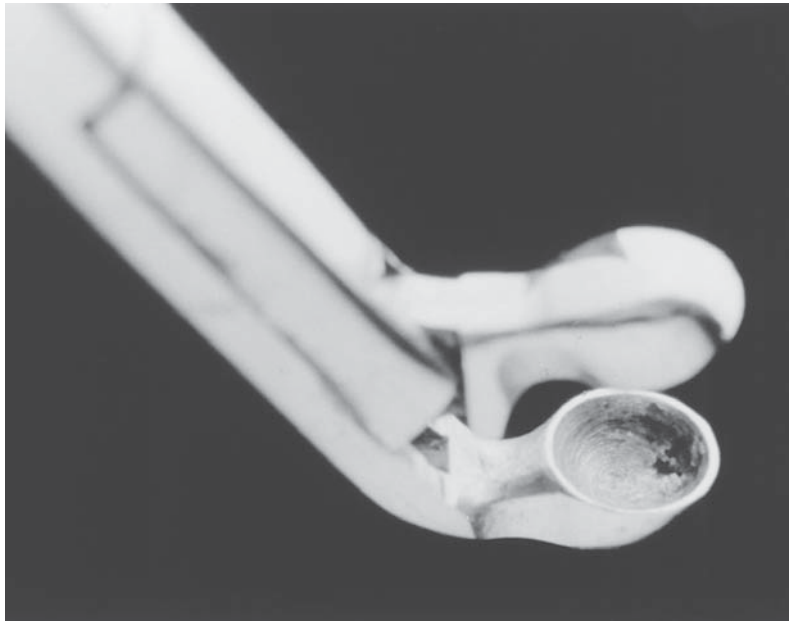


Fig. 2. Close-up of the biopsy forceps used for obtaining mucosal biopsies of the rectum.

2. Prepare 1.5 *M* NaCl (10X) by dissolving 87.66 g of sodium chloride in 1 L of distilled water (*see* **Note 6**).
3. Prepare TBS by adding 100 mL of 10X Tris to 100 mL of 10× NaCl and diluting the mixture with distilled water to 1 L (final concentration of 0.05 *M* Tris in 0.15 *M* saline) (*see* **Note 6**).

3.3.2. Indirect Peroxidase Technique

1. Fix air-dried cryostat sections in cold acetone (4°C) for 10 min and then air-dry (*see* **Note 7**).
2. Incubate (*see* **Note 8**) the sections for 30 min with the primary mouse MAbs against either CD3, TCRγδ, CD68, or HLA-DR at dilutions of 1/15–1/100 in TBS.
3. Wash the slides in fresh TBS for 2 min, and remove excess fluid from around the sections.
4. Treat sections with PxRAM immunoglobulins in 1/25 dilution in TBS and incubate for 30 min.
5. Wash the sections in fresh TBS for 2 min and dry around the sections.
6. Apply DAB (*see* **Subheading 2.2.2.**) by filtering fresh solution directly onto the sections and incubate for 7–10 min.
7. Wash the sections in fresh TBS for 5 min.
8. Wash the sections in tap water.

9. Counterstain with Mayer's hematoxylin for 2 min, and then wash under tap water.
10. Wipe off excess water on the slides and mount sections in Apathy's aqueous medium.

3.3.3. APAAP Technique

1. Fix air-dried sections in cold acetone for 10 min and then air-dry.
2. Dilute the primary mouse MAbs CD15 and IL-2R in 1/50 in TBS in a glass tube and apply to slides for 30 min.
3. Wash the sections in fresh TBS for 2 min, and dry the slides around the sections.
4. Incubate the sections with secondary (link) RAM immunoglobulins (1/50 dilution in TBS) with normal human serum (1/10 dilution in TBS) for 30 min.
5. Wash in fresh TBS for 2 min and dry around the sections.
6. Incubate the sections with APAAP complex (1/50 dilution in TBS) for 30 min.
7. Wash in fresh TBS for 2 min and dry around the sections.
8. Repeat **steps 4** and **6** (10 min for each step) to enhance the intensity of final staining.
9. Prepare the APAAP substrate, Fast Red (*see* **Subheading 2.2.2.**), and filter onto the sections and incubate for 15 min.
10. Wash the sections in fresh TBS for 5 min and then wash in tap water.
11. Counterstain the slides with Mayer's hematoxylin for 2 min.
12. Wash the sections under running tap water.
13. Mount the slides with Apathy's aqueous mounting medium.

*3.3.4. Double Immunostaining (*see ***Notes 9*** *and* ***10****)*

1. Perform the indirect peroxidase technique (*see* **Subheading 3.3.2.**) using either CD3, CD68, or HLA-DR as primary antibodies until **step 7**.
2. Apply the APAAP technique (*see* **Subheading 3.3.3.**, **steps 1–13**) on to the slides already stained by the indirect peroxidase technique, using either CD15 or IL-2R as primary antibodies.
3. Perform double labeling for the following combinations:
   a. Apply indirect peroxidase (*see* **Subheading 3.3.2.**, **steps 1–7**) using CD3 (dilution 1/50 in TBS) and then apply APAAP (*see* **Subheading 3.3.3.**, **steps 1–13**) using CD15 (dilution 1/50 in TBS).
   b. Apply indirect peroxidase (*see* **Subheading 3.3.2.**, **steps 1–7**) using CD3 (dilution 1/50 in TBS) and then apply APAAP (*see* **Subheading 3.3.3.**, **steps 1–13**) using IL-2R (dilution 1/50 in TBS).
   c. Apply indirect peroxidase (*see* **Subheading 3.3.2.**, **steps 1–7**) using CD68 (dilution 1/50 in TBS) and then apply APAAP (*see* **Subheading 3.3.3.**, **steps 1–13**) using IL-2R (dilution 1/50 in TBS).
   d. Apply indirect peroxidase (*see* **Subheading 3.3.2.**, **steps 1–7**) using HLA-DR (dilution 1/50 in TBS) and then apply APAAP (*see* **Subheading 3.3.3.**, **steps 1–13**) using IL-2R (dilution 1/50 in TBS).
4. Use nickel chloride enhancement for less prominent antibodies to obtain a darker color (*see* **Note 11**).

3.4. Quantitative Methods

3.4.1. How Much Tissue Should Be Analyzed?

1. Count positive cells along a unit length of 200 μm of muscularis mucosae, with a calibrated ocular graticule.
2. Repeat procedure, until minimum measurements necessary for constant mean have been achieved (*see* **Fig. 3**).
3. Repeat procedure for other specimens, for each antibody used, for each time interval (if experimental challenge is being observed over a time course) (*see* **Note 12**).

3.4.2. Determining Effective Section Thickness

The number of cells contained within any volume compartment of the mucosa do not simply reflect their profiles, when counted in a series of histological sections, since each profile represents only a fraction of the whole cell. It has been shown that any nucleus of radius r, whose profile appears in a section of finite thickness, t, is, in reality, contained within a superslice of thickness $(t + 2r)$μm, where $2r$ = mean cell (nuclear) diameter. In other words, all nuclear profiles observed in a section of thickness t μm have their centers within the superslice $D + t$ μm (*see* **Fig. 4C**, **D**) and conversely, those profiles whose centers lie outside it (*see* **Fig. 4A**, **B**) do not exhibit profiles in that section of thickness t.

The thickness of the superslice within which all the observed profiles lie is termed the *effective section thickness* (EST). To determine EST, and hence the absolute numbers of each cell type within a given mucosal compartment, it is necessary to determine the actual range and mean nuclear diameters (D) for each cell type from which EST = $(t + D)$ is calculated.

These principles are presented as diagrams (*see* **Fig. 5**) because of their relevance and importance to the measurements used in our studies.

Once D and EST are known, calculate the number of cells contained (within a defined tissue compartment) relative to the 10^4 μm^2 test area of muscularis mucosae. The absolute number (N) of nuclei overlying the standard (100 × 100 μm) test square of muscularis mucosae is therefore: $N = n \times 100 \times 100/T$, where n = summated profiles counted per 1000 μm (linear) of muscularis mucosae (**Fig. 5**). Therefore, taking the mean nuclear diameter, D, to be 5 μm, then, for both frozen and wax sections (where t is 6 μm), EST = (6 + 5) or 11 μm. Hence, the actual number (N) of lymphocytes per 100 × 100 μm^2 muscularis mucosae (or 10,000 linear μm) for any profile count, n, is $N = 10/11 \cdot n$. Therefore, $N = 0.91 \cdot n$.

3.5. Summary

Morphometric analysis allowed us to quantitatively define the normal constituents of the rectal mucosa and establish confidence limits, and thus reference ranges, for the various cell types observed. The main value of morpho-

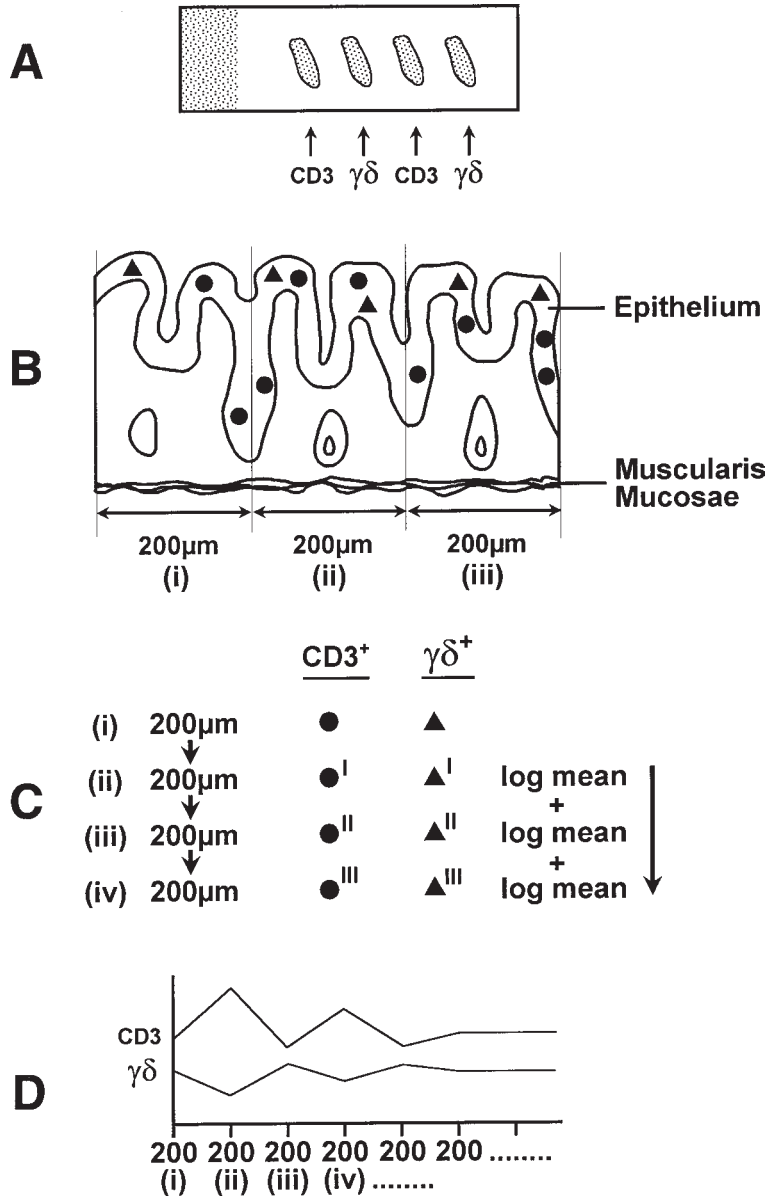


Fig. 3. Four frozen sections are applied to slide and stained alternately with anti-CD3 and anti-γδ mAbs. Count each type of cell that lies over successive 200-µm lengths of muscularis **(B)**, and calculate the new mean as each new set of data are accumulated **(C)**. Plot the means, for each cell type, as illustrated **(D)** and thus determine the minimum length of muscularis that needs to be surveyed to obtain a constant mean.

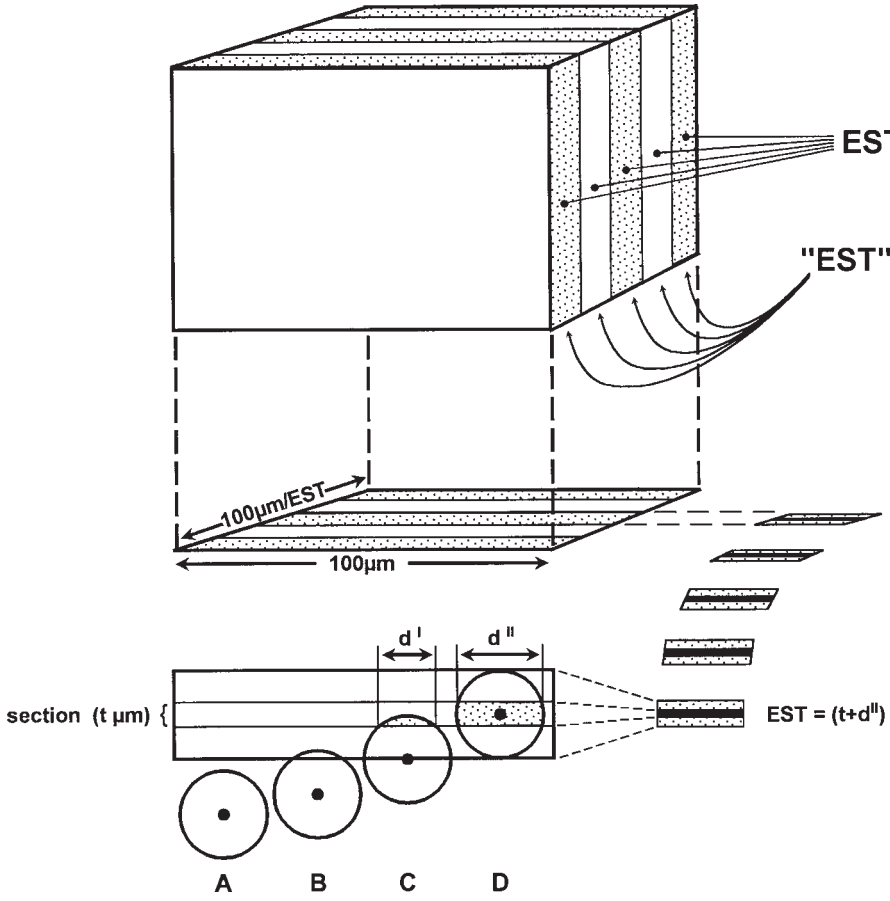


Fig. 4. EST is that superslice of tissue that contains any particle of interest (e.g., cell nucleus). For any particle, of diameter D µm, the width of tissue on either side of an actual section is equal to the radius of that particle, if it is to appear (i.e., **B, C, D**) in the section. Thus, EST = $D + t$µm, where t = actual section thickness. To determine how many such particles theoretically overlie the test area, one side must be divided by EST. The count will involve 100 × (100/EST) actual particles.

metric analysis is likely to be in clinical research in which an accurate quantitation of cell types in the rectal mucosa may be useful to define milder morphological alterations. This is likely to be most true for disorders characterized by abnormalities in cell number without much distortion of mucosal architecture. Then, by correlating histology with function the significance of mild abnormalities can be determined. The use of computerized analysis of quantitative histological data is an exciting new approach to the study of micro-

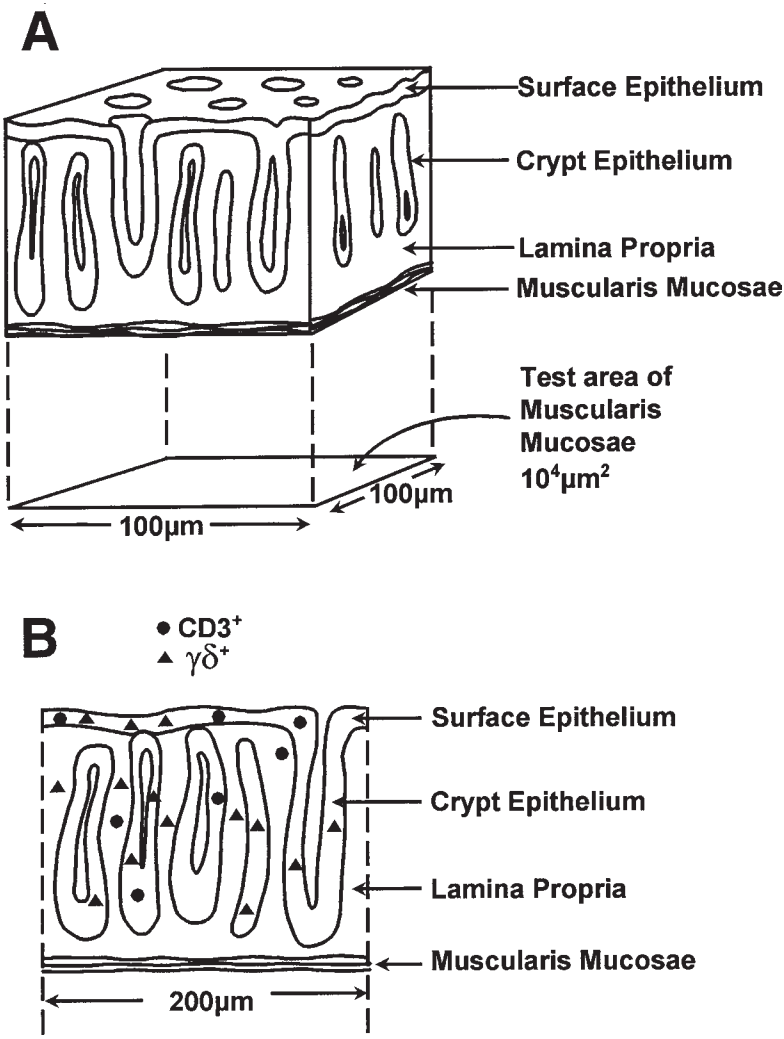


Fig. 5. **(A)** A 3D representation of a mucosal biopsy with reference to the standard test area (10^4 µm^2). **(B)** A typical section indicating that a linear 200-µm segment of muscularis mucosae can more usefully be scanned at the microscope when counting specific cellular profiles, i.e., CD3 or $\gamma\delta^+$ lymphocytes.

scopic pathology. It can yield valuable information of practical clinical importance in improving the discrimination between normality and mild degrees of inflammatory change, improve the diagnosis and classification of inflammatory diseases of the GI tract, and shed new light on mucosal changes and pathogenetic processes.

4. Notes

1. The biopsy forceps (as used in our studies) should not take up too much muscle, so that the specimen does not curl up.
2. Before mounting the biopsy specimen on a thin strip of carrot, it should be pulled to both sides (but never stretched) in order to have the muscularis mucosa smoothed out horizontally. Then the biopsy should be placed on the carrot so that the mucosal surface is upward. A little amount of OCT embedding material is carefully poured into the aluminum foil boat first and the biopsy, mounted on carrot is then placed into OCT, and the remainder of the boat filled with OCT. This should be done very gently, so that the OCT does not change the position of the biopsy specimen and no air bubbles are produced. Thus prepared, the fresh specimen should be frozen in Isopentane (–160°C) as rapidly as possible, so as not to cause any tissue artifact. It is important that a small piece of carrot stays outside the OCT; this is necessary for handling the block while sectioning.
3. The main concern here is to ensure that the tissue is frozen and melted under optimum conditions to avoid tissue damage. While sectioning the cylindrical tissue block (approx 1 cm high), it should be mounted on the cryostat chuck very tightly. Next, the first trims should be made with great care since the tissue block can easily be knocked off the chuck if the attachment is insecure.
4. To obtain the best vertical plane of rectal mucosa, sufficient trimming should be performed until the whole surface of the biopsy specimen is reached (this should be checked by taking control sections and staining with toluidine blue). In this way, good orientation of the tissue block, with precise sectioning perpendicular to the mucosal surface, can be routinely achieved. Such sectioning precision is of critical importance for the subsequent counting and analysis of stained cellular profiles at the microscope.
5. Allow the sections to reach room temperature before staining. Also let each solution or freeze-dried antibody/chemical used in staining come to room temperature since the frozen weight of these substances can be different, leading to calculation errors while making the dilutions.
6. The stock solution of 0.5 *M* Tris-HCl (10×) and 1.5 *M* NaCl (10×) can be stored at room temperature for up to 6 mo whereas TBS solution can stay at room temperature for 1 mo.
7. Acetone fixation can be done either immediately after sectioning (before the sections are placed in the freezer) or immediately before the staining procedure is commenced. The most important thing about acetone fixation is to make sure that fresh acetone is used each time and that it is kept at 4°C (hence, cold acetone). Ten to fifteen minutes of fixation is necessary for good tissue preservation.
8. All the incubations are performed in a moist chamber at room temperature.
9. Depending on the staining technique used, some cells stain brown by the DAB-indirect peroxidase technique, whereas Fast Red-APAAP gives a bright red end product.

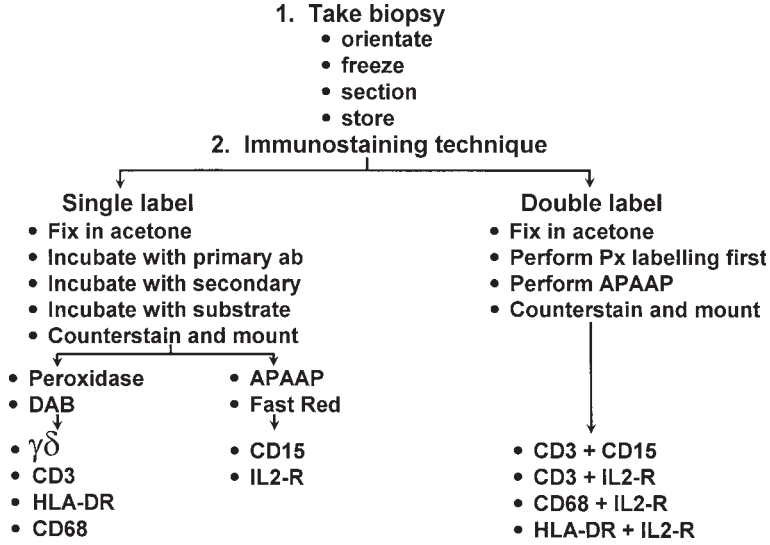


Fig. 6. Summary of immunohistochemical procedures, and their uses, as described in the text.

10. After initial preliminary stainings, the best results were achieved by employing indirect peroxidase-DAB for the first enzymatic reaction and APAAP-Fast Red as the second enzyme-substrate combination (*see* **Fig. 6**).
11. Strongly expressed antibodies were used with Fast Red whereas those less prominent were used with DAB with nickel chloride enhancement. This is done by adding 1 mg of nickel chloride to 1 mL of DAB solution ***(35)*** to obtain a darker color.
12. The number of observations (i.e., counts per unit blocks of 200 μm) made on each set of biopsies varied between 40 and 500 owing to variations in the size of each biopsy. However, the plots showed that approx 5–20 consecutive observations were necessary to obtain a constant for each one of the biopsies. Hence, 1000- to 4000-μm lengths of muscularis mucosae necessarily required analysis, as a minimum, in order to obtain a constant result. However, since the coefficient of variation for the observations between the tenth and twentieth observations was <5%, it was concluded that an average of 10 observations, or an equivalent 2000-μm length of muscularis mucosae, was required to yield a constant cell count per specimen.

References

1. Allen, D. C., Hamilton, P. W., Watt, P. C. H., and Biggart, J. D. (1987) Morphometrical analysis in ulcerative colitis with dysplasia and carcinoma. *Histopathology* **11,** 913–926.

2. Allen, D. C., Hamilton, P. W., Watt, P. C. H., and Biggart, J. D. (1988) Architectural morphometry in ulcerative colitis with dysplasia. *Histopathology* **12,** 611–621.
3. Hamilton, P. W., Allen, D. C., Watt, P. C. H., Patterson, C. C., and Biggart, J. D. (1987) Classification of normal colorectal mucosa and adenocarcinoma by morphometry. *Histopathology* **11,** 901–911.
4. Thompson, E. M., Price, A. B., Altman, D. G., Sowter, C., and Slavin, G. (1985) Quantitation in inflammatory bowel disease using computerized interactive image analysis. *J. Clin. Pathol.* **38,** 631–638.
5. Scott, B. B., Goodall, A., Stephenson, P., and Jenkins, D. (1983) Rectal mucosal plasma cells in inflammatory bowel disease. *Gut* **24,** 519–524.
6. Jenkins, D. (1988) Computing and the histopathology of intestinal inflammation, in *Computers in Gastroenterology* (Vicary, F. R., ed.), Springer-Verlag, London, pp. 193–204.
7. Hamilton, P. W., Williamson, K. E., Grimes, J., Arthur, K., and Wilson, R. H. (1994) Three-dimensional computerised analysis of epithelial cell proliferation in the gastrointestinal tract. *Br. J. Cancer* **69,** 1027–1031.
8. Jenkins, D., Goodall, A., Drew, K., and Scott, B. B. (1988) What is colitis? Statistical approach to distinguishing clinically important inflammatory change in rectal biopsy specimens. *J. Clin. Pathol.* **41,** 72–79.
9. Ruifrok, A. C. (1997) Quantification of immunohistochemical staining by color translation and automated thresholding. *Analyt. Quant. Cytol. Histol.* **19,** 107–113.
10. El-Salhy, M., Sandström, O., Nasström, E., Mustajbasic, M., and Zachrisson, S. (1997) Application of computer image analysis in endocrine cell quantification. *Histochem. J.* **29,** 249–256.
11. Korelitz, B. I. and Sommers, S. C. (1974) Differential diagnosis of ulcerative and granulomatous colitis by sigmoidoscopy, rectal biopsy and cell counts of rectal mucosa. *Am. J. Gastroenterol.* **61,** 460–469.
12. Rubio, C. A., Johansson, C., Uribe, A., and Kock, Y. (1984) A quantitative method of estimating inflammation in the rectal mucosa. *Scand. J. Gastroenterol.* **19,** 525–530.
13. Lee, E., Schiller, L. R., and Fordtran, J. S. (1988) Quantification of colonic lamina propria cells by means of a morphometric point-counting method. *Gastroenterology* **94,** 409–418.
14. Marsh, M. N. and Hinde, J. (1986) Morphometric analysis of small intestinal mucosa. III. The quantitation of crypt epithelial volumes and lymphoid cell infiltrates, with reference to coeliac sprue mucosae. *Virchows Arch. (Pathol. Anat.)* **409,** 11–22.
15. Guix, M., Skinner, J. M., and Whitehead, R. (1979) Measuring intraepithelial lymphocytes, surface area and volume of lamina propria in the jejunal mucosa of coeliac patients. *Gut* **20,** 275–278.
16. Ishido, T., Itabashi, M., Ochiai, A., Hirota, T., Yokota, T., and Saito, D. (1994) Morphometric analysis of colorectal dysplasia with image processing. *Arch. Pathol. Lab. Med.* **118,** 619–623.

17. Verhest, A., Kiss, R., d'Olne, D., Larsimont, D., and Salmon, I. (1990) Characterization of human colorectal mucosa, polyps and cancers by means of computerized morphonuclear image analyses. *Cancer* **65,** 2047–2054.
18. Puglisi, R. N., Whalen, T. V., and Doolin, E. J. (1995) Computer analyzed histology of ischemic injury to the gut. *J. Pediatr. Surg.* **30,** 839–844.
19. Minimo, C., McCue, P. A., Pindzola, A., Brennan, J., and Bibbo, M. (1996) Role of computed quantitation of immunohistochemical staining of Ki-67 antigen in diagnosing ampullary lesions. *Analyt. Quant. Cytol. Histol.* **18,** 400–404.
20. Furgal, A. and Litwin, J. A. (1997) Distribution of eosinophils along and across successive segments of the rat digestive tract: a quantitative study. Folia. *Histochemica Cytobiologica* **35,** 155–163.
21. Jenkins, D., Goodall, A., Gillet, F. R., and Scott, B. B. (1985) Defining duodenitis: quantitative histological study of mucosal responses and their correlations. *J. Clin. Pathol.* **38,** 1119–1126.
22. Niazi, N. M., Leigh, R., Crowe, P. T., and Marsh, M. N. (1984) Morphometric analysis of small intestinal mucosa. I. Methodology, epithelial volume compartments and enumeration of inter-epithelial space lymphocytes. *Virchows Arch. (Pathol. Anat.)* **404,** 49–60.
23. Grant, N. J., Van Kruiningen, H. J., Haque, S., and West, A. B. (1997) Mucosal inflammation in Pediatric Diversion Colitis: a quantitative analysis. *J. Pediatr. Gastroenterol. Nutr.* **25,** 273–280.
24. Marsh, M. N. (1992) Mucosal pathology in gluten sensitivity, in *Coeliac Disease* (Marsh, M. N., ed.), Blackwell Scientific, London, pp. 136–191.
25. Weibel, E. R. (1979) *Stereological Methods*, Academic, New York.
26. Crowe, P. T. and Marsh, M. N. (1994) Morphometric analysis of intestinal mucosa. VI. Principles of enumerating intra-epithelial lymphocytes. *Virchows Arch.* **424,** 301–306.
27. Loft, D. E., Marsh, M. N., Sandle, G. I., Crowe, P. T., Garner, V., Gordon, D., and Baker, R. (1989) Studies of intestinal lymphoid tissue. XII. Epithelial lymphocyte and mucosal responses to rectal gluten challenge in coeliac sprue. *Gastroenterology* **97,** 29–37.
28. Dhesi, I., Marsh, M. N., Kelly, C., and Crowe, P. (1984) Morphometric analysis of small intestinal mucosa. II. Determination of lamina propria volumes, plasma cell and neutrophil populations within control and coeliac disease mucosae. *Virchows Arch. (Pathol. Anat.)* **403,** 173–180.
29. Leigh, R. J., Marsh, M. N., Crowe, P., Kelly, C., Garner, V., and Gordon, D. (1985) Studies of intestinal lymphoid tissue IX. Dose-dependent, gluten-induced lymphoid infiltration of coeliac jejunal epithelium. *Scand. J. Gastroenterol.* **20,** 715–719.
30. Loft, D. E. (1990) *Coeliac Disease: The Structural and Functional Response of Large Intestinal Mucosa to Local Gluten Challenge*, MS thesis, University of Birmingham, Birmingham, UK.
31. Ensari, A., Marsh, M. N., Loft, D. E., Morgan, S., and Moriarty, K. (1993) Morphometric analysis of intestinal mucosa V. Quantitative histological and immunohistochemical studies of rectal mucosae in gluten sensitivity. *Gut* **34,** 1225–1229.

32. Ensari, A., Ager, A., Marsh, M. N., Morgan, S., and Moriarty, K. (1993) Time-course of adhesion molecule expression in rectal mucosa of gluten-sensitive subjects. *Clin. Exp. Immunol.* **92,** 303–307.
33. Ensari, A. (1995) *Gluten Sensitivity: The Cellular and Molecular Biology of Mucosal Inflammation*, PhD thesis, University of Manchester, Manchester, UK.
34. Marsh, M. N. and Ensari, A. (1995) The gut-associated lymphoid tissue and immune system, in *Gastrointestinal and Oesophageal Pathology*, 2nd ed. (Whitehead, R., ed.), Churchill Livingstone, London, pp. 201–225.
35. Mason, D. Y. and Woolston, R.-E. (1982) Double immunoenzymatic labelling, in *Techniques in Immunohistochemistry*, vol 1. (Bullock, G. and Petruzs, P., eds.), Academic, London, pp. 136–153.

12

Organ Culture of Rectal Mucosa

In Vitro Challenge with Gluten in Celiac Disease

Giuseppe Mazzarella, Francesco Paparo, Maria Maglio, and Riccardo Troncone

1. Introduction

Celiac disease is sustained by an immunological process that mainly affects the jejunal mucosa ***(1)***. Nonetheless, jejunum is not the only site of the gastrointestinal tract that is involved in celiac disease. In recent years, Ensari and colleagues ***(2,3)***, by using immunohistochemical analysis and computerized image analysis for numerical quantitation, have significantly contributed to a definitive and clear demonstration of a celiac disease–associated "proctitis," and its gluten dependence. Morphometry has shown increased populations of plasma cells, lymphocytes, and mast cells in the rectal mucosa of untreated patients, with these changes being reverted, with the sole exception of mast cells, by dietary treatment ***(2)***. The immunohistochemical approach has demonstrated highly significant increases in $CD3^+$ and $\gamma\delta^+$ lymphocytes within both the lamina propria and the epithelium. Mononuclear cells, both lymphocytes ($CD3^+$) and macrophages ($CD68^+$) expressing interleukin-2 (IL-2) receptors ($CD25^+$), have been found to be increased in the lamina propria, usually immediately below the basal lamina. Enterocytes have been noted to be positive for major histocompatibility complex class II display, a pattern usually absent in normal colon. Furthermore, increased expression of vascular cell adhesion molecule-1 (VCAM-1) molecules in the rectal mucosa of untreated, compared to either treated celiac rectum or control mucosae, has been reported ***(3)***. As a whole, these data suggest, analogously to jeunum, an ongoing T-cell-dependent, cell-mediated immune response in the rectal mucosa.

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

Because rectal mucosa in untreated celiac disease has been demonstrated to be a site of activated mucosal cell-mediated immunity, and because the rectum provides a site of easy access for obtaining multiple mucosal samples, the investigation of the response of rectal mucosa to local gluten challenge has become a very important issue. Loft and colleagues ***(4)*** have extensively investigated the consequences of challenging the rectal mucosa with a peptic-tryptic digest of gluten. The data indicate that, within 1 h of gluten instillation, a rapid increase of lamina propria volume occurs; this swelling is owing to increased microvasculature permeability and is likely to be the result of mast cell discharge. The rapid fall in mast cell number is followed by a rapid influx of neutrophils that achieves a maximum at 8 h. At 4–6 h from challenge, lymphocytes also emigrate to the lamina propria; in the epithelium a peak is reached at 6–8 h. When adhesion molecules have been investigated, the rectal mucosa of celiac disease patients has revealed after local gluten challenge increased expression of endothelial adhesion molecule-1 (ELAM-1) and VCAM-1, but not intercellular adhesion molecule-1 (ICAM-1). ELAM-1 has been proposed as a key endothelial adhesion molecule mediating neutrophil infiltration, and, in fact, the results of time-course experiments support a major role in neutrophil recruitment. VCAM-1 expression increased at 8 h after challenge together with a rise of CD3 cells within the lamina propria and epithelium; its expression, analogously to what is described in experimental models of cell-mediated activated mucosal immunity, is the result of T-cell activation. Some of these observations have been recently reproduced in our laboratory ***(5)***. A substantial infiltration of lymphocytes was noted in the epithelium 6 h after challenge with a peptic-tryptic digest of gliadin. These data were paralleled by immunohistochemical analysis showing a recruitment of CD3 and $\gamma\delta^+$ lymphocytes. All the phenomena observed in celiac disease patients appeared quite specific for the antigen gliadin, an unrelated protein (ovalbumin, bovine serum albumin) producing no change whatsoever.

Most of our information on the immunological consequences of gluten challenge at the mucosal level has been obtained from in vitro studies using organ culture of jejunal biopsies (*see* Chapter 10 on organ culture of jejunal mucosa). Taking advantage of the experience gained in our laboratory on the organ culture system, we have extended data from in vivo rectal challenge studies, and we have established an in vitro system, which is described in detail in this chapter. Rectal biopsies taken from treated celiac disease patients and controls are cultured in the presence of a peptic-tryptic digest of gliadin, and the phenomena observed compared with those occurring in paired fragments cultured in the presence of medium alone, or with an unrelated control protein, such as ovalbumin. At the end of the culture period, biopsies are snap-frozen and an immunohistochemical analysis is carried out. In the lamina propria of celiac

biopsies cultured in the presence of peptic-tryptic digest of gliadin, but not in those from controls, the number of cells expressing IL-2 receptor is significantly higher than in biopsies cultured in medium alone; similarly, the expression of VCAM-1 molecules is also enhanced ***(6)***. Another phenomenon observed in rectal biopsies cultured with gliadin is the increased density of intraepithelial $CD3^+$ cells ***(6)***. This observation also parallels previous in vivo data ***(5)***, as well as data obtained in in vitro cultures of treated small intestinal celiac mucosa with gliadin ***(7)***. As a whole, our data confirm that gliadin is able to activate mucosal cell-mediated immunity in the rectal mucosa in celiac disease patients.

In this chapter, we mainly report on the immunohistological analysis of rectal biopsy fragments cultured for 24 h in the presence of gliadin. However, it is clear that a series of other observations can be made in the system; antibodies, cytokines, and other soluble factors are released in culture supernatants, and by means of enzyme-linked immunosorbent assay techniques, can be easily measured. Biopsy fragments can also be utilized for the quantitation of mRNA for cytokines and other factors released by T-cells, macrophages, and other inflammatory cells, which may be responsible for mucosal damage. The suitability of the system for obtaining the latter information has been recently assessed by Nilsen et al. ***(8)*** in the organ culture of jejunal mucosa. In conclusion, the in vitro organ culture of rectal mucosa is particularly suitable for assessing the intestinal immune response to gliadin in celiac disease, but it could be equally suitable for investigating the mucosal immune response to any other dietary antigens (e.g., in the diagnostic approach of patients with suspected food allergy).

As far as celiac disease is concerned, in vitro rectal challenge studies may significantly contribute to an understanding of processes triggered by the contact of the antigen gliadin with the celiac intestine, the characterization of antigenic epitopes of cereal proteins, and the identification of gluten-sensitized subjects. Insofar as these studies contributes to an understanding of the pathogenesis of celiac disease, the cascade of events triggered by contact with the antigen gliadin seems to resemble what happens in the jejunum. Furthermore, this system represents an essential tool to assess the antigenic properties of different cereals as well as different gliadin-derived peptides ***(9)***. Finally, this test may also prove important from a diagnostic point of view. In fact, a combination of immunohistochemical data in a discriminatory equation ***(10)*** has allowed the correct classification of 100% of cases, both celiacs and disease controls. Our data suggest that it allows the identification not only of celiac disease patients but also those subjects with an abnormal mucosal immune response to gliadin. In fact, recent studies have suggested gluten sensitization as a condition of abnormal immunity to gluten restricted to genetically predis-

posed individuals, but not exclusive to subjects presenting with a flat mucosa on a gluten-containing diet ***(11)***. We have recently performed in vitro rectal gluten challenge in siblings of celiac disease children ***(6)***, and the data are consistent with the hypothesis that a significant proportion of first-degree relatives are sensitized to gliadin. In vitro rectal gluten challenge, which is relatively less invasive being based on the study of a rectal biopsy fragment, then, has great potential for defining the epidemiology of gluten sensitivity, particularly in at-risk groups, such as first-degree relatives of celiac disease patients, and patients affected by other autoimmune diseases (e.g., insulin-dependent diabetes).

2. Materials

2.1. Preparation of Peptic-Tryptic Digest of Gliadin

1. Wheat flour (cultivar Sagittario, a kind gift from Dr. La Fiandra, Università della Tuscia, Viterbo, Italy).
2. Tubes for dialysis (cutoff 5000 mol wt) (Dasit, Milan, Italy).
3. Pepsin from porcine stomach mucosa (Sigma Aldrich, Milan, Italy) (store at –20°C).
4. Trypsin from bovine pancreas (Sigma) (store at –20°C).

2.2. Rectal Biopsies

1. Watson intestinal biopsy capsule (D. J. Allen Engineering, London, UK).

2.3. Organ Culture

1. Sterile scalpel blades (Aesculap, Frankfurt, Germany).
2. Sterile dishes (Falcon, Becton Dickinson, Milan, Italy).
3. Sterile steel mesh (Falcon).
4. Modular-incubator chamber (ICN Biomedicals, Milan, Italy).
5. Castro-Viejo scissors (Dimart, Germany), or any other scissors for microsurgery.
6. 0.22-μm filters (Millipore, Milan, Italy).
7. Trowells T8 medium (1X) (Gibco-BRL, Life Technologies, Milan, Italy) (store at 4°C).
8. National Collection of Type Cultures-135 (NCTC-135) medium with L-glutamine (1X) (Gibco-BRL) (store at 4°C).
9. Fetal bovine serum (heat inactivated) (Sigma): The serum should be aliquoted and stored at –20°C.
10. Lyophilized penicillin/streptomycin (Gibco-BRL) (store at 4°C): Prepare with 10,000 U/mL of penicillin G (sodium salt) and 10,000 μg/mL of streptomycin sulfate. Rehydrate with 20 mL of sterile distilled water. After rehydration, aliquot 300 μL in sterile cryovials and store at –20°C.
11. Gentamicin liquid (10 mg/mL) (Gibco-BRL) (store at room temperature).
12. 0.5 *M* HCl: Filter and store in sterile vials at room temperature.
13. 0.5 *M* NaOH: Filter and store in sterile vials at room temperature. **Table 1** gives the composition of the culture medium.

Table 1
Composition of Culture Medium (per 10 mL)

Component	Volume
Trowell	6.5 mL
NCTC-135	2.5 mL
FCS[a]	1.0 mL
Penicillin	100 U/mL
Streptomycin	100 µg/mL
Gentamicin	50 µg/mL

[a]Fetal calf serum.

2.4. Immunohistochemistry

1. OCT compound (mixture of polyvinyl alcohol, polyethylene glycol, dimethyl benzyl ammonium chloride) (Tissue Tek, Elkhart, IN) (store at room temperature).
2. Aminopropyltriethoxysilane (APES) (Merck, Rahway, NJ).
3. 0.15 *M* Tris-buffered saline (TBS) (Sigma): For 1 L of 0.15 *M* TBS, mix 6.055 g of Tris with 4.38 g of sodium chloride in <1 L of deionized water. Add 3 mL of 12 *M* HCl. Check and adjust, if necessary, to pH 7.4, and make up the volume to 1 L by adding more deionized water (store at room temperature).
4. Acetone (Merck) (store at 4°C).
5. Normal rabbit serum (Dako, Copenhagen, Denmark) (store at 4°C).
6. Rabbit antimouse immunoglobulin (secondary antibody) (Dako) (store at 4°C).
7. Alkaline phosphatase-antialkaline phosphatase (APAAP) (Dako) (store at 4°C).
8. Naphtol-AS/biphosphate (Sigma) (store at –20°C).
9. *N*-*N*-dimethylformamide (Merck) (store at room temperature).
10. New fuchsin (Sigma) (store at room temperature).
11. Levamisole (Sigma) (store at room temperature).
12. Sodium nitrite (Sigma): Prepare 4% of sodium nitrite in distilled water (store at 4°C).
13. Mayer's hematoxylin (Sigma) (store at room temperature).
14. Aquamount (BDH, Poole, England).

3. Methods

3.1. Preparation of Peptic-Tryptic Digest of Gliadin

1. Extract 100 g of finely ground wheat flour at 4°C for 3 h in a shaker with 1 L of the following solution: 5.68 g of sodium phosphate (0.04 *M*) and 237.6 g of ammonium sulfate (1.8 *M*) in <1 L of deionized water. Adjust to pH 7.0 by adding 1 *M* NaOH and make up the volume to 1 L by adding more deionized water.
2. Centrifuge at 16,000*g* for 15 min and discard the supernatant.
3. Extract the pellet at 4°C for 3 h in a shaker with 1 L of the following solution: 5.68 g of sodium phosphate (0.04 *M*) and 52.84 g of ammonium sulfate (0.4 *M*) in

<1 L of deionized water. Adjust to pH 7.0 by adding 1 *M* NaOH and make up the volume to 1 L by adding more deionized water.
4. Add 0.04 *M* sodium phosphate/0.4 *M* ammonium sulfate pH 7.0.
5. Centrifuge at 16,000*g* for 15 min and discard the supernatant.
6. Extract the pellet at room temperature for 3 h in a shaker with 300 mL of 60% ethanol.
7. Centrifuge at 16,000*g* for 15 min and collect the supernatant containing the gliadin fraction.
8. Dialyze the fraction against distilled water for 48 h at 4°C.
9. Centrifuge at 15,000*g* and freeze-dry the pellet representing the gliadin fraction.
10. Digest 100 g of gliadin in 1 L of 0.2 *M* HCl with 2 g of pepsin for 2 h at 37°C by vigorous stirring.
11. Adjust the pH to 8.0 with 2 *M* NaOH.
12. Digest with 2 g of trypsin for 4 h at 37°C by vigorous stirring.
13. Destroy protease activity by heating at 100°C for 30 min. Freeze-dry the sample.

3.2. Preparation of Fresh Substrate Solution for Immunohistochemistry

1. Dissolve 20 mg of Naphtol-AS/biphosphate in 0.5 mL of *N*-*N*-dimethylformamide.
2. Add 40 mL of 0.15 *M* TBS (pH 8.7).
3. Immediately before use, mix 80 µL of 5% new fuchsin in 1 *M* HCl, and 200 µL of 4% sodium nitrite in distilled water. Allow to stand for 1 min.
4. Add the mixture to the substrate solution; add 17.5 mg of levamisole. Filter before use.

3.3. Rectal Biopsies

1. Position Watson capsule at 10 cm from the anal verge. Patients are not prepared for the procedure but are asked to void the rectum before the investigation.
2. Wash the fragment immediately in a sterile tube with 3 mL of 0.15 *M* sodium chloride supplemented with the antibiotic solution as used for the culture medium (*see* **Note 1**).
3. Repeat the procedure if more fragments are required. Biopsies should be transferred in 2 mL of culture medium in a sterile tube, in ice, to the laboratory as soon as possible. Culture fragments no later than 10 min from excision.

3.4. Organ Culture

1. Under the dissecting microscope, examine and slice each biopsy with a sterile scalpel blade into two approximately equal-sized pieces (*see* **Note 2**). Process one fragment immediately as baseline. For immunohistochemistry, orient specimens and embed in OCT compound, snap-freeze in isopentane (cooled in liquid nitrogen), and store at –70°C until cryosectioning.
2. Place the remaining pieces carefully on a stainless steel mesh positioned over the central well of an organ culture dish with the mucosal surface of the biopsies uppermost.

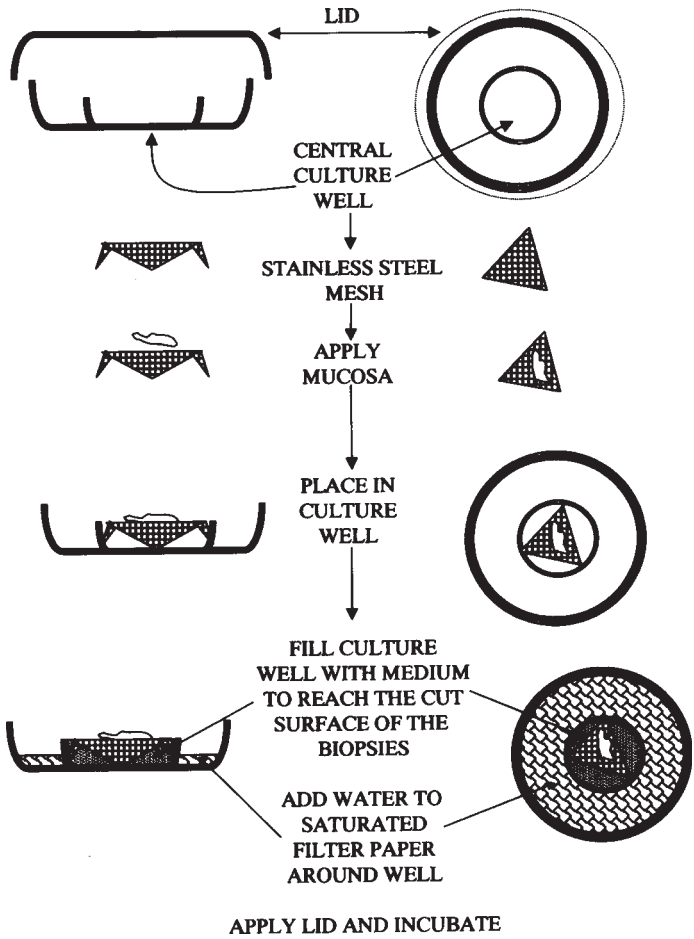


Fig. 1. Detailed methodology for setting up culture of rectal mucosa in Falcon tissue dish.

3. Fill the well with culture medium (approx 1 mL), so as just to reach the cut surface of the biopsies. Place an additional drop of medium over the surface of the biopsy (*see* **Note 3**).
4. Put filter paper saturated with several drops of sterile distilled water around the central well, to maintain a humid environment (*see* **Fig. 1**).
5. Place the dishes carefully in the modular-incubator chamber.
6. Gas with a mixture of 95% O_2/5% CO_2 (*see* **Note 4**).
7. Place the modular-incubator chamber in an incubator at 37°C for the period of culture (*see* **Note 5**).
8. After the organ culture period, under sterile flow hood, open the chamber, remove the sample carefully from the stainless steel mesh utilizing a sterile needle and

Table 2
Monoclonal Antibodies Used for Immunohistochemical Analysis

Designation	Specificity	Isotype	Working dilution	Source
T3-4B5	CD3	IgG1	1:200	Dakopatts
ACT-1	CD25	IgG1	1:25	Dakopatts
3C5	ICAM-1	IgG1	1:500	Genzyme
4B9	VCAM-1	IgG1	1:500	Genzyme
BB-11	ELAM-1	IgG1	1:500	Genzyme

sterile scalpel blade, and process the specimens in accordance with the selected technique. In the case of immunohistochemistry, embed in OCT compound, snap-freeze in isopentane (cooled in liquid nitrogen), and store at –70°C until cryosectioning.

9. Collect supernatant from the well, filter-sterilize, and keep at –70°C before analysis.

3.5. Immunohistochemistry

1. Cut frozen sections at 5 μm. Put the sections on APES-coated slides.
2. Air-dry the sections overnight at room temperature.
3. Fix the sections in acetone at 4°C for 20 min.
4. Air-dry the sections.
5. Proceed with immunostaining or store the sections at –20°C. Sections stored at –20°C should be wrapped in parafilm foil for protection until required (*see* **Note 6**).
6. Rehydrate the frozen sections (including positive and negative controls; *see* **Note 7**) in TBS.
7. Wipe off excess TBS, incubate sections in a humid chamber for 20 min, at room temperature, with normal rabbit serum (1:100 in TBS).
8. Drain off the normal rabbit serum and incubate in a humid chamber for 1 h at room temperature with optimally diluted primary antibodies (*see* **Table 2**).
9. Wash in TBS for 10 min.
10. Wipe off excess TBS, incubate the sections in a humid chamber for 40 min, at room temperature, with 1:30 rabbit antimouse immunoglobulin diluted with TBS.
11. Wash in TBS for 15 min.
12. Wipe off excess TBS and incubate the sections at room temperature for 30 min in 1:40 mouse APAAP diluted with TBS.
13. Wash in TBS for 15 min.
14. Incubate the sections with substrate solution (*see* **Subheading 3.2.**) in a Coplin jar and agitate gently for approx 5 min (*see* **Note 8**).
15. Wash in water.
16. Counterstain with Mayer's hematoxylin for 3 min.
17. Wash the slides gently in running water for 2 min.
18. Mount in aquamount.

3.6. Morphometric Evaluation

1. Determine the density of cells expressing CD3 in the surface epithelium by counting the number of stained cells relative to 100 enterocytes.
2. Enumerate the $CD25^+$ cells within a total area of 1 mm^2 of lamina propria, using a microscope with a calibrated ocular aligned parallel to the muscularis mucosae.
3. Evaluate the expression of adhesion molecules in terms of staining intensity, and grade on an arbitrary scale: nil staining (–) = 0, weak staining (+) = 1, moderate staining (++) = 2, strong staining (+++) = 3.

4. Notes

1. Washing of samples in a sterile tube with 3 mL of 0.15 *M* sodium chloride supplemented with the antibiotic solution as used for the culture medium must be performed at least twice, gently shaking for 1 min during each washing. This operation is critical to reduce the transfer of bacteria to the culture system, thereby minimizing the risk of contamination. Note that the concentration of antibiotics used for the organ culture of rectal mucosa is about 10 times higher than that used for the culture of small intestinal mucosa.
2. Mucosal fragments should be critically assessed with the dissecting microscope; damaged biopsies or fragments smaller than 2 × 2 mm should not be cultured. On the other hand, biopsies should be not so large that their bulk impedes the diffusion of nutrients. The optimal size of the mucosal fragments is 3 × 3 mm. If the biopsy contains parts of submucosal or lymphoid tissue (e.g., caused by a deep cut of the capsule), these must be removed by using sterile needles and Castro-Viejo scissors.
3. The biopsy specimens were cultured in the either absence or presence of the drugs, or proteins, under investigation. In our studies, we used 1 mg/mL of peptic-tryptic digest of gliadin. Following the addition of drugs or protein, the pH of the culture medium was checked and could be adjusted to 7.3–7.4 by adding either a few drops of sterile 0.5 *M* HCl or 0.5 *M* NaOH.
4. Gas the chamber with a moderate flow (0.5 bar pressure). After closing the exit valve of the chamber, wait for a few seconds before closing the inlet valve.
5. The time of culture can vary in accordance with the studies selected. When culture exceeds 24 h, it is necessary to change the culture medium and to repeat the gassing procedure (*see* **Subheading 3.4.**, **step 6**).
6. If stored sections are used, thaw in cold acetone for 2 to 3 min and then air-dry for at least 30 min at room temperature.
7. Sections should include slides known to contain the antigen under investigation, to confirm that the reagents and methods are all in working order. Negative control preparations of the tissue being studied, in which the primary antibody is either omitted or replaced by an inappropriate primary antibody, are also required, to indicate the level of background labeling present in that preparation, in addition to any owing to the primary antibody.
8. The reaction is allowed to develop to the point at which there is a reasonable distinction between a positive signal and background noise. This must be determined by trial and error.

Acknowledgments

This work was supported by grants from Regione Campania (Protocollo per programmi di diagnosi, cura e riabilitazione delle malattie croniche dell'apparato digerente e della nutrizione dell'infanzia), and Istituto Superiore di Sanita' (Progetto prevenzione dei fattori di rischio nella salute materno-infantile). We are grateful to C. Meccariello (Istituto di Scienze dell'Alimentazione CNR, Avellino, Italy) for technical help.

References

1. Branski, D. and Troncone, R. (1998) Celiac disease: a reappraisal. *J. Pediatr.* **133,** 181–187.
2. Ensari, A., Marsh, M. N., Loft, D. E., Morgan, S., and Moriarty, K. (1993) Morphometric analysis of intestinal mucosa. V. Quantitative histological and immunocytochemical studies of rectal mucosae in gluten sensitivity. *Gut* **34,** 1225–1229.
3. Ensari, A., Ager, A., Marsh, M. N., Morgan, S., and Moriarty, K. J. (1993) Time-course of adhesion molecule expression in rectal mucosa of gluten-sensitive subjects after gluten challenge. *Clin. Exp. Immunol.* **92,** 303–307.
4. Loft, D. E., Marsh, M. N., Sandle, G. I., Crowe, P. T., Gardner, V., Gordon, D., and Baker, R. (1989) Studies of intestinal lymphoid tissue. XII. Epithelial lymphocyte and mucosal responses to rectal challenge in celiac sprue. *Gastroenterology* **97,** 29–37.
5. Troncone, R., Greco, L., Mayer, M., Mazzarella, G., Maiuri, L., Congia, M., Frau, F., De Virgiliis, S., and Auricchio, S. (1996) In siblings of coeliac children rectal gluten challenge reveals sensitisation to gluten not restricted to coeliac HLA. *Gastroenterology* **111,** 318–324.
6. Troncone, R., Mazzarella, G., Leone, A., Mayer, M., De Vincenzi, M., Greco, L., and Auricchio, S. (1998) Gliadin activates mucosal cell-mediated immunity in in vitro cultured rectal mucosa from coeliac patients and a subset of their siblings. *Gut* **43,** 484–489.
7. Maiuri, L., Picarelli, A., Boirivant, M., Coletta, S., Mazzilli, M. C., De Vincenzi, M., Londei, M., and Auricchio, S. (1996) Definition of the immunological modifications upon in vitro gliadin challenge in the small intestine of coeliac patients. *Gastroenterology* **110,** 1368–1378.
8. Nilsen, E. M., Jahnsen, F. L., Lundin, K. E. A., Johansen, F. E., Fausa, O., Sollid, L. M., Johansen, J., Scott, H., and Brandtzaeg, P. (1998) Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology* **115,** 551–563.
9. Maiuri, L., Troncone, R., Mayer, M., Coletta, S., Picarelli, A., De Vincenzi, M., Pavone, V., and Auricchio, S. (1996) In vitro activities of A-gliadin related synthetic peptides: damaging effect on the atrophic coeliac mucosa and activation of mucosal immune response in the treated coeliac mucosa. *Scand. J. Gastroenterol.* **31,** 247–253.

10. Ensari, A., Marsh, M. N., Morgan, S., Moriarty, K., Lobley, R., and Unsworth, D. (1995) A comparative prospective study of rectal gluten challenge in the diagnosis of gluten-sensitivity. *Gastroenterology* **108,** A816.
11. Troncone, R., Greco, L., Mayer, M., Paparo, F., Caputo, N., Micillo, M., Mugione, P., and Auricchio, S. (1996) Latent and potential coeliac disease. *Acta Paediatr.* **85(Suppl. 412),** 10–14.

13

In Situ Hybridization

Conrad G. Beckett and Paul J. Ciclitira

1. Introduction

In situ hybridization permits specific identification of genes and gene expression without removing the target sequence from its topographical surroundings. The technique was described simultaneously in 1960 by two groups ***(1,2)***. It depends on the hybridization of a labeled nucleic acid probe to a complementary sequence of tissue mRNA or DNA.

Table 1 highlights the advantages and disadvantages of the three common types of probes used. The probes are designed as complementary sequences to certain regions of the cytokine gene of interest and are chemically synthesized. All probes are based on the antisense sequence and purified by high-performance liquid chromatography or polyacrylamide gel electrophoresis.

There are several methods of labeling the probes employing radioactive and nonradioactive techniques. Until recently, it was felt that the nonradioactive methods, such as digoxigenin and fluorescein, were not as sensitive as the radioactive methods. However, recent improvements in methodology mean that in skilled hands this may not be the case and that they have the advantages of being safer, easier to prepare, and quicker. The ^{32}P and ^{35}S isotopes have been used; the latter is safer and has a longer half-life, thus enabling longer storage and better resolution and sensitivity.

Radioactive *in situ* hybridization can be used on most tissue preparations. It is important that the sample be firmly adherent to the support medium, such as glass slides. Depending on the type of fixation, unmasking of the target nucleic acid sequences may be required. This is most easily achieved using a protease under carefully controlled conditions.

The hybridization solution contains a complex mixture of reagents that have a profound influence on the specificity (stringency) of hybrid formation.

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

Table 1
Types of Probes Used for *In Situ* Hybridization

Probe	Advantage	Disadvantage
Double-stranded DNA	Able to produce long sequences	Poor hybridization efficiency
Single-stranded RNA	High hybridization efficiency	Difficult to produce
Oligonucleotides	High hybridization efficiency	Insensitive unless a cocktail of several sequences used

The determination of the correct balance of hybridization solution reagents is an essential step in obtaining good hybridization. Microscopic autoradiography is employed for the detection of the radioactive hybrids using a dipping emulsion technique.

It should be borne in mind that the detection of mRNA does not necessarily equate to active protein because there is posttranscriptional regulation and activation. We employed immunohistochemistry as well as *in situ* hybridization to provide an independent method for assessing cytokine production in histological sections because the former method detects protein and the latter mRNA.

We used *in situ* hybridization as part of the investigation of interleukin-4 (IL-4), IL-10, and inducible nitric oxide synthase (iNOS). We have previously employed this method to investigate the roles of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-6, and IL-2 in the small intestine of patients with celiac disease. The former probes were obtained commercially whereas the latter cytokine probes were synthesized in-house. Details are given in **Subheading 4.** (*see* **Note 1**).

2. Materials

2.1. Labeling and Purification

1. Oligonucleotide probe.
2. Sense probe (commercially available).
3. Oligo dT probe (store at –70°C).
4. DNase and RNase.
5. 5X Buffer: 500 m*M* cacodylate buffer, pH 6.8, 5 m*M* $CoCl_2$, 0.5 m*M* dithiothreitol, 500 mg/mL bovine serum albumin.
6. Terminal deoxynucleotidyl transferase (Promega Corp., Madison, WI).
7. α-^{35}S dATP (250 μC) (Amersham Pharmacia, Piscataway, NJ) (lasts 4–6 wk at –70°C).
8. Q water: 0.1% diethylpyrocarbonate (DEPC) mixed well with distilled water in a fume cupboard and left overnight. The solution is then autoclaved and allowed to cool before use.
9. Rackbeta counter.
10. Centrifuge.

2.2. Removal and Incorporation of Label

1. Sephadex G-50 DNA column (Pharmacia).
2. Whatman DC81 filter paper.
3. Scintillation fluid.
4. Elution buffer: 150 m*M* NaCl, 10 m*M* EDTA, 50 m*M* Tris, pH 7.5.

2.3. Hybridization

1. 4% Paraformaldehyde: Dissolve paraformaldehyde in PBS and add 100 mL of 5 *M* NaOH under stirring at 60°C.
2. Triton-X 100.
3. 15% Sucrose (store at 4 °C).
4. Slides: 12 slides maximum per run with four sections per slide.
5. Proteinase K (store at –20°C): Final concentration is 1 μg/mL in 0.1 *M* Tris + 50 m*M* EDTA, pH 7.5.
6. 0.4% Paraformaldehyde.
7. 0.25% Acetic anhydride in 0.1 *M* triethanolamine.
8. 2X saline sodium citrate (SSC) + 50% formamide + 1X Denhardt's solution.
9. 2X SSC + 50% formamide + 10% dextran sulfate (mol wt 500,000) + 400 μg/mL salmon sperm DNA.
10. Incubator.

2.4. Posthybridization Washes

1. 2X SSC.
2. 1X SSC + 50% formamide.
3. 0.1X SSC.
4. 95 and 99% ethanol.

2.5. Visualization of Hybridization Signal

1. Ilford K5 emulsion gel (light sensitive; stored at 4°C) (Ilford Scientific, Cheshire, UK).
2. Water bath.
3. Silica gel.
4. Lightproof box.
5. 44.7 g/L Dektol solution (Ilford Scientific).
6. 30% Sodium thiosulfate fixing solution.
7. Ice.
8. Mayer's hematoxylin.

2.6. Tissue Sections

1. Small intestinal biopsies are taken at upper gastrointestinal endoscopy and immediately orientated and embedded in optimal cutting temperature compound, snap-frozen in liquid nitrogen-cooled isopentane, and stored in liquid nitrogen until processed.

2. Cryostat sections are cut at 5 μm on a Bright open-top cryostat at –20°C and collected on to glass slides previously washed in diethylpyrocarbonate (DEPC)-treated water (as described in **Subheading 2.1.**, **item 8**) and precoated with poly-L-lysine. The sections are briefly fixed in acetone for 5 min and stored at –20°C.

3. Methods

3.1. Labeling and Purification of Probes

1. Label 4 pmol of each oligonucleotide (*see* **Note 2**) by the addition of a ^{35}S dATP (Amersham) "tail" to the 3' terminus using terminal deoxynucleotidyl transferase, according to the supplier's protocol (Promega). This enzyme catalyzes the repetitive addition of mononucleotides from dATP to the terminal 3'-OH of a DNA initiator, accompanied by the release of inorganic phosphate.
2. Make up the reaction solution as follows: 8 μL of 5X buffer, 4 pmol of oligonucleotide probe (1 μL), 3.2 μL of [α-^{35}S] dATP, 2.5 μL of terminal deoxynucleotidyl transferase (15 U/mL).
3. Add deionized distilled water (25.3 μL) to make up the final volume to 40 μL and mix thoroughly (*see* **Note 3**).
4. Incubate the solution at 37°C overnight.
5. Stop the reaction by placing on ice.

3.1.1. Removal of Unincorporated Label

Following the labeling reaction, the probes are purified by passing them through a prepacked Sephadex G-50 DNA grade column (Pharmacia). This removes the unincorporated label.

1. Wash the Sephadex column twice with elution buffer
2. Place the column in a plastic centrifuge tube and centrifuge at 1400 rpm for 4 min. Remove the column and check for cracks. If there are no cracks, resuspend in elution buffer and recentrifuge.
3. Add 40 μL of elution buffer to the labeled probe to make a total volume of 80 μL. Carefully pipet in the middle of the top of the Sephadex column and avoid touching the sides of the column.
4. Firmly place a sterile Eppendorf tube with the top cut off in a clean centrifuge tube. Place the loaded Sephadex column in it and centrifuge for 5 min at 1400 rpm.
5. Collect the purified labeled probe from the Eppendorf tube and measure its volume.

3.1.2. Assessment of Specific Activity of Labeled Probes

1. Spot 3 μL of probe cocktail in duplicate on to Whatman DE81 2.3-cm circular filters made up in a 1:100 dilution of 0.2 *M* EDTA.
2. Dry the filters briefly under a heat lamp. Keep one filter and use directly for the determination of counts per minute (cpm) in the sample.
3. Wash the other filter in 50 mL of 0.5 *M* Na_2HPO_4, pH 6.8, twice for 5 min to remove the unincorporated cpm.

4. Dry the washed filter under a heat lamp.
5. Add 3 mL of scintillation fluid to each filter and count activity on a Rackbeta scintillation counter.
6. Calculate specific activity as follows:

$$\frac{\text{Incorporated cpm}}{\text{unincorporated cpm}} \times 100 = \%\ \text{incorporation}$$

$$\frac{\%\ \text{incorporation} \times \text{total cpm in reaction}}{\text{mg of DNA substrate in reaction (cpm/mg)}} = \text{specific activity of each probe cocktail}$$

The specific activity of the probes should not be less than 1×10^8 cpm/mg in a successful labeling reaction. If it is found to be less, repeat (*see* **Note 4**).

3.2. Hybridization Procedure

3.2.1. Pretreatment of Tissue Sections

The method used to pretreat tissue sections is a modification of that previously employed in colonic ***(3)*** and bronchial ***(4)*** tissue. The prehybridization steps optimize probe access and reduce background signal. All steps are carried out at room temperature unless otherwise stated.

1. Air-dry sections previously stored at –20°C for at least 1 h.
2. Fix slides in freshly prepared 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 15 min.
3. Wash the sections twice in 15% (w/v) sucrose in PBS for 10 min each.
4. Incubate the sections with 0.3% (v/v) Triton X-100 in PBS for 10 min.
5. Incubate the sections at 37°C with 1 µg/mL of proteinase K (made up in 0.1 *M* Tris + 50 m*M* EDTA, pH 7.5) for 30 min to unmask target nucleic acid sequences (*see* **Note 5**).
6. Achieve postfixation by immersing the slides in 0.4% (w/v) paraformaldehyde for 10 min followed by treatment with 0.1 *M* triethanolamine containing 0.25% (v/v) acetic anhydride for 10 min to ensure that target sequences are not lost during hybridization.
7. Prehybridize the sections in a mixture of 2X SSC buffer, 50% (w/v) formamide, and 1X Denhardt's solution at 37°C for 1 h (*see* **Note 6**).

3.2.2. Hybridization (see ***Notes 7*** *and* ***8)***

1. Perform hybridization overnight with 2 ng per section (30 µL) of labeled probe at 37°C in a solution containing 2X SSC, 50% (w/v) formamide, 400 µg/mL of denatured (boiled for 5 min) salmon sperm DNA (*see* **Note 9**), and 10% (w/v) dextran sulfate (mol wt 500,000).

3.2.3. Posthybridization Washes

1. Carry out posthybridization stringency washes at 39°C, for 30 min each, in 2X SSC, 1X SSC + 50% formamide, and finally 0.1X SSC (*see* **Note 10**).
2. Dehydrate sections serially in 60, 90, and 95% ethanol and dry at room temperature.

3.3. Visualization of Hybridization Signal

3.3.1. Autoradiography

The following steps are performed in a dark room:

1. Melt Ilford K5 photographic gel emulsion (Kodak) (Eastman Kodak Co., Rochester, NY) by leaving it in a water bath and then dilute 1:1 in prewarmed distilled water at 40°C for about 15 min (*see* **Note 11**).
2. Cover the slides with the emulsion taking care to avoid the entrapment of bubbles.
3. Leave the slides to dry in a dark room for approx 2 h and then incubate at 4°C in a lightproof box containing silica gel for 5 d (*see* **Note 12**).

3.3.2. Development and Final Washes

1. Develop the slides in 44.7 g/L of Dektol solution (Kodak) for 2 min. Then wash briefly in distilled water and fix in 30% (w/v) sodium thiosulfate (on ice) for 15 min.
2. Thoroughly rinse the slides in running tap water and then counterstain with Mayer's hematoxylin.
3. Dehydrate the slides in ethanol and xylene before mounting.

3.4. Controls

Treat the slides with either RNase-free DNase, which has no effect on hybridization, or DNase-free RNase, which results in no hybridization: these serve as controls. Following the proteinase K treatment, sections are incubated at 37°C with either of the two enzymes at a concentration of 100 mg/mL for 30 min. Oligonucleotides containing sequences complementary to the IL-4, IL-10, and iNOS probes (sense probes) are used as negative controls. An oligo dT probe is used for the detection of total mRNA as a positive control. The inclusion of tissue known to contain the target sequence is often useful, and tonsil tissue is usually useful when employing cytokine probes.

3.5. Quantification

Count the number of positive cells per unit area within the lamina propria using a ×10 eyepiece graticule under ×40 objective. Assess the lamina propria from the tip of the villus down to the deeper lamina propria on duplicate sections for each cytokine/cell phenotype. The eyepiece graticule consists of 100 squares, which enables the villous lamina propria to be counted as a fraction of a complete grid, thus avoiding crypt epithelium in the deeper lamina propria. In total, count at least five complete graticule areas per section. The results

are expressed as the median number of positive cells in the lamina propria per unit area (0.02 mm^2). Positive cells in the epithelium are expressed relative to 100 epithelial cells per section: at least 500 epithelial cells must be counted per section. The analysis is performed blindly by two independent observers.

4. Notes

1. The IL-4, IL-10 ***(5)***, and iNOS ***(6)*** probe cocktails are obtained commercially (R&D Systems Europe, Abingdon, Oxon, UK), which does not allow access to their exact sequences. They may be successfully used on frozen sections (data supplied by R&D systems). Previous workers in our laboratory synthesized DNA oligonucleotide probes using a Beckman synthesizer. Probes were synthesized for IFN-γ, TNF-α, IL-6, and IL-2 ***(7)***. A mixture of three probes, each 30 bases long, with cDNA sequences, coded by three different exon regions of the cytokine gene, were synthesized. The gene sequences were obtained from published databases. The specificity of the probes was confirmed by Northern blotting of mRNA coding for IFN-γ, TNF-α, IL-6, and IL-2 isolated from blood mononuclear cells that had been cultured in the presence of 1 mg/mL of phytohemagglutinin or 10 mg/mL of concanavalin A ***(8)***. The sequences of the probes were compared with known genomic sequences by computer analysis using the DNASIS program. Northern blot analysis also determined their ability to hybridize to complementary mRNA. The reactivity of the probes was tested by *in situ* hybridization of mononuclear cells activated with phytohemagglutinin or concanavalin A. The size of the oligonucleotides (30–40 bases), the cytosine-guanine content (55–65%), as well as the use of several oligonucleotides are important determinants of effectiveness.
2. The α-^{35}S dATP is decanted in to 3.2-mL aliquots on receipt. All work with the radioactive label is performed on a designated workbench employing standard laboratory procedures for handling radioactive substances.
3. Make all solutions in Q water. The treatment with DEPC renders the distilled water RNase free.
4. Once the probes are labeled, they may be stored at –20°C overnight so that the hybridization phase can start the following day.
5. It is important to ensure that the solutions for the proteinase K and the stringency wash steps are prewarmed to their correct temperatures prior to immersing the sections. Other pretreatment steps that may be used include acetylation to reduce background staining.
6. The prehybridization step helps reduce nonspecific probe binding.
7. The components of the hybridization solution play a major role in the stringency of hybrid formation. This is an important area for fine tuning if results are not satisfactory. The stringency is influenced by the concentration of the probe, salt, and formamide concentration as well as the duration and temperature of incubation. The formamide reduces the effective temperature of hybridization and the inclusion of dextran sulfate increases the rate of hybrid formation and reduces nonspecific ionic staining.

8. It is essential to prevent mixing of different hybridization solutions as they are pipetted onto the sections. Ensure that all the sections are covered with hybridization solution.
9. Once the salmon sperm DNA is denatured by boiling for 5 min, it is immediately placed on ice.
10. The posthybridization washes improve the stringency of hybrid formation. Some groups report that if the conditions of hybridization are carefully controlled, the pre- and posthybridization steps may not be required.
11. If the water bath used in the darkroom has a thermostat light, this should be masked. The silica gel should be dried the day before use. Occasionally, spurious nonradioactive-induced reaction products are seen with the liquid emulsion. It is thought to be caused by the interaction of heavy metal ions with the emulsion. This can be minimized by the avoidance of heavy metals from fixatives, exposure to emulsion at 4°C, and the maintenance of a constant temperature during processing.
12. The whole method usually runs over 5 d. The samples are then left for a further 5 d before development. If the radioactive label is not fresh, another day may be required. Other texts provide useful background and detailed information ***(9,10)***.

If we were to extend our work in this area, we would like to develop nonradioactive labeling methods, and employ image analysis in the quantification of our results.

References

1. Buongiorno-Nardelli, M. and Amaldi, F. (1969) Autoradiographic detection of molecular hybrids between rRNA and DNA in tissue sections. *Nature* **225,** 946,947.
2. Gall, G. and Pardue, M. L. (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proc. Natl. Acad. Sci. USA* **63,** 378–381.
3. Cappello, M., Keshav, S., Prince, C., Jewell, D. P., and Gordon, S. (1992) Detection of mRNAs for macrophage products in inflammatory bowel disease by in situ hybridisation. *Gut* **33,** 1214–1219.
4. Hamid, Q., Azzawi, M., Yind, S., Moqbel, R., Wardlaw, A. J., Corrigan, C. J., Bradley, B., Durham, S. R., Collins, J. V., Jeffery, P. K., Quint, D. J., and Kay, A. B. (1991) Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J. Clin. Invest.* **87,** 1541–1546.
5. Beckett, C. G., Dell'Olio, D., Kontakou, M., Przemioslo, R., Rosen-Bronson, S., and Ciclitira, P. J. (1996) The analysis of interleukin-4 and interleukin-10 and their relationship to the lymphocytic infiltrate in the small intestine of patients with coeliac disease. *Gut* **39,** 818–823.
6. Beckett, C. G., Dell'Olio, D., Ellis, H. J., Rosen-Bronson, S., and Ciclitira, P. J. (1998) Detection of inducible nitric oxide synthase in the small intestine of patients with coeliac disease and the identification of the major cellular sources of production. *Eur. J. Gastroenterol. Hepatol.* **10,** 641–647.

7. Kontakou, M., Przemioslo, R., Sturgess, R., Limb, G. A., Ellis, H. J., Day, P., and Ciclitira, P. J. (1995) Cytokine mRNA expression in the mucosa of treated coeliac patients after wheat peptide challenge. *Gut* **37,** 52–57.
8. Sambrook, J., Fritsch, E., and Maniatis, T., eds. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY, p. 7.4.
9. Sirinathsinghi, D. L. S. and Dunnett, S. B. (1993) *Molecular Imaging in Neuroscience: A Practical Approach* (Sharif, N. A., ed.), IRL Press, Oxford, UK, pp. 43–70.
10. Hofler, H. (1990) *In Situ Hybridisation: Principles and Practice* (Polak, J. M. and McGee, J. O'. D., eds.), Oxford Science, Oxford, UK, pp. 15–30.

14

Measurements of Cytokine mRNA Expression by Quantitative Polymerase Chain Reaction in Studies of Celiac Disease

Ellen M. Nilsen, Finn-Eirik Johansen, and Per Brandtzaeg

1. Introduction

Cytokines are known to play a key regulatory role in immune responses. The onset or progression of immunopathology in various diseases is often associated with aberrant production of one or more cytokines. It is therefore of considerable interest to characterize cytokine "profiles" associated with disease processes. Many methods employed for identification and quantification of cytokines produced by different cell types rely on the responsiveness of indicator cell lines. Such bioassays are technically restrictive owing to the time required for performance and because of sensitivity and specificity problems. Enzyme-linked immunosorbent assays (ELISAs), on the other hand, detect both biologically active and inactive cytokines without discrimination. These assays are easy to use, but the commercial kits are usually expensive. Both bioassays and ELISAs are unable to identify actual cytokine production and do not account for cytokines consumed by cells. In addition, the minute amounts of cytokine protein often produced in autocrine or paracrine microenvironments may not be easily detectable in a sample, especially when tissue or cells are available in only small quantities ***(1)***. Furthermore, although cells producing cytokine protein may be detected by immunocyto/histochemistry, only a limited number of antibodies with good performance are available ***(2)***, and the possibility of confusing synthesis with cellular uptake of cytokines exists.

As an alternative (or supplement) to cytokine protein measurement, an indication of cytokine production can be obtained by quantification of mRNA for the actual product. To this end, quantitative polymerase chain reaction (PCR) is the method of choice (**Table 1**). PCR is widely used and has proven to be the

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

Table 1
Assays for Measurement of Cytokine Levels and Their Advantages and Disadvantages

Bioassay
Requires a lot of time
Detects only biologically active form of cytokines
Has sensitivity and specificity problems
Requires neutralization to confirm specificity
Has a limited number of available responder cells
Immunoassay
Requires only a few hours to obtain results
Detects both biologically active and inactive cytokines
Has a limited number of available kits
Immunocyto/histochemistry
Detects cytokine-containing cells
Has a limited number of available antibodies that are useful
May be difficult to distinguish between cytokines that occupy cellular receptors and those cytokines that are actually produced by the cell
RT-PCR
Detects cytokine mRNA
Provides no information about the cellular source of mRNA unless performed on isolated and phenotyped cells
Provides no proof of protein translation, even after detection of large amounts of mRNA
Requires an internal standard to obtain quantitative results

molecular technique of choice for the detection and analysis of minute amounts of DNA *(3)*. It is an in vitro method for enzymatically driven synthesis of defined sequences of DNA; the reaction employs two oligonucleotide primers that hybridize to opposite strands and flank the target DNA sequence that is to be amplified. A repetitive series of cycles involving template denaturation, primer annealing, and extension of the annealed primers by a thermostable DNA polymerase results in exponential accumulation of a specific DNA fragment. The length of the products generated during PCR is equal to the sum of the lengths of the two primers plus the distance in the target DNA between the primers.

RNA cannot serve as a template for PCR, so amplification of RNA molecules is performed by a method that combines reverse transcriptase (RT) to turn RNA into a complementary DNA (cDNA) strand with PCR colloquially referred to as RT-PCR. However, one drawback inherent in RT-PCR is that this method is at best semiquantitative, and can only be used to provide quali-

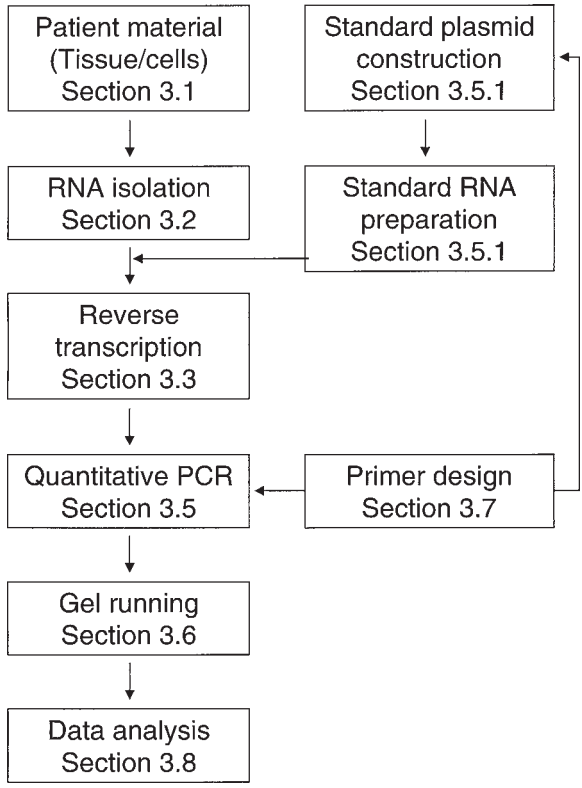


Fig. 1. Overview of the RT-PCR method with reference to sections in the text. Total RNA is prepared from, e.g., an intestinal biopsy specimen added to a dilution series of an exogenous RNA standard, with both samples reverse transcribed and then coamplified in a single PCR reaction mixture. Following gel electrophoresis, the relative amount of target RNA vs standard RNA is measured by direct scanning of an ethidium-stained agarose gel. When target and standard RNAs are present at equal concentrations, coamplification results in equal amounts of both products with bands of equal density. Because the concentration of the standard is known, this gives the concentration of the target (unknown) RNA.

tative results. Because amplification is (at least initially) an exponential process, small differences in any of the variables that control the reaction rate will dramatically affect the yield of the PCR product. We describe herein a technique that obviates these problems and allows precise quantitation of specific mRNA species.

Figure 1 illustrates the major steps involved in quantitative RT-PCR. By this approach, an exogenous RNA standard is added to the target RNA sample

and amplified simultaneously with the target transcript in a single PCR reaction mixture. The standard and target sequences compete for the same primers and, therefore, for amplification. The relative amount of target RNA vs competitor RNA can be measured by direct scanning of ethidium-stained agarose gels. Because the starting concentration of the standard is known, the initial concentration of the target RNA can be determined. We demonstrate herein that this method can rapidly and accurately detect cytokine mRNA in biopsy specimens taken from the small intestinal mucosa of celiac disease patients, and can be applied to the quantification of several cytokines (and chemokines) expressed at low levels (≥1000 transcripts/μg of total RNA) in the mucosa.

This competitive PCR approach has been shown to be highly sensitive and specific when compared with Northern blot analysis or dot-blot analysis ***(4,5)***, and it is considered to be the best method for determination of gene expression at low levels such as for cytokine genes. The application of this approach has extended the use of PCR to, e.g., analysis of mRNA induction in response to exogenous cellular stimuli.

2. Materials

2.1. Patient Material

1. Obtain mucosal specimens by the use of an Olympus endoscope (GIF IT20) with biopsy forceps (Olympus America, Melville, NY, cat. no. FB13K).

2.2. Isolation of RNA

1. CsCl (5.7 *M* CsCl and 0.01 *M* EDTA in a final volume of 50 mL of autoclaved deionized H_2O). Store at room temperature.
2. Guanidine isothiocyanate (GTC) with β-mercaptoethanol (5 *M* GTC, 25 m*M* sodium citrate, 0.5% sodium lauroyl sarcosine, 0.1 *M* β-mercaptoethanol added fresh). Store at 4°C.

2.3. Reverse Transcription

1. 5X First-strand buffer: 250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$ (Gibco, Paisley, UK). Store at –20°C.
2. Superscript™ in storage buffer: 20 m*M* Tris-HCl, pH 7.5, 100 m*M* NaCl, 0.1 m*M* EDTA, 1 m*M* dithiothreitol (DTT), 0.01% (v/v) Nonidet P-40 (NP-40), 50% (v/v) glycerol (Gibco) at 200 U/μL. Store at –20°C.
3. RNase inhibitor (RNasin) (Promega, Madison, WI) at 40 U/μL. Store at –20°C.
4. DTT, 100 m*M* stock (Gibco). Store at –20°C.
5. Oligo$(dT)_{16}$, 100 pmol/μL in TE (Gibco). Store at –20°C.
6. dNTP mixture (Pharmacia, Uppsala, Sweden): Prepare a 10X stock solution of 10 m*M* each of dATP, dCTP, dGTP, and dTTP. Store in aliquots at –20°C.

2.4. Polymerase Chain Reaction

1. 10X *Taq* DNA polymerase reaction buffer: 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl (Perkin-Elmer, Branchburg, NJ). Store at –20°C.
2. $MgCl_2$, 25 m*M* (Perkin-Elmer). Store at –20°C.
3. *Taq* DNA polymerase in storage buffer: 20 m*M* Tris-HCl, pH 8.0, 100 m*M* KCl, 0.1 m*M* EDTA, 1 m*M* DTT, 50% (v/v) glycerol, 0.5% Tween-20, 0.5% NP-40 (Perkin-Elmer) at 5 U/μL. Store at –20°C.
4. PCR primers: 18–25 bases in length, 100 pmol/μL in TE (Gibco). Store at –20°C (*see* **Subheading 2.7.**).
5. Light white mineral oil (Sigma, St. Louis, MO).
6. Microfuge tubes (Sarstedt Ltd., Germany): Use only tubes specified for use in the Thermal Cycler, because ill-fitting tubes will result in inconsistent and inefficient amplifications.
7. Pipets and cotton-plugged pipet tips.

2.5. Quantitative PCR

To quantify the amounts of different cytokine transcripts, we use standard RNA generated from the plasmids pHCQ1, pHCQ2, and pHCQ3 (courtesy of Dr. M. F. Kagnoff, University of California, San Diego). The construction of these plasmids has been previously described *(6)*.

2.6. Gel Running and Data Analysis

1. 6X Loading buffer: 0.25% xylene cyanol and 30% glycerol in a final volume of 20 mL of autoclaved deionized H_2O. Store at room temperature.
2. SeaKem LE agarose (FMC Bioproducts, Rockland, ME).
3. DNA marker: 123-bp ladder (Gibco). Store at 4°C.
4. 1X TBE buffer, pH 8.3 (0.045 *M* Tris-borate, 0.001 *M* EDTA). Prepare a 5X stock. Store at room temperature.
5. Gel apparatus: horizontal slab gel casting/electrophoresis chamber/power supply.

2.7. Selection of Primers (Primer Design)

Primer selection for PCR is most often undertaken by computer programs designed to take into account variables such as G:C ratio, sequence homology, the probability of primer-dimer or "hairpin" formation, and anchor sequence characteristics. Many PCR primer sequences are available from published sources; most can be used with confidence. Alternatively, suitable primers and primer pairs may be designed with the aid of commercially available computer software (e.g., Primer Designer 1.0). Many national molecular biology nodes on the World Wide Web have available software, e.g., embnet. no. PRIMER. Primers that are used in combination should have a similar T_m value.

3. Methods

3.1. Patient Material

1. Obtain mucosal specimens by the use of an Olympus endoscope (GIF IT20) with biopsy forceps (Olympus FB13K).
2. Snap-freeze the biopsy specimens in liquid nitrogen, and store at –70°C (*see* **Note 1**). Alternatively, place the biopsy specimens on iron grids in tissue culture chambers in a tight container at 37°C with 5% CO_2 and 95% O_2 (Costar, Cambridge, MA), and stimulate for different time intervals with 5 g/L of a peptic-tryptic digest of commercial gluten (Sigma) in RPMI 1640 containing inactivated pooled normal human serum (NHS) and antibiotics *(7)*. Incubate control specimens in parallel with only medium and NHS. Then snap-freeze the biopsy specimens in liquid nitrogen and store at –70°C.

3.2. Isolation of RNA

RNA may be prepared from tissue or cells in several ways. We have isolated total RNA by the GTC method *(8)*. *Please follow the instructions that are provided with the RNA isolation method you choose*. Numerous commercial kits are available, and we have successfully used RNAzol (Biogenesis, Poole, UK) and RNAesy (Qiagen, Hilden, Germany).

1. Remove frozen biopsy specimens from liquid nitrogen and homogenize in GTC by the stroke of a tightly fitting pestle in a glass Dounce homogenizer (Kontes, Vineland, NJ). We use 3.5 mL of GTC to one biopsy specimen with a size corresponding to 25–35 mg of wet wt.
2. Isolate total RNA. We most often isolate RNA according to MacDonald et al. *(8)*.
3. Dissolve the precipitated RNA in 10–20 μL of diethylpyrocarbonate (DEPC)-treated water (*see* **Note 2**).
4. Determine the yield and purity of the RNA by measuring the OD_{260}:OD_{280} ratio. RNA purified by this method should result in an OD_{260}:OD_{280} ratio of >1.7 (*see* **Note 3**).
5. At this point, keep the isolated RNA on ice when being handled, or store at –70°C.

3.3. Reverse Transcription

Because RNA cannot serve as a template for PCR, reverse transcription is first performed to make RNA into cDNA suitable for PCR. As previously defined, the combination of both techniques is referred to as RT-PCR. Total RNA (0.5–1 μg) is reverse-transcribed into cDNA according to a standard protocol (Gibco). *Please follow the instructions that are provided with the protocol and for the solutions you choose*.

1. Synthesize cDNA by RT in a 20-μL reaction mixture at 42°C.
2. Centrifuge briefly (≥8000*g* for 5–10 s) all reagents (*see* **Subheading 2.3.**) before beginning the procedure.
3. Keep all solutions and the reaction mixture on ice during the preparation.

4. For each reverse transcription, mix the following components in a sterile microfuge tube (*see* **Note 4**): 4.0 µL of 5X first-strand buffer, 2.0 µL of 0.1 *M* DTT, 1.0 µL of 10 m*M* dNTP mix, 1.0 µL of 20 pmol/µL of oligo(dT)$_{16}$ (*see* **Note 5**), 0.5 µL of RNasin (40 U/µL), 9.0 µL of autoclaved deionized H_2O, 0.5 µL of Superscript (200 U/µL) (*see* **Note 6**), and 2.0 µL of total RNA (500 ng/µL).
5. Gently tap the tubes to mix all the reagents.
6. After brief centrifugation (8000*g* for 5–10 s) to deposit all the liquid to the bottom of the reaction tube, place the tubes into a PCR thermal cycler.
7. Incubate at 42°C for 60 min, followed by heat inactivation at 99°C for 10 min (*see* **Note 7**).
8. Store cDNA at –20°C until required for use.

3.4. Polymerase Chain Reaction (see Notes 8–10)

PCR is carried out in a 25-µL reaction mixture according to a standard protocol (Perkin-Elmer). *Please follow the instructions that are provided with the protocol and for the solutions you choose.*

1. Centrifuge briefly (8000*g* for 5–10 s) all reagents (*see* **Subheading 2.4.**) before beginning the procedure.
2. Keep all solutions on ice, and during the preparation of reaction mixtures.
3. For each reaction, mix the following components in a sterile microfuge tube: 2.5 µL of 10X buffer, 1.5 µL of 25 m*M* $MgCl_2$ (*see* **Note 11**), 0.5 µL of 10 m*M* dNTP (*see* **Note 12**), 1.0 µL of 10 pmol/µL of forward primer, 1.0 µL of 10 pmol/µL of reverse primer (*see* **Note 13**), 16.5 µL of autoclaved deionized H_2O, 1.0 µL of *Taq* DNA polymerase (5 U/µL, diluted 1/8) (*see* **Note 14**), and 1.0 µL of cDNA (*see* **Note 15**).
4. Gently tap the tubes to mix all the reagents. Layer 25 µL of mineral oil on top of the PCR solution to prevent evaporation of liquid during thermal cycling.
5. After brief centrifugation (8000*g* for 5–10 s) to deposit all of the liquid to the bottom of the reaction tube, place the tubes into a PCR thermal cycler. Follow the manufacturer's instructions for programming.
6. For an initial denaturing step, incubate at 95°C for 4 min. A typical temperature profile is denaturation (95°C for 1 min), annealing (50–72°C for 30 s), and elongation (72°C for 1 min) (*see* **Notes 16** and **17**).
7. Repeat these steps for 25–35 cycles (*see* **Notes 18–20**).
8. For the final step, incubate at 72°C for 10 min to promote completion of partial extension products and annealing of single-stranded complementary products.
9. Keep PCR products at 4°C until they are analyzed.

3.5. Quantitative PCR

One drawback of RT-PCR is that this method at best is semiquantitative (*see* **Notes 21–23**). In an attempt to correct for tube-to-tube variations in both the cDNA synthesis and PCR amplification efficiency, it is recommended that internal RNA standards are used (*see* **Note 24**). By this approach, an exog-

enous RNA standard is added to the target sample and amplified simultaneously with the target transcript in a single PCR reaction mixture. The standard and target sequences compete for the same primers and, therefore, for amplification. A dilution series is made of either the target sequence or the standard sequence, and a constant amount of the other component is added to each of the reactions. Quantification is performed after competitive amplification of the entire series of reactions and is achieved by distinguishing the two PCR products in each tube by differences in size.

1. Carry quantitative PCR in a 25-μL reaction mixture (*see* **Subheading 3.4.**). Reverse-transcribe a constant amount of total RNA (we use 500 ng to 1 μg) from each sample into cDNA together with serial dilutions (i.e., two- or threefold dilutions) of standard RNA transcripts in the same reaction volume and coamplify by PCR. We typically start at 2–10 pg of standard/μg of total RNA.

3.5.1. RNA Standards for Quantification of Cytokines

Since the first study by Wang et al. ***(4)*** that describes a synthetic RNA used as an internal standard, several reports have described the construction of exogenous RNA and DNA internal standards that differ from target sequences only by the presence or absence of small introns or restriction sites ***(6,9–11)***. In these cases, there is little doubt that the amplification efficiencies of the standard and target sequences will be the same. To quantify the amounts of different cytokine transcripts, we used standard RNA generated from the plasmids pHCQ1, pHCQ2, and pHCQ3. The construction of these plasmids has been previously described ***(6)***. The arrangement of priming sites are designed to yield PCR products that differ in size from those of the target RNA. Because the same primer set is used in the PCR amplification on both templates, differences in primer annealing efficiency and hence PCR efficiency are minimized.

3.6. Gel Running

After amplification, separate the PCR products generated by standard and target RNA by agarose gel electrophoresis and visualize the bands by ethidium bromide staining. Horizontal agarose gels are usually run in an electrical field at constant strength and direction. Prepare the gel for loading as follows:

1. Seal the open ends of the plastic tray supplied with the electrophoresis apparatus (or a glass plate) with autoclave tape to form a mold.
2. Add the correct amount of powdered agarose (*see* **Notes 25** and **26**) to a measured quantity of electrophoresis buffer in a glass bottle. Heat in a microwave oven until the agarose dissolves. Heat the solution for the minimum time required to allow all grains of agarose to dissolve. Swirl the bottle from time to time.

Check that the volume of the solution has not decreased by evaporation during boiling; replenish with water if necessary.

3. Cool the solution to 60°C, add ethidium bromide to a final concentration of 0.5 μg/mL, and mix thoroughly (*see* **Notes 27** and **28**).
4. Pour the agarose solution into the mold and position the comb 0.5–1.0 mm above the plate so that a complete well is formed when the agarose is added. The gel should be between 3 and 5 mm thick. Make sure that no air bubbles are retained under or between the teeth of the comb.
5. After the gel is completely set (20–30 min at room temperature), carefully remove the autoclave tape and mount the gel in the electrophoresis tank.
6. Add just enough electrophoresis buffer to cover the gel, and carefully remove the comb.
7. Mix the sample of DNA with the gel-loading buffer. Slowly load the mixture (15–25 μL is used for analysis) into the slots of the submerged gel with a pipet.
8. Load a DNA marker (123-bp ladder) into one slot of the gel. This ladder allows verification of predicted size of PCR products and makes it easier to determine the sizes of unknown DNAs if any systematic distortion of the gel should occur during electrophoresis (*see* **Note 29**).
9. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the anode (red lead). Apply a voltage of 1–5 V/cm.
10. Run the gel until the bands have migrated the appropriate distance, or until the bands for standard and target are appropriately separated.
11. Turn off the electric current and remove the leads and lid from the gel tank. If ethidium bromide was present in the gel, examine the gel by ultraviolet (UV) light (*see* **Note 27**).

3.7. Selection of Primers

A critical requirement in the selection of primers is that the extension products of each primer extend far enough through the target region to include the sequences of the other flanking primer. In this way, each extension product made in one cycle can serve as a template for extension in the next cycle; this results in an exponential increase in PCR product as a function of cycle number. After the first few cycles, the major product is a DNA fragment that is exactly equal in length to the sum of the lengths of the two primers and the intervening target DNA (*see* **Notes 30–35**).

3.8. Data Analysis

1. Photograph the gels with Polaroid 665 film and use the negatives to quantify band intensities by a Nikon Scantouch 110 (Nikon, Tokyo, Japan).
2. Analyze the data. We use Phoretix Array Advanced (Phoretix, Newcastle upon Tyne, UK).

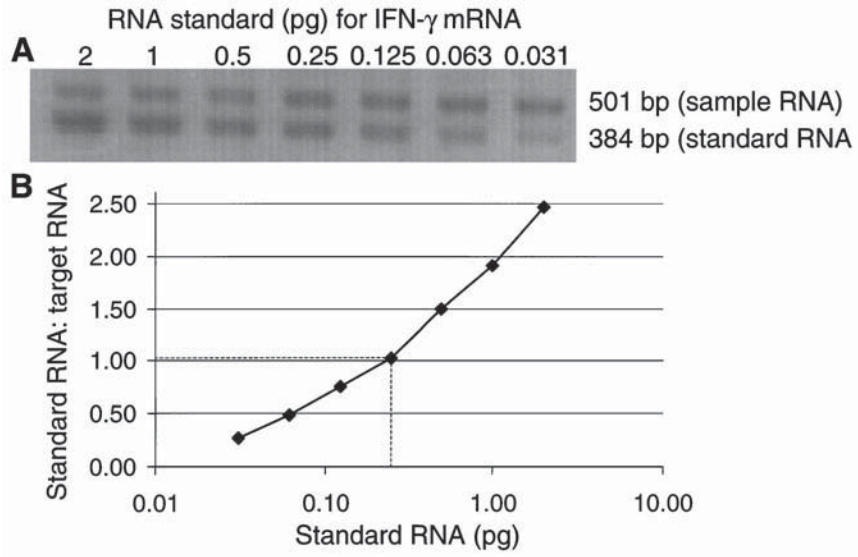


Fig. 2. **(A)** Competitive quantitative analysis of IFN-γ transcripts in RNA extracted from a duodenal biopsy specimen isolated from an untreated celiac disease patient. A master mixture was prepared containing IFN-γ primers, and 17 μL of the master mixture was aliquoted into each of seven tubes. Sample RNA (2 μL, 1 μg) was added together with serial dilutions of the standard (competitor). The final concentration of standard RNA in each tube was 2, 1, 0.5, 0.25, 0.125, 0.063, and 0.031 pg, respectively. After reverse transcription and 30 cycles of PCR on 1 μL of cDNA, the PCR products generated by standard (384 bp) and sample (501 bp) were electrophoresed on an agarose gel. Polaroid 665 negatives were scanned by a Nikon Scantouch and analyzed by Phoretix Array Advanced. **(B)** Quantitative analysis of amplified IFN-γ mRNA products. The ratio of standard IFN-γ mRNA per target IFN-γ mRNA was plotted as a function of the amount of known standard IFN-γ mRNA. The level of IFN-γ mRNA was determined by calculating how much of the standard was required to achieve equal amounts of products. The point of equivalence (1:1 ratio, marked with broken line), indicates where target IFN-γ mRNA equals standard IFN-γ mRNA and represents the concentration of RNA in the unknown sample.

3.9. Applications: Use of Quantitative PCR for Detection of Cytokine Expression in Biopsy Specimens of Celiac Mucosa

Quantification of interferon-γ (IFN-γ) transcripts in duodenal/jejunal biopsy specimens from untreated celiac disease is performed with the HCQ1 competitive template, as shown in **Fig. 2**.

1. Isolate RNA (*see* **Subheading 3.2.**) from biopsy specimens (*see* **Subheading 3.1.**).
2. Perform reverse transcription (*see* **Subheading 3.3.**).

3. Prepare the master mix (*see* **Note 4**), and add 17 μL to each of seven (or more if necessary) marked tubes, e.g., A–G (*see* **Note 36**).
4. Add 1 μL of standard RNA at a known concentration in a dilution series (usually two- or threefold dilution) to each tube (*see* **Notes 36–38**).
5. Add 1 to 2 μL (1 μg of RNA) of the sample to each tube.
6. Perform reverse transcription (*see* **Subheading 3.3.**).
7. Perform PCR (*see* **Subheading 3.4.**).
8. Mix the sample of DNA with the gel-loading buffer, and load 25 μL/well on a 1.6% ethidium-stained SeaKem LE agarose gel.
9. Photograph and analyze the gel as described (*see* **Subheading 3.8.**).
10. Plot standard mRNA per target mRNA as a function of the amount of standard mRNA. The point of equivalence is where target mRNA equals standard mRNA (i.e., ratio 1:1) and thus represents the concentration of RNA in the unknown sample (**Fig. 2B**) (*see* **Notes 39** and **40**).

4. Notes

1. The critical factor in isolating RNA from eukaryotic tissues is inactivation of endogenous RNases and avoidance of RNases from external sources. RNA is extremely unstable in samples once removed from the body, so it is critical to quick-freeze the tissue in liquid nitrogen.
2. For RNA isolation, extreme care must be taken to avoid RNases. All solutions should be prepared by use of RNase-free glassware, autoclaved DEPC-treated water, and chemicals reserved for work with RNA. Nondisposable glassware should be baked at 150°C for 4 h to destroy RNases. RNA is extremely susceptible to degradation by endogenous RNases after cell lysis and before cDNA synthesis.
3. Total RNA can be used directly for production of cDNA without the need of mRNA isolation; this may be an advantage because the low amounts of available mRNA (5–10% of total RNA) are difficult to quantify. A solution whose $OD_{260} = 1$ contains approx 40 μg/mL of RNA.
4. When multiple samples are to be analyzed for the content of a given mRNA, a master mix may be prepared containing all the components except the RNA source. Use of such mixtures will minimize reagent loss during pipetting, increase accuracy, and reduce the number of reagent transfers. The master mix will eliminate the need for repeated pipetting of small volumes and increase consistency among samples.
5. Three types of primers may be used for reverse transcription:
   a. $Oligo(dT)_{12\text{-}18}$ binds to the endogenous poly(A) tail at the 3' end of mammalian mRNA. This primer most frequently produces a full-length cDNA product, but the 5' end of some mRNAs may be reverse-transcribed inefficiently owing to the secondary structure of the mRNA.
   b. Random hexanucleotides can bind to mRNA templates at any complementary site and will give partial length (short) cDNAs.
   c. Specific oligonucleotide sequences can be used to prime selectively the RNA of interest.

6. Superscript RT is a DNA polymerase that synthesizes cDNA from single-stranded RNA or DNA. We have also successfully used the enzyme MuLV (murine leukemia virus) RT (Perkin-Elmer) for cDNA synthesis.
7. RT binds to cDNA and is thereby inhibitory to PCR amplification. Under the conditions provided (12.5 U/μL), incubation at 99°C for 10 min inactivates the RT and removes the inhibitory effect.
8. For most PCR applications, it is essential that the only DNA entering the reaction is the added template. The ideal way to ensure that PCR is performed only with the desired template is to maintain a DNA-free clean environment. It is suggested that a selected laboratory area, preferably a laminar flow hood, be dedicated to performance of PCRs. No amplified product should ever be brought into this hood. A set of pipettors, racks, tubes, cotton-plugged pipet tips, and so forth should be confined to the preparation hood and used only to mix the reactions. Autoclaved deionized water, sterile mineral oil, and buffers should be aliquoted and stored in small amounts, enough for an average single preparation. Use autoclaved tubes and solutions whenever possible. Always wear gloves to avoid nuclease contamination from fingers. It is recommended to prepare your own sets of reagents and store them in small aliquots. Do not apply these reagents for other purposes. Use new glassware, plasticware, and pipets that have not been exposed to DNAs in the laboratory. After use, discard aliquots of reagents; do not return them to storage.
9. RT-PCR is a very powerful technique for the detection of small amounts of mRNA; it appears ideally suited for use on cells that cannot be grown in large numbers, or tissue that is available in only minute quantities. However, it is important to be aware that the high sensitivity of mRNA transcript detection may lead to false conclusions regarding protein translation. Even detection of large amounts of mRNA is no proof of protein translation. Several examples of posttranscriptional regulation of protein expression have been described in the literature ***(12,13)***.
10. Each new PCR application is likely to require optimization. Some problems often encountered include no detectable product or low yield of the desired product, the presence of nonspecific background bands owing to mispriming or misextension of the primers, the formation of "primer-dimers" that compete for amplification with the desired product, and mutations or heterogeneity owing to misincorporation.
11. It is beneficial to optimize the magnesium ion concentration. The magnesium concentration may affect all of the following: primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer-dimer artifacts, and enzyme activity and fidelity. *Taq* DNA polymerase requires free magnesium on top of that bound by template DNA, primers, and dNTPs. Accordingly, PCRs should contain 0.5–2.5 m*M* magnesium ***(14)***.
12. The nucleotide concentration must be sufficient to saturate the enzyme, but not so high or imbalanced as to promote misincorporation. The primer concentration must be high enough to anneal rapidly to the single-stranded target and, in later stages of the reaction, faster than target-target reassociation.

13. Primer concentrations that are too low will result in little or no PCR product, whereas concentrations that are too high may result in amplification of nontarget sequences. Primer concentrations in the range of 0.1–0.5 μ*M* will work for most PCR amplifications ***(14)***.
14. The isolation of a heat-resistant DNA polymerase from *Thermus aquaticus* (*Taq*) allows primer annealing and extension to be carried out at an elevated temperature ***(15)***, thereby reducing mismatched annealing to nontarget sequences. This added selectivity results in the production of large amounts of virtually pure target DNA. Another important advantage of *Taq* polymerase is that it escapes inactivation during each cycle, unlike the Klenow enzyme, which must be added after every denaturation step. This has allowed automation of PCR in machines that have controlled heating and cooling capability. The *Taq* polymerase has measurable activity at room temperature and at almost all of the temperatures up to the DNA denaturation temperature ***(14)***. For optimal specificity, the highest possible annealing temperature should be used to reduce nonspecific primer extension. We have also successfully used other thermostable polymerases, such as Vent (New England Biolabs, Beverly, MA), Dynazyme (Finnzyme, Espoo, Finland), and Amplitaq Gold (Perkin-Elmer).
15. Always include a control that contains all the components of the PCR reaction except the template DNA. A negative control tube is necessary to monitor possible DNA contamination and estimate background levels. A cDNA synthesis reaction with all components including RNA except RT is a good negative control. Whenever possible, include a positive control.
16. Temperature control and timing are important. The temperatures used must maximize specific primer annealing and polymerase elongation but not sacrifice yield by reducing primer-template hybridization. Denaturation must be efficient, but the temperature should neither be too high nor maintained too long because the *Taq* polymerase, although heat resistant, is not indefinitely stable. The range of enzyme activity varies by 2 orders of magnitude between 20 and 85°C ***(14)***. The temperature and length of time required for primer annealing depend on the base composition, length, and concentration of the amplification primers. An applicable annealing temperature is 5°C below the true T_m of the amplification primers. Annealing temperatures in the range of 55–70°C generally yield the best results. Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces misextension of incorrect nucleotides at the 3' end of primers. Therefore, stringent annealing temperatures will help increase specificity.
17. For some primer-template pairs, two-temperature rather than three-temperature cycles may work when the annealing temperature is above 60°C; the extension is then completed at the annealing temperature. The slower extension rate at lower temperatures may require optimization of the time for such combined anneal-extend steps. We have often successfully used a two-step reaction in which the annealing and extension steps are carried out at the same temperature, as has also been reported by other laboratories ***(6)***.

18. The number of PCR cycles required depends on the level of the target. Usually, 20–40 cycles are used, but the optimal number should be determined for each reaction (*see* **Note 19**). Use the smallest number of PCR cycles that provides the "cleanest" result. Increasing the number of cycles often produces more nonspecific amplification. Also, with a large number of amplification cycles, contamination problems are more likely to occur.
19. Optimizing the number of PCR cycles is the best way to avoid the problems in reaching the plateau phase. This can be performed by preparing a master mix, with trace radioactive dCTP. PCR is then performed for different numbers of cycles (e.g., between 15 and 40 cycles). After separation on a 5% acrylamide gel (approx 30 mL of 5% acrylamide gel solution with 300 μL of 10% ammonium persulfate and 20 μL of TEMED are used for a 14-well gel), the radioactivity is quantified as described under **Subheading 3.8.** Calculate the PCR product yield as a function of cycle numbers. Always use cycle numbers below the plateau phase.
20. Limiting amplification to the desired target can also be enhanced with a nesting strategy that involves two different rounds of PCR. After amplification with one set of primers, a small aliquot is taken and amplified in a second round, either with two new primers internal to those used in the first round (nesting) ***(16)*** or with one new internal primer and one of the original primers ("heminesting") ***(17)***. This strategy works because, unlike the desired target, any nontarget sequences amplified in the first round cannot be further amplified with the internal target-specific primer(s) used in the second round.
21. If every template molecule in the sample is completely extended at each cycle, then the amplification efficiency is 100%. In practice, efficiencies can vary quite significantly throughout the course of amplification. For example, the amount of product produced at each cycle eventually levels off. This plateau can be explained by two phenomena. First, as the concentration of double-stranded product reaches high levels, competition increases between annealing of the template (PCR product) to primer and reannealing of the complementary template strands. Second, the amount of enzyme is finite and eventually there is not enough to extend all of the primer-template complexes during the allotted time. The efficiency may also depend on the amount of original target and the properties of the target that are not well understood, such as the likelihood of secondary structure in the single-stranded template. In general, long targets are less efficiently amplified than shorter ones, but there are exceptions to this rule, which could be owing to the actual sequence involved.
22. It is often useful to include an endogenous mRNA sequence, e.g., for β-actin or glyceraldehyde phosphate dehydrogenase, known to be present at constant levels throughout a series of samples as an internal control for a semiquantitative analysis. To ensure reliability, the level of expression of this control gene must be the same in each sample to be compared and must not change as a result of the experimental treatment. Unfortunately, few if any genes are expressed in a strictly constitutive manner. This is even the case for many "housekeeping" genes,

including β-actin ***(5,18)***. Therefore, such data must be examined very carefully for constancy among all experimental conditions studied. Because the amplification is initially exponential, any tube-to-tube variation or differences in the rate of amplification of the different templates will be magnified over the course of the reaction. Furthermore, this method does not allow comparison of mRNA levels for two different genes (e.g., IFN-γ and interleukin-4) to be made, because the efficiency of each reaction may vary significantly. Consequently, the amount of the unknown template may be over- or underestimated.

23. To account for cytokine differences caused by a varying number of T-cells in the tissue samples, T-cell-derived mRNA can be quantified by the use of T-cell receptor α-chain-specific primers. This is particularly important when the specimens in question contain variable proportions of different cell types with presumably variable amounts of the transcripts of interest.
24. It is a clear advantage to use RNA standards instead of DNA standards. In this way the efficiency of reverse transcription is controlled.
25. Agarose gels have a lower resolving power than polyacrylamide gels but have a greater range of separation. DNAs from 200 bp to approx 50 kb in length can be separated on agarose gels of various concentrations ***(19)***. We generally use SeaKem LE agarose to separate the PCR products. This is an agarose often used for routine nucleic acid electrophoresis of fragments between 100 and 23,000 bp. In addition, we have also successfully used the NuSieve GTG agarose (FMC Bioproducts) (*see* **Note 26**). We recommend 1.6% ethidium-stained SeaKem LE agarose gel made in 1X TBE buffer.
26. To obtain a better resolution (separation) of the PCR products (between standard and template) and sharper gel bands, we have successfully used the NuSieve GTG Agarose.
27. During electrophoresis, ethidium bromide migrates toward the cathode (in the direction opposite that of DNA). Extended electrophoresis can remove much of the ethidium bromide from the gel, making detection of small fragments difficult. If this occurs, restain the gel by soaking it for 30–45 min in a solution of ethidium bromide. The presence of ethidium bromide allows the gel to be examined by UV illumination at any stage during electrophoresis. However, some researchers feel that sharper bands of DNA are obtained when the gel is run in the absence of this dye. In that case, stain the gel after electrophoresis.
28. Ethidium bromide is a carcinogen and should be handled with care.
29. Primary confirmation of PCR product identity is obtained by the anticipated molecular weight. A single band of the expected size will most likely be the appropriate product. To confirm the identity of the PCR product, one method that can be used is restriction fragment mapping. The sequence of the PCR product can be examined for the presence of single sites of restriction enzyme activity, leading to the generation of digestion products of specific size that can then be resolved on an agarose gel. Other techniques include sequencing the PCR product, thereby directly confirming the identity of the band, or transferring the PCR product to a suitable membrane via Southern blotting and probing the product(s) by use of an oligo-probe.

30. The length of the primers (usually 15–30 bases) must be sufficient to overcome the statistical likelihood that their sequence would occur randomly in the overwhelmingly large number of nontarget DNA sequences present in the sample.
31. The G+C percentage of primers should be about 50%, to maximize annealing efficiency.
32. Primers should not be self-complementary, complementary to each other, or have secondary structure because this will inhibit the specific PCR reaction.
33. Whenever possible, primers used for amplifying cDNAs of RNAs should be derived from separate exons, thus spanning one or more introns. Amplification products of genomic DNA can then be easily distinguished from those of reverse-transcribed mRNA; only minute amounts of contaminating genomic DNA may give a false-positive signal in this type of assay. It is often useful to test the primers with genomic DNA as template.
34. Optimization of reactions for each primer-template pair is necessary and can be achieved by varying $MgCl_2$ concentration, primer concentration, and anneal-extend temperature. The effect of these variations can be monitored by examining the intensity and distribution of product samples electrophoresed on agarose gels. It may be necessary to lower or raise (in the range of 37–75°C) the anneal-extend temperature for the primer-template pairs. Higher anneal-extend temperatures generally result in a much more specific product ***(20)***.
35. Various DNAs differ in purity, GC content, chemical modification, secondary structure, or other variables that can inhibit PCR. Certain primer-template combinations have characteristics that cause poor PCR amplification in the "standard" buffer supplied with *Taq* DNA polymerase. The Opti-Prime PCR optimization kit (Stratagene, La Jolla, CA) provides a convenient method for determining which PCR buffers and adjuncts are most effective for a specific template and primer set. The kit consists of a matrix of 12 buffers that vary in pH and final concentrations of $MgCl_2$ and potassium. Six different adjuncts or cosolvents known to affect PCR are also included. We have used this kit successfully.
36. An example of a reaction mixture typically used to quantify IFN-γ mRNA is as follows: tube A: 2 pg/1 μg; tube B: 1 pg/1 μg; tube C: 0.5 pg/1 μg; tube D: 0.25 pg/1 μg; tube E: 0.125 pg/1 μg; tube F: 0.063 pg/1 μg; tube G: 0.031 pg/1 μg.
37. The ratio of competitive template per unknown template plotted against competitive template is a hyperbolic relationship that approaches an asymptote when one species is present in vast excess. For this reason, the most accurate results are obtained when competitive template and unknown template are amplified at nearly equivalent concentrations.
38. It is often advantageous to perform an initial titration in log increments to determine the approximate concentration of the unknown RNA. Then a finer titration can be performed to obtain the most accurate results.
39. When target and standard RNA are at equal concentrations, coamplification results in equal amounts of both products with bands of equal density. Because the concentration of the standard is known, this gives the concentration of the target (unknown) RNA (**Fig. 2A**). A point is determined where the starting num-

ber of standard RNA transcripts is equal to the starting number of target RNA transcripts. To determine this point, the ratios of the band intensities of the PCR products from the standard RNA and target RNA (i.e., ratio of standard RNA:target RNA band intensity) is plotted against the starting number of standard RNA molecules on a semilogarithmic scale (**Fig. 2B**).

40. As shown by the broken line in **Fig. 2B**, the unknown sample contains 0.25 pg of mRNA/μg of total RNA. To convert this picogram value into the number of transcripts (often a more useful figure), we apply the following equation:

$$(\text{g RNA} \times 6 \times 10^{23})/[350\ (\text{mol wt/base}) \times \text{number of nucleotides}]$$

$$0.25\ \text{pg} \times 10^{-12}\ \text{g/pg} \times 6 \times 10^{23}/350 \times 840 = 5 \times 10^{5}\ \text{transcripts/μg total RNA:}$$

The unknown RNA sample is then found to contain 5×10^5 IFN-γ transcripts/μg of total RNA (*see* **Note 41**).

41. Although we found that all transcripts examined could be detected at a level even below 10^3 transcripts/μg of total RNA, this value was chosen as a lower limit for the quantitative PCR analysis.

Acknowledgment

We thank Erik Kulø Hagen for help with the drawings. This work was supported by the Norwegian Cancer Society, the Research Council of Norway, Anders Jahre's Fund, the Norwegian Coeliac Disease Association, and the Medinnova Governmental Research Organization, Rikshospitalet.

References

1. Nilsen, E. M., Jahnsen, F. L., Lundin, K. E. A., Johansen, F.-E., Fausa, O., Sollid, L. M., Jahnsen, J., Scott, H., and Brandtzaeg, P. (1998) Gluten induces an intestinal cytokine response strongly dominated by interferon-gamma in patients with celiac disease. *Gastroenterology* **115,** 551–563.
2. Andersson, J., Abrams, J., Björk, L., Funa, K., Litton, M., and Ågren, K. (1994) Concomitant in vivo production of 19 different cytokines in human tonsils. *Immunology* **83,** 16–24.
3. Erlich, H. A., Gelfand, D., and Sninsky, J. J. (1991) Recent advances in the polymerase chain reaction. *Science* **252,** 1643–1651.
4. Wang, A. M., Doyle, M. V., and Mark, D. F. (1989) Quantitation of mRNA by the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **86,** 9717–9721.
5. Siebert, P. D. and Larrick, J. W. (1992) Competitive PCR. *Nature* **359,** 557–558.
6. Jung, H. C., Eckmann, L., Yang, S. K., Panja, A., Fierer, J., Morzycka-Wroblewska, E., and Kagnoff, M. F. (1995) A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J. Clin. Invest.* **95,** 55–65.
7. Halstensen, T. S., Scott, H., Fausa, O., and Brandtzaeg, P. (1993) Gluten stimulation of coeliac mucosa in vitro induces activation (CD25) of lamina propria CD4+ T cells and macrophages but no crypt-cell hyperplasia. *Scand. J. Immunol.* **38,** 581–590.

8. MacDonald, R. J., Swift, G. H., Przybyla, A. E., Rutters, W. J., and Chirgwin, J. M. (1979) Isolation of RNA using guanidium salts. *Methods Enzymol.* **152,** 219–234.
9. Kanangat, S., Solomon, A., and Rouse, B. T. (1992) Use of quantitative polymerase chain reaction to quantitate cytokine messenger RNA molecules. *Mol. Immunol.* **29,** 1229–1236.
10. Huang, S. K., Essayan, D. M., Krishnaswamy, G., Yi, M., Kumai, M., Su, S. N., Xiao, H. Q., Lichtenstein, L. M., and Liu, M. C. (1994) Detection of allergen- and mitogen-induced human cytokine transcripts using a competitive polymerase chain reaction. *J. Immunol. Methods* **168,** 167–181.
11. Sun, B., Wells, J., Goldmuntz, E., Silver, P., Remmers, E. F., Wilder, R. L., and Caspi, R. R. (1996) A simplified, competitive RT-PCR method for measuring rat IFN-γ mRNA expression. *J. Immunol. Methods* **195,** 139–148.
12. Neel, H., Gondran, P., Weil, D., and Dautry, F. (1995) Regulation of pre-mRNA processing by src. *Curr. Biol.* **5,** 413–422.
13. Bamford, R. N., Tagaya, Y., and Waldmann, T. A. (1997) Interleukin 15—what it does and how it is controlled. *Immunologist* **5,** 52–56.
14. Innis, M. A. and Gelfand, D. H. (1990) Optimization of PCRs, in *PCR Protocols: A Guide to Methods and Applications* (Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J., eds.), Academic, San Diego, pp. 3–12.
15. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239,** 487–491.
16. Mullis, K. B. and Faloona, F. A. (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* **155,** 335–350.
17. Li, H., Cui, X., and Arnheim, N. (1990) Direct electrophoretic detection of the allelic state of single DNA molecules in human sperm by using the polymerase chain reaction. *Proc. Natl. Acad. Sci. USA* **87,** 4580–4584.
18. Elder, P. K., French, C. L., Subramaniam, M., Schmidt, L. J., and Getz, M. J. (1988) Evidence that the functional β-actin gene is single copy in most mice and is associated with 5' sequences capable of conferring serum- and cycloheximide-dependent regulation. *Mol. Cell. Biol.* **8,** 480–485.
19. Sambrook, J., Fritsch, E. F., and Maniatis, T., eds. (1989) Gel electrophoresis of DNA, in *Molecular Cloning: A Laboratory Manual* (Ford, N., Nolan, C., and Ferguson, M., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 6.1–6.19.
20. Sambrook, J., Fritsch, E. F., and Maniatis, T., eds. (1989) In vitro amplification of DNA by the polymerase chain reaction, in *Molecular Cloning: A laboratory Manual* (Ford, N., Nolan, C., and Ferguson, M., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 14.1–14.35.

15

Immunohistochemistry in Research and Diagnosis of Celiac Disease

Per Brandtzaeg and Frode L. Jahnsen

1. Introduction

1.1. Basis of Immunostaining

Immunohistochemistry comprises methods used to recognize tissue components as antigens *in situ* by means of directly or indirectly labeled antibodies, usually (but not always) derived from another species. When applied to cell preparations, the same methods are called immunocytochemistry (**Fig. 1**), although some authors also use this term for immunostaining of cellular components in tissue sections. Note that compared with immunostaining of vital cells in suspension or cultured monolayers, the sensitivity of immunohistochemical cell-surface staining is considerably reduced because of the decreased amounts of marker antigen represented by the cross-section of the plasma membrane. Also, for certain cellular markers examined in a tissue section, truly peripheral staining may be difficult to distinguish from a rim of cytoplasmic antigen expression.

Scientists not appreciating the power of immunological probing in tissue sections, often consider it to be an inferior research tool. However, it is usually unjustified when immunohistochemistry is referred to as "merely descriptive." Admittedly, the acquisition of mechanistic information largely must rely on in vitro experiments, but tissue studies of functional cell markers are necessary to generate "maps" that depict the real situation. Regarding disease in humans, one may be led completely astray by in vitro results and animal studies if they do not comply with still pictures obtained from the ongoing process in human tissues. The informative scientific and clinical value of performing parallel studies in vitro and *in situ* cannot be overemphasized. In fact, immunohistochemi-

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

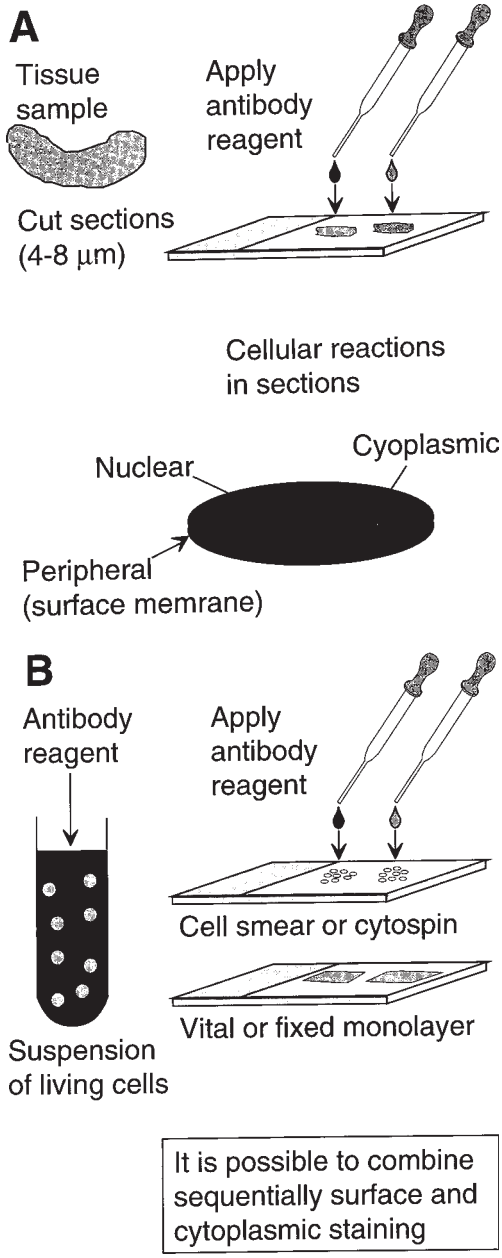


Fig. 1. Immunohistochemistry **(A)** is performed on tissue sections whereas immunocytochemistry **(B)** is performed on cells in suspensions, smears, or monolayers. Antibodies do not penetrate the surface membrane of living cells; therefore, unequivocal peripheral staining is obtained only for cells in suspension or vital monolayers. With tissue sections or dried (and fixed) cells, it is difficult to distinguish peripheral from cytoplasmic immunostaining unless the antigen is a distinct surface membrane marker.

cal information obtained in advance is often necessary to design the correct in vitro experiments. Therefore, *in situ* studies can often be considered the cornerstones of biomedical research, and the power of immunohistochemistry is steadily increasing.

The human mind seems to be the only limitation to the application of these powerful probing methods, which can be used for localization of antigens ranging from amino acids and proteins to infectious agents and subsets of leukocytes. However, despite being simple in principle, immunostaining methods can be difficult to perform and they pose many pitfalls; the results should always be interpreted with great caution.

1.2. Choice of Immunostaining Method

Since its introduction in the early 1940s, immunostaining technology has developed remarkably ***(1)***. There has been an apparently exponential rise in the number of immunohistochemical and immunocytochemical publications, but the descriptions of the applied methods are often so poor that it is difficult to evaluate the results. The availability of monoclonal antibodies (mAbs) raised by hybridoma technology is an important advancement, but this has not solved all methodological problems.

Because of their flexibility, convenience, and economic applicability, most currently used immunostaining protocols are indirect and therefore involve several incubation steps. By means of immunological, biotin-avidin, or enzymatic amplification principles (**Fig. 2**), high sensitivity can be obtained, but the various steps may also be a source of unwanted interactions resulting in spurious results. It is important in the planning that all reagent sequences be carefully designed, and flowcharts must be prepared and studied in advance to design appropriate combinations of antibodies. Immunological expertise should preferably (and hopefully) be a guarantee against the risk of introducing unwanted interspecies cross-reactivity. More important, the quality and selection of commercial antibody reagents have been steadily increasing over the last decade. Moreover, a large variety of immunofluorescence and immunoenzyme staining protocols have been proposed, as recently reviewed ***(2)***.

Both the desired immunohistochemical information and the practical methodological performance are decisive for the choice of method (**Table 1**). Thus, immunoenzyme staining is often attractive because of its applicability for light microscopy; the colored product of most enzyme substrates contrasts well with histological nuclear counterstain (usually hematoxylin), thereby facilitating simultaneous immunological and morphological evaluation. Also, potentially problematic autofluorescence is avoided by this approach, and immunoenzyme methods are more economical with regard to reagent consumption, therefore making them ideal for use in semiautomatic staining machines ***(1)***. However,

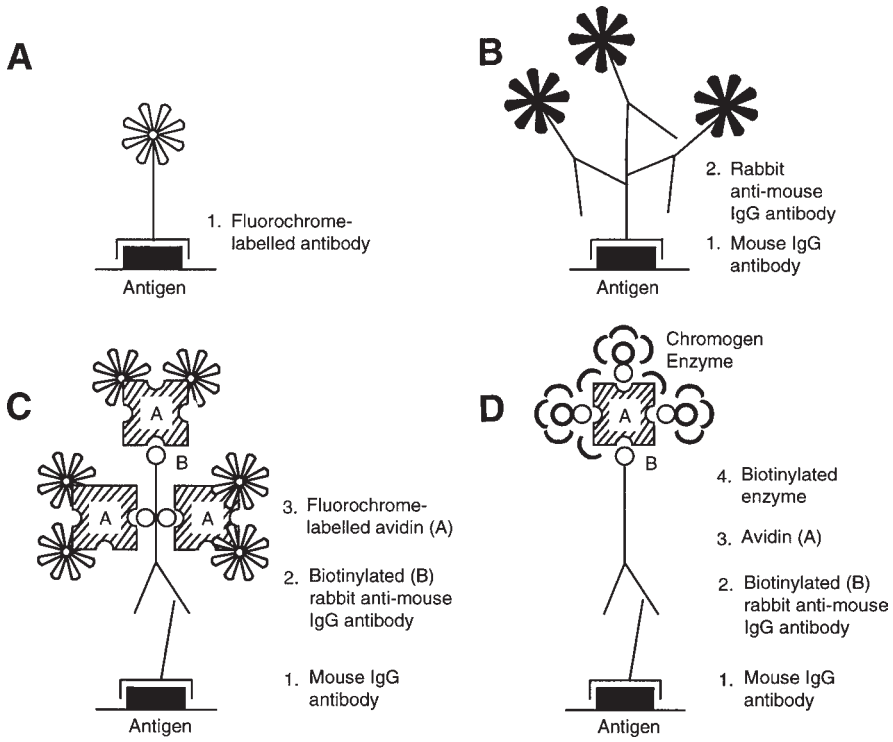


Fig. 2. Amplification principles in immunohistochemistry direct method with no amplification **(A)**; indirect or sandwich method **(B)**; biotin/avidin-enhanced method **(C)**; and biotin/avidin- and enzyme/substrate-enhanced method **(D)**. Color signal amplification provided by secondary antibody **(B–D)** or avidin-biotin **(C,D)** interactions is indicated in an arbitrary manner and will, in reality, be much more extensive, depending on epitopes available on the primary antibody and the extent of biotinylation of the secondary antibody. Also, the intensity of the color signal provided by the enzyme-driven chromogen precipitate is indicated arbitrarily and can be enhanced by variables such as enzyme and substrate concentrations, enzyme reaction time and temperature, as well as the degree of biotinylation of secondary antibody and enzyme.

immunofluorescence remains the most powerful and reliable immunohistochemical method for multicolor staining to evaluate colocalization of two or more marker antigens in an objective manner. Notably, multicolor immunoenzyme staining provides an easily obtainable and reliable result only when the markers are known *a priori* to be separately located (different cells or different cellular compartments/structures), both because of technical problems and because imbalanced color mixing is difficult to evaluate in the light microscope ***(2–5)***. Finally, the fluorescent color signal exhibits a relatively consis-

Table 1
Comparison of Immunofluorescence and Immunoenzyme Staining Methods

Immunofluorescence	Immunoenzyme
Specialized microscope	Ordinary light microscope
Reagents used at relatively high concentrations; most economic in manual applications	Reagents often extensively diluted, well suited for automated staining machines
Technically simple and often less time-consuming	Includes additional steps and often technically more complex
Possible for staining result to be disturbed by autofluorescence	Possible for staining result to be disturbed by endogenous enzymes
Easy to adapt for multicolor staining with two or three fluorochromes	Multicolor staining not always reliable when different antigens are colocalized at same site
Sometimes difficult morphological orientation	Possible for staining signals to be related to morphology
Difficult to discuss without photography or image analysis	Easy to demonstrate and discuss with colleagues
Preparations not permanent	Preparations better suited for storage

tent relationship to the actual antigen concentration in the test preparation and is hence better suited for quantitative computerized image analysis than light-microscopic observation of immunoenzyme staining ***(2,6)***.

This chapter describes some of our experience with immunohistochemical methods as applied to studies of celiac disease, including double or triple staining. Simultaneous visualization *in situ* of more than one antigen by multicolor localization is often desirable or even necessary, both for studies of leukocyte phenotypes and to explore spatial marker relationships (topography) of functional significance, e.g., the production of immunoglobulin (Ig) isotype by individual immunocytes (B-cell blasts and plasma cells), which generally show phenotypic restriction. The results obtained depend on many variables that are often interdependent, such as tissue preparation (fixation and processing), pretreatment of sections, incubation procedures and reagent specificity, as well as the general quality of the immunostaining method, including performance specificity, efficiency, sensitivity, accuracy, and precision (**Fig. 3**). It is particularly important in the planning of indirect methods that the staining sequences be carefully designed; flowcharts should be prepared in order to minimize the risk of introducing unwanted interspecies crossreactivity with endogenous Ig in

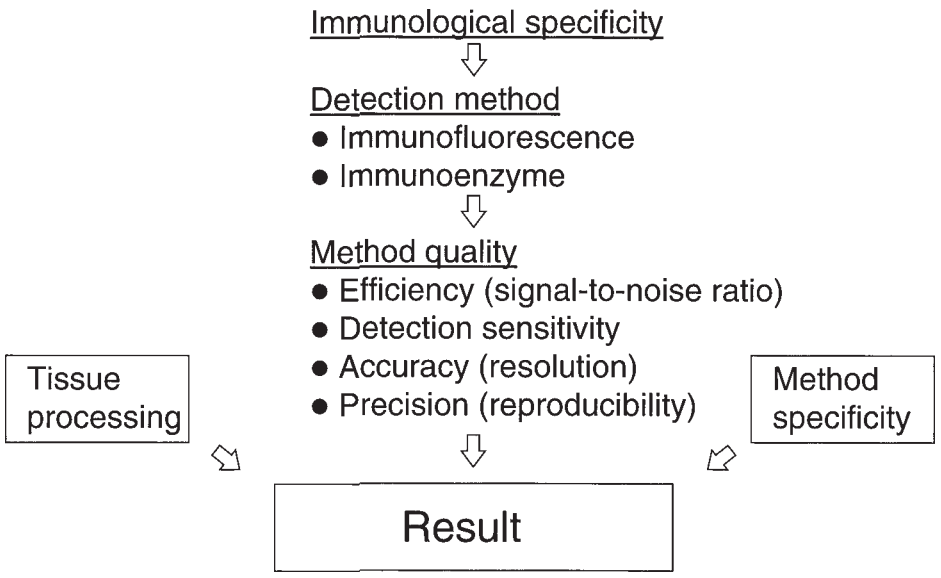


Fig. 3. Basis of immunohistochemistry. The immunohistochemical result depends on both immunological specificity and methodological variables, including the specificity of the color signal (e.g., that it depends on an immune reaction), in addition to the many variables involved in different tissue-processing techniques.

the test preparation or between the different sequences in multicolor experiments. A variety of immunostaining protocols have been published ***(2,4,5,7–9)*** and are available from several commercial companies (DAKO, Glostrup, Denmark; Jackson ImmunoResearch, West Grove, PA; Vector, Burlingame, CA). We provide herein some of the protocols used in our laboratory.

2. Materials

2.1. Preparation of Human Tissue

2.1.1. Sampling of Intestinal Mucosa

1. Obtain duodenal/jejunal specimens by using an Olympus endoscope (GIF IT20) equipped with biopsy forceps (Olympus, Melville, NY, cat. no. FB 13K).
2. Make bedside decision on how to proceed with the preparation of the tissue specimen, depending on the molecules/marker antigens to be studied by immunohistochemistry (*see* **Note 1**).
3. Prepare one or more representative specimens by routine formalin fixation and paraffin embedding for histological evaluation by hematoxylin and eosin (H&E) staining. For certain purposes (*see* **Subheading 3.5.**), routine paraffin sections may be used for immunohistochemistry after antigen retrieval (*see* **Note 2**).

2.1.2. Tissue Preparation for Detection of Sensitive Cell-Surface Markers

1. Prepare ice-chilled periodate/lysine/0.5% paraformaldehyde (PLP): A, 13.6 g of lysine (Sigma, St. Louis, MO) and 12.2 g of Trisma® base (Sigma) in 5 mL of deionized water; B, 20 g of paraformaldehyde (FERAK, Laborat GmbH, Berlin, Germany) dissolved at 60°C in 500 mL of deionized water and cleared by dropwise addition of 1 *N* NaOH (keep overnight at room temperature); C, 3.3 g of sodium *m*-periodate (Sigma) in 30 mL of deionized water. Mix 25 mL of A, 6.75 mL of B, 18.25 mL of isotonic phosphate-buffered saline (PBS), pH 7.5, and 1 mL of C to make 0.5% PLP and adjust pH to 7.5 with 1 *N* NaOH (can be stored in the dark at 4°C for 3 wk).
2. Orient the tissue sample on a thin slice of carrot, embed Tissue-Tek® O.C.T. 4583 Compound (Sakura Finetek U.S.A., Inc., Torrance, CA); Miles, Elkhart, IN) contained in a small chamber made of aluminum foil, preferably snap-freeze at bedside in liquid nitrogen, and store at –70°C.
3. Alternatively, place the tissue sample in ice-chilled tissue culture medium (RPMI 1640, Gibco, Paisley, UK) until freezing for rapid transport to the laboratory, or fix immediately in 0.5% PLP for 2–6 h before freezing.
4. Cut serial cryosections (preferably from the whole block) at 4 μm, place them on coated glass slides (*see* **Note 3**), dry overnight at room temperature, fix in acetone for 10 min, wrap in aluminum foil, and store at –20°C until use.
5. When taking the slides out of the freezer, do not remove the foil before the whole rack has reached room temperature (to avoid moisture on the sections).
6. Isolate sections for subsequent application of immunoreagent by encircling with a ring made with nail varnish or a water-repellent marker (PAP Pen; Daido Sangyo, Tokyo, Japan).

2.1.3. Tissue Preparation or Detection of Cellular Immunoglobulins and Extracellular Immune Deposits

1. Stretch the biopsy specimen immediately on a piece of filter paper (connective tissue downward) and transport it as soon as possible to the laboratory, either in ice-cold PBS or ice-cold RPMI 1640 tissue culture medium.
2. Wrap the specimen in cheesecloth under cold PBS, put it in an appropriately labeled perforated embedding cassette (Dudek Plast, Vaerloese, Denmark), and wash at 4°C with gentle agitation in a large volume (approx 1 L) of PBS for 24–48 h (*see* **Note 4**).
3. Place the cassette in 96% ethanol at 4°C overnight for fixation.
4. Thereafter, pass the cassette through two baths of absolute ethanol at 4°C for dehydration (2 h each), two baths of xylene at 4°C for clearing (1 h and overnight), then (after 30 min at room temperature in xylene) through three containers with low-melting paraffin wax at 56°C for embedding (altogether 3–4 h), and cast the paraffin block in the cassette after removing the lid and cheesecloth.
5. Cut serial paraffin sections at 4 μm and place them on clean glass slides coated with adhesive (*see* **Note 3**).

6. Dry the sections for 30 min at 37°C and store overnight at 4°C.
7. Dewax the sections in xylene at room temperature (two times for 5 min each), wash (three times for 1 min each) in graded baths of ethanol (absolute, 95 and 70%), rinse briefly in PBS and deionized water, drain, and air-dry as speedily as possible.
8. Encircle the sections by using a ring made with nail varnish or a water-repellent marker (PAP Pen) before application of the immunoreagent.

2.1.4. Tissue Preparation for Detection of Cellular Cytokines

1. Dissolve paraformaldehyde (8 g) in PBS (40 mL) at 65°C for 1 h with intervening stirring. Filter the solution through a 0.22-μm filter and add 160 mL of PBS to obtain a final concentration of 4% (w/v) paraformaldehyde (can be stored in the dark for 1 wk at 4°C).
2. Immediately orient the biopsy specimen on a thin slice of carrot, place it in a chamber of aluminum foil filled with O.C.T., snap-freeze in liquid nitrogen, and store at –70°C (*see* **Subheading 2.1.2.**).
3. Cut serial cryosections at 8 μm, place on coated glass slides (*see* **Note 3**), and let dry for 10 min in the cryostat.
4. Fix in buffered 4% paraformaldehyde for 15 min at 4°C, wash in PBS at room temperature for 5 min, and rinse briefly in deionized water.
5. Air-dry sections at room temperature for at least 2 h.
6. Apply immunostaining reagents to the sections immediately, or store them at –20°C until use. We fix sections before freezing, but it is possible to freeze cryosections directly and fix in buffered 4% paraformaldehyde before staining.

2.2. Preparation of Tissue Substrate for Detection of IgA Endomysial Antibodies (IgA-EMAs)

1. Obtain fresh or frozen esophagus from an animal stable where rhesus monkeys are sacrificed for experimental work (*see* **Note 5** and **Fig. 4**).
2. Snap-freeze transverse blocks of the tube (0.5–1 cm long), and store at –70°C until serially cut in the cryostat (*see* **Subheading 2.1.2.**).
3. Cut a whole block as one series, and place two parallel sections on each coated glass slide (*see* **Note 2**) for storage at –20°C until use.

2.3. Mounting Media for Immunostained Tissue Sections

2.3.1. Immunofluorescence Preparations

1. To prepare semisolid Tris-buffered polyvinyl alcohol (PVA) mounting medium (*see* **Note 6**), mix 5 mL of 1 *M* Tris buffer (pH 9.0) with 75 mL of deionized water.
2. Heat in a water bath until nearly boiling.
3. Add slowly 20 g of PVA (Sigma type II) while stirring.
4. Add 10 mL of glycerol and 1 mL of 1% (w/v) Thimerosal® (Sigma).
5. Aliquot in 10-mL glass tubes with screw caps and store in the dark at 4°C.
6. Before use, readjust pH weekly (1 *N* NaOH).

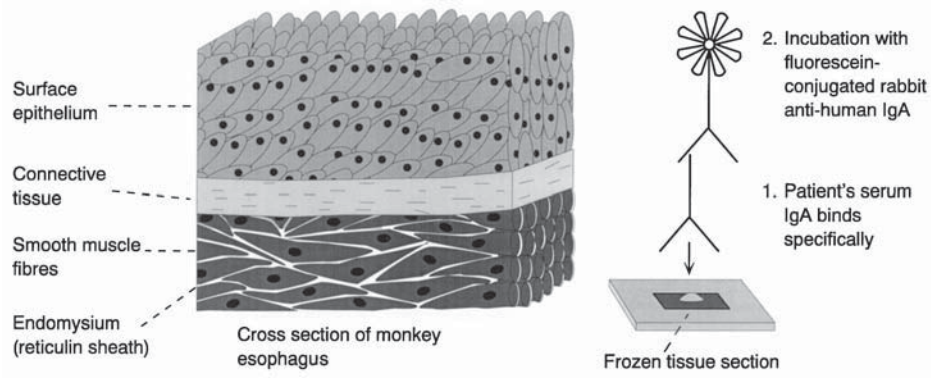


Fig. 4. Schematic depiction of indirect immunohistochemical detection of IgA antibodies to endomysium (IgA-EMA test).

2.3.2. Immunoenzyme Preparations

1. Mount preparations with color products resisting organic solvents (**Table 2**) in Eukitt® (O. Kindler GmbH, Freiburg, Germany).
2. Mount preparations with color products that dissolve in organic solutions (**Table 2**) in an aqueous mountant such as PVA.

3. Methods

3.1. Direct Two-Color Immunofluorescence Staining of Intestinal Immunocytes

1. Prepare serial paraffin sections of washed and ethanol-fixed tissue for detection of Ig-producing cells or immune deposits (*see* **Subheading 2.1.3.**)
2. Apply paired fluorochrome conjugates in various combinations (**Table 3**) of contrasting colors (*see* **Notes 7–9**), appropriately diluted in PBS containing bovine serum albumin (BSA) (1.25 g/L).
3. Incubate in a humidified chamber for 1 or 20 h at room temperature, depending on the working concentration (dilution) determined by performance testing (*see* **Note 10**). Incubate appropriate control sections in parallel (*see* **Notes 11–13**).
4. Rinse the sections for 10 min in PBS with slow agitation.
5. If enhanced morphological orientation is desired, nuclear staining may be performed at this stage by briefly rinsing the section in PBS containing the fluorescent (blue) Hoechst dye (bisbenzimide H 33254 fluorochrome, 0.05 μg/mL; Calbiochem-Behring, La Jolla, CA). This may also facilitate precise cell counting (*see* **Note 14**).
6. Rinse briefly in deionized water, drain without prolonged drying, and mount the sections in PVA (*see* **Subheading 2.3.1.**).
7. Observe in a fluorescence microscope with epi-illumination and a beam splitter (*see* **Notes 15–18**).

Table 2
Enzymes and Corresponding Chromogenic Substrates Most Commonly Used as Immunohistochemical Labels

Enzyme label	Substrate (chromogen)	Color reaction	Mountant
Horseradish peroxidase	Diaminobenzidine	Brown	Organic
	3-Amino-9-ethylcarbazole	Reddish	Aqueous
Alkaline phosphatase	Fast Blue BB salt and naphthol AS-MX	Blue	Aqueous
	Ventana Blue®	Blue	Organic
	Fast Red salt	Red	Aqueous
	New Fuchsin	Red	Organic
	Ventana Red®	Red	Organic
	Vector Red®	Red	Organic

Table 3
Heavy-Chain Specificities of Paired Fluorochrome Conjugates Used for Immunostaining of Ig-Containing Cells in Human Intestinal Mucosa[a]

Serial section	Rhodamine (red) conjugates	Fluorescein (green) conjugates
1	γ-chain (IgG)	μ-chain (IgM)
2	μ-chain (IgM)	α-chain (IgA)
3	γ-chain (IgG)	α-chain (IgA)
4	α-chain (IgA)	γ-chain (IgG)

[a]Section adjacent to no. 1 in the series is stained with H&E for morphological orientation.

8. When appropriate computerized equipment is available, capture digitized photographs, e.g., by means of a Hamamatsu C-5810 3-CCD cooled video camera (Hamamatsu Photonics KK, Hamamatsu City, Shizuoka-ken, Japan) connected to a PC employing Photoshop (Adobe Systems, WA) and Photostation (Interfoto A.S., Høvik, Norway).

3.2. Indirect Immunofluorescence Staining to Screen Human Sera for IgA-EMA

1. Prepare serial frozen sections of monkey esophagus (*see* **Subheading 2.2.**).
2. Use one slide with two parallel tissue sections for each serum sample (diluted 1/10 and 1/50), and incubate in a humidified chamber for 1 h at room temperature. Include a positive control serum in every run.

3. After washing for 3 min in PBS with slow agitation and a brief rinse in deionized water to remove salt, drain and air-dry the sections.
4. Immediately thereafter, apply a fluorescein isothiocyanate (FITC)-labeled rabbit IgG conjugate specific for human IgA (1/30; DAKO) for 30 min at room temperature.
5. Wash sections in PBS, rinse briefly in deionized water, drain, dry, and mount in PVA (*see* **Subheading 2.3.1.**).
6. Observe in a fluorescence microscope (*see* **Note 15**). Strongly positive sera give a distinct fluorescent pattern (**Fig. 4**) at both 1/10 and 1/50, whereas weakly positive sera show distinct reactivity only at 1/10.

3.3. Indirect Two- or Three-Color Immunofluorescence Staining

3.3.1. Primary Antibodies from Different Species to Show Coexpression of Interleukin-8 and von Willebrand Factor in Intestinal Microvascular Endothelial Cells (**Fig. 5**)

1. Make appropriate antibody dilutions based on performance testing according to the chosen staining method (*see* **Notes 19–22**).
2. Prepare intestinal tissue sections as for staining of cytokines (*see* **Subheading 2.1.4.**).
3. Wash the sections (three times for 3 min each) in PBS/0.1% pure saponin (SERVA, Electrophoresis GmbH, Heidelberg, Germany).
4. Incubate the sections with mAb to interleukin-8 (IL-8) (clone 2A2; purified IgG_1, 10 μg/mL; courtesy of Dr. C. Mackay, LeukoSite, Cambridge, MA) containing 0.1% saponin and 12.5 g/L of BSA for 20 h at 4°C in a humidified chamber.
5. Wash the sections (three times for 3 min each) in PBS/0.1% saponin.
6. Incubate the sections for 3 h at room temperature with rabbit antihuman von Willebrand factor (IgG fraction, 1/1400; DAKO) combined with secondary biotinylated horse antimouse IgG (5 μg/mL; Vector) containing 0.1% saponin, 125 g/mL of BSA, and 10% normal human serum (NHS) (*see* **Note 20**).
7. Wash the sections (three times for 3 min each) in PBS/0.1% saponin.
8. Incubate with FITC-labeled swine antirabbit IgG (1/50; DAKO) combined with streptavidin-Texas Red (3 μg/mL; Gibco) containing 0.1% saponin and 12.5 g/L of BSA for 30 min.
9. Wash in PBS without saponin for 5–10 min (*see* **Note 21**).
10. Rinse briefly in deionized water and mount with PVA (*see* **Subheading 2.3.1.**).
11. Observe in a fluorescence microscope with epi-illumination and a beam splitter (*see* **Notes 15–18**), and preferably capture digitized photographs (*see* **Subheading 3.1.**).

3.3.2. Primary Antibodies of Different Murine Isotypes to Detect Cell Surface Markers (**Fig. 6**)

1. Select the necessary primary mAbs for the desired purpose (*see* **Note 23** and **Table 4**).
2. Prepare cryosections (*see* **Subheading 2.1.2.**).

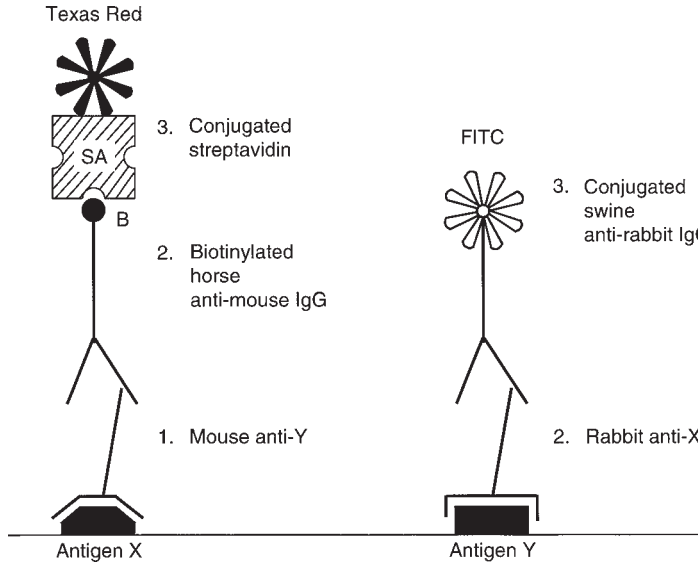


Fig. 5. Schematic illustration of two-color immunofluorescence staining combining the three-step streptavidin (SA)/biotin (B)-enhanced method with an indirect two-step method; incubation steps 2 and 3 are performed in parallel.

3. Incubate with appropriate mixtures of mAbs of differing isotype in a humidified chamber for 1 h at room temperature, followed by a 3-min rinse in PBS with slow agitation and one change.
4. After a brief rinse in deionized water to remove salts, drain the sections, and without prolonged drying, incubate with appropriate secondary reagents (**Table 5**) for 1.5 h.
5. After rinsing as in **steps 3** and **4**, incubate with tertiary conjugates (**Table 5**) for 30 min, rinse again as in **steps 3** and **4**, and mount in PVA (*see* **Subheading 2.3.1.**).
6. Obtain negative controls by replacing primary mAbs with irrelevant isotype- and concentration-matched mAbs (e.g., against keyhole limpet hemocyanin) and by totally omitting primary mAbs (*see* **Note 24**).
7. Observe in a fluorescence microscope with epi-illumination and a beam splitter (*see* **Notes 15–18**), and preferably capture digitized photographs (*see* **Subheading 3.1.**).

3.4. Immunoenzyme Staining of Cytokines in Celiac Mucosa

1. Prepare cryosections as described (*see* **Subheading 2.1.4.**) and select immunoenzyme staining method (*see* **Note 25** and **Table 2**).
2. Inhibit endogenous peroxidase with blocking reagent (*see* **Note 26**).
3. Wash the sections (three times for 3 min each) in PBS with 0.1% pure saponin (SERVA) and store at 4°C.

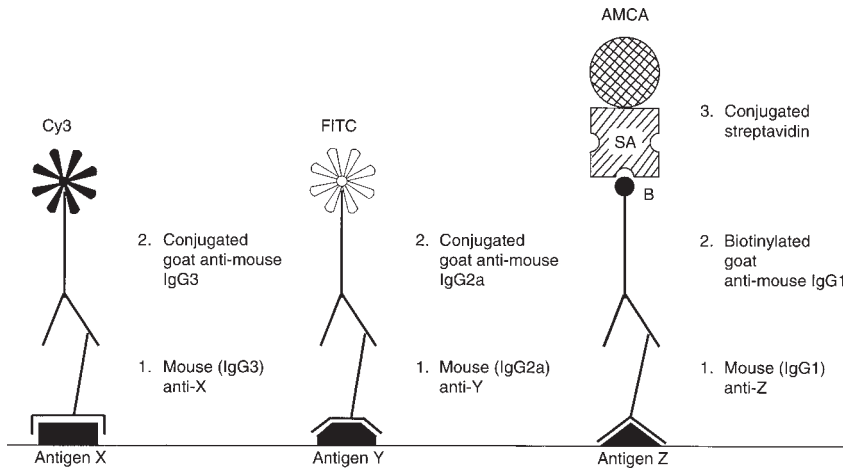


Fig. 6. Schematic illustration of three-color immunofluorescence staining based on primary murine antibodies of different IgG isotypes applied in parallel and secondary subclass-specific reagents, two of which are conjugated with fluorochromes of contrasting colors (red and green, respectively), while the third is biotinylated (B) to provide enhancement with AMCA (blue)-conjugated streptavidin (SA). It is advantageous that the secondary reagents are from the same animal species to avoid unwanted interactions between the parallel sequences.

4. Incubate the sections with appropriately diluted primary antibody (*see* **Note 20**) containing 0.1% saponin for 20 h at 4°C in humidified chambers. Do not allow sections to dry out.
5. Wash the sections (three times for 3 min each) in PBS/0.1% saponin (*see* **Note 21**).
6. Incubate the sections with secondary biotinylated antibody (*see* **Notes 20** and **22**) containing 0.1% saponin for 3 h at room temperature.
7. Wash the sections (three times for 3 min each) in Tris-buffered saline (TBS) containing 0.05 *M* Tris (TBS) and 0.1% saponin.
8. Mix nine parts of streptavidin-peroxidase conjugate (Ventana Medical Systems, Tucson, AZ) with one part of 1% saponin, and incubate the sections for 8 min at 37°C.
9. Wash in TBS without saponin for 5–10 min (*see* **Note 21**).
10. Mix one drop of 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution with one drop of H_2O_2 (Ventana detection kit) on the sections and incubate for 8 min at 37°C.
11. Wash in TBS without saponin for 5–10 min.
12. Counterstain with hematoxylin for 1 min and dehydrate to absolute ethanol.
13. Mount the sections in Eukitt.
14. Observe in a light microscope (*see* **Note 27**).

Table 4
Primary Reagents Used for Three-Color Immunofluorescence *In Situ* Staining of Leukocyte Markers and Adhesion Molecules in Human Intestinal Mucosa

Designation	Specificity	Isotype	Final working concentration (dilution or μg/mL)[a]	Source
βF1	TcRα/β	IgG_1	Purified (1/40)	T-Cell Sciences, Cambridge, MA
TcRδ1	TcRγ/δ	IgG_1	Purified (1/20)	T-Cell Sciences
δTCS1	Vδ1/Jδ1	IgG_1	Purified (1/20)	T-Cell Sciences
Anti-Leu-4	CD3	IgG_1	Purified (2.5 μg/mL)	Becton Dickinson, Mountain View, CA
BMA-180	CD3	IgG_{2a}	Purified (1/40)	Behringwerke, Marburg, Germany
RIV9	CD3	IgG_3	Supernatant (1/50)	Bioprobe, Amstelveen, The Netherlands
Anti-Leu-3a&b	CD4	IgG_1	Purified (5.0 μg/mL)	Becton Dickinson
RIV6	CD4	IgG_{2a}	Purified (1/5)	Sanbio, Am Uden, The Netherlands
Anti-Leu-2a	CD8α	IgG_1	Purified (2.5 μg/mL)	Becton Dickinson
BMA-081	CD8α	IgG_{2a}	Purified (1/40)	Behringwerke
Anti-Leu-2b	CD8α	IgG_{2a}	Purified (2.5 μg/mL)	Becton Dickinson
FP 298A3	CD15	IgM	Purified (1/20)	Biosys, Compiègne, France
HD37	CD19	IgG_1	Supernatant (1/10)	DAKO
L26	CD20	IgG_{2a}	Supernatant (1/40)	DAKO
ACT-1	CD25	IgG_1	Supernatant (1/10)	DAKO
Anti-Leu-18	CD45RA	IgG_1	Purified (1/20)	Becton Dickinson

PD/7/16	CD45RB	IgG_1	Supernatant (1/20)	DAKO
UCHL-1	CD45R0	IgG_{2a}	Supernatant (1/10)	DAKO
L243	HLA-DR	IgG_{2a}	Purified (2.5 μg/mL)	Becton Dickinson
BerAct8	αEβ7	IgG_1	Supernatant (1/50)	Dr. H. Stein, Berlin, Germany
#10704	E-cadherin	IgG_1	Purified (1/10)	Organon Teknika, Asker, Norway
BBIG-E4	E-selectin	IgG_1	Purified (10 μg/mL)	R&D Systems, Abingdon, UK
ICAM-1	ICAM-1	IgG_1	Purified (1 μg/mL)	Genzyme, Cambridge, MA
BBIG-V1	VCAM-1	IgG_1	Purified (1 μg/mL)	R&D Systems
X39	KLH[b]	IgG_1	Purified (5.0 μg/mL)	Becton Dickinson
X40	KLH[b]	IgG_{2a}	Purified (5.0 μg/mL)	Becton Dickinson

[a]Diluted in PBS containing BSA (12.5 g/L).
[b]Keyhole limpet hemocyanin.

Table 5
Secondary Antibody Reagents and Tertiary Avidin Conjugates Used Three-Color for Immunofluorescence *In Situ* Staining of Leukocyte Markers and Adhesion Molecules in Human Intestinal Mucosa

Binding specificity	Label or conjugate	Final working concentration (μg/mL)[a]	Species	Source
Mouse IgM	Biotin	20	Goat	Southern Biotechnology, Birmingham, AL
Mouse IgG_1	Biotin	10	Goat	Southern Biotechnology
Mouse IgG_1	Cy-3	2	Goat	Southern Biotechnology
Mouse IgG_1	TRITC	50	Goat	Southern Biotechnology
Mouse IgG_{2a}	Cy-3	2	Goat	Southern Biotechnology
Mouse IgG_{2a}	TRITC	50	Goat	Southern Biotechnology
Mouse IgG_{2a}	FITC	50	Goat	Southern Biotechnology
Mouse IgG_3	Cy-3	2	Goat	Southern Biotechnology
Mouse IgG_3	FITC	50	Goat	Southern Biotechnology
Biotin	Streptavidin-Texas Red	3	*Streptomyces avidinii*	Gibco-BRL, Gaithersburg, MD
Biotin	Streptavidin-Cy2	1	*S. avidinii*	Amersham, Galesbury, UK
Biotin	Streptavidin-AMCA	20	*S. avidinii*	Vector

[a]Diluted in PBS containing BSA (125 g/L) and human IgG (0.8 g/L) when necessary to avoid crossreaction with tissue elements.

3.5. Sequential Three-Color Immunoenzyme Staining of Intestinal Immunocytes

1. Cut serial paraffin sections of routinely formalin-fixed biopsy specimens at 5 μm and place on clean glass slides coated with adhesive (*see* **Note 3**).
2. Dewax the sections (*see* **Subheading 2.1.3.**).
3. Place the sections in citrate buffer (pH 6.0) and boil in a microwave oven at maximum efficiency for 3 min (*see* **Note 2**).
4. Continue boiling at 10% efficiency (power level 1) for 20 min.
5. Let the sections cool at room temperature, wash in PBS and deionized water, dry, and apply code tags according to the staining protocol (Ventana).
6. Inhibit endogenous peroxidase with blocking reagent (*see* **Note 26**).
7. Apply appropriately diluted (*see* **Note 20**) primary rabbit antibody reagents (DAKO; 37°C, 30 min) to IgA (α-chain), IgM (μ-chain), and IgG (γ-chain) sequentially (**Fig. 7**) in the Ventana Medical Systems (VMS) automated immunostaining instrument (Ventana) employing the required secondary and developing reagents as described by the manufacturer (*see* **Notes 28** and **29**).

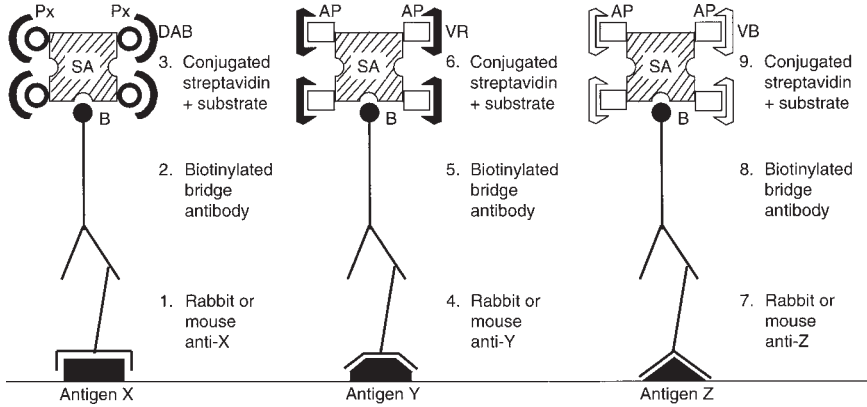


Fig. 7. Schematic depiction of nine-step sequential three-color immunoenzyme LSAB method with primary rabbit (or murine) antibody reagents. The first sequence employs streptavidin (SA) conjugated with peroxidase (Px) and developed with DAB as chromogen (brown), whereas the two next sequences are based on alkaline phosphatase (AP) conjugates developed with Ventana Red (VR) and Ventana Blue (VB), respectively. The dense DAB precipitate shields the first sequence from subsequent unwanted interactions. The second sequence is denatured by microwaving before the third primary antibody is applied. With these precautions, antibody from any animal species can be adopted as the primary reagent. B, biotinylated.

4. Notes

4.1. Tissue and Section Preparation

1. Tissue processing is one of the cornerstones in immunohistochemistry (**Fig. 3**) but remains problematic. Note that the final result significantly depends on the preparation of the tissue specimen, regardless of the quality of the applied immunostaining method. Regrettably, it is unrealistic to hope for a standard procedure that, at the same time, can immobilize all types of antigens by fixation, preserve optimally and equally their antigenic epitopes, provide optimal access of the applied antibodies, and retain satisfactorily the structural integrity of tissues and cells. For sensitive epitopes such as cellular cluster of differentiation (CD) cell-surface markers and adhesion molecules, the best results are usually obtained with cryosections. It is advantageous to collect the intestinal biopsy specimen at bedside for immediate freezing or PLP fixation. Frozen tissue blocks are neither permanent nor easily handled, especially when sections need to be cut from the same specimen on repeated occasions. Because cryostat temperatures favor formation of ice crystal artifacts and membrane damage, it is important that a sufficiently large number of cryosections are prepared in each series and stored at –20°C after drying and acetone fixation. The use of fixed and permanently

embedded tissue (e.g., paraffin blocks) offers the advantage of relatively good morphological preservation together with convenient handling and storage of the specimens. From a morphological point of view, crosslinking fixatives (e.g., formaldehyde based) are preferable, but they induce variable degrees of antigenic masking ***(10–12)***. Limited reactivity after routine fixation in 10% buffered (neutral) formalin (4% formaldehyde) and paraffin embedding is especially encountered with mAbs. However, the availability of mAbs reactive with robust epitopes increases steadily, thereby expanding the possibilities for probing on sections cut from diagnostic paraffin blocks, particularly after antigen retrieval procedures (*see* **Note 2**). Small peptides (such as cytokines) generally tolerate quite well stabilization by crosslinking with fixatives such as formaldehyde. Large glycoproteins, cytoskeletal elements, and cell membrane component constituents are usually better preserved by precipitating fixatives such as acetone or ethanol, but PLP is an alternative crosslinking fixative that is less denaturing to protein epitopes than formaldehyde.

2. Antigen retrieval by treating sections of formalin-fixed paraffin-embedded tissue with various proteolytic enzymes (pronase, trypsin, or pepsin) has been practiced for more than two decades. The striking beneficial result apparently reflects breakdown of protein crosslinking, thereby providing enhanced antibody access to relevant epitopes as well as freedom for protein antigens to assume a more reactive conformation. However, such digestion is a relatively unpredictable procedure because the degree of crosslinking depends on variables that are often uncontrolled, including the concentration of formaldehyde reaching the center of large tissue specimens, fixation temperature and pH, time of exposure to fixative, and partial reversal of methylene bridges by rinsing before paraffin embedding. Moreover, a balance must be found between proteolytic antigen retrieval and loss of sensitive epitopes as well as deterioration of the tissue structure ***(4,10,12–14)***. The same limitations afflict other methods used for antigen retrieval, such as detergents combined with denaturing agents ***(15)*** and heating by microwaving. The latter approach has become increasingly popular since it was proposed by Shi et al. ***(16)*** in 1991. Because of violent boiling with extensive evaporation of the buffer in which the sections are immersed, monitoring of the procedure and repeated bursts (usually two times for 5 min each) are generally required. Plain boiling at 120°C by autoclaving in plastic Coplin jars filled with citrate buffer (pH 6.0) for 5–10 min ***(17)***, or boiling in a pressure cooker for 2–5 min ***(18)***, has been proposed as a simpler method with larger capacity. Microwaving is usually carried out in the same buffer, sometimes combined with urea or heavy metals, but Tris-HCl or sodium acetate buffer at pH 8.0–9.0 may improve the reactivity of most antigens ***(19)***. For CD3 T-cell marker we often obtain an improved result by ordinary boiling under pressure (116°C) in this buffer. However, the choice of retrieval method should be based on performance testing; tailoring a combination of heating and short proteolytic digestion may sometimes be useful, and some epitopes seem to tolerate proteolytic enzymes better than heating ***(20)***. Nevertheless, microwaving has turned out to have a remarkably wide applicability

for antigen retrieval ***(21)***. Furthermore, prolonged incubation of primary antibodies or application at raised temperature (37–40°C) facilitates their reactivity, especially with partially denatured epitopes ***(22)***.

3. For coating of glass slides, we use gelatin dissolved in water (20 g/L) by heating and stabilized by mixing with an equal volume of potassium chromium sulfate (10 g/L) before use. The slides are briefly immersed in this solution, drained, and air-dried. Other adhesives may also be used such as 2% 3-aminopropyltriethoxysilane (A-3648, Sigma) in acetone or absolute ethanol.
4. Lymphoid cells, especially those located outside organized lymphoid structures such as in the intestinal lamina propria, are generally surrounded by very high levels of extracellular Ig components (IgG > IgA > IgM) that are retained in directly fixed tissue specimens ***(12,13,23)***. Thus, when subjected to ethanol fixation, the lamina propria of the gut shows strong background staining, which interferes with the visualization of Ig-expressing cells and immune deposits ***(12,23)***. Therefore, Ig localization poses interpretative problems not encountered with antigens occurring at only low concentrations in interstitial tissue fluid. Removal of diffusible Ig by washing the tissue specimen before ethanol fixation may therefore be required for reliable detection of various Ig isotypes expressed on or in B-cells ***(12,14,23)***. Immune deposits can also be clearly demonstrated after this tissue preparation method ***(12,24–26)***. However, the procedure will, to some extent, destroy structural details. After formaldehyde fixation, antigenic masking is extensive because of crosslinking between Ig components and bystander proteins ***(10,14)***. Consequently, Ig isotypes expressed on B-cells become unreactive. Antigen retrieval methods (*see* **Note 2**) will lead to antigenic unmasking and may thereby again increase the Ig background staining, but usually less than that of cytoplasmic Ig. Therefore, the isotype distribution pattern of intestinal immunocytes can usually be evaluated in routine paraffin sections (*see* **Subheading 3.5.**). Cellular Ig staining may also be performed in cryosections (*see* **Subheading 2.1.2.**) from which diffusible extracellular Ig, to a variable extent, has leached, thereby revealing the isotype expression on the surface membrane; however, too much background staining often remains in the lamina propria. Furthermore, leaching also affects cytoplasmic Ig, resulting in staining of immunocytes of poor quality.
5. The IgA-EMA test is currently the most specific serological test for celiac disease ***(27)***. The best substrate is cryosections of monkey esophagus, in which the endomysium is defined as the reticulin sheet covering smooth-muscle fibers of the muscularis mucosae below the epithelium. Its presence should be documented by light-microscopic examination (H&E staining) of at least the first and last section in a series, because the muscularis mucosae is often scant or totally lacking in the proximal part. Commercial monkey slides are apparently not always controlled in this critical manner. Because the monkey is an endangered species, alternative substrates for IgA-EMA determinations are being sought. Human esophagus appears to be quite satisfactory ***(28)*** but is difficult to use for ethical reasons, especially when it comes to the distribution of commercial substrate.

Human umbilical cord may be a better choice, as recommended by a task force group organized jointly by the European Medical Research Council and the European Society for Paediatric Gastroenterology and Nutrition (Maikammer, Germany, Nov. 1994), for serological screening of celiac disease. Although positive staining in our hands is detected with cryostat sections of human umbilical cord vein for most IgA-EMA-positive sera, the staining intensity is considerably weaker than in our monkey esophagus sections ***(27)***. Thus, some sera weakly positive for IgA-EMA are deemed to be negative with umbilical cord substrate, and the microscopic reading takes more time than with monkey esophagus substrate. In the future, similar screening may be based on enzyme-linked immunoassay because a major component of the IgA-EMA antigen appears to be tissue transglutaminase ***(29)***.

6. In our laboratory, PVA has always been used as a mounting medium for immunofluorescence ***(30,31)***; it is practical, requires no sealing of the cover slips, and limits deterioration in staining intensity when adjusted to pH 8.5–9.0. Fading of immunofluorescence is a continuing methodological problem, especially when FITC conjugates are used. Although various chemical compounds have been introduced for the preservation of fluorescence, they reduced the initial emission intensity and thereby the staining efficiency ***(32)***. We compared freshly prepared buffered (pH 8.7) PVA containing paraphenylenediamine (PPD), *n*-propyl gallate (NPG), and 1,4-diazobicyclo (2,2,2)-octane with PVA alone or with buffered glycerol (pH 8.7) in terms of preservation of FITC emission. At a concentration of 0.2–2.0 and 6 g/L, respectively, PPD and NPG were shown to retard effectively fluorescence fading, but they had to be rather fresh to avoid a substantial decrease in the initial emission intensity. For ordinary PVA and buffered glycerol, the relative degree of fading was comparable and most pronounced during the first 45 s, but the initial intensity was significantly lower with buffered glycerol than with PVA ***(32)***. In our opinion, PPD at a concentration of approx 1 g/L is most useful when added to the PVA mounting medium. This addition is helpful for prolonged microscopy (e.g., cell counting) at high magnifications and for photographic documentation in special instances. However, the mounted preparations must be examined within a few days, and remounting in ordinary PVA is necessary for prolonged storage of the sections ***(32)***. Even FITC-labeled preparations can be stored in PVA at 4°C in the dark for several years without any notable decrease in fluorescence intensity.

4.2. Immunofluorescence Staining Methodology

7. The IgG fraction of an antiserum, affinity-purified polyclonal IgG antibody, purified mAb, and $F(ab')_2$ or Fab fragments of IgG antibody can all be conjugated with fluorescent compounds. However, in our experience, mAb conjugates provide insufficient sensitivity to be used in immunohistochemistry. The most common fluorochromes employed for polyclonal (rabbit, goat, and so forth) conjugates have been FITC, which emits apple-green fluorescence when excited by ultraviolet (UV) or preferentially blue light, and tetramethylrhodamine isothio-

cyanate (TRITC), which emits orange fluorescence when excited by UV or preferentially green light ***(33)***. Several other fluorochromes are currently used for commercially available antibody or avidin conjugates: the carbocyanine dye Cy2®, the carboxyrorhodol dye Rhodol Green®, and difluoroboradiazaindacene (BODIPY®), which all emit green fluorescence under the same optical conditions as FITC; Lissamine® rhodamine B sulfonyl chloride (RB200SC) and its derivative Texas Red®, indocarbocyanine (Cy3®), or indodicarbocyanide (Cy5®), which all emit orange to red fluorescence ***(34)***; and Cascade Blue® and aminomethylcoumarin acetic acid (AMCA), which both emit blue fluorescence ***(35)***. A new series of highly sensitive Alexa® fluorochromes provides conjugates with various emission colors (Molecular Probes, Eugene, OR). Note that the approximate absorption maxima (λ_{max}) for all fluorochromes are generally shifted slightly (1–10 nm) to a higher wavelength after conjugation, depending on the degree of labelling ***(36)***. R-phycoerythrin, one of several light-harvesting phycobiliproteins from cyanobacteria and eukaryotic algae exploited as fluorescent labels, has a broad absorption range for uptake of energy and may provide a signal 5–10 times more intense than FITC; however, because of its large size and rapid fading, it is rarely used for immunohistochemistry ***(37)***.

8. Conjugation with fluorochromes is simple and can be performed by different procedures in a reproducible manner ***(13,36)***. We now use mainly commercial conjugates because several companies during the last two decades have specialized in the production of fluorochromes and/or their conjugation to antibodies (Amersham, Buckinghamshire, UK; Biological Detection Systems, Pittsburg, PA; DAKO; Jackson ImmunoResearch; Molecular Probes; Sigma; Southern Biotechnology, Birmingham, AL). Excess free fluorochrome is easily removed by gel filtration, and thereafter the conjugate can be characterized. The optical density (OD) ratio ($OD_{280\ nm}$:$OD_{\lambda max}$), as proposed by Cebra and Goldstein ***(38)***, may be used as a simple and generally applicable estimate of the degree of labeling, in which OD at 280 nm mainly reflects the protein (IgG) content of the conjugate. However, some producers prefer to give the reverse ratio: $OD_{\lambda max}$:$OD_{280\ nm}$. When a pure standard of free fluorochrome is available, both types of estimate can conveniently be converted to the actual molar fluorochrome-to-protein (F:P) ratio ***(36)***. The F:P ratio of a conjugate obtained after removal of free fluorochrome is merely an average estimate because considerable heterogeneity exists for most fluorescent reagents. The negative charge introduced by bound fluorochrome will reflect the degree of labeling when a conjugate is subjected to anionic-exchange chromatography ***(36,39)***. Such separation provides fractions with a relatively homogeneous F:P ratio ***(36)*** and has been adopted by most companies to remove over- as well as underlabeled protein from fluorochrome conjugates. Free fluorochromes no longer represent a problem in commercial reagents, but overlabeled conjugates cause considerable problems with unwanted staining of various tissue elements, whereas underlabeled reagents provide poor color signals. Dealers should provide such specification. It cannot be overemphasized that the best precaution against nonspecific fluorescence stain-

ing is to use fractionated and well-characterized fluorochrome conjugates at the lowest applicable working concentration ***(2,39)***. Moderately labeled IgG antibody fractions (F:P ratio of 1.5–3.0) usually cause few nonspecific problems. However, commercial conjugates are often highly labeled (F:P ratios of 3.0–8.0) to produce strong color signals; although such reagents generally perform well when applied on living cells (e.g., in flow cytometry), they are quite likely to cause nonspecific staining in tissue sections. Indeed, it is sometimes difficult to obtain a strong and specific immunohistochemical signal because the conjugate does not work at a sufficiently low concentration; this problem may be circumvented by affinity purification of the antibody or by a new round of hyperimmunization to produce a reagent of higher affinity.

9. By combining two primary conjugates, either in direct immunofluorescence (or direct immunoenzyme staining), interaction between the reagents is usually no problem. The conjugates can therefore be applied simultaneously, which is clearly the simplest approach for paired staining when the antigen is present in relatively large amounts such as cytoplasmic Ig in plasma cells ***(23)***. We have used this immunofluorescence staining method extensively to map mucosal Ig production in celiac disease ***(40–44)*** in prewashed tissue specimens (*see* **Subheading 2.1.3.**). If one or both of the conjugates have been absorbed with soluble antigens, excess of this absorbent can exert unwanted blocking effects; the immunostaining then has to be performed by sequential incubations with intermediate rinsing. Conjugates from different species may also be mixed provided interspecies interactions are avoided by appropriate absorption. With mAbs such direct immunohistochemical staining is usually not feasible because of faint color signals ***(45)***.
10. An essential feature of an immunohistochemical conjugate is its actual working concentration as determined by performance testing on appropriate tissue substrate and with a chosen incubation time. A higher dilution (>10X) can usually be applied by prolonging the incubation overnight ***(22)*** or raising the incubation temperature to 37°C (*see* **Note 29**). The concentration of labeled protein can be satisfactorily estimated by OD with $E_{280\ nm,\ 1\ cm} = 14$ for IgG or $F(ab')_2$ when a correction factor is introduced, depending on the fluorochrome ***(36,38)***. Information about both the degree of labeling and the protein concentration must be available when the optimal working conditions of a conjugate are determined, with the investigator taking both its specific and nonspecific staining properties into consideration ***(13,39)***. The immunohistochemical results obtained cannot be appropriately evaluated without knowledge about such characteristics of the conjugate ***(2)***.
11. The antibody specificity of immunostaining can only be ensured by performance studies with known antigens present in a relevant preparation. For immunohistochemical applications, the antibody reagents must be tested on tissue sections, even when the specificity has been established by other methods such as radioimmunoassay (RIA). A specific RIA relies merely on the competitive interaction between radiolabeled and unlabeled purified antigen, whereas a specific immunohistochemical signal depends on restricted binding of antibody to the correspond-

ing tissue antigen and may be severely jeopardized by unknown antibody-antigen interactions ***(46)***. Flow cytometric analysis of cell surface antigens is likewise insufficient for immunohistochemical specificity testing because the sectioning may disclose previously undetected crossreacting antigens ***(47)***. Therefore, even the application of presumably monospecific primary mAbs to tissue sections can produce quite surprising results ***(48)***.

12. Immunohistochemical performance testing over a wide range of antibody dilutions on both positive and negative tissue preparations is necessary to ensure that unwanted antibodies or crossreacting specificities are absent from the primary reagent. This applies equally to immunofluorescence and immunoenzyme methods. Although vendors generally indicate appropriate working concentrations of commercial reagents, serial dilutions should be made for every new batch to establish its actual immunohistochemical performance. The absorption of polyclonal reagents with defined purified antigen (not the immunogen itself) is an additional desirable control; however, it does not necessarily establish immunological specificity and often cannot be performed owing to lack of appropriate antigen ***(4)***. Moreover, this control is of less value in terms of absolute antigen specificity of mAbs, which, by definition, react only with a single epitope. Therefore, it is not possible to remove crossreactivity of mAbs without neutralizing all antibody activity, and isotype- as well as concentration-matched control mAbs should be included in every staining experiment.
13. When mucous membranes are studied by immunofluorescence, several tissue elements, such as eosinophils, squamous epithelium, goblet cells, and columnar epithelium, are prone to show nonspecific staining, usually in that order ***(13,39)***. It may be quite difficult to distinguish between specifically fluorescent lymphoid cells and nonspecific granulocyte staining. For example, eosinophils can interfere with the detection of Ig-producing plasma cells, particularly when highly labeled FITC conjugates are used ***(49)***. There are several methods to block or identify such nonspecific staining. For example, the tissue sections can be pretreated with benzidine reagents and H_2O_2 ***(50)*** or Lendrum's phenol chromotrope 2-R stain ***(51)***, and staining with FITC-labeled BSA, which selectively binds to the eosinophils, can be paired with a specific rhodamine conjugate ***(50)***.
14. In addition to nuclear counterstain, enhanced localization of cellular immunofluorescence in relation to the mucosal epithelium or basement membrane can be obtained by the inclusion of antibody to cytokeratin, laminin, β4 integrin, or various types of collagen in two- or three-color staining ***(52–55)***.

4.3. Fluorescence Microscopy

15. Modern fluorescence microscopes employ incident excitation light (epi-illumination) provided by a Ploem-type vertical beam splitter that contains several interchangeable dichroic mirrors ***(56)***. The combined excitation and barrier filter sets are constructed according to the Stokes shifts of the individual fluorochromes and are mounted in removable holders (Omega® Optical, Brattleboro, VT). Three and preferably more filter holders are placed in sliders or turrets, which allows

rapid switching of the optical conditions; these can be selected with bandwidths that are more or less selective for the isolation of green, red, or blue emission colors. Thus, by changing the filter sets, each fluorescent signal from a morphological element can be observed one after the other. Simultaneous photographic recording of double or triple immunofluorescence staining (also called fluorescent overlay antigen mapping [FOAM]) can be achieved by blocking the film transport ***(23)***. However, instead of such rather cumbersome multicolor documentation with inherent problematic balancing of the exposure times, photographic recording of multicolor staining can now more conveniently be obtained either as digitized images with a videocamera and an attached computerized photounit ***(1)***, with a complete image analysis system ***(2,52,53)***, or with confocal laser microscopy ***(57)***.

16. When FOAM is based on a Ploem-type epi-illuminator, several technical variables may disturb the obtained images. These errors include problems with image shift differences (geometrical errors) and lack of color fidelity. The main reason for the latter is "bleed-through" of contrasting exmission colors or autofluorescence, often caused by inappropriate filter set combinations or photographic overexposure. To avoid geometrical errors, it is crucial that the images be completely overlapping, and the emission colors should ideally reflect the true distribution of different marker antigens; these problems concern both photographic recording of multicolor staining and visual inspection by rapid filter switching. When three markers are analyzed, the results are usually best recorded as digitized images ***(1,2,53)***. Multicolor immunofluorescence staining can often be satisfactorily depicted by parallel black-and-white illustrations of the separated fluorescent signals ***(1)***, which is an advantage compared to immunoenzyme staining that requires expensive color illustrations for convincing documentation.
17. Simultaneous visualization of different fluorescent colors by double- and triple-band filter sets (Omega® Optical) has recently made direct microscopic inspection of FOAM feasible. In theory, this outstanding advancement of practical immunofluorescence microscopy should eliminate the need for photographic double or triple exposures and thereby the problem with image shifting ***(58)***. However, inherent limitations of the interference coatings on the filters decrease, to some extent, the possibilities of producing satisfactory images within the visible spectrum. Therefore, the multiband filter sets provide lower emission intensities than the ideal single color sets, and hence faint or imbalanced color mixing may be difficult to recognize. In fact, the single color dichroic filter sets remain the "gold standard" for verification of antigen colocalization and for ensuring lack of unwanted bleed-through of contrasting emission signals. Also, photographic documentation of FOAM may require fine tuning of individual images by adapting the exposure times one after the other.
18. In practice, counting of fluorescent cells and photographic double or triple exposures can usually be performed satisfactorily after mounting in ordinary buffered PVA, provided that adequate filter and microscopy conditions are at hand ***(40–44,52,53)***. Instead of FITC conjugates for green signals, it is advantageous

to use reagents labeled with Cy2, Rhodol Green, or BODIPY when possible because these fluorochromes show relatively good photostability and rather low pH sensitivity in the physiological range ***(59)***. Rhodamine fluorochromes, which in contrast to FITC do not exhibit strongly pH-dependent characteristics, likewise show relatively little fading, and preparations that are single stained with such conjugates can in fact tolerate ordinary organic mounting media. Also, one important advantage during fluorescence photography is that 100% of the emission light reaches the film.

4.4. Indirect Multicolor Immunofluorescence Methods

19. Simultaneous visualization of two or more marker antigens can be achieved by a variety of combinations of the different immunohistochemical methods and their modifications. Whether the immunoreagents are applied sequentially, in parallel, or partly simultaneously, avoidance of unwanted interactions between the different staining sequences is imperative. Several approaches may be successful to this end, but many pitfalls exist ***(2,5)***, particularly when it comes to detecting three or more rather than two antigens in the same tissue section. The technical problems are mainly related to the availability of relevant primary antibodies from only one animal species, which often turns out to be the case. By applying primary antibodies from different animal species, two indirect staining sequences can be conveniently combined based on immunofluorescence (**Fig. 5**) or immunoenzyme methods. This principle can be extended to three antigens employing FITC (green), TRITC or Cy3 (red), and AMCA (blue) as labels ***(1,60)***, but the availability of primary antibodies from different species limits its applicability. We often use this approach for two- or three-color indirect immunofluorescence staining by combining a rabbit antiserum with murine mAbs ***(52,53,61)***. Note, however, that despite the use of primary antibodies from different animal species, troublesome interspecies reactions may occur in multicolor staining protocols ***(60)***. Interactions between the two staining sequences can be avoided by appropriate absorptions with IgG or normal serum from the species that shows unwanted binding (*see* **Note 20**), or preferably by using secondary antibodies from the same species.
20. With indirect immunostaining methods, the primary reagents are usually extensively diluted. We use PBS containing 0.1% (w/v) sodium azide (FERAK) stored at 4°C, and with the addition of 12.5 g/L of BSA for primary antibodies and 125 g/mL of BSA for secondary antibody reagents (e.g., biotinylated horse antimouse IgG, 5 μg/mL; Vector). Nonspecific staining is most often caused by bridging antibodies ***(62)*** or immune complexes ***(63,64)***. Pretreatment with normal animal serum (for which there must be no wanted antibody activity in the staining sequence), or dilution of the reagents with defatted NHS or another protein-rich solution (e.g., BSA), may reduce the nonspecific interactions but does not always abolish them. Progressive dilution of the primary reagent until the color signal is omitted is therefore an important preliminary control for method specificity, as in all types of indirect immunostaining. Precautions must espe-

cially be taken to avoid crossreactions of secondary antibodies with endogenous Ig present in the tissue section. We have, e.g., observed that a commercial horse antimouse Ig reagent that was claimed to show <1% activity with human Ig by RIA, nevertheless crossreacted sufficiently to visualize distinctly Ig-producing plasma cells when applied to human tissue sections. When appropriate, we therefore add 8 g/L of human IgG or 10% NHS to secondary reagents. Omission of the primary antibodies is imperative to control for unwanted interactions in indirect staining methods. Also, always include parallel incubation with isotype-matched irrelevant primary mAb at the same working concentration as the relevant one.

21. To identify cytokine- or chemokine-producing cells in biopsy specimens from patients with celiac disease, we use the fixation and permeabilization technique established for cytokines by Andersson et al. ***(65)***. Chemokines are a relatively new family of small secretory molecules (chemoattractant cytokines) that have important functions in the immune system. It has recently been shown that the Andersson method is useful also for detecting these signaling molecules, such as IL-8 ***(61,66)***. The protocol is based on the assumption that small intracellular secretory peptides must be stabilized with crosslinking fixatives to remain in place during the staining procedure. After fixation, tissue and cells must be permeabilized to allow antibodies to penetrate from the section surface all the way through intracellular membranes. By including saponin in all antibody incubations, this detergent permeabilizes membranes in a reversible way; but after the application of all immunostaining reagents, it is important to "close" the substrate to retain the labeled precipitates. In our experience, it is not necessary to block for endogenous biotin activity when streptavidin is used (*see* **Note 22**). However, if there are problems with background staining, appropriate blocking buffers should be tested. To control for immunological specificity, two independent mAbs (different clones) should preferably be included for each cytokine or chemokine and show similar staining results ***(67)***. In addition, preincubation with an excess of the relevant cytokine or chemokine should block the reactivity to exclude unwanted (nonimmunological) staining ***(67)***.
22. Avidin is a 68-kDa basic glycoprotein from egg white that has an extraordinarily high affinity ($Ka > 10^{15}\ M^{-1}$) for the small coenzyme biotin (part of the vitamin B complex), and this property is exploited extensively in immunohistochemistry ***(68)***. One molecule of avidin will bind four molecules of biotin by a noncovalent interaction that is essentially irreversible because it is at least 10^6 times stronger than most antibody-antigen reactions. Both avidin and biotin can be conjugated with fluorochromes ***(69)*** or enzymes ***(68)*** without interfering with the binding affinities. The use of streptavidin may reduce spurious staining produced by avidin-binding sites present in some tissues ***(70)***. This homotetramer from *S. avidinii* shows an affinity for biotin that is several orders of magnitude lower than that of egg white avidin, but its fairly neutral isoelectric point and unglycosylated state favor immunohistochemical performance.
23. By combining primary antibodies of different classes (e.g., murine IgG and IgM), light chains (murine κ- and λ-chains), or subclasses (murine IgG_1, IgG_{2a}, IgG_{2b},

and IgG_3) from the same species (**Table 4**), multicolor staining can be performed provided truly isotype-specific polyclonal secondary antibodies are available ***(3,53)***. We have used this approach extensively for multicolor immunofluorescence staining of leukocyte markers in healthy and diseased intestinal mucosa ***(52,53,71-80)***. Because the number of unique isotype epitopes on the primary mAb may be extremely small, an amplifying avidin-biotin sequence sometimes has to be introduced to obtain a sufficiently strong color signal (**Table 5**, **Fig. 6**). For appropriate selection of mAb combinations, several clones must be subjected to immunohistochemical performance testing. Other limitations of this approach are that the appropriate secondary antibodies are relatively expensive and that they occasionally show unwanted crossreactions. This problem can best be avoided when the secondaries are derived from the same animal species. In addition, most murine mAbs are of the IgG_1 subclass, and murine IgM often performs poorly in immunohistochemistry. For three-color staining, it may therefore be practical to combine two murine mAbs with, e.g., one primary rabbit antibody ***(53)***.

24. Multicolor immunofluorescence provides inherent controls by displaying different staining patterns depending on the primary mAb, but the staining efficiency may be reduced because certain tissue elements emit primary fluorescence, mainly blue or blue-green. Such autofluorescence generally shows a broad emission spectrum combined with a short Stokes shift. Thus, it can often be distinguished from fluorochrome emission because of bleed-through with several filter sets and can be reduced if fluorochromes and filter sets are adapted for a large Stokes shift. Crosslinking fixatives, particularly glutaraldehyde, tend to increase the bluish autofluorescence induced by UV excitation of proteins in general, and this phenomenon often worsens during storage of the tissue preparations. AMCA-stained sections should therefore be studied and recorded as soon as possible. Especially organized collagen and elastic fibers may produce very strong autofluorescence. In addition, eosinophils, hepatocytes, carotenoids, vitamin A, and lipofuscin are autofluorescent. Lipofuscin granules with strong yellow emission are common in hepatocytes, glandular ducts, and certain macrophages. Autofluorescence may be exploited for morphological orientation, and the primary fluorescence of certain counterstains has also been used to this end. However, the latter approach is not recommended because immunoreactivity may be masked ***(4)***, but nuclear staining may be useful (*see* **Note 14**).

4.5. Immunoenzyme Staining Methodology

25. Various enzymes and their chromogens can be used as immunohistochemical labels (**Table 2**). Horseradish peroxidase is most commonly applied together with its substrate DAB, which polymerizes on oxidation with H_2O_2. The color precipitate is brown, which contrasts well with nuclear hematoxylin staining ***(81)***. If necessary, the DAB reaction may be further enhanced by a variety of agents such as osmium ***(82)***, $CuSO_4$, $CoCl_2$, $NiCl_2$ ***(22)***, imidazole ***(83)***, thioglycolic acid-silver nitrate ***(84)***, or ferric ferricyanide ***(85,86)***. Such enhancers are also com-

mercially available (Vector). Several alternatives to DAB (which is potentially carcinogenic) as a peroxidase substrate have been developed, such as 3-amino-9-ethylcarbazole (AEC) ***(87)***. Tetramethyl benzidine has also been found to be an extremely sensitive chromogene, but crystallization on the tissue section may be a problem ***(88)***. Benzidine dihydrochloride produces a blue color that contrasts well with the brown DAB reaction product ***(89)***. An alternative chromogene with a blue-black permanent end product is *p*-phenylenediamine-HCl in combination with pyrocatechol ***(90)***. Also 4-chloro-1-naphthol (CN), which generates a similar color, may be useful for paired immunoenzyme staining ***(91)***. Alkaline phosphatase (AP) has been increasingly used in immunohistochemistry, particularly after the introduction of paired immunoenzyme staining techniques ***(92)***. This label was originally visualized with a substrate of Fast Blue BB salt and naphthol AS-MX, giving a blue reaction product. We often use, instead, Fast Red salt and particularly the alternative substrates New Fuchsin ***(7)***, Vector Red (Vector) or Ventana Red (Ventana, Tucson, AZ), which provide excellent contrast with hematoxylin counterstain.

26. Endogenous peroxidase is formalin resistant and can therefore interfere with specific immunostaining even in routine paraffin sections. Disturbing enzymatic activity may be blocked by methanol-H_2O_2 ***(93)***, phenylhydrazine ***(94)***, or periodate oxidation ***(95)***; but the possibility of destroying antigenic epitopes by this pretreatment must always be considered ***(96)***. We use an efficient commercial blocking kit (Ventana). Nevertheless, in cryosections disturbing endogenous peroxidase reactivity (e.g., in eosinophils) often remains after blocking, and alternative enzymes may then be preferred as label. Endogenous AP staining is generally abolished by routine formalin fixation and paraffin embedding, but isoenzyme activity often occurs in frozen or ethanol-fixed preparations of various tissues. Several blocking agents can be used, such as levamisole ***(97)***, acetic acid ***(98)***, or periodate oxidation ***(99)***, because they have little effect on AP from calf intestine (or from *Escherichia coli*), which is most frequently used as label. Notably, human intestinal AP is not blocked by levamisole; although the other agents we have mentioned may be useful ***(100)***, we often prefer peroxidase staining or immunofluorescence on sections of gut mucosa, particularly for intraepithelial immunostaining.
27. The staining pattern is dependent on the intracellular distribution of the actual cytokine of interest. For example, interferon-γ (IFN-γ) positive cells show a characteristic local paranuclear expression pattern, reflecting that this cytokine is processed and accumulates within the endoplasmic reticulum and the Golgi organelles. The most critical step in this method is the selection of suitable cytokine-specific antibodies. Andersson et al. ***(65)*** have shown that only some mAbs work in this method, and they have made great efforts in identifying suitable reagents to detect a range of cytokines *in situ*. The reader should therefore be aware that successful immunostaining of cytokines with this technique is critically dependent on the application of appropriate mAbs. We have used the following reagents: mAb to human IFN-γ (clone 7B6; purified IgG, 1.5 μg/mL;

Mabtech AB, Nacka, Sweden); and mAb to human IL-4 (clone 1-41-1; purified IgG, 1.5 μg/mL; courtesy of Dr. F. Kalthoff, Sandoz Forschungsinstitut, Vienna, Austria). Another problem suggested in the literature is that the secretion of cytokines from certain cells such as T-lymphocytes may be so rapid in vivo that cytoplasmic immunostaining is difficult to obtain *in situ* ***(101)***.

28. With sequential immunoenzyme methods, primary and secondary antibodies from the first sequence can be eluted from the tissue section without removing the colored end product; unwanted interactions may thereby be avoided when the next sequence is applied. Such stepwise staining was introduced by Nakane ***(91)***, when he reported simultaneous visualization of three antigens by consecutive immunoperoxidase sequences with different peroxidase substrates (DAB, alpha-naphthol purpure, and CN); the section was incubated at low pH between each sequence. Many methods have been reported for similar elution, but in practice they are cumbersome and unreliable. An alternative approach is to denature the antibodies applied in the first sequence. Thus, heating tissue sections twice for 5 min in a microwave oven was found to denature efficiently antibody reactivity as well as antigenicity and enzyme activity of immunoenzyme staining sequences ***(102)***. By employing intervening microwaving in sequential alkaline phosphatase-antialkaline phosphatase (blue) and peroxidase antiperoxidase staining, the latter developed stepwise with different chromogens (AEC, red; DAB, brown; and DAB plus 1% nickel ammonium sulfate, black), the authors were able to decorate simultaneously three or four antigens located at separate sites (cell membrane, cytoplasm, nucleus, and basement membrane). However, in cryosections the microwaving denatured not only the applied reagents but also cell-surface antigens that had not been decorated by the first staining sequence ***(102)***.
29. Simplified and enhanced immunoenzyme staining protocols are based on the labeled streptavidin-biotin (LSAB) method including application of biotinylated bridge antibody and streptavidin conjugated with peroxidase (*see* **Subheading 3.4.**) or AP. Such conjugates are available commercially (DAKO; Vector; Ventana) and have potentially more available binding sites for the bridge antibody than provided in the avidin-biotin complex method. Similar conjugates of modified avidin can also be purchased (ExtrAvidin®, Sigma). We have applied successfully a sequential three-color LSAB (Ventana) method with intervening microwaving after the second sequence (*see* **Note 28** and **Fig. 7**) for distinct visualization of three marker antigens expressed by separate cells or different cellular structures ***(1,2)***. Because of the numerous steps involved, this method is best performed in an automated immunoenzyme staining instrument such as the VMS (Ventana). In the VMS instrument, all reagents are applied by a computerized program at elevated temperature (37°C) with continuous vibration ("shake and bake" principle), thereby allowing high dilutions and short incubation times (4–32 min for primary and ≤12 min for secondary reagents, the latter standardized according to the actual protocol). Similar two- or three-color immunoenzyme staining can be performed manually, but this would be extremely time-consuming. Polyclonal primary reagents or murine mAbs (disregarding their isotype) are

used in the first step of each sequence, or a combination of polyclonal or monoclonal antibodies. Note that in addition to intervening microwaving, blocking of the avidin-binding activity (*see* **Note 22**) of every preceding biotin-based step may be necessary to avoid disturbing color mixing. The most reactive marker should preferably be stained first with peroxidase-DAB because an extensively developed brown product shields all reagents of the first sequence (**Fig. 7**). DAB shielding can be further enhanced if necessary by heavy metal salts (*see* **Note 25**). Notably, reliable colocalization of two marker antigens at the same site cannot be achieved by this multicolor immunoenzyme staining method ***(2,5,103,104)***.

Acknowledgments

Hege E. Bryne is thanked for excellent secretarial assistance and Erik Kulø Hagen for the drawings. Studies in the authors' laboratory have been supported by the Norwegian Cancer Society, the Research Council of Norway, and Anders Jahres Fund.

References

1. Brandtzaeg, P. (1998) The increasing power of immunohistochemistry and immunocytochemistry. *J. Immunol. Methods* **216,** 49–67.
2. Brandtzaeg, P., Halstensen, T. S., Huitfeldt, H., and Valnes, K. (1997) Immunofluorescence and immunoenzyme histochemistry, in *Immunochemistry 2—A Practical Approach* (Johnstone, A. P. and Turner, M. W., eds.), Oxford University Press, Oxford, UK, pp. 71–130.
3. Mason, D. Y., Abdulaziz, Z., Falini, B., and Stein, H. (1983) Double immunoenzymatic labelling, in *Immunocytochemistry: Practical Applications in Pathology and Biology* (Polak, J. M. and van Noorden, S., eds.), Wright & Sons, Boston, pp. 113–128.
4. Larsson, L.-I., ed. (1988) *Immunocytochemistry: Theory and Practice*. CRC Press, Boca Raton, FL.
5. van der Loos, C. M., Becker, A. E., and van den Oord, J. J. (1993) Practical suggestions for successful immunoenzyme double-staining experiments. *Histochem. J.* **25,** 1–13.
6. Brandtzaeg, P. (1972) Evaluation of immunofluorescence with artificial sections of selected antigenicity. *Immunology* **22,** 177–183.
7. Mason, D. Y. (1985) Immunocytochemical labelling of monoclonal antibodies by the APAAP immunoalkaline phosphatase technique, in *Techniques in Immunocytochemistry*, vol. 3. (Bulloch, G. R. and Petrusz, P., eds.), Academic, London, pp. 25–42.
8. Plenat, F., Martinet, Y., Martinet, N., and Vignaud, J. M. (1994) Immunohistochemical methods for studying mononuclear phagocytes in tissue sections. *J. Immunol. Methods* **174,** 133–154.
9. Falini, B., Abdulaziz, Z., Gerdes, J., Canino, S., Ciani, C., Cordell, J. L., Knight, P. M., Stein, H., Grignani, F., Martelli, M. F., and Mason, D. Y. (1986)

Description of a sequential staining procedure for double immunoenzymatic staining of pairs of antigens using monoclonal antibodies. *J. Immunol. Methods* **93,** 265–273.

10. Brandtzaeg, P. and Rognum, T. O. (1984) Evaluation of nine different fixatives. 1. Preservation of immunoglobulin isotypes, J chain, and secretory component in human tissues. *Pathol. Res. Pract.* **179,** 250–266.
11. Brandtzaeg, P. and Rognum, T. O. (1984) Evaluation of nine different fixatives. 2. Preservation of IgG, IgA and secretory component in an artificial immunohistochemical test substrate. *Histochemistry* **81,** 213–219.
12. Brandtzaeg, P. (1982) Tissue preparation methods for immunohistochemistry, in *Techniques in Immunocytochemistry*, vol. 1 (Bullock, G. R. and Petrusz, P., eds.), Academic, London, pp. 1–75.
13. Brandtzaeg, P. (1982) Immunofluorescence studies of mucous membranes and exocrine glands, in *Immunofluorescence Technology: Selected Theoretical and Clinical Aspects* (Wick, G., Traill, K. N., and Schauenstein, K., eds.), Elsevier Biomedical, Amsterdam, pp. 167–217.
14. Brandtzaeg, P. and Rognum, T. O. (1983) Evaluation of tissue preparation methods and paired immunofluorescence staining for immunocytochemistry of lymphomas. *Histochem. J.* **15,** 655–689.
15. Peränen, J., Rikkonen, M., and Kääriäinen, L. (1993) A method for exposing hidden antigenic sites in paraformaldehyde-fixed cultured cells, applied to initially unreactive antibodies. *J. Histochem. Cytochem.* **41,** 447–454.
16. Shi, S.-R., Key, M. E., and Kalra, K. L. (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* **39,** 741–748.
17. Bankfalvi, A., Navabi, H., Bier, B., Böcker, W., Jasani, B., and Schmid, K. W. (1994) Wet autoclave pretreatment for antigen retrieval in diagnostic immunohistochemistry. *J. Pathol.* **174,** 223–228.
18. Piffko, J., Bankfalvi, A., Öfner, D., Joos, U., Böcker, W., and Schmid, K. W. (1995) Immunohistochemical detection of p53 protein in archival tissues from squamous cell carcinomas of the oral cavity using wet autoclave antigen retrieval. *J. Pathol.* **176,** 69–75.
19. Shi, S., Imam, S. A., Young, L., Cote, R. J., and Taylor, C. R. (1995) Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. *J. Histochem. Cytochem.* **43,** 193–201.
20. Cattoretti, G., Pileri, S., Parravicini, C., Becker, M. H. G., Poggi, S., Bifulco, C., et al. (1993) Antigen unmasking on formalin-fixed, paraffin-embedded tissue sections. *J. Pathol.* **171,** 83–98.
21. Brown, R. W. and Chirala, R. (1995) Utility of microwave-citrate antigen retrieval in diagnostic immunohistochemistry. *Mod. Pathol.* **8,** 515–520.
22. Brandtzaeg, P. (1981) Prolonged incubation time in immunohistochemistry: effects on fluorescence staining of immunoglobulins and epithelial components in ethanol- and formaldehyde-fixed paraffin-embedded tissues. *J. Histochem. Cytochem.* **29,** 1302–1315.

23. Brandtzaeg, P. (1974) Mucosal and glandular distribution of immunoglobulin components: immunohistochemistry with a cold ethanol-fixation technique. *Immunology* **26,** 1101–1114.
24. Halstensen, T. S., Mollnes, T. E., and Brandtzaeg, P. (1989) Persistent complement activation in submucosal blood vessels of active inflammatory bowel disease: immunohistochemical evidence. *Gastroenterology* **97,** 10–19.
25. Halstensen, T. S., Mollnes, T. E., Garred, P., Fausa, O., and Brandtzaeg, P. (1990) Epithelial deposition of immunoglobulin G1 and activated complement (C3b and terminal complement complex) in ulcerative colitis. *Gastroenterology* **98,** 1264–1271.
26. Halstensen, T. S., Hvatum, M., Scott, H., Fausa, O., and Brandtzaeg, P. (1992) Association of subepithelial deposition of activated complement and immunoglobulin G and M response to gluten in celiac disease. *Gastroenterology* **102,** 751–759.
27. Scott, H. and Brandtzaeg, P. (1996) Endomysial autoantibodies, in *Autoantibodies* (Peter, J. B. and Shoenfeld, Y., eds.), Elsevier, Amsterdam, pp. 237–244.
28. Uibo, O., Lambrechts, A., and Mascart-Lemone, F. (1995) Human oesophagus: a convenient antigenic substrate for the determination of antiendomysium antibodies in the serological diagnosis of coeliac disease. *Eur. J. Gastroenterol. Hepatol.* **7,** 37–40.
29. Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E. O., and Schuppan, D. (1997) Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat. Med.* **3,** 797–801.
30. Rodriguez, J. and Deinhardt, F. (1960) Preparation of a semipermanent mounting medium for fluorescent antibody studies. *Virology* **12,** 316, 317.
31. Lennette, D. A. (1978) An improved mounting medium for immunofluorescence microscopy. *Am. J. Clin. Pathol.* **69,** 647, 648.
32. Valnes, K. and Brandtzaeg, P. (1985) Retardation of immunofluorescence fading during microscopy. *J. Histochem. Cytochem.* **33,** 755–761.
33. Riggs, J. L., Seiwald, R. J., Burckhalter, J. H., Downs, C. M., and Metcalf, T. G. (1958) Isothiocyanate compounds as fluorescent labeling agents for immune serum. *Am. J. Pathol.* **34,** 1081–1087.
34. Titus, J. A., Haugland, R., Sharrow, S. O., and Segal, D. M. (1982) Texas red, a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies. *J. Immunol. Methods* **50,** 193–204.
35. Khalfan, H., Abuknesha, R., Rand-Weaver, M., Price, R. G., and Robinson, D. (1986) Aminomethyl coumarin acetic acid: a new fluorescent labelling agent for proteins. *Histochem. J.* **18,** 497–499.
36. Brandtzaeg, P. (1973) Conjugates of immunoglobulin G with different fluorochromes. I. Characterization by anionic-exchange chromatography. *Scand. J. Immunol.* **2,** 273–290.
37. Pizzolo, G. and Chilosi, M. (1984) Double immunostaining of lymph node sections by monoclonal antibodies using phycoerythrin labeling and haptenated reagents. *Am. J. Clin. Pathol.* **82,** 44–47.

38. Cebra, J. J. and Goldstein, G. (1965) Chromatographic purification of tetramethylrhodamine-immune globulin conjugates and their use in the cellular localization of rabbit—globulin polypeptide chains. *J. Immunol.* **95,** 230–245.
39. Brandtzaeg, P. (1973) Conjugates of immunoglobulin G with different fluorochromes. II. Specific and non-specific binding properties. *Scand. J. Immunol.* **2,** 333–348.
40. Brandtzaeg, P. and Baklien, K. (1976) Immunohistochemical studies of the formation and epithelial transport of immunoglobulins in normal and diseased human intestinal mucosa. *Scand. J. Gastroenterol.* **11(Suppl. 36),** 1–45.
41. Baklien, K., Brandtzaeg, P., and Fausa, O. (1977) Immunoglobulins in jejunal mucosa and serum from patients with adult coeliac disease. *Scand. J. Gastroenterol.* **12,** 149–159.
42. Baklien, K., Fausa, O., Thune, P. O., and Gjone, E. (1977) Immunoglobulins in jejunal mucosa and serum from patients with dermatitis herpetiformis. *Scand. J. Gastroenterol.* **12,** 161–168.
43. Brandtzaeg, P. and Baklien, K. (1977) Characterization of the IgA-immunocyte population and its product in a patient with excessive intestinal formation of IgA. *Clin. Exp. Immunol.* **30,** 77–88.
44. Scott, H., Ek, J., Baklien, K., and Brandtzaeg, P. (1980) Immunoglobulin-producing cells in jejunal mucosa of children with coeliac disease on a gluten-free diet and after gluten challenge. *Scand. J. Gastroenterol.* **15,** 81–88.
45. Goding, J. W. (1980) Antibody production by hybridomas. *J. Immunol. Methods* **39,** 285–308.
46. Petrusz, P., Ordronneau, P., and Finley, J. C. W. (1980) Criteria of reliability for light microscopic immunocytochemical staining. *Histochem. J.* **12,** 333–348.
47. Ghosh, S. and Campbell, A. M. (1986) Multispecific monoclonal antibodies. *Immunol. Today* **7,** 217–222.
48. Zola, H. (1985) Speaking personally: monoclonal antibodies as diagnostic reagents. *Pathology* **17,** 53–56.
49. Brandtzaeg, P. and Baklien, K. (1976) Inconclusive immunohistochemistry of human IgE in mucosal pathology. *Lancet* **i,** 1297, 1298.
50. Valnes, K. and Brandtzaeg, P. (1981) Selective inhibition of nonspecific eosinophil staining or identification of eosinophilic granulocytes by paired counterstaining in immunofluorescence studies. *J. Histochem. Cytochem.* **29,** 595–600.
51. Johnston, N. W. and Bienenstock, J. (1974) Abolition of nonspecific fluorescent staining of eosinophils. *J. Immunol. Methods* **4,** 189–194.
52. Halstensen, T. S., Scott, H., and Brandtzaeg, P. (1990) Human $CD8^+$ intraepithelial T lymphocytes are mainly CD45RA RB^+ and show increased co-expression of CD45R0 in celiac disease. *Eur. J. Immunol.* **20,** 1825–1830.
53. Halstensen, T. S., Scott, H., Farstad, I. N., Michaelsen, T. E., and Brandtzaeg, P. (1992) In situ two- and three-color immunofluorescence staining of mucosal T-cells in celiac disease: increase of $TCR\gamma/\delta^+CD8^-$ and $TCR\alpha/\beta^+CD45R0^+$ intraepithelial lymphocytes and IL-$2R^+$ $TCR\alpha/\beta^+CD4^+CD45R0^+$ lamina propria lymphocytes. *Prog. Histochem. Cytochem.* **26,** 201–210.

54. Jahnsen, F. L., Farstad, I. N., Aanesen, J. P., and Brandtzaeg, P. (1998) Phenotypic distribution of T cells in human nasal mucosa differs from that in the gut. *Am. J. Respir. Cell Mol. Biol.* **18,** 392–401.
55. Bruins, S., De Jong, M. C., Heeres, K., Wilkinson, M. H., Jonkman, M. F., and Van der Meer, J. B. (1995) Fluorescence overlay antigen mapping of the epidermal basement membrane zone: III. Topographic staining and effective resolution. *J. Histochem. Cytochem.* **43,** 649–656.
56. Ploem, J. S. (1967) The use of a vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. *Z. Wissensch. Mikr.* **68,** 129–142.
57. Johnson, G. D. (1997) Confocal laser scanning microscopy, in *Immunochemistry 2—A Practical Approach* (Johnstone, A. P. and Turner, M. W., eds.), Oxford University Press, Oxford, UK, pp. 131–148.
58. Bruins, S., de Jong, M. C., Heeres, K., Wilkinson, M. H., Jonkman, M. F., and van der Meer, J. B. (1994) Fluorescence overlay antigen mapping of the epidermal basement membrane zone: I. Geometric errors. *J. Histochem. Cytochem.* **42,** 555–560.
59. Haugland, R. P. (1996) *Handbook of Fluorescent Probes and Research Chemicals,* 6th ed., in *Molecular Probes* (Spence, M. T. Z. and Johnson, I. D., eds.), Eugene, OR, p. 679.
60. Staines, W. A., Meister, B., Melander, T., Nagy, J. I., and Hökfelt, T. (1988) Three-color immunofluorescence histochemistry allowing triple labeling within a single section. *J. Histochem. Cytochem.* **36,** 145–151.
61. Utgaard, J. O., Jahnsen, F. L., Bakka, A., Brandtzaeg, P., and Haraldsen, G. (1998) Rapid secretion of prestored interleukin-8 from microvascular endothelial cells. *J. Exp. Med.* **188,** 1751–1756.
62. Bergroth, V., Reitamo, S., Konttinen, Y. T., and Tolvanen, E. (1982) Fixation-dependent cytoplasmic false-positive staining with an immunoperoxidase method. *Histochemistry* **73,** 509–513.
63. Childs, G. and Unabia, G. (1982) Application of the avidin-biotin-peroxidase complex (ABC) method to the light microscopic localization of pituitary hormones. *J. Histochem. Cytochem.* **30,** 713–716.
64. Hsu, S.-M., Cossman, J., and Jaffe, E. S. (1983) A comparison of ABC, unlabeled antibody and conjugated immunohistochemical methods with monoclonal and polyclonal antibodies—an examination of germinal center of tonsils. *Am. J. Clin. Pathol.* **80,** 429–435.
65. Andersson, J., Abrams, J., Bjork, L., Funa, K., Litton, M., Agren, K., and Andersson, U. (1994) Concomitant in vivo production of 19 different cytokines in human tonsils. *Immunology* **83,** 16–24.
66. Lore, K., Sonnerborg, A., Spetz, A. L., Andersson, U., and Andersson, J. (1998) Immunocytochemical detection of cytokines and chemokines in Langerhans cells and in vitro derived dendritic cells [erratum]. *J. Immunol. Methods* **218,** 173–187.
67. Nilsen, E. M., Jahnsen, F. L., Lundin, K. E. A., Johansen, F.-E., Fausa, O., Sollid, L. M., Jahnsen, J., Scott, H., and Brandtzaeg, P. (1998) Gluten induces

an intestinal cytokine response strongly dominated by interferon-gamma in patients with celiac disease. *Gastroenterology* **115,** 551–563.
68. Guesdon, J.-L., Ternynck, T., and Avrameas, S. (1979) The use of avidin-biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem.* **27,** 1131–1139.
69. Berman, J. W. and Basch, R. S. (1980) Amplification of the biotin-avidin immunofluorescence technique. *J. Immunol. Methods* **36,** 335–338.
70. Buckland, R. M. (1986) Strong signals from streptavidin-biotin. *Nature* **320,** 557, 558.
71. Halstensen, T. S., Scott, H., and Brandtzaeg, P. (1989) Intraepithelial T cells of the TcR γ/δ^+CD8^- and $V\delta1^+/J\delta1^+$ phenotypes are increased in coeliac disease. *Scand. J. Immunol.* **30,** 665–672.
72. Halstensen, T. S., Farstad, I. N., Scott, H., Fausa, O., and Brandtzaeg, P. (1990) Intraepithelial TcR α/β^+ lymphocytes express CD45R0 more often than the TcR γ/δ^+ counterparts in coeliac disease. *Immunology* **71,** 460–466.
73. Halstensen, T. S. and Brandtzaeg, P. (1993) Activated T lymphocytes in the celiac lesion: non-proliferative activation (CD25) of $CD4^+$ α/β cells in the lamina propria but proliferation (Ki-67) of α/β and γ/δ cells in the epithelium. *Eur. J. Immunol.* **23,** 505–510.
74. Halstensen, T. S., Scott, H., Fausa, O., and Brandtzaeg, P. (1993) Gluten stimulation of coeliac mucosa *in vitro* induces activation (CD25) of lamina propria $CD4^+$ T cells and macrophages but no crypt cell hyperplasia. *Scand. J. Immunol.* **38,** 581–590.
75. Farstad, I. N., Halstensen, T. S., Fausa, O., and Brandtzaeg, P. (1993) Do human Peyer's patches contribute to the intestinal γ/δ T-cell population? *Scand. J. Immunol.* **38,** 451–458.
76. Farstad, I. N., Halstensen, T. S., Fausa, O., and Brandtzaeg, P. (1994) Heterogeneity of M-cell-associated B and T cells in human Peyer's patches. *Immunology* **83,** 457–464.
77. Farstad, I. N., Halstensen, T. S., Lazarovits, A. I., Norstein, J., Fausa, O., and Brandtzaeg, P. (1995) Human intestinal B-cell blasts and plasma cells express the mucosal homing receptor integrin $\alpha4\beta7$. *Scand. J. Immunol.* **42,** 662–672.
78. Farstad, I. N., Halstensen, T. S., Lien, B., Kilshaw, P. J., Lazarovits, A. I., and Brandtzaeg, P. (1996) Distribution of $\beta7$ integrins in human intestinal mucosa and organized gut-associated lymphoid tissue. *Immunology* **89,** 227–237.
79. Farstad, I. N., Halstensen, T. S., Kvale, D., Fausa, O., and Brandtzaeg, P. (1997) Topographic distribution of homing receptors on B and T cells in human gut-associated lymphoid tissue: relation of L-selectin and integrin $\alpha4\beta7$ to naive and memory phenotypes. *Am. J. Pathol.* **150,** 187–199.
80. Farstad, I. N., Norstein, J., and Brandtzaeg, P. (1997) Phenotypes of B and T cells in human intestinal and mesenteric lymph. *Gastroenterology* **112,** 163–173.
81. Nakane, P. K. and Pierce, G. B. (1966) Enzyme-labeled antibodies: preparation and application for the localization of antigens. *J. Histochem. Cytochem.* **14,** 929–931.
82. Busachi, C. A., Ray, M. B., and Desmet, V. J. (1978) An immunoperoxidase technique for demonstrating membrane localized HBsAg in paraffin sections of liver biopsies. *J. Histochem. Cytochem.* **19,** 95–99.

83. Hsu, S.-M. and Soban, E. (1982) Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. *J. Histochem. Cytochem.* **30,** 1079–1082.
84. Straus, W. (1972) Phenylhydrazine as inhibitor of horseradish peroxidase for use in immunoperoxidase procedures. *J. Histochem. Cytochem.* **20,** 949–951.
85. Gallyas, F., Görcs, T., and Merchenthaler, I. (1982) High-grade intensification of the end-product of the diaminobenzidine reaction for peroxidase histochemistry. *J. Histochem. Cytochem.* **30,** 183–184.
86. Nemes, Z. (1987) Intensification of 3,3'-diaminobenzidine precipitation using the ferric ferricyanide reaction, and its application in the double-immunoperoxidase technique. *Histochemistry* **86,** 415–419.
87. Graham, R. C., Lundholm, U., and Karnovsky, M. J. (1965) Cytochemical demonstration of peroxidase activity with 3-amino-9-ethylcarbazole. *J. Histochem. Cytochem.* **13,** 150–152.
88. Mesulam, M.-M. (1978) Tetramethyl benzidine for horseradish peroxidase neurohistochemistry: a non-carcinogenic blue reaction-product with superior sensitivity for visualizing neural afferents and efferents. *J. Histochem. Cytochem.* **26,** 106–117.
89. Levey, A. I., Bolam, J. P., Rye, D. B., Hallanger, A. E., Demuth, R. M., Mesulam, M.-M., and Wainer, B. H. (1986) A light and electron microscopic procedure for sequential double antigen localization using diaminobenzidine and benzidine dihydrochloride. *J. Histochem. Cytochem.* **34,** 1449–1457.
90. Hanker, J. S., Yates, P. E., Metz, C. B., and Rustioni, A. (1977) A new specific, sensitive and non-carcinogenic reagent for the demonstration of horseradish peroxidase. *Histochem. J.* **9,** 789–792.
91. Nakane, P. (1968) Simultaneous localization of multiple tissue antigens using the peroxidase-labeled antibody method: a study on pituitary glands of the rat. *J. Histochem. Cytochem.* **16,** 557–560.
92. Mason, D. Y. and Sammons, R. (1978) Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. *J. Clin. Pathol.* **31,** 454–460.
93. Streefkerk, J. G. (1972) Inhibition of erythrocyte pseudoperoxidase activity by treatment with hydrogen peroxide following methanol. *J. Histochem. Cytochem.* **20,** 829–831.
94. Straus, W. (1979) Peroxidase procedures: technical problems encountered during their application. *J. Histochem. Cytochem.* **27,** 1349–1351.
95. Heyderman, E. and Neville, A. M. (1977) A shorter immunoperoxidase technique for the demonstration of carcinoembryonic antigen and other cell products. *J. Clin. Pathol.* **30,** 138–140.
96. Fink, B., Loepfe, E., and Wyler, R. (1979) Demonstration of viral antigen in cryostat sections by a new immunoperoxidase procedure eliminating endogenous peroxidase activity. *J. Histochem. Cytochem.* **27,** 1299–1301.
97. Borgers, M. (1973) The cytochemical application of new potent inhibitors of alkaline phosphatases. *J. Histochem. Cytochem.* **21,** 812–824.

98. Dearnaley, D. P., Sloane, J. P., Ormerod, M. G., Steele, K., Coombes, R. C., Clink, H. McD, Powles, T. J., Ford, H. T., Gazet, J.-C., and Neville, A. M. (1981) Increased detection of mammary carcinoma cells in marrow smears using antisera to epithelial membrane antigen. *Br. J. Cancer* **44,** 85–90.
99. Bulman, A. S. and Heyderman, E. (1981) Alkaline phosphatase for immunocytochemical labelling: problems with endogenous enzyme activity. *J. Clin. Pathol.* **34,** 1349–1351.
100. Van Noorden, S. (1986) Tissue preparation and immunostaining techniques for light microscopy, in *Immunocytochemistry: Modern Methods and Applications*, 2nd ed. (Polak, J. M. and van Noorden, S., eds.), Wright, Bristol, UK, pp. 26–53.
101. Ying, S., Humbert, M., Barkans, J., Corrigan, C. J., Pfister, R., Menz, G., Larche, M., Robinson, D. S., Durham, S. R., and Kay, A. B. (1997) Expression of IL4 and IL5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J. Immunol.* **158,** 3539–3544.
102. Lan, H. Y., Mu, W., Nikolic-Paterson, D. J., and Atkins, R. C. (1995) A novel, simple, reliable, and sensitive method for multiple immunoenzyme staining: use of microwave oven heating to block antibody crossreactivity and retrieve antigens. *J. Histochem. Cytochem.* **43,** 97–102.
103. Valnes, K. and Brandtzaeg, P. (1982) Comparison of paired immunofluorescence and paired immunoenzyme staining methods based on primary antisera from the same species. *J. Histochem. Cytochem.* **30,** 518–525.
104. Valnes, K. and Brandtzaeg, P. (1984) Paired indirect immunoenzyme staining with primary antibodies from the same species: application of horseradish peroxidase and alkaline phosphatase as sequential labels. *Histochem. J.* **16,** 477–487.

16

Immunoassay for Detection of IgA Antitissue Transglutaminase in Patients with Celiac Disease

Walburga Dieterich, Ernst Otto Riecken, and Detlef Schuppan

1. Introduction

Celiac disease is characterized by mucosal changes in the small intestine, ranging from increased numbers of intraepithelial lymphocytes to complete villus effacement with various signs of malabsorption ***(1,2)***. Celiac disease is diagnosed by the demonstration of an altered intestinal mucosa in a jejunal biopsy specimen ***(3)***. Celiac disease patients invariably have antibodies directed against gliadin and endomysium, a structural component of the extracellular matrix: both antibodies disappear under a gluten-free diet. Therefore, these antibodies are useful tools for diagnosis, and in the dietary control of coeliac disease ***(3–7)***. Serum IgA antibodies against endomysial antibodies (EMAs) are especially considered to be sensitive and highly specific markers for celiac disease ***(8,9)***. Previously, EMAs were detected by indirect immunofluorescence on tissue slides of monkey esophagus or human umbilical cord ***(10–12)***.

Recently, we identified the endomysial autoantigen as tissue transglutaminase (tTG) ***(13)***. Since IgA from celiacs did not bind to tTG in Western blotting, these antibodies appear to recognize only the native or calcium-activated enzyme tTG. Therefore, we developed an enzyme-linked immunosorbent assay (ELISA) for detection of serum IgA anti-tTG to allow for a sensitive, objective, and quantitative determination of antibody titers in serum.

2. Materials

2.1. Immunochemicals

1. Because of the high degree of identity between human tTG and tTG from guinea pig liver ***(14)***, the ELISA is performed with commercially available rodent enzyme (Sigma, Deisenhofen, Germany).

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

2. Microtiter plates (Greiner Labortechnik, Frickenhausen, Germany).
3. Peroxidase-conjugated goat IgG antihuman IgA (Dianova, Hamburg, Germany).

2.2. Buffers

All buffer reagents (Sigma, Deisenhofen, Germany) are of the highest purity available. Perhydrol and *o*-phenylenediamine-hydrochloride were from Merck, Darmstadt, Germany.

1. Buffer A—coating buffer: 0.05 *M* Tris-HCl, 0.15 *M* NaCl, 5 m*M* $CaCl_2$, pH 7.5. This buffer should be prepared freshly or stored at 4°C for a maximum of 1 wk.
2. Buffer B—dilution and wash buffer: 0.05 *M* Tris-HCl, 0.15 *M* NaCl, 0.01 *M* EDTA, 0.1% Tween-20, pH 7.4.
3. Buffer C—citrate buffer: 0.1 *M* citrate, 1 mg/mL of *o*-phenylenediamine, 0.06% H_2O_2, pH 4.2.

2.3. ELISA Plate Absorbance Measurement

1. The absorbance was read at 450 nm on an ELISA reader MRX (Dynex Technologies GmbH, Denkendorf, Germany).

2.4. Handling of Sera

1. Sera were stored at –20°C. Repeated thawing and freezing cycles should be avoided.
2. Since tTG is reported to crosslink fibronectin and fibrinogen ***(15–17)***, which are present in serum, the samples were diluted in EDTA-containing buffer to prevent unintended reactions with the coated enzyme.

3. Method

3.1. Preparation of Plates

1. Coat the plates with tTG in 50 m*M* Tris-HCl and 150 m*M* NaCl, containing 5 m*M* $CaCl_2$, pH 7.5 (buffer A) (*see* **Note 1**) for 2 h at 37°C. Other coating procedures were less sensitive (*see* **Note 2**). The optimal quantity of tTG, per well, is ≈1 µg of solid tTG (equivalent to 0.00167 U of tTG).
2. After coating, remove unbound antigen by washing the wells three times with 250 µL of buffer B, and use immediately or store the plate overnight at 4°C in buffer B.
3. Prevent all nonspecific binding sites on the microtiter plates by adding Tween-20 in all buffers subsequently used (buffer B) (*see* **Note 3**). Tween-20 is quite useful in this situation to eliminate background reactions and to increase the sensitivity of positive reactions ***(20)***.

3.2. Performing the Test

1. Add 100 µL of 1/25 diluted serum (buffer B; *see* **Note 4**) to each well. Test each serum in duplicate and add positive and negative reference sera. High-

titer sera must be diluted appropriately (1/100 or 1/400) to get optical densities below 2.5.
2. Incubate the sera at room temperature for 1 h.
3. After incubation, wash the plates three times with 250 µL of buffer B.
4. Incubate the wells with peroxidase-conjugated goat IgG antihuman IgA.
5. Remove surplus antibodies by intensive washing with 250 µL of buffer B.
6. Develop color reaction with *o*-phenylenediamine-hydrochloride and 0.06% H_2O_2 in citrate buffer (buffer C) in the dark for 30 min.
7. Read absorbances at 450 nm on a plate reader.
8. Calculate the titer by subtracting the background value (OD ≈ 0.08) and multiplying by the serum dilution factor.

3.3. Summary

This ELISA technique was used to check 106 sera of celiac patients with known EMA titers, 43 sera of patients under a gluten-free diet, and 114 sera of controls, including various chronic inflammatory diseases. The Spearman test showed a positive correlation between the obtained IgA anti-tTG titers and the EMA data with $r = 0.862$ and $p < 0.0001$. In addition, patients with celiac disease showed significantly elevated IgA anti-tTG titers compared with patients on a gluten-free diet, or with controls. The sensitivity and specificity of this ELISA was 98.1 and 94.7%, respectively ***(18)***. Similar results were obtained by a group from Finland ***(19)***. Therefore, this test seems to be a useful tool in screening and controlling therapy of patients with celiac disease.

4. Notes

1. The addition of 5 m*M* calcium to the coating buffer is crucial for a successful test, but calcium is not necessary in the washing or storage buffers. All further steps should be done *without* calcium to prevent crosslinking reactions of the Ca-activated tTG.
2. The optimal buffer was found to be buffer A (*see* **Subheading 2.2.**, **item 1**). Nil or weak reactivity was obtained if tTG was coated in phosphate-buffered saline or in 50 m*M* Tris-HCl and 150 m*M* NaCl with 10 m*M* EDTA, pH 7.5. This was not owing to differences in the coating efficiency, which were checked by the addition of radioactively labeled tTG as tracer, since similar coating efficiencies of 23–29.4% were obtained in all tested buffer systems when 1 µg of tTG (0.00167 U) was coated. The addition of more than 1 µg of tTG to the microtiter plates resulted in no further improvement of the ELISA.
3. Care must be taken with blocking. Bovine serum albumin should be avoided, because some patients with celiac disease, and also some controls, have circulating antibodies to this food protein.
4. Sera should be diluted in EDTA-containing buffer to prevent crosslinking reactions with the coated tTG.

References

1. Trier, J. S. (1991) Celiac sprue. *N. Engl. J. Med.* **325,** 1709–1719.
2. Marsh, M. N. (1992) Gluten, major histocompatibility complex, and the small intestine: a molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* **102,** 330–354.
3. Walker-Smith, J. A., Guandalini, S., Schmitz, J., Shmerling, D. H., and Visakorpi, J. K. (1990) Revised criteria for diagnosis of coeliac disease. *Arch. Dis. Child.* **65,** 909–911.
4. Bürgin-Wolff, A., Gaze, H., Hadziselimovic, F., Huber, H., Lentze, M. J., Nusslé, D., and Reymond-Berthet, C. (1991) Antigliadin and antiendomysium antibody determination for coeliac disease. *Arch. Dis. Child.* **66,** 941–947.
5. McMillan, S. A., Haughton, D. J., Biggart, J. D., Edgar, J. D., Porter, K. G, and McNeill, T. A. (1991) Predictive value for coeliac disease of antibodies to gliadin, endomysium, and jejunum in patients attending for jejunal biopsy. *Br. Med. J.* **303,** 1163–1165.
6. Lindquist, B. L., Rogozinski, T., Moi, H., Danielsson, D., and Olcen, P. (1994) Endomysium and gliadin IgA antibodies in children with coeliac disease. *Scand. J. Gastroenterol.* **29,** 452–456.
7. Corrao, G., Corazza, G. R., Andreani, M. L., Torchio, P., Valentini, R. A., Galatola, G., Quaglino, D., Gasbarrini, G., and di Orio, F. (1994) Serological screening of coeliac disease: choosing the optimal procedure according to various prevalence values. *Gut* **35,** 771–775.
8. Volta, U., Molinaro, N., Fusconi, M., Cassani, F., and Bianchi, F. B. (1991) IgA antiendomysial antibody test: a step forward in celiac disease screening. *Dig. Dis. Sci.* **36,** 752–756.
9. Lerner, A., Kumar, V., and Iancu, T. C. (1994) Immunological diagnosis of childhood coeliac disease: comparison between antigliadin, antireticulin and antiendomysial antibodies. *Clin. Exp. Immunol.* **95,** 78–82.
10. Chorzelski, T. P., Beutner, E. H., Sulej, J., Tchorzewska, H., Jablonska, S., Kumar, V., and Kapuscinska, A. (1984) IgA anti-endomysium antibody: a new immunological marker of dermatitis herpetiformis and coeliac disease. *Br. J. Dermatol.* **111,** 395–402.
11. Ladinser, B., Rossipal, E., and Pittschieler, K. (1994) Endomysium antibodies in coeliac disease: an improved method. *Gut* **35,** 776–778.
12. Sategna-Guidetti, C., Bruna Grosso, S., Bruno, M., and Grosso, S. (1997) Is human umbilical cord the most suitable substrate for the detection of endomysium antibodies in the screening and follow-up of coeliac disease? *Eur. J. Gastroenterol. Hepatol.* **9,** 657–660.
13. Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E. O., and Schuppan, D. (1997) Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat. Med.* **3,** 797–801.
14. Gentile, V., Saydak, M., Chiocca, E. A., Akande, O., Birckbichler, P. J., Lee, K. N., Stein, J. P., and Davies, P. J. A. (1991) Isolation and characterization of cDNA clones to mouse macrophage and human endothelial cell tissue transglutaminases. *J. Biol. Chem.* **266,** 478–483.

15. LeMosy, E. K., Erickson, H. P., Beyer, W. F., Radek, J. T., Jeong, J. M., Murthy, S. N. P., and Lorand, L. (1992) Visualization of purified fibronectin-transglutaminase complexes. *J. Biol. Chem.* **267,** 7880–7885.
16. Barsigian, C., Stern, A. M., and Martinez, J. (1991) Tissue (type II) transglutaminase covalently incorporates itself, fibrinogen, or fibronectin into high molecular weight complexes on the extracellular surface of isolated hepatocytes. *J. Biol. Chem.* **266,** 22,501–22,509.
17. Achyuthan, K. E., Goodell, R. J., Kennedye, J. R., Lee, K. N., Henley, A., Stiefer, J. R., and Birckbichler, P. J. (1995) Immunochemical analyses of human plasma fibronectin-cytosolic transglutaminase interactions. *J. Immunol. Methods* **180,** 69–79.
18. Dieterich, W., Laag, E., Schöpper, H., Volta, U., Ferguson, A., Gillet, A., Riecken, E. O., and Schuppan, D. (1998) Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* **115,** 1–6.
19. Sulkanen, S., Halttunen, T., Laurrila, K., Kolho, K. L., Korponay-Szabo, I. R., Sanilahti, E., Lollin, P., and Mäki, M. (1998) Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology* **115,** 6–13.
20. Bullock, S. L. and Walls, K. W. (1977) Evaluation of some of the parameters of the enzyme-linked immunospecific assay. *J. Infect. Dis.* **136(Suppl.),** S279–S285.

17

Antiendomysial and Antigliadin Antibody Tests and Diagnosis of Celiac Disease

David J. Unsworth

1. Introduction

Celiac disease and the related condition dermatitis herpetiformis are caused by ingestion of wheat gluten (gliadin being the active moiety) and certain other cereal proteins.

A strict gluten-free diet is curative for both the skin and gut lesions. Support for the autoimmune hypothesis includes the presence of autoantibodies to connective tissue–related autoantigens. These so-called antiendomysial autoantibodies (AEAs) ***(1)*** are closely associated with gluten-sensitive enteropathy, providing a fascinating model for a form of autoimmunity, predictably induced by a defined environmental agent (gluten), and fully treatable/reversible by dietary exclusion. Pathogenesis may be owing to autosensitization caused when gliadin binds to "reticulin" connective tissue fibrils—a phenomenon that is clearly demonstrable in vitro ***(2)***. Certainly, AEAs cannot be sustained if gluten is withdrawn. Tissue transglutaminase (tTG), an enzyme involved in tissue repair and widely distributed as a normal constituent of connective tissue, appears to be the major autoantigen ***(3)*** accounting for AEAs. tTG and anti-tTG antibodies are discussed in detail in Chapter 16. In time, anti-tTG tests may replace AEA as the serological "gold standard" for diagnostic celiac disease serology.

AEAs of the IgA isotype are pathognomonic for gluten-sensitive enteropathy (GSE), disappearing on a gluten-free diet and returning postgluten challenge. Serological tests for celiac disease are thus based on detecting IgA rather than the less specific IgG isotype ***(4)***. The established detection method in popular use is indirect immunofluorescence (IIF). IgA AEAs provide a powerful diagnostic tool, with a sensitivity and specificity for celiac disease of more

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

than 90 and 95%, respectively ***(4)***. The specificity for dermatitis herpetiformis is again excellent, at about 95%, but sensitivity is poorer, at about 50%.

1.1. IIF and AEA Detection

R1 antireticulin autoantibodies, detected on composite rat liver, kidney, and stomach tissue sections, were the first celiac disease–related autoantibodies to be reported ***(5)***. Changes in methodology since 1971, including the use of monkey esophagus or, more recently, human umbilical cord ***(6)***, rather than the less satisfactory rodent tissue sections, have improved sensitivity and disease specificity enormously. However, perhaps the most significant contributor to improved specificity has been the realization that IgA but not IgG autoantibodies are disease specific ***(4)***.

The term *antireticulin antibody* (ARA) has increasingly been replaced by *antiendomysial antibody*. ARA and AEA are essentially different descriptive terms for the same autoantibody factor ***(4)***, giving different IIF patterns of reactivity on rat (ARA) or monkey (AEA) tissue sections, respectively. Longitudinal smooth muscle fibers within monkey esophagus, cut in transection, are seen to be arranged in bundles, enclosed within a "perimysial" connective tissue sheath, itself enclosing smaller "endomysial" fiber bundles. AEAs in IIF tests react strongly with this "endomysial" connective tissue (**Fig. 1**). These tests have helped show that celiac disease is underdiagnosed ***(7)***, can exist as an occult problem in apparently healthy persons ***(8)***, and, most interestingly, can predict (GSE) in predisposed persons with "latent celiac disease" ***(9)***.

1.2. Enzyme-Linked Immunosorbent Assay Testing for Antigliadin Antibody

In contrast to AEA, serum antiwheat protein antibodies (including antigliadin antibody [AGA]) and other food antibodies are often present in any disease featuring a damaged or "leaky" small bowel (e.g., Crohn's disease, cow's milk enteropathy, Giardia infection). AEAs are absent from these nongluten-sensitive disease states ***(10)***, hence the far superior disease specificity data. Nonetheless, AGA has been popular, in part because parallel testing for both IgA and IgG AGA provides excellent sensitivity for celiac disease ***(4)***. Few cases of untreated celiac disease show both IgA and IgG AGA in the normal range ***(4)***. Negative AGA testing (for both IgA and IgG isotypes) can thus virtually exclude celiac disease. IgA-deficient individuals with celiac disease are missed by IgA-based AEA tests but tend, e.g., to be IgG AGA positive ***(11)***. IgA AGAs are more likely than IgG AGAs to be associated with celiac disease ***(4)***, and IgG AGAs show poor disease specificity. We are now seeing a trend away from AGA testing.

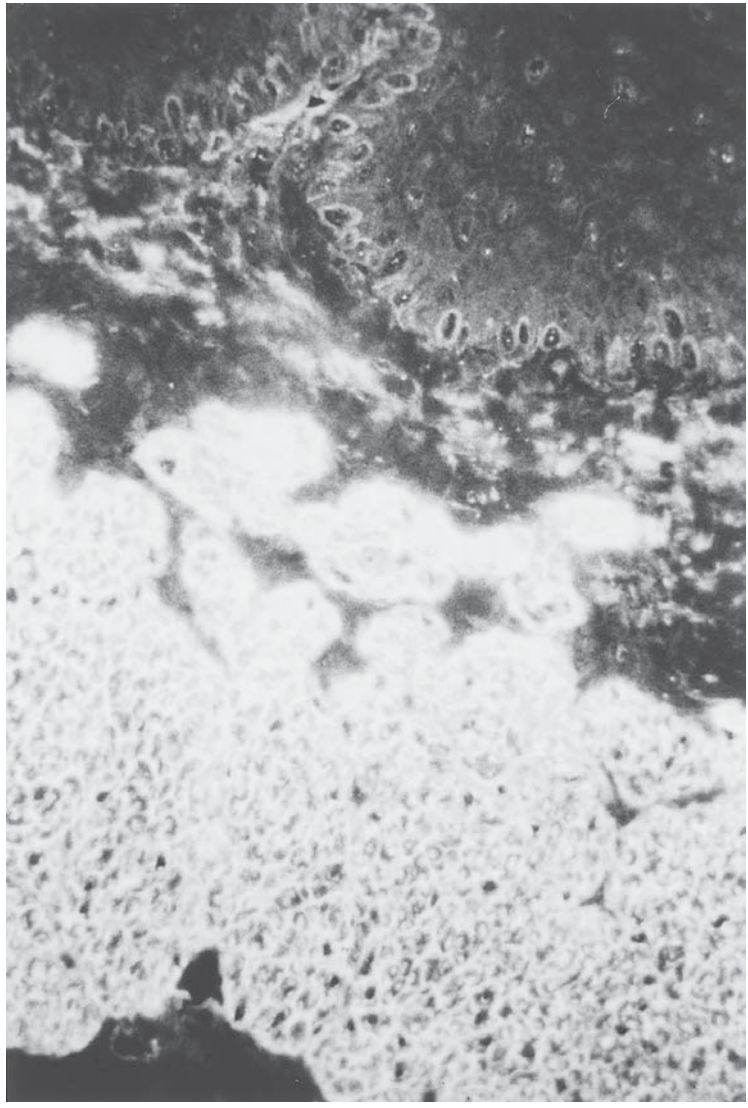


Fig. 1. IgA AEA reactivity on unfixed monkey esophagus. Negative epidermis (**top**), and below a lacework "endomysial" pattern of immunofluorescence staining around the smooth muscle bundles in the lamina propria.

Many different AGA detection methods are available, including IIF tests using tissue sections pretreated with gliadin solution ***(2)***, hemagglutination methods ***(12)***, and commercial nephelometry methods. The most common

method used is enzyme-linked immunosorbent assay (ELISA), and is described herein ***(13)***. As to choice of antigen, some authors have advocated the use of purified subfractions of gliadin such as alpha gliadin. There is little evidence, in my view, to suggest that this is useful or worthwhile ***(14)***.

2. Materials

2.1. Indirect Immunofluorescence (see Note 1)

1. Tissue sections: Use unfixed, frozen, cryostat sections, cut 5 μm thick of either monkey esophagus (Biodiagnostics, Worcester, UK) or human umbilical cord (The Binding Site, Birmingham, UK).
2. Mount the sections on Multispot slides (Henley, Essex, UK). If cut in-house, either cut the sections immediately before use or cut, air-dry under a fan for 30 min, and store (wrapped in silver foil) at –70°C (*see* **Note 2**).
3. IgA AEA positive control sera: Obtain a good positive control serum (from a biopsy-proven case of celiac disease, sampled before the patient starts on a gluten-free diet) from a colleague (*see* **Note 3**).
4. Phosphate-buffered saline (PBS) pH 7.4. Use a commercial concentrate (Clintech, Essex, UK, produces a 20X-strength product).
5. Fluorescein isothiocyanate (FITC) conjugated antihuman IgA isotype-specific reagent (FITC anti-IgA): Use Dako reagent (High Wycombe, UK). The appropriate dilution, in PBS, is determined by checkerboard titration. For Dako reagents, this is of the order of 1/50–1/100.
6. Mounting: Obtain cover slips (Clarence, Whorley, UK) of the same size (22 × 64 mm) as the microscope slide. Use 10% glycerol/90% PBS (v/v) to mount.

2.2. ELISA Test for AGA

1. Use Immunolon 4 micro-ELISA plates (Dynatech, Billinghurst, West Sussex, UK).
2. Gliadin: Dissolve crude gliadin (Sigma, St. Louis, MO) in 70% ethanol/distilled water (final concentration of 50 μg/mL) to prepare the antigen plate coating solution (*see* **Note 4**).
3. Use PBS with added detergent Tween-20 (Sigma) rather than ordinary PBS for all washing procedures. Add 0.05% by volume (thus 500 μL in 1 L of PBS). One liter is needed per ELISA plate per experiment.
4. Enzyme-conjugated antihuman immunoglobulin reagents: Use horseradish peroxidase (HRP) conjugated reagents (Dako) at a working dilution of between 1/350 and 1/1000 for the anti-IgA-specific reagent.
5. HRP enzyme substrate: Dissolve a single tablet (35 mg weight) of ortho-phenylene-diamine (OPD) (Sigma) in 100 mL of substrate buffer. Make the buffer by adding 50 mL of distilled water, 25.7 mL of 0.2 *M* NaHPO, and 24.3 mL of 0.1 *M* citric acid. Prepare fresh (*see* **Note 5**), and in the last step, add 20 μL of hydrogen peroxide (30% by volume), mixing by gentle inversion. Use without delay (*see* **Note 5**).
6. Stop solution: 4 *M* sulfuric acid (*see* **Note 5**).

3. Methods

3.1. IIF Indirect Immunofluorescence for AEA

1. Perform all steps at room temperature.
2. Air-dry tissue sections of either monkey esophagus or human umbilical cord for 30 min under a fan before use.
3. Apply 50 µL of patient serum (diluted at 1/5 in PBS) to each section. Test up to 12 sera per slide. Always include a positive control serum (*see* **Note 3**). Incubate in a humidity chamber for 30 min.
4. Prepare dilution of FITC anti-IgA in PBS for use in **step 6**.
5. Wash (30 min) in a PBS wash bath, and carefully dry off the areas around the sections with a paper towel (*see* **Note 7**).
6. Without delay, apply 50-µL volumes of FITC anti-IgA to each section. Incubate for 30 min (*see* **Note 8**).
7. Wash ((*see* **Note 9**)).
8. Without delay, apply 50-µL volumes of mounting fluid (*see* **Subheading 2.1.**, **item 6**) and apply the cover slips.
9. Store the slides in the dark at room temperature.

3.2. ELISA for AGA

1. Coat ELISA plates, adding 100 µL of gliadin solution per well overnight (at least 12 h) at 4°C. Ideally, coat half the ELISA plate (rows 1–6) with gliadin, and the other half of the plate with 70% ethanol/water solution lacking gliadin. Cover the plates with a lid to prevent dehydration. Alternatively, remove precoated ELISA plates from the freezer, and thaw (under a fan) for 15 min) (*see* **Note 10**).
2. After coating the plates, perform all subsequent steps at room temperature.
3. Prepare dilutions of patient's sera, in PBS-Tween-20 (*see* **Note 11**), approx 1/100 for IgA detection and 1/1000 for IgG antibody (depends on reagent used).
4. Thoroughly wash the plates, using either an automated machine washer or manually using generous amounts of PBS-Tween-20 dispensed via a plastic squeeze bottle. Repeat two more times. Dry off the plates by tapping out vigorously against a pad of paper towels (*see* **Note 8**).
5. Apply 100-µL volumes of diluted patient's sera, in duplicate, to both gliadin-coated and uncoated wells. Incubate for 2 h, covering the ELISA plates with a lid at room temperature (*see* **Note 12**).
6. Prepare dilutions of HRP-conjugated antihuman IgA (or anti-IgG as appropriate), by diluting in PBS-Tween-20 (I suggest 20 µL of antiserum in 10 mL of PBS-Tween-20) for use in **step 8**.
7. Wash three times as in **step 4** and blot dry.
8. Apply 100 µL of diluted HRP conjugate to each well, cover the plate, and incubate for 2 h.
9. Ten minutes before actually required, prepare the HRP substrate solution in a darkened bottle, and place in the dark until required (*see* **Note 5**).
10. Wash the plates three times as in **step 4**.

11. Start the stop clock. Apply 100 μL of enzyme substrate per well. Place the ELISA plate in the dark throughout the 10 to 20-min incubation time.
12. Stop the reaction by adding 25 μL of sulfuric acid (mixing with a pipet within the ELISA well) to each well (*see* **Note 5**). Reactions should be stopped after a fixed time interval (*sees* **Note 13** and **14**).
13. Read the optical density (OD) at 495 nm within the next 30 min. By including dilutions of a standard positive control serum, and allowing a defined reaction time (say 10 min), a standard curve can be drawn, with results expressed in OD units.

4. Notes

1. Basic laboratory equipment required includes a fluorescence microscope allowing indirect ultraviolet light illumination. I use a Nikon Labophot. Both low- (×250) and high-power (×400) magnifications are helpful.
2. For in-house tissue section production, obtain (with ethical permission) freshly discarded human obstetric material, or use Macaque monkeys as a source of monkey esophagus. I use human umbilical cord routinely with no problems. In-house cut sections require fresh tissue within 1 h of childbirth (or fresh postmortem animal tissue) snap frozen in liquid nitrogen as soon as possible. Frozen uncut tissue can be stored for at least 1 yr at –70°C. Cut transverse sections through the full thickness of umbilicus/esophagus wall. If cutting homemade sections of monkey esophagus, remember that only the lower third of the esophagus (gastric end) is suitable. Macaque monkey tissue is acceptable. I know of certain commercially available slides in which this requirement has been ignored. This can lead to false-negative results. Any commercial source should be checked in-house by using defined prototype control sera. Interpretation of results depends on the quality of the tissue sections cut. This can be checked before proceeding with the full method, by air-drying a sample slide and staining with methylene blue before mounting and viewing under ordinary light, to check morphology. These sample slides are then discarded.
3. Always include a positive and a separate negative control serum on each test run. True positive results are characterized by fibrillar staining of the connective tissue "endomysial" bundles scattered through the lamina propria of monkey esophagus (*see* **Fig. 1**). Beware the occasional IgA antismooth muscle autoantibody that gives false-positive staining. *See* **Figs. 2** and **3** for true and false (smooth muscle antibody) reactivity patterns on human umbilical cord.
4. Many researchers use gliadin "dissolved" in saline to coat ELISA plates. In fact, gliadin is quite difficult to dissolve in saline, and I specifically choose and prefer 70% ethanol/distilled water, because, by definition, gliadins will dissolve easily. The use of saline suspensions to coat can lead to variable efficiency of coating, and therefore variable results.
5. Always prepare fresh enzyme substrate, and keep in a light-shielded bottle. Within 10 min, light-accelerated decay occurs, generating color product, which affects assay interpretation. **Caution:** OPD is *toxic*—use gloves and prepare solution within a fume cupboard. NB: Sulfuric acid (stop solution) is also toxic.

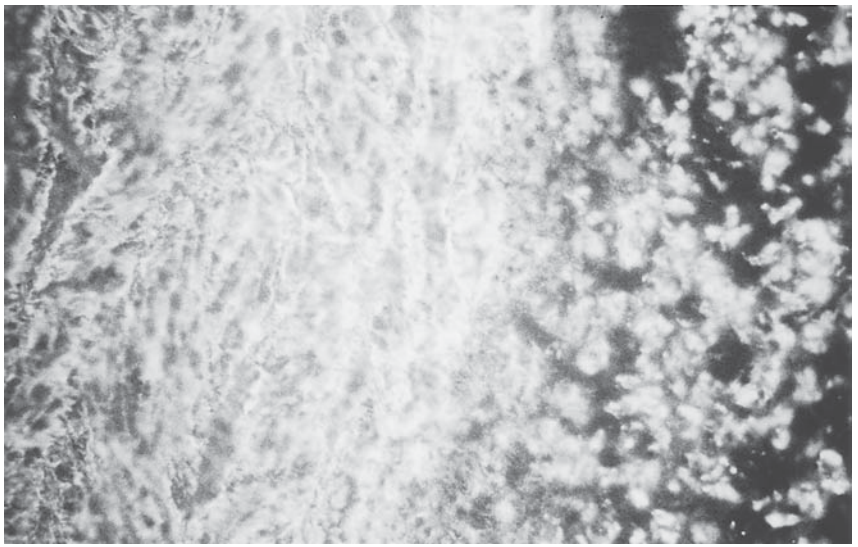


Fig. 2. IgA AEA reactivity (same serum as **Fig. 1**) on unfixed human umbilical cord. Note connective tissue reactivity in between muscle bundles (endomysial type reactivity on the left) and staining of individual mesenchymal cells within Wharton's jelly on the right.

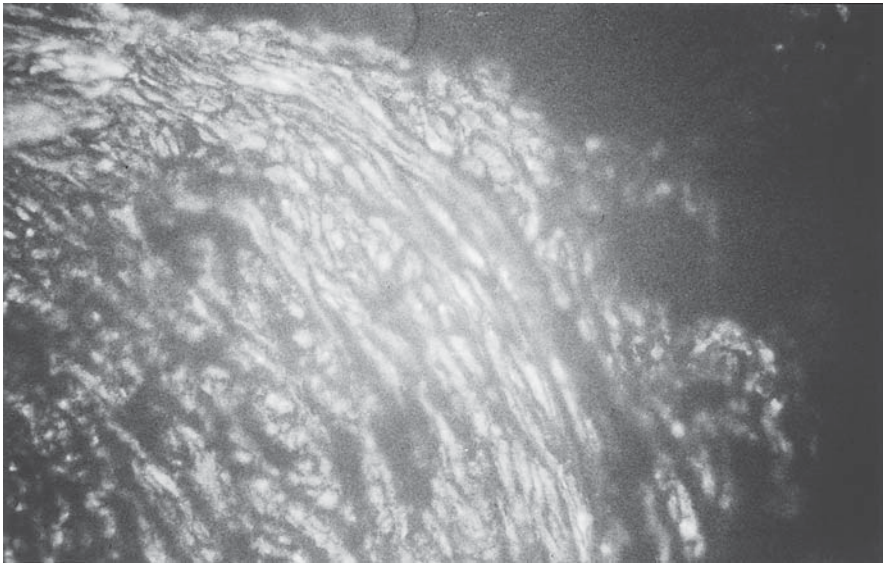


Fig. 3. False positive on umbilical cord, owing to IgA antismooth muscle autoantibody reactivity. This is not related to gluten sensitivity and has no known clinical significance.

6. To avoid artifactual staining, it is vital to ensure that sections do not dry out at any point during the IIF method. Drying out is most likely at the stages when slides are withdrawn from the wash bath, and excess fluid is removed by using a small folded piece of paper towel to dry in between the sections. This process must be carefully done, but speedily completed. If delays occur, it is best to replace the slides in the wash bath and start again.
7. A wash bath, large enough to allow slides to be fully immersed (drying out must not be allowed) is also required. I use a bath equipped with a submersible microscope slide holder. The leg supports beneath the holder allow a small magnetic stirrer to spin harmlessly within the bath but underneath the slide holder.
8. Glycerol/PBS mountant has a long shelf life (at least 3 mo).
9. Immunofluorescence slides can be stored for up to 24 h before interpretation under the microscope, without significant diminution of fluorescence intensity.
10. If negative control wells (no gliadin on the plate) show up as false positives after (*see* **Note 8**)developing the assay, suspect either failure to add correctly Tween-20 to the wash buffer or poor washing technique.
11. The most common pitfall with ELISA is the failure to wash plates properly. Washing must be thorough and vigorous, using copious quantities of PBS-Tween-20. Tween-20 prevents nonspecific binding to the plastic plates, but will allow antigen/antibody interaction. If washing manually (rather than using a dedicated ELISA washer), ensure that each well is flushed to overflowing before patting (aggressively) onto dry filter paper until no evidence of residual fluid is seen (replace the filter paper with new dry pieces and repeat patting until no new damp spots appear on dry filter paper).
12. Note that the majority of normal individuals show detectable AGA (especially IgG type), so that only by diluting test sera (1/100 is usually sufficient as described in the method) do we simply and reliably distinguish between normal and high-titer (usually celiac disease) positives.
13. Inclusion of a blank (gliadin-uncoated well) allows test results to be corrected by subtraction of the blank OD value.
14. For quantitative purposes, either titer sera to their end point or, better, construct a standard curve, using dilutions of a known positive sample plotted against OD values.

References

1. Chorzelski, T. P., Beutner, E. H., Sulej, J., Chorzewska, H., Jablonska, S., Kumar, V., and Kapuscinska, A. (1984) IgA anti endomysium antibody. A new immunological marker of dermatitis herpetiformis and coeliac disease. *Br. J. Dermatol.* **111,** 395–402.
2. Unsworth, D. J., Manuel, P. D., Walker-Smith, J. A., Campbell, C. A., Johnson, G. D., and Holborow, E. J. (1981) A new immunofluorescence blood test for gluten sensitivity. *Arch. Dis. Child. Health* **56,** 864–868.
3. Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E. O., and Schuppan, D. (1997) Identification of tissue transglutaminase as the autoantigen in celiac disease. *Nat. Med.* **3(7),** 797–801.

4. Unsworth, D. J. (1996) Serological diagnosis of gluten-sensitive enteropathy. *J. Clin. Pathol.* **49,** 704–711.
5. Seah, P. P., Fry, L., Hoffbrand, A. V., and Holborow E. J. (1971) Tissue autoantibodies in dermatitis herpetiformis and adult coeliac disease. *Lancet* **i,** 834–836.
6. Ladinser, B., Rossipal, E., and Pittschieler, K. (1994) Endomysium antibodies in coeliac disease: an improved method. *Gut* **35,** 776–778.
7. Unsworth, D. J. and Brown, D. L. (1994) Serological screening suggests that adult coeliac disease is underdiagnosed in the UK and increases the incidence by up to 12%. *Gut* **35,** 61–64.
8. Maki, M., Holm, K., Lipsanen, V., Hallstrom, O., Viander, M., Collin, P., Savilahti, E., and Koskimies, S. (1991) Serological markers and HLA genes among healthy first-degree relatives of patients with coeliac disease. *Lancet* **ii,** 1350–1353.
9. Maki, M., Holan, K., Koskimies, S., Hallstrom, O., and Visakorpi, J. K. (1990) Normal small bowel biopsy followed by coeliac disease. *Arch. Dis. Child.* **65,** 1137–1141.
10. Unsworth, D. J., Walker-Smith, J. A., and Holborow, E. J. (1983) Gliadin and reticulin antibodies in childhood coelic disease. *Br. Med. J.* **288,** 69,70.
11. Lock, R. J. and Unsworth, D. J. (1999) Identifying IgA deficient children and adults does not necessarily help the serological diagnosis of coeliac disease. *J. Paediatr. Gastroenterol. Nutr.* **28,** 81–83.
12. Unsworth, D. J., Kieffer, M., Holborrow, E. J., Coombs, R. R. A., and Walker-Smith, J. A. (1981) Association of IgA anti-gliadin with gut lesion in coeliac disease. *Clin. Exp. Immunol.* **46,** 286–293.
13. Unsworth, D. J., Leonard, J. N., McMinn, R. M. H., Swain, A. F., Holborow, E. J., and Fry, L. Anti-gliadin antibodies and small intestinal mucosal damage in dermatitis herpetiformis. *Br. J. Dermatol.* **105,** 563–568.
14. Unsworth, D. J., Holborow, E. J., Kumar, P. J., Ellis, A., and McConnell, R. B. (1981) Gliadin antigens and screening tests for coeliac disease. *Br. Med. J.* **288,** 69,70.

18

Whole Gut Lavage Fluid Analysis

A Minimally Invasive Method for Study of Mucosal Immunity and Inflammation

Subrata Ghosh, Anna Dahele, Hazel E. Drummond, Syed S. Hoque, Kenneth Humphreys, and Ian D. R. Arnott

1. Introduction

Studies of intestinal mucosal immunity and inflammation are limited by the relative inaccessibility of most of the small intestine. Any new method of studying mucosal immunity and inflammation in patients should be minimally invasive, cost-effective, and provide information not readily available using current methods. Gaspari et al. ***(1)*** described gut lavage with 3 to 4 L of nonabsorbable, commercially available polyethylene glycol (PEG)–based bowel cleansing fluid as a method for analyzing human intestinal secretions for antibody content. Peroral gastrointestinal (GI) lavage is widely used to cleanse the GI tract prior to colonoscopy, barium enema examination, or colonic surgery. Whole gut lavage fluid (WGLF) therefore often becomes available without subjecting a patient to any additional investigation. This method is also often more acceptable to healthy volunteers than almost any other method of studying small intestinal secretions. Over the past decade, we have found that WGLFs from patients (both adults and children) suffering from a variety of intestinal diseases contain immunoglobulins (Igs) and antibodies, hemoglobin, plasma-derived proteins, cytokines, inflammatory cells, and their granule-derived proteins and growth factors ***(2,3)***. In this chapter, we describe our experience of the use of WGLF to study mucosal immunity and inflammation.

From: *Methods in Molecular Medicine, Vol. 41: Celiac Disease: Methods and Protocols*
Edited by: M. N. Marsh

2. Materials

2.1. Lavage

The lavage ingredients (available commercially as Klean-Prep®, Norgine, Oxford, UK) are available in sachets. Each sachet contains: 59 g of PEG with a molecular weight of 3350; 1.45 g of NaCl; 1.63 g of $NaHCO_3$; 5.68 g of Na_2SO_4; and 0.75 g of KCl.

2.2. Reagents for Lavage Fluid Processing

1. Phosphate-buffered saline (PBS) (Sigma): 1 tablet dissolved in 200 mL distilled water.
2. Soybean–tryspin inhibitor (SBTI) (Sigma): 0.1% wt vol in PBS.
3. Disodium EDTA (EDH): add 11 g to 100 mL distilled water. Dissolve to form a saturated solution. Store at room temperature.
4. Phenylmethylsulphonylfluoride (PMSF) (Sigma): 200 *M* in 95% alcohol.
5. Sodium azide: 2% wt vol distilled water.

2.3. Reagents for WGLF IgG Assay

1. Carbonate/bicarbonate coating buffer: 0.05 *M*, pH 9.6, at 25°C (10 capsules in 1 L of distilled water; Sigma; cat. no. C-3041). Store at 4°C.
2. Coating antibodies: Goat antihuman IgG, Fc specific, affinity purified (Sigma; cat. no. I-2136).
3. Enzyme-linked immunosorbent assay (ELISA) wash: 0.9% saline with 0.05% Tween-20 (polyoxyethylene-sorbitan monolaurate; Sigma; cat. no. P-1379). Store at +4°C.
4. ELISA diluent: 0.9% saline with 0.05% Tween-20 and 1% adult bovine serum (ABS) (SAPU, Law Hospital, Carluke, Lanarkshire, Scotland; product no. S026-220).
5. Alkaline phosphatase (AP) conjugate: Goat antihuman IgG (Sigma; cat. no. A-9544).
6. Diethanolamine liquid: Working reagent of 100 mL of diethanolamine (BDH; no. 10393 4J), 0.102 g of magnesium chloride ($MgCl_26H_2O$), and 0.2 g of sodium azide (NaN_3) adjusted to pH 9.8 with 6 *N* HCl and made up to 1 L.
7. *p*-Nitrophenyl phosphate (Sigma; cat. no. 104-105 phosphatase substrate tablets; store at –20°C). One tablet is used per 5 mL of diethanolamine working reagent (5 mg/tablet).
8. Standard: protein calibrant SPS 01 (Protein Reference Unit). Store at 4°C for 1 mo when open. Top standard for IgG assay = 250 ng/mL.

2.4. Reagents for WGLF Albumin and α_1-Antitrypsin Assays

1. PEG 6000 reagent: 40 g of PEG 6000 with 6 g of Tris, 2 g of Tween-20, and 1 g of sodium azide, adjusted with dilute HCl to pH 7.0 and made up to 1 L with distilled water (reagents obtained from BDH, Lutterworth, UK, prod. no. 44271).
2. Antibody reagent: Just prior to analysis, dilute antihuman (sheep) albumin (SAPU; prod. no. S034-205) 1 in 40 with filtered PEG 6000 or dilute antihuman

(goat) α_1-antitrypsin (Protein Reference Unit, Central Antiserum Procurement Unit, Sheffield, UK) 1 in 40 with filtered PEG 6000.
3. Standard diluent: 9 g of sodium chloride, 48 g of PEG 3350 (Sigma), and 1 g of sodium azide in 1 L of distilled water.
4. Standard: Serum SPS 01 (Protein Reference Unit).

2.5. Reagents for WGLF Interleukin-8 Assay

1. CLB Pelikine Compact Human interleukin-8 (IL-8) ELISA kit (supplied by Eurogenetics, Hampton, UK). All vials are centrifuged at 3000*g* before use. Additional reagents used include the following:
2. Coating buffer: 0.1 *M* carbonate/bicarbonate buffer, pH 9.6.
3. Washing buffer: PBS/0.005% Tween-20.
4. Stop solution: 1.8 *M* H_2SO_4.
5. Substrate solution: K-blue substrate, Bionostics (North York, Ontario, Canada).
6. Blocking buffer: 500 µL of blocking reagent to 25 mL of PBS.

2.6. Reagents for WGLF Granulocyte Elastase

1. Assay buffer: 12.1 Tris with 56.2 g of NaCl dissolved in 800 mL distilled water, adjusted with approx 50 mL of 1 *N* Hcl to pH 8.3 and made with distilled water up to 1 L.
2. Substrate S-2484 (Quadratec, Epsom, UK). Dissolve 25 mg of substrate in 7 mL of dimethyl sulfoxide (DMSO). Further dilute this stock solution with 3 vol of distilled water for use in the assay.
3. 20% Acetic acid.
4. Triton X-100.

2.7. Reagents for IgA Antigliadin Antibody Assay

1. Diethanolamine (DEA) substrate buffer: 500 mL of concentrated DEA (BDH; Analar reagent product no. 10393 4J), 0.51 g of $Mg_2Cl_26H_2O$, 1.0 g of NaN_3, and 4 L of sterile water. Adjust pH to 9.8 with 6 *N* HCl.
2. *p*-Nitrophenylphosphate: 5 mg of phosphatase substrate tablets (Sigma; product no. 104-105).
3. AP substrate: Constitute fresh substrate 30 min before required. Dissolve 1 tablet of phosphatase substrate per 5 mL of DEA buffer.
4. Diluent (0.9% saline, 0.05% Tween-20, 1% adult bovine serum), adult bovine serum (SAPU; product no. S026-220).
5. Wash solution (0.9% saline + 0.05% Tween-20).
6. Carbonate-bicarbonate coating buffer (0.05 *M*, pH 9.6, at 25°C).

2.8. Reagents and Materials for WGLF IgA Antiendomysial Antibody

1. Collect umbilical cord as soon after delivery as possible, cut transversely into 5-mm tissue blocks, embed in embedding compound, and freeze rapidly using either cryospray or dry ice. Store the embedded tissue blocks in an ice cube tray

at –70°C. Send a sample of cord blood anonymously for human immunodeficiency virus (HIV) and hepatitis testing.
2. Acetone: Dried Analar grade, product no. 10475 (BDH, Merck, Leicester, UK). Chloroform: Analar grade, product no. 10077 6B, BDH, Merck.
3. Cryospray 22: Order code B1029, Bright Instrument (Cambridgeshire, UK). Embedding compound: Bright Cryo-M-Bed (113 mL), Bright Instrument.
4. Glycerol gelatin (Sigma; product no. GG-1).
5. PBS tablets (Sigma; product no. P4417).
6. Adult bovine serum albumin (ABSA) (SAPU; prod. no. S026-220).
7. ELISA diluent = 10 mL of ABSA + 500 µL of Tween-20 + 1 L of 0.9% NaCl.
8. Fluorescein isothiocyanate (FITC)-conjugated rabbit antihuman IgA (SAPU; prod. no. S173-201). Add 100 µL to 2 mL of diluent.
9. 0.01% Poly-L-lysine (Sigma; prod. no. P8920).

2.9. Reagents for WGLF Antitissue Transglutaminase Assay

1. Tissue transglutaminase (tTG) from guinea pig liver (Sigma; T5398) is available in dried powder form.
2. Carbonate-bicarbonate coating buffer (Sigma carbonate-bicarbonate buffer capsules—10 capsules in 1 L of distilled water—0.05 *M*, pH 9.6, at 25°C).
3. Immulon 2 plates (Dynex, Ashford, UK), ELISA Reader (Dynatech MR 5000).
4. PBS diluent (1 L of distilled water, 0.05% Tween-20).
5. tTG wash solution (1 L of 0.9% NaCl, 0.05% Tween-20), ELISA washer (Dynatech MRW).
6. BSA fraction V (Sigma BSA, prod. no. A-7906).
7. Sigma goat antihuman IgA (α-chain specific) AP conjugate.
8. DEA substrate buffer: 500 mL of DEA concentrated (BDH; Analar reagent product no. 10393 4J), 0.51 g of $Mg_2Cl_26H_2O$, 1.0 g of NaN_3, and 4 L of sterile water. Adjust pH to 9.8 with 6 *N* HCl.
9. AP substrate (*p*-nitrophenylphosphate; Sigma; prod. no. 104-105). Dissolve one tablet of phosphatase substrate per 5 mL of DEA buffer.

3. Methods

All assays (described in **Subheadings 3.3.–3.8.**) are performed on thawed, filtered, processed WGLF as described in **Subheading 3.2.**, unless otherwise specified.

3.1. Obtaining Whole Gut Lavage Fluid

One sachet is dissolved in 1 L of drinking water to give an osmolality of 260 mosm/L. After an overnight fast, the patients drink the gut lavage solution aiming for a rate of 250 mL every 15 min. This is supervised and monitored by an experienced nurse. All liquid stools and fecal stained fluids passed per rectum are discarded. Clear fluid, resembling urine, passed per rectum is collected. Generally the first clear fluid passed per rectum is processed as described below. The volume of such clear fluid required will depend on the number and

nature of assays planned, but generally 20–30 mL should suffice for most routine use. With this protocol the perfusion rate, after correction for addition of processing reagents, is 20 mL/min, i.e., 28.8 L/24 h (*see* **Note 1**).

The acceptability of whole gut lavage in 252 patients (age range 12–86 yr) was high, and the procedure was successful in providing a satisfactory specimen in 241 patients (95.6%). It is more often successful in patients aged 40 yr or older compared with younger patients (98.3% vs 88.2%; $p < 0.05$). The mean volume of fluid required was 3.9 L (SD, 1.4 L), and the mean duration of the procedure was 4.7 h (SD, 2.0 h) ***(4)***. The rate of output of effluent from the rectum significantly paralleled intake irrespective of the duration of the gut lavage ***(5)***. Concentrations of various proteins, such as IgG assayed in up to five sequentially collected specimens starting with the first clear effluent, showed no significant variation with time, thus confirming that peroral gut lavage under the conditions mentioned above is akin to a whole gut perfusion system. Assays of Igs (including IgG), albumin, and isotype-specific antibodies in matched samples of feces and WGLF from 20 patients showed that the amount of protein detected in feces varied from 0.01–35.5% (estimated daily output) of the amount known to be produced in the gut from results of assays on WGLF ***(6)*** (*see* **Notes 2** and **3**).

3.2. Lavage Fluid Processing

For most assays, WGLF is processed as described as follows by the addition of protease-inhibiting reagents. For a few assays, specimens are processed differently and these are described with the individual assay methods (*see* also **Notes 4** and **5**). WGLF should be processed within 10–15 min after obtaining the specimen to prevent degradation of unstable proteins.

1. A specimen of clear WGLF is filtered through Whatman GF/A 12.5 glass microfiber filters (Whatman, Maidstone, England). To 5 mL of the specimen, the following reagents are sequentially added with mixing after each addition (final concentration in parentheses):
2. 0.5 mL of soya bean trypsin inhibitor in phosphate-buffered saline (PBS) (80 μg/mL); 0.28 mL of sodium EDTA (BDH, Poole, England; cat. no. 10093) in PBS (15 m*M*); 0.12 mL of phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO; cat. no. 7626) in 95% ethanol (2 m*M*); 0.06 mL of sodium azide (1 m*M*), and leave for 2 min; and 0.3 mL of newborn calf serum (Sigma; cat. no. N4762; heat inactivated) (5% v/v).
3. Aliquots of processed WGLF (250 μL to 1 mL) are then stored at –70°C for later analysis in batches.

3.3. Immunoglobulin G

IgG is absorbed by class-specific antibodies bound to the solid phase (ELISA plate). An antiglobulin conjugate, which is enzyme labeled, is added to bind to

the specific Ig. Sample analysis for IgG is by ELISA using affinity-purified goat antihuman IgG (Sigma) and, as standard, human reference serum (Protein Reference Unit).

3.3.1. Quantitating IgG

Between batch coefficient of variation (CV) is 12.3% at 19.8 μg/mL. The upper limit of normal based on results for immunologically normal patients and volunteers is <10 μg/mL.

1. Coat the Immulon 1 flat-bottomed plate (Dynatech, UK) with a 1/5000 dilution of coating antibody in coating buffer and store overnight at 4°C.
2. The following day, wash the plates three times with ELISA wash.
3. Fill the wells with ELISA diluent and incubate at 20°C for 1 h.
4. All standard, sample, and quality control (QC) dilutions were made in ELISA diluent. The WGLF samples required a 1/25 dilution. A sample (processed and stored at –70°C) from a patient with active inflammatory bowel disease was used as QC (prior consent of the patient is required for testing of hepatitis B and HIV).
5. Drain the plates, load with samples, standard material, and QC, all in duplicate, and incubate overnight at 4°C.
6. The following day, wash the plates three times. Add the conjugate to all wells at a dilution of 1/5000 and incubate the plates for 3 h at room temperature.
7. Wash the plates another three times prior to addition of the substrate.
8. The plates should stand on the bench for 3 min prior to their placement on the shaker, with manual checking of the optical density (OD) of the top standard. When the OD reaches 1.0, read the plate on the Microplate Dynatech Reader MR 5000 at 405 nm.
9. Construct a standard curve and calculate the sample values. The correlation coefficient required is 0.985. The QC values also must fall within 95% of the current QC mean value for the rest of the samples to be accepted.

3.4. Albumin

The immunoturbimetric method is used and antihuman albumin was supplied by the Scottish Antibody Production Unit. When human albumin reacts with an antibody in the presence of PEG, precipitating immunocomplexes are formed that produce a turbidity when the antibody is in excess. This can be measured by flow through a spectrophotometer. Albumin in gut lavage is unstable prior to processing and 10–80% may be lost within 2 h from the production of the specimen. Hence, it is important to process the WGLF within 10–15 min after obtaining the specimen.

3.4.1. Quantitating Albumin

1. Centrifuge filtered, processed WGLF samples at 1500*g* for 10 min. Using a Dilugil dispenser, dilute 0.05 mL of diluent, standard, and test samples in dupli-

Table 1
Detection of Cytokines and Adhesion Molecules in WGLF

Detectable in WGLF	Not reliably detectable in WGLF
IL-1β, IL-8, IL-1RA, sIL2R, ICAM-1	TNF-α, IL-5, IL-10, IFN-γ, IL-6, TGF-β,

cate into two rows of blank and tests with 0.95 mL of PEG reagent in 2-mL polystyrene tubes (LIP, Shipley, UK).
2. Read turbidity at 340 nm after 15 min and plot the standard curve after blank subtraction. QC used is the same as in the IgG ELISA (*see* **Subheading 3.3.1., item 4**).
3. Repeat analysis within a batch gives a CV of 8.5%. Patients' results are accepted only if the QC value is within the 17% range of the expected value. The upper limit of normal based on results from immunologically normal patients and adults is <26 μg/mL.

3.5. α_1-Antitrypsin

The immunoturbimetric method is used and antihuman α_1-antitrypsin was obtained from the Protein Reference Unit. The principle is identical to the albumin assay described in **Subheading 3.2.** α_1-Antitrypsin is stable in lavage fluid for up to 2 h prior to processing.

3.5.1. Quantitating α_1-Antitrypsin

1. The procedure for quantitating α_1-antitrypsin is identical to that described for albumin assay (*see* **Subheading 3.2.1.**).
2. Repeat analysis within a batch gives a CV of 10%, and the sample results are accepted only if the QC value is within the 20% range. The upper limit of normal from results obtained in immunologically normal patients and volunteers is <19 μg/mL. The clinical usefulness of lavage protein measurement has been reported by us previously *(7,8)*.

3.6. Cytokines

Proinflammatory cytokines such as IL-1β and chemokines such as IL-8 are produced in an inflamed gut epithelium, and hence raised WGLF concentrations of these cytokines are a feature of active inflammatory bowel disease. **Table 1** presents our experience in detecting common cytokines in WGLF. Assays to detect some of the common cytokines are described in the following sections.

3.6.1. Interleukin-1β

IL-1β can be detected using a commercial ELISA kit and filtered, processed WGLF. The assay is run in duplicate. Since the WGLF test samples contain

PEG, modifications to the assay are made as described in **Subheadings 3.6.1., item 1** and **3.6.1., item 2** to make test samples and standards equivalent. A commercial sandwich ELISA kit (Cistron Biotechnology, Pine Brook, NJ, 03-HS96, supplied by Eurogenetics) is used and the manufacturer's recommendations are followed for preparation of the reagent.

3.6.1.1. Quantitating IL-1β

1. The plates are supplied ready coated. Prepare the standards in a 50:50 solution of PEG (with added protease inhibitors as in **Subheading 3.2.**): PBS/Tween-20, 0.02%. Place 100 μL of each standard concentration in the appropriate wells.
2. Fill the sample wells with 50 μL of PBS/Tween-20 followed by 50 μL of thawed test sample of WGLF. The samples are thus in the same matrix as standards but now diluted 1 in 2.
3. Use a QC WGLF sample from a patient with active ulcerative colitis with each plate.
4. Then incubate the plates for 1 h (37°C), wash three times, and add 100 μL of polyclonal anti-IL-1β antiserum (rabbit).
5. After further incubation for 20 min (37°C) and washing three times, add 100 μL of antirabbit IgG-horseradish peroxidase.
6. Leave the plates at room temperature for 20 min, and after a further three washes, add a peroxidase substrate system (100 μL).
7. We found that less than 10 min of reaction time is adequate. Then, stop the reaction by adding 50 μL of 4 *N* sulfuric acid in each well and read the plate at 450 nm within 15 min.
8. The lower limit of detection was 2 pg/mL and the interassay CV was 12%. The normal range was calculated from a group of patients with irritable bowel syndrome or constipation with immunologically normal gut who underwent whole gut lavage. The normal range was taken as 2 SDs of the mean in this group and was calculated as 2–12 pg/mL. **Figure 1** depicts WGLF IL-1β concentrations in a cohort of patients presenting with gastrointestinal symptoms.

3.6.2. Interleukin-8

The process of filtration significantly reduces the concentration of IL-8, and hence an unfiltered specimen, processed as in **Subheading 3.2.** by the addition of protease inhibitors, is preferable for assay of IL-8. The assay is run in duplicate, and a commercial ELISA kit with minor modifications is used.

3.6.2.1. Quantitating IL-8

The lower limit of detection of the assay is 3.0 pg/mL, and the normal range from the same immunologically normal gut cohort as in **Subheading 3.2.** is 3–60 pg/mL. **Figure 2** presents WGLF IL-8 concentrations in a large cohort of patients presenting with GI symptoms.

1. Add a solution of coating antibody (100 μL) to each well and incubate the plate overnight at room temperature.

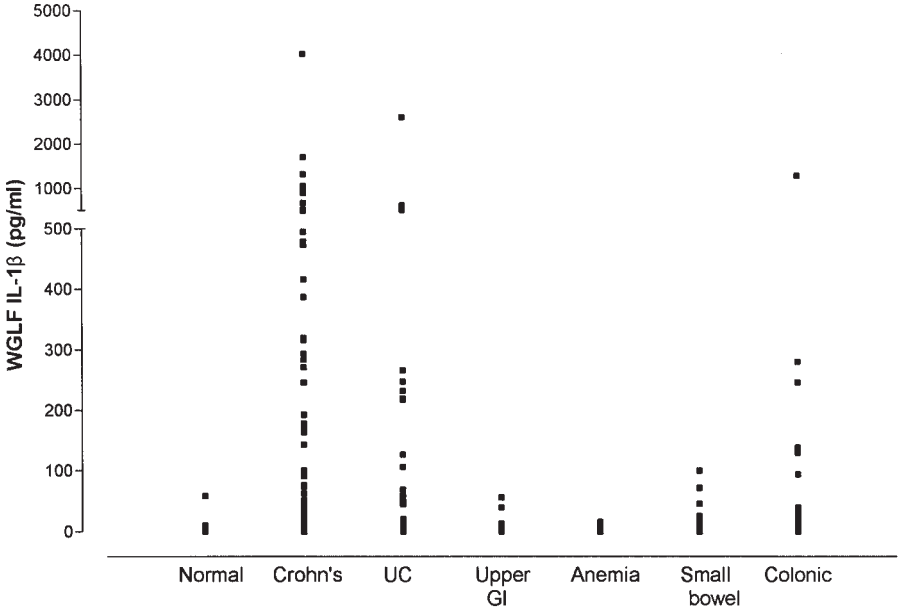


Fig. 1. WGLF IL-1β concentrations in a cohort of patients presenting with GI symptoms.

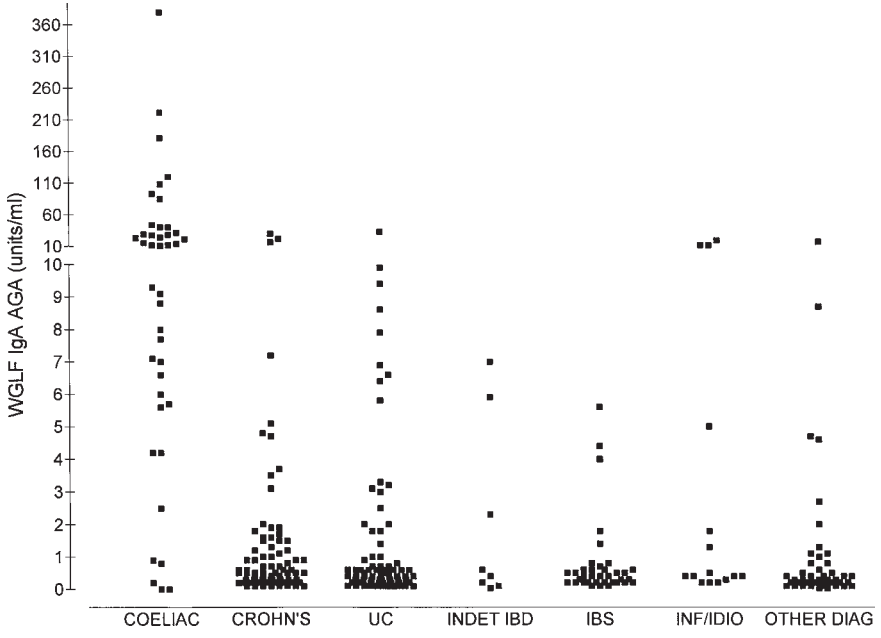


Fig. 2. WGLF IL-8 concentrations in a cohort of patients presenting with GI symptoms.

2. Wash the plate with PBS alone five times and then add 200 µL of blocking buffer followed by further incubation (1 h at room temperature) and washing five times.
3. Prepare the standards in a 50:50 solution of kit diluent and PEG (with added protease inhibitors as in **Subheading 3.2.**). Add 100 µL of each standard to the wells.
4. Add 50 µL of kit diluent to each well and add 50 µL of the thawed WGLF test sample. This modification keeps the conditions in the test sample and standard wells as uniform as possible. In this assay, samples have to be prediluted 1/5 before use. The rest of the assay follows the kit's guidelines. However, again we have found that less than 15 min of final incubation time is necessary before the addition of stop solution.

3.6.3. Eotaxin (Eosinophil-Specific Chemokine)

Eotaxin is determined on filtered, processed WGLF using a high-sensitivity human eotaxin immunoassay kit (R&D Systems, Abingdon, Oxon, UK, cat. no. DTX00). The principle is the same as for the sandwich ELISA described in **Subheading 3.6.2.1.**

3.6.3.1. Quantitating Eotaxin

The interassay CV is 12%. The lower limit of detection of the assay is 5 pg/mL.

1. Prepare standards in 0.02% PBS/Tween-20/1% ABS.
2. Prepare sample diluent as 10 mL (75% PBS/Tween-20/ABS) by adding 5 mL of PBS/Tween-20/ABS to 5 mL of 50:50 PBS/Tween-20/ABS:PEG with protease inhibitors.
3. Prepare standard diluent as 4 mL (75% PEG with protease inhibitors) by adding 2 mL of PEG (with protease inhibitors) to 2 mL of 50:50 PBS/Tween-20/ABS:PEG with protease inhibitors.
4. Blanks = PBS/Tween-20/ABS 50 µL + 100 µL of standard dilution.
5. Add 100 µL of standard diluent and 100 µL of sample diluent to the standard and sample wells, respectively.
6. Add 50 µL of standard or test WGLF sample to the appropriate wells and cover the microplate at room temperature for 2 h.
7. After washing three times, add 200 µL of eotaxin conjugate to each well, cover, and incubate for 1 h at room temperature.
8. After washing three times, add 200 µL of substrate solution to each well and incubate for 30 min.
9. Add 50 µL of stop solution to each well and read within 30 min at 450 nm.

3.7. Granulocyte-Derived Enzymatic Markers

Cytology of WGLF can detect neutrophils and eosinophils derived from inflamed mucosa and other cells derived from the epithelial lining and lumen such as enterocytes and bacteria ***(9)***. Cytology, however, needs to be performed on fresh specimens and is laborious. An experienced cytologist

is also required. Assaying WGLF for a granulocyte-derived enzyme has been shown to correlate well with WGLF cell count ***(10)*** in patients with active inflammation and hence serves as a useful surrogate marker of luminal migration of neutrophils and eosinophils.

3.7.1. Granulocyte Elastase

Filtration removes granulocytes, and protease inhibitors interfere with the detection of elastase. Hence, it is appropriate to use unfiltered, unprocessed WGLF for the detection of granulocyte elastase (GE). Unlike plasma, gut-derived GE is not complexed with α_1-antitrypsin, and hence ELISA methods that detect this complex are not appropriate for use with WGLF. GE activity is determined by its amidolytic effect on the substrate pyroGlu-Pro-Val-pNA. The released pNA is measured photometrically at 405 nm after the reaction has been stopped with acetic acid.

3.7.1.1. Quantitating GE

GE is assayed by an enzyme-substrate reaction using a specific chromogenic substrate *p*-pyroglutamyl-L-propyl-L-valine-*p*-nitroanilide. We used to sonicate each sample at 0°C for 1 min three times to disrupt the granulocytes and release the enzyme ***(10)***. However, we have discovered that sonication reduces the concentration of GE detectable, perhaps by denaturation of the protein. Hence, we have now abandoned this practice. Each laboratory needs to establish normal ranges (*see* **Notes 6** and **7**), but GE is generally undetectable in subjects with an immunologically normal gut. Detection of high concentrations of GE is a feature of active colonic inflammation, but not active small bowel inflammation ***(10)***.

1. Dissolve the substrate in DMSO and dilute in water to give a concentration of 2 mmol/L. Mix 1 µL of Triton-X (Sigma) into 600 µL of thawed, unfiltered, unprocessed WGLF test sample in a plastic (LIP, cat. no. 912700) (*see* **Note 8**).
2. Add 250 µL of test sample to 250 µL of buffer (Tris 0.1 mol/L, NaCl 0.96 mol/L, pH 8.3), and warm the mixture at 37°C for 2 to 3 min.
3. Add 250 µL of substrate at 37°C, and after exactly 3 min stop the reaction with 250 µL of acetic acid.
4. Run blank tubes for all samples in an identical way except add the acetic acid prior to the substrate.
5. Use a crude extract of human granulocytes from blood as positive control.
6. Measure absorbance at 405 nm in a spectrophotometer using 1-cm semi-microcuvets. The amount of GE activity present in each sample is calculated as follows: (Test A405 – blank A405) × 2.31 µkat/L. (The correlation between absorbance and GE activity is linear in the 0.1–1.5 µkat/L range.)

3.7.2. Eosinophil Cationic Protein

A commercial double antibody radioimmunoassay (RIA) kit is used where in which eosinophil cationic protein (ECP) in the sample competes with a fixed amount of ^{125}I labeled ECP for the binding sites of specific antibody. Bound and free ECP are separated by the addition of a second antibody immunosorbent followed by centrifugation and decanting. The radioactivity in the pellet is then measured and is inversely proportional to the quantity of ECP in the sample. Unfiltered, but processed WGLF is used without dilution. The assay is performed with the ECP RIA, Kabi Pharmacia (Uppsala, Sweden).

3.7.2.1. Quantitating ECP

1. Samples and standards are assayed in duplicate. Add 50 μL of standards and samples are added to polystyrene centrifuge tubes.
2. Add 50 μL of ECP^{125}I (blue) and 50 μL of anti-ECP (yellow) to the tubes (resultant green color), mix, cover, and incubate for 3 h at room temperature.
3. Mix the decanting fluid well and add 2 mL with further incubation for 30 min at room temperature.
4. Centrifuge all tubes at 1500*g* for 10 min and immediately decant. Leave the tubes to stand for 30 s upside down on absorbent paper.
5. Then measure the radioactivity of the pellet and express the mean count of samples and standards as a percentage of the mean counts of the zero standard. Construct a standard curve and read the concentration of the unknown samples from the standard curve.

3.8. IgA and IgM: Total and Specific Antibodies

IgA and IgM assays are similar in principle to that described for WGLF IgG. The standard for IgA is human IgA purified Ig from colostrum (Sigma; I-2636) at top standard = 1000 ng/mL. The coating antibody used for IgA is goat antihuman IgA, α-chain specific (Sigma; no. I-2261), and the conjugated antibody is goat antihuman IgA, AP conjugated (Sigma; A3036). Standard for the IgM ELISA is SPS 01 (Department of Immunology) at top standard 1000 ng/mL. The coating antibody used is goat antihuman IgM, μ-chain specific (Sigma; no. I-2386), and the conjugated antibody is goat antihuman IgM, AP conjugated (Sigma; no. A3437).

3.8.1. IgA Antigliadin Antibody

Gut mucosal production of IgA antigliadin antibody (AGA) can be confirmed by using an indirect ELISA technique to estimate the antigen-specific Ig in WGLF measured against a known high-titer human specimen.

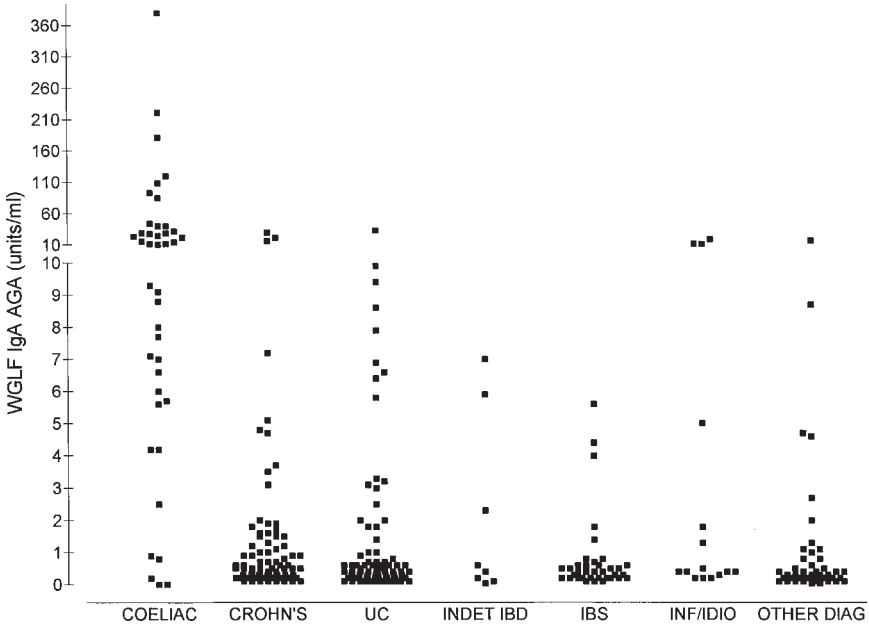


Fig. 3. WGLF IgA AGAs in GI diseases.

3.8.2.1. Quantitating IgA AGA Antibody

Celiac WGLF samples have significantly higher levels of IgA AGA than disease controls (*see* **Fig. 3**).

1. Coat Immulon 2 flat-bottomed γ-irradiated plates with gliadin dissolved in coating buffer (25 μg/mL) (Gliadin extract, Sigma, cat. no. G-3375). Dispense 100 μL of coating solution to each well of the ELISA plate and incubate at 22°C for 5 h (*see* **Notes 9** and **10**).
2. Either manually or using the Multi-Reagent Washer, wash the plate three times and then add 250 μL of diluent followed by incubation for 2 h.
3. For WGLF IgA, standard should be doubly diluted (in duplicate) to obtain a linear curve (*see* **Note 11**).
4. High and low serum QC should be used at 1/100.
5. Test WGLF samples should be diluted at 1/2 and 1/4 (in duplicate), added to the appropriate wells, and incubated overnight in a covered, moist box.
6. Wash and add 100 μL of conjugate to each well and incubate at 22°C for 5 h.
7. Wash and dispense 100 μL of substrate to each well of the ELISA plate. The absorbance end point for IgA is when the top standard reaches 1.0. The test results

are technically acceptable if the QC absorbance values lie within 95% of the current QC absorbance values and the duplicate values differ by no more than 10% (*see* **Notes 12** and **13**).

3.8.3. IgA Antiendomysial Antibody

IgA class antiendomysial antibodies are highly sensitive and specific for diagnosis of celiac disease while patients are on a normal gluten-containing diet. Mucosal production of IgA class antiendomysial antibodies can be detected by WGLF analysis. Antiendomysial antibodies can be detected using smooth muscle tissue of primates or humans. Original work used primate esophagus, but we use human umbilical cord. The test sample is added to human umbilical cord and the antigen/antibody complex in the smooth muscle surrounding the blood vessels is reacted with anti-IgA conjugated with FITC. The complex is visualized using a fluorescence microscope. Reporting is qualitative.

3.8.3.1. Assaying WGLF IgA Antiendomysial Antibody

1. Cut 8-μ thick cryostat sections of human umbilical cord and place on microscope; multispot slides precoated with poly-L-lysine. Store at –20°C, wrapped in aluminum foil.
2. Bring precryostatted sections to room temperature for 30 min and unwrap. Fix the sections by placing the slides into a Coplin jar containing precooled dried acetone and leave for 10 min at –20°C.
3. Transfer the sections immediately to a Coplin jar containing chloroform and leave for 30 min at room temperature.
4. Remove the sections and allow to dry at room temperature. Draw a line between each section on the slide using a PAP pen (Sigma, cat. no. Z377821) to create a wax barrier and prevent cross contamination.
5. Add sufficient diluent to cover each section to block nonspecific binding. Leave for 10 min in a damp staining box. Drain the slides and dry between each section with a tissue.
6. Add 50 μL of test WGLF diluted 1:4 with ELISA diluent to each section. Leave for 60 min in a damp staining box at room temperature.
7. Rinse the sections in phosphate-buffered saline (PBS) and wash in a Coplin jar on a rocking table. Add 50 μL of prepared conjugate to each section. Leave for 60 min and protect from light.
8. Place a bottle of glycerol gelatin on a hot plate to liquify. Place the slides into a Coplin jar containing PBS and rinse with running water for 5 min. Dry each slide and mount onto cover slips with glycerol gelatin.
9. View the slides using the ×10 objective lens. Endomysial antibodies attached to the connective tissue surrounding the muscle cells fluoresce brightly in a honeycomb pattern. This specific fluorescence indicates a positive result. A minimum of one positive and one negative control must be included in each batch (*see* **Notes 14–16**).

3.8.4. Antitissue Transglutaminase Antibody

tTG has been recently found to be the autoantigen with which antiendomysial antibodies interact in celiac sera ***(12)***. The enzyme is highly conserved among the species and is widely distributed in body fluids and tissues including the gut submucosa ***(13)***. Homogenized guinea pig liver is the main source of the enzyme and shares 80% sequence homology with the human molecule ***(14)***. A recombinant human form of the enzyme has been developed that is reported to have a higher sensitivity than the guinea pig source; however, this recombinant form is not currently available commercially ***(15)***. tTG catalyzes the reaction between protein-bound glutamine and lysine residues forming an ε-(γ-glutamyl)lysine bond and releasing ammonia ***(14)***. These bonds are quite resistant to proteolysis, thereby increasing the resistance of tissues to injury ***(16)***. It has been shown that human jejunal tTG enzyme activity is maximal at a pH of 9.0 and has an absolute requirement for calcium (optimal activity at 10 mmol/L). Untreated celiac patients have an increased jejunal TG activity ***(17)***. Gliadin is a favored substrate for the enzyme because it contains about 40% of glutamine residues. The gliadin-tTG interaction proposed by Molberg et al. ***(18)*** provides a plausible mechanism for the immunologically mediated damage in celiac mucosa. However, tTG also has an affinity for fibronectin, fibrin, and type 1 collagen and therefore could bind to these extracellular matrix proteins after its release from injured cells ***(19)***.

This finding has led to the development of a semiquantitative ELISA technique for detecting tissue antibodies in celiac patients. Dietary gliadin undergoes peptin-trypsin digestion and may then interact with submucosal tTG to produce predominantly IgA class anti-tTG antibodies in genetically susceptible individuals. WGLF provides an ideal material for an ELISA method to study the secretion of these antibodies into the gut lumen.

3.8.4.1. Quantitating Anti-tTG Antibody

The test results are technically acceptable if the QC absorbance values lie within 95% of the current QC absorbance values and the duplicate values differ by no more than 10% (*see* **Notes 17–20**). WGLF anti-tTG antibodies are significantly higher in untreated celiac patients than in other GI diseases (*see* **Fig. 4**).

1. Coat Immulon 2 flat-bottomed γ-irradiated plates with 100 µL per well of 10 µg/mL solution of Sigma guinea pig liver transglutaminase (cat. no. T5398). Calculate the protein content from the units/milligram protein data supplied (*see* **Notes 21** and **22**). Incubate overnight (approx 15 h) at 4°C.
2. Either manually or using the Multi-Reagent Washer, wash the plate three times.

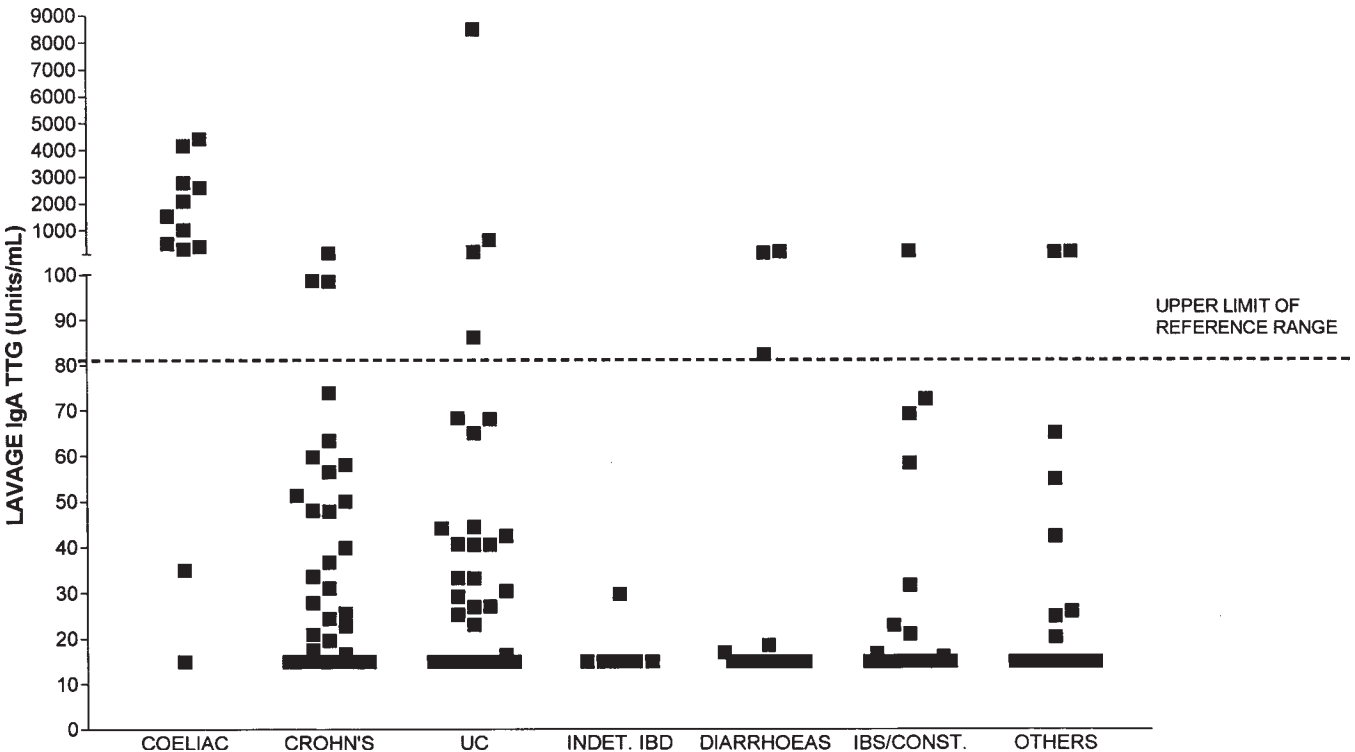


Fig. 4. WGLF IgA antitissue transglutaminase antibody in GI disease.

3. Add 250 μL per well of blocking solution (PBS, 1% BSA, 0.05% Tween-20) followed by incubation for 2 h at 24°C. Then freeze the plates at –20°C until use.
4. A serum reference standard (an untreated celiac patient with high antibody levels) should be doubly diluted from 1/50 (in duplicate) to obtain a linear curve. High and low serum QCs should be diluted in PBS diluent at 1/50 and 1/100 (in duplicate).
5. Test WGLF samples (filtered, processed) should be diluted at 1/2 and 1/4 (in duplicate), added to the appropriate wells, and incubated overnight in a covered, moist box at 4°C.
6. Wash and add 100 μL of 1:800 dilution of conjugate in PBS diluent to each well and incubate at 22°C for 5 h.
7. Wash and dispense 100 μL of substrate to each well of the ELISA plate. The absorbance end point for IgA is when the top standard reaches 1.0.

3.9. Summary

Both gut protein loss in mucosal inflammation and local mucosal secretion of cytokines and antibodies can be detected and studied by the technique of WGLF analysis. This technique can be easily combined with colonoscopy or barium enema, but an experienced nurse is usually required to monitor the patient. The information obtained can be used to exclude active inflammation as a cause of symptom in complex cases of inflammatory bowel disease or to exclude a GI cause of blood loss (by detection of hemoglobin). Use of this technique has clearly shown the dissociation between systemic and mucosal humoral immune responses in celiac disease ***(20)***. Complement components and leukotrienes such as LTB_4 are also detectable in WGLF.

4. Notes

1. If the WGLF is hazy, it may be worthwhile to centrifuge at 1400*g* for 5 min prior to filtering. This will speed up filtration. If the fluid is still hazy, it is unsuitable for analysis. If possible, the gut lavage should be continued till the effluent becomes clear.
2. For patients finding difficulty in drinking the volume recommended, a nasogastric tube may well make the procedure easier. The lavage solution (Klean Prep) should be administered as bolus through the nasogastric tube using a syringe at the rate of 200 mL every 12 min.
3. Nausea can generally be dealt with by slowing the lavage rate for 30 min and administering metoclopramide (10 mg) orally. Frank vomiting is uncommon if the patient has fasted overnight as recommended.
4. For unfiltered, unprocessed WGLF sample (e.g., for GE assay), 20 μL of sodium azide was added to a 1-mL aliquot of lavage fluid before storage at –70°C. The lavage fluid is neither filtered nor treated with protease inhibitors. Though granulocytes may be identified by cytology in such a sample, we have consistently failed to identify lymphocytes.

5. For unfiltered but processed WGLF (e.g., for IL-8 assay), 5 mL of unfiltered fluid should be treated exactly as described in **Subheading 3.2.** for filtered WGLF, i.e., the protease inhibitors added.
6. We have found some variability in the results obtained using different batches of the chromogenic substrate S-2484. It would therefore be advisable to ensure that sufficient substrate of a certain batch is obtained initially to allow completion of a proposed study.
7. The WGLF GE assay is extremely temperature sensitive. The same reliable thermometer should be used consistently.
8. For GE assay, Triton X-100 is used to aid disruption of cell membranes and also reduce the nonspecific adherence of GE to the assay tubes. The precise concentration of Triton X-100 is not critical, but its presence is essential for optimum GE availability in the assay.
9. Gliadin is difficult to dissolve and heating does not hasten this process. We have found that overnight mixing is desirable.
10. Gliadin-coated plates are stable frozen at –20°C in a covered box, and it is most convenient to make sufficiently large batches to last at least 1 mo.
11. Serum standard and QC material may be used for the AGA assay instead of WGLF because antibody levels in lavage fluid may not be stable for prolonged periods and supplies of WGLF may not be sufficient. It also provides the same reference material to compare paired serum and lavage AGA titers.
12. For most assays test samples and reference material should be aliquoted and used only once if possible. Aliquot tubes should be wide enough to permit easy pipetting of small volumes. Approximately 200 μL is adequate to perform both the AGA and antiendomysial antibody assay.
13. For AGA assays, all high samples tested at the initial dilution of 1/2 and 1/4 should be diluted to reach the end point.
14. It is recommended that two experienced individuals read all antiendomysial antibody slides independently and blinded to the final diagnosis (if available) because the interpretation is subjective.
15. Antiendomysial antibody slides should be labeled with the date and a list of test samples. They can be kept for many months for future reference but should be stored covered and in a dark room.
16. The long incubation periods during the antiendomysial antibody assay permit the AGA assay to be performed in parallel on the same day, thereby conserving both lavage test material and time.
17. There is no significant difference in the results if the anti-tTG ELISA is performed in the morning or afternoon, i.e., after 16 or 20 h coating.
18. WGLF IgG anti-tTG is not detectable using this method, which is not surprising because we have observed that celiac patients seldom have an elevated WGLF total IgG.
19. It is valid to use a serum standard and QC material for the determination of WGLF anti-tTG antibodies because the dilution curves are parallel (*see* **Fig. 5**).

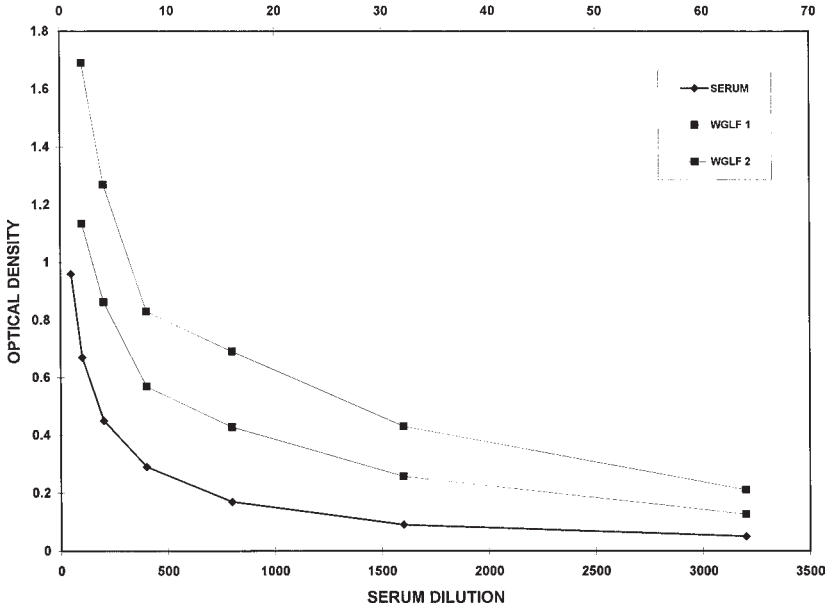


Fig. 5. Serum vs WGLF IgA antitissue transglutaminase antibody OD readings.

20. Other tTG ELISA methods using serum have been described ***(19)*** in which 5 mmol/L of $CaCl_2$ is added during the coating stage and BSA is omitted during the blocking stage. This is thought to increase the sensitivity of the assay. In our experience, adding calcium reduces the OD readings at our coating pH of 9.6, but increases the OD readings if coating is performed at pH 7.5. Calcium is not needed to detect anti-tTG antibodies, and its use in the anti-tTG ELISA is pH dependent.
21. For anti-tTG ELISA, tTG protein content may vary from batch to batch. It is necessary to calculate the milligrams/protein content of each batch before use. It would be wise to purchase a sufficient supply of a single batch, to last for many months.
22. Antigen preparation: Sigma guinea pig liver tTG is currently available in 0.5-, 1-, and 2-U bottles. A 2-U bottle is sufficient to coat nine plates. Storing the antigen prediluted in coating buffer is not suitable because the reconstituted enzyme is not stable. Batches of plates can be prepared and stored at –20°C for up to 1 mo without loss in OD readings, although, in practice, it may be wise to make fresh batches weekly.

References

1. Gaspari, M. M., Brennan, P. T., Soloman, S. M., and Elson, C. O. (1988) A method of obtaining, processing, and analysing human intestinal secretions for antibody content. *J. Immunol. Methods* **110,** 85–91.
2. Ferguson, A., Ghosh, S., Handy, L. M., Choudari, C., Mwantembe, O., and McIntyre, M. A. (1994) Analysis of disease distribution, activity and complications in the patient with inflammatory bowel disease. *Scand. J. Gastroenterol.* **29(Suppl. 203),** 15–19.
3. Ferguson, A. and Ghosh, S. (1998) Mucosal immunity and immune-mediated injury in Crohn's disease: relevance of disease distribution, in *Systemic Manifestations of IBD: The Pending Challenge for Subtle Diagnosis and Treatment* (Lembcke, B., Kruis, W., and Sartor, R. B., eds.), Kluwer Academic, Lancaster, UK, pp. 306–312.
4. Ferguson, A., Ghosh, S., and Brydon, W. G. (1996) Use of whole gut lavage fluid to measure occult GI bleeding and protein-losing enteropathy. *Z. Gastroenterol.* **34(Suppl. 4),** 60–64.
5. Ferguson, A., Sallam, J., O'Mahoney, S., and Poxton, I. (1995) Clinical investigation of gut immune responses: review on non-parenteral vaccines. *Adv. Drug Delivery Rev.* **18,** 53–71.
6. Ferguson, A., Croft, N., and Humphreys, K. (1995) Technical report: results of immunological tests on faecal extracts are likely to be extremely misleading. *Clin. Exp. Immunol.* **99,** 70–75.
7. O'Mahoney, S., Choudari, C. P., Barton, J. R., Walker, S., and Ferguson, A. (1991) Gut lavage fluid proteins as markers of activity of inflammatory bowel disease. *Scand. J. Gastroenterol.* **26,** 940–944.
8. Choudari, C. P., O'Mahoney, S., Brydon, G., Mwantembe, O., and Ferguson, A. (1993) Concentrations of immunoglobulin G, albumin and alpha-1 antitrypsin in whole gut lavage fluid: objective measures of disease activity in inflammatory bowel disease. *Gastroenterology* **104,** 1064–1071.
9. Handy, L. M., Ghosh, S., and Ferguson, A. (1995) Investigation of neutrophil migration into the gut by cytology of whole gut lavage fluid. *Eur. J. Gastroenterol. Hepatol.* **7,** 53–58.
10. Handy, L. M., Ghosh, S., and Ferguson, A. (1996) Investigation of neutrophils in the gut lumen by assay of granulocyte elastase in whole-gut lavage fluid. *Scand. J. Gastroenterol.* **31,** 700–705.
11. Barclay, G. R. (1990) Antibodies to endotoxin in health and disease. *Rev. Med. Microbiol.* **1,** 133–142.
12. Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E., and Schuppan, D. (1997) Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat. Med.* **3(7),** 797–801.
13. Ichinose, A., Bottenus, R., and Davie, E. (1990) Structure of transglutaminases: minireview. *J. Biol. Chem.* **265(23),** 13,411–13,414.
14. Folk, J. (1972) Structure and catalytic properties of hepatic transglutaminase. *Ann. NY Acad. Sci.* **202,** 59–76.

15. Sblattero, D., Berti, I., Trevisiol, C., Marzari, R., Bradbury, A., Not, T., Fasano, A., and Ventura, A. (1999) Human tissue transglutaminase ELISA: a powerful mass screening diagnostic assay for coeliac disease. Abstract in *Proceedings of the Eighth International Symposium on Coeliac Disease*, Jean Gilder Congressi, Naples, Italy, p. 34.
16. Greenberg, C., Birckbichler, P., and Rice, R. (1991) Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues. *FASEB J.* **5,** 3071–3077.
17. Bruce, S., Bjarnason, I., and Peters, T. (1985) Human jejunal transglutaminase: demonstration of activity, enzyme kinetics and substrate specificity with special relation to gliadin and coeliac disease. *Clin. Sci.* **68,** 573–579.
18. Molberg, O., McAdam, S., Korner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Noren, O., Roepstorff, P., Lundin, K., Sjostrom, H., and Sollid, L. (1998) Tissue transglutaminase selectively modifies gliadin peptides that are recognised by gut-derived T cells in celiac disease. *Nat. Med.* **4(6),** 713–717.
19. Sulkanen, S., Halttunen, T., Laurila, K., Kolho, K., Korponay-Szabo, R., Sarnesto, A., Savilahti, E., Collin, P., and Maki, M. (1998) Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. *Gastroenterology* **115,** 1322–1328.
20. O'Mahoney, S., Arranz, E., Barton, J. R., and Ferguson, A. (1991) Dissociation between systemic and mucosal humoral immune responses in coeliac disease. *Gut* **32,** 29–35.

Index

METHODS IN MOLECULAR MEDICINE™

Series Editor: *John M. Walker*

Celiac Disease

Methods and Protocols

Edited by

Michael N. Marsh, MD, DSc, FRCP

Department of Medicine, University of Manchester, Salford, UK

With the recent development of powerful bioanalytical techniques, research on gluten sensitivity has entered an exciting new phase. In *Celiac Disease: Methods and Protocols,* Professor Michael N. Marsh, a recognized world authority on this condition, together with a team of other expert laboratory/clinical investigators from around the world, present a collection of these state-of-the-art techniques for studying the biology and immunopathology of celiac disease. Both novice and experienced researchers will find in this collection detailed step-by-step methods for cloning lymphocytes, creating gene/peptide libraries, and performing genotyping, linkage, and positional cloning. Also included are techniques for determining the peptide structure of HLA-bound material (tandem mass spectroscopy), computerized morphometry, *in situ* hybridization, organ culture, and monoclonal AB assays.

Comprehensive and cutting-edge, *Celiac Disease: Methods and Protocols* provides all experimental and clinical investigators with the essential core of readily reproducible methods necessary for successful work on celiac disease today.

Features

- **Comprehensive collection of biomedical, molecular, and genetic techniques**
- **Detailed step-by-step methods ensuring experimental success and robust results**
- **Numerous hints on potential pitfalls and how to avoid them**
- **The first complete collection of cutting-edge methods for the study of celiac disease**

Contents

ISBN 0-89603-650-2

9 0 0 0 0 >

9 780896 036505

Methods in Molecular Medicine™
Celiac Disease: *Methods and Protocols*
ISBN: 0-89603-650-2