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PREFACE

Working with my collaborators, contributors, and colleagues on these volumes is a continuous joy and learning experience. The scope and scholarship of all of the segments connected with the creation and production of this series are personally and professionally rewarding. The recent association with two renowned and talented regional associate editors, along with a distinguished editorial board, continues to be an invaluable asset in providing a sustained quality publication.

This volume has several foci. The six chapters consist of chapters on two different aspects of apoptosis, a chapter on DNA genotyping, one on noncompetitive immunoassay for small molecules, a review of CSF fluid, and a review of biochemical hematology. It was our editorial intent to present the science and reflect the style as well as the philosophies of each author.

As the boundaries of this discipline move ever outward, the editors will try to continually meet the practical and scholarly interests of our readership. The next volume is already in preparation. Our objective is to maintain a mission of relevance and responsiveness. As has been our policy, we invite inquiry, suggestions for future chapters, and possible authorship from the worldwide audience these volumes are privileged to enjoy.

I once again thank my wife, Joanne, my friend and greatest advocate. I also acknowledge the very able staff of Academic Press. Their help, abilities, and cooperative spirit maximize the creation and production of all of the elements that constitute each of these volumes.

HERBERT E. SPIEGEL

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CEREBROSPINAL FLUID

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1. Introduction

Cerebrospinal fluid (liquor cerebrospinalis, CSF) belongs to the class of transcellular fluids. It has a low cell count and is hypooncotic and isoosmotic, even with the different composition of ions relative to plasma (lower concentration of Na^+, K^+, Ca^{2+}, inorganic phosphates, and HCO^- in contrast to a higher concentration of Mg^{2+} and Cl^-). The ion composition of CSF is stable; changes occur with large plasmatic variations.

CSF fills the intracerebral (intraventricular; 20%) and extracerebral (subarachnoidal; 80%) space. CSF originates from plasma (ultrafiltration) as well as from choroid plexus (active secretion) in the ventricles, flows through cisternae and the subarachnoid space, and finally drains through the arachnoid villi into venous blood. Equilition processes establish a physiological ratio between composition and resorption of CSF. CSF flow starts around the time of birth and reaches its maximum rate at four months after birth, following the complete maturation of the arachnoid villi.

The function of CSF includes:

- mechanical protection of the brain and spinal cord
- a drain function of the CNS (like the lymphatic system for removing metabolic agents from the tissues)
- influx of nutrients, hormones, and neurotransmitters
- homeostatic function (regulation of volume, pressure, osmolality, pH, and ion composition) to provide an optimal medium for the cells of the CNS

The constant transport of substances among blood, CSF, and brain cells is influenced by the blood–brain barrier (BBB) (Section 3.2.1). The transport function of the BBB depends on the concentration gradient, molecular weight, ion composition, and liposolubility of compounds as well as on the presence of specific transmitters (i.e., glucose and amino acids).

Thus, the relations and changes among CSF, interstitial space, and the BBB reflect pathological processes of nervous tissue (metabolic and degradation affections of cells), immunological reactions, and BBB dysfunction.

2. Routine Biochemistry of the CSF

2.1. Indications of CSF Drawing

CSF is obtained mainly by a lumbar or, less often, by a suboccipital or ventricular puncture. In the course of the puncture, the appearance and pressure of the CSF are evaluated, together with maneuvers to fully appreciate the CSF pathways.

The indications of lumbar puncture include:

- spinal cord disorders (compressive syndrome)
- inflammation (meningitis, encephalitis, myelitis)
- inflammatory autoimmune diseases [multiple sclerosis (MS), Guillain–Barré syndrome (GBS)]
- systemic autoimmune disorders (vasculitis, systemic lupus erythematosus)
- acute brain vascular affections (ischemia, hemorrhage, and traumatic affections)
- degenerative processes (Alzheimer's syndrome, amyotrophic lateral sclerosis, Parkinson's syndrome)
- tumors
- other psychiatric and neurological diseases or cases of some secondary affections of the CNS (diabetes mellitus, hypothyrosis, renal and hepatic failures, etc.)

To appreciate the BBB and intrathecal (ith) synthesis of immunoglobulins, a blood specimen should be taken as well.

2.2. Appearance of CSF

Under physiological conditions, CSF is clear and colorless. It may be pink or red if many red blood cells (RBCs) are present, and cloudy if more than 400 elements/mm^3 are present, white blood cells (WBCs) are present, or the protein content is especially high. When blood has been present in the CSF for more than four hours, xanthochromia may occur owing to the presence of hemoglobin pigment from lysed red blood cells. Also, high protein levels (>1.5 g L^{-1}) may produce a yellowish color that can simulate xanthochromia of red blood cell origin.

Xanthochromia in centrifuged CSF is an important finding in the diagnosis of vascular neurological diseases. The xanthochromia of centrifuged CSF has

TABLE 1
EVALUATION OF GLUCOSE AND LACTATE IN SEVERAL CSF DISEASES

Parameter	Normal value	Low clinical diagnosis	High clinical diagnosis
Glucose	>50% of the serum values	Acute bacterial meningitis Tuberculosis meningitis Cryptococcal meningitis *Listeria* meningitis Neurosarcoidosis	
Lactate	<2.1 mmol L^{-1}		Acute bacterial, fungal, and tuberculosis meningitis CNS tissue destruction

also been generally accepted as a sign of a hemorrhagic disorder. However, CSF xanthochromia may also be observed in patients with high CSF protein concentration (>1.5 g L^{-1}), jaundice, and traumatic lumbar puncture bleeding.

2.3. QUANTITATIVE BIOCHEMICAL MARKERS OF CSF

2.3.1. *Total Protein*

See Section 3.2.4.

2.3.2. *Glucose*

Of the large number of biochemical parameters in CSF, just a few are suitable for routine diagnostic procedures. Their concentration in CSF depends on the state of the blood–brain barrier (Table 1).

Glucose is the basic source of energy of CNS cells. The reference range for glucose is <4.5 mmol L^{-1}. It is necessary to determine glucose levels in the blood and the CSF simultaneously because the blood glucose level affects CSF glucose levels. Potentially, intravenous glucose therapy in diabetic patients or hypoglycemia can influence glucose levels in CSF. On the other hand, only 60–80% of children with acute bacterial meningitis have CSF glucose levels below the reference range.

2.3.3. *Lactate*

Lactate is a useful parameter of cerebral metabolism. The level of lactate is not influenced by plasmatic concentration. Lactate is produced only in small amounts in CSF in the case of anaerobic glycolysis. Under physiological conditions, lactate penetrates the blood–brain barrier in very low concentrations. In bacterial meningitis, the main source of lactate is brain tissue. Different mechanisms are included in the production of lactate: first, brain edema with reduction of cerebral blood flow, ischemia, and (consequently) anaerobic metabolism; then, production

of cytokines, inducing an invasion of granulocytes into the subarachnoid space with rising lactate levels owing to glycolysis.

In general, patients with acute bacterial, tuberculous, and fungal meningitis have elevated CSF lactate levels, whereas patients with viral meningitis are usually below twice the reference range (G2). In comparison to the CSF/glucose ratio in the diagnosis of bacterial meningitis following neurosurgery, lactate level has proved to be a very sensitive and specific discriminatory factor (L2).

2.3.4. *Chlorides*

Currently, chloride evaluation appears to be obsolete. However, a lower concentration of chlorides can have clinical significance in the appreciation of the BBB. It can signal a hard affection of the BBB in the case of tuberculous meningitis, purulent meningitis, mycotic meningitis, or malignant meningeal infiltration.

2.4. Spectrophotometry of CSF

The most important examination for evaluating the xanthochromic CSF components is spectrophotometry. Xanthochromia may be caused by one of three substances: bilirubin, oxyhemoglobin, or methemoglobin. Spectrophotometry of cerebrospinal fluid should be used in suspected intracranial vascular disorders and appears to be a good marker in the early stages of hemorrhage when pathological changes in cytology are not present. In comparison to computer tomography imaging (CT), it is good, reliable, and complementary and can establish a specific diagnosis in every patient (K8).

Bilirubin and oxyhemoglobin have been shown to cause CSF xanthochromia in subarachnoid hemorrhage (B1). Methemoglobin has been detected in cases of hematoma, indicating the oxidation of the Fe^{2+} of oxyhemoglobin to the Fe^{3+} of methemoglobin.

3. CSF Proteins and Evaluation of the CSF Proteinogram

3.1. Overview

3.1.1. *A Four-Compartment Model as a Basis for CSF Diagnosis of CNS*

The complex anatomy of the brain requires a schematic model for interpreting serum and CSF findings (Fig. 1). The assumption of the four-compartment model suffices for the laboratory diagnosis of CNS diseases. These compartments are as follows:

- intravascular compartment: lumen of the capillaries and venules of the parenchyma, the choroid plexus, and the leptomeninges

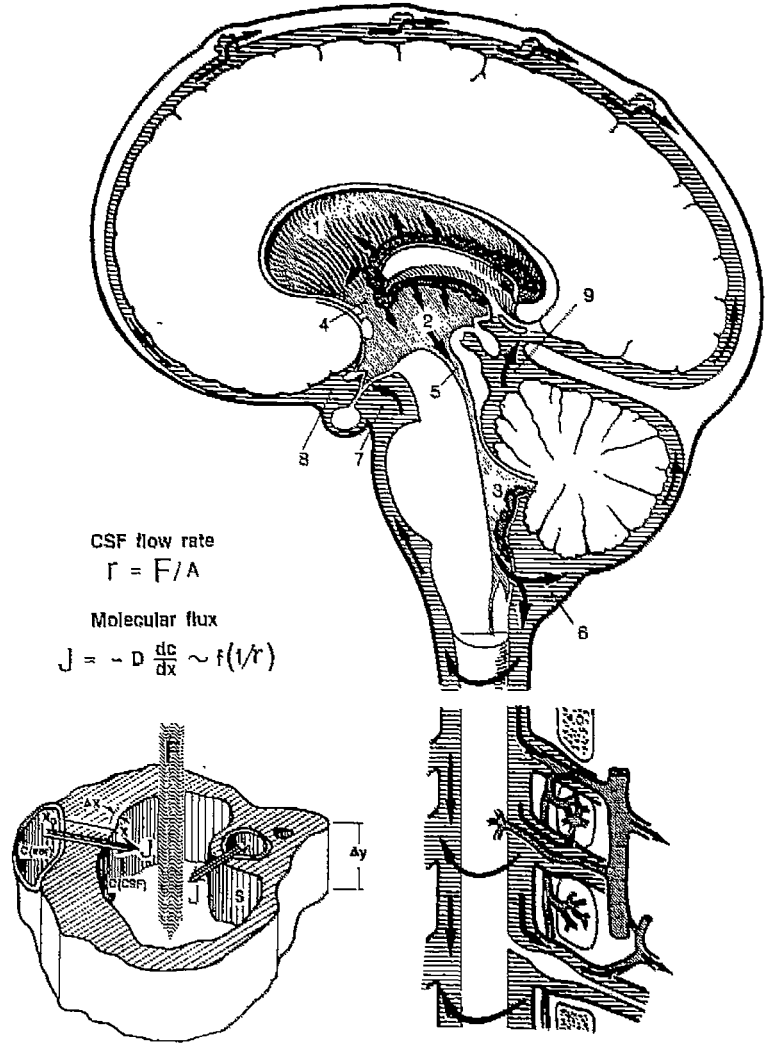

FIG. 1. Model of subarachnoidal space, CSF flow, and molecular flux (N1). After CSF production in choroid plexus of the ventricles (1, 2, 3), CSF passes the aperture (4, 5), reaches the cisternae (6–9), and divides into a cortical and a lumbar branch of the subarachnoidal space. Finally, CSF drains through the arachnoid villi into venous blood. The illustration represents an idealized cross section through the subarachnoid space. Molecules diffuse from serum with a concentration $C_{(ser)}$ through tissue along the diffusion path x into the subarachnoid space with a concentration $C_{(CSF)}$. The molecular flux J depends on the local gradient $\Delta c/\Delta x$ or dc/dx and the diffusion constant D. The CSF concentration increases with decreasing volume exchange, i.e., decreasing CSF volume bulk flow ($F = 500$ ml/day). The flow rate of a molecule in CSF is $r = F/A$, where A is the varying cross section of the subarachnoid space.

- intracellular space of the nerve and glial system
- extravascular labyrinth of gaps between the interwoven processes of nerve and glial cells (open to the CSF space)
- CSF compartment: ventricles, basal cisternae, subarachnoid space (CSF flow), and the narrow zone of adjacent extracellular space (diffusion)

Among the four compartments, there is an equilibrium based on an exchange regulated by the following barriers:

- blood–brain barrier: tight junction (zonulae occludentes) of the brain capillaries; preferred transendoepithelial exchange of lipophilic substances; various specific carriers
- blood–CSF barrier: filtration of the primary cerebrospinal fluid in the plexus (fenestrated capillaries, leaky epithelial junctions); permanent flow through the ventricles and subarachnoid space to the Pacchioni granulations; various secondary alterations due to *de novo* intrathecal synthesis, active transport, and specific absorption
- intra/extracellular barrier: preferred lipophilic transmembranous exchange; specific carriers

Between the free CSF space and the extracellular space there is a diffusion equilibrium for macromolecules. The intra/extracellular barrier and the blood–brain barrier are essentially lipid barriers, retaining small hydrophilic molecules but allowing the passage of lipophilic molecules up to approximately 500 Da (F2, R6, T2).

3.1.2. *CSF Diagnosis of Neurological Diseases*

The exclusion of organic disease as the cause of a neuropsychiatric syndrome can be difficult. In this context, among other tests, lumbar puncture is indicated because many diseases of the brain are associated with a barrier disorder or an increased cell count. Both findings, sensitive yet nonspecific, are suggestive of central nervous system disease. Apart from these two nonspecific alterations, targeted CSF analysis is an important additional tool, depending on the clinical setting (F4, R6, T2).

3.2. CSF Barrier

3.2.1. *Physiology and Pathophysiology of CSF and Blood–CSF Barrier Function*

Cerebrospinal fluid originates from the choroid plexus in the ventricles, flows through the cisternae and subarachnoid space, and finally drains through the arachnoid villi into the venous blood. CSF flow starts at around the time of birth and reaches a maximum rate about four months after birth, with complete maturation of the arachnoid villi.

Blood proteins enter the CSF along its way between the ventricles and lumbar subarachnoid space, inducing a 2.5-fold increase of total protein concentration between ventricular and lumbar CSF.

Protein transfer from the brain into CSF, and from blood into CSF, follows the law of diffusion as a function of molecular size. The diffusion-related transfer of proteins into CSF is the cause for molecular size–dependent discrimination (i.e., selectivity) of the barrier function. As a consequence, we have larger CSF/serum quotients for the smaller molecules: QAlb > QIgG > QIgA > QIgM. Again, the smaller albumin molecule equilibrates faster between blood and CSF than do the larger molecules of IgG, IgA, or IgM.

The absolute concentration of blood-derived proteins is modulated by the CSF flow rate. In general, the actual CSF protein concentration is determined by the following variables: individual serum concentration and individual diffusion pathway, age, site of puncture, and volume of extraction.

The blood–CSF barrier for proteins is defined functionally by nonlinear interaction of the molecular flux and CSF flow rate.

A blood–CSF barrier dysfunction means decreased CSF flow rate. The structures along the diffusion pathway for proteins between blood and CSF are conservative (no "leakage").

A blood–CSF barrier dysfunction (i.e., pathologically reduced CSF flow) can have different causes: reduced CSF production rate, restricted flow in the subarachnoid space, or restricted passage through the arachnoid villi (F2, R5).

3.2.2. *Filtration Concept of the Blood–CSF Barrier*

The blood–CSF barrier is relatively permeable to hydrophilic macromolecules, (i.e., α_2-macroglobulin and IgM). In addition, the passage of smaller molecules, which are larger than 500 Da, is facilitated by lipophilicity (i.e., by antibiotics and cytostatic drugs). The composition of the extracellular fluid of the brain parenchyma is unknown. It resembles CSF only in a narrow margin of a few millimeters adjacent to the free CSF space, a zone where a limited diffusion of water-soluble molecules is possible (F2). The composition of CSF is well known because the subarachnoid space can be tapped at its lowest point. Despite the great distance from the site of production, the choroid plexus, it shows all of the characteristics of a filtrate, even in the lumbar sac.

Thus, for hydrophilic molecules, there is a clear correlation between the CSF/serum ratio and the hydrodynamic radius of the molecule. This is applicable only in the presence of a steady-state equilibrium (i.e., when the serum concentration is stable and the exchange conditions at the blood–CSF barrier are undisturbed) (T1b). The ratio for water is by definition 1.0. The concentration of the smaller chloride ion is higher in CSF than in serum; therefore, in barrier dysfunction, it decreases in comparison to larger molecules. For most amino acids, active

transport across the blood–CSF barrier is apparently negligible. Barrier dysfunction is associated with a decrease in filtration selectivity (i.e., the CSF concentration of proteins increases more steeply than in smaller serum components) Albumin, with a radius of 35.8 Å and a CSF/serum ratio of 4×10^{-3}, is used as a barrier marker.

Substances with an unexpectedly low CSF concentration are either selectively removed from the CSF between the ventricles and lumbar sac (e.g., glycine) or are metabolized (e.g., sorbitol). Substances with an unexpectedly high CSF concentration (e.g. ascorbate, β_2-microglobulin, or neuron-specific enolase) either originate from the central nervous system or reach the CSF space by carrier-mediated transport. Through the albumin ratio (R_{Alb}), the size of a locally synthesized (i.e., intrathecal) fraction can be calculated (F2, R3).

3.2.3. *Numerical Evaluation of CSF Protein Data*

In the case of barrier dysfunction, the serum protein proportion gradually increases and the percentage of CSF protein of local origin decreases. The serum/CSF ratio of total protein is suitable as an approximate indication when the more reliable albumin ratio is not available. In many laboratories, however, total protein is used primarily as an indicator of dilution for immunoglobulin quantification.

Albumin is an excellent marker of the blood–CSF barrier because it originates exclusively in serum. The first attempts to correlate the increase of albumin and IgG assumed a linear rise of both proteins independent of the extent of the barrier alteration.

This way, it is possible to differentiate the increased concentration of IgG in CSF, which is based on the penetration of this protein from serum under conditions of increased serum concentrations or failure of the blood–CSF barrier.

The simplest method is the calculation using the Delpech–Lichtblau quotient, which is based on parallel determinations of concentrations of IgG and albumin in cerebrospinal fluid and serum. The mathematical structure of the formula is as follows:

$$(IgG_{CSF} \times A_S)/(IgG_S \times A_{CSF}) \qquad \text{or} \qquad Q_{IgG}/Q_{Alb} \qquad \text{(see Table 2)}$$

If the quotient exceeds 0.7, the situation suggests intrathecal synthesis of IgG. However, this formula is largely empirical and is not mathematically sound. Since the end of the 1970s, numerous more complex equations have been published for the calculation of intrathecal synthesis of IgG (as well as IgM and IgA) that take into account the parameters of diffusion, depending on the dimensions of the molecule. Their description exceeds the scope of this review.

Nowadays, mathematical formulas by H. Reiber are usually used. They have undergone a complicated development and their current form is presented below (the definition of terms appears in Table 2).

TABLE 2
DEFINITION OF TERMS FOR NUMERIC EVALUATION OF CSF BARRIER

Terms	Definition of terms
IgG_{CSF}	Concentration of IgG in cerebrospinal fluid in mg L^{-1}
A_S	Concentration of albumin in blood serum in mg L^{-1}
IgG_S	Concentration of IgG in blood serum in g L^{-1} (or in mg L^{-1})
A_{CSF}	Concentration of albumin in cerebrospinal fluid in mg L^{-1}
Q_{IgG}	Quotient of IgG
Q_{Alb}	Quotient of albumin
$Qlim_{IgG}$	Mathematical limit to the quotient of IgG (similar to IgA and IgM)
IgG_{LOC}	Quantity of IgG produced in CNS (LOC = local)
IgG_{ith}	Quantity of intrathecally produced IgG out of the total quantity of IgG in CSF (%)

Note: Using the individual terms, it is always necessary to work with identical units (everything in g L^{-1} or in mg L^{-1}).

Reiber's scheme as based on an upper threshold index of 1.0:

1. $Q_{lim}(IgG) = 0.93\sqrt{(Q_{Alb})^2 + 6 \times 10^{-6}} - 1.7 \times 10^{-3}$
   $Q_{lim}(IgA) = 0.77\sqrt{(Q_{Alb})^2 + 23 \times 10^{-6}} - 3.1 \times 10^{-3}$
   $Q_{lim}(IgM) = 0.67\sqrt{(Q_{Alb})^2 + 120 \times 10^{-6}} - 7.1 \times 10^{-3}$
2. $Ig_{LOC} = (Q_{Ig} - Q_{lim(Ig)} \cdot Ig_S)$ [mg L^{-1}]
3. $Ig_{ith} = (1 - Q_{lim(Ig)}/Q_{Ig}) \times 100$ [%]

The calculated intrathecal synthesis of IgG based on Reiber's formula is more precise in its latest version (see the recommended literature) and is recommended for the examination of cerebrospinal fluid in many countries. Physiological values for this formula are negative or equal to zero (software used for this purpose is able to recognize this). Negative values are considered to be equal to zero. Positive values indicate evidence of intrathecal synthesis of immunoglobulins (F3, R7).

3.2.4. *Total Protein*

Concentration of total protein in cerebrospinal fluid is an essential biochemical parameter. It is impossible to agree with the opinion of some clinicians (many of whom are fascinated by the latest examination methods) that this is an unnecessary parameter. It is true that the concentration of total protein in cerebrospinal fluid varies with respect to the reference range. It is known that the concentration of total protein in cerebrospinal fluid exceeding $\sim$1 g L^{-1} represents a principal challenge to the diagnosis of some CNS diseases (e.g., multiple sclerosis). This is fully in accordance with the results of a study (A24) in which the highest detectable level of total protein was 0.92 g L^{-1}.

The concentration of total protein increases slightly with age. In multiple sclerosis, we have not proved any dependence of the level of total protein in cerebrospinal fluid on other clinical parameters.

Although the examination of total protein in cerebrospinal fluid is quite valuable, it is necessary to mention that this parameter does not provide exact information on the function of the blood–CSF barrier. This is easy to understand. The increased concentration of total protein in cerebrospinal fluid can be based both on the failure of the barrier with a subsequent increase in the concentration of albumin and of other proteins originating from serum and on a more significant intrathecal synthesis of immunoglobulins, especially in levels of IgG.

3.2.5. *Albumin*

The albumin quotient is the most precise, routinely used criterion for assessment of the function of the blood–CSF barrier because albumin in cerebrospinal fluid originates exclusively from serum. Its parallel determination during the monitoring of any CSF protein is necessary because this is the only way to differentiate its increased concentration in cerebrospinal fluid due to passive penetration of the respective serum protein from a more specific increase in the concentration of the monitored protein. It is based on its intrathecal synthesis or on a specific transport mechanism for the given protein across the blood–CSF barrier. Unfortunately, some clinicians disregard this recommendation, and this elementary fact is not sufficiently emphasized in publications on cerebrospinal fluid (A22).

The CSF/serum ratio of albumin during mid-adult life is less than 7×10^{-3}. If the barrier permeability increases (as seen in purulent meningitis), the albumin ratio can increase to values greater than 100×10^{-3} (barrier breakdown). The albumin ratio is not dependent only on the barrier's permeability, but also on the fluid turnover, which normally is 14% per hour of the total CSF space volume. If turnover decreases, as in the presence of a spinal cord tumor, the albumin ratio can increase to values $<100 \times 10^{-3}$ (CSF block).

Between the albumin ratio (R), the barrier permeability (P), and fluid turnover (T), the following relationship exists:

$$R = \frac{P}{T + P}$$

Increasing permeability and decreasing turnover will result in the CSF concentration of albumin approaching its serum concentration because of the passive transfer of albumin through the barrier (F1). Identical concentrations ($R = 1$) are not quite reached, but the albumin ratio can increase to values $<100 \times 10^{-3}$.

In the case of space-occupying processes (i.e., tumors, hemorrhage, prolapse of intervertebral discs), the extent of barrier dysfunction depends on the location and extent of the underlying process. The more CSF flow is impaired

TABLE 3
CLASSIFICATION OF BARRIER DYSFUNCTION INDEPENDENT OF CAUSE (F4)

Barrier dysfunction	Possible diseases
Slight	Multiple sclerosis
(R_{Alb} up 10×10^{-3})	Chronic HIV encephalitis
	Herpes zoster ganglionitis
	Alcoholic polyneuropathy
	Amyotrophic lateral sclerosis
Moderate	Viral meningitis
(R_{Alb} up 20×10^{-3})	Opportunistic meningoencephalitis
	Diabetic polyneuropathy
	Brain infarction
	Cortical atrophy
Severe	Guillain–Barré polyneuritis
(R_{Alb} up $10–50 \times 10^{-3}$)	Lyme meningopolyneuritis
	Herpes simplex encephalitis
($R_{Alb} < 20 \times 10^{-3}$)	Tuberculous meningitis
	Purulent meningitis

between the ventricles and the lumbar sac, the higher the increase in protein concentration.

The classification of barrier dysfunction is shown in Table 3.

3.2.6. *The Blood–CSF Barrier in Relation to Age*

The blood–CSF barrier of newborns is significantly more permeable than that of adults (F2, R5, S11). The albumin ratios continuously decrease during the course of the first month of life, reaching the lowest values between 1 and 3 years of age, and then slowly rise again (Table 4). It is therefore advisable to consider age when assessing the blood–CSF barrier, especially during infancy and old age.

TABLE 4
CSF/SERUM RATIO OF ALBUMIN DEPENDENT ON AGE (F2)

Age	$R_{Alb} \times 10^{-3}$
30th week of gestation	50
At birth	25
1 month	15
6 month	5
20 years	5
40 years	7
60 years	8

3.3. CSF Proteins

3.3.1. *Proteins Originating from Plasma*

3.3.1.1. *Albumin.* See Section 3.2.5.

3.3.1.2. *α_1-Acid Glycoprotein (AAG, Orosomucoid).* The follow-up of the orosomucoid levels in CSF biochemical syndromes was not specific for certain groups of diseases. However, cytological syndromes showed much higher specificity for the orosomucoid levels in respective groups of diseases. Significantly higher levels of orosomucoid in CSF were found in granulocytous cytological syndromes typical for inflammatory diseases and in tumorous cytological syndromes positive for malignancy.

In cases of increased apolipoproteins [known nowadays as markers of tissue destruction (A3)], there were correlations of orosomucoid with Apo A-I and Apo A-II. This fact supports expectations about the presence of tissue destruction in inflammatory and tumorous diseases of the CNS.

Levels of AAG were significantly higher in cases of patients with a cytologically positive tumorous diagnosis (i.e., in presence of tumorous oligocytosis and pleocytosis, especially in patients with granulocytous pleocytosis) in CSF than in other cytological syndromes. Serum levels of AAG were significantly higher as well (A15, A19).

As yet, a phenomenon has not been observed in CSF. Orosomucoid in CSF can be considered to be a reliable tumor marker. Assessment of CSF orosomucoid levels has become a routine method in CSF laboratories and has become a mandatory parameter of the CSF protein status.

3.3.1.3. *Antitrypsin (AAT).* The α_1-antitrypsin (AAT) level in serum is one of the nonspecific markers of the activity of various tumors. Studies discuss the importance of evaluation of the AAT level in CSF in patients with various CNS neoplasias.

A high incidence of malignant elements detected in CSF is of great diagnostic value in CNS malignancies of hematological origin. The levels of AAT in CSF were statistically processed in patients with various cytological tumorous syndromes, cytologically nonspecific findings, and in normal cytology. The levels of AAT in CSF of cytologically positive patients were significantly elevated as opposed to findings of the control group. This demonstrates the importance and possible diagnostic value of the assessment of acute-phase proteins in CSF (P5).

3.3.1.4. *Hemopexin.* Hemopexin originates in CSF as a marker of meningeal carcinomatosis (F4).

3.3.1.5. *α_2-HS Glycoprotein.* Its biological role has not been defined and it is not routinely measured.

3.3.1.6. *Transferrin.* Transferrin produces specific biological functions. It behaves as a substantial inflammatory marker, and its concentration rises in

inflammatory diseases of the CNS. In patients with malignancies of the CNS, a marked decrease in the concentration of transferrin in serum is observed.

Concentration of transferrin in CSF does not correlate with serum levels. This suggests consideration of the presence of a specific transport system for transferrin in the blood–CSF barrier. This transport system may be similar to that, for instance, for immunoglobulins (transcytosis). Only as an epiphenomenon is there a significant decrease of serum concentrations of transferrin, as in cases of ulcerous meningitis and malignant meningeal infiltration. In this case other methods are required to determine the levels with any degree of precision (A24, Z1).

Assessment of transferrin in cerebrospinal fluid is considered technically easy, a quick and practical auxiliary investigative method that is accepted in a large number of laboratories. The efficacy of these investigations for timely diagnostics of inflammatory disorders of the CNS is undoubted.

3.3.1.7. *Carcinoembryonic Antigen (CEA).* Locally synthesized CEA is a marker of malignant CNS tumors. An intrathecal fraction occurs in 90% of all carcinomatous meningeal involvements and in 45% of all intraparenchymal metastases.

The chance of identifying an intraparenchymal metastasis by means of CSF CEA declines with increasing distance from the ventricles. Although intracortical metastases communicate with the subarachnoid space of the pallium, the latter is connected only in the basal (temporal) sections with the lumbar sac. The largest portion of the supracortical CSF space (frontoparietal) drains directly to the blood via the Pacchioni granulation. The intact dura is impermeable to proteins (R5).

3.3.2. *Proteins Originating in Both Plasma and CSF*

3.3.2.1. *Immunoglobulins.*

3.3.2.1.1. *Neuroimmunological reactions.* The humoral immune response in CNS is different from the immune response usually observed in blood. As a main difference, we find no switch from the IgG-class response to a more specific IgG-class response in the course of inflammatory disease. The pattern of intrathecal IgG/IgA/IgM synthesis remains rather constant and depends on the causes, pathophysiology, and localization of the disease process (R4).

As a second difference in neuroimmunological processes, we find a slow, long-lasting decay of intrathecal antibody synthesis, sometimes detectable 10–15 years after sufficient treatment (neurosyphilis, neuroborelliosis, or HSV encephalitis).

Both aspects, the lack of an IgM-to-IgG switch and the slow normalization of intrathecal antibody synthesis, could be consequences of the same problem: the handicapped regulation of the intrathecal immune response. Given the barrier-dependent low immunoglobulin concentration in CNS and the local (perivascular) invasion of relatively few immunocompetent cells, we might calculate a 10^5 lower probability for the encounter of cells and antibodies compared to blood. Irrespective

TABLE 5
IMMUNOGLOBULIN PATTERNS FOUND IN CERTAIN DISEASES (F4)

Pattern	Diseases
Strong predominance of IgG, IgA < 20%, IgM < 50%	Multiple sclerosis Herpes simplex encephalitis Neurosyphilis Chronic HIV encephalitis
Reaction in two Ig classes	
IgG + IgA	Purulent meningitis Neurotuberculosis
IgG + IgM	Early summer meningoencephalitis Progressive paralysis
Reaction in three Ig classes	
IgG + IgM + IgA	Neuroborreliosis Mumps encephalitis Opportunistic infections

of the discontinuous distribution of immunocompetent cells, this figure is suggestive of a compartmental regulation problem. From a diagnostic point of view, the lack of an IgM-to-IgG switch in CNS is the opportunity to characterize disease-related instead of acuity-related patterns. However, this also presents a diagnostic disadvantage—the characterization of the acuity of disease (see below).

Patterns of immunoglobulins found in certain diseases are listed in Table 5.

3.3.2.1.2. *Relevance of CSF analysis.* The combined analysis of IgA, IgG, and IgM in CSF, together with CSF turnover (R_{Alb}), is now widely recognized as a helpful tool for the differentiation diagnosis of neurological diseases. Of course, there is no case in which CSF analysis can be used as the only source of information for a secure diagnosis. CSF analysis cannot now and never will replace a good clinical history. However, in the framework of incisive, clinically well-founded questions, a clear and concise history (reported to the laboratory) along with a CSF protein analysis offers relevant information for the confirmation or discrimination of many differential diagnostic alternatives.

In addition, and independent of the quotient patterns, IgG and IgA quotients are needed to calculate the most sensitive detection of intrathecal synthesis of specific antibodies for the diagnostics of certain diseases (e.g., VZV-induced infections). Experience indicates that the CSF data's helpfulness to physicians is directly proportional to the physician's clinical expertise (F4, R4) (see Sections 3.2.3. and 3.2.5).

3.3.2.1.3. *IgG.* The detection of locally (intrathecally) synthesized IgG is based on a comparison with serum immunoglobulins. Normal serum and CSF immunoglobulins are polyclonal and reflect the practically infinite heterogeneity of the individual antibodies (i.e., the end product of a patient's numerous immune

responses). In contrast, intrathecal antibodies reflect the isolated response to a microorganism or autoantigen and thus differ from those in the serum by the kappa/lambda ratio, the pattern of electrophoretic charge, the IgG class composition, and the antigen specificity. Within the small CSF compartment, these immunoglobulins dominate those originating from the serum, showing limited "oligoclonal" heterogeneity in the spectrum of specific antibodies (R4). Because of their preponderance, they can be recognized as additional bands not present in the serum, especially in the alkaline range on isoelectric focusing. The number and location of these additional bands are of no differential diagnostic significance.

Possible causes underlying the appearance of intrathecal antibodies are:

- acute CNS infections with a very specific immune response to a single microorganism (virus, bacterium, parasite)
- infections in the distant past with a persisting anamnestic immune response to some microorganisms (TPHA antibodies in neurosyphilis)
- a polyspecific response associated with CNS autoimmune diseases [antibodies against measles, rubella, and zoster virus (MRZ reaction)], in multiple sclerosis as well as lupus erythematosus, without the presence of the corresponding antigens

3.3.2.1.4. *IgA.* Evidence describing the role of IgA in cerebrospinal fluid is abundant. Some references do exist for IgA patterns in CSF for some neurological and infectious disorders, user files. They are, however, nearly always about a very limited number of patients, making the precise statistical appreciation of that set practically useless.

Most of the publications cover multiple sclerosis, for which intrathecal synthesis of immunoglobulins is possible owing to their clonal restriction. Uniquely, IgA is insinuated in CSF for polyradiculoneuritis Guillain–Barré, Down's syndrome, Alzheimer's disease, multi-infarctional dementia, and in cases of nonpurulent, tumor, and autoaggressive disorders of the CNS. Frequently, immune proteins, including IgA, were observed in purulent inflammatory disorders (i.e., bacterial and tuberculous meningitis). Also, the influence of age on the concentration of IgA in CSF was cited.

CSF and serum levels of IgA are routinely evaluated in patients with neurological diseases. Simultaneously, another 17 protein fractions were recommended in the investigation of CSF and serum: albumin, immunoglobulins (IgA, IgG), acute-phase reactants (prealbumin, α_1-antitrypsin, orosomucoid, transferrin, haptoglobin, fibrinogen, CRP), complement components (C3, C4), apolipoproteins (Apo A-1, Apo A-2, Apo B), and other protein fractions (antithrombin III, α_1-microglobulin). The results obtained were correlated with another set of 17 protein fractions and with biochemical CSF findings, both in the group as a whole and after its division according to the presence of cytological and biochemical CSF syndromes. Specific biological behavior of CSF IgA has been proved and the increase of its level is expected, both in inflammatory diseases of the CNS and to a lesser

extent in MS patients. Increase in CSF IgA levels can also be caused by its leakage through the blood–CSF barrier and/or by its intrathecal oligoclonal synthesis.

3.3.2.1.5. *IgM.* A large body of literature on the monitoring of IgM levels in cerebrospinal fluid is currently available, addressing topics such as autoimmune disease, multiple sclerosis, infectious diseases of the central nervous system, and dementias. Although most groups of patients described are large enough, what is badly needed is a comparison of IgM with the behavior of other protein fractions in CSF in statistically appropriate groups. To date, no convincing correlations with cytological CSF findings have been demonstrated.

In patients with normal CSF findings, just a few statistically significant correlations were demonstrated between IgM and other CSF protein fractions. In the groups of patients with pathological CSF findings, significant correlations between CSF IgM and other immunoglobulins, complement fractions, and the rate of intrathecal synthesis of immunoglobulins were found. Surprisingly, correlations were found between CSF IgM and CSF antithrombin III and α_1-microglobulin. Correlations between CSF IgM and CSF apolipoproteins support the theory of CNS tissue destruction, as demonstrated whenever the concentrations of CSF apolipoproteins are elevated. The data obtained could substantially contribute to establishing the diagnosis in patients with neurological disease. Simultaneous measurement of a large number of CSF proteins is becoming inevitable for a reasonable assessment of the CSF proteinic status.

The conclusions suggest that determination of CSF IgM levels alone, however clarifying it may be for the diagnosis or the specificity of infectious diseases, is absolutely insufficient. It is imperative to assess IgM concentrations, quotients, and the degree of intrathecal synthesis with other protein fractions of CSF and serum. Today, laboratories routinely determine 17 protein fractions in CSF and serum (some in plasma). The term "CSF proteinic status" can be used for a comprehensive assessment of topical changes in the CSF proteinogram. The protein fractions that are monitored routinely in terms of function can be divided into markers of inflammation (F4, R3), markers of potential malignancies (F1, R7), markers of tissue destruction (R8), and compression markers; moreover, the degree of intrathecal synthesis of IgG, IgA, and IgM is always calculated.

3.3.2.1.6. *IgD, IgE.* Their significance in CSF is not known.

3.3.2.2. *Inflammatory Markers.*

3.3.2.2.1. *CRP.* Assessment of CSF CRP may become a useful parameter for distinguishing between septic and aseptic meningitis. Concentrations significantly increase in most cases of bacterial meningitis and are useful in separating acute bacterial from acute viral infections. When CSF CRP concentrations exceed the level of 120 μg L^{-1}, the probability of bacterial infection is very high (C2). CRP shows a sensitivity of 80% in the diagnosis of bacterial meningitis (S13).

3.3.2.2.2. *α_1-Microglobulin (AMG).* AMG, a protein whose biological relevance has not yet been established, has not been investigated to date in CSF,

except by Preiningerová and colleagues (P5). The data suggest the possibility of an important role played by this protein in the function of the blood–CSF barrier. Because of the statistical correlations of AMG levels and those of immunoglobulin fractions and complement components in CSF, a very significant role of AMG in the inflammatory diseases of the nervous system is quite probable.

A low correlation was demonstrated between CSF AMG levels and its serum levels. The correlation related to the patient's gender was surprisingly marked. Interesting findings included those of a marked correlation between AMG levels in CSF and serum levels in females in the presence of a pathological finding in CSF. No such correlation was demonstrated in males. Under physiological conditions, a marked correlation between CSF AMG levels and total protein levels in CSF can be seen, whereas no such correlation was found in pathological cases. However, after dividing the group by gender, a surprisingly high correlation of AMG levels in CSF with CSF total protein levels in males was observed; the correlation in females was not statistically significant. A correlation is obvious in the whole group between AMG levels and IgA in CSF. This is most likely due to the mutual binding of these proteins in serum. Regarding immunoglobulins, a most significant correlation was demonstrated with IgM. In the group of acute-phase proteins, a close correlation was found, especially with haptoglobin, orosomucoid, and C-reactive protein (CRP). A clear correlation in CSF was also found between AMG and C3 and C4 complement components (P5).

The high correlation with apolipoproteins might be associated with the component of tissue destruction in inflammatory disease, further supporting correlations with the presence of lipophagic elements in the cytological picture (these elements phagocytizing CNS necrotic tissue) (Fig. 8, see color insert). For the above reasons, AMG in CSF seems to be a most promising subject of future research and likely to become an important diagnostic tool in clinical practice. As it is, this protein can be used today in practical CSF study as a marker of inflammation. Hence, its determination in CSF seems to be fully substantiated, especially in inflammatory disease and neurological infections, particularly in cases where the component of tissue destruction is also present (D3, P5).

3.3.2.2.3. *Haptoglobin.* The specific biological function of haptoglobin was observed. As a substantial inflammatory marker, an increase of its concentration is present in inflammatory diseases of CNS. In patients with malignancies of the CNS, a marked increase in the concentration of haptoglobin in serum was also observed (K1).

3.3.2.2.4. *Transferrin.* See Section 3.3.1.6.

3.3.2.2.5. *C4 complement.* C4 complement (along with C3) provides an assessment in CSF, becoming one of the acute-phase proteins, as a prognostic marker of inflammatory disorders.

Relative to concentrations of other protein fractions in patients with normal cytological and biochemical findings and in cases with the lymphocytic cytological

syndromes (i.e., lymphocyte pleocytosis and oligocytosis), concentrations of C4 can signal CSF syndromes in the presence of nonpurulent neuroinfective diseases, usually in viral infections and in patients with multiple sclerosis.

In patients with lymphocytic CSF cytological syndromes, elevation of CSF C4 concentrations was observed. Leakage of several proteins across the blood–CSF barrier was also found. Leakage of C4 complement into CSF depends on the functional state of the barrier to a certain extent, being partially selective. Under pathological circumstances, the rate of penetration of protein fractions across the blood–CSF barrier can be modified selectively, which has been proved in CSF acute-phase reactants. They are highly influenced by the production of cytokines. These considerations evoke the question as to whether similar mechanisms of penetration can be expected in cytokines. Elucidation of the pharmacokinetics of interferons in CSF could substantially influence our approach not only to MS patients but to others as well (A18).

3.3.2.2.6. *ICAM-1.* An increased CSF/serum ratio (i.e., barrier dysfunction with a CSF increase of all serum proteins) can be caused by:

- an increase in permeability (i.e., as seen in viral meningitis)
- a CSF flow obstruction (i.e., associated with a spinal cord tumor).

Possibly, the determination of sICAM-1, allows the differentiation between these two causes.

The intracellular adhesion molecule-1 (ICAM-1) is an important protein of endothelial cells. Its expression is induced by the proinflammatory cytokines IL-1, TNF-α, and INF-γ during the early phase of the inflammatory process. Under these conditions, its soluble form, sICAM-1, is released intrathecally at a high rate, probably originating from the endothelial cells of the inflamed tissue (i.e., the meninges and adjacent brain parenchymal tissue).

During inflammation, permeability increases in the blood–CSF barrier, elevating levels of sICAM in the CSF. Thus, in settings of acute meningitis, multiple sclerosis, and Guillain–Barré polyradiculoneuritis, CSF concentrations of 44 μg L^{-1}, 4.5 μg L^{-1}, and 16.2 μg L^{-1}, respectively, were measured (R8). A precise, quantitative differentiation between the serum portion and the intrathecal fraction, comparable to the immunoglobulins, is still not possible; therefore, the ICAM index represents the best approximation:

$$\text{ICAM} = \frac{\text{ICAM}_{(\text{CSF})} \times \text{albumin}_{(\text{serum})}}{\text{ICAM}_{(\text{serum})} \times \text{albumin}_{(\text{CSF})}}$$

The average values of the ICAM index in various settings are:

- 0.7 in cases of noninflammatory diseases of the central nervous system
- 1.0 in multiple sclerosis
- 1.5–1.7 in cases of meningitis

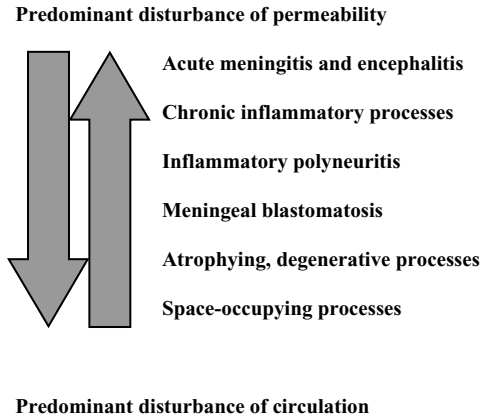

FIG. 2. Causes of blood–CSF barrier dysfunction (F5)

A cutoff index level for inflammatory diseases of the CNS could not be calculated. Therefore the concept depicted in Fig. 2 continues to be valid (i.e., a CSF increase of all serum proteins can be caused either by an increase in permeability or by a disorder in circulation such as flow obstruction) (F5).

3.3.2.2.7. *Antithrombin III (AT III).* There are only a few references concerning the evaluation of antithrombin III (AT III) in cerebrospinal fluid. Extension of evaluated groups of patients is not sufficient; a comparison with other CSF protein fractions and with CSF cytological findings was not done. Some experimental works describe the vasorelaxant effect of AT III on brain arteries, and the inhibitory influence of AT III in subarachnoid hemorrhage on onset of vasospasms is expected (W2). AT III also plays a possible role in etiopathogenesis of ischemic stroke (A19, W2).

In addition to CSF cytological findings, higher levels of CSF AT III were observed when lipophagic macrophages were present. Levels of CSF AT III were substantially higher in pleocytic CSF cytological syndromes than in oligocytic syndromes.

Antithrombin III in cerebrospinal fluid can be easily denoted as an inflammatory marker. Correlations with levels of immunoglobulins, their intrathecal oligoclonal synthesis, complement components, and acute-phase reactants confirm such concepts. Correlations with apolipoproteins and with the presence of lipophagic macrophages in cytological preparations confirm the elevation of CSF AT III levels when a destructive lesion of the CNS is present.

AT III in CSF has become a routinely measured parameter in laboratories, especially in cases of inflammatory diseases of the CNS and in the differential diagnosis of these illnesses.

3.3.2.2.8. *Markers of microglia/macrophage system (β_2-microglobulin and neopterin).* β_2-Microglobulin levels reflect the activation of T cells, whereas neopterin represents the activation of macrophages. The CSF concentrations are a useful indication of the functional activity of the microglia/macrophage system. Less than 1% of CSF β_2-microglobulin originates in plasma, with more than 99% being of intrathecal origin. In the case of neopterin, 2.5% and 97.5% are of plasma and intrathecal origins, respectively (F4).

During the course of chronic inflammatory processes of the central nervous system, the concentration of neopterin and β_2-microglobulin probably reflects microglial activation and the extent of the intraparenchymal macrophage activation. Thus, as reported in one study (M4), patients with HIV infection without clinical neurological symptoms showed an inflammatory CSF reaction with an increase in β_2-microglobulin and most notably in neopterin. Patients with HIV had higher values than did asymptomatic ones.

In general, presuming no ongoing opportunistic CSN infection, β_2-microglobulin and neopterin are good markers for central nervous system involvement in HIV infection.

3.3.2.2.9. *Markers of immune activation and process.* Acting with TNF-α–mRNA, sICAM-1, IL-10–mRNA, and sTNF-R, these proteins and nucleic acids can be used as markers of the immunologically mediated inflammatory process seen in multiple sclerosis (M4). This disorder is associated with deregulation of cytokine expression, especially in regard to TNF-α.

- TNF-α: Synthesized by monocytes/macrophages and vascular endothelium during the proinflammatory phase leading to activation of the inflammatory reaction. Its synthesis is most reliable by means of the TNF-α mRNA expression in mononuclear cells.
- ICAM-1: An adhesion protein of the capillary endothelium. Its synthesis is increased following stimulation by TNF-α. It is related in the blood and CSF as "soluble" ICAM-1 (sICAM-1). The brain capillaries are rich in ICAM-1, and even a single lesion visible by magnetic resonance imaging results in elevated serum concentrations.
- IL-10: A cytokine inhibitory factor synthesized by TH_2 cells, macrophages, and B cells. IL-10 inhibits the formation of inflammatory cytokines. Its synthesis is most reliably determined by means of IL-10 mRNA expression in mononuclear cells.
- TNF-R: The effect of TNF-α is mediated via a number of receptors, which are synthesized at an increased rate following stimulation by TNF-α and are subsequently released into the blood and CSF as soluble TNF-R (sTNF-R).

In multiple sclerosis, the four parameters mentioned are indicators of the course of the disease with intermittent remission and exacerbation. ICAM-1 reflects the

size of lesions caused by multiple sclerosis, assuming extracerebral endothelial activation can be excluded. Serum concentrations, followed over a long period, are indicative of the activity of the inflammatory process.

3.3.2.3. *Tumor Markers.*

3.3.2.3.1. *C3 complement.* Until now, the biological role of C3 in cerebrospinal fluid has not been studied in a sufficiently large group of patients with neurological diseases (A14). C3 complement compares (simultaneously with the measurement of levels of immunoglobulins, albumin, acute-phase proteins, apolipoproteins, antithrombin III, C4, and α_1-microglobulin) this confers clinical significance, as both an inflammatory and tumor marker. In all cases, the simultaneous appreciation of cytological investigation of CSF should be done. Cytological findings are classified according to the morphological and functional prevalence of the cellular elements. The collective results compared in accordance with the presence of the biochemical and cytological CSF syndromes may play a role in correct diagnostics.

Significantly high CSF C3 levels were observed in inflammatory processes of the CNS while less significant elevation was found in patients with malignancies of the CNS. Measurement of CSF C3 levels can easily be used as a routine diagnostic method (A14).

3.3.2.3.2. *α_1-Acid glycoprotein.* See Section 3.3.1.2.

3.3.2.3.3. *Neuron-specific enolase (NSE).* NSE is useful for monitoring the outcome of treatment and the course of disease in patients with neuroendocrine tumors, in particular small cell lung cancer and neuroblastoma. The test is not suitable as a screening or adjunct to diagnosis because of low clinical sensitivity and specificity. Elevated serum NSE concentrations are found in patients with:

- benign pulmonary diseases
- cerebral diseases, in particular in the cerebrospinal fluid of patients with cerebrovascular meningitis, spinocerebellar degeneration, cerebral ischemia and infarction, intracerebral hematomas, subarachnoid hemorrhages, head injury, inflammatory cerebral diseases, organic convulsive disorders, Guillain–Barré, schizophrenia, and Creutzfeld–Jacob disease (J1, V2).
- neuroblastoma-increased serum NSE levels are present in about 60% of children with neuroblastoma depending on the stage of disease. In comparison with neuroblastoma in patients with Wilm's tumor, lower levels may be present.

3.3.2.3.4. *Mucin-like cancer-associated antigens (MCA).* MCA findings in CSF are denoted as a marker of metastatic breast cancer.

3.3.2.4. *CNS Compression Markers.*

3.3.2.4.1. *Fibrinogen.* Serum protein, together with the mechanism of hemocoagulation, is present in normal CSF only in trace concentrations, probably due to its high molecular weight. An increase in CSF concentrations is always connected with severe damage of the blood–CSF barrier or mechanical obstruction in the

spinal canal. An increase in fibrinogen concentration is frequently accompanied with the appearance of "Froin syndrome—xanthochromia and spontaneous coagulation of the CSF sample." In neuroinfections, an increase in fibrinogen is documented in neurotuberculosis. Nowadays it belongs to the routinely assessed markers (A19).

3.3.2.4.2. *Albumin.* See Section 3.2.5.

3.3.2.5. *Markers of Tissue Destruction.*

3.3.2.5.1. *Apolipoprotein A-I.* Apolipoproteins are routinely analyzed parameters in serum, and the evidence of their investigation in cerebrospinal fluid remains unique. The biological relevance of these analytes in serum is well known.

Apo A-I is the main structural apolipoprotein on HDL particles; it is synthesized in hepatic and enteric cells. Bound phosphatidylcholine and sphingomyelin participate in the creation of protein–phospholipid complexes.

Apolipoprotein A-II is a component of HDL and VHDL particles, and serves as a cofactor of enzymatic reactions that take part in esterification of cholesterol and mediate its transport.

Apo B is structural apolipoprotein for chylomicrons and for VLDL and LDL particles. It is synthesized in enteric and hepatic cells. It is important for cholesterol transport to cells via interaction with LDL receptors.

These apolipoproteins are present in normal cerebrospinal fluid, but only in low concentrations. In the presence of lipophagic elements in CSF cytology, the findings of the concentrations of apolipoproteins in cerebrospinal fluid were clearly improved.

Dealing with phenomena with a high reciprocal correlation, it is known that the lipophagic–macrophagic elements contain lipid substances whose origin lies in the metabolic degradation of structural lipids of the CNS. These cells, under various pathological conditions, phagocytize the necrotic tissue of the CNS. An increase of apolipoprotein concentration in cerebrospinal fluid indicates the presence of tissue destruction and, likewise, the presence of lipophagic elements in cytological findings. This phenomenon is probably based on the binding process of lipid metabolites to transport proteins: apoproteins creating apolipoproteins. Thus they are responsible for the transfer of lipid metabolites from the CNS and from cerebrospinal fluid.

As perspective and sensitive markers for the presence of tissue destruction in CNS from apolipoproteins, the apolipoprotein A-I and Apo B are related. These analytes are already routinely analyzed, with some laboratories using laser nephelometry (A16, T2).

3.3.2.5.2. *Apolipoprotein A-II.* In cytological findings indicating the presence of lipophages in CSF elevation of CSF Apo A-II concentrations can be observed. The probable function of Apo A-II in CSF is the transport of lipid substances formed during the degradation of CNS structural lipids by macrophages. Similar mechanisms have been described for the apolipoprotein Apo A-I. Assessment

of CSF Apo A-II concentration can be used in routine practice to identify CNS structural lesions (A23, T3).

3.3.2.5.3. *Apolipoprotein B.* Apo B is a structural apolipoprotein for chylomicrons, VLDL, and LDL particles. It is synthesized in enteric and hepatic cells. It is important for cholesterol transport to cells via interaction with LDL receptors. Nowadays, it seems its clinical relevance in CSF investigation is near Apo A-I and Apo A-II, but in current studies some varieties can be found (A23, T3).

3.3.2.5.4. *Apolipoprotein H.* Apo H (previously called β_2-glycoprotein 1) seems to be of a minor interest, and inthathecal synthesis is not suspected (S5).

3.3.2.5.5. *Apolipoprotein E.* Apo E is involved in peripheral myelin repair and synthesized by astrocytes. Its very low blood/CSF ratio suggests an intrathecal synthesis. Recent studies shows that Apo E derives largely (80–90%) from ith synthesis. In patients with multiple sclerosis, a slight increase in serum Apo E contrasts with a significant decrease in CSF Apo E (i.e., of its ith synthesis rate). Such a decrease seems significantly linked to the duration of MS: the intrathecal synthesis mean is significantly lower 10 years after the onset of the disease. This new finding suggests a defect in lipid turnover, which may slow (or even block) myelin repair (S5).

3.3.3. *Proteins Originating from CSF*

3.3.3.1. *Prostaglandin-D Synthetase (β-Trace Protein, β-TP).* Cerebrospinal fluid fistulae, after head injuries or operations, may cause inflammation of the central nervous system via nasal or otogenic entrance of pathogens. Sensitive methods are needed to detect traces of CSF in nasopharyngeal or otogenous secretions to differentiate rhinorrhea and otorrhea. CNS-specific constituents are suitable for detecting CSF diluted in secretions because other CSF constituents are nonspecific and inaccurate (i.e., glucose, potassium, total protein, or prealbumin) when showing concentrations found in CSF. β-Trace proteins (β-TP) and β_2-transferrin (β_2-TF, τ-fraction), as well as asialo-transferrin (a-TF), separated electrophoretically, are recommended as CNS-specific parameters to detect CSF in rhinorrhea or otorrhea. Here, different mixtures of a human CSF pool with a human serum pool were analyzed for β-TP and a-TF to evaluate optimum conditions for CSF detection in the secretions. Moreover, two different absorbents in use for secretion collection were studied, with the mixtures representing models for rhinorrhea and otorrhea.

Thus, complementary use of β-TP and a-TF assay for CSF detection in rhinorrhea and otorrhea is recommended (K9).

3.3.3.2. *Transthyretin (Formerly Prealbumin).* An increase in transthyretin concentrations is usually found in inflammatory processes and neurodegenerative diseases (A19).

3.3.3.3. *β_2-Transferrin (a-TF, β_2-TF, τ-Fraction, τ-Transferrin).* See Section 3.3.3.1 (K9).

3.3.3.4. *Cystatin C (Formerly Post-γ-globulin, γ-Trace Protein).* Cystatin C is a new parameter in a spinal fluid and serum (plasma), originating from glial elements and belonging to so-called trace proteins. Its increase in CSF is considered to be a marker of tissue destruction. Assessment of cystatin C in serum (plasma) is a marker of renal glomerular filtration.

3.3.3.5. *Adrenomedullin (AM).* Adrenomedullin is a polypeptide produced by vascular smooth muscle, nerve, heart, and other organs. An increased serum concentration has been reported in patients with essential hypertension, heart failure, and renal failure. There have been few reports of AM in CSF, as related to cerebrovascular disease. The concentrations in CSF can be significantly higher in patients with acute subarachnoidal hemorrhage than in those with nonacute cerebrovascular disease (K7).

3.3.3.6. *Amyloid β_{1-42}-peptide ($A\beta_{1-42}$).* Serum amyloid A is an acute-phase protein. Using a monoclonal ELISA, it can be established in the serum and CSF and its intrathecal synthesis seems very common in AIDS patients and in some other inflammatory processes (S5).

3.3.3.7. *τ-Protein.* Amyloid β_{1-42} peptide and τ-protein have a clinical significance as early diagnostic markers in Alzheimer's disease (AD), and its evaluation is mandatory for efficient treatment. The mean levels of $A\beta_{1-42}$ and τ-protein in CSF were significantly reduced (with high specificity and sensitivity) in patients in comparison to normal findings (M6).

3.3.3.8. *S 100b Protein.* S 100b protein is synthesized in astroglial cells and thus can be used as a reliable and sensitive marker for central nervous system damage. Measurements of S 100b protein are useful in the diagnosis and prognosis of acute stroke and in the estimation of ischemic brain damage during cardiac surgery (K4).

3.3.3.9. *Tetranectin (TN).* Tetranectin is a plasma protein of about 80 kDa. A reduction in the serum TN level has been described in malignant disease and in other conditions with tissue remodeling, whereas it does not seem to exhibit acute-phase reactant behavior. Using a polyclonal ELISA to determine the TN concentrations in pair of CSF/serum quotient in pair of CSF and serum, it was found that the CSF/serum quotient compared to the Q_{Alb} was compatible with intrathecal TN synthesis. In clinically definite multiple sclerosis a reduced CSF/serum quotient was found, suggesting that the hypothesis of a reduced TN level as a marker of tissue remodeling may be extended to the CNS (C5).

4. Isoelectric Focusing of the CSF

4.1. Overview

Classical electrophoresis of CSF has largely been abandoned. In physiological conditions, in comparison with serum, the expression of prealbumin, β-globulin,

and a trace of γ-fraction is present. In a large affection of the blood–brain barrier or in the case of hemorrhage within a liquor space, the levels of certain fractions are nearer to those of serum. The meaningful polyclonal increase of γ-fraction is typical in Guillain–Barré syndrome.

Classical electrophoresis is not suitable for the detection of intrathecal synthesis of immunoglobulins.

4.2. The Intrathecal Synthesis of Immunoglobulins

Normal serum and CSF immunoglobulins are polyclonal and reflect the practically infinite heterogeneity of individual antibodies as the end product of a patient's numerous immune responses. The intrathecal antibodies reflect the isolated response to a microorganism or autoantigen and thus differ from those in the serum by the kappa/lambda ratio, the pattern of electrophoretic charge, the Ig-class composition, and the antigen specificity. Within the small CSF compartment, these immunoglobulins dominate those originating from serum and show a limited "oligoclonal" heterogeneity in the spectrum of specific antibodies (F2).

4.2.1. *Possible Causes of Intrathecal Antibodies*

4.2.1.1. *Acute Central Nervous System Infection.* A specific immune response is typical for acute central nervous system infection by a present microorganism. The first sign of primary antigenic stimulation is IgM production. Intrathecal synthesis of IgM is found in various infectious diseases such as neurosyphilis, acute aseptic meningoencephalitis, herpes encephalitis, or HIV infection. Intrathecal synthesis of IgM is an important sign for the early diagnosis of inflammatory neurological diseases and for monitoring the effects of therapy. Persistent IgM production in the central nervous system indicates continuous antigenic stimulation (A17, E1, K6, L1, M1, S9).

An increased IgA index is found in aseptic meningitis in 40% of cases and in herpes encephalitis in almost 100% of cases as a result of virus-specific IgA antibody production. In neurotuberculosis, an increased IgA index is often accompanied by normal IgG and IgM indices (L1).

4.2.1.2. *Infection in the Distant Past with a Persisting "Anamnestic" Immune Response.* There are, for example, TPHA antibodies in neurosyphilis (K6, L1, L3).

4.2.1.3. *Humoral Immune Response in Chronic Inflammatory Processes.* The specificity of the diagnostically relevant intrathecal antibodies depends on the underlying cause of the disorder. In the case of chronic infective processes, the antibodies are exclusively targeted against the causative organism (F5). Increased IgG levels are found in about 30% of patients with chronic meningitis or encephalitis from various causes, such as bacteria, virus and protozoa, and in diseases like polyradiculitis, sarcoidosis, and chronic myelopathy (L1).

4.2.1.4. *Polyspecific Response Associated with CNS Autoimmune Diseases.* The oligoclonal, intrathecally synthesized IgG contains numerous specific antibodies and autoantibodies. Antibodies are frequently found with specificities against measles, the rubella virus and the varicella-zoster virus, but seldom against the herpes simplex virus. The occurrence of one, two, or three of these antibodies is referred to as the MRZ reaction. The corresponding antigens are not present in these cases. The MRZ reaction is typical of multiple sclerosis as well as cerebral lupus erythematosus and is a chronically evolving immune process (F5, K10, S16).

An increased IgG index is found in more than 80% of patients with multiple sclerosis. However, there seems to be no relationship in multiple sclerosis between the severity or the activity of the disease and the intrathecal production of IgG (F5, L1, S8, V1). The intrathecal IgA synthesis is found in only 12% of patients with multiple sclerosis (L1).

An increased IgM index is seen in cerebral lupus erythematosus and in 30–60% of multiple sclerosis patients with a short disease duration (A20, A24, L1). An increase in the IgM index is usually associated with oligoclonal IgM bands on electrophoresis. This indicates an oligoclonal aspect of intrathecally produced IgM. Cerebral IgM production is related to the activity of multiple sclerosis, and the early detection of oligoclonal IgM in patients with acute isolated lesions of the brainstem and spinal cord has more predictive value for the development of multiple sclerosis than does detection of oligoclonal IgG bands (L1).

4.3. Evaluation of the Intrathecal Synthesis of Immunoglobulin G

The absolute value of the IgG concentration in CSF depends on the IgG concentration in serum, blood–brain barrier function, age of the patient, volume of CSF extracted, and local IgG synthesis in the central nervous system. Older studies used the IgG/TP ratio or IgG/albumin CSF ratio to estimate intrathecal production of IgG in CNS. To achieve a quantification of IgG intrathecal production, an empirical Tourtelotte's formula derived from three sources was proposed:

(1) IgG produced in the CNS
(2) the expected amount of IgG in CSF that would normally arise from plasma and enter the CSF from the blood via the normal blood/CSF barrier
(3) the plasma IgG that enters the CNS via the damaged blood/CSF barrier (C1, S10).

See Table 6.

Another method to detect locally synthesized IgG is based on CSF/serum concentration quotients:

$$\text{IgG index} = \frac{\text{IgG}_{\text{CSF}} \times \text{albumin}_{\text{S}}}{\text{IgG}_{\text{S}} \times \text{albumin}_{\text{CSF}}}$$

TABLE 6
EVALUATION OF IgG SYNTHESIS

Terms	Definition
$\frac{IgG_S}{369}$	IgG normally expected to enter the CSF from serum
$\frac{alb_S}{230}$	Proportion of CSF albumin that normally enters the CSF in intact blood–brain barrier
$\left(Alb_{CSF} - \frac{Alb_S}{230}\right)$	Excess albumin in the CSF that has crossed a damaged blood–brain barrier
$\left(\frac{IgG_S}{Alb_S}\right)(0.43)$	Converts the quantity for excess CSF albumin to that for excess CSF IgG that has crossed a damaged blood–brain barrier with albumin
$\frac{5\ mg}{day}$	Multiplying by this ratio yields the results in milligrams of IgG per day

The CSF/serum ratio of IgG eliminates the individual variation of serum IgG. The quotient of IgG (CSF/serum) to albumin (CSF/serum) eliminates the variation of the IgG quotient by the individual blood–CSF barrier function. Intrathecal IgG is total CSF IgG minus transudative IgG. The first formulas were based on a linear relationship between Q_{alb} and Q_{IgG} (C1, K3, L1, S4). More recent formulas make use of a hyperbolic or exponential function. The application of the latter two formulas reduces the number of false-positive results in the cases of blood–brain barrier disturbances, while sensitivity is maintained. Soeverijn compared Reiber's hyperbolic formula to five other formulas and showed that Reiber's formula produced the best agreement with the IEF gold standard (L1). For the latest modification of the IgG, IgA, and IgM subclasses of immunoglobulins, see Section 3.2.3.

4.4. ISOELECTRIC FOCUSING

4.4.1. *Principles of Isoelectric Focusing*

Isoelectric focusing can be described as an electrophoresis in a pH gradient set up between a cathode and anode with the cathode at a higher pH than the anode. Because of the amino acids in proteins, they have amphoteric propertites and will be positively charged at pH values below their isoelectric point (Ip) and negatively charged at pH values above their isoelectric point. This means that proteins will migrate toward their IpH. Under the influence of the electrical force, the pH gradient will be established by the carrier ampholytes, and the protein species will migrate and focus at their IpH. The proteins will thus concentrate at the position where pH = IpH.

Three major types of isoelectric focusing first dimensions are used currently (S7):

(1) The method developed by O'Farrell uses ampholytes, which form the pH gradient and carry the proteins along this gradient. Gels are cast in a tube and the pH gradient is formed in prefocusing. Following prefocusing, proteins are loaded on the basic end of the gel at the cathode end, and then focused for several hours.
(2) Nonequilibrium pH gel electrophoresis (NEPHGE) uses a pH gradient created by soluble ampholytes. However, the proteins are loaded on the acid end and then electrophoresed. The pH gradient does not really form in a uniform manner and proteins do not focus to a particular location. This method is useful in separating more basic proteins.
(3) The method using immobilized pH gradients (IPG) was developed by D. Hochstrasser and offers many advantages over the O'Farrell and NEPHGE first-dimension system. The pH gradient is immobilized in the acrylamide gel. This allows better consistency between runs, better resolution of proteins throughout the entire pH range, and a choice between linear and nonlinear gradients.

4.4.2. *pH Gradients and Gels*

The pH gradient is formed from amphoteric substances collectively called carrier ampholytes. The established pH gradient is maintained by hundreds to thousands of carrier ampholytes molecules lined up in order of the IpH with partially overlapping distributions. A large number of carrier ampholyte mixtures are available giving differing pH gradients. The optimal pH gradient will depend on the purpose of the experiment. For screening purposes, a broad range interval (e.g., pH 3–10) should be used. A narrow pH range interval is useful for careful IpH determinations or when analyzing proteins with very similar IpH points. Generally, one should not use a narrower gradient than necessary because the shallower gradient will lead to longer focusing times and more diffuse bands.

Gels are usually made of polyacrylamide or agarose. A number of amphoteric buffer solutions and premade gels are available, covering broad and narrow pH ranges. High resolution is obtained when narrow pH ranges are created. In polyacrylamide gels, pore size can be accurately controlled by the total acrylamide concentration and the degree of crosslinking available by appropriate relationship between acrylamide and bisacrylamide. When crosslinking is kept constant and total concentration increases, pore size will decrease and diffusion is reduced. On the other hand, agarose has some important advantages over polyacrylamide for isoelectric focusing. The agarose gels are formed from a single, stable, nontoxic polymer brought into solution within minutes, mixed with ampholytes, and gelled. The gels do not exclude large macromolecules. Ampholytes are removed from reacted agarose gel plates within minutes, and the agarose gels are easily dried,

stained, and cleared. In contrast, polyacrylamide does not permit the separation of many macromolecules, which cannot penetrate the gel. The polyacrylamide gels can only be used 12 or more hours after polymerization. Finally, acrylamide is known to be toxic to laboratory workers (S1).

4.4.3. *Analysis of IgG Oligoclonal Bands in the CSF*

Since the late 1960s and early 1970s, CSF protein electrophoresis has found an important application in the diagnosis of some diseases of the central nervous system. The CSF γ-globulins from patients with multiple sclerosis migrate to form a pattern of a restricted number of discrete oligoclonal bands or zones in the gel. IgG isolated from brain tissue of patients with multiple sclerosis also shows this electrophoretic pattern. But the presence of oligoclonal bands in the CSF is not specific for multiple sclerosis, because it also occurs in a variety of other central nervous system inflammatory disorders. Nevertheless, the proportion of patients with definite multiple sclerosis that demonstrate oligoclonal bands is in the range of 80%, and these bands may be demonstrable even when the CSF γ-globulin level is not elevated (B3, D2, L1, O1, S3, S10).

A variety of techniques are used to analyze oligoclonal bands, including electrophoresis on cellulose acetate, agar, and agarose gels, in sodium dodecyl sulfate–polyacrylamide gels; isoelectric focusing in polyacrylamide gel; isoelectric focusing in agarose gel; and disc polyacrylamide gel electrophoresis (L1, S10, S12). Many studies describe electrophoretic investigations, including isoelectric focusing, of concentrated CSF with various stains for IgG oligoclonal bands detection, i.e., Coomassie brilliant blue, Ponceau S or Amido black (A25, B3, C6, F7, D2, G3, O1, S6, W5). In these cases, it is necessary to start with 5–10 ml of CSF and then concentrate it 50-fold or more before applying it to the gel (S10). Therefore, electrophoretic analysis of concentrated CSF presents a serious drawback, for example, when CSF from newborns, infants and childern has to be analyzed (G1, H3, O1). The majority of recent studies and some of the older studies present electrophoretic analysis of unconcentrated CSF and diluted sera with the identification of oligoclonal IgG bands in CSF. Isoelectric focusing on agarose or polyacrylamide provides greater sensitivity and resolution when compared with conventional electrophoretic methods (D2, G1, L1, S10). There are two basic ways to routinely detect separated protein fractions:

- immunofixation with specific antihuman IgG serum and silver staining (P1, S6, S12, W6)
- immunoblot on a nitrocellulose membrane and immunoenzymatic staining (B2, G3, K5, K11, L1, S15, S16, S10)

4.4.4. *Evaluation of the IgG Oligoclonal Bands in CSF*

The claim for the presence of oligoclonal IgG is generally made when two or more IgG bands are detected in CSF (H2, K5, K11, O1, P2, S10, W6). This

definition is essential in both agarose electrophoresis and isoelectric focusing when the procedures do not include immunofixation, since γ-protein migrates within the immunoglobulin's region of CSF (S12).

Following the evaluation of isoelectric focusing, the results of unconcentrated CSF and sera, diluted to the corresponding concentrations, are classified into four categories by their banding patterns (S12):

Normal banding pattern:
- bands only in serum

Pathological banding patterns:
- abnormal bands equally intense in both CSF and serum ("mirror" pattern)
- bands more intense in CSF compared to serum
- bands only in CSF

Oligoclonal IgG bands are defined as bands found exclusively or more intensely in CSF compared to serum. They are usually observed in the region of the pH gradient between 6.5 (and if need be, 5.5) and 9.5 by isoelectric focusing. Some oligoclonal bands have an IpH in the very alkaline part to pH 10.5 (D2, G3, H1, K11, O1). The most frequent occurrence of the oligoclonal bands in CSF of patients with multiple sclerosis is found in the pH gradient between 7.8 and 8.6. In these cases, the immune process with IgG synthesis is extended to the CNS. Detection of "oligoclonal" bands in CSF supports the diagnosis of demyelinating disease. In multiple sclerosis, oligocloclonal IgG bands are observed in more than 90% of cases.

In patients with chronic infection of the central nervous system (bacteria, viruses, fungi), IgG oligoclonal bands are found in approximately 40% of cases, such as herpes, varicella, toxoplasmosis, mumps, rubella, tuberculous bacilli, borrelia, or aspergillus. Oligoclonal IgG bands with antibody activity against the responsible agent are observed in the CSF in herpes encephalitis, herpes zoster meningoradiculitis, subacute sclerotic panencephalitis, HIV infection, neurobrucellosis, neurosyphilis, neuroborreliosis, tuberculous meningitis, neurosarcoidosis and neuro-Behcet's syndrome. Oligoclonal abnormalities are also found regularly in other inflammatory diseases such as neurolupus (C1, L1, S12, T4, W6).

Oligoclonal bands may also be present in serum. If the CSF bands exibit a "mirror" pattern of the serum bands, the process is limited to the systemic compartment. A particular case is a paraprotein pattern. It is seen both in CSF and serum, either due to myeloma or so-called "benign" paraprotein (L1).

4.4.5. *Other Ways of Using Isoelectric Focusing by the Investigation of CSF*

The specific labeled separated protein fractions blotted on a nitrocellulose membrane or specific immunofixation-separated protein fractions in polyacrylamide after isoelectric focusing make it possible to detect some additional bands in CSF, i.e., IgM, IgA, free kappa or lambda light chains of specific antibodies (i.e., anti-herpes, anti-borrelia, or anti-HIV) (L1, M3).

Oligoclonal IgM bands are observed in multiple sclerosis (in 40% of cases), neurosyphilis (22%), meningitis (44%), and encephalitis (22%). Oligoclonal IgM disappears again rapidly, in contrast to oligoclonal IgG (L1).

The IgA banding patterns are seen in the pH 4.5–6.0 region in polyacrylamide gel after protein separation by isoelectric focusing. It was observed in the CSF of multiple sclerosis patients that the intrathecally synthesized viral antibodies of IgG and IgM classes possess oligoclonal characteristics, whereas IgA antibodies appeared to be polyclonal (M3).

The presence of oligoclonal free kappa and lambda chains in CSF is a sensitive indication for recent antigenic immune response within the central nervous system, comparable with IgM. The detection of oligoclonal free kappa chains in CSF supports the diagnosis of multiple sclerosis. In addition, free light chains can also be found in the CSF of patients having inflammatory diseases of the central nervous system (L1).

Particular uses of isoelectric focusing with transferrin-specific immunofixation detects asialotransferrin in nasopharyngeal or otogenous secretions to differentiate rhinorrhea and otorrhea (K9).

5. Multiple Sclerosis

5.1. Introduction

Currently multiple sclerosis is classified in the group of diseases with an autoimmune etiology. From the aspect of cytology of cerebrospinal fluid, this disease can be referred to as a serous inflammatory process of a chronic nature, usually with a less expressed cellular response. The typical finding includes lymphocytic oligocytosis or lymphocytic pleocytosis, usually only of a mild degree. If the element count is higher, exceeding 100/3 μl (35/μl) in the Fuchs–Rosenthal chamber, it is advisable to question the principal diagnosis of multiple sclerosis. Frequently, the element count is completely normal, and then the assessment of cytological specimens is made more difficult by a low number of detected elements that are suitable for evaluation.

5.2. Biochemical Parameters in Multiple Sclerosis

Concentration of total protein in cerebrospinal fluid is the essential biochemical parameter. It is true that the concentration of total protein in cerebrospinal fluid varies with a mild to significant increase with regard to the reference range. It is well known that the concentration of total protein in cerebrospinal fluid exceeding $\sim$1 g L^{-1} means a principal challenge to the diagnosis of multiple sclerosis. In multiple sclerosis, the levels of total protein are either normal or only insignificantly

elevated. The concentration of total protein increases slightly with age. Although the examination of total protein in cerebrospinal fluid is in general quite invaluable, it is necessary to mention that this parameter does not provide exact information on the function of the blood–CSF barrier.

The albumin quotient is the most precise routinely used criterion for assessment of the function of the blood–CSF barrier, because albumin in cerebrospinal fluid originates exclusively from serum. Its parallel determination during the monitoring of any CSF protein is necessary, since this is the only way to differentiate its increased concentration in cerebrospinal fluid due to passive penetration of the respective protein originating in serum from a more specific increase in the concentration of the monitored protein. It is based on its intrathecal synthesis or on a specific transport mechanism for the given protein in the blood–CSF barrier. In patients with multiple sclerosis, the failure of the blood–CSF barrier (assessed according to the albumin quotient) is a relatively rare finding. In the literature it is reported on average in 12% of patients with this disease.

5.3. Immunoglobulins

The most characteristic abnormality in patients with multiple sclerosis is certainly the intrathecal synthesis of IgG. It can be demonstrated—with different sensitivity—by various methods, which can be divided into qualitative and quantitative methods. The gold standard for the demonstration of intrathecal synthesis of IgG is the detection of oligoclonal bands, which are not present in CSF, in the appropriately diluted serum (i.e., to the same concentration of IgG) by isoelectric focusing. This is a qualitative method and the description of its different modifications and interpretations goes beyond the scope of this chapter. This method is by far the most sensitive, and its sensitivity is reported between 90 and 100%. Here it is suitable to repeat that the detection of plasmocytic forms in cerebrospinal fluid may also be regarded as qualitative proof of intrathecal synthesis of immunoglobulins—although in this case the proof is obviously not specific for IgG from the theoretical point of view.

Quantitative methods are based on the determination of the concentrations of IgG and albumin correspondingly in cerebrospinal fluid and in serum. This way, it is possible to differentiate the increased concentration of IgG in CSF, which is based on the penetration of this protein from serum under the conditions of increased serum concentrations or failure of the blood–CSF barrier (Reiber's formulas; see Section 3.2.3).

Physiological values of this formula are negative or equal to zero (software used for this purpose is able to recognize this fact); the negative values are considered to be equal to zero. Positive values indicate intrathecal synthesis of immunoglobulins and are demonstrated in 60–80% of the patients with a diagnosis of multiple sclerosis.

In patients with multiple sclerosis, a qualitative cytological examination should always be carried out. Besides the finding of plasmocytic forms, which are considered to be one of the proofs of intrathecal synthesis of immunoglobulins, this examination also provides invaluable information concerning the reaction of the monocyte–macrophage system in the CSF compartment. It should be noted on the scope of biochemical examinations of cerebrospinal fluid in multiple sclerosis that it is most important to return to the simple and inexpensive method.

The optimum combination of all available methods, including the determination of albumin and IgG by laser nephelometry or by another suitable method (ELISA or particle-enhanced immunonephelometric determination if IgM and IgA), will help in the future to significantly increase the sensitivity of the assays in the CSF diagnostics of multiple sclerosis, thereby improving the correlations between individual examinations.

In multiple sclerosis, it is also advisable to determine the other protein fractions of cererbrospinal fluid and serum (plasma). Complex evaluation of proteins in cerebrospinal fluid of patients with multiple sclerosis provides very useful information of clinical relevance. Therefore, routine examination of a complete proteinogram is recommended for patients with multiple sclerosis. Monitoring of C3 and C4 components of complement, transferrin, orosomucoid, and antithrombin III should be considered obligatory in clinical practice, because these parameters demonstrate the severity of the process. It is also beneficial to monitor prealbumin, haptoglobin, fibrinogen, and apolipoproteins A-I, A-II, and B, which could be used for monitoring the therapy as well. Monitoring of α_1-antitrypsin could be considered optional, though very important owing to its possible role as a proteinase inhibitor in the pathogenesis of multiple sclerosis, which has been suggested by some literature references.

During the monitoring of any protein in cerebrospinal fluid, it is necessary to bear in mind the functional status of the blood–CSF barrier, serum concentration of the respective protein, and the dimensions of the molecule (or the information based on the molecular weight). These data principally influence the resulting concentration in cerebrospinal fluid. It must also be emphasized again that the parallel determination of albumin in cerebrospinal fluid and serum is necessary.

In order to present a complete picture, in the case of suspected multiple sclerosis, it is mandatory to demonstrate intrathecal oligoclonal synthesis of immunoglobulin G using isoelectric focusing and, if possible, by specific immunodetection of IgG (by immunofixation with subsequent silver stain or Coomassie blue stain or using affinity immune blotting for the detection of IgG). The latter can be performed in numerous modifications. From the aspect of differential diagnostics, it is necessary to mention the need for a simultaneous determination of antibodies against borrelia and neurotropic viruses in cerebrospinal fluid and in serum with the calculation by Reiber's formula. Under optimum conditions, the detection of specific antibodies by isoelectric focusing is advisable as well.

5.4. Cytological Findings in Multiple Sclerosis

In a cytological manifestation, it is possible to observe activated lymphocytic forms or at least solitary plasma cells, but their proportion can sometimes be remarkably high. However, if the remission of the disease lasts a long time, the numerical proportion of activated lymphocytic forms decreases and even the plasma cells may disappear temporarily; thus the cytological finding appears normal. On the other hand, the higher element count, representing lymphocytic pleocytosis (although usually mild), can be observed during an acute attack of the disease. Lymphocytic activated forms are usually plentiful, and soon plasma cells appear as well. In multiple sclerosis, it is also possible to observe the presence of granulocytes, which are usually in lower numbers. Eosinophilic granulocytes are generally present more often, and even in these cases they are considered to be signs of chronicity of the ongoing pathological process. Neutrophilic granulocytes may occur more frequently during an acute attack of the disease. After the administration of pharmaceuticals into the spinal canal, their numbers increase together with the numbers of activated monocytes. The elements of the monocytic series show signs of activation very frequently. It is also possible to observe the transformation of the elements into macrophagic ones. Lymphophages may also be present, which belong to the most frequently occurring macrophagic elements in this condition. Leukophages are rare and they phagocyte neutrophilic and eosinophilic granulocytes, are present in small numbers. Less frequently, it is also possible to observe the presence of lipophages. They are observed during an acute attack of the disease when demyelinization of the white matter takes place and are phagocytosed by lipophages. With regard to the usually larger number of elements in cerebrospinal fluid during an acute attack, there is a greater probability of detection of lipophages in cytological specimens. Special staining methods must be used to demonstrate the initial forms of lipophages.

In multiple sclerosis patients who are receiving intrathecal administration of corticoids or cytostatic drugs, the cytological appearance is significantly changed. In such cases, the numerical increase in the elements of the monocytic series is usually observed together with their activation. Therefore the appearance of monocytic oligocytosis to mild monocytic pleocytosis is present. These conditions are reactions to the presence of chemical and mechanical irritation in the spinal canal caused by the crystals of administered drugs with a slow-release effect. Subsequently, neutrophilic granulocytes can occur increasingly as well; however, their numerical proportion will never exceed that of the elements of the monocytic series.

5.4.1. *Development of Cytological Stages in Multiple Sclerosis*

• Lymphocytic oligocytosis or lymphocytic pleocytosis, usually to a mild degree—simultaneously a plasmocytic response occurs.

• Normal cytological finding—does not rule out the diagnosis of multiple sclerosis; however, under practical conditions, normalization of the finding happens less frequently and usually only for a transient period.

5.4.2. *Differential Diagnosis*

• Neuroborreliosis—especially in the initial stages, the numerical proportion of neutrophils is higher; in the chronic stage, the cytological differentiation from multiple sclerosis is practically impossible.

• Serous neuroinfections—the appearance of lymphocytic pleocytosis or lymphocytic oligocytosis is present. In this case, the element count is higher than 100/3 μl (35/μl) and the probability of the diagnosis of multiple sclerosis is very low.

• Monocytic oligocytosis and monocytic pleocytosis—in multiple sclerosis this is practically always a reactive manifestation after administration of intrathecal drugs.

5.5. Our Experiences

We investigated 55 samples of CSF in patients with multiple sclerosis. The clinical diagnosis of multiple sclerosis was confirmed, according to common diagnostic criteria, by MRI finding and oligoclonal bands of IgG in CSF (S8).

5.5.1. *Material and Methods*

The following investigations of CSF were performed:

• Basic biochemical analysis, cell count in CSF, and CSF qualitative cytological examination

TABLE 7
Multiple Sclerosis: Overview of Pathological Findings

Parameters	Pathological findings
Cytology	Mostly lymphocyte pleocytosis and oligocytosis
Biochemistry	Syndrome of proteinocytological association
Acute-phase proteins	Above all: orosomucoid
Immunoglobulins	Most frequent elevation of IgA, IgG, ith synthesis, most probably in both IgM and IgG
Complement	Both C3 and C4
Markers of destruction	Apo A-I
Blood–CSF barrier	A higher quotient of albumin is frequent owing to a higher permeability of blood–CSF barrier in inflammation

TABLE 8
MULTIPLE SCLEROSIS ($N = 55$): ABNORMAL BIOCHEMICAL FINDINGS (S8)

Pathological syndrome	*n*	%
APC—Proteinocytological association syndrome	20	36.4
DPC—Proteinocytological dissociation syndrome	12	21.8
DCP—Cytoproteinic dissociation syndrome	5	9.1
Elevated total protein	32	58.2
Elevated number of cells	25	45.5
Impaired blood–CSF barrier	13	23.6
Pathological syndrome total	41	74.5

TABLE 9
MULTIPLE SCLEROSIS ($N = 55$): ABNORMAL CYTOLOGY (S8)

Pathological syndrome	*n*	%
LO—Lymphocytic oligocytosis	14	25.5
MO—Monocytic oligocytosis	11	20
LP—Lymphocytic pleocytosis	24	43.6
MP—Monocytic pleocytosis	1	1.8
GO—Granulocytic oligocytosis	1	1.8
GP—Granulocytic pleocytosis	0	0
Lipophages	23	41.8
Plasma cells	23	41.8
Pathological findings total	51	92.7

TABLE 10
MULTIPLE SCLEROSIS ($N = 55$): ELEVATED LEVELS OF IMMUNOGLOBULINS (S8)

Parameters	*n* (CSF)	%	*n* (S)	%
IgA	27	49.1	6	10.9
IgG	24	43.6	11	20
IgM	20	36.4	11	20

TABLE 11
MULTIPLE SCLEROSIS ($N = 55$): INTRATHECAL SYNTHESIS OF IMMUNOGLOBULINS ACCORDING TO REIBER'S FORMULA (S8)

Parameters	Number (ith)	%
IgM	25	45.5
IgG	25	45.5
IgA	5	9.1
Intrathecal synthesis in minimally one class	36	65.5

• Determination of acute-phase proteins (CRP, orosomucoid, haptoglobin, transferrin, prealbumin), immunoglobulins (IgA, IgG, IgM), compressive markers (albumin, fibrinogen), markers of tissue destruction (Apo A-I, A-II, Apo B), components of complement (C3, C4), proteinase inhibitors (antithrombin III, α_1-antitrypsin). The measurement was performed simultaneously in CSF and in serum (plasma) by a laser nephelometric method. The functional state of the blood–CSF barrier was evaluated numerically with the help of the quotient $Q = Alb_{CSF/S}$ and also by the intrathecal synthesis of immunoglobulins according to Reiber's formula and for each class—IgG, IgM, IgA.

5.5.2. *Conclusions*

Examination of the most frequent complex of abnormal CSF parameters may be defined as in Table 7. According to this study, we concluded that generally the inflammatory changes are not so evident as in, say, neuroborreliosis (S8) (see Tables 8–12).

In summary, complete examination of CSF, including basic biochemical analysis and qualitative cytology with the determination of specific CSF proteins and isoelectric focusing, provides very sensitive diagnostic imformation concerning serous inflammatory processes in the central nervous system, including diseases of autoimmune origin such as multiple sclerosis.

TABLE 12
MULTIPLE SCLEROSIS ($N = 55$): ELEVATED LEVELS OF MONITORED PROTEINS (S8)

Parameters		*n* (CSF)	%	*n* (S)	%
Immunoglobulins	IgG	24	43.6	11	20
	IgA	27	49.1	6	10.9
	IgM	20	36.4	11	20
Inflammatory markers	CRP	1	1.8	16	29.1
	Haptoglobin	9	16.4	9	16.4
	Transferrin	14	25.5	9	16.4
	Prealbumin	16	29.1	21	38.2
	Orosomucoid	23	41.8	4	7.3
Compressive markers	Albumin	22	40	35	63.6
	Fibrinogen	5	9.1	11	20
Markers of tissue destruction	Apo A-I	16	29.1	31	56.4
	Apo A-II	5	9.1	2	3.6
	Apo B	2	3.6	11	20
Complement	C3	9	16.4	0	0
	C4	7	12.7	15	27.3
Proteinase inhibitors	Antitrypsin	6	10.9	9	16.4
	Antithrombin III	6	10.9	11	20

TABLE 13
ETIOLOGY AND EPIDEMIOLOGY OF BORRELIOSIS

Etiology agents	Continent	Epidemiology agents
Borrelia burgdorferi	North America	*Ixodes dammini, Ixodes pacificus, Ixodes scapularis*
Borrelia garini	Europe	*Ixodes ricinus, Ixodes persulcatus*
	Asia	*Ixodes ovatus, Ixodes dermatocentor*
Borrelia afzelii	Europe	*Ixodes ricinus, Ixodes persulcatus*
	Asia	*Ixodes ovatus, Ixodes dermatocentor*
Borrelia japonica	Europe	*Ixodes ricinus, Ixodes persulcatus*
	Asia	*Ixodes ovatus, Ixodes dermatocentor*

6. Neuroborreliosis

6.1. INTRODUCTION

Borreliosis is a disease that frequently and typically affects both the central and peripheral nervous systems (Table 13; for a historical overview, see Table 14). Owing to the frequent occurrence in our area, it is possible to observe highly variable clinical manifestations as well as different types of pathological appearances in cerebrospinal fluid. Current methods used in neuroborreliosis diagnosis include the evaluation of specific antibodies: IgM, IgG detected by ELISA, Western blot, and PCR.

The clinical stages of borreliosis are as follows:

- early localized infection: erythema chronicum migrans, myalgia, arthralgia, cephalea, lymphadenopathy
- early disseminated infection: acute multiorgan inflammation, including infection of CNS—meningitis, encephalitis, polyradiculoneuritis (Garin–Bujadoux–Bannwarth syndrome), polyneuritis cranialis multiplex (PCM), retrobulbar neuritis
- late persistent infection: chronic organ changes (e.g., encephalomyelitis)

TABLE 14
HISTORICAL OVERVIEW IN DIAGNOSTICS OF BORRELIOSIS

Date	Diagnoses	Authors
1883	Acrodermatitis chronica atroficans (ACA)	Described by Buchwald in Europe
1909	Erythema chronicum migrans (ECM)	Described by Afzelius
1922	Description of neurological syndromes	Garin, Bujadoux, and Bannwarth
1975	Seasonal arthritis in children	Old Lyme, Connecticut
1981	Isolation of new spirochetal bacteria	Burgdorfer

6.2. Biochemical Findings in Neuroborreliosis

The concentration of total protein is a problematic parameter; it may vary from normal to slightly increased levels up to a very significant increase, especially in polyradiculoneuritic syndromes caused by neuroborreliosis.

It is often observed that the concentration of some other protein markers in cerebrospinal fluid is increased. In this respect, it is worth mentioning the increase in some acute-phase proteins, including transferrin, haptoglobin, and orosmucoid. Very frequently an increased concentration of the C3 component is observed in cerebrospinal fluid. Increased concentrations of apolipoproteins in cerebrospinal fluid are frequently seen as well, especially Apo A-I and, less distinctly, Apo B. Although these findings are common, they are also rather nonspecific, suggesting only that the destruction of tissue is present—in this case, the destruction of the central and peripheral myelin.

Changes of immunoglobulin concentrations in cerebrospinal fluid are very common. In neuroborreliosis the increase occurs very frequently in the IgM class, for which it is possible (using Reiber's formula) to demonstrate the presence of intrathecal synthesis.

Considerable problems in the determination of a differential diagnosis for neuroborreliosis are caused by the presence of the intrathecal synthesis in IgG class, which is otherwise a very common finding in multiple sclerosis. This synthesis can be demonstrated very well by Reiber's formula. However, in multiple sclerosis, the typical finding is intrathecal synthesis of an oligoclonal type, with a typical manifestation of oligoclonal bands in isoelectric focusing in the alkaline or strongly alkaline areas. On the contrary, in neuroborreliosis the intrathecal synthesis is of a polyclonal type and therefore the oligoclonal bands are not typically expressed. Nevertheless, in some patients with neuroborreliosis the oligoclonal bands may be also present, but they are more frequently located in slightly neutral or paraneutral areas.

The demonstration of specific antiborrelia antibodies in cerebrospinal fluid will certainly be far more important and specific in the future, as will the exact determination of the presence (or the absence) of their possible intrathecal synthesis in CNS and in cerebrospinal fluid.

6.3. CSF Cytology of Neuroborreliosis

Cytological findings in cerebrospinal fluid in neuroborreliosis may be quite variable with each sample. It is possible to observe different types of inflammatory processes, which may indirectly suggest that this disease is present. Neuroborreliosis belongs among the diseases with the manifestation of serous inflammation; however, in the early stages of the disease, the findings may be quite different. The

reason for their less frequent detection is that often the development of the disease is not apparent and the cerebrospinal fluid is examined only after the early stage of the disease has subsided. The element count is also quite variable. It is usually not significantly increased and only in exceptional cases will the values reach 1000/3 μl (350/μl). More frequently the pleocytosis, which is present, reaches the cell count of approximately 10/3 μl (3–4/μl) to 100/3 μl (35/μl).

In the initial stages of neuroborreliosis, it is sometimes possible to observe mixed pleocytosis with prevailing neutrophils. The pleocytosis is usually mild. During the course of the disease the numbers of lymphocytic elements increase, and the same applies to the monocytic elements, although to a smaller extent. In later chronic stages of the disease, plasma cells occur, sometimes in quite large numbers. Thus, initially, granulocytic pleocytosis may change into predominantly lymphocytic pleocytosis. The element count need not necessarily be increased when lymphocytic pleocytosis is observed. Initially, granulocytic pleocytosis is changed into lymphocytic pleocytosis due to a chronic manifestation of the disease, which is typical for this disease if no therapy is applied. Furthermore, the transformation may also be accelerated by the application of antibiotic therapy. If a normal or only insignificantly higher element count is present, the cytological finding in neuroborreliosis may easily imitate the findings of multiple sclerosis, where it is also possible to observe lymphocytic oligocytosis or mild lymphocytic pleocytosis with plasmocytic reaction. In neuroborreliosis, there may be frequent cases when the element count is low at the beginning. In this situation it is possible to find more often lymphocytic oligocytosis (which otherwise occurs in chronic stages); activated lymphocytic forms are also frequently found, as are plasma cells, which occur when the disease becomes chronic. Rarely during the initial stage is it possible to observe the manifestation of granulocytic oligocytosis, forming in this situation a continuous transition toward granulocytic oligocytosis, which is usually mild in appearance, during the initial stages. In the chronic stage, solitary neutrophilic granulocytes may still be present; moreover, eosinophilic granulocytes may be infrequently observed.

The finding of monocytic oligocytosis does not rate at all in neuroborreliosis. This phenomenon can be explained by the fact that borreliosis quite frequently imitates the appearance of polyradiculoneuritis Guillain–Barré, and cytological findings in such cases are usually fairly similar as well. Even a completely normal finding in cerebrospinal fluid cannot completely rule out the diagnosis of neuroborreliosis, because the disease may be subsiding due to therapy or even spontaneously and then can recur after some time.

6.3.1. *Development of Cytological Findings in Neuroborreliosis*

• Mild mixed pleocytosis with prevailing segments or granulocytic oligocytosis—it may or may not progress rapidly into the next stage.

• Lymphocytic pleocytosis—usual count of elements is 10/3 μl to 100/3 μl (3–4/μl to 35/μl).

• Lymphocytic oligocytosis—in its chronic stage plasmocytic response is usually present.

• Normalization of cytological finding.

Note: Occasionally, the manifestation of monocytic oligocytosis is present, which mimics the cytological findings in Guillain–Barré syndrome.

6.3.2. *Differential Diagnosis*

• Other serous neuroinfections—the differentiation is possible only through a serologically detectable antibody response; morphological identification of the agent is unusual.

• Tuberculous meningitis—clinical manifestation is completely different, but otherwise the differentiation is more feasible using biochemical rather than cytological parameters. Sporadically, the agent is detected by staining for acid-fast mycobacteria.

• Polyradiculoneuritis Guillain–Barré—the manifestation of monocytous oligocytosis is present and is practically indistinguishable from certain forms of neuroborreliosis; the syndrome of proteinocytological dissociation is also present, and it is usually distinctly expressed. The best differentiation can be obtained by the presence of an antibody response in cerebrospinal fluid in neuroborreliosis.

• Other neurological diseases accompanied by the manifestation of monocytic oligocytosis; this is the most complicated issue of cytological diagnostics in CSF. Borreliosis can be differentiated again by the presence of an antibody response in cerebrospinal fluid.

TABLE 15
NEUROBORRELIOSIS: OVERVIEW TO PATHOLOGICAL FINDINGS (S9)

Parameters	Pathological findings
Cytology	Mostly lymphocyte pleocytosis
Biochemistry	Syndrome of proteinocytological association
Acute-phase proteins	Above all: orosomucoid and transferrin; to a lesser extent, haptoglobin, prealbumin
Immunoglobulins	Most frequent elevation of IgA, ith synthesis, most probably in IgM
Complement	both C3 and C4
Markers of destruction	Apo A-I
Blood–CSF barrier	A higher quotient of albumin is frequently due to a higher permeability of blood–CSF barrier in inflammation

TABLE 16
NEUROBORRELIOSIS ($N = 53$): ABNORMAL BIOCHEMICAL FINDINGS (S9)

Pathological syndrome	*n*	%
APC—Proteinocytological association syndrome	30	56.6
DPC—Proteinocytological dissociation syndrome	7	13.2
DCP—Cytoproteinic dissociation syndrome	7	13.2
Elevated total protein	37	69.8
Elevated number of cells	37	69.8
Impaired blood–CSF barrier	25	47.2
Pathological syndrome total	45	84.9

6.4. OUR EXPERIENCES

We investigated 53 samples of cerebrospinal fluid (CSF) in patients with neuroborreliosis. The clinical diagnosis of neuroborreliosis was confirmed by positive antibody titers and intrathecal synthesis of immunoglobulins in CSF examined in the IgM and IgG classes by the ELISA method or Western blot or confirmed by direct detection by PCR.

6.4.1. *Material and Methods*

The following investigations of CSF were performed:

- Basic biochemical analysis, cell count in CSF, and CSF qualitative cytological examination.
- Determination of acute-phase proteins (CRP, orosomucoid, haptoglobin, transferrin, prealbumin), immunoglobulins (IgA, IgG, IgM), compressive markers (albumin, fibrinogen), markers of tissue destruction (Apo A-I, A-II, Apo B), components of complement (C3, C4), proteinase inhibitors (antithrombin III, α_1-antitrypsin). The measurement was performed simultaneously in CSF and in serum

TABLE 17
NEUROBORRELIOSIS ($N = 53$): ABNORMAL CYTOLOGY (S9)

Pathological syndrome	*n*	%
LO—Lymphocytic oligocytosis	1	1.9
MO—Monocytic oligocytosis	10	18.9
LP—Lymphocytic pleocytosis	32	60.4
MP—Monocytic pleocytosis	5	9.4
GO—Granulocytic oligocytosis	0	0
GP—Granulocytic pleocytosis	0	0
Lipophages	13	24,5
Plasma cells	23	43.4
Pathological findings total	48	90.6

TABLE 18
NEUROBORRELIOSIS ($N = 53$): ELEVATED LEVELS OF IMMUNOGLOBULINS (S9)

Parameters	*n* (CSF)	%	*n* (S)	%
IgA	38	71.7	12	22.6
IgG	28	52.8	16	30.2
IgM	35	66	8	15.1

TABLE 19
NEUROBORRELIOSIS ($N = 53$): INTRATHECAL SYNTHESIS OF IMMUNOGLOBULINS ACCORDING TO REIBER'S FORMULA (S9)

Parameters	*n* (ith)	%
IgM	43	81.1
IgG	28	52.8
IgA	14	26.4
Intrathecal synthesis in minimally one class	53	100

TABLE 20
NEUROBORRELIOSIS ($N = 53$): ELEVATED LEVELS OF MONITORED PROTEINS (S9)

Parameters		*n* (CSF)	%	*n* (S)	%
Immunoglobulins	IgG	28	52.8	16	30.2
	IgA	38	71.7	12	22.6
	IgM	35	66	8	15.1
Inflammatory markers	CRP	4	7.5	22	41.5
	Haptoglobin	24	45.3	13	24.5
	Transferrin	28	52.8	12	22.6
	Prealbumin	26	49.1	15	28.3
	Orosomucoid	35	66	8	15.1
Compressive markers	Albumin	31	58.5	29	54.7
	Fibrinogen	15	28.3	17	32.1
Markers of tissue destruction	Apo A-I	24	45.3	26	49.1
	Apo A-II	16	30.2	2	3.8
	Apo B	10	18.9	16	30.2
Complement	C3	25	47.2	0	0
	C4	24	45.3	25	47.2
Proteinase inhibitors	Antitrypsin	25	47.2	16	30.2
	Antithrombin III	13	24.5	12	22.6

(plasma) by the laser nephelometric method. The functional state of the blood–CSF barrier was evaluated numerically with the help of the quotient $Q = Alb_{CSF/S}$ and further by the intrathecal synthesis of immunoglobulins according to Reiber's formula and that for each class of immunoglobulins—IgG, IgM, IgA.

6.4.2. *Conclusions*

According to this study, the most frequent complex of abnormal CSF parameters may be defined as in Table 15. Generally, it is possible to identify inflammatory changes in the cytological as well as in the basic biochemical examination.

Out of the examined CSF protein fractions, the elevation of levels occurs especially in acute-phase proteins and in immunoglobulins: the destruction of nervous tissue is indicated as well as blood–CSF barrier dysfunction.

As to the differences between normal plasma levels and elevated concentrations in CSF of many of the monitored proteins (IgA, C3 compound of complement, orosomucoid, etc.), we suspect active transport of these proteins to the CSF due to an inflammatory process in the compartment of the CNS (Tables 16–20).

7. CSF Cytology

7.1. Introduction

The description of cytological findings in cerebrospinal fluid is very inconsistent in the literature since no generally recognized uniform classification of these findings has been proposed to date. The need for developing such a classification system becomes quite obvious against the background of the renaissance in CSF cytology that our country is currently experiencing. A *conditio sine qua non* for developing a uniform classification system is its general applicability and recognition as well as a capacity to be established using precisely formulated conclusions.

Our draft classification is one used by a team of physicians working in the CSF Laboratory of the Department of Clinical Biochemistry at Homolka Hospital in Prague. The classification employed is based on monitoring the pathology in the cytological picture according to the presence of the prevailing cellular population in CSF and the activation in elements of the lymphocyte and monocyte lines. We were able to combine both criteria into a single viable system, expressing the current status of cellular response in CSF. The presence of a pathological cytological finding provides the basis for defining individual cytological CSF syndromes closely related to the etiological diagnosis of the patient, which in the great majority of cases makes it possible to formulate the diagnostic conclusion. The classification employed can establish the diagnosis in diseases manifesting themselves by at least a mild alteration of the cytological picture. In general, it is useful for classifying inflammatory and neoplastic diseases, intermeningeal hemorrhage, and morphological manifestations of CNS tissue destruction. A distinct advantage is

the plausible classification of cytological findings in oligocellular CSF specimens, which to date has been difficult to make owing to the low numbers of cellular elements detected in samples.

In the cytological examination of CSF, the parameters evaluated include, in addition to the number of elements, qualitative representation of individual cellular lines. When evaluating the monocytomacrophage system and/or the reticuloendothelial system, attention is focused on the proportions of activated monocytes and particularly on the presence of macrophages showing a specific substrate of phagocytosis. It is according to this substrate that macrophages are further divided into erythrophages, siderophages, lipophages, lymphophages, leukophages, mycophages, etc. To vizualize a substrate, it is often necessary to use additional staining, i.e., staining by oil red for lipids, Berlin blue for iron, and so on.

If intermeningeal hemorrhage is suspected, monitoring of the phagocytosis of red blood cells and hematogenic pigments allows us to determine the approximate age and course of the bleeding. Monitoring of lipophagocytosis and visualizing the scavenging response on CNS parenchymal damage also has a number of potential applications.

As the number of CSF examinations increases, the number of cells being detected increases proportionately. This is true especially of diseases involving the presence of primary or secondary neoplastic processes right in the CNS or in the vicinity of CSF pathways. The currently employed cytological methods of CSF examination, whenever malignant elements were detected, have only made it possible to establish the presence of a tumor disease in general. For instance, monitoring of the functional status of nucleoli, PAS positivity, or the presence of adipose droplets in the cytoplasm suggest only indirectly an increased metabolic activity of the cells monitored. Other morphological markers of atypical cells (polymorphy of cells, nuclei, polynuclear elements, cytoplasm basophilia, atypical mitoses, etc.) may raise suspicion of the presence of a tumorous process, but not identify the cellular system they belong to. Another problem, which is by no means negligible, is the low number of cells detected.

As a result, we started to study the mode of reaction of atypical elements with certain monoclonal antibodies binding to individual antigens, tumor markers specific for the respective cellular populations. Moreover, the method can be used to determine the degree of their maturity, presence of individual receptors, and the state of activation in the course of their cellular cycle.

7.2. Classification of Cytological CSF Findings

There is currently no generally recognized classification system of cytological CSF findings. Moreover, descriptions of cytological findings appearing in the relevant literature are inconsistent and do not allow a syndromologic conclusion or

TABLE 21
CSF ELEMENTS

Lymphocytes
Monocytes
Granulocytes
Macrophages
Epitheloid lining cells of CSF pathways
Erythrocytes
Atypical cells
Tumor cells
Leukemic cells

the establishment of an etiological diagnosis. A team of physicians working in the CSF Laboratory at the Department of Neurology of Prague's Charles University employs a uniform classification scheme, allowing an exact formulation of cytological findings by determining the cytological CSF syndrome which, in most cases, makes it possible to establish an exact diagnosis of patients examined.

The classification is based on several criteria of the existing cellular alteration, which may be either a pathological numerical prevalence of a certain cellular population or signs of activation in the numerically prevalent line(s). Another aspect is the number of elements in CSF, where up to 10/3 elements in the Fuchs–Rosenthal chamber represents oligocellular CSF (oligocytosis of varying types) and, in the case of several cells in CSF, reference is made to pleocytosis (A6, A7). For a more detailed classification, a normal cytological finding must be mentioned with a prevalence of lymphocyte elements (65–80%), and the remainder made up of elements of the monocyte line, with both populations represented by quiescent elements (Tables 21–24).

Oligocellular and pleocytic CSF can be divided by their cytological composition into several groups, with individual cytological CSF defined syndromes. In the presence of pleocytosis, CSF findings can be easily classified.

TABLE 22
LYMPHOCYTIC ELEMENTS IN CSF

Lymphocytes
Small
Naked—nucleated
Medium-sized
Large, i.e., lymphoid cells
Lymphoplasmocytes
Plasma cells

TABLE 23
MONOCYTIC ELEMENTS OF CSF

Monocytic elements	Subtypes	Diagnosis
(1) Quiescent (nonactivated) monocytes		
(2) Activated monocytes		
(3) Macrophages	Erythrophages	Recent hemorrhages
	Siderophages	Older hemorrhages
	Leukophages	Mostly in purulent inflammations
	Lymphophages	Serous inflammations and multiple sclerosis
	Lipophages	Tissue destruction of CNS
	So-called bacteriophages	Phagocytosis of bacteria (predominantly mediated by neutrophiles)
	Mycophages	Mycotic diseases of CNS
(4) Degenerative forms of monocytic cells	Ring-shaped cells	

7.2.1. *Syndromes with Pleocytosis*

See Table 25.

7.2.1.1. *Granulocytic Pleocytosis.* Granulocytic pleocytosis is indicated by a prevailing representation of granulocytes, usually neutrophils and, much less often, eosinophils. Using this criterion, granulocyte pleocytosis can be further divided into two more subgroups:

- Granulocytic pleocytosis with a prevalence of neutrophils (neutrophilic pleocytosis): This is a typical picture of purulent inflammations in CSF, such as that occuring in bacterial meningitis.
- Granulocytic pleocytosis with a prevalence of eosinophils (eosinophilic pleocytosis): This is a relatively rare picture of so-called "eosinophilic meningitis," which is not an inflammatory infective disease but a general severe allergic reaction of the body.

7.2.1.2. *Lymphocytic Pleocytosis.* Lymphocytic pleocytosis is indicated by a prevailing representation of lymphocytic line elements and a high representation of activated forms which, in the event of a chronic course of the lesion, evolve (in B-system elements) into plasma cells. This picture is quite typically associated

TABLE 24
MYELOID ELEMENTS—GRANULOCYTES

(1) Neutrophils
(2) Eosinophils
(3) Basophils

TABLE 25
TYPES OF CSF PLEOCYTOSIS

Pleocytosis	Type of cells	Diagnoses
(1) Granulocytic pleocytosis (i.e., polynuclear)	Neutrophilic granulocytes	Mostly in bacterial neuroinfections
	Eosinophilic granulocytes	Parasitary, mycotic, allergic, and autoagressive diseases
(2) Mononuclear pleocytosis		
Lymphocytic pleocytosis	Activated lymphocytes	Serous neuroinfections
	Activated lymphocytes with presence of plasma cells	Chronic neuroinfections, multiple sclerosis (lymphocyte oligocytosis is more frequent)
Monocytic pleocytosis	Very complicated differential diagnosis	Compressive syndromes—disc herniations, tumors Systemic vasculitis affecting CNS Brain ischemia Guillain–Barré syndrome Terminal phases of neuroinfections with scavenger reaction
Tumorous pleocytosis	Presence of malignant cells, accompanying cellular reaction differs highly, predominantly of monocyte type	

with nonpurulent inflammatory diseases (serous inflammation) whose pathogens are usually viral agents; bacterial spirochetal disease (borreliosis, leptospirosis, and syphilis) may also be involved. The presence of other bacterial agents is suggested by purulent inflammation manifesting itself as granulocytic pleocytosis.

7.2.1.3. *Monocytic Pleocytosis.* Monocytic pleocytosis is indicated by a prevailing representation of monocytic line elements. These elements usually show signs of activation; phagocytosis mediated by activated monocytic elements (macrophages) is quite frequent. Provided macrophagic elements are present, the etiological diagnosis is usually easy to establish. Macrophages can be classified by the specific substrate of phagocytosis whose presence reflects individual pathological states. Erythrophages appear in recent intermeningeal hemorrhage (however, in these cases, the prevalent cellular response is only rarely monocytic), while siderophages are usually present in intermeningeal hemorrhage of an older age. Leukophages (i.e., macrophages phagocytosing granulocytes, especially neutrophilic granulocytes) are present in the terminal stages of purulent inflammations. Lymphophages, phagocytes, and lymphocytic line elements are typically present in the end stages of nonpurulent inflammations. A characteristic feature of so-called lipophages is the presence of adipose droplets in the cytoplasm; these elements

phagocytose necrotic CNS tissue, and can be seen as part of the monocyte cellular response in cerebral ischemia and in degenerative disease.

7.2.1.4. *Tumorous Pleocytosis.* Tumorous pleocytosis is indicated by the sample showing malignant elements; while the picture of the accompanying cellular response may be different, it is usually monocyte. Phagocytosis of malignant elements in CSF is quite frequent.

Evaluation of cytological findings in oligocyte CSFs is fraught with a number of problems. The most serious problem is the low number of elements detected in samples. The term "pathological oligocytosis" can be used to refer to the presence of a pathological cytological finding with an otherwise normal number of cellular elements in CSF. While some types of pathological oligocytosis can be regarded as a smooth transition to pleocytoses of the same types, other oligocytoses take on another functional relevance.

7.2.2. *Syndromes with Oligocytosis*

See Table 26.

7.2.2.1. *Granulocytic Oligocytosis.* Granulocytic oligocytosis is associated with a prevalence of neutrophilic granulocytes; granulocytic oligocytosis does not make a transition to granulocytic pleocytosis and, unlike the former, appears in the initial stages of nonpurulent inflammations and in the early stage of cerebral ischemia.

7.2.2.2. *Lymphocytic Oligocytosis.* Lymphocytic oligocytosis is characterized by the presence of a larger number of activated lymphocytic elements and, in the case of a chronic course, even of plasma cells. It appears in association with multiple

TABLE 26
TYPES OF PATHOLOGICAL OLIGOCYTOSIS IN CSF

Pathological oligocytosis	Type of cells	Diagnoses
Lymphocytic oligocytosis	Activated forms of lymphocytes with possible presence of plasma cells	Chronic neuroinfections and multiple sclerosis
Monocytic oligocytosis	Prevalence of monocytes or marks of their activation present; phagocytosis also possible	Usually noninflammatory diseases or terminal phases of inflammations
Granulocytic oligocytosis	Neutrophilic oligocytosis	Frequent in early stages of inflammations
	Eosinophilic oligocytosis	Rarer affection, some autoimmune diseases, chronic affections as a whole
Tumorous oligocytosis		Presence of malignant cells accompanying monocyte reaction is usual

sclerosis and in some serous neurological infections. This type of oligocytosis is a smooth transition to lymphocytic pleocytosis.

7.2.2.3. *Monocytic Oligocytosis.* Monocytic oligocytosis is characterized by a numerical prevalence of monocytic line elements and signs of their activation by at least one of the above phenomena. Cytological findings are very hard to evaluate because of the generally low rate of detection of cellular elements and because of difficult evidence of phagocytes. However, if macrophagic elements are present at least occasionally, the etiological classification of these findings is usually easier. Otherwise, monocytic oligocytosis occurs in the end stages of all neurological infections. A specific substrate of phagocytosis can often be demonstrated; if absent, the entity is called residual monocytic stage, which may persist in the cytological picture for quite a long time after neurological infections. Monocytic oligocytosis is also a frequent cytological finding in Guillain–Barré polyradiculoneuritis. Along with the accompanying lipophagocytic reaction, it is also fairly often seen in CNS destruction.

7.2.2.4. *Tumorous Oligocytosis.* Tumorous findings can make a fully smooth transition from oligocytosis to pleocytosis. The criterion of importance in this classification is the detection of malignant elements. An accompanying cellular reaction may often be of quite a different type; however, it is usually a monocytic cellular reaction. Rarely, the presence of phagocytosis of malignant elements can be observed.

The classification proposed has been employed by our team as a binding one. In addition to internal use within the department, it is used to describe cytological conclusions to other departments. Syndromological cytological conclusions and diagnostic analysis may be regarded as an integral part of every investigation of CSF. It would be especially helpful to use a uniform and standard classification in each center.

7.3. Diagnostic Use of Macrophagic Elements in CSF

In addition to determining the total number of elements, cytological evaluation of CSF involves the qualititative representation of individual cell lines. Cells of the monocytomacrophagic (or possibly reticuloendothelial) system are represented both by quiescent elements and by activated cells whose number should physiologically exceed 10% of the total number of cells of the particular line. The finding of macrophages, i.e., monocytes with a clear phagocytic substrate, is a pathological finding (P3) (see Table 23). In the course of activation, most metabolic changes involve the spectrum of enzymes in the cytoplasm, the number of receptors on the membrane, the antigenic structure, or secretion of some substances. We can observe morphological manifestations of activation, including the rounding of roll-shaped nuclei, expansion of the cytoplasm volume, and formation of pseudopodia, as well as the onset of phagocytosis of a specific substrate and its

changes in the course of digestion. Depending on the character of the substrate, macrophages are further classified into erythrophages, siderophages, lipophages, lymphophages, leukophages, mycophages, and so on.

Activation of the monocytic line is a sign of primary noninflammatory processes. It is only in oligocellular CSF samples that this evaluation may be of principal importance for establishing a correct diagnosis.

Monocyte-line activation occurs in the course of infectious (both serous and purulent) diseases of the CNS. The macrophages present (or, alternatively, lymphophages and leukophages) are indicative of an advanced stage of the disease. In this case, lipophagocytosis is usually associated with a focal finding in the objective neurological examination and hence suspicion of meningoencephalitis.

Of essential importance is the evaluation of macrophages on the suspicion of intermeningeal hemorrhage (A8) (Table 27). Here the specific substrate are red blood cells whose phagocytosis by so-called erythrophages (Fig. 3, see color insert.) occurs in the initial stage not earlier than 4–6 hours after the start of bleeding. This is followed 2–3 days later by their digestion, manifesting itself by the formation of a halo (a bright circle around phagocytosed red blood cells) and their progressive discoloration until empty vacuoles are left in the macrophage. In the ensuing stage, hematogenic pigments (hemosiderin and hematoidin crystals) start to be scavenged. Hemosiderin, because of its trivalent iron content, is readily visualized with Berlin blue, enhancing its diffuse and granular nature; it cannot be seen until 4–5 days later. As a result, it is a reliable sign of a previous intermeningeal hemorrhage. Hematoidin that no longer contains Fe^{3+} and presents in the form of yellow-ochre crystals in macrophagic cytoplasm appears still later, on about

TABLE 27
CYTOLOGICAL PICTURE OF INTERMENINGEAL HEMORRHAGE

Intermeningeal hemorrhage	Cytological picture
(1) Phagocytosis of erythrocytes	Recent hemorrhage, presence of macrophages called erythrophages
(2) Digestion of phagocytosed erythrocytes	Decoloration and destruction of erythrocytes; optically empty vacuoles
(3) Hemosiderin	An iron-containing hematogenous pigment, present in so-called siderophages
(4) Hematoidin	Hematogenous pigment forming rhombic crystals in macrophages, then present extracellularly (after decline of macrophages)

Adhesion of erythrocytes—also detectable *in vitro.*

day 13. Later, it can also be noted extracellularly as late as 6 months after a hemorrhage. The presence of several of these stages concurrently enables us to detect protracted or repeated intermeningeal hemorrhage.

An important part is the evaluation of lipophagocytosis (A2, A3, A4, A5) (Fig. 4, see color insert). With basic staining, the nonspecific-looking foamy cytoplasm escapes attention. Oil red O staining (or Sudan black B or scarlet R) of lipids provides excellent visualization, which is why oil red is the second most frequently used stain after basic stain with May–Grünwald/Giemsa–Romanowski in our laboratory. Lipophagocytosis occurs as a scavenging response of the monocyte system of damage to and breakup of cerebral parenchyma for a number of causes. It is consequently a parameter with a wide area of applications.

A typical finding is that of monocytic oligocytosis or pleocytosis in cerebral ischemia. The degree of pleocytosis, which is a frequent finding in cerebral ischemia, cannot be regarded as a measure of parenchymal damage since the distance of the ischemic focus to CSF pathways space makes a difference. A diagnosis with lipophagosis, also a regular finding, allows us to assess the activity of a disease like vasculitis with CNS damage.

7.4. Use of Monoclonal Antibodies in CSF Cytology

A finding that continues to be frequent is that of tumor cells in CSF (A1, T5) (Table 28; Figs. 5–7, see color inserts.). Recently, there has even been an increase in the rate of detection of tumor elements, especially in hematological malignancies, where cytology is a frequent indication with respect to possible leukemic meningeal

TABLE 28
Malignant Cells in CSF and Criteria of Malignancy

Malignant cells in CSF	Criteria of malignancy
(1) Tumor cells—problematically distinguishable in Fuchs–Rosenthal chamber	(1) Polymorphism of cells
(2) Leukemic cells—resemble mononuclear cells in Fuchs–Rosenthal chamber	(2) Polymorphism of nuclei
	(3) Numerous and activated nucleoli
	(4) Giant cells
	(5) Multinucleated cells
	(6) Increased nucleus/cytoplasm ratio
	(7) Increased stainability
	(8) Numerous mitoses
	(9) Atypical mitoses
	(10) Basophilia of cytoplasma
	(11) Formation of syncytia
	(12) Polychromasia

infiltration. In other cases, malignant elements appear in CSF in the presence of metastases into the brain, the spinal canal, and in vertebral body destruction by the malignant process. A less frequent finding is that of malignant elements in CSF in primary tumor processes involving the CNS. In some forms of carcinomas, tumor cells can occasionally be detected even without the presence of metastases. On meningeal infiltration, if the tumor is in the vicinity of CSF pathways or intraventricular, the maximum rate of detection of malignant elements is 40–50%. Provided no malignant elements have been detected in the cytological preparation, the presence of a tumor process can be indirectly suggested by the finding of monocytic pleocytosis or monocytic oligocytosis.

In some cases, it may be difficult to distinguish malignant cells from normal cells. For instance, when counting elements according to the Fuchs–Rosenthal chamber, it is not easy to distinguish cells from the CSF pathway lining or common mononuclears. Some potential for misidentifiction in the cytological picture with neurological infection exists, especially with so-called leukemic meningeal infiltrations. It is therefore reasonable to assess, in the cytological preparation, the functional status of nucleoli stained with toluidine blue (staining according to Smetana), PAS positivity, or the presence of adipose droplets in the cytoplasm as markers of increased metabolic activity. Other usual criteria of malignancy include cellular polymorphy, nuclear polymorphy, multiple and activated nuclears, giant cells, polynuclear elements, considerable size of nuclei *vis-à-vis* cytoplasmic volume, increased tincture properties, frequent mitoses, nontypically dividing elements, cytoplasmic basophilia, syncytial formation or polychromasia.

Essentially, classification of malignant elements in CSF is extremely difficult because of the low number of cells detected in this manner, and also because of the considerable morphological changes occurring in these cells upon crossing into CSF and their presence therein. These changes include the loss of typical morphological markers and the rounding up of malignant cells.

If the number of suspicious cells in the cytological picture is low, mostly in oligocellular CSF samples, it is possible to multiply malignant or controversial cellular elements using the methods of tissue culture and further identification.

In such cases, a more exact diagnosis is possible only when using specific monoclonal antibodies against specific tumor markers—antigens (Table 29; Fig. 8, see color insert.). Individual cells or whole-cell populations can thus be assigned, using monoclonal antibodies, to the respective their cellular systems to. The degree of their maturity, the presence of some receptors or products of their secretion, the status of activation, or the degree during their cell cycle can also be determined.

Cytological findings are thus divided into several groups. The first group embraces tumors with no signs of invasive growth. In this case, evidence of tumor elements is rare on account of the primarily "benign" nature of tumor growing in this manner. The presence of these cells in meningiomas or neurinomas is a very rare finding. A more frequent finding is that of completely benign tumor cells

TABLE 29
SIGNIFICANT TUMOROUS MARKERS DETECTABLE IN CSF

Definitions	Diagnoses
GFAP—Glial fibrillary acidic protein	Majority of glial tumors
HMB-45—Human melanoblastoma	Malignant melanomas
CEA—Carcinoembryonic antigen	Mostly in tumors of gastrointestinal system
α_1-Fetoprotein	Expressed in seminomas penetrating to CNS
Vimentin	Mesenchymal tumors
C-erbB-2 oncoprotein	Nonspecific marker, more frequent in breast carcinoma
L-26—CD 26	B-lymphomas
BLA-36—HDLM-3	Hodgkin lymphoma
PCNA—Proliferating cell nuclear antigen	Breast carcinoma; also in other epithelial tumors
UCHL—IL-2 dependent T-cell line	T lymphomas
CD 43	T lymphomas
Ki-1—CD 30	Lymphomas as a whole
CD 14 a CD 68—KP-1	Histiomonocytic malignancies
CD 71	Proliferating cells as a whole (marker is a transferrin receptor)
MLA—Mucose lymphocyte antigen	Hairy cell leukemia
LCA—Leukocyte common antigen, CD 45 RB	All lymphomas
OPD 4—Helper/inducer phenotype	T lymphomas
Cytokeratin	Epithelial tumors

in ependymomas and papillomas of the choroid plexus mainly because of their presence in the vicinity of the CSF pathways.

The other group comprises tumors with signs of invasive growth. These tumors include especially malignant gliomas and metastatic tumors. In this group, malignant cells are a more frequent finding, phagocytosis of tumor cells is more frequent, and activation of the lymphatic line or pleocytosis can be seen more often (neutrophilic pleocytosis less often and eosinophilic pleocytosis very rarely). The picture of phagocytosis partly covers the contact mechanism between macrophages and lymphocytes.

The actual response of monoclonal antibodies with individual cells is usually visualized either directly (typically using fluorescent stains) or indirectly [using the reaction of antibody labeled with horseradish peroxidase (HRP) or other enzymes] with diaminobenzidine (DAB) (or other substrate while using other enzymes) under the microscope or in the flow cytometer. The latter, however, is not employed routinely in CSF immunocytology, although it has an advantage in clinical hematology.

The main advantage of evaluating the cytological preparation under the microscope is that it allows better assessment of cellular morphology in oligocellular CSF specimens. A limitation of immunotyping is the relatively high cost of the

procedure. In hematologic indications, 5% of malignant cells must be present as a minimum. Another major obstacle is the difficulty in distinguishing reactive granulocytosis with a shift to the left and reactive monocytosis from neoplastic states with small well differentiated cells, such as in chronic myeloid leukemia.

Still, we believe that widespread use of monoclonal antibodies in CSF immunocytology in tumor disease as part of the arsenal of routine techniques of examination will markedly improve the prognosis of patients because it offers the possibility of establishing the diagnosis early and prompt initiation of specific therapy.

8. Conclusions

After years of development, CSF or "liquorology" has become a consistent laboratory discipline, encompassing clinical cytology, biochemistry, immunology, and immunochemistry. In spite of the other laboratory disciplines, it still preserves its original character: Cooperation with the clinician is necessary in many clinical cases and its multidisciplinary character requires the cooperation of a number of specialists. In the last few decades, a boom in CSF has been observed, as witness innumerable publications concerning this rather complicated but still novel and fascinating topic. The author would not dare to predict the future of this discipline and its further development, but its clinincal applications and future findings should be highly promising in the field of CSF neurology and clinical medicine.

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APOPTOSIS, PART I: BIOCHEMICAL ASSESSMENT

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1. Apoptosis: An Overview

In Greek, *apò* means "from" and *ptòsis,* "a fall." The term apoptosis has been used to describe the falling off of petals from flowers or leaves from trees (K5). The widespread significance of apoptosis in biology and medicine was first recognized in 1972 (K5). Apoptosis is the most prevalent form of physiological cell death during development and other stages, permitting the elimination of unwanted cells (Table 1). The mechanisms for apoptosis in all cell types of multicellular and unicellular organisms are genetically encoded and readily expressed. All cell types are capable of undergoing apoptosis and each cell type must use one apoptotic pathway preferentially to others, indicating that the choice of cell-specific apoptotic pathways is predetermined genetically. Many environmental, pharmacological, or physiological stimuli—including DNA damage, radiation, chemotherapy, developmental signals, growth factor deprivation, hypoxia, heat, loss of adhesion, spindle disruption, ceramide, Fas ligand (FasL), tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL), glucocorticoids, and nonsteroidal anti-inflammatory drugs (NSAIDs)—can trigger apoptosis (K9, W15, W16, Z2). Abnormal apoptosis can lead to the development of various diseases. Although many authors use the terms apoptosis and programmed cell death (PCD) interchangeably (C6), it seems appropriate to distinguish between the two. Apoptosis denotes purposeful cell death with morphologically and biochemically apoptotic characteristics, whereas PCD describes the on-schedule cell death during ontogenic development. This article reviews the cell biology and clinical significance of apoptosis together with the updated biochemical methods for its assessment.

TABLE 1
PHYSIOLOGICAL ROLES OF APOPTOSIS

Function	Example
Developmental plasticity and selection of the fittest cells	Brain development (O5)
	Immune system development (H2)
Sculpting the body during embryogenesis	Disappearance of the human tail (F2)
	Digit formation of the limbs (G1)
Homeostasis of tissues	Hematopoietic system (M9)
	Ovarian follicle atresia (K1)
	Remodeling of endometrium during menstruation (T1)
Protection of organisms from threats created by deleterious cells	Inactivation of active immune cells after an immune response (V6)
	Host defense against tumorigenesis and viral infections (P2, W3)

TABLE 2
MORPHOLOGICAL AND BIOCHEMICAL DIFFERENCES BETWEEN NECROSIS AND APOPTOSIS

Necrosis	Apoptosis
Morphological features	
Begins with swelling of cytoplasm and mitochondria	Begins with shrinking of cytoplasm and condensation of nucleus
Loss of membrane integrity	Membrane blebbing without loss of integrity
	Aggregation of chromatin at the nuclear membrane
No vesicle formation, complete lysis	Formation of membrane-bound vesicles (apoptotic bodies)
Disintegration (swelling) of organelles	Mitochondria become leaky owing to pore formation involving proteins of the Bcl-2 family
Ends with total cell lysis	Ends with fragmentation of cell into smaller bodies
Biochemical features	
Loss of ion homeostasis	Highly regulated process involving activation and many enzymatic steps
Random digestion of DNA causing smear of DNA after agarose gel electrophoresis	Active process, energy (ATP)-dependent
	Cannot occur at 4°C
Passive process without the need for energy	Nonrandom mono- and oligonucleosomal length, fragmentation of DNA (ladder pattern after agarose gel eletrophoresis)
Occurs at 4°C	
Postlytic DNA fragmentation at late event of death	Prelytic DNA fragmentation
	Release of various factor (e.g., cytochrome C) into cytoplasm by mitochondria
	Caspase cascade is activated
	Alterations in membrane asymmetry (e.g., translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane)

1.1. APOPTOSIS AND NECROSIS

Eukaryotic cell death can occur via two distinct mechanisms: necrosis and apoptosis (M1, W19). There are many observable morphological and biochemical differences between necrosis and apoptosis (Table 2).

The term *necrosis* derives from the Greek *nekros* (dead) and *osis* (condition), meaning "deadness." It has recently been used to describe the status of death in cells and tissues irrespective of the mechanism of death. Nevertheless, necrosis ("accidental" cell death) is a pathological process consequent to groups of contiguous cells' exposure to nonphysiological disturbance from hypothermia, hypoxia, complement attack, chemical challenges, lytic viral infection, ischemia, or metabolic poisons resulting in damage to the plasma membrane. It begins with an impairment of the ability of cells to maintain homeostasis, leading to an influx of water and extracellular ions. The entire cell, including its intracellular organelles

(e.g., mitochondria and granules), swells and disrupts to release the cytoplasmic and organellic contents such as lysosomal enzymes or other cytotoxic proteins to the extracellular fluids (W19). Therefore, necrotic cell death is often associated with extensive tissue damage and an inflammatory response.

In contrast, *apoptosis* is a physiological process by which unwanted or useless cells are eliminated during growth, development, and other normal biological processes (see Table 1). It is induced by physiological stimuli such as lack of growth factors or change in hormone environment or immunological status. It often occurs during normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system, inflammation, and endocrine-dependent tissue atrophy. Apoptotic cells show characteristic morphological and biochemical features including chromatin aggregation, nuclear and cytoplasmic condensation, DNA fragmentation at the nucleosome linkage regions, cell shrinkage, and partition of cytoplasm and nucleus into apoptotic bodies (W17). These apoptotic cells are rapidly recognized and phagocytosed by either macrophages or epithelial cells before they lyse and cause inflammation (W19).

The main differences between necrosis and apoptosis are in the triggers (accidental *vs.* physiological), the process (energy-independent *vs.* dependent), and the outcomes (with *vs.* without inflammation). However, apoptosis and/or necrosis can be induced by the same causes in some cases (K16). Alteration of mitochondrial permeability is involved in both apoptosis and necrosis (K16). Both apoptosis and necrosis are found in conditions such as stroke and myocardial infraction (F5), and necrosis can occur secondary to apoptosis (T4). To preserve the usefulness of the two terms for denoting different modes of cell death while still recognizing possible overlap of the two processes (H7), some more descriptive terms have been proposed: primary necrosis (oncosis, ischemic cell death) (M1) and secondary necrosis (apoptotic necrosis, necrosis secondary to apoptosis) (K15).

1.2. Hallmarks of Apoptosis

1.2.1. *Death Receptors*

Many death-triggering signals are related to the TNF family, including FasL, TNF-α, lymphotoxin, CD30 ligand, CD40 ligand, CD27 ligand, and TNF-related apoptosis-inducing ligand (TRAIL) (N1). Apoptosis can be induced via the stimulation of several different cell surface receptors in association with caspase activation (e.g., caspase-8) (W13). For example, the Fas/CD95 receptor ligand system is a critical mediator of several physiological and pathophysiological processes such as cytotoxic T cell–mediated apoptosis. Fas receptor initiates a signal transduction cascade that leads to caspase-dependent apoptosis (K11).

1.2.2. *Alterations in Cell Membrane and Mitochondria*

Changes occur at the cell surface and plasma membrane in the early stages of apoptosis. One of the major plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer for external exposure (F1). This change of exposure requires the activation of caspase-3, a Ca^{2+} flux over the plasma membrane, and a change in Bcl-2 family (B8, B13, M6).

During apoptosis, the mitochondrial permeability is altered and apoptosis-specific protease activators are released from this organelle. The discontinuity of the outer mitochondrial membrane results in the release of cytochrome C to the cytosol followed by subsequent depolarization of the inner mitochondrial membrane (C5, P1). The release of cytochrome C further promotes activation of caspases, which are important molecules for initiating apoptosis (T6). Apoptosis inducing factor (AIF), another molecule released into the cytoplasm, has proteolytic activity and is by itself sufficient to induce apoptosis.

1.2.3. *Protease Cascade and DNA Fragmentation*

Signals of apoptosis lead to the activation of a family of intracellular cysteinyl aspartic acid proteases (caspases; see Section 3.3) to play a pivotal role in the initiation and execution of apoptosis induced by various stimuli. Fourteen caspases in mammalian cell have been identified (W13).

The biochemical hallmark of apoptosis is the fragmentation of the genomic DNA, an irreversible event that commits the cells to die. This fragmentation in many cell types has been shown to result from the activation of an endogenous Ca^{2+}- and Mg^{2+}-dependent nuclear endonuclease (W18). This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA), generating mono- and oligonucleosomal DNA fragments.

1.3. Recognition and Phagocytosis of Apoptotic Cells

The normal fate of cells undergoing apoptosis is their recognition, uptake, and degradation by phagocytes, thereby preventing the leakage of toxic cell contents [e.g., eosinophilic cationic protein (ECP) and major basic protein (MBP) in eosinophils, and perforin and granzyme B in cytotoxic T lymphocytes (CTL)]. Cell clearance by macrophage-mediated phagocytosis is fast, efficient, and injury-limiting (S4). Studies have partially defined a number of mechanisms by which apoptotic cells may be recognized by phagocytes via uncharacterized phagocyte receptors, including loss of sialic acid resulting in exposure of side-chain sugars (M12), increases in thrombospondin binding moiety (S5), and PS exposure (F1). It has been shown that the involvement of phagocyte receptors includes the lectins,

thrombospondin receptors $\alpha_v\beta_3$ and CD36, PS receptors, scavenger receptors, the 61D3 antigen, and the murine macrophage ABC-1 transporter. It has also been suggested that recognition mechanisms may be ordered in a hierarchy of "backups," each recognizing cells at different stages of the death program (S4). Further studies showed that phagocyte receptors might need to cooperate to achieve phagocytosis; for example, $\alpha_v\beta_3$ cooperates with CD36 to achieve high-efficiency phagocytosis (S5). The deficiencies in phagocyte clearance of apoptotic cells may contribute to the pathogenesis of persistent inflammatory and other immunological injuries.

1.4. Apoptosis and Cellular Response to Injury

DNA injury can initiate apoptosis by a powerful, early activated mechanism mediated by the nuclear phosphoprotein p53. This protein is activated by both transcriptional and posttranslational means and is critical in the cellular response to double-strand DNA breaks, which can be induced by high-energy radiation such as UV light. Although the detailed mechanisms are not well understood, p53 apparently plays a regulatory role whereby the cell is directed either toward the completion of repair or to apoptosis (W20). In fact, p53 was proven to be essential for the induction of apoptosis of some cells treated with DNA alkylating a gent (W19).

Injury to cell plasma membrane can activate acid sphingomyelinase to break down membrane lipid sphingomyelin and generate the second messenger ceramide, a complex lipid, to initiate the apoptosis (H1). Ceramide, perhaps through intracellular mitogen-activated protein kinases (MAPK), can alter cellular susceptibility to TNF-α, FasL, and ionizing radiation–induced apoptosis (H1, W11).

Injury to mitochondria can result in depolarization of their membrane, a characteristic early event in apoptosis. The depolarization is linked to the release of proapoptotic factors such as AIF and cytochrome C for the activation of caspases, thereby triggering apoptosis (K14).

CTL-mediated cell killing (e.g., removal of active immune cells after an immune response) is effected by at least two major pathways. This includes Fas-mediated apoptosis (R6) and the release of granular perforin (which increases the permeability of the target cell membrane) and a group of proteases (e.g., serine protease granzyme B). Granzyme B can directly activate the target cell caspases (G8).

2. Apoptosis and Disease

Homeostasis of cell numbers in the human body is a delicate balance between cell proliferation and apoptosis. Either deficient or excessive apoptosis may result in the development of different diseases (Table 3) (C2, T4, W4).

TABLE 3
APOPTOSIS-RELATED DISEASES

Diseases associated with decreased apoptosis	Diseases associated with increased apoptosis
Autoimmune diseases	Infectious diseases
Autoimmune diabetes	Acquired immunodeficiency syndrome (AIDS)
Autoimmune lymphoproliferative syndromes	Chemically induced diseases
Immune-mediated glomerulonephritis	Alcohol-related liver disorders
Cancer	Decreased blood production
Gene-dependent tumor formation:	Myelodysplastic syndromes
Bax, p53, retinoblastoma protein (Rb) mutation	Aplastic anemia
B cell lymphoma-2 (Bcl-2) overexpression	Heart diseases
Hormone-dependent tumors:	Idiopathic dilated cardiomyopathy
Breast, ovary, prostate	Myocardial infarction
Viral infections	Reperfusion injury
Adenovirus	Neurodegenerative disorders
Epstein–Barr virus	Alzheimer's disease
Herpesvirus	Amyotrophic lateral sclerosis
Poxvirus	Cerebellar degeneration
Allergic inflammation	Parkinson's disease
Delayed apoptosis of eosinophils	Retinitis pigmentosa
Others	Huntington disease
Endometriosis	Physically induced conditions
	Sunburn

2.1. DISEASES ASSOCIATED WITH INHIBITION OF APOPTOSIS

Diseases associated with inhibition of apoptosis include autoimmune diseases, cancer, viral infection, and allergic inflammation.

Apoptosis is essential for removing potentially autoreactive lymphocytes during development. Failure to remove these autoimmune cells can result in autoimmune diseases such as autoimmune diabetes (B3) and systemic lupus erythematosis (SLE) (R4, S1). SLE is a complex autoimmune disorder characterized by multiple immunological abnormalities, including an increased number of circulating activated B lymphocytes producing large quantities of autoreactive antibodies (K10). It has been suggested that B cell proliferation in SLE is T cell–dependent, and the persistence of autoreactive B and T lymphocytes is thought to be responsible for hypergammaglobulinemia and production of autoantibodies in SLE (B5). Apoptosis is the mechanism by which autoreactive lymphocytes are normally eliminated; the dysregulation of apoptosis therefore contributes to the development of SLE (R4, S1).

The accumulation of cancer cells is the net effect of cell proliferation and apoptosis (T4). The inhibition of apoptosis by activation of the proto-oncogene

(e.g., BRCA1 in breast cancer, Bcl-2 in leukemia) (V7, W17), mutation of tumor suppressor genes [e.g., Bax (M10), p53 (W3, W20), or Rb (L5, S3)], stimulation of antiapoptotic pathways by ectopic hormones (e.g., estrogen) (H8, J2), or a combination thereof is critical for tumorigenesis (H6).

Viruses with lytic cycles express antiapoptotic proteins to inhibit apoptosis of host cells for viral proliferation. The antiapoptotic proteins include inhibitors of apoptosis proteins (IAPs) and p35 synthesized by baculovirus (C8), E1B-19k from adenoviruses (D1), BHRF1 from Epstein–Barr virus (H3), the cowpox serpin protein CrmA (T2), and the Fas-associated death-domain protein (FADD)–like interleukin-1β converting enzyme (FLICE) inhibitory proteins from Kaposi's sarcoma–associated human herpes virus-8 (T3). Among the host cell reactions to the presence of viral infection is the activation of apoptosis, but viral synthesis of IAPs may forestall host cell death long enough to permit initiation of viral replication and synthesis of coat proteins for the completion of the viral infective cycle. The Epstein–Barr virus codes for two antiapoptotic proteins—BHRF-1, a Bcl-2 analog, and LMP-1, which appears to block signaling by the TNF receptor pathway (M13). The papilloma virus early gene product HPV16 E6 and the adenoviral E1A 55-kDa protein inactivate p53, while the adenoviral E1A 19-kDa protein enhances the intracellular effectiveness of members of the Bcl-2 family by binding their inhibitors (W12).

It is probable that the transforming genes of RNA viruses possess both replicative and antiapoptotic functions. Therefore, v-src, v-abl (C7), and probably the transforming ras genes (K2) inhibit apoptosis in addition to activating replication. The proto-oncogene tyrosine kinase abl has been shown to play a specific role in cells that have DNA injury, binding to and being phosphorylated by the DNA damage recognition protein ATM (B14). Although the precise role of abl in the cellular response to injury is not yet clear, it seems probable that this is part of a survival response in the injured cells.

In recent years, the incidence and prevalence of allergic diseases such as asthma and allergic rhinitis have been increasing worldwide and much effort has been devoted to the study of their pathogenesis (I1). Eosinophils are the principal effector cells for causing allergic inflammation. In allergic rhinitis, asthma, and atopic dermatitis, inhibition of eosinophil apoptosis has been proposed as a key mechanism for the development of blood and tissue eosinophilia. Subsequent release of cytotoxic granular proteins from activated eosinophils (ECP, MBP, and eosinophil peroxidase) causes allergic inflammation (S7). Therefore, it is important that eosinophils be effectively and rapidly destroyed concomitant with the removal of an inflammatory stimulus to avoid the excess tissue damage or chronic inflammation. Elucidation of the intracellular mechanisms responsible for restoring eosinophil apoptosis and preserving their phagocytic clearance should facilitate the development of therapies for inducing apoptosis of eosinophils *in vivo,* thereby allowing their rapid removal by macrophages or neutrophils to prevent their accumulation and release of cytotoxic proteins (W15, W16, Z2).

2.2. Diseases Associated with Increased Apoptosis

Among the diseases associated with increased apoptosis are acquired immunodeficiency syndrome (AIDS), hemopoietic diseases, heart diseases, and neurodegenerative diseases.

AIDS, caused by the human immunodeficiency virus (HIV), represents the most dramatic example of viral-induced cell depletion (A6). The FasL of T cells in AIDS patients is upregulated by two HIV gene products: Tat and gp120 for the depletion of CD4 T helper lymphocytes (A6). Tat is secreted by HIV-infected cells and can penetrate noninfected T cells to upregulate FasL expression in the affected cells and may thus facilitate Fas-mediated apoptosis. In addition, gp120 can sensitize T cells for CD95-mediated apoptosis (A6).

Various cytokines are required for the development of cell lineages in the hematopoietic system. Mainly, cytokines appear to act as survival factors to inhibit the apoptosis of these cells. The fact that hematopoietic stem cells in which apoptosis is suppressed by overexpressed Bcl-2 can differentiate in the absence of extracellular growth factors or cell division supports this hypothesis (T4). Myelodysplastic syndromes and some forms of aplastic anemia are associated with increased apoptosis of hematopoietic stem cells (T4).

Apoptosis is an important contributor to the integrity and complete development of the cardiovascular system and to the adaptation of the cardiovascular system to its continually changing demands (e.g., stress, sports, and illness). Therefore, increased apoptosis of vascular and myocardial cells is an important feature in human cardiac diseases including ischemic and nonischemic heart failure (e.g., myocardial infarction, idiopathic dilated cardiomyopathy, ischemic cardiomyopathy, and arrhythmias) (M2, O4, V5). Among the most potent stimuli that elicit cardiomyocyte apoptosis are oxygen radicals (e.g., nitric oxide), cytokines (Fas/TNF-α receptor signaling), stress conditions (chemical or physical, e.g., radiation), sphingolipid metabolites (ceramide), and autocoids (e.g., angiotensin II). Increased apoptosis of cardiac myocytes may contribute to progressive pumpfailure, arrhythmias, and cardiac remodeling (F5).

Accelerated apoptotic cell death has been observed in many neurodegenerative diseases such as Alzheimer's disease (K7), Huntington disease (W9), amyotrophic lateral sclerosis (K12), cerebellar degeneration (T4), neuron degeneration in Down's syndrome (B16), Parkinson's disease (J1), and retinitis pigmentosa (R1).

3. Biological Functions of Intracellular Regulators of Apoptosis

Apoptosis is one of a number of phenotypic responses that may occur as a result of a signal transduction pathways occurring in the cell. Clustering of cellular receptors is a commonly observed first step in the mechanism of signal transduction pathways to result in apoptosis. Receptor-clustered cytoplasmic domains trigger

subsequent steps in signal transduction pathways. An important link in this system is provided by signal molecules which bind directly to the intracellular domains of receptors or within a receptor signal complex. A schematic diagram of the major pathways for the intracellular regulation of apoptosis is shown in Fig. 1.

3.1. Fas and FasL

Many death-triggering signals are related to the TNF family, including FasL, TNF-α, lymphotoxin, CD30 ligand, CD40 ligand, CD27 ligand, and TRAIL (N1). Fas (Apo-1, CD95) is the receptor for FasL and a member of the TNF receptor family, e.g., TNFR1, TNFR2, lymphotoxin-βR, nerve growth factor receptor (NGFR), CD40, CD27, CD30, and death receptor 3 (DR-3). Binding of FasL to Fas results in clustering of the receptors' death domains (A5), or crosslinking Fas with agonistic antibodies induces the activation of the transcription factor NF-κB and apoptosis. Fas can also be induced by many cytotoxic drugs (F7). Fas and FasL play an important role in three types of physiological apoptosis (N1): (1) peripheral deletion of activated mature T cells at the end of an immune response; (2) killing of targets such as virus-infected cells or cancer cells by CTL and natural killer cells; (3) killing of inflammatory cells at "immune-privileged" sites such as the eye.

However, Fas/FasL interaction does not necessarily result in apoptosis, because downstream regulatory factors can suppress Fas/FasL death signaling (M14). Recently, a new family of six viral inhibitors (V-FLIPs for FLICE-inhibitory proteins) has been described. They interfere with the apoptosis signal through death receptors and are present in several herpes viruses including Kaposi's sarcoma–associated human herpes virus-8 and tumorigenic human molluscipox virus (K11). These V-FLIPs contain two death-effector domains that interact with the adaptor protein FADD, thereby inhibiting the recruitment and activation of the protease FLICE by the CD95 death receptor (N1). Protection of virus-infected cells against death-receptor–induced apoptosis may contribute to the oncogenicity of several FLIP-encoding viruses.

3.2. The Bcl-2 Family

It has been well established that Bcl-2 prevents most forms of apoptotic cell death as well as certain forms of necrotic cell death (T6). A large number of Bcl-2–related proteins have been isolated and divided into three categories (T6): (1) antiapoptotic members such as Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, A1 (Bfl-1), and Boo, all of which exert anti–cell death activity; (2) proapoptotic members including Bax, Bak, Bad, Mtd, and Diva; and (3) proapoptotic proteins, which include Bik, Bid, Bim, Hrk, Blk, Bnip3, and Bnip3L and share sequence homology only in Bcl-2 homology (BH)3.

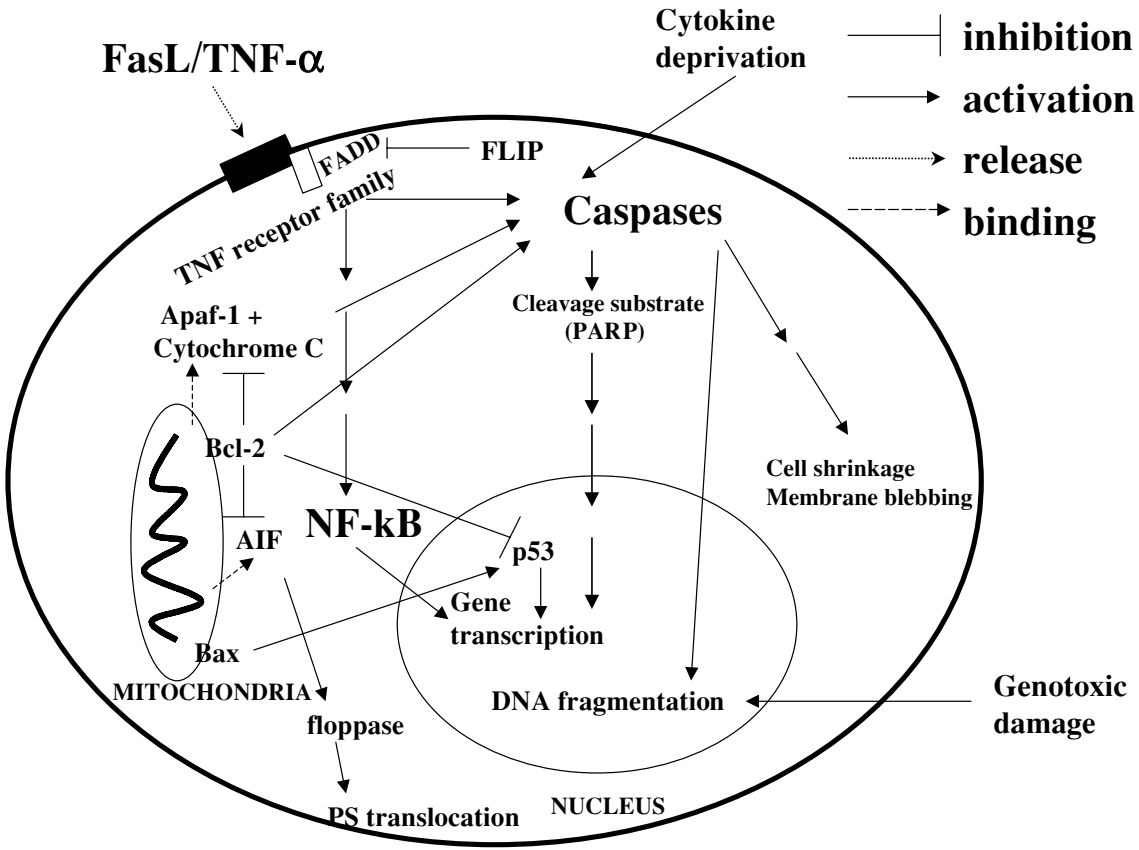


FIG. 1. Schematic diagram of the major pathways for the intracellular regulation of apoptosis.

The Bcl-X gene is closely related to Bcl-2 (B8), and its transcripts are alternatively spliced into long (L) and short (S) forms. The protein product of the long form functionally resembles Bcl-2 as a potent inhibitor of cell death. The Bcl-X_S splice variant antagonizes inhibition of cell death by the Bcl-X_L and Bcl-2 products (B8).

The action of Bcl-2–related proteins constitutes a major life-or-death decision of the mitochondria that depends on the ratio of the antiapoptotic and proapoptotic proteins (Fig. 1). It has been shown that mitochondria undergo a transition of permeability characterized by a breakdown of membrane potential followed by an outflow of AIF and cytochrome C into the cytoplasm. It seems that Bcl-2 and Bcl-X_L act against this permeability transition, whereas Bax and Bak suppress this antiapoptotic activity of Bcl-2 and Bcl-X_L (T6).

The Bcl-2 family has been extensively investigated and has become the target of many approaches to modulate its expression and function. Bcl-2 antisense oligonucleotides are able to induce apoptosis in leukemia cell line and primary cells, and enhance chemotherapy-induced apoptosis (K4). This approach has already been implemented successfully in a phase I study in treating lymphoma patients (W7).

3.3. Caspases

Caspases, a group of cytosolic aspartate-specific proteases, are probably the most important effector molecules that induce apoptosis (A1). In 1993, the *C. elegans* cell death gene *ced-3* was found to contain a remarkable sequence similarity to interleukin (IL)-1β-converting enzyme (caspase-1), a mammalian proteinase responsible for proteolytic maturation of pro-IL-1β (Y1). This discovery delineated the first member of the caspase family (caspase-1) and suggested that these proteinases might regulate apoptosis (Y1). Fourteen known mammalian members of the caspase family have been identified (W13). Activation of caspases correlates with the onset of apoptosis and their inhibition attenuates apoptosis. They are synthesized as inactive precursors (latent zymogens) and activated by autocatalytic cleavage (autoactivation), transactivation, or proteolysis due to other proteases (W13).

In response to extracellular apoptosis-inducing ligands, caspase-8 is activated in a complex associated with the FADD through self-cleavage (A5). FADD probably activates caspase-10 through similar mechanisms (V10). In response to agents that trigger release of cytochrome C from the mitochondria, procaspase-9 undergoes oligomerization for facilitating subsequent autoactivation (G7). Caspase-3 appears to amplify caspase-8 and -9 signals into full-fledged commitment to disassembly (T5). In general, the apoptotic initiators (caspase-2, -8, -9, and -10) generally act upstream of the small prodomain apoptotic executioners (caspase-3, -6,

and -7) (W13). On the other hand, caspase-1 and -11 function predominantly as cytokine processors (G6). Less is known about caspase-4, -5, -12, -13, and -14, but these caspases demonstrate a higher degree of sequence similarity to caspase-1 than to the apoptotic caspases (V3). Therefore, these caspases are grouped with the cytokine processors. Caspase substrate specificity, prodomain length, and prodomain sequence determine caspase function. Targets of activated caspases include poly(ADP-ribose) polymerase (PARP), DNase, and DNA-dependent protein kinase, which are involved in DNA damage and repair (E1); other targets include U1 ribonucleoprotein, nuclear laminins protein kinase C-delta, and cytoskeleton components such as actin (W13). Figure 2 summarizes the caspase activation mechanisms.

Recent studies have indicated that the activated caspases play a role in the formation of apoptotic morphology by proteolytic cleavages of a set of key proteins (Table 4). Although the exact mechanism whereby the degradation of these proteins results in apoptotic morphology remains unknown, many target proteins of caspases participate in the formation and regulation of the membrane-associated cortical microfilament cytoskeleton, which is an important determinant of the cell shape (Table 4). Insufficient apoptosis because of caspase inactivation may promote oncogenesis by allowing cell accumulation (M5). On the other hand, caspase overreactivity promotes cellular suicide, and this may be the basis for degenerative conditions such as Huntington's disease and Alzheimer's disease (G2, W8). Further investigation of the caspase system will provide a biochemical basis for the treatment of apoptosis-related diseases in the future.

3.4. Roles of AIF and Cytochrome C; p53, Protein Kinase B (Akt), and TRAIL

During the process of apoptosis, several mitochondrial proteins are released into the cytoplasm, including AIF and cytochrome C for the activation of proteases (L4). AIF, a flavoprotein, can induce apoptotic morphological changes of the nucleus in a caspase-independent manner (M7, S8). Cytochrome C probably executes apoptosis by interaction with cytoplasmic protein Apaf-1 and direct activation of caspases (L2). Since the release of AIF and cytochrome C is regulated by the proteins of the Bcl-2 family, Bcl-2 can inhibit apoptosis by retention of cytochrome C in the mitochondria (T6).

The p53 protein is essential for the induction of apoptosis as a response to chromosomal damage (e.g., γ-irradiation). It acts by blocking DNA replication of damaged cells. Cells deficient in p53 replicate in spite of the DNA damage to accumulate further mutations, thereby producing effects similar to overexpression of Bcl-2 to favor the accumulation of further mutations and reduce the efficiency of drugs for chemotherapy (E2).

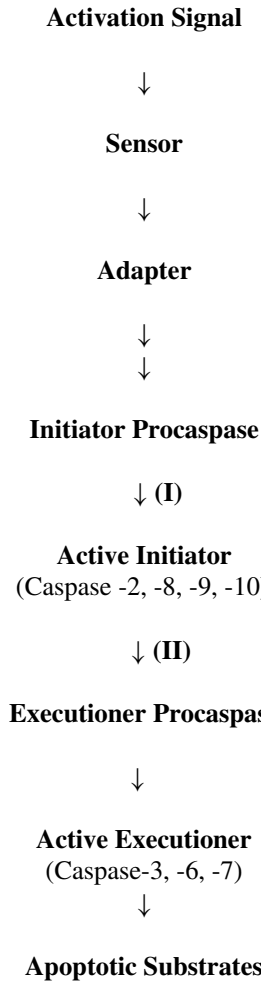


FIG. 2. Activation mechanism of caspases. Receptors convey activating signals to adapters that facilitate oligomerization and subsequent autoactivation of long prodomain caspases (I). Caspases transactivate other procaspases upon activation (II).

Protein kinase B (Akt) is a serine/threonine, mitogen-regulated protein kinase involved in the protection of cells from apoptosis as well as the promotion of cell proliferation and diverse metabolic responses. It is activated upon binding of phospholipids and phosphorylation at residues Thr and Ser by upstream kinases such as phosphoinositide-dependent protein kinase 1 and 2 (M15). Activation of

TABLE 4
STRUCTURAL PROTEINS PROCESSED BY CASPASES ASSOCIATED WITH APOPTOTIC CELL MORPHOLOGY

Protein	Function/Localization
Actin	Mircofilament-forming protein with various localizations and function, i.e., regulation of cell shape in the cortical cytoskeleton (B15)
Beta-catenin	Intracellular attachment protein in cell-to-cell junction sites (B12)
FAK	Regulation cell adhesion at cell–matrix and cell–cell contact sites (W10)
Gas2	Microfilament-organizing protein (B11)
Gelsolin	Microfilament-fragmenting protein (K13)
Keratins 18 and 19	Intermediate filament protein in keratinocytes (C4)
Lamin A and B	Intermediate filament that forms the nuclear lamina (O6)
MEKK-1	Regulation of cell survival and morphology at cell–matrix and cell–cell contact sites (C1)
NuMa	Mediator of nuclear chromatin–matrix protein interaction (C3)
PAK2	Protein kinase involved in regulation of cytoskeleton (R7)
Rabaptin 5	Membrane protein that regulates intracellular vesicle traffic (S9)
Spectrin/fodrin	Actin crosslinking protein in cortical cytoskeleton (W1)

Akt results in phosphorylation and inactivation of a number of proteins involved in apoptosis such as Bad and caspase-9 (C12).

The TNF-related apoptosis-inducing ligand (TRAIL) (TRAIL/Apo-2 ligand) is a recently described member of the TNF family that can induce apoptosis in many cell lines. Different receptors of TRAIL have recently been identified (D2). Two of these receptors, TRAIL-R1 and TRAIL-R2, contain classical cytoplasmic death domains and are able to transduce an apoptotic signal. The others lack functional death domains and are not able to promote cell death. In fact, it has been speculated that TRAIL-R3 counteracts the activity of TRAIL-R1 and TRAIL-R2 by competing with these molecules for binding to the ligand (A5). More recently, several other receptors for TRAIL have also been identified: DR4, DR5, DcR1, and DcR2. DR4 and DR5 were found to activate caspases through FLICE 2 (FADD-like IL-1β-converting enzyme 2). Subsequently, nonsignaling decoy receptors (DcR1, DcR2) were identified in normal human tissue but not in most cancer cell lines. Their recognition of TRAIL may prevent TRAIL from binding to functional TRAIL receptors, thereby blocking and not transducing the cell death signal (G3). However, the definitive role(s) of TRAIL in apoptosis remains to be determined.

3.5. MITOGEN-ACTIVATED PROTEIN KINASES (MAPK)

MAPK are serine and threonine kinases that are activated by an upstream dual-specificity kinase cascade in response to various stimuli (e.g., growth factors, proinflammatory cytokines) through tyrosine kinase receptors, cytokine receptors, and G-protein–coupled receptors in many cell types. Three structurally related

MAPK subfamilies have been identified in mammalian cells. The extracellular signal-regulated kinase (ERK) group ($MAPK^{p42\ and\ p44}$), also designated ERK2 and ERK1, is activated by growth factors including insulin, usually by means of a Ras/Raf-1–dependent cascade (C9, M4), whereas c-Jun NH_2-terminal protein kinase (JNK)/stress-activated protein kinase (JNK/SAPK) and p38 MAPK are strongly activated by UV irradiation, osmotic stress, and the proinflammatory cytokines TNF-α and IL-1 (D3). Therefore, these three MAPK subfamilies are activated in response to different extracellular stimuli to act on different downstream targets to perform different functions.

Previous studies have reported that ERKs are characteristically associated with cell proliferation and protection from apoptosis (B1, X1), while activation of JNK and p38 MAPK can promote apoptosis in many systems, including B lymphocytes (G5), cerebellar granule cells (K3), hematopoietic cells (K8), and neuronal cells (M3, X1). On the other hand, a recent report found that a pyridinyl imidazole, SB 202190, the specific inhibitor of p38 MAPK, by itself was sufficient to induce apoptosis in T lymphocyte Jurkat cells (N2). Moreover, Th-2–derived cytokine IL-5, the ERK activator and antiapoptotic factor for eosinophils, could also activate p38 MAPK in human eosinophils (B10). We recently reported that cytokine IL-3, IL-5, and GM-CSF could prolong survival of human eosinophilic leukemic (EoL-1) cells through the transient activation of ERK (W15). On the other hand, activation of p38 MAPK in EoL-1 cells by the NSAID sodium salicylate (NaSal) could lead to apoptosis (W15). We also found that the suppression of ERK using ERK antisense phosphorothioate oligodeoxynucleotides could promote the apoptosis of peripheral blood eosinophils (W16). Moreover, we found that dexamethasone-induced apoptosis and activation of JNK and p38 MAPK activity in eosinophils are regulated by caspases (Z2).

4. Biochemical Assessment of Apoptosis

As explained earlier, apoptosis is a fundamental process in cell biology that is critical for tissue and organ development, physiological adaptation, and disease. Therefore, detection of early apoptosis in different tissues is crucial for the diagnosis and monitoring of the disease activity, for example, in cardiovascular diseases (V5) and Alzheimer's disease (B7, H4). The assay of apoptosis is also very useful for elucidating the pathogenetic mechanisms of diseases (e.g., cancer, SLE, and allergic asthma) and in the design of new drugs for treating apoptosis-related diseases. Finally, assessment of apoptosis is also required for optimizing chemotherapy and radiotherapy so as to minimize the apoptosis-inducing effect of normal tissues.

Many biochemical methods have been developed for the assessment of apoptosis. They are based on the analysis of cellular morphology, DNA fragmentation, or

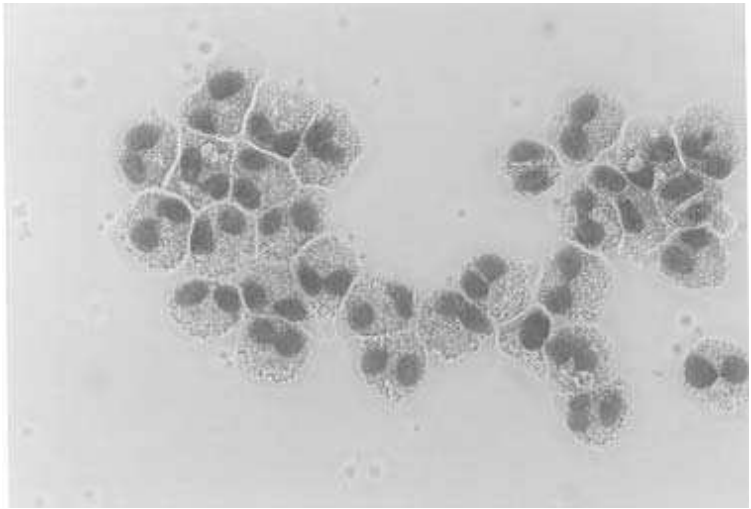
cytoplasmic and membrane changes. Below is a review of their principles, merits, and limitations.

4.1. Microscopic Analysis of Cellular Morphology

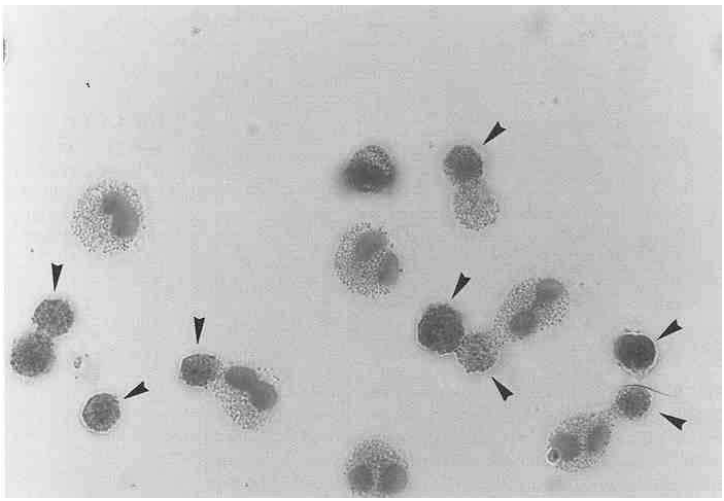
Microscopic analysis is the earliest technique to detect apoptosis by studying changes in cell morphology. Membrane blebbing (zeiosis), shrinkage of the cell, nuclear fragmentation, and chromatin condensation are general apoptotic characteristics that can be visualized with a Hemacolor Rapid Blood Smear Staining Set (E. Merck Diagnostica, Darmstadt, Germany) using light microscopy (LM) (Fig. 3). Briefly, the onset of apoptosis is characterized by shrinkage of the cell and the nucleus as well as condensation of nuclear chromatin into sharply delineated masses that become marginated against the nuclear membranes. Later, the nucleus progressively condenses and breaks up (karyorrhexis). The cell starts to detach from the surrounding tissue and its outline becomes convoluted and forms extensions. The term *budding* has been coined for a process whereby the extensions separate and the plasma membrane seals to form a separate membrane around the detached solid cellular material. These apoptotic bodies are crowded with closely packed cellular organelles and fragments of the nucleus. However, membrane and mitochondria are preserved inside the bodies (S2). The apoptotic bodies are rapidly phagocytosed into neighboring cells, including macrophages and parenchymal cells. Apoptotic bodies can be recognized inside these cells, but eventually they become degraded by enzymes. If the fragmented cell is not phagocytosed, it will undergo degradation that resembles necrosis in a process called secondary necrosis (K5). The previously mentioned apoptotic characteristics can be visualized using LM, which is suitable for screening a large number of cells. A disadvantage of LM is its low sensitivity (K5). Electron microscopy (EM) has the advantage of high specificity, permitting detection of morphological changes at a subcellular level (Fig. 4). In fact, the definition of apoptosis was first based on a distinct sequence of morphological features by EM, as described by Kerr *et al.* in 1972 (K5). However, it is unsuitable for routine use owing to the tedious preparation of the material and the limited number of cells that can be studied. Therefore, EM is considered to be a valuable, specific, and sensitive but qualitative special method for detecting the loss of intact intracellular structures during apoptosis (G4).

4.2. Annexin V Apoptosis Assay

In normal viable cells, PS localizes predominantly in membrane leaflets facing the cytosol (S6). Fadok *et al.* established the fundamentals for a novel apoptosis detection methodology by showing that apoptotic cells expose PS at their outer leaflet of cell surface during early apoptosis (F1). This change of exposure



(a) Normal eosinophils
(X 400)



(b) Eosinophils
NaSal (20 mM)
(X 400)

FIG. 3. Micrograph demonstrating the effect of NaSal on morphological changes in eosinophils (W16). After eosinophils were treated for 12 h (a) without or (b) with 20 m*M* NaSal, which is an apoptosis-inducing agent, cells were harvested and stained with Hemacolor Rapid blood smear staining set. The stained cells were examined by light microscopy. The arrows depict the apoptotic eosinophils

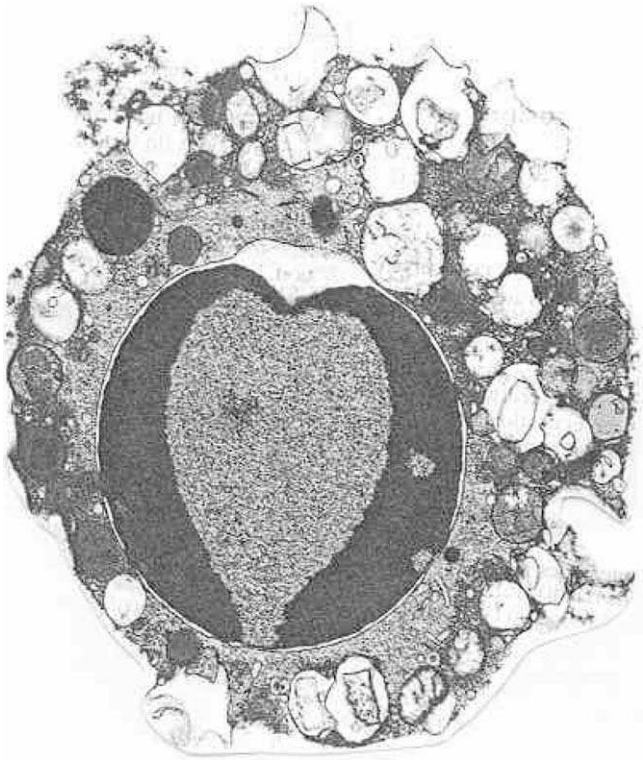


FIG. 4. An apoptotic peripheral blood eosinophil observed under EM. The apoptotic cell displays the characteristic condensation of nuclear chromatin into large, electron-dense masses surrounding the central, relatively electron-lucent nuclear matrix. The swelling and breakage of plasma and perinuclear membranes and the release of the contents of granules from swollen, enlarged, electron-lucent granule containers within secretory cells are also observed. A single osmiophilic lipid body is found in the cytoplasm (×18,500) (D6). Reproduced with permission from Dvorak, A. M., Images in clinical medicine, an apoptotic eosinophil. *N. Engl. J. Med.* **340,** 437 (1999).

requires the activation of caspase-3, a Ca^{2+} flux over the plasma membrane, and the change in Bcl-2 family (B8, B13, M6). Annexin V, a 32-kDa endogenous human protein, was originally isolated from the human umbilical cord artery by virtue of its anticoagulant activity (R2). It is able to bind to PS of apoptotic cells in a calcium-dependent manner (A4). Conjugation of annexin V to fluorescein, biotin,

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with reduction in cell size and increased chromatin condensation. Reproduced with permission from Wong, C. K., Zhang, J. P., Lam, C. W. K., Ho, C. Y., and Hjelm, N. M., Sodium salicylate-induced apoptosis of human peripheral blood eosinophils is independent of the activation of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase. *Int. Arch. Allergy Immunol.* **121,** 44–52 (2000) and S. Karger AG, Basel.

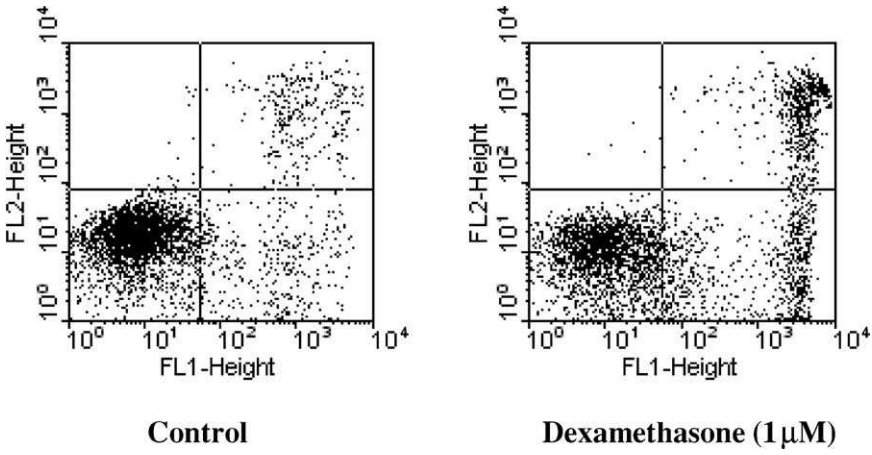


FIG. 5. Dot plots of lymphocytes treated with dexamethasone, a synthetic glucocorticoid, using annexin V–FITC and –PI by flow cytometry (Z1). Apoptosis of lymphocytes was assessed by TACS^{TM} annexin V–FITC/PI staining kit (Trevigen Inc., Maryland). Cells (1×10^6) cells from 24-h cultures were washed with cold PBS and resuspended in annexin V incubation reagent (100 μl). After incubation in the dark for 15 min at room temperature, binding buffer (400 μl) was added to samples and two-color immunofluorescence analysis was performed using a FACScan flow cytometer (Becton Dickinson). A total of 5000 events were gated on FSC versus SSC to exclude cell debris. Dot plots show early apoptotic cells in the lower right quadrant (annexin V–FITC-positive), late apoptotic (necrotic) cells in the upper right quadrant (annexin V–FITC-positive and –PI-positive), and viable cells in the lower left quadrant (double negative). Dexamethasone was found to induce a higher degree of apoptosis.

or horseradish peroxidase can be used to detect apoptotic cells with PS exposure (V8) using microscopy or flow cytometry. The sensitivity and specificity of the annexin V–based assay depends on the biological properties of PS and the physicochemical property of annexin V to bind to PS. Using flow cytometry, the annexin V binding assay with propidium iodide (PI) has become a widely used quantitative method for detecting apoptotic and necrotic cells (Fig. 5). Many companies (e.g., Trevigen Inc., Maryland Roche Diagnostics Ltd., Germany; R & D Systems, Minnesota; and Zymed Laboratory Ltd., California) commercially provide annexin V–binding assay kits. This assay detects the unique marker for apoptosis-related plasma membrane changes and allows the simultaneous labeling of other cell surface antigens and analysis by flow cytometry, fluorescence microscopy, or light microscopy. In conjunction with annexin V/biotin, it allows fixation following annexin V binding for further analysis of additional cellular parameters. However, annexin V assay has the following shortcomings. It requires many cells (10^6/test) and cannot be used on fixed samples. It is also not specific for apoptosis since annexin V can also stain the inner membrane of ruptured cells. Therefore, apoptotic cells must be distinguished from necrotic cells with an additional DNA stain [e.g., PI and 7-aminoactinomycin D (7-AAD)].

Propidium iodide can be used to assess plasma membrane integrity in annexin V apoptosis assays. It does not cross the plasma membrane of cells that are viable or in the early stages of apoptosis because of their plasma membrane integrity. In contrast, cells in the late stages of apoptosis or already dead have lost plasma membrane integrity and are permeable to PI for DNA staining (Fig. 5). In flow cytometric assays, another nucleic acid dye that can be used in place of PI for the exclusion of nonviable cells is 7-AAD. The advantage of 7-AAD over PI is its ability to be used in conjunction with phycoerythrin (PE)- and FITC-labeled monoclonal antibodies with minimal spectral overlap between the 7-AAD, PE, and FITC fluorescence emissions.

First developed for cells in suspension, the annexin V assay was later shown to be useful in adherent cell types (V4, V9) and in tissues (V2). It can also be used *in vivo* by injecting biotin enzyme–conjugated or radiolabeled annexin V into the bloodstream of living mouse embryos (O1, V1). In the murine ischemia and reperfusion model, administration of fluorescence-labeled annexin V to the circulation results in the visualization of apoptotic cells in the area at risk (V5). Recently, annexin V has been used as an apoptosis marker of cardiovascular tissues (V5) and noninvasive diagnosis of acute heart or lungtransplant rejection (B6). In some situations, however, cells can expose PS independent of an apoptotic process. First, myoblasts in the embryo express PS (P3). Second, embryonic megakaryoblasts expose PS before they fragment into multiple platelets (V1). Third, murine syncytiotrophoblasts in the placenta possess annexin V binding sites, suggesting the existence of viable cells with surface-exposed PS (W5). Because annexin V is widely distributed in mammals, it is possible that administration of exogenous annexin V may also interfere with physiological processes in which annexin V is involved, such as the blood coagulation, inflammation, and phagocytosis.

4.3. Apoptotic DNA Ladder Assay

During apoptosis, the activation of the endogenous Ca^{2+}/Mg^{2+}-dependent nuclear endonuclease leads to multiple breaks at the internucleosomal linker within the DNA, ultimately generating DNA oligomers whose size is proportional to that of a nucleosome with about 180 bp. The DNA fragments contain blunt ends (A1) and single-base 3′ overhangs (D4). Internucleosomal fragmentation has been demonstrated with well-characterized apoptotic morphology in a wide variety of situations and cell types (B9). Such DNA fragmentation in cells or homogenized tissues can be detected qualitatively using DNA extraction followed by agarose gel electrophoresis (C11) (Fig. 6). The advantages of the apoptotic DNA ladder assay are as follows: (1) It offers a definitive marker of apoptosis; (2) it allows demonstration of the mono- and oligonucleosomal DNA fragments (180-bp multimers); (3) no prelabeling of the cells is required; and (4) it is not limited to cells that proliferate *in vitro*. The major limitation is that it is not sensitive enough to detect

FIG. 6. Apoptotic DNA ladder pattern of eosinophils treated with dexamethasone (Dexa, 2 μM) for 18 h (Z1). DNA was extracted from cells with ethanol (P4) and electrophoresed on 1% agarose gel in 1 × TAE (Tris acetate–EDTA) buffer (pH 8.0). After electrophoresis, the gel was soaked in 1 × TAE buffer containing 0.5 μg/ml ethidium bromide, and DNA was visualized by an ultraviolet illuminator. Reproduced with permission from Zhang, J. P., Wong, C. K., Lam, C. W. K., Ho, C. Y., and Hjelm, N. M., Biochemical assessment of apoptosis. *Chinese J. Lab. Med. Clin. Sci.* **1,** 27–28 (2000).

small quantitative differences for quantitative measurement. Moreover, it is labor-intensive and time-consuming because only a few tests may be performed simultaneously. Commercial DNA ladder assay kits are available from Trevigen and Roche.

4.4. TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE–MEDIATED dUTP NICK END LABELING (TUNEL) ASSAY

This fast, sensitive method is used for assessing endonuclease-induced DNA strand breaks in single cell suspension or tissue section during apoptosis. Cleavage of the DNA in apoptotic cells yields double- and single-strand breaks. Apoptotic cells are fixed with formaldehyde and subsequently permeabilized. Using terminal deoxynucleotidyl transferase (TdT), these breaks in apoptotic cells can be detected by enzymatic labeling of the free 3′-OH termini with modified nucleotides (X-dUTP; X = biotin, DIG, fluorescein, or bromide). The principle of the TUNEL assay is shown schematically in Fig. 7. The apoptotic cells with DNA strand breaks can be visualized with colorimetric staining using LM (Fig. 8), or

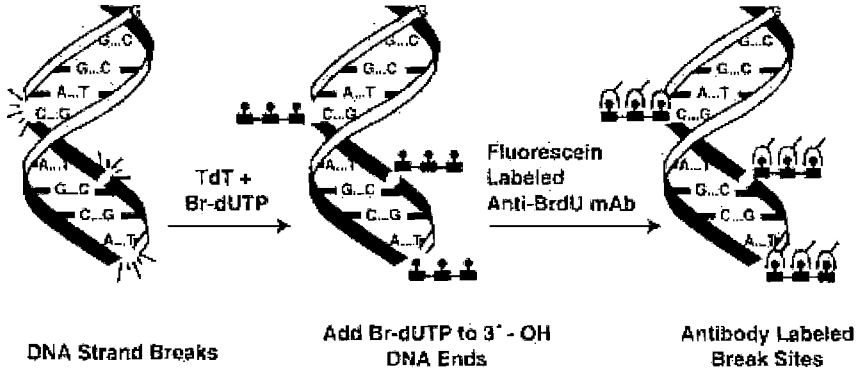
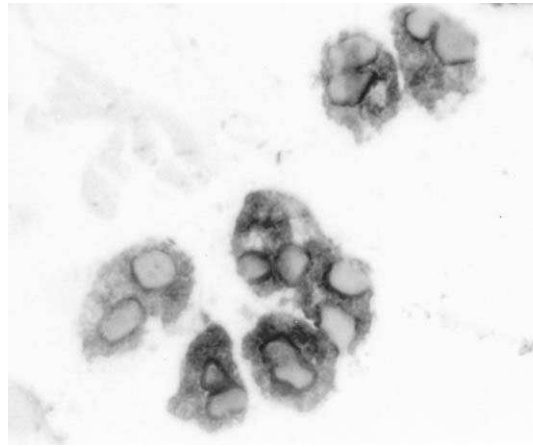


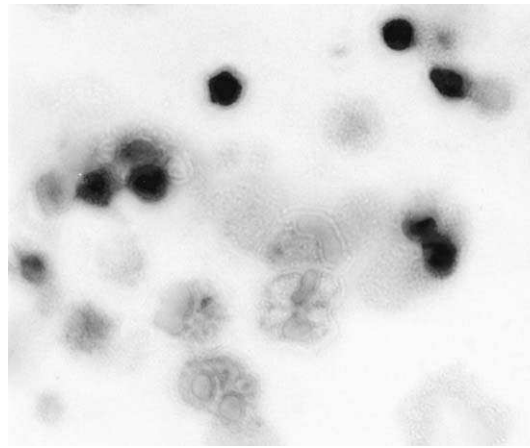
FIG. 7. Schematic representation of the principle of TUNEL assay. The enzyme TdT catalyzes a template-independent addition of bromolated deoxyuridine triphosphates (Br-dUTP) to the 3′-OH ends of double- and single-stranded DNA. After Br-dUTP incorporation, DNA break sites are identified by an FITC-labeled anti-BrdU monoclonal antibody.

quantitated with fluorescence using flow cytometry (Fig. 9). Many companies (e.g., Trevigen, Roche, R & D Systems, and Zymed) market the TUNEL assay kit. Since this method is based on the detection of DNA strand breaks, it can give a false negative result in rare situations when apoptosis is induced without DNA degradation. Moreover, extensive DNA degradation, even that specific to the internucleosomal linker DNA, may accompany necrosis. Thus one should always use another independent assay to confirm and characterize apoptosis. One advantage of this method is that counterstaining with DNA fluorochrome (profile of DNA content) allows the cell cycle specificity of apoptosis to be studied. Other advantages include (1) identification of apoptosis at a molecular level (DNA strand breaks), (2) suitability for tissue sections, (3) fast kinetics of dUTP incorporation in comparison with the DNA polymerase method, (4) high sensitivity for apoptosis using direct detection by fluorescein–dUTP without a secondary detection system, and (5) fewer working steps with the direct TUNEL labeling assay.

The combination of annexin V binding assay and the TUNEL method can reveal the presence of three subpopulations of apoptotic cells in tissues: (1) annexin V–positive/TUNEL-negative cells which are in the early phase of apoptosis, (2) both TUNEL- and annexin V–positive cells which are in the late phase, and (3) annexin V–negative/ TUNEL-positive cells which are located in phagolysosomes (V2). In summary, the TUNEL assay can be used for: (1) detection of individual apoptotic cells in frozen and formalin-fixed tissue sections for basic research and diagnostic pathology, (2) determination of the sensitivity of malignant cells to drug-induced apoptosis in cancer research and clinical oncology, and (3) typing of cells undergoing cell death in a heterogeneous population by double-staining procedures.



(a) Normal eosinophils
(X 400)



(b) Eosinophils
Dexamethasone (2 μM)
(X 400)

FIG. 8. Morphological changes of apoptotic eosinophils induced by dexamethasone (Z2). After eosinophils were treated (a) without or (b) with dexamethasone (2 μM) for 12 h, cells were harvested and detected by TUNEL assay using the *In Situ* Cell Death Detection Kit (Boehringer Mannheim). Briefly, cells were fixed with 4% paraformaldehyde and permeabilized by proteinase K and incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT). After washing to remove unbound enzyme conjugated antibody, the horseradish peroxidase retained in the immune complex was visualized by a substrate reaction with diaminobenzidine. The cell nucleus was counterstained with methanol green. Apoptotic eosinophils with nuclear DNA breaks were seen to stain dark brown using a Nikon Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan) in Fig. 8b.

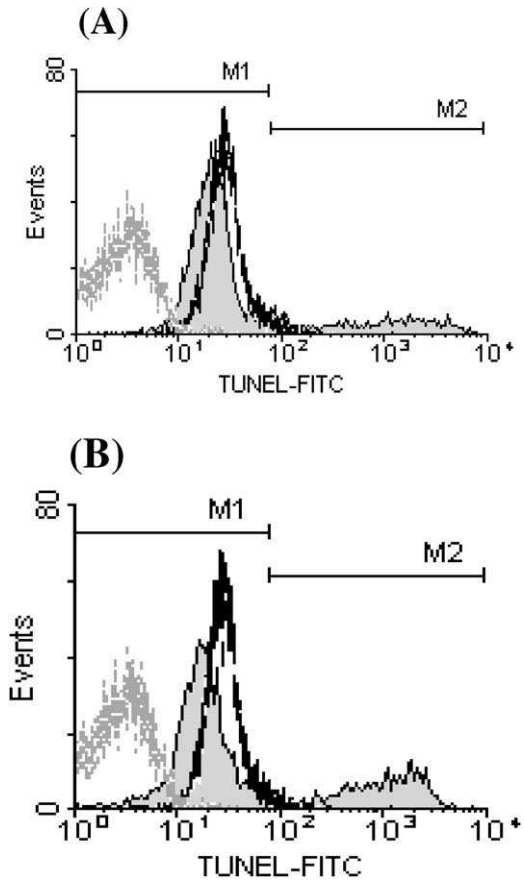


FIG. 9. Flow analysis of apoptotic human peripheral blood lymphocytes using direct TUNEL assay. Human peripheral blood lymphocytes (1×10^6) treated (A) without or (B) with dexamethasone ($0.1\ \mu M$) for 16 h were transferred to a 15-ml tube. Paraformaldehyde (2%) was added to cells with shaking and incubated for 10–15 min in ice with occasional shaking. Cells were washed with PBS with 1% BSA and 3–4 ml cold acetone was then added to cells. After 2–3 min incubation on ice with occasional shaking, cells were washed twice and TUNEL reaction mixture including enzyme TdT and fluorescein-labeled anti-dUTP antibody was added to cells (■). For the negative control group, only label solution without TdT was added to cells (□). Cells without any addition of reaction or label solution were used for assessment of the autofluorescence (□). The cell mixture was incubated 1 h at 37°C in the dark. The result of the apoptosis after flow analysis was expressed as a histogram using software CellQuest (Becton Dickinson). In Fig. 9A: M1 (nonapoptotic cells), 86%; M2 (apoptotic cells), 14%. In Fig. 9B: M1 (nonapoptotic cells), 78%; M2 (apoptotic cells), 22% (our unpublished data).

Reproduced with permission from Zhang, J. P., Wong, C. K., and Lam, C. W. K. Role of caspases in dexamethasone-induced apoptosis and activation of c-Jun NH_2-terminal kinase and p38 mitogen-activated protein kinase in human eosinophils. *Clin. Exp. Immunol.* **122,** 20–27 (2000) and Blackwell Science Ltd.

TABLE 5
EFFECT OF DIFFERENT TREATMENT OF EoL-1 CELLS ON APOPTOSIS (W14)

Treatment	Enrichment factor
Control	1.00
NaSal (5 m*M*)	2.81
NaSal (20 m*M*)	7.72
Anisomycin (10 μg/ml)	8.13
Dexamethasone (10 μM)	5.34
Anti-Fas antibody (5 μg/ml)	3.53
Hydrocortisone (10 μM)	2.81

EoL-1 cells (1×10^4/0.1 ml) were incubated with NaSal (5 and 20 m*M*), anisomycin (10 μg/ml), dexamethasone (10 μM), anti-Fas antibody (5 μg/ml), or hydrocortisone (10 μM) for 6 h at 37°C in a 96-well microtiter plate (MTP). MTP was centrifuged and supernatant was removed. The fragmented nucleosomes were quantitated using the Cell Death Detection ELISA^{PLUS} kit (Boehringer Mannheim, Germany). Briefly, after cells were incubated with lysis buffer, the cell lysate was transferred to streptavidin-coated MTP. The lysate was incubated with the immunoreagent containing antihistone and anti-DNA for 2 h at room temperature. After plate washing, a 2,2′-azino-di-3-ethylbenzthiazoline-sulfonate substrate solution was added to wells and incubated for 15 min at room temperature. Absorbance was measured at 405 nm using an ELISA plate reader. Results were expressed as the enrichment factor which is the ratio of absorbance of control and treated cells.

4.5. ELISA OF HISTONE-COMPLEXED DNA FRAGMENTS AND CELLULAR DNA FRAGMENTATION

This assay is a rapid one-step sandwich colorimetric immunoassay for quantitating histone-complexed DNA fragments (mono- and oligonucleosomes) with the size of discrete multiples of 180 bp in different types of cells in a microtiter plate (Boehringer Mannheim GmbH, Mannheim, Germany).

Because this sensitive nonradioactive kit does not require prelabeling of cells, it can detect internucleosomal degradation of genomic DNA during apoptosis even in nonproliferating cells. Moreover, it detects DNA and histones in one immunoassay demonstrating mono- and oligonucleosomal DNA fragments. However, samples must be analyzed immediately because storage reduces ELISA signals; therefore, the assay is not recommended for tissue homogenates owing to increased background by the activation of nucleases during sample preparation. Table 5 shows the results of the quantitation of apoptosis in human eosinophilic leukemia EoL-1 cells (W14).

The ELISA of cellular DNA fragmentation is a sandwich ELISA that measures apoptosis by quantitating the fragmentation and/or release of BrdU-labeled DNA. The commercial kit (Roche) can detect DNA fragments in the cell-free supernatants from cultured cells or cytoplasmic lysate of apoptotic cells prelabeled with BrdU

using two mouse monoclonal antibodies: one directed against DNA and the other against BrdU. This assay provides an alternative and specific choice to ELISA of histone-complexed DNA fragments for the assessment of apoptosis.

4.6. DNA Analysis by Flow Cytometry

Flow cytometric measurement of the percentage of apoptotic nuclei with PI staining has been shown to be a rapid, simple, reproducible method for assessing apoptosis of specific cell populations in heterogeneous tissues such as bone marrow, thymus, and lymph nodes (N3). The reduced DNA content of apoptotic nuclei resulted in an unequivocal hypodiploid DNA peak in the red fluorescence channels; therefore, apoptotic nuclei appear as a broad hypodiploid DNA peak (Sub G_0) that is easily discriminable from the narrow peak with normal (diploid) DNA content (G_0/G_1) (Fig. 10). Besides PI, acridine orange and Hoechest 33342 can also be used for staining of DNA content. The results from flow analysis have shown that the percentage of hypodiploid cells in both time-course and dose–response experimemts correlated well with the results of the classical DNA fragmentation assay (N3). If the flow cytometry pattern of the DNA and the expression of the surface antigens recognized by FITC-conjugated monoclonal antibodies are analyzed

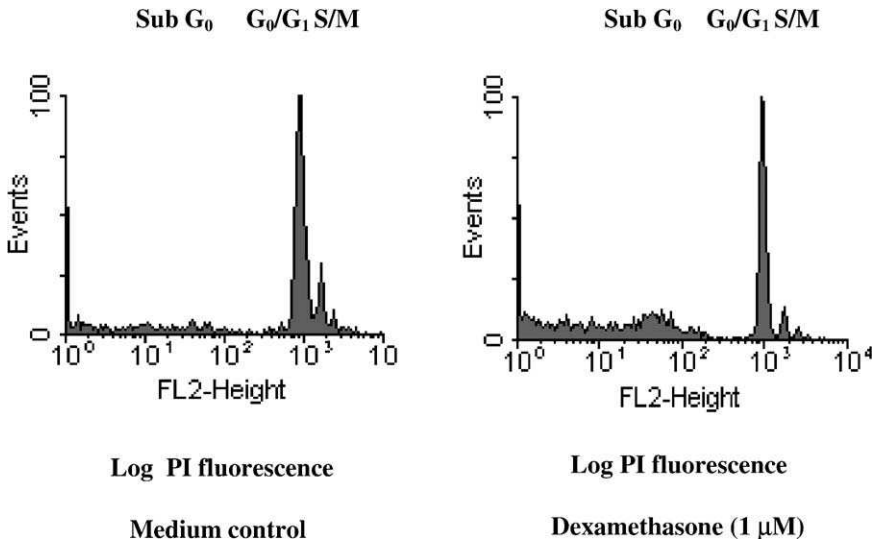


FIG. 10. DNA fluorescence flow cytometric profiles of PI-stained lymphocytes after 12 h incubation with 1 μM dexamethasone (Z1). Cells were fixed with 0.25% paraformaldehyde and treated with 70% ethanol at 4°C for 1 h to increase the cell membrane permeability. PI (50 μg/ml) was used to stain DNA for 30 min. Results of flow cytometer (FACSCAN, Becton Dickinson, California) were expressed as a histogram. Dexamethasone-treated lymphocytes showed a small peak before the G_0/G_1 peak of diploid cells. This characteristic sub-G_0 peak represents the DNA in apoptotic cells.

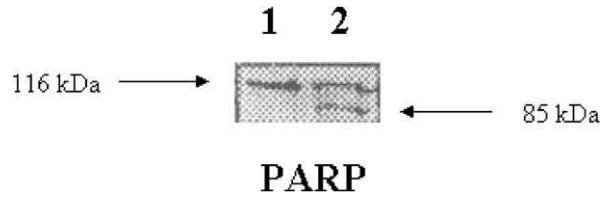


FIG. 11. Detection of cleaved PARP in cell extracts of apoptotic Jurkat-16 cells. Serum-starved cells were incubated in 10 μM apoptosis-inducing agent C2-ceramide. Cell extracts of treated or untreated cells were fractionated on 10% SDS–polyacrylamide gel. After electrophoresis, proteins on the gel were transferred to a PVDF membrane by electroblotting and the blot was blocked with 5% powdered milk. The blocked membrane was incubated with a 1:3000 dilution of anti-PARP. Subsequent incubations with a peroxidase-conjugated anti–rabbit secondary antibody and a peroxidase substrate indicated the presence of PARP cleavage products on the blot. Note that the antibody recognizes both cleaved PARP (116 kDa) and the larger cleavage fragment (85 kDa) (H5). Lane 1: Untreated control cells; Lane 2: Cells treated with 10 μM C2-ceramide. Reproduced with permission from Herr, I., Wilhelm, D., Bohler, T., Angel, P., and Debatin, K. M. Activation of CD95 (APO-1/Fas) signaling by ceramide mediates cancer therapy-induced apoptosis. *EMBO J.* **16,** 6200–6208 (1997) and by permission of the Oxford University Press.

simultaneously (N3), the combined method should prove useful for assessing apoptosis of specific cell types in different tissues (N5).

4.7. CASPASE ASSAYS

A variety of assays are available.

Assays utilizing anti-PARP antibody (R & D Systems, Roche) depend on the ability of the antibody to recognize PARP, a 113-kD DNA repair enzyme that binds DNA specifically at strand breaks. PARP is a substrate for certain caspases (e.g., caspase-3 and -7) in early apoptosis. These caspases cleave PARP into fragments of approximately 85 kDa and 24 kDa (Fig. 11). It appears that cleavage of PARP facilitates the degradation of cellular DNA. Detection of the 85-kDa degraded PARP (PARP p85) fragment with anti-PARP (Promega) using Western blot thus serves as an early marker of apoptosis (K6). PARP p85 in the cytoplasm can also be localized by immunocytochemical staining. This nonradioactive method is flexible and has application for many different cell types without prelabeling of cells. However, it is insensitive, labor-intensive, and time consuming.

ELISA has been used for measuring caspase activity. For the ELISA of intracellular caspase activity at the very early stages of apoptosis, apoptotic cells are first lysed to isolate their intracellular contents. Different caspase activities in the cell lysate can then be determined by the addition of a caspase-specific tetrapeptide substrate that is conjugated to the color reporter molecule *p*-nitroanilide (pNA) (e.g., DEVD-pNA for caspase-3 and IETD-pNA for caspase-8). The cleavage of the substrate peptide by the caspase releases the chromophore pNA, which can be

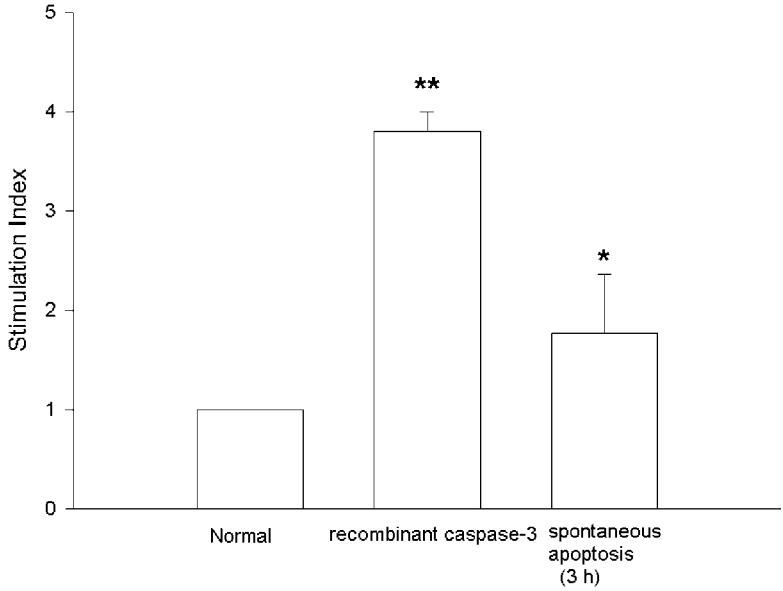


FIG. 12. Effect of spontaneous apoptosis on the activation of caspase-3 in human eosinophils. Eosinophils (2×10^6/ml) were cultured for 3 h. Cells were lysed and caspase-3 activity was measured by caspase-3 colorimetric assay kit (R & D Systems). Enzymatic products were measured at 405 nm with BIOTEK EL340 ELISA microplate reader (BIO-TEK Instrument Inc., Vermont). Human recombinant caspase-3 (5 U) (Calbiochem, California) was used as a positive control. The stimulation index was determined by direct comparison to the level of the normal control. Background readings from cell lysates and buffers were substracted from the readings of both induced and uninduced samples samples before calculating the stimulation index in caspase-3 activity. The differences between control and treated groups were assessed by Student's *t*-test. $^{*}P < 0.05$; $^{**}P < 0.001$ (Z2).

quantitated by the ELISA plate reader. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction (N4). It is a specific assay for individual caspase without cross reactions with other members of the caspase family. However, this assay requires quite high cell numbers (10^6/assay). The ELISA kit for caspase assay is commercially available from Biosource Europe, Nivelles, Belgium; R & D Systems; Roche; and MBL Co., Nagoya, Japan. Figure 12 shows the result of a caspase-3 ELISA assay for the assessment of apoptosis in human eosinophils (Z2).

The FITC-VAD-FMK *in situ* assay is based on the use of FITC-VAD-FMK *in situ* marker (Promega Corp., Wisconsin) which is an FITC conjugate of the caspase inhibitor Z-Val-Ala-Asp (OMe)-CH_2F (Z-VAD-FMK) which irreversibly inhibits caspase-1, -3, -4, and -7. FMK allows delivery of the inhibitor into the cell where it irreversibly binds to activated caspases (F3). The FITC label allows for a direct, one-step assay of caspase activity in living cells or tissue section. The inhibitor

can be added to cells in culture to block apoptosis, then used to detect qualitatively the presence of caspases using fluorescence microscopy. Recent studies have also shown that methods based on demonstration of caspase activation can be applied to detect apoptotic cells in tissue sections by LM (S2). However, sufficient quantitative accuracy and reproducibility of these assays remain to be established.

Finally, the use of anti–active caspase-3 antibody is based on its targeting. Caspase-3 (CPP32, Yama, or apopain) has been implicated as a key protease that is activated during the early stages of apoptosis (T5). Caspase-3 exists in cells as an inactive 32-kDa proenzyme, called procaspase-3. Active caspase-3, found in cells undergoing apoptosis, consists of a heterodimer of 17- and 12-kDa subunits which are cleaved from the 32-kDa proenzyme by upstream proteases such as caspase-6 and -8 and granzyme B. The downstream substrates of caspase-3 include PARP and others (W13). Active caspase-3 proteolytically cleaves and activates other caspases, as well as other targets in the cytoplasm, e.g., PARP, sterol regulatory element binding proteins (SREBPs), and nuclear lamins (W13). The overexpression of caspase-3 can result in apoptosis. Antibodies (R & D Systems) are available against an active human caspase-3 fragment and bind to a conformational epitope which is exposed by activation-induced cleavage or denaturation of the inactive enzyme (procaspase-3). These antibodies are useful for flow cytometry, for immunohistochemistry of acetone-fixed, frozen cytospins or tissue sections, and for immunoprecipitation. However, the antibody may cross-react with procaspase-3 at high concentrations or in techniques where procaspase-3 is denatured (e.g., Western blotting).

4.8. Comet Assay

Rydberg and Johanson (R8) introduced single-cell gel electrophoresis as a method to measure DNA damage in individual cells. Subsequently, greater understanding of the biological details of apoptosis and advancements in digital imaging have established the comet assay as one of the better methods for measuring cellular DNA damage. The comet assay is a single-cell gel electrophoresis assay that can evaluate cellular DNA damage (F6). Cells are immobilized on a slide that is covered with low-melting-point agarose and then gently lysed. Following treatment to (1) denature DNA, (2) hydrolyze DNA at sites of damage, (3) electrophorese degraded DNA, and (4) stain separated DNA with fluorescent dye (e.g., ethidium bromide), the sample is analyzed by microscopy. In apoptotic cells, the DNA strand breaks damage the higher order, tightly packed structure of DNA to allow a mass migration of the DNA out of the nucleus and cell body (the comet head) into the surrounding agarose (the comet tail) (Fig. 13). This assay was named for the characteristic shape seen when DNA exits the nucleus and cell body and migrates toward the anode. The amount of DNA damage can be quantified using a digital comet image by combining tail length and the distribution of DNA in the tail. Assay kits are available commercially from R & D Systems and Promega.

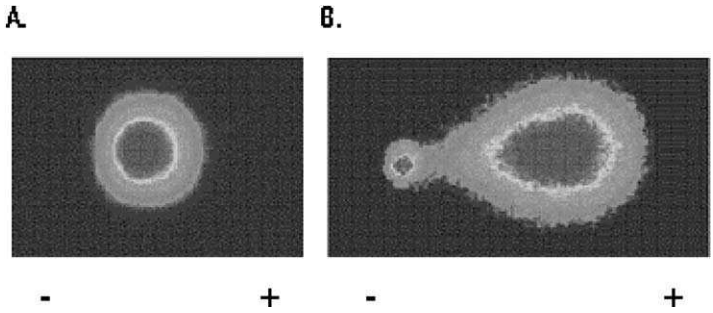


FIG. 13. Digital images of HL-60 cells embedded in agarose and stained with PI using comet assay (B2). Migration is left to right of the DNA in the electric field. (A) Normal cell with an undetectable degree of DNA fragmentation, resulting in the cellular DNA remaining in the nucleus. (B) Apoptotic cell showing severe fragmentation of cellular DNA, evidenced by a mass migration of the DNA out of the cell body (comet head) and into the surrounding agarose (comet tail).

The principal advantage of the comet assay is that it measures the response of individual cells, thus allowing the study of heterogeneity within a cell population. The ability to evaluate heterogeneity of response has potential value in many research areas, e.g., tissue-to-tissue variations in cellular response to ionizing radiation to optimize radiation therapy. Another obvious advantage is in the study of apoptosis on a cell-by-cell basis. Different assay conditions allow the study of either single-strand or double-strand DNA damage. Alkaline treatment facilitates the unwinding and denaturation of the DNA molecules for the sensitive detection of single-strand damage. Neutral assay conditions do not induce denaturation and allow the detection of only double-strand damage. The ability to analyze these two endpoints separately is another advantage of these assays (O3, R5).

The best way to quantify the amount of DNA damage using a digital comet image is by combining tail length and the distribution of DNA in the tail. These two important comet quantities define the "tail moment." The tail moment is calculated as a true physical tail moment about the center of a comet head. Intensity integrals for each column of pixel data are weighted by multiplying by their horizontal distance (in pixels) from the center of the comet head. These individual moments are then summed for the entire comet tail and normalized to the total comet intensity to give the tail moment. If the DNA intensities in pixel values represent mass, the tail moment is the distance from the center of the comet head to the tail's center of mass multiplied by the fraction of the DNA in the tail. Therefore, calculating the tail moment can include not only factors in the amount of DNA that migrates into the comet tail, but also the distance it migrates. The combination of comet assay with either annexin V apoptosis assay or TUNEL assay can provide a way first to screen for whether apoptosis has occurred and then to assess the heterogeneity of cells in a given cell population.

4.9. Mitochondrial Membrane Assays

Three techniques are discussed.

In the mitochondrial membrane permeability assay, advantage is taken of the fact that the mitochondrial transmembrane potential changes during early apoptosis, thereby causing altered membrane permeability (G7). A lipophilic cationic dye is now commercially available (Clontech Laboratories Inc., USA; R & D Systems), which is sensitive to these changes in mitochondrial membrane permeability by showing different fluorescences in apoptotic and nonapoptotic cells. In apoptotic cells, this cationic dye (e.g., MitoSensor™ from Clontech) cannot accumulate in the mitochondria because of the altered mitochondrial membrane permeability. Therefore, the dye remains in monomeric form in cytoplasm to give a green fluorescence. In normal cells, the dye is taken up and kept in mitochondria, where it forms aggregates that show an intense orange fluorescence. The fluorescence signals are easily distinguished by fluorescence microscopy or flow cytometry. Therefore, this sensitive and quantitative assay is particularly useful for studies of changes in mitochondrial membrane potential during apoptosis and other cellular process involving the alteration of mitochondrial membrane. This is a rapid method that takes just 20 minutes to perform and requires only small amounts of cells.

Another assay is the Apo2.7 assay. This assay is a simple staining procedure for detecting the newly described protein Apo2.7 (7A6 antigen), which is exposed on the mitochondrial membrane of apoptotic cells using fluorescence dye–conjugated anti-Apo2.7 monoclonal antibody (Coulter-Immunotech GmbH, Krefeld, Germany; Clontech). The apoptotic cells can be quantitated by flow cytometry (L3). Although the expression of Apo2.7 is a sensitive marker of cell death, it may not be specific for apoptosis alone as it can be detected in cells treated with cell membrane–permeabilizing viscotoxins (B17). In conjunction with annexin V labeling, two independent apoptotic events can be detected with one kit: the exposure of the Apo2.7 antigen on the mitochondrial membrane and the exposure of PS on the plasma membrane. It is a simple, quick method providing easily interpretable results.

The immunocytochemical staining of cytochrome C offers another alternative since, upon exposure to apoptotic stimuli, cytochrome C is rapidly released into the cytosol, an event that may be required for the completion of apoptosis in some systems (L2). The effect of cytosolic cytochrome C is thought to be the activation of caspases. The immunocytochemical staining of cytochrome C localized in mitochondria in healthy cells or diffused in the cell cytoplasm with monoclonal antibody (Promega) after induction of apoptosis, as detected by fluorescence microscopy, can be used for monitoring apoptosis (L2, M7, S8, T6). It is also a simple, rapid, specific method for quantitative assessment of apoptotic cells.

Pharmingen also provides two antibodies to cytochrome C which may be used for immunoprecipitation or Western blotting of cell and tissue extracts.

4.10. Antibodies against Apoptosis-Related Signal Proteins

As mentioned before, apoptosis is one of the cellular responses that may occur as a result of signal transduction pathways. Different antibodies have been developed for recognizing different apoptosis-related signal proteins, e.g., the Bcl-2 family including Bcl-2, Bcl-X, Bad, Bak, and Bax, as well as Fas, FasL, FADD, TRAIL, Rb, p53, receptor-interacting protein (RIP), TNF receptor–associated death domain (TRADD), TNF receptor–associated factors (TRAFs), and IAPs (Pharmingen, Zymed, R & D Systems). The detection of these apoptosis-associated proteins using immunocytochemical staining, flow cytometry, Western blot, mRNA expression array or immunoprecipitation is useful for the assessment of apoptosis.

4.11. Ribonuclease Protection Assay (RPA)

The RPA is a highly sensitive and specific method for the detection and quantitation of mRNA species. Pharmingen has developed a multiprobe RPA system to generate a series of apoptosis-related gene templates, each of distinct length and each representing a sequence in a distinct mRNA species. The templates are assembled into biologically relevant sets (caspases, Bcl-2, death receptors, FasL) to be used for the T7 polymerase–directed synthesis of a high–specific-activity, ^{32}P-labeled antisense RNA probe set. The probe set is hybridized in excess to target RNA in solution, after which free probe and other single-stranded RNAs are digested with RNases. The remaining "RNase-protected" probes are purified, resolved on denaturing polyacrylamide gel, and quantitated by autoradiography or phosphorimaging. The quantity of each mRNA species in the original RNA sample can then be determined based on the intensity of the appropriately sized, protected probe fragment (M11).

This multiprobe RPA offers the advantages of its sensitivity and capacity to simultaneously quantitate several mRNA species in a single sample of total RNA. This allows comparative analysis of different mRNA species within samples, moreover, by incorporating probes for housekeeping gene transcripts, the levels of individual mRNA species can be compared between samples. Furthermore, the assay is highly specific and quantitative owing to the RNase sensitivity of mismatched base pairs and the use of solution-phase hybridization driven toward completion by excess probe. Finally, the multiprobe RPA can be performed on total RNA preparations derived by standard methods from either frozen tissues or cultured cells.

Because different apoptotic characteristics (e.g., activation of caspases, DNA fragmentation) appear at different times after the initiation of apoptosis, different

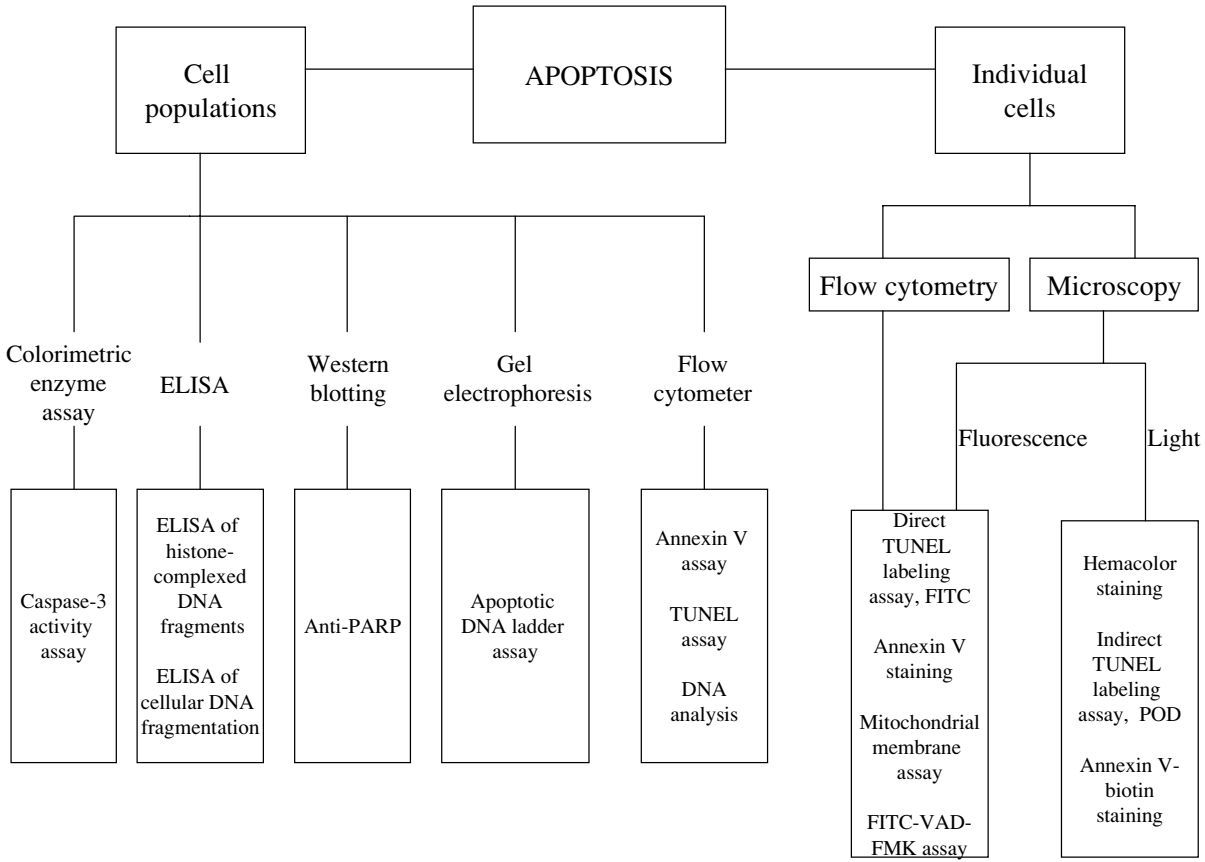


FIG. 14. Selection guide of suitable apoptosis assessment methods based on different cell types and methodologies available in the laboratory.

TABLE 6
PROGRESSIVE STAGES AT DIFFERENT TIMES OF ANTI-FAS–INDUCED APOPTOSIS IN JURKAT CELLS (A3)

Event	Time after induction by anti-Fas (h)	Detection method
Activation of caspases	1.2	Colorimetric enzyme assay of caspase activity
PARP cleavage	1.6	Anti-PARP antibody
PS externalization	2.8	Annexin V apoptosis assay
DNA fragmentation	4.8	Apoptotic DNA ladder assay TUNEL assay
Morphological change	8.0	Microscopy
Plasma membrane leakage	10.0	PI staining

detection methods for apoptosis must be considered at various times. Table 6 lists the general progressive stages of apoptosis at different times upon treatment with an apoptosis-inducing agent, anti-Fas, and the different apoptosis assessment methods for each stage (A3). Figure 14 is a selection guide of different apoptosis methods.

5. Conclusions and Perspective

A wide variety of methods have been developed for biochemical assessment of apoptosis. In addition to the above methods, the gene expression of apoptosis-related molecules (e.g., p53, Rb, Bcl-2, Bax, Bcl-X_L, c-myc, Fas, and FasL) detected by reverse transcription–polymerase chain reaction or cDNA expression arrays can also be used for the investigation of apoptosis. However, a general disadvantage of the current methods is that many of them are single-endpoint measurements, which do not provide information on the dynamics of the apoptotic process. Consequently, different assays assess apoptotic events occurring at different times after the onset of apoptosis (D5) (Table 6). Moreover, assessment of only one parameter will, in most cases, result in insufficient sensitivity and specificity. This can be improved by measuring multiple parameters (e.g., apoptotic events in mitochondrial and plasma membranes). It would also be very useful if techniques could measure parameters (e.g., caspase activity, alteration of mitochondrial membrane potential) active at the transition from the induction to the propagation phase of apoptosis. Therefore, further technological development is required for the wider application of assessing apoptosis in disease.

Understanding the role of apoptosis-related molecules (MAPK, caspases, NF-κB, the Bcl-2 family, etc.) will have important implications for the development of novel therapeutic modalities that target critical apoptosis-related molecules for apoptosis-related diseases (L1, W9, W15, W16, Z2) and for the development

of more specific and sensitive apoptosis assays for clinical utilization. The differences and similarities between different cell types (e.g., eosinophils and neutrophils, tumor cells and normal cells) demonstrate that there is a potential for the development of agents with specificity to remove different cell types selectively. Therefore, a similar strategy might prove useful in the development of compounds for the selective deletion of cancer cells resulting in the dissolution of tumors without damaging healthy nonmalignant tissues. In fact, several antiapoptotic agents that may provide cardioprotection due to an antiapoptotic mechanism have been reported: (1) p38 MAPK inhibitors, e.g., SB 203580; (2) caspase inhibitors, e.g., Z-VAD-FMK; (3) β-adrenergic receptor blockers; and (4) antioxidants and growth factors that regulate c-Jun N-terminal kinase (JNK) (O2, W2). Moreover, Z-VAD-FMK has been shown to serve as a potential drug for neurodegenerative disease (W9, T5).

The development of cDNA microarray or DNA-chip technology for investigating changes in gene expression during apoptosis, together with the development of animal models using transgenic or knockout mice, will widen our knowledge of intracellular mechanisms for regulating apoptosis and hence facilitate further development of novel methods for the assessment of apoptosis (V11). For example, experiments using Bcl-2 transgenic mice have provided proof of the concept that enhanced expression of antiapoptotic pathways may provide protection from ischemic injury (K9). Animal models using mice with mutation of the Fas gene (*lpr*) or the FasL gene (C3H*gld*) (C10) and transgenic mice for overexpression of the antiapoptotic molecule Bcl-2 develop prolonged B cell survival and other common pathological features of SLE (M8).

In summary, using suitable methods for qualitative and quantitative assessment of apoptosis is useful for investigating the pathogenesis and physiological mediators of apoptosis-related diseases; serving as diagnostic markers; monitoring efficacy of treatment; estimating the effect of drugs, radiation, or other cytotoxins on a cell population or tissue; and the development of novel specific and effective therapeutic intervention for various apoptosis-related diseases (Table 2) (R3, W6).

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APOPTOSIS, PART II: THE ROLE OF MUTATED Fas GENES IN TUMORIGENESIS

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1. Introduction

Homeostasis of the normal number of cells in various organs is the result of a balance between cell proliferation, cell differentiation, and cell death via apoptosis. In contrast, one feature of neoplasms is an extremely high tumor cell burden due to one or more failures of these three components. Although accelerated proliferation

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with dedifferentiation is thought to play a central role in tumorigenesis, it has been shown that the rate of leukemic cell proliferation is not always higher than that of their normal cell counterparts (M3). This implies that the accumulation of leukemia cells is due to their escape from elimination by apoptosis.

Cell death can occur by either of two morphologically and biologically distinct modes: necrosis and apoptosis (M3). Apoptosis, an active form of cell suicide, is a mechanism that maintains total cell numbers within physiologically appropriate ranges. Unlike necrotic cell death, which follows abnormal environmental injuries due to heat, poisons, microorganisms, or hypoxia, apoptosis is a physiological mode of cell death in which the cell actively participates by providing the necessary endogenous enzymes and energy for the process under the control of normal internal genetic programs. These apoptotic processes are actually directed by a combination of internal gene expression and external signals from cell–cell contacts, for example, through signal transduction systems mediated by cell-surface receptors and their ligands.

External signals, at first, are mainly initiated through ligation with surface receptors such as tumor necrosis factor receptor (TNFR) and Fas receptor. Studies of Fas receptor (also known as APO-1 and CD95), a member of the TNFR and nerve-growth receptor superfamily, have greatly contributed to our understanding of the relationship between abnormal apoptosis and the pathology of incurable diseases such as cancers and immunological and degenerative disorders.

The Fas receptor is a 45-kD transmembrane protein that can induce programmed cell death, i.e., apoptosis, when crosslinked by natural ligand or specific antibodies. Human Fas consists of 325 amino acids with a single transmembrane domain, including signal peptides. The extracellular domain is rich in cysteine residues and shows similarity to human TNF receptor, human nerve growth factor receptor, and human B-cell antigen CD40 (I2). The intracytoplasmic domain (about 70 amino acids) designated as the death domain is highly conserved between Fas and TNF receptor and is necessary for the transduction of the apoptotic signal. A deletion of 15 amino acids from the C terminus enhances the Fas antibody-induced killing activity, whereas a further deletion abolishes the activity (I1). Fas can exist as a cell-surface (mFas) and as a soluble (sFas) protein, both of which are generated by alternative splicing. While mFas anchored by a transmembrane domain induces apoptosis, sFas protects cells from apoptosis by a decoy action against mFas (C1, C3, P1).

The Fas/Fas ligand system has been recognized as a major pathway for the induction of apoptosis in cells and appears to play an important role in the normal development of T lymphocytes in the thymus by elimination of self-reactive lymphocytes and in the deletion of virus-infected cells and malignant cells (L7, N1, P1).

In particular, since tumors are in part characterized by abnormal accumulation of malignant cells which survive for long time by escaping apoptosis, recent attention has been focused on the role of apoptosis in hematopoietic neoplasms.

These finding require further investigation to determine the relationship between tumor biology and defects of Fas-mediated apoptosis. Indeed, Fas gene mutations were first reported in *lpr* and *lpr*^{cg} mice (A2, L5, W1) and were recently reported in several human diseases, including the congenital nonmalignant diseases, autoimmune lymphoproliferative syndrome (ALPS), and Canale–Smith syndrome (CSS) (B7, D1, F3, H1, M2, P2, R1, V1) as well as acquired hematopoietic (B4, G1, L1, M1, T2, T4) and nonhematopoietic malignancies (L2, L3, L4, S2).

Adult T-cell leukemia (ATL) is derived from an uncontrolled clonal expansion of T cells infected with human T-cell leukemia virus type 1 (HTLV-1) integrated randomly into the genome. Leukemic ATL cells are characterized by a mature CD4-positive T-cell phenotype showing abundant Fas antigens and low dividing cells (B3, K1, K3). ATL, however, has the characteristic feature of high tumor burden, suggesting that cells probably prolong their lives by escaping from apoptosis (K2). On the other hand, ATL cells are usually sensitive to Fas-mediated apoptosis *in vitro* after activation by agonistic anti-Fas antibodies (M1, S3). The clinical features of ATL are quite diverse, so that the disease is usually classified into the four subtypes of acute, lymphoma, chronic, and smoldering according to the criteria proposed by the Lymphoma Study Group in Japan (S1). A part of the diversity, especially of malignant behavior, may result from disruption of Fas-mediated apoptosis. These facts suggest that ATL represents an "experiment of nature" that is convenient and useful for understanding the role of apoptosis in oncology.

Accordingly, this section will review the role of apoptosis mediated by aberrant Fas, and the resultant clinical or biological implications in tumors, referring to our recent results derived from studies on ATL.

2. Fas Gene and Its Two Main Transcripts Encoding Membrane and Soluble Isoforms

The Fas gene discovered by Itoh and Nagata *et al.* (N2) has been mapped to chromosome 10q24.1 and consists of nine exons encoding extracellular (EC), transmembrane (TM), and intracytoplasmic (IC) domains. The Fas gene, moreover, normally encodes two main types of transcripts: full-length Fas mRNA and alternatively spliced mRNA. The former is translated into the membrane Fas isoform (mFas), which is in turn anchored on the cell membrane by the TM domain; the latter lacks exon 6 (FasΔTM mRNA) and is translated into soluble Fas isoform (sFas) which lacks the TM domain (C3, P1). Figure 1 shows a schematic representation of the Fas gene and its mRNA transcripts—the full-length and alternatively spliced forms. The transcripts in normal T cells consist of approximately 60% full-length and 40% FasΔTM mRNAs. The two isoforms translated from these transcripts are probably functional *in vivo;* intact mFas transduces the signaling after ligation with Fas ligand, while sFas acts negatively against Fas-mediated apoptosis *in vivo* through the decoy mechanism either by interfering with ligation

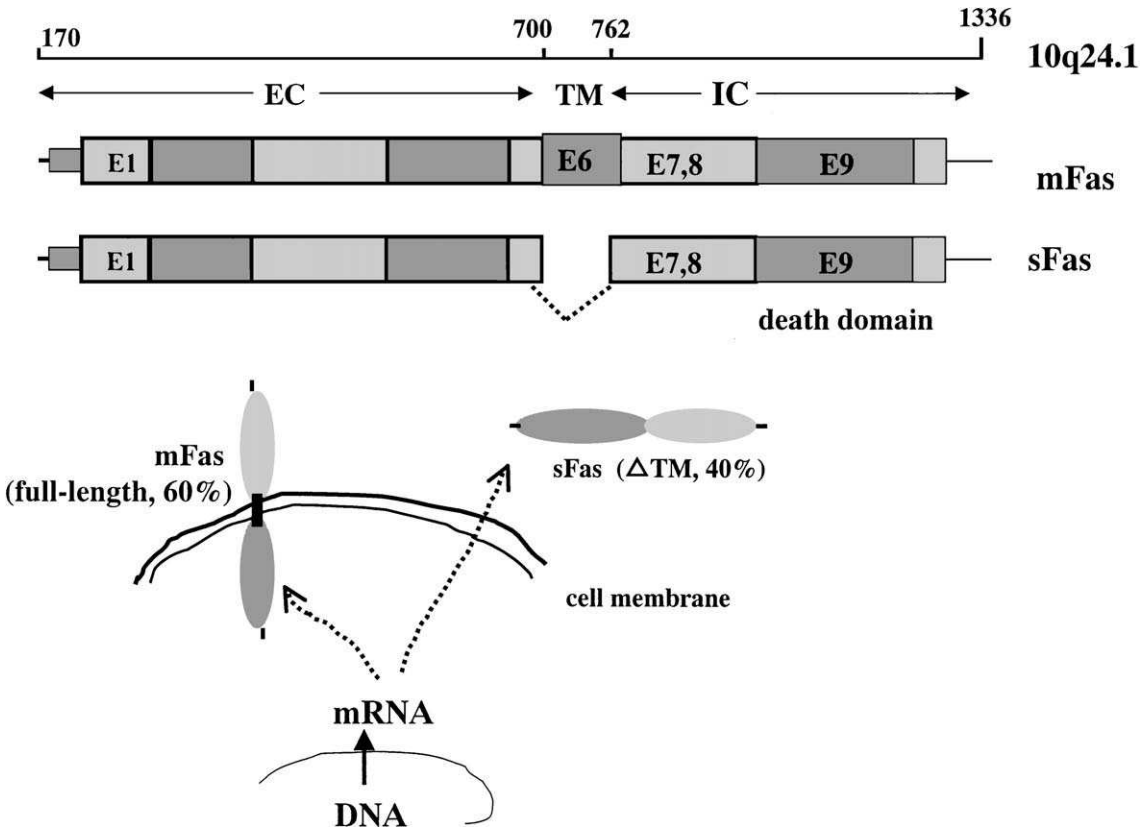


FIG. 1. Fas gene structure containing nine exons corresponding to the three domains EC, TM, and IC, and the main two alternatively spliced transcripts of full-length Fas mRNA and ΔTM mRNA, which are translated into membrane (mFas) and soluble (sFas) isoforms, respectively.

to Fas ligand or by causing incomplete trimerization between intact mFas and sFas via a mechanism similar to that of soluble TNFR (H2, V2).

3. Defects of Fas Expression in Tumors

Since it is usually ubiquitous in a variety of hematopoietic and nonhematopoietic cells, initial studies of mFas mainly focused on the relationship between the downregulation of mFas and disruption of Fas-mediated apoptosis by which tumor cells may escape elimination. Indeed, some reports support such ideas (L5). However, it was been found that quantitative downregulation of mFas is not always positively associated with tumorigenic properties. Therefore, to understand these issues as they relate to Fas, we consider below the two subjects of quantitatively abnormal regulation of Fas isoforms and qualitative changes due to somatic Fas mutations.

3.1. Both mFas and sFas Are Aberrantly Expressed on ATL Cells

Most studies concerning Fas and tumor biology have examined only downregulated or mutated mFas on the cell surface because mFas is directly involved in Fas-mediated apoptosis. However, both normal and leukemic T cells can produce intact membrane and soluble isoforms of Fas by alternative splicing, as shown in Fig. 1, and this regulation has the potential to control Fas-mediated apoptosis *in vivo.* Indeed, upregulation of mFas and unbalanced production of Fas isoforms have been noted in a few solid and hematopoietic malignancies, implying that not only mFas but also sFas is involved in apoptosis of tumor cells (S3, T1).

For example, as shown in Fig. 2, ATL cells derived from about 90% of patients with ATL express not only mFas but also sFas at 2–5-fold higher density than normal controls, i.e., a membrane density of 3470 antibody-binding capacity units (equivalent to Fas molecules/cell) and a serum sFas level of 1.54 ± 0.48 ng/ml (K2, S3). Furthermore, some ATL cell lines express only mFas abundantly, with about 3–10-fold higher density than normal controls, but express no detectable sFas. These isoforms can be measured using flow cytometric quantitative assays and enzyme-linked immunosorbent assays (K2). mFas expressed by malignant cells is functional, at least *in vitro,* after triggering by agonistic anti-Fas monoclonal antibodies (mAb) and recombinant Fas ligand (K2). This means that ATL cells expressing mFas are in a primed state *in vivo* for apoptosis via Fas signaling mediated by the mutual actions of both mFas and sFas, which is probably important for tumor characteristics.

On the other hand, about 10% of ATL patients have no detectable mFas on the cell surface but have a high sFas level in their sera, with no significant correlation between the mFas and sFas levels. These upregulated sFas properties in ATL consist

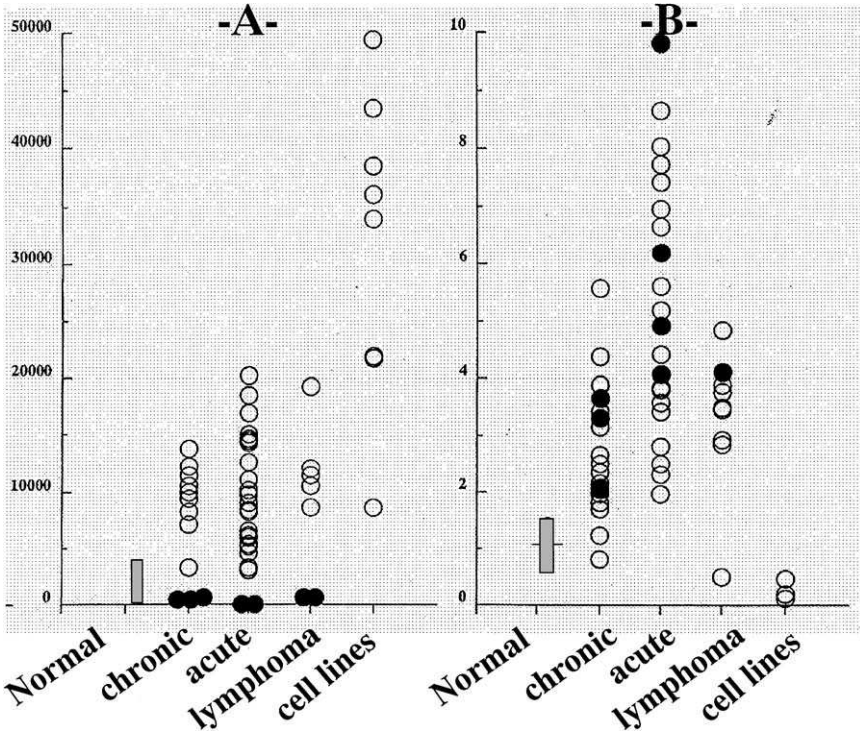


FIG. 2. mFas (A) and sFas (B) expression levels in various ATL cell lines and ATL subtypes, which are subclassified into chronic, acute, and lymphoma subtypes (K2, S3). The majority of ATL cases prominently express both isoforms of Fas, while the density in a few cases (filled circles) is discrepant, with undetectable mFas and high levels of sFas. (A) *y*-Axis, antibody-binding capacity (ABC) equivalent to mFas molecules/cell; (B) *y*-Axis, serum sFas level (ng/ml).

of both intact and mutated sFas proteins produced by different mechanisms of aberrant splicing and mutations, respectively. Consequently, either mFas or sFas, and frequently both, in ATL cells are usually upregulated and seem to have the potential to control clonal expansion through regulation of Fas-mediated apoptosis.

3.2. FAS ISOFORMS ARE REGULATED AT THE TRANSCRIPTIONAL LEVEL

Fas transcripts are known to exist in normal cells in two differently spliced forms—the full-length or alternatively spliced variants encoding mFas and sFas, respectively. Moreover at least seven variants of alternatively spliced Fas mRNA have been reported in peripheral blood mononuclear cells (PBMC) harvested from normal volunteers. Notably, these variants, encoding mainly sFas, are expressed

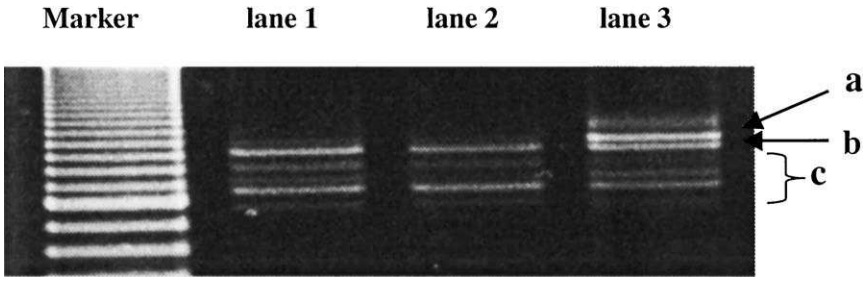
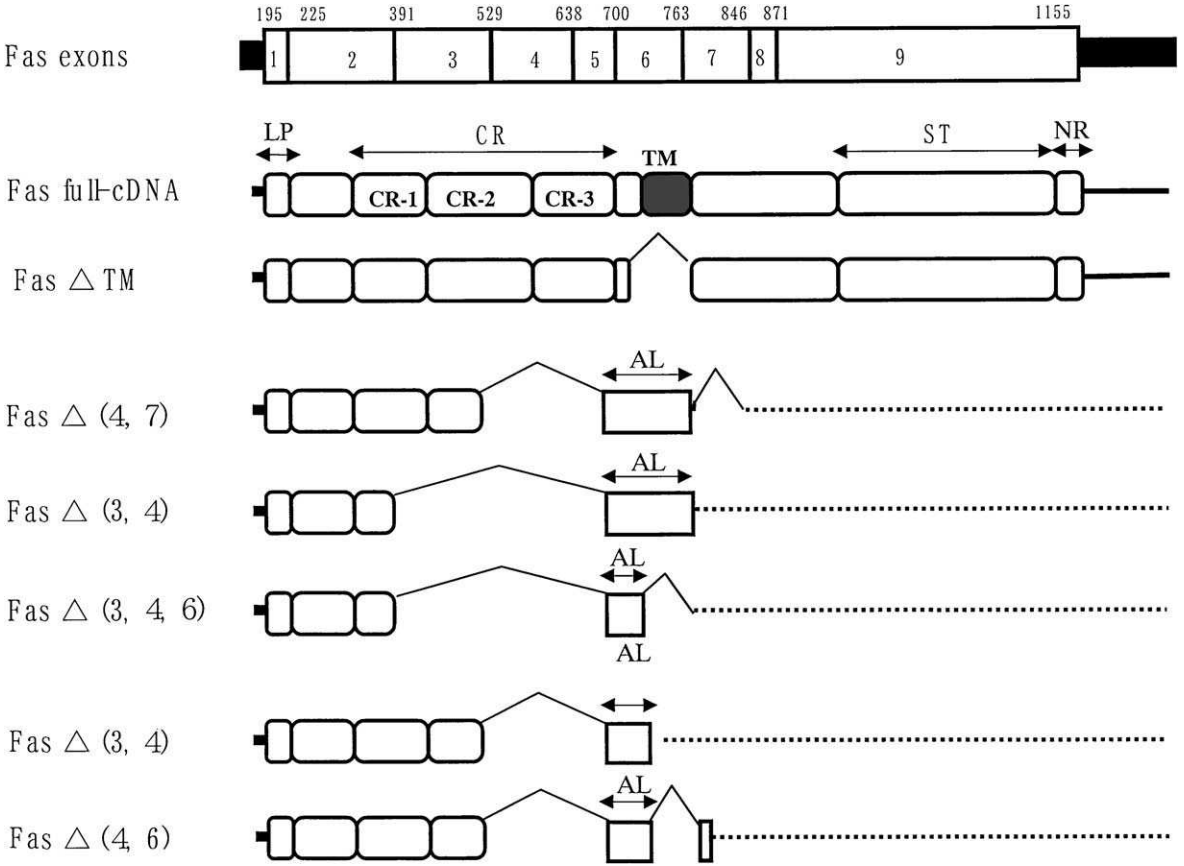


FIG. 3. Various Fas transcripts analyzed by RT-PCR. In addition to the two main bands of 1167 (a) and 1104 (b) base pairs corresponding to Fas full-cDNA and FasΔTM-cDNA, at least four to five extra bands (c) corresponding to splicing variants are observed in normal and leukemic T cells. Lanes 1 and 2: Weakly mFas-expressing ATL cells characterized by a faint band of full-length Fas cDNA and strong bands of the other splicing variants. Lane 3: Normal lymphocytes with dominant expression of full-length Fas cDNA.

more abundantly by malignant tumors than in normal tissues (K2, S3). Our results using Cheng's primers (C3) in RT-PCR analysis of ATL cells and normal resting lymphocytes, as shown in Fig. 3, show the two a and b bands of 1167- and 1104-bp products corresponding to full-length Fas cDNA and alternatively spliced Fas (FasΔTM) cDNA, as well as other variant bands, including smaller bands of at least five transcripts. These smaller transcripts have been reported to be alternatively spliced forms of Fas mRNA, whose deduced amino acid sequences (C1, L6, P1) are compared in Fig. 4. In addition, all of these truncated Fas proteins are expected to be soluble because of splicing out of the TM domain, by which Fas is anchored to the cell surface. The ratio of full-length Fas mRNA to FasΔTM closely parallels the respective isoform levels in normal T cells, *de novo* ATL cells, and ATL cell lines. That is, the ratio by semiquantitative RT-PCR analysis is about 1.14 in normal T cells; in other words, full-length Fas mRNA is slightly more abundant, in agreement with the fact that the density of mFas proteins is 3400 molecules/cell and the serum sFas level is 1.54 ng/ml in normal T cells (K2, S3). The balance between production of mFas and sFas can change depending on the condition of the PBMC. It can depend, for example, on whether they are in the resting, active, transformed state, since Liu *et al.* (L6) and we have reported that, on activation of the PBMC, the cells can switch from the usual balanced pattern to a more mFas-dominant pattern in production, with similar changes of the levels of each mRNA.

Also, as with many biologically important membrane molecules, the expression of the soluble isoform is also tightly regulated at the level of mRNA transcripts. Consequently, either overproduction or imbalance in density of Fas isoforms is likely to play an important role in the control of Fas-mediated apoptosis *in vivo.*

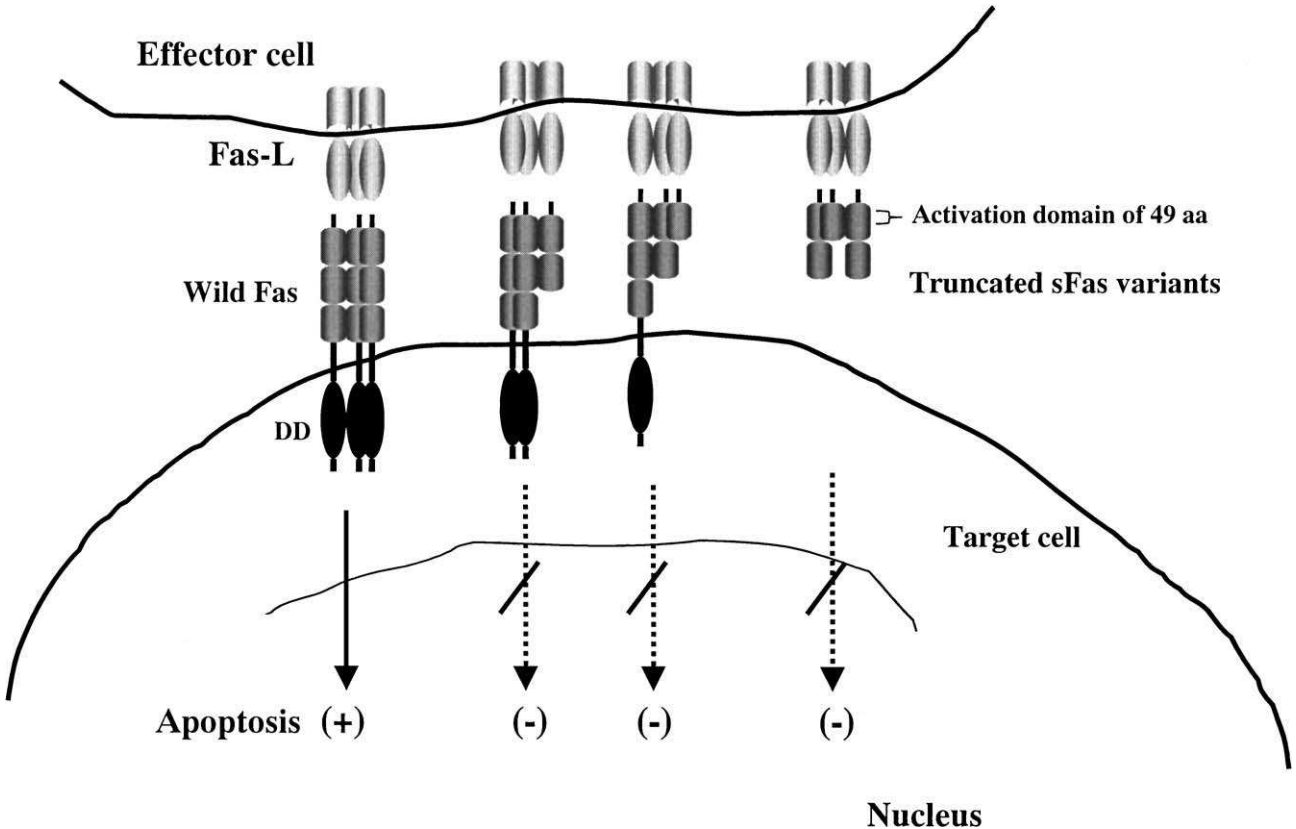

Fas exons
195
225
391
529
638
700
763
846
871
1155
1
2
3
4
5
6
7
8
9
LP
CR
TM
ST
NR
Fas full-cDNA
CR-1
CR-2
CR-3
Fas △ TM
AL
Fas △ (4, 7)
Fas △ (3, 4)
Fas △ (3, 4, 6)
Fas △ (3, 4)
Fas △ (4, 6)

3.3. Upregulated sFas on ATL Cells Is Responsible for Wild-Type and Novel Splicing Variants of Fas Transcripts

In freshly isolated ATL cells, in addition to the alternative splicing variants observed in normal PBMC, four novel variants of alternatively spliced forms, which are translated into sFas, have been found in our laboratory and designated FasΔ(6, 7), FasΔ(4, 6, 7), FasΔ(3, 4, 6, 7), and FasΔ(3, 4, 6, 7, 8). The concomitant presence of these additional novel transcripts may contribute to the dominant expression of sFas relative to mFas, especially in ATL patients with low levels of mFas and high levels of sFas. On the other hand, there have been a few reports (C2, M1) on Fas splicing variants encoding truncated mFas that contains the TM domain but lacks the intracellular death-signaling domain. Such truncated mFas can negatively inhibit apoptotic cell death in a dominant negative fashion, whereas the intact mFas on the majority of ATL cells appears to be functional, at least *in vitro,* because agonistic anti-Fas mAb can induce cell death of freshly isolated ATL cells. Despite the presence of intact abundant mFas on ATL cell surfaces, however, the cells can survive *in vivo* and cause leukemia, suggesting attenuation of mFas function *in vivo,* perhaps by autoregulation of Fas-mediated apoptosis via a decoy action by the soluble isoform (T3). Upregulated sFas in ATL results from overexpression of intact and aberrant splicing variants of the transcripts. Similar unbalanced production of Fas isoforms, with a shift from mFas to sFas, has been noted in a few hematopoietic malignancies and cell lines, implying that not only mFas but also sFas is involved in apoptosis of tumor cells (K2, K5, S3).

As to the functional role of sFas variants, there have been several reports based on apoptosis inhibition tests (C1, J1, L6, P1). According to these studies, all sFas variants observed in normal donors are able to block the apoptosis induced by either an agonistic antibody or the natural Fas ligand in Fas-positive cells. Injection of sFas into mice significantly alters lymphocyte development (C3). Although the functional machinery remains controversial, Papoff *et al.* (P1) have proposed an attractive hypothetical model of structure/function relationships for the Fas molecule, as shown in Fig. 5. This model appears to be supported by the fact that all sFas variants have N-terminal ends containing the activation domain of 49 amino acids, which is required for proximal Fas ligand-mediated events.

Fig. 4. A schematic representation of alternatively spliced Fas mRNA variants and the proteins they encode in normal PBMC. The upper three variants of FasΔ(4, 7), FasΔ(3, 4), and FasΔ(3, 4, 6) and the lower two variants of FasΔ(3, 4), and FasΔ(4, 6) were reported by Liu *et al.* (L6) and Papoff *et al.* (P1), respectively. The solid line indicates regions lacking in Fas mRNAs. The regions that are translated or not translated into proteins are indicated by boxes and broken lines, respectively. LP, leader peptide; CR, cysteine-rich subdomain; TM, transmembrane domain; ST, signal-transducing domain; NR, negative regulation domain; AL, altered amino acid region.


Effector cell
Fas-L
Activation domain of 49 aa
Truncated sFas variants
Wild Fas
DD
Target cell
Apoptosis (+)
(-)
(-)
(-)
Nucleus

3.4. Flexibility in Production of Fas Isoforms

In ATL cells, mFas is abundantly expressed at a 3-fold elevated density of 9800 molecules/cell compared with the density in normal lymphocytes, as shown in Fig. 2. sFas also is upregulated and then secreted into the serum by ATL cells under the control of the Fas mRNA level. Notably, some patients with ATL (approximately 10%) have weak or no expression of mFas but a high level of sFas, in parallel with the expression levels of each mRNA. Thus, the sFas level in ATL is independent of the mFas status. However, most ATL cells with undetectable mFas also normally preserve both full-length and truncated Fas mRNAs, corresponding to mFas and sFas (K1, K2, S3). Accordingly, ATL cells are expected to have the potential for flexible production patterns of Fas isoforms similar to that of normal T cells. Indeed, when ATL cells with weak mFas expression are cultured for several days, mFas expression becomes strong while sFas becomes weak. Given this phenomenon, it is instructive that ATL cell lines, even those having high expression of both isoforms in the original cells, acquire a characteristic feature of extremely strong mFas but no sFas expression (K2, S3). On the other hand, the expression level of each transcript is proportional to the respective protein level of isoforms on successive days in culture. In particular, the ratio of full-length Fas mRNA to FasΔTM corresponds well with the pattern of production of both isoforms in normal T cells, *de novo* ATL cells, and ATL cell lines.

A similar phenomenon has also been reported in B cells derived from chronic lymphoid leukemia (CLL) patients in which mFas is deficient or defective. Fresh *de novo* CLL cells, which express low levels of mFas, are resistant to agonistic anti-Fas mAb-mediated apoptosis, while CLL cells that acquire abundant mFas after short-term culture become sensitive (T4) to such induction of apoptosis. This flexibility in production of the isoforms is expected to provide a target for selective molecular therapy of tumors on the basis of manipulating decoy action of sFas.

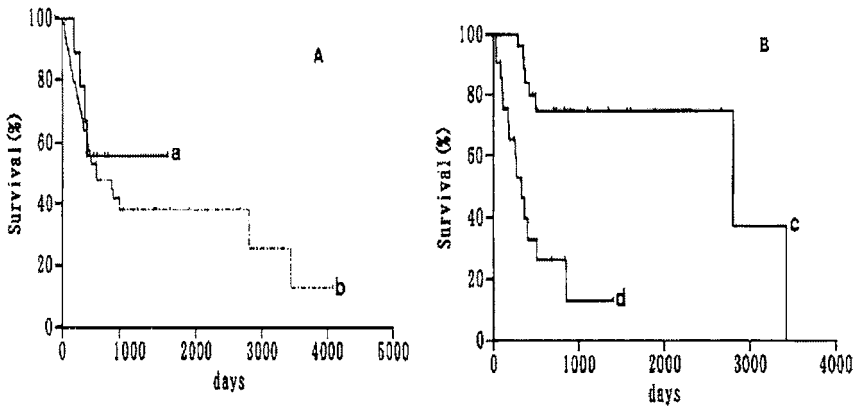
3.5. sFas Level Is Related to Tumor Biology and Is a Biomarker for Poor Prognosis in ATL

Loss or downregulation of mFas on the cell surface of tumor cells is reported to be positively associated with malignant behavior of the tumors because mFas is ubiquitously present on neoplastic and normal cells. In contrast to such tumor cells that downregulate mFas, ATL cells usually express abundant mFas and yet survive *in vivo.* This suggests that it may be informative to consider the biological

Fig. 5. A hypothetical model of the structure/function relationship between wild-type mFas and sFas variants. A requirement for the normal signaling process is to form homotrimers of the complete mFas molecules. sFas can also trimerize with wild-type mFas, suggesting that such an aberrant trimerization results in disruption of the signaling. Fas-L, Fas ligand; DD, death domain; aa, amino acid.

role *in vivo* of sFas which is overproduced in serum, since the decoy action of the upregulated sFas may influence the tumor biology of ATL. Although ATL in general shows a diverse clinical course, some cases show spontaneous regression of the tumor, suggesting apoptotic elimination of the tumor cells. Such a mechanism of eliminating tumor cells may occur *in vivo* by accidental triggering of mFas or escape from the decoy action of sFas. If either mFas or sFas function in the tumor biology of ATL is significant, the expression levels of each isoform will affect the biological behavior of the ATL cells *in vivo,* and will consequently affect the natural history of the clinical manifestation and prognosis. Unexpectedly, however, sFas, not mFas, has been found to influence the malignant behavior of ATL, suggesting that upregulated sFas may act dominantly as a decoy *in vivo* against mFas. In fact, the findings from studies of aberrant mFas and sFas in ATL suggest that high tumor burden, reflected by large lymph nodes and a high count of ATL cells in the blood, and highly malignant behavior, manifested by serum lactate dehydrogenase activity, hypercalcemia, and a poor 3-year survival rate (3y SR), are not related to mFas. In contrast, a high sFas level is apparently associated with high tumor burden and highly malignant behavior, especially an extremely short survival time (Fig. 6), suggesting the decoy function of sFas in the blood. Thus, in ATL pathology, sFas appears to be more important than mFas in determining clinical aspects of tumors.

Like these ATL studies, other interesting studies (B2, N4) have been instructive about the relationship between other Fas isoforms and tumor biology. These studies showed that the presence of alternative splicing isoforms, such as sFas with deleted



A: mFas- ATL (a) vs mFas+ ATL(b) **B: sFas^{low} (c) vs sFas^{high} (d).**

FIG. 6. Survival curves of ATL groups classified according to mFas (A) and sFas status (B). Prognosis could not be predicted by mFas status (a *vs.* b), but could be predicted by sFas status (c *vs.* d), suggesting that the sFas isoform plays a more important role in tumor biology than mFas in some ATL cases.

TM, leads to resistance to Fas-mediated apoptosis by neutralizing the function of anti-Fas or Fas ligand.

4. Fas Mutations and Various Disorders in Experimental Animals and Humans

Concerning the relationship between Fas gene mutations and various disorders, it is notable that Fas gene mutations showing a 2-bp insertion in intron 2 and a missense point mutation in the intracytoplasmic region were found in *lpr* and *lpr*^{cg} mice, which were experimental models of autoimmune disease. These mice have characteristic features of hypergammaglobulinemia, autoantibodies, rheumatoid factor, circulating immune complexes, lymphadenopathy, and splenomegaly (A2, N3, W1). All of these clinical features are probably due to the failure to eliminate self-reactive T cells with defective Fas owing to the mutation of the Fas gene, resulting in the accumulation and expansion of a large number of nonneoplastic polyclonal T cells in the lymph node, peripheral blood, and spleen.

Soon after the reports of Fas gene mutations in *lpr* and *lpr*^{cg} experimental mice, several studies identified function-ablating mutations of the Fas gene in patients with a human congenital lupus-like syndrome, designated autoimmune lymphoproliferative syndrome (ALPS) or Canale–Smith syndrome (CSS), which produces symptoms similar to the *lpr* mouse phenotype. As shown in Table 1, at least 29 distinct Fas gene mutations have been reported in patients with congenital ALPS or CSS disorders. These germline mutations are concentrated in exon 9, which forms the death domain of Fas proteins, and consist mainly of point mutations causing premature termination or missense mutations. More interestingly, it is instructive that some patients with ALPS or CSS develop a lymphoid neoplasm, malignant lymphoma, during their lives. This has encouraged many researchers to study tumor biology related to defective Fas-mediated apoptosis in acquired incurable disorders. In fact, acquired malignancies have also been reported to harbor somatically mutated Fas genes, as shown by analysis of clonal neoplastic cells. These findings support the theory that mutated Fas causes tumors, as suggested by studies by Beltinger *et al.* (B4) about acute lymphoblastic leukemia (ALL), Landowski *et al.* (L1) about myeloma, Tamiya *et al.* (T2) about ATL, and von Reyher *et al.* (V3) about colon carcinoma. Somatic Fas mutations and tumorigenesis will be discussed in detail below.

4.1. Somatic Mutated mFas and Its Function in Tumorigenesis

In addition to germline Fas mutations in the human congenital disorders ALPS and CSS corresponding to the phenotypes of *lpr* and *lpr*^{cg} experimental mice,

TABLE 1
GERMLINE MUTATIONS OF FAS GENE

Authors	Diagnosis	Codon	Region	Nucleotide	Type of mutations	Alteration of amino acid
Rieux-Laucat	ALPS		Exon 9		Deletion (290 bp)	Frameshift → premature termination
et al. (R1)	ALPS	253, 254	Exon 9	1001–1002	Deletion (2 bp, CA)	Frameshift → premature termination
Fisher	ALPS	63	Exon 3	429	Deletion (1bp, G)	Frameshift → premature termination
et al. (F3)	ALPS		Intron 3 (5′ splice site)		Duplication (t)	Aberrant splicing → premature termination
	ALPS	225	Exon 9	915	Point mutation (ACA → CCA)	Missense mutation (Thr → Pro)
	ALPS		Intron 6 (3′ splice site)		Point mutation (ag → cg)	Aberrant splicing → premature termination
	ALPS	257	Exon 9	1011	Point mutation (CAG → TAG)	Missense mutation (Gln → Stop)
Drappa	CSS	215	Exon 9	887	Insertion (1 bp, T)	Frameshift → premature termination
et al. (D1)	CSS	244	Exon 9	972	Point mutation (GAT → TAT)	Missense mutation (Arg → Tyr)
	CSS	234	Exon 9	942	Point mutation (CGA → TGA)	Missense mutation (Gln → Stop)
Bettinardi	ALPS	105	Exon 4	555	Point mutation (CGG → TGG)	Missense mutation (Arg → Trp)
et al. (B7)	ALPS	216	Exon 9	889	Point mutation (TAT → TGT)	Missense mutation (Tyr → Cys)
Haas	CSS	268	Exon 9	997	Point mutation (CAA → CCA)	Missense mutation (Gln → Pro)
et al. (H1)	+T-cell lymphoma					

Vaishnaw	ALPS	66	Exon 3	438	Point mutation (TGC → CGC)	Missense mutation (Cys → Arg)
et al. (V1)	ALPS		Intron 8		Point mutation (ctatttttag → ctagttttag)	Aberrant splicing → premature termination
	ALPS		Exon 2	360–366	Duplication (7 bp, GGCCAAT)	Frameshift → premature termination
	ALPS	244	Exon 9	973	Point mutation (GAT → GGT)	Missense mutation (Asp → Gly)
	ALPS	234	Exon 9	943	Point mutation (CGA → CCA)	Missense mutation (Arg → Pro)
	ALPS	254	Exon 9	1003	Point mutation (ACA → ATA)	Missense mutation (Thr → Ile)
	ALPS		Intron 7		Point mutation (gtaggt → gaaggt)	Aberrant splicing → premature termination
	ALPS	SP	Exon 2	240–241	Deletion (2 bp, GC)	Frameshift → premature termination
Martin	ALPS	241	Exon 9	964	Point mutation (GCC → GAC)	Missense mutation (Ala → Asp)
et al. (M2)	ALPS	294	Exon 9	1123	Point mutation (ATT → AGT)	Missense mutation (Ile → Ser)
	ALPS	244	Exon 9	973	Point mutation (GAT → GTT)	Missense mutation (Asp → Val)
	ALPS	234	Exon 9	943	Point mutation (CGA → CAA)	Missense mutation (Arg → Gln)
	ALPS	278	Exon 9	1074	Deletion (TTG ATT → TGA TT)	Premature termination (Leu → Stop)
	ALPS	234	Exon 9	943	Point mutation (CGA → CCA)	Missense mutation (Arg → Pro)
	ALPS	260	Exon 9	1020	Point mutation (CAA → TAA)	Premature termination (Gln → Stop)
Peters	CSS	256	Exon 9	1009	Point mutation (GAA → GGA)	Missense mutation (Glu → Gly)
et al. (P2)						

ALPS = autoimmune lymphoproliferative syndrome; CSS = Canale–Smith syndrome.

TABLE 2
SOMATIC MUTATIONS OF FAS GENE

Authors	Diagnosis	Codon	Region	Nucleotide	Type of mutations	Alteration of amino acid
Landowski	MM (2 cases)	253	Exon 9	999	Point mutation (GAC → TAC)	Missense mutation (Asp → Tyr)
et al. (L1)	MM	235	Exon 9	946	Point mutation (AAG → AGG)	Missense mutation (Lys → Arg)
	MM	264	Exon 9	1032	Point mutation (AAT → CAT)	Missense mutation (Asn → His)
Tamiya	ATL	5–7	Exon 2	257–262	Deletion (5bp, AAGTG) and insertion (1bp, T)	Frameshift → premature termination
et al. (T2)						
Beltinger	T-ALL	68	Exon 3	445	Point mutation (CCC → CTC)	Missense mutation (Pro → Leu)
et al. (B4)	T-ALL		Promoter		Deletion (3bp) and insertion (1bp, T)	Destructon of the AP-2 binding site
Gronbaek	B-CLL	SP	Exon 2	240	Point mutation (GCT → ACT)	Missense mutation (Ala → Thr)
et al. (G1)	FCC+DLC-B(TRB)	119	Exon 4	598	Insertion (1bp, T)	Frameshift → premature termination
	DLC-B	164	Exon 6	732	Point mutation (CTT → TTT)	Missense mutation (Leu → Phe)
	ALCL null	167	Exon 6	742	Point mutation (CCA → CTA)	Missense mutation (Pro → Leu)
	DLC-B(TRB)	182	Exon 7	787	Point mutation (ACA → ATA)	Missense mutation (Thr → Ile)
	DLC-B	199	Exon 7	838	Point mutation (TTA → TAA)	Premature termination (Leu → Stop)
	DLC-B		Intron 7 (3′ splice site)		Point mutation (ag → gg)	Aberrant splicing → premature termination
	MALT		Intron 8 (5′ splice site)		Point mutation (gtaagt → gtaaat)	Aberrant splicing → premature termination
	MALT		Intron 8 (5′ splice site)		Point mutation (gtaagt → gtaact)	Aberrant splicing → premature termination
	DLC-B	208	Exon 8	865	Point mutation (TTA → TGA)	Premature termination (Leu → Stop)
	DLC-B	244	Exon 9	973	Point mutation (GAT → GTT)	Missense mutation (Asp → Val)

	DLC-B	248	Exon 9	986	Point mutation (AAT → AAA)	Missense mutation (Asn → Lys)
	MALT	248	Exon 9	986	Point mutation (AAT → AAG)	Missense mutation (Asn → Lys)
	FCC	256	Exon 9	1008	Point mutation (GAA → AAA)	Missense mutation (Glu → Lys)
	DLC-B	262	Exon 9	1026	Point mutation (CTT → TTT)	Missense mutation (Leu → Phe)
	DLC-B	283	Exon 9	1091	Point mutation (AAA → AAT)	Missense mutation (Lys → Asn)
Maeda *et al.* (M1)	ATL	213–219	Exon 9	880–899	Deletion (20bp)	Frameshift → premature termination
Lee	LC	250	Exon 9	991	Point mutation (AAT → AGT)	Missense mutation (Asn → Ser)
et al. (L4)	LC	269	Exon 9	1048	Point mutation (CAT → CGT)	Missense mutation (His → Arg)
	LC	251	Exon 9	993	Point mutation (GTC → ATC)	Missense mutation (Val → Ile)
	LC	255	Exon 9	1006	Point mutation (GCA → GAA)	Missense mutation (Ala → Glu)
	LC	162	Exon 6	726	Point mutation (TGT → CGT)	Missense mutation (Cys → Arg)
Shin	CMM	241	Exon 9	963	Point mutation (GCC → ACC)	Missense mutation (Ala → Thr)
et al. (S2)	CMM	250	Exon 9	991	Point mutation (AAT → AGT)	Missense mutation (Asn → Ser)
	CMM	251	Exon 9	993	Point mutation (GTC → ATC)	Missense mutation (Val → Ile)
Lee	TCC (2 cases)	162	Exon 6	726	Point mutation (TGT → CGT)	Missense mutation (Cys → Arg)
et al. (L3)	TCC (8 cases)	251	Exon 9	993	Point mutation (GTC → ATC)	Missense mutation (Val → Ile)
	TCC	236	Exon 9	950	Point mutation (AAT → AAG)	Missense mutation (Asn → Lys)
	TCC	244	Exon 9	973	Insertion (1bp, C)	Frameshift → premature termination
Lee	CSCC	239	Exon 9	957	Point mutation (AAT → GAT)	Missense mutation (Asn → Asp)
et al. (L2)		162	Exon 6	726	Point mutation (TGT → CGT)	Missense mutation (Cys → Arg)
		102	Exon 4	547	Point mutation (AAC → AGC)	Missense mutation (Asn → Ser)

MM = multiple myeloma; ATL = adult T-cell leukemia; ALL = acute lymphoblastic leukemia; CLL = chronic lymphocytic leukemia; FCC = follicular center cell lymphoma; DLC = diffuse large cell lymphoma; TRB = T-cell rich B-cell lymphoma; ALCL = anaplastic large cell lymphoma; MALT = mucosa-associated lymphoid tissue-type lymphoma; LC = lung cancer; CMM = cutaneous malignant melanoma; TCC = transitional cell carcinoma; CSCC = cutaneous squamous cell carcinoma.

recently, even in acquired cancers, such as myeloma, ALL, colon cancer, and hepatoma, somatic mutations of the Fas gene have also been demonstrated in the neoplastic cells, implying that mutations of the Fas gene contribute in part to tumorigenesis. There are reports of a spectrum of mFas behavior on tumor cells harboring mutated Fas genes, varying from complete lack or deficiency to normal or even rare cases of upregulated density. In the case of the loss of mFas, it is expected that there is no signaling mediated by the receptors, which should be favorable for expanding the neoplastic cell population and consequently forming tumors. In contrast, aberrant mFas fixed on the cell surface has been reported to function mainly in a dominant negative manner because mFas is heterogeneous, consisting of the wild-type and mutant forms due to heterozygous somatic mutation of the Fas gene.

If Fas gene mutations are significant in tumor biology, mutations may be clustered in the most important region of the gene. Accordingly, the hot spot of the mutations in cancers and the relationship between the mutation domain and the functional impairment are discussed in this section.

4.2. Hot Spot in Fas Mutations

Fas gene mutations in acquired diseases have been reported mainly in lymphoid-lineage malignancies and rarely in nonlymphoid malignancies. In the first reported studies of lymphoid malignancies, Fas gene mutations, including silent mutations, were detected in 6 of 48 patients with multiple myeloma (MM) by RT-PCR and single-stranded conformation polymorphism (SSCP) analyses followed by sequencing analyses (L1). Later, various types of mutations were reported in patients with lymphoid tumors, such as ATL (M1, T2), T-lineage acute lymphoblastic leukemia (T-ALL) (B4), non-Hodgkin's lymphoma (NHL), and chronic lymphocytic leukemia (CLL) (B6, G1). In addition, mutations were reported in patients with solid tumors, such as non–small cell lung cancer (L4), cutaneous malignant melanoma (S2), transitional cell carcinoma of the urinary bladder (L3), and cutaneous squamous cell carcinoma of burn scars (L2), but not in patients with colorectal cancer (A1), B-lineage acute lymphoblastic leukemia (B-ALL) (B3), some B-cell lymphomas (B6), or ovarian cancer cell lines (B5). However, the frequency of the mutations in each disease is usually low, ranging from 2.5 to 28%, suggesting that Fas gene mutations play a critical role in tumorigenesis or in disease progression of only a limited fraction of cases in the respective diseases.

The majority of these somatic mutations are heterozygous and located in the intracytoplasmic (IC) domain, which contains the death domain, as summarized in Table 2. The literature shows that 89.5% of the mutations are detected in exons: 5.3% in exon 2, 3.9% in exon 3, 3.9% in exon 4, 7.9% in exon 6, 2.6% in exon 7, 1.3% in exon 8, and 64.5% in exon 9. In contrast, only 10.5% of the mutations are reported to occur in introns. All mutations in introns reportedly occur at 5′ or 3′ splice sites and cause probably aberrant splicing, resulting in premature termination

and production of truncated Fas antigen. The mutations in exons or introns consist of 82.9% point mutations, 7.9% deletions, 6.6% insertions, and 2.6% others.

Overall, as shown in Fig. 7, the mutations are concentrated in exon 9 which encodes the IC region containing the death domain; this is necessary for transduction of the apoptotic signal. The region with the second-highest concentration is in exon 6, which encodes the TM domain, which is anchored in the cell membrane. The positions of the codons mutated most frequently are around 162 and 250, which are located in exons 6 and 9, suggesting that these are potential hot spots for mutations. Indeed, Lee *et al.* have reported eight cases having a G → A substitution at 993 bp, corresponding to codon 251, and stressed that this is a potential hot spot in transitional cell carcinoma of the urinary bladder (L3). Interestingly, since no mutations have been reported at codon 251 in hematological malignancies or ALPS, mutations in this codon might be specific for solid tumors.

4.3. Different Mechanisms of Attenuating the Signaling by Aberrant mFas Depending on the Region or Type of Mutations

The decoy function of aberrant and intact sFas was discussed above; now we will review the attenuating mechanism of aberrant mFas for Fas-mediated apoptosis. Data from our ATL studies and the literature indicate several different mechanisms for attenuating Fas-mediated signaling depending on the aberrant structure of the Fas molecule produced by the mutated Fas gene, as shown in Fig. 8.

The first type of functionally aberrant mFas, designated as a membrane-binding decoy receptor, which carries a mutation within the EC or IC domains, especially within the death-signaling domain, can be normally anchored to the cell membrane as a nontruncated or truncated form caused by the generation of premature termination (Fig. 8a), because such aberrant mFas properties retain at least an intact TM domain. Although this type of aberrant mFas molecule can bind to the Fas ligand on the cell surface *in vivo,* this model predicts interference with the the trimerization of residual intact mFas molecules, i.e., a dominant negative mechanism, resulting in the impairment of signal transduction. Based on the reported mutations of the Fas gene (see Table 2), the majority of aberrant mFas molecules are expected to be classified into this type because the majority of mutations occur in exon 9. In fact, a dominant negative interfering effect has been experimentally demonstrated by transfection analysis of an IC domain–mutated Fas gene in such cases (F3, M1, M2, V1). This phenomenon is accounted for by the fact that the trimerization of Fas is essential for signal transduction in the process of Fas-mediated apoptosis (K4).

The second type of aberrant Fas, designated as the membrane-binding decorative receptor, which has substitutions of amino acid(s) within the EC domain, leading to an alteration of the conformation of Fas-ligand binding site (B1), especially in cysteine-rich domains, can be anchored in the cell membrane. However, this

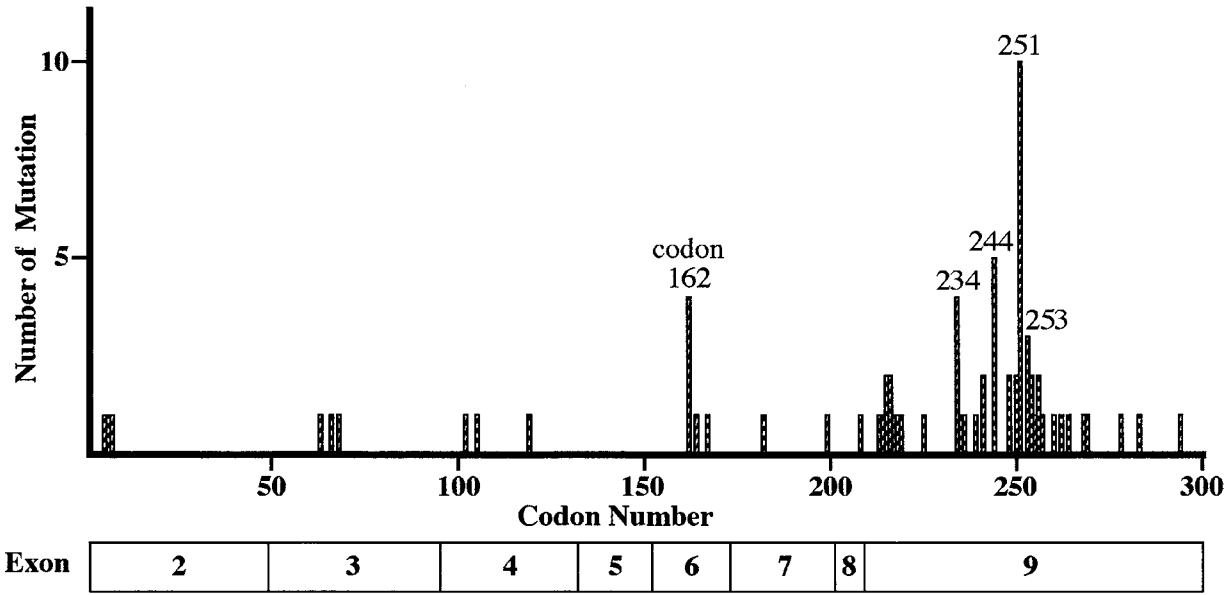


FIG. 7. The distribution of Fas gene mutations within the coding region. The codon number indicated on the *x*-axis corresponds to the lower horizontal boxes, which indicate the exon numbers of the Fas gene. Mutations are obviously concentrated in exons 6 and 9, which encode the transmembrane domain and the intracytoplasmic region containing the death-signaling domain, respectively. No mutations have been reported in exons 1 or 5. The numbers on top of the bars (162, 234, 244, 251, and 253) show codons that are mutated at high frequency.

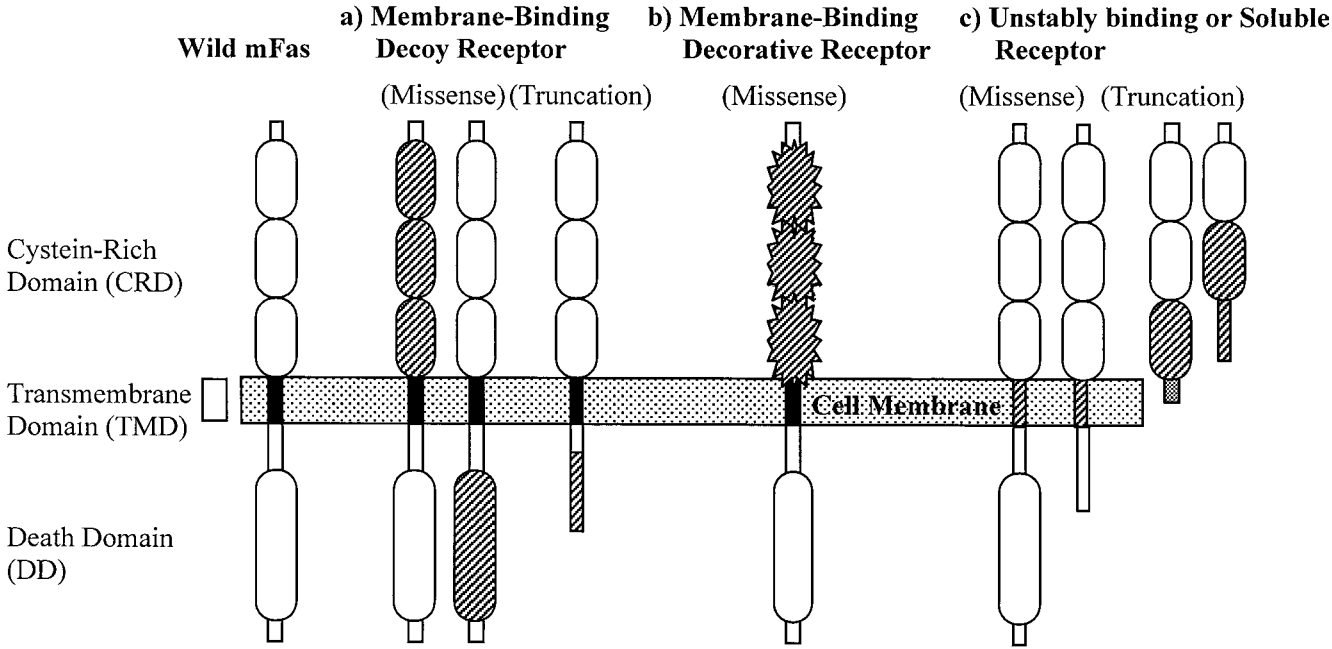


FIG. 8. Schematic representation of aberrant mFas, which is expected to attenuate the Fas-mediated signaling. Aberrant mFas is functionally and structurally classified into main three types: (a) the membrane-binding decoy receptor, (b) the membrane-binding decorative receptor, and (c) the membrane-unstable or soluble receptor. Although both mFas in models (a) and (b) are normally fixed on the membrane, the mFas in the former can bind Fas ligand, but is defective for trimerization, whereas the mFas in the latter would have no ability to bind Fas ligand *in vivo* because of conformational alteration. Like model (a), the mFas in model (c) can be reactive for Fas ligand, but it cannot transduce the apoptotic signal into the cytoplasmic death cascade because of incomplete trimerization due to an abnormal TM domain or truncation of the IC domain. The hatched and jagged markings indicate deduced alterations of amino acid sequence or three-dimensional structure, respectively.

type likely cannot transduce the apoptotic signal into the cytoplasm because this decorative receptor is expected to have no ability to bind Fas-ligand *in vivo* owing to alteration of the conformation, as shown in Fig. 8b and the following section.

Finally, the third type, designated as unstably membrane-binding or soluble receptor, which has mutations affecting the EC domain or TM domain, is incompletely anchored in the cell membrane or may be rarely secreted outside cells along with normal sFas (Fig. 8c). This aberrant Fas molecule is suggested to exert a protective effect against ligation with Fas ligand as well as soluble Fas *in vivo* because such aberrant Fas retains the ability to bind to Fas ligand. Indeed, it has been reported that the soluble variants of Fas generated by alternative splicing are secreted and possess the potential to inhibit apoptosis mediated by anti-Fas mAb (C1, C3, P1).

4.4. A Mutated mFas with a Dominant Negative Effect in ATL

We have established an interesting ATL cell line, designated KOB, which harbors a mutation of the Fas gene encoding a truncated mFas without the death domain. The mutation in KOB cells is heterozygous; i.e., mutated Fas and intact Fas coexist in the cells. The ATL cells from which KOB cells originated have also been demonstrated to be positive for the mutation. Now, as described above, there have been only two reports about Fas mutations in ATL: one by Tamiya *et al.* (T2) and the other by our group (M1). Our case was shown experimentally to have a dominant negative defect in Fas-induced apoptosis in the KOB cell line cells and the *de novo* ATL cells from a patient with ATL. Although all of our established ATL cell lines, including KOB, were shown by flow cytometry to express mFas, only KOB cells showed resistance to induction of cell death by agonistic anti-Fas mAb using the annexin-V method, as shown in Fig. 9. This figure shows that the KOB cells, despite abundant mFas expression, are not killed by agonistic anti-Fas antibody.

How can they escape from Fas-mediated cell death? First, in our case, molecular analysis of the RT-PCR products from not only KOB cells but also their original *de novo* cells showed a 20-bp deletion in exon 9, resulting in a frameshift and the generation of a premature stop codon at amino acid 239, encoding truncated mFas without the death domain, as shown in Fig. 10 (see color insert). Retrovirus-mediated gene transfer of the truncated Fas into Jurkat cells, which are susceptible to Fas-mediated apoptosis, renders the cells resistant to apoptosis, as shown in Fig. 11. The Jurkat cells transfected with the mutated Fas express both endogenous wild-type and transfected mutant Fas on the cell surface, so that the mechanism of escape from apoptosis is considered to be due to dominant negative interference by the transfected mutant Fas against the endogenous wild-type Fas. This dominant interference, as shown in Fig. 8a, is probably due to incomplete trimerization of the death-signaling domain that is essential for signal transduction in the process of Fas-mediated apoptosis (K4), because heterotrimers between the transfected truncated Fas and the endogenous normal Fas cannot transduce the apoptotic signal.

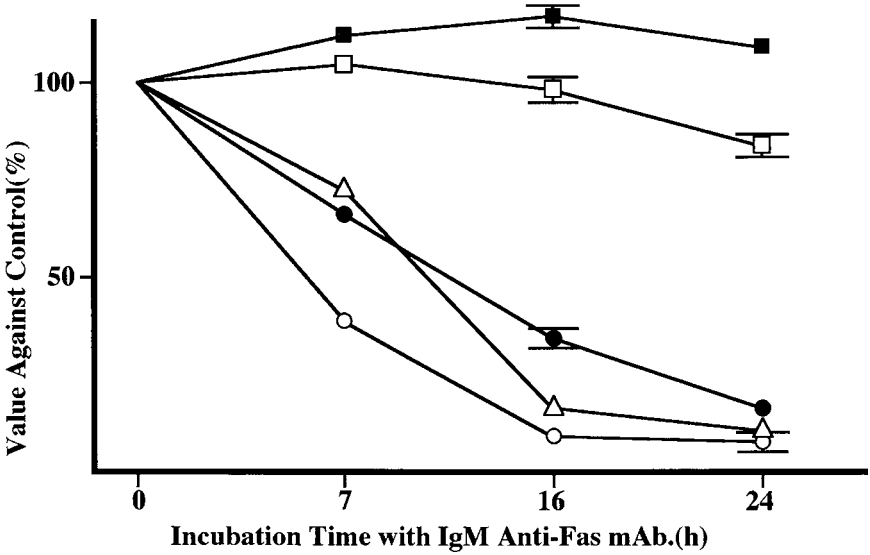


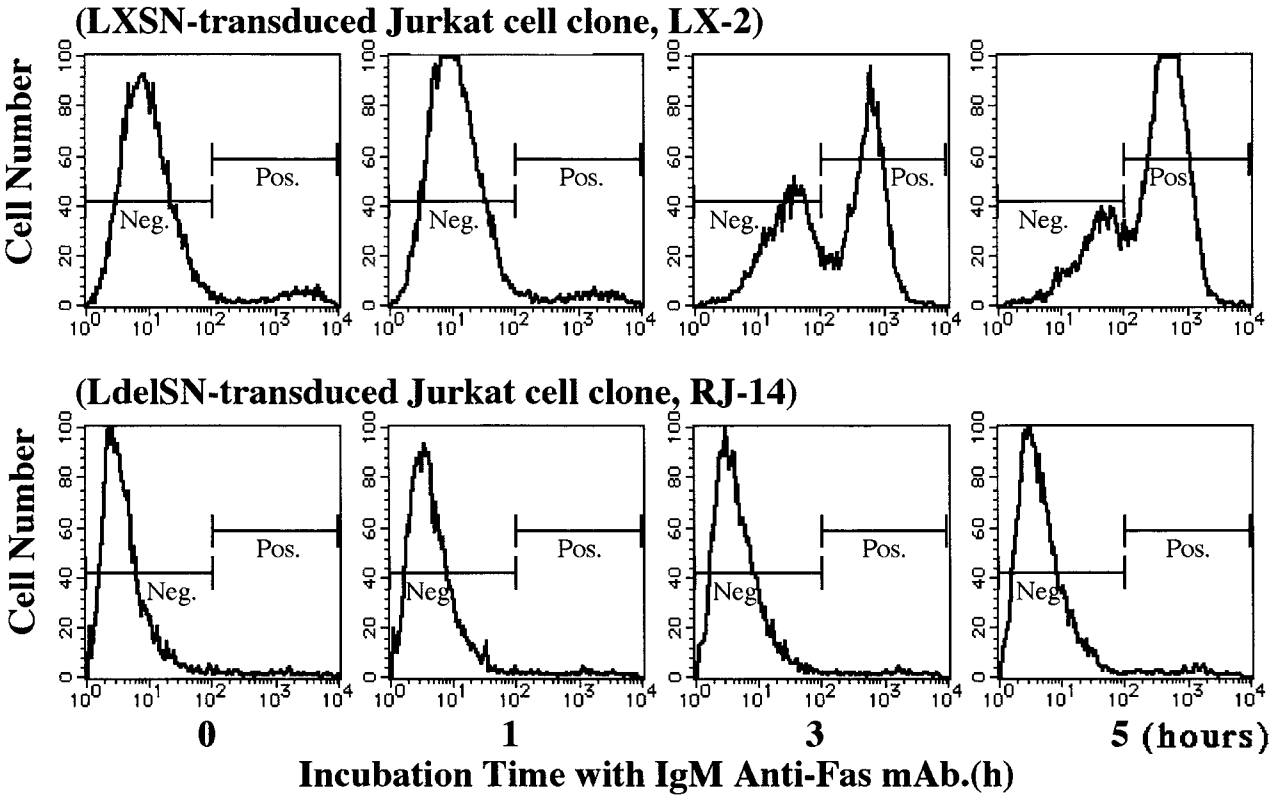
FIG. 9. Evaluation of viability status after treatment with IgM anti-Fas mAb (M1). The viability of three ATL cell lines—SO4 (open circles), KK1 (filled circles), and ST1 (filled triangles)—was strongly suppressed to 7.7, 16.8, and 10.8% of the control level at 24 h after treatment, respectively, whereas KOB (open squares) and K562 (filled squares) cells were not affected. Data are the mean ± SD from three independent experiments. SO4, KK1, and ST1 express abundant wild-type mFas, while the KOB and K562 express aberrant mFas and no mFas, respectively.

Thus, the KOB cells have been demonstrated *ex vivo* to prevent apoptosis by expression on their surfaces of function-ablating Fas antigen, which lacks the entire death domain. These aberrant Fas molecules probably behave as decoy receptors and interfere with the trimerization of normal Fas in a dominant negative manner, resulting in the impairment of signal transduction.

More interestingly, in this case, ATL cells in the lymph node at the disease onset contained the wild-type Fas gene, and later an ATL subclone acquired a Fas mutation in the crisis phase, leading to malignant behavior, enabling the subclone to escape from apoptosis mediated by the Fas signaling system and proliferate in the body. This indicates that Fas gene mutation is likely to be responsible for a late stage of tumor development.

4.5. A Missense Mutation of the EC Domain Involving Alteration of the Fas Three-Dimensional Structure in ATL

Why missense mutations of the EC domain, except in the death domain, cause Fas-mediated signaling defects remains to be elucidated. We have established interesting ATL cell lines, designated SO4 and RSO4, that are instructive regarding


(LXSN-transduced Jurkat cell clone, LX-2)
Cell Number
Neg.
Pos.
(LdelSN-transduced Jurkat cell clone, RJ-14)
Cell Number
Neg.
Pos.
0
1
3
5 (hours)
Incubation Time with IgM Anti-Fas mAb.(h)

the function-ablating mechanism in mutated Fas. The RSO4 cell line was subcloned from the SO4 cell line; the former is resistant and the latter is sensitive for Fas-mediated cell death induced by anti-Fas mAb.

Molecular analysis has revealed that the SO4 cells harbor the wild-type Fas gene, while the RSO4 cells harbor mutated Fas with the transition of A to G at nucleotide 373 in exon 2 (corresponding to the EC domain), resulting in substitution of arginine (R) for histidine (H) at codon 44. Interestingly, the substitution in RSO4 cells is thought to generate a significant alteration of the three-dimensional structure, as predicted using computer modeling analysis (Fig. 12; see color insert), which probably causes the signaling defect.

Agonistic anti-Fas mAbs, such as CH-11, ZB4, VB3, WB, and CBE, are known to be capable of binding the linear domain of amino acids (aa) 126–135 in the EC domain of Fas (F1, F2), called the antibody-binding loop, as shown in Fig. 12. Thus, the substitution position of H (44) in the RSO4 cells occurs outside the antibody-binding loop, where most agonistic anti-Fas mAbs can bind. Apoptosis mediated by anti-Fas IgM mAb CH-11 is thought to be initiated by at least two steps: The first is binding of CH-11 with the antibody-binding loop, and the second is trimerization of the Fas molecule, which mainly involves the EC domain (M3).

The RSO4 cells can react with anti-Fas mAb, but are not killed, suggesting incomplete trimerization between aberrant mFas and intact mFas. Recently, three-dimensional analysis using computer modeling has provided new insights into the structure and function of aberrant proteins (H3).

5. Conclusions

Many publications and our ATL studies concerning aberrant Fas in tumors and its role in the development and progression of neoplasms were reviewed, and Fas mutations were shown to be in part responsible for impairment of Fas-mediated apoptosis. Moreover, most of such impairment results from a qualitatively and quantitatively aberrant Fas molecules of either the membrane or soluble isoforms generated by somatic Fas gene mutations. Furthermore, we showed that studies concerning aberrant Fas and tumor biology ought to provide new insights into not only wild-type but also oncogenic mechanisms of Fas-mediated apoptosis; thus, it

FIG. 11. Flow cytometric detection of apoptotic cell death using the annexin V method after treatment with IgM anti-Fas mAb. The vacant retrovirus vector (LXSN)–transfected Jurkat cell clone of LX-2 clearly exhibits annexin V binding at 3 h after treatment (56.8%), and the binding cell population is further increased at 5 h (77.2%), whereas only faint annexin V binding is observed in LdelSN-transfected Jurkat cell clone RJ-14. This means that endogenous wild-type mFas in the LX-2 cells is functional in Fas-mediated apoptosis, but transfected aberrant mFas in the RJ-14 cells interferes with the signaling in a dominant negative manner.

is expected that, in the near future, such studies will lead to new selective molecular therapies for incurable diseases caused by tumors.

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NONCOMPETITIVE IMMUNOASSAYS FOR SMALL MOLECULES WITH HIGH SENSITIVITY AND SPECIFICITY

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Abbreviations: AGI, angiotensin I; AGII, angiotensin II; ALP, alkaline phosphatase; BSA, bovine serum albumin; 1,25D_3, 1α,25-dihydroxyvitamin D_3; DMFO, α-(difluoromethyl)ornithine; E_2, estradiol; ELISA, enzyme-linked immunosorbent assay; FIA, flow injection analysis; GAL, β-galactosidase; HPLC, high-performance liquid chromatography; HPPA, 3-(*p*-hydroxyphenyl)propionic acid; HRP, horseradish peroxidase; IEMA, immunoenzymometric assay; IRMA, immunoradiometric assay; KLH, keyhole limpet hemocyanin; MW, molecular weight; SD, standard deviation; SPIE-IA, solid-phase immobilized epitope immunoassay; T_3, triiodothyronine; T_4, thyroxine; THC, tetrahydrocannabinol; TRH, thyroliberin; UDCA 7-NAG, ursodeoxycholic acid 7-*N*-acetylglucosaminide.

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1. Introduction: Principle and Advantages of Noncompetitive Immunoassays

Trace characterization of physiologically active substances with a low molecular weight (e.g., steroids, catecholamines, eicosanoids, thyroid hormones, and synthetic drugs) is an important subject in biomedical analyses. Immunoassays are now widely used for this purpose because of their excellent specificity and feasibility and because they exhibit higher sensitivity than other common analytical methods used in this field. Conventionally, such small molecules, which are immunochemically classified as haptens, are determined exclusively with competitive immunoassays. The assay format is based on the competitive reaction between a variable amount of unlabeled hapten (the analytical target, or analyte) and a fixed amount of labeled hapten with a signal-generating group (i.e., radioisotopes, enzymes, or fluorescent and chemiluminescent dyes) against a fixed and limited amount of anti-hapten antibody. Figure 1 illustrates a typical competitive immunoassay procedure using a solid-phase immobilized antibody. Once equilibrium has been reached, the hapten–antibody complex is separated from the free haptens (bound/free separation) and the signal intensity from the bound or free fraction is measured. The bound signal intensity is inversely proportional to the amount of analyte, as shown by the dose–response curve in Fig. 2. The sensitivity of such competitive immunoassays is, however, essentially limited by

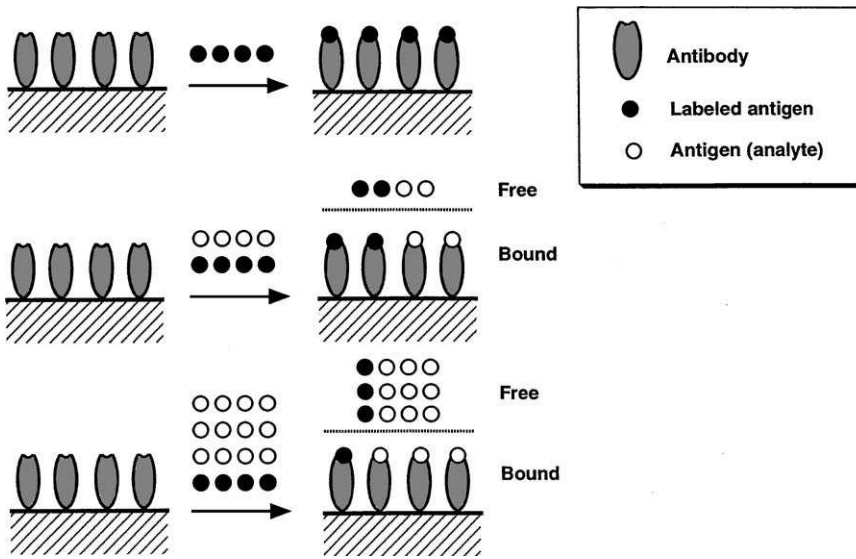


FIG. 1. Schematic representation of a typical competitive immunoassay procedure.

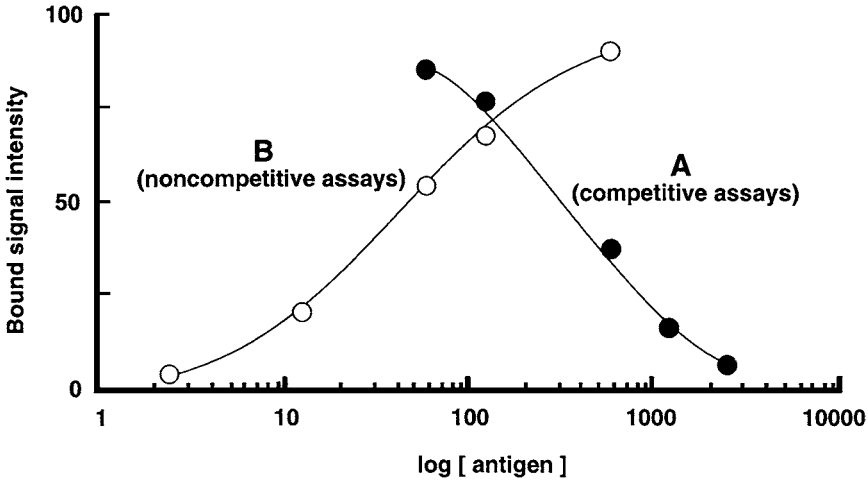


FIG. 2. Typical dose–response curves of (A) competitive immunoassays and (B) noncompetitive immunoassays.

the affinity of the anti-hapten antibody (E1, J1), which hardly exceeds the range of $10^{10}\ M^{-1}$ as the affinity constant (K_a). To date, the monoclonal anti-hapten antibody exhibiting the largest K_a value, which has been generated via conventional immunization and officially reported, would be an anti-digoxin antibody with the K_a value of $1.7 \times 10^{12}\ M^{-1}$ (M2). Very recently, a mutant antibody fragment (single-chain Fv fragment) having a very high affinity against fluorescein hapten (reported K_d, 48 fM; corresponds to K_a, $\sim 2 \times 10^{13}\ M^{-1}$) has been generated by an antibody-engineering technique called "DNA shuffling" (B5). However, a general and reliable strategy for producing such an anti-hapten antibody whose affinity exceeds $10^{12}\ M^{-1}$ is not yet available; consequently, it has been very difficult to measure subfemtomole levels of hapten molecules with competitive immunoassays.

Noncompetitive immunoassays have been considered to be potentially more sensitive than competitive assays, an advantage that has been shown by mathematical modeling (J1). Noncompetitive assays, also known as "immunometric assays," are classified as two-site immunometric assays (M1) (Fig. 3A) and single-antibody (single-epitope) immunometric assays (S2) (Fig. 3B). The common feature of these assays is the reaction of analyte versus excess amount of antibodies. Excess antibodies promote the antigen–antibody reaction, which follows the law of mass action; hence the antibody can efficiently capture even trace amounts of antigen. This explains why the noncompetitive immunoassays are more sensitive than the competitive immunoassays. The resulting antibody–antibody complex, whose quantity should be proportionally related to the amount of the analyte, is selectively and directly determined, being distinguished from coexisting free

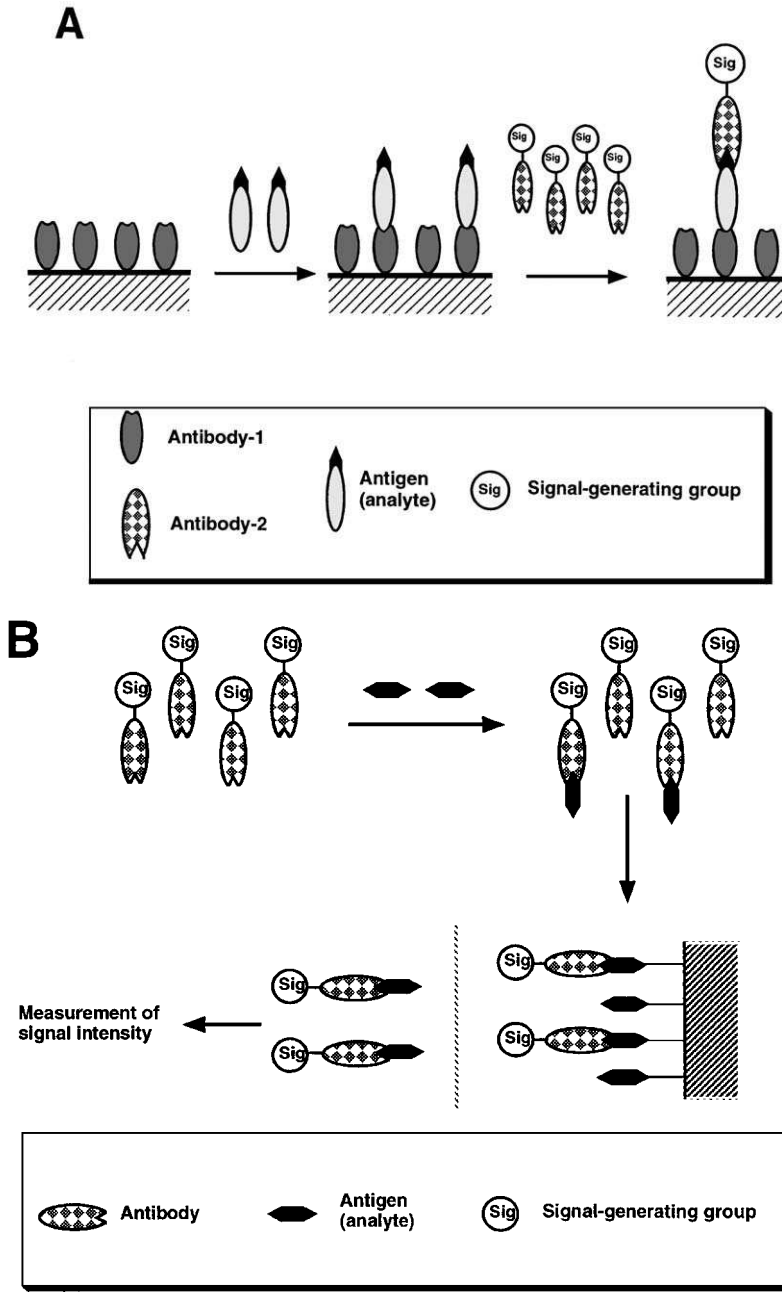


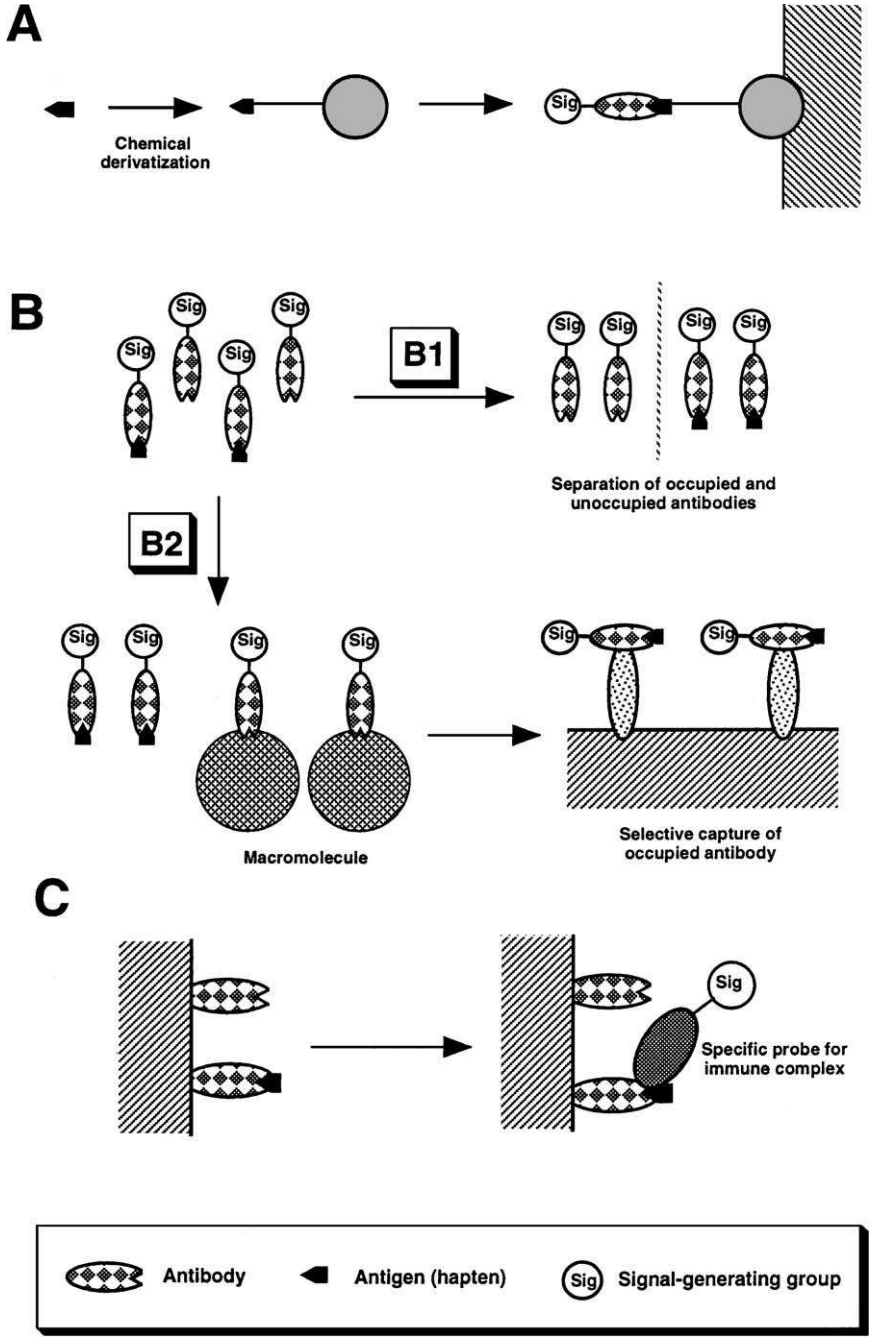
FIG. 3. Schematic representation of noncompetitive immunoassays. (A) Two-site immunometric assays (sandwich assays) and (B) single-antibody (single-epitope) immunometric assays.

antibodies and antigens. Thus, the noncompetitive assays measure the analyte-bound sites, while the competitive assays determine the analyte-unbound sites (S5); this corresponds to the difference in the dose–response curves of these assays (Fig. 2). The antigen-specific antibodies are often directly labeled with the signal-generating group to allow simple and sensitive measurement of the antigen–antibody complex. The noncompetitive assays employing radioisotope-labeled antibody and the assay using enzyme-labeled antibody are termed "immunoradiometric assay" (IRMA) and "immunoenzymometric assay" (IEMA), respectively.

These noncompetitive assays have additional advantages, including, higher precision (less susceptibility of pipetting error) and wider working range, both of which are also due to the use of excess antibody. Furthermore, such noncompetitive assays can usually require shorter incubation times than the competitive assays because a large excess of antibody accelerates the antigen–antibody reaction to form the immune complex quantitatively.

Two-site immunometric assays, also called "sandwich assays," are suitable for measuring macromolecules such as proteins having more than two antigenic determinants (epitopes) (M1). Indeed, Ishikawa and colleagues (H2, I1, I2, R1) reported the sandwich-type IEMA systems for various peptides of high molecular weight with subattomole-range sensitivity. Figure 3A illustrates a typical assay procedure. The analyte is effectively captured on a solid phase via an excess amount of immobilized antibody. The captured analyte is detected with the aid of another antibody recognizing a different epitope, which has been labeled with a suitable signal-generating group. In the case of these two-site assays, one can obtain a higher specificity owing to double recognition of the analyte with the immobilized antibody and the labeled antibody. One more advantage of these assays is lower susceptibility to interfering substances from biological specimens because the washing step, interpolated prior to measurement of signal intensity, removes such impurities. However, these assay principles have not been applicable for measuring haptens because their low molecular mass prohibits simultaneous binding by two antibody molecules. To date, the smallest molecule, which has been determined by the two-site immunometric assay, would be angiotensin II (AGII; MW 1046) (G2).

On the other hand, the single-antibody immunometric assays should be applicable to both macromolecules and haptens (S2). Thus, the analyte is reacted with the excess amount of labeled specific antibody and unoccupied labeled antibodies are removed by adsorption onto an immobilized antigen (Fig. 3B). The signal intensity from the immune complex remaining in the assay medium is then measured. This assay principle, however, has not yet been widely applied to haptens. The major factor hampering its applicability is the difficulty of removing unoccupied antibodies almost perfectly to reduce the background signal. Furthermore, it is not easy to supply either the immobilized antigen continuously in a relatively large amount or the labeled antibody with extremely high purity. The latter problem,


A
Chemical derivatization
Sig
B
B1
Separation of occupied and unoccupied antibodies
B2
Macromolecule
Selective capture of occupied antibody
C
Specific probe for immune complex
Antibody
Antigen (hapten)
Signal-generating group

however, has recently been becoming easier owing to the invention of monoclonal antibodies and excellent labeling methods.

During the last decade, various attempts have been made to overcome such limitations and establish novel noncompetitive immunoassay methods applicable to haptens. The methods reported so far are based on one of the principles illustrated in Fig. 4. This review surveys these new assay methods, which may lead to a breakthrough for subattomole-range determination of various hapten molecules. Figure 5 shows the chemical structures and molecular weights of the analytes (haptens) referred to in this article (A, peptides; B, steroids; C, other compounds). We have attempted to show the assay sensitivity (detection limit) of each method described below using units of mole/assay, which is convenient for comparison, even if it was not given in the original paper. Values estimated from dose-response curve or converted from values described in units of weight/liter (or milliliter) are marked with an asterisk (e.g., 10 fmol*/assay).

2. Noncompetitive Hapten Immunoassays Employing Chemical Derivatization of an Analyte Permitting Sandwich-Type Determination

The assay procedures described in this section include chemical derivatization of a target hapten molecule to enable a variation of the sandwich-type assay.

2.1. Sandwich-Type Assay Based on Biotinylation of a Target Hapten

Ishikawa and colleagues (reviewed in Refs. I4 and I5) reported one of the earliest noncompetitive hapten immunoassays, which is based on principle A in Fig. 4. In this procedure, a target hapten having a primary amino group was quantitatively converted into a biotin adduct by reaction with a biotinylation reagent equipped with a carboxylic acid *N*-succinimidyl ester as a reacting group (Fig. 6). The chemical linker combining the hapten and biotin groups is long enough to allow simultaneous binding of an anti-hapten antibody and avidin. In the typical assay procedure, the biotinylated hapten in a reaction mixture is affinity-purified

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Fig. 4. Classification of reported noncompetitive immunoassays for haptens based on the assay principle. (A) Assays that include a chemical modification of hapten to allow sandwich-type detection. (B1) Improved single-antibody immunometric assays that separate immune complex and excess labeled antibody, either by using a hapten-immobilized affinity column or based on differences in their physical properties. (B2) A variation of single-antibody immunometric assays based on masking of unoccupied antibody by an immunoreactive macromolecule followed by selective capture and detection of the hapten-occupied antibody. (C) Assays employing a probe molecule specific to a hapten–antibody complex.

using an immobilized anti-analyte antibody to remove unreacted biotin reagent (Fig. 6, steps i and ii). The purified adduct was then reacted with an enzyme-labeled Fab′ fragment of the anti-hapten antibody (step iii), whereupon the resulting complex is captured on the avidin-coated polystyrene balls (step iv). This procedure was successfully applied to angiotensin I (AGI) (I4, I5, T2, T3) and

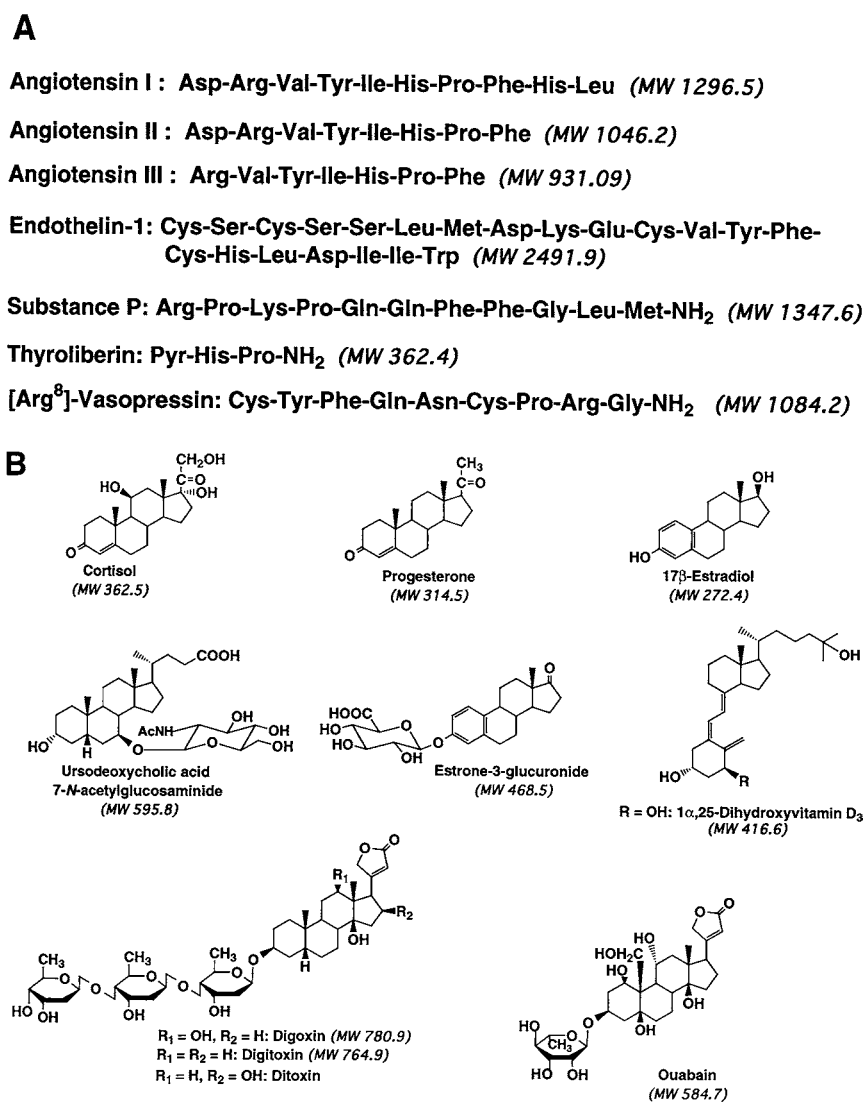

FIG. 5. Chemical structures of the haptens covered in this chapter: (A) peptides, (B) steroids, and (C) the other compounds.

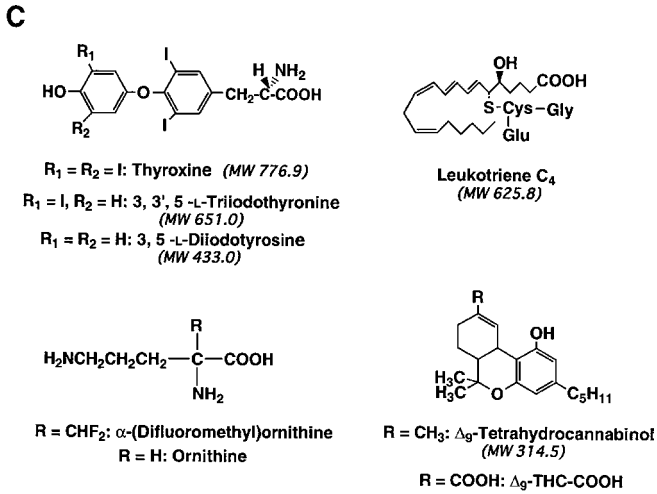

FIG. 5. (*continued*)

[Arg^8]-vasopressin (I4, T1) (Fig. 5A), both of which are low-molecular-weight peptides having one primary amino group as the N-terminal. The detection limits of these assays were 10 amol (AGI) and 50 amol ([Arg^8]-vasopressin) per assay, the former being 100-fold lower than that of the competitive radioimmunoassay using the same antibody with an ^{125}I-labeled antigen (I4). An additional derivatization step was, however, essential to measure a smaller molecule, e.g., thyroxine (T_4; Fig. 5C). Thus, the hapten was first conjugated with glutathione via a bifunctional reagent (*N*-hydroxysuccinimidyl-6-maleimidohexanoate) reacting with the amino group of T_4 and the mercapto group of glutathione, then the N-terminal amino group of glutathione was reacted with the biotinylation reagent. This "indirect" derivatization (I4, T1, T4) lengthens the distance between the hapten and biotin, thereby avoiding steric interference between an anti-T_4 antibody and avidin and providing a detection limit (0.1 fmol) 50-fold lower than that of the competitive enzyme immunoassay. The indirect derivatization also improved the assay sensitivity of such small peptides as [Arg^8]-vasopressin (detection limit reduced to 10 amol) (I4). Furthermore, the authors reported the detection of 1 amol of [Arg^8]-vasopressin (H3) by combining their original technique, called "immune complex transfer," which reduces nonspecific binding of an enzyme-labeled molecule.

Although the assay principle provided the most sensitive noncompetitive hapten immunoassays among those reported so far, it essentially requires that the target hapten possess a reactive amino group. This principle has not been applied to the haptens having other common functional group (e.g., hydroxyl or carboxyl group) owing to the lack of a quantitative derivatization reaction. It should be noted that the same assay principle based on derivatization into a biotin adduct was also

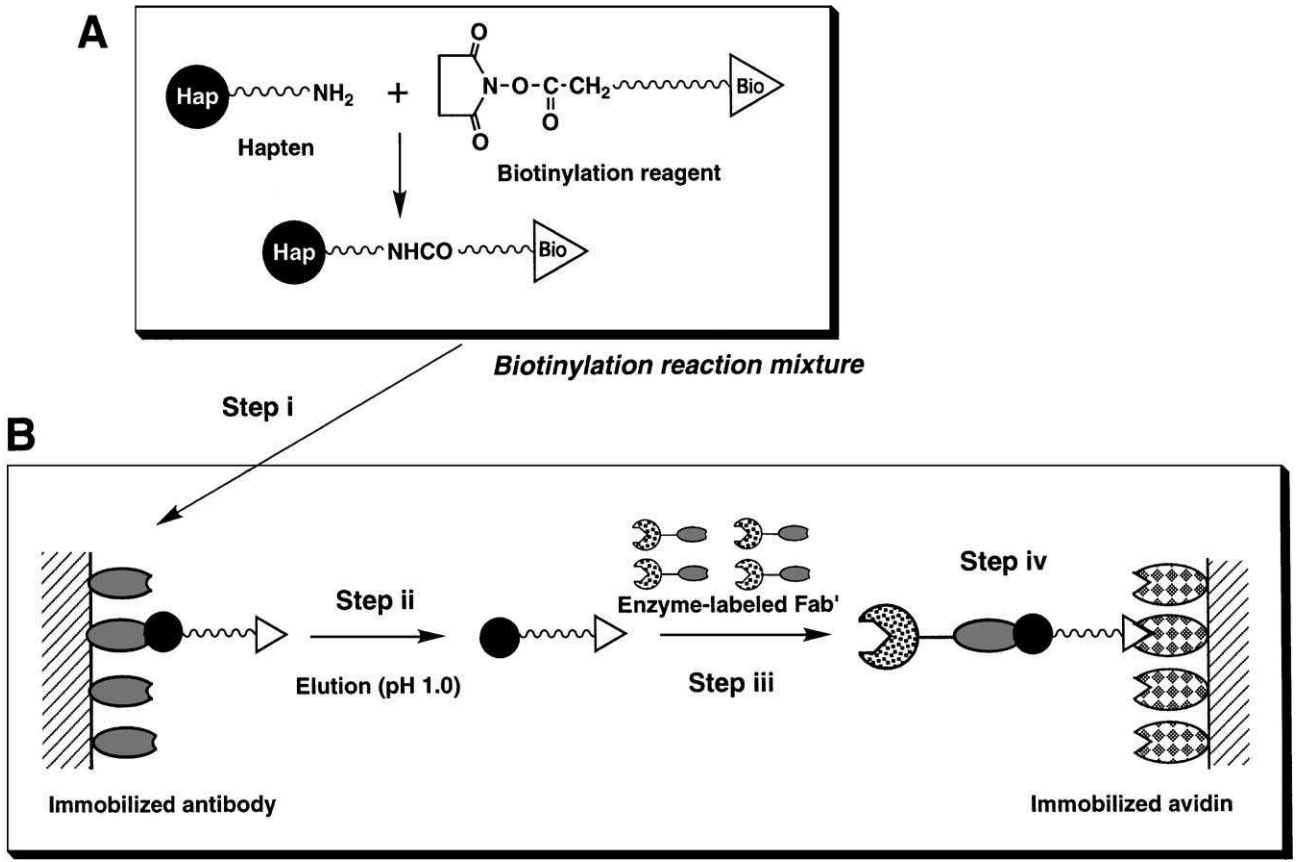

FIG. 6. Principle of a noncompetitive hapten immunoassay based on quantitative biotinylation of the target molecule: (A) biotinylation of a hapten with reactive amino group(s) and subsequent (B) sandwich-type immunoassay procedure.

proposed by Von Grünigen *et al.* (V2), in which biotinylated gastrin was used as a model conjugated antigen.

2.2. Solid-Phase Immobilized Epitope Immunoassays

More recently, Pradelles and colleagues devised a unique noncompetitive assay principle which is also based on chemical derivatization (reviewed in Ref. G2). They called this assay "solid-phase immobilized epitope immunoassay" (SPIE-IA), whose typical procedure is illustrated in Fig. 7. A target hapten having primary amino group(s) is first captured onto a microtiter plate previously coated with an excess amount of anti-hapten antibody, then subsequently blocked using an adequate protein solution (Fig. 7A). This immunological reaction was done at 4°C for 18 h (P2). The captured hapten is then covalently immobilized to the anti-hapten antibody on the solid phase via its lysine residues using a homobifunctional cross-linking agent (e.g., glutaraldehyde or disuccinimidyl suberate) that reacts with amino groups (Fig. 7B). The immobilized hapten is released from the paratope of the antibody as an epitope by treatment with a dissociating/denaturing agent (acid or alkaline solution) (Fig. 7C), and is subsequently detected with an excess amount of acetylcholine esterase–labeled antibody (Fig. 7D). The bound-enzyme activity on the plate (measured by colorimetry using Ellman's reagent as a substrate) was proportionally related to the amount of the hapten molecules to be measured. This SPIE-IA has advantages and limitations similar to those of the previous assays (Section 2.1). Thus, these procedures require that the target compounds have reactive amino group(s), but only one kind of anti-hapten antibody is necessary, in contrast with two-site immunometric assays.

This assay procedure has been applied to various haptens having a primary amino group, including leukotriene C_4 (V1), T_4 (P2), substance P (P2), endothelin (P2), and AGII (G2) (for structures, see Fig. 5A and 5C). These noncompetitive assays were 10–300-fold more sensitive than corresponding competitive assays (G2). The minimal detectable concentrations described in these papers were as follows: 2 pg/ml (∼0.3 fmol/assay) for leukotriene C_4, 14 pg/ml (∼0.2 fmol/assay) for T_4, 6 pg/ml (∼0.4 fmol/assay) for substance P, and 20 pg/ml (∼0.8 fmol/assay) for endothelin.

Two haptens without amino groups were also determined by SPIE-IA using a different immobilization method. In the measurement of thyroliberin (TRH; Fig. 5A) (E2), a primary amino group was introduced by a chemical derivatization [a reaction of the histidine side chain of TRH with diazotized 2-(4-aminophenyl)ethylamine]. 17β-Estradiol (E_2; Fig. 5B) was covalently linked to the anti-E_2 antibody on a solid phase by ultraviolet irradiation (B6) or by the addition of a Fenton-like reagent which generates free-radical species (B7). These crosslinking methods might extend the applicability of the SPIE-IA procedure to haptens lacking primary amino groups.

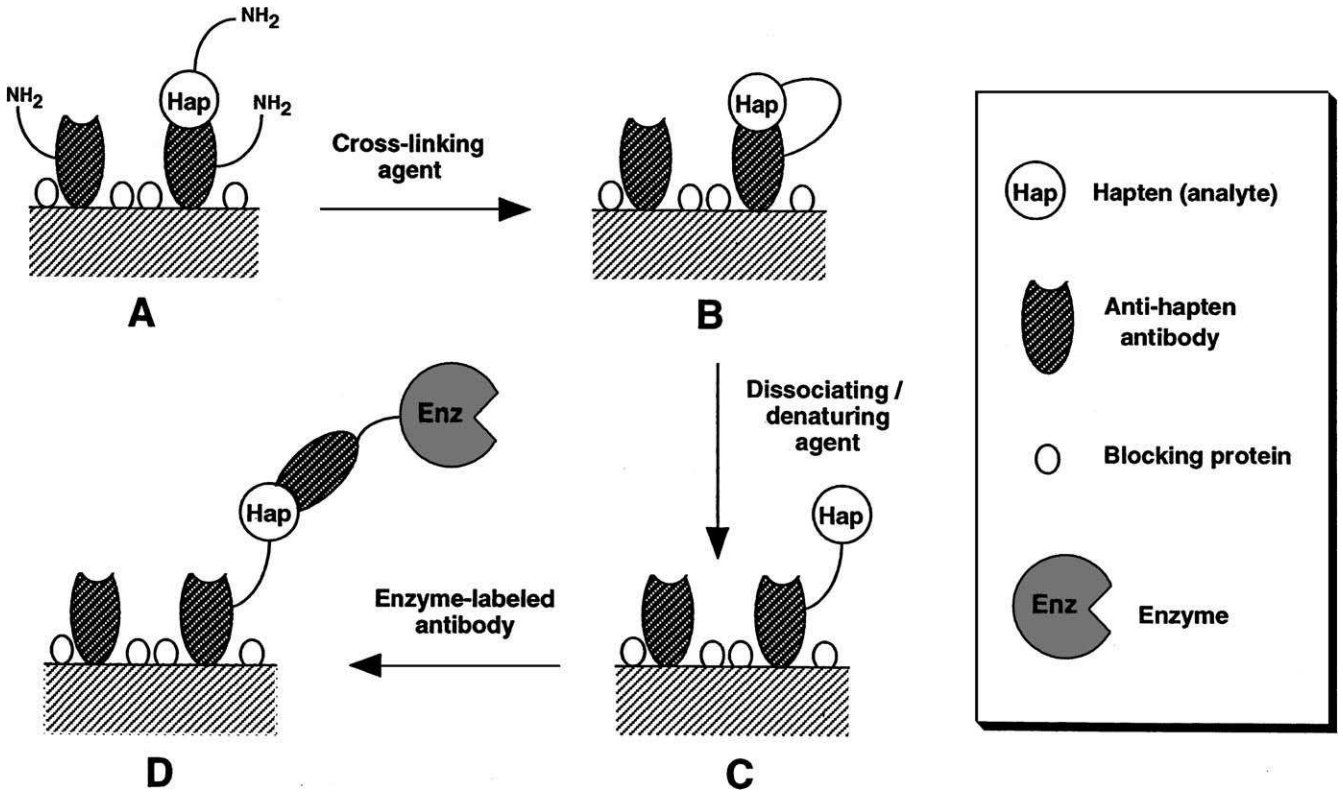

FIG. 7. Principle of the solid-phase immobilized epitope immunoassay (SPIE-IA).

3. Improved Single-Antibody Immunometric Assays

Several noncompetitive assays for haptens reported so far can be regarded as variations of conventional single-antibody immunometric assays, the majority of which (the assays in Sections 3.1 and 3.2) are based on principle B1 in Fig. 4. In many cases, these methods employ an automated flow system to simplify the assay procedure and minimize the time and labor required for the analysis.

3.1. Immunometric Assays Using an Automated Flow System Equipped with a Hapten-Immobilizing Affinity Column

Some immunometric assays have been reported in which excess unoccupied antibodies are eliminated by delivering the antigen (hapten)–antibody reaction mixture automatically through a hapten-immobilizing affinity column using a flow system (Fig. 8) . The signal intensity generated from the hapten-bound labeled antibody, which should be in the flow-through fraction, is also recorded automatically. Thus, these assay systems are variations of flow injection immunoassays (FIA-IA), which have been reviewed by Fintschenko and Wilson (F1).

In 1984, a fully automated IEMA of digoxin (Fig. 5B) was reported (F2, F3) in which an affinity-column immobilizing ouabain (Fig. 5B) was employed for removing excess labeled antibody (named "affinity column–mediated immunoenzymometric assay"). The analyte, digoxin was mixed with a β-galactosidase (GAL)–labeled anti-digoxin antibody and incubated at room temperature for 10 min. An aliquot of this reaction mixture was aspirated into the instrument and pumped through the affinity column (flow rate 34 μl/s), and the enzyme activity that passed through was measured colorimatrically using *o*-nitrophenyl β-D-galactoside as a substrate (F3). All these procedures were done automatically using a clinical analyzer. The assay sensitivity was reported to be 0.2 μg/liter (corresponding to 77 fmol*/assay).

During the establishment of this assay method, the authors investigated some important parameters for optimizing such single-antibody immunometric assays (F3). Two kinds of GAL-labeled anti-hapten antibodies were prepared using the monovalent Fab′ fragment and the divalent $F(ab')_2$ fragment, which were then compared in terms of their overall assay performance. It is expected that the monovalent labeled Fab′ fragment would afford somewhat better results, because the divalent labeled $F(ab')_2$ fragment, when it binds only one hapten molecule (so that one of the two antigen-binding sites is unoccupied), would be adsorbed on the affinity column, resulting in the loss of specific signal intensity. The authors reported that the labeled Fab′ fragment yielded more sensitive dose–response curves that were linear over a wider range, while the labeled $F(ab')_2$ fragment provided the assay with adequate sensitivity and extremely good precision; furthermore, the $F(ab')_2$ fragment was generally easier to prepare reproducibly. In addition, digoxin

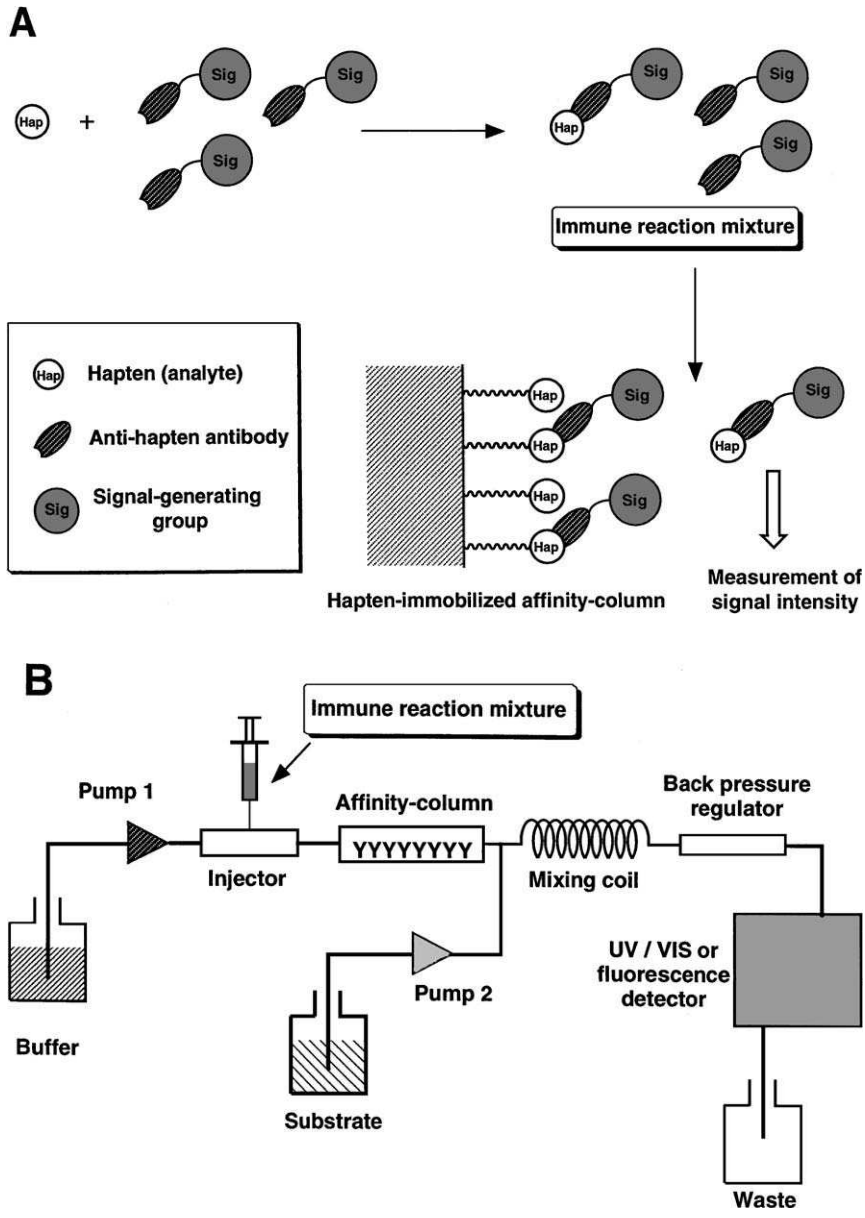

FIG. 8. Schematic representation of (A) the principle and (B) a system of flow injection immunometric assay (G4).

(the target hapten itself) and ouabain (an analog of digoxin) were compared as a ligand of the affinity column. The ouabain-immobilized affinity column afforded higher sensitivity and much better within-run precision, while the digoxin column sometimes showed lower background levels. Although the authors did not clearly state the reasons for these better results, the analogous hapten, showing lower affinity to the antibody than digoxin, would be more inactive for substituting with already-bound digoxin to the antibody.

Another IEMA based on the FIA system was developed for measuring an anticancer drug, α-(difluoromethyl)ornithine (DMFO; Fig. 5C) (G4). The standard sample or biological specimen containing DMFO was incubated (room temperature, 15 min) with excess anti-DMFO antibody labeled with horseradish peroxidase (HRP). The antibody used here was elicited in rabbit (thus, polyclonal antibody), affinity-purified against the hapten, and reduced to cleave interchain disulfide bonds to provide a half IgG molecule, which is monovalent and thus advantageous for gaining larger signal intensity due to hapten binding. The reaction mixture was then injected into an FIA system equipped with an affinity column immobilizing a large excess of DMFO hapten linked with bovine serum albumin (BSA). Unlike the previous method by Freytag *et al.* (F2, F3), the affinity column immobilizing the analyte itself (DMFO) afforded a successful result. The HRP activity due to the eluted immune complex was fluorometrically determined using 3-(*p*-hydroxyphenyl)propionic acid (HPPA) and hydrogen peroxide (H_2O_2) as a substrate. The hapten-immobilizing column could be used for 12–15 samples successively, then regenerated by washing with an acidic buffer (pH 2.2). The assay sensitivity was 200 amol, and showed no interference from other structurally similar endogenous amines (ornithine and putrescine).

Various parameters for optimizing such flow injection IEMA for haptens have been systematically investigated using a theoretical model system (L2). The parameters evaluated were as follows: affinity, concentration, homogeneity, and purity of labeled antibody; incubation time for the reaction between hapten and labeled antibody; and residence time of the reaction mixture in immobilized antigen for removing excess antibody. The detection method of the enzyme activity was also investigated: namely, the flow rate through a reaction coil in which the enzyme-labeled antibody is mixed with a substrate; and the concentrations of the substrate, enzyme species, and so on. Based on the results obtained, the authors established the IEMA of digoxigenin using an alkaline phosphatase (ALP)–labeled Fab fragment of sheep anti-digoxigenin antibody, whose enzyme activity was fluorometrically measured using 4-methylumbelliferyl phosphate as a substrate. The assay system afforded a linear calibration curve covering 0.38–7.7 fmol and high sample throughput: 15 samples were analyzed per hour, and more than 60 injections were possible before regeneration of the affinity column was necessary.

Such a hapten-immobilizing affinity column was also operated on-line as part of a high-performance liquid chromatography (HPLC) system (I3). This is a

reversed-phase HPLC system employing an automated immunofluorometric assay as a detector. The mixture of digoxin and digoxigenin was initially submitted to an analytical ODS column to effect separation, and the effluent containing the hapten was automatically mixed with a solution of a fluorescein-labeled antibody (Fab fragment; showing similar reactivity to both compounds) in a reaction coil. The resulting mixture was then delivered into an affinity column packed with a digoxin-immobilizing hydrazine support, where the excess unoccupied Fab fragment was adsorbed. The passed immune complex was introduced into a fluorescence detector, and the signal due to fluorescein molecules on the antibody was determined. The immunoreaction was in equilibrium after ~1 min, resulting in peak broadening comparable to that in standard post-column derivatization systems. Both digoxin and digoxigenin can be determined in urine (injected directly) and plasma (injected after deproteinization with acetonitrile) with detection limits of 4 and 1 n*M* (corresponding to 200 and 50 fmol* per injection), respectively.

3.2. On-Line Immunometric Assays Based on Nonimmunological Removal of an Unoccupied Antibody

Another immunometric assay was reported (H1) in which a hapten (T_4)–antibody complex and unoccupied antibody were separated using a cation-exchanging column. Human serum containing T_4 (5 μl) and excess HRP-labeled anti-T_4 monoclonal antibody (Fab′ fragment) (50 μl) was incubated (37°C, 3 min), and the reaction mixture (10 μl) was then directly analyzed by an HPLC equipped with a Wakopack CQK-30S (sulfopropyl type, 7.5 mm i.d. ×75 mm). The immune complex and the unoccupied labeled Fab′ fragment were separately eluted (t_R 10.3 and 13.5 min, respectively) at a flow rate of 1.0 ml/min. Then the enzyme activity due to these components was determined fluorometrically after mixing with the substrate (HPPA and H_2O_2) in a post-column flow-through coil. This assay system afforded human serum T_4 levels correlating well with the assay values obtained with a commercially available EIA kit, with an assay sensitivity of 3.9 n*M* (19.5 fmol*/assay).

In automated immunometric assays using the flow system described above, the limitation concerning constant supply of immobilized antigen is mitigated or perfectly eliminated. Thus, the FIA-based assays (Section 3.1) employed a repeatedly usable immunosorbent, and the HPLC-based assay (Section 3.2) was independent of the immunosorbent for removing excess unoccupied antibody. Furthermore, these systems permitted quick separation of the complex of hapten-labeled antibody and excess antibody without disturbing the equilibrium of the antigen–antibody reaction. Such assays have not afforded sensitivities as high as those of the methods described in the Section 2, however, they are attractive because the assay principle seems to be widely applicable to any hapten and because they inherently have the potential for full automation, with the promise of highly prompt and successive measurement of many specimens.

3.3. Immunometric Assays Based on Masking Unoccupied Antibody Binding Sites

The modified single-antibody immunometric assays discussed below are based on principle B2 in Fig. 4. In these assays, unoccupied antibody-binding sites are masked by reaction with a multiply hapten-labeled macromolecule to permit subsequent selective determination of hapten-occupied antibodies.

Piran *et al.* (P1) reported an assay procedure involving the following three incubation steps, each of which was carried out at 37°C for 2.5 min, and automated using an ACS : 180 instrument (Fig. 9). First, the analyte 3,3′,5-L-triiodothyronine (T_3; Fig. 5C) was reacted with an excess amount of anti-T_3 antibody labeled with acridinium ester (AE), a chemiluminescent dye (L1) (step i). Second, unoccupied antibody was captured on controlled-pore glass particles (CPG) immobilizing 3,5-diiodothyronine (T_2; Fig. 5C) via bovine γ globulin (step ii). Third, paramagnetic particles (PMP) coated with an anti-AE antibody were added to the reaction mixture to isolate the complex of T_3 and the AE-labeled antibody (step iii). This selectivity was achieved by virtue of steric hindrance between PMP and CPG. Finally, chemiluminescence from AE molecules captured on PMP was determined. The minimal detectable T_3 concentration, defined as 2 standard deviations (SD) of the zero calibrator, was 0.005 μg/liter (corresponding to $\sim$ 70 amol*/assay), which was $\sim$10-fold more sensitive than a competitive assay performed with the same AE-labeled anti-T_3 antibody.

This immunometric assay employed an analog of the analyte (T_2) as a ligand of immunosorbent, as in the example discussed in Section 3.1. The authors mentioned that the use of T_2 molecules having low intrinsic affinity to the antibody but high avidity in the immobilized form on the CPG, through the use of divalent binding, obviated the need to prepare a monovalent labeled antibody.

A prominent advantage of this assay procedure is the feature that the complex of hapten and labeled antibody was captured on a solid phase (PMP) and separated from the reaction medium before signal determination. This additional step not only reduces interference due to biological specimens but also eliminates the tedious transfer of supernatant, which is essential in conventional immunometric assays. This immunometric assay provided somewhat improved specificity in terms of the cross-reactivities with T_2 and reverse T_3 (3,3′,5′-L-triiodothyronine). The authors speculated that the dissociation rate of the antibody–cross-reactant complex would be faster than that of an antibody–analyte complex; thus the former binding would be preferentially substituted by T_2 immobilized on CPG.

More recently, a somewhat similar assay principle has been reported (G1) determining cortisol (Fig. 5B) as a model hapten, in which a polydentate ligand [multiply cortisol-substituted poly(L-lysine) with a high MW (418,400)] was used as the masking reagent (Fig. 10). First, the analyte (cortisol) and the polydentate ligand were simultaneously added to the wells on which rabbit anti-cortisol antibody had been immobilized (step i). After incubation (room

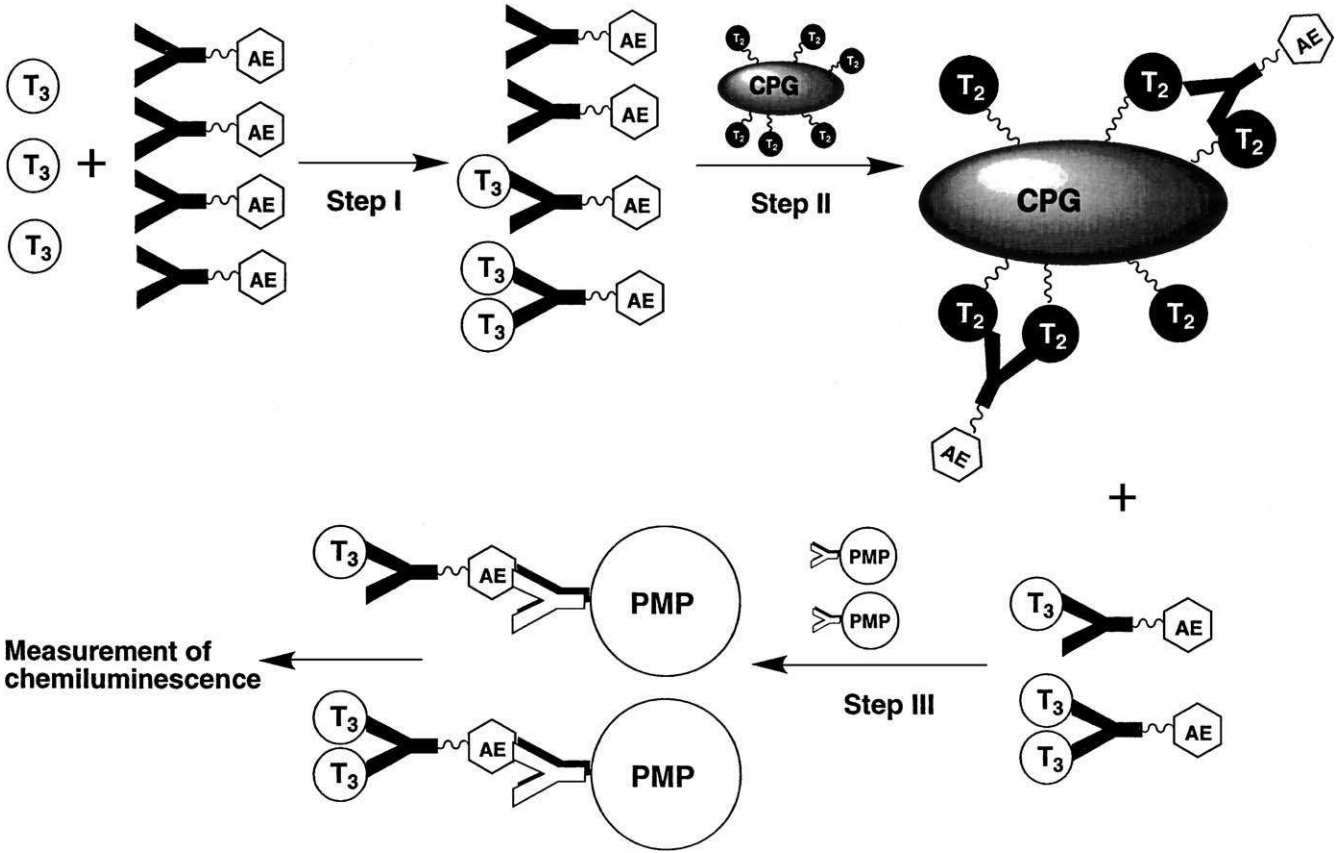

FIG. 9. Schematic representation of the noncompetitive immunoassay of triiodothyronine using a solid-phase immobilized hapten for masking an unoccupied antibody.

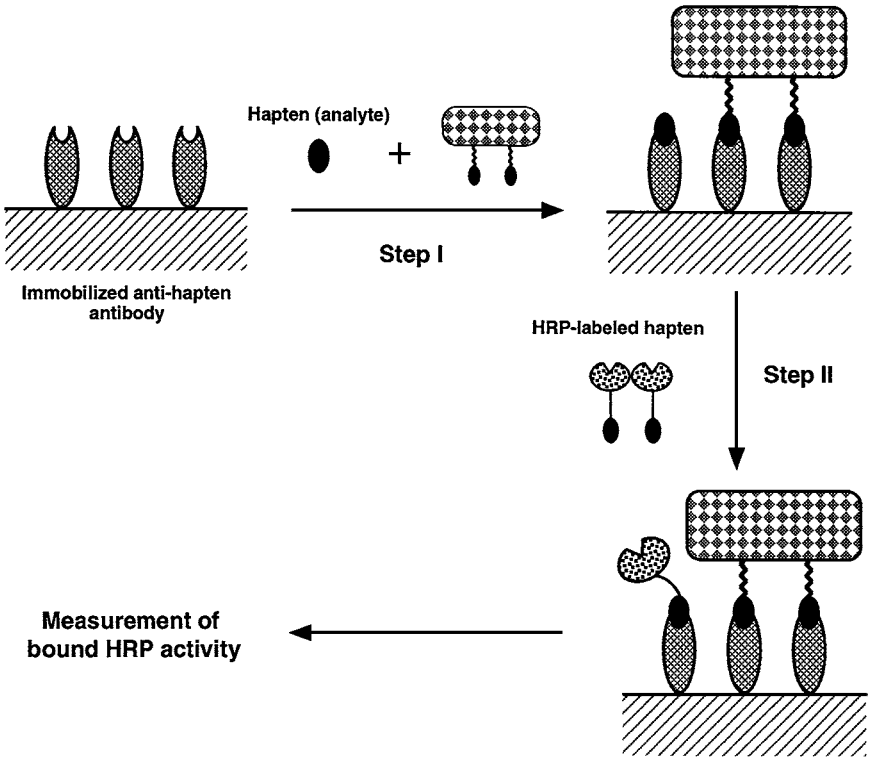

FIG. 10. Schematic representation of a noncompetitive immunoassay of cortisol using a polydentate ligand for masking an unoccupied antibody.

temperature, 2 h) and subsequent washing, a cortisol–HRP conjugate was added and incubated (room temperature, 10 min) to displace free cortisol bound by the antibody (step ii). Consequently, the bound HRP activity increased proportionally with the increase of the amount of added cortisol, which was colorimetrically determined using tetramethylbenzidine and H_2O_2. The noncompetitive format was achievable under an optimal condition, in which the cortisol–HRP conjugate quickly replaces the bound free cortisol while the bound polydentate ligand was resistant to the substitution, presumably because of the avidity effect and steric hindrance between the poly-L-lysine backbone and HRP molecule. This noncompetitive assay afforded a lower detection limit (0.15 ng/ml; 41 fmol*/assay) than that of the competitive assay using the same antibody (0.72 ng/ml).

The methods discussed in Section 3.3 still require the constant supply of a hapten-immobilized macromolecule (or solid phase) and thus require a haptenic derivative having an activated functional group, which is not easy to synthesize. It

would, however, be of interest to demonstrate whether this noncompetitive assay format is generally applicable to various haptens.

4. Noncompetitive Hapten Immunoassays Using New-Generation Antibody Reagents

The invention of B cell hybridoma technology (K3) has allowed the generation of various kinds of useful antibodies, even very minor or rare antibody species elicited in serum by the conventional procedure, as a pure immunochemical reagent in almost unlimited amounts. Among such new-generation monoclonal antibodies, anti-idiotype antibodies and anti-immune complex (anti-metatype) antibodies have been successfully introduced as key reagents enabling noncompetitive hapten immunoassays (Fig. 11).

4.1. Assay Systems Based on Anti-idiotype Antibodies

Anti-idiotype antibodies are the "second antibodies" whose specificity is directed to the epitopes ("idiotopes") located on the variable regions of a particular "primary antibody" (J2). Although several methods have been proposed to categorize the anti-idiotype antibodies, they are simply classified into two groups, α-type and β-type antibodies (B1, J2, S1) (Fig. 11). The α-type anti-idiotype antibodies are those recognizing the framework of the variable region, permitting simultaneous binding of the antigen to the paratope. The β-type antibodies bind to the paratope and compete with binding of the original antigen. Such anti-idiotype antibodies are usually generated by the immunization of an animal with a purified

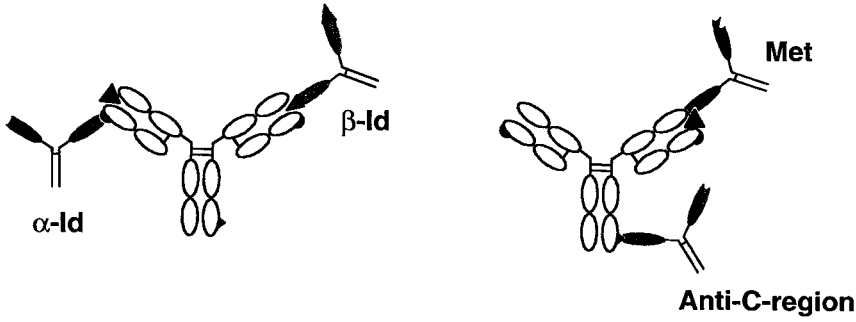

Fig. 11. Schematic representation of the binding properties of anti-idiotype antibodies and anti-metatype antibodies. ▼, hapten; α-Id, α-type anti-idiotype antibody; β-Id, β-type anti-idiotype antibody; Met, anti-metatype antibody; anti-C-region, antibodies recognizing the constant region of a primary antibody (recognizing isotype or allotype).

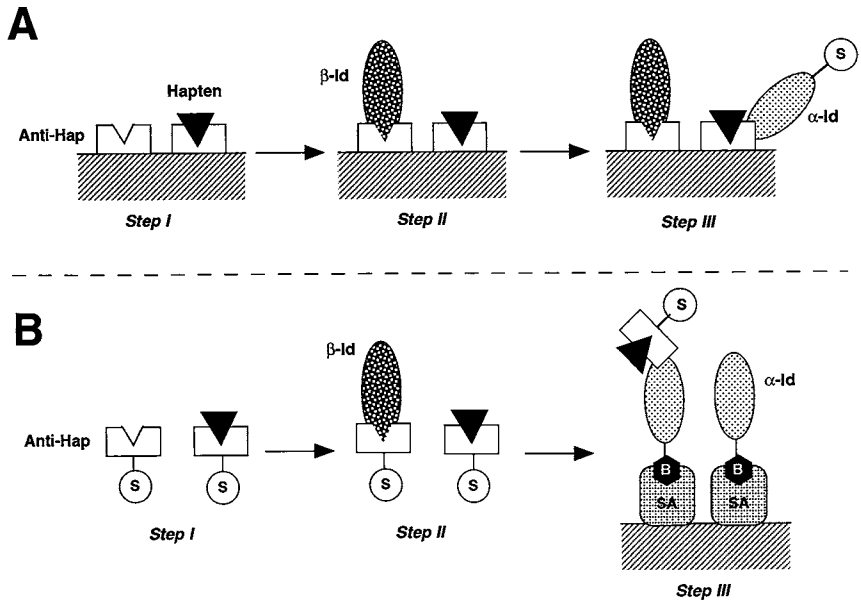

FIG. 12. Noncompetitive hapten immunoassay procedures (A and B) using a combination of the α-type and β-type anti-idiotype antibodies, each recognizing the framework and paratope of the anti-hapten antibody. Anti-hap, anti-hapten antibody (primary antibody); α-Id, α-type anti-idiotype antibody; β-Id, β-type anti-idiotype antibody; S, signal-generating group; B, biotin; SA, streptavidin.

primary antibody against the target molecule, which is sometimes conjugated with a strongly immunogenic protein [e.g., keyhole limpet hemocyanin (KLH)] as a carrier (B3, B4, S1).

Self (S4) first proposed the concept of noncompetitive assay for haptens utilizing an adequate combination of an α-type and a β-type anti-idiotype antibody, in which he used the term, "selective antibody" for the α-type antibodies. Then, Barnard and Cohen (B1) applied this assay principle for the determination of serum E_2, naming the assay system an "idiometric assay." Figure 12A illustrates the assay procedure of the idiometric assay of E_2. The target hapten is captured by excess anti-E_2 antibody immobilized on microtiter strips by incubation at room temperature for 1 h (step i). After washing the strips, the β-type anti-idiotype antibody was added in order to saturate (or block) the unoccupied paratope of the anti-E_2 antibody (incubation, room temperature for 30 min) (step ii). The α-type anti-idiotype antibody, which has been labeled with a europium chelate (H4), was then added to the plate and incubated at room temperature for a further 2 h (step iii). Finally, fluorescence intensity due to bound europium was measured with a time-resolved fluorometer. Because of large steric hindrance around the bound β-type antibody (MW $\sim$150,000), the labeled α-type antibody would,

if it recognizes an idiotope located close to the paratope, preferentially bind to the framework region of the anti-E_2 antibody, which captures E_2 but not the antibody bound by the β-type anti-idiotype antibody (step iii). Consequently, bound fluorescence intensity elevates proportionally with the increase of the amount of E_2 molecules captured on the anti-E_2 antibody. The authors also applied the idiometric assay to the measurement of two more endogenous steroids, estrone 3-glucuronide (E_1-3G) (B2, B3) and progesterone (B4) (Fig. 5B). A different reaction sequence was employed in these assays (Fig. 12 B), in which the hapten is first captured by excess europium-labeled anti-steroid antibody (step i). After blocking the unoccupied paratope with the β-type anti-idiotype antibodies (step ii), the hapten–antibody complex was selectively bound by a biotin-labeled α-type anti-idiotype antibody, and the resulting complex was then captured on avidin-coated or (B3) anti-biotin antibody-coated (B4) microtiter strips (step iii). Both procedures in Fig. 12 are essentially based on the same mechanism (i.e., masking of unoccupied paratope by the β-type antibody followed by selective detection of hapten-occupied paratope by the α-type antibody), which is based on principle B2 in Fig. 4. Thus, the idiometric assays could be regarded as variations of the single-antibody immunometric assay, although they employ three kinds of antibody. It should be noted that the assay specificity should be supplied from the recognition by the anti-hapten antibody, thus differing from the two-site immunometric assays for a macromolecule, where the double recognition mechanism is expected.

Although these assay procedures for steroids were successfully applied to clinical samples, the sensitivity was not extensively improved, as shown by their femtomole range detection limits: ~ 2 fmol*/assay for E_2, 0.32 nmol/liter (40 fmol*/assay) for progesterone, and 0.4 nmol/liter (8 fmol*/assay) for E_1-3G, respectively. Difficulty in obtaining a higher sensitivity would come from the competitive substitution of the bound hapten with the β-type antibody and/or imperfect selectivity of the α-type antibody toward the hapten–antibody complex.

We (K1) attempted to develop a noncompetitive assay based on the anti-idiotype antibodies for a conjugated bile acid metabolite, ursodeoxycholic acid 7-*N*-acetylglucosaminide (UDCA 7-NAG), which is expected to serve as a diagnostic index for an autoimmune disease, primary biliary cirrhosis. In our assay, the hapten UDCA 7-NAG, a β-type antibody, and a biotin-labeled α-type antibody were simultaneously added to a microtiter plate coated with an $F(ab')_2$ fragment of a specific anti-UDCA 7-NAG antibody, then incubated at room temperature for 8 h. Bound biotin was then detected with HRP-labeled streptavidin, whose enzyme activity was measured using *o*-phenylenediamine/H_2O_2 as a substrate. This noncompetitive assay system provided a subfemtomole-order sensitivity (detection limit 118 amol) that was 7 times lower than the competitive immunoassay using the same anti-hapten antibody (K2), even with a common colorimetric detection (Fig. 13). Somewhat improved specificity was also obtained: namely, better

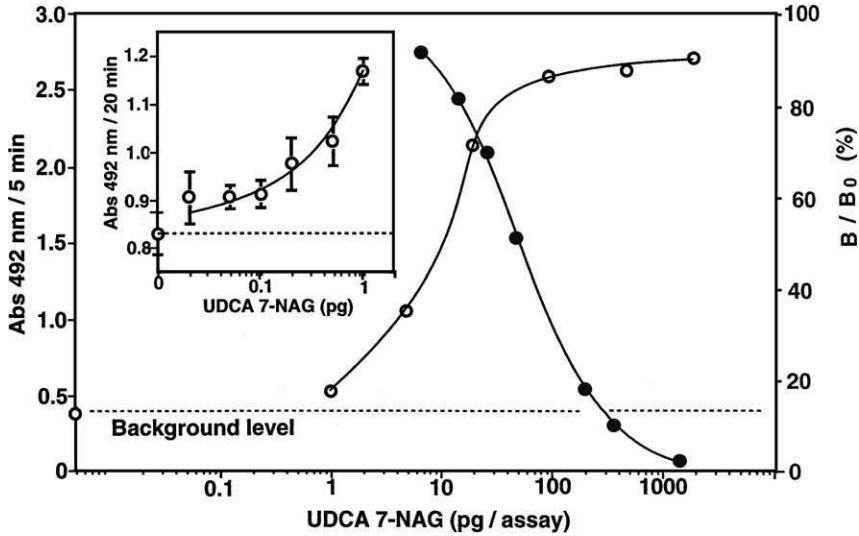

FIG. 13. Dose–response curves of (○) noncompetitive and (●) competitive immunoassays of UDCA 7-NAG.

group-specificity to nonamidated UDCA 7-NAG and its glycine and taurine conjugate (GUDCA 7-NAG and TUDCA 7-NAG) (cross-reactivity 100%, 97%, and 72%, respectively), and lower cross-reactivity with UDCA 7-glucoside (1.4%) and UDCA 3-NAG (0.12%) than the competitive assay (Table 1). This noncompetitive assay enabled direct measurement (without any pretreatment of specimens) of urine UDCA 7-NAG levels of both healthy volunteers and patients with liver diseases. Although a long incubation time (8 h) was adopted for the antigen–antibody reaction to achieve the best sensitivity in this system, 2 hours of incubation was found to be sufficient to obtain a practical sensitivity for clinical study.

Development of such idiotype-dependent assays requires much labor and a long period because two-step antibody production is necessary: first, immunization with a hapten–carrier conjugate to produce a specific anti-hapten antibody, then a second immunization with the anti-hapten antibody for generating two types of anti-idiotype antibodies. Furthermore, a suitable combination between α-type and β-type antibodies, whose binding to the anti-hapten antibody is competitive, has to be found.

The assay principle should, however, be applicable to any target hapten, unlike assays based on a chemical modification. Cloning efficiency of the hybridoma-secreting anti-idiotype antibodies would be in a practical range, and much higher than that of anti-metatype antibodies. We (K1) established four kinds of α-type and two kinds of β-type anti-idiotype antibodies after three fusion experiments, each using spleen cells from one immunized mouse. Barnard *et al.* (B1, B3, B4)

TABLE 1
CROSS-REACTION OF NONCOMPETITIVE AND COMPETITIVE IMMUNOASSAYS FOR UDCA 7-NAG

Bile acid	Assay method (%)	
	Noncompetitive[a]	Competitive[b]
UDCA 7-NAG	100	100
GUDCA 7-NAG	97	45
TUDCA 7-NAG	72	50
UDCA 7-glucoside	1.4	4.3
UDCA 3-NAG	0.12	0.36
UDCA	<0.001	<0.01
UDCA 3-sulfate	<0.001	<0.01
UDCA 3-glucuronide	<0.01	<0.02
GUDCA	<0.001	<0.01
GUDCA 3-NAG	0.16	0.29
Cholic acid	<0.001	<0.01
Glycocholic acid	<0.001	<0.1

[a] Calculated by the amount giving the defined absorbance (background level +1.0).
[b] Calculated by 50% displacement method.

obtained these antibodies in better efficiency: 3–4 kinds of α-type and 3–8 kinds of "strong" β-type anti-idiotype antibodies after a single fusion experiment.

As reported by Self (S6), the β-type antibody can be replaced with a macromolecule multiply labeled with the target hapten, such as hapten–carrier conjugates used for immunogen. The noncompetitive assay for cortisol (G1), which was discussed in Section 3.3, is based on this principle, but employs a different method for selective detection of hapten-occupied antibody.

4.2. ASSAY SYSTEMS BASED ON ANTI-METATYPE ANTIBODIES

Using an antibody specifically recognizing the antigen–antibody complex, more direct noncompetitive hapten immunoassays, which can be regarded as "semi" two-site immunometric assay, could be established (S3). Figure 14 depicts two typical procedures of noncompetitive assays using anti-metatype antibodies, which are based on principle C in Fig. 4.

Voss *et al.* (V3, V4) succeeded in isolating the antibody, which strongly bound to the immune complex of fluorescein and anti-fluorescein antibody (dubbed the "liganded" antibody) but did not recognize the free hapten or the free antibody. The authors proposed the term "metatype" for such anti-complex antibodies: This means an antigen-induced conformational state of the antigen-binding site, which can be distinguished from the idiotype. They have been extensively studying the

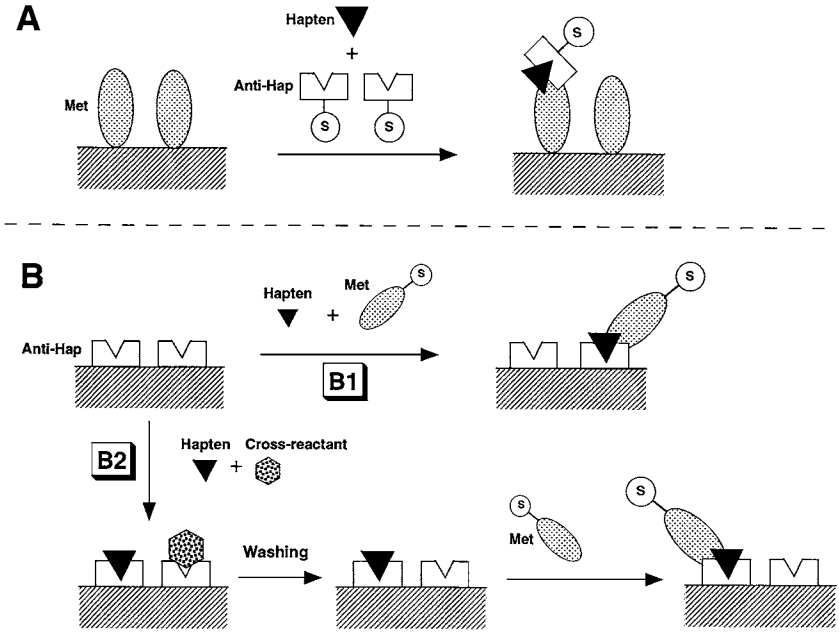

FIG. 14. Noncompetitive hapten immunoassay procedures (A, B1, and B2) using an anti-metatype antibody recognizing the immune complex between a hapten and the anti-hapten antibody. Anti-hap, anti-hapten antibody (primary antibody); Met, anti-metatype antibody; S, signal-generating group.

role of these antibodies in the immune network, and recently published a review article (V5) written with a view to utility in immunoassays.

In 1993, Ullman *et al.* (U1) immunized mice with an anti-Δ^9-tetrahydrocannabinol (Δ^9-THC; Fig. 5C) antibody, which was affinity-labeled with a Δ^9-THC derivative (thus, a "covalently anchored" antigen–antibody complex). This affinity labeling was intended to keep the hapten–antibody complex bound during circulation in the body after immunization and reduce by-production of anti-idiotype antibodies. After five fusion experiments using the resulting immune spleen cells, one hybridoma clone, secreting a desired anti-metatype antibody (they called it an "anti-immune complex antibody"), was established among very many clones secreting the α-type (130 clones) and β-type anti-idiotype antibodies (67 clones). Using the anti-metatype antibody, the following noncompetitive-type ELISA was performed (Fig. 14A). First, the anti-metatype antibody was indirectly immobilized on a microtiter plate via a rabbit anti-mouse IgG antibody. Then the analyte Δ^9-THC and an HRP-labeled anti-Δ^9-THC monoclonal antibody were added, followed by incubation at room temperature for 30 min. After washing the plate, the bound HRP activity was measured colorimetrically using 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a hydrogen donor. The

dose–response curve showed a typical feature of noncompetitive assays, in which the bound signal increases with an increase of the added Δ^9-THC. Although the assay sensitivity was similar to that of competitive assays (assumed to be $\sim$ 10 fmol* per assay), somewhat improved specificity was observed. The noncompetitive assay discriminates the Δ^9-THC analog having a carboxyl group (Δ^9-THC-COOH; Fig. 5C), which exhibited the same affinity against the anti-Δ^9-THC antibody (thus, the competitive assay cannot distinguish these two compounds).

A practical noncompetitive assay was then reported by Towbin *et al.* (T5), in which AGII was determined. Four kinds of anti-metatype antibodies were established from two fusion experiments using the spleen cells from mice immunized with the complex of AGII, anti-AGII antibody, and KLH, which were randomly crosslinked with glutaraldehyde. It should be noted that underivatized anti-AGII antibody was simultaneously injected at the immunization, which treatment was effective for obtaining the anti-metatype antibodies. The authors speculated that the antibody might have tolerized mice against an immune response for generating anti-idiotype antibodies, or the antibodies generated *in vivo* may have been adsorbed. Very sensitive dose–response curve was obtained (detection limit, 1 pg/ml) ($\sim$50 amol*/assay) with the following noncompetitive assay system (called a "metatypic assay"). AGII and an anti-AGII antibody, which had been labeled with a chemiluminescent dye [an acridinium ester derivative (L1)], were simultaneously added to a white microtiter plate coated with the anti-metatype antibody (Fig. 14A). After incubation for 16 h, the plate was washed and bound chemiluminescence, emitted by the addition of H_2O_2, was determined using a plate luminometer. In the metatypic assay, the cross-reactivity with angiotensin III (the AGII analog lacking the N-terminal aspartic acid) (Fig. 5A) dramatically decreased (cross-reactivity 5.5%), while it exhibited the same reactivity (100%) in the competitive radioimmunoassay using the same anti-AGII antibody. This result, as well as the result in the Δ^9-THC assay, is ascribable to the double recognition due to the use of two kinds of antibodies.

Self and colleagues reported (S5, S6, W1) a series of studies on developing a noncompetitive assay of digoxin (called an "anti-complex assay") using an excellent anti-metatype monoclonal antibody recognizing digoxin-bound primary antibody whose affinity was >2000-fold greater than its binding to the primary antibody alone (S5). Digoxin (standard or clinical samples) and an ALP-labeled anti-metatype antibody were added to a microtiter plate coated with the primary antibody, which was incubated at room temperature for 10 min ("simultaneous anti-complex assay") (Fig. 14B1). After washing the plate, bound ALP activity was determined colorimetrically using *p*-nitrophenyl phosphate or an enzyme cycling reaction (using an AMPAK™ kit), which provided a detection limit of 0.1 μg/liter and 0.03 μg/liter (the latter value corresponds to $\sim$1 fmol*/assay), respectively (S5). It was also shown that the incubation period could be shortened to 1 min without serious loss of sensitivity (S5). This noncompetitive assay system

also exhibited excellent specificity, as indicated by the absence of interference from digoxin-like immunoreactive factor. Accuracy of the assay was satisfactory, and the assay results for clinical samples correlated well with the values obtained with a conventional assay method.

This assay system has been improved to achieve higher specificity (S6, W1). Simply interposing a wash step between the addition of analyte and the labeled anti-metatype antibody ("sequential anti-complex assay") (Fig. 14B2) dramatically enhanced the assay specificity. Thus, the improved assay exhibited almost negligible cross-reactivities with digoxigenin, digitoxin (Fig. 5B), digitoxigenin, and acetylstrophanthidin, while retaining the original assay sensitivity. Although the cross-reactivity with ditoxin remained as before, the authors succeeded in reducing it to negligible level by a further modification of the assay system in which two kinds of anti-digoxin antibodies, each recognizing a different partial structure on the digoxin, were employed (S6).

One more successful result has recently been reported (N1), in which microcystin-LR (MCLR; MW $\sim$1000) (structure not shown), a cyclic heptapeptide hepatotoxin produced by cyanobacteria, was determined. Three anti-metatype antibodies were established from four fusion experiments using spleen cells from mice immunized with an immune complex of MCLR and a high-affinity anti-microcystin monoclonal antibody without affinity labeling or conjugation with carrier protein. The best anti-metatype antibody used in the assay showed an affinity to the immune complex about 300-fold higher than to the anti-microcystin antibody alone. This assay was performed according to the procedure in Fig. 14B1, but MCLR and a biotinylated anti-metatype antibody were sequentially added to the primary antibody-coated plate, employing overnight incubation (at 4°C) for each step. The detection limit was 2 pg/ml of MCLR ($\sim$50 amol*/assay).

Although a dramatically improved sensitivity has not always been achieved, the anti-metatype antibodies promise to be one of the simplest and most attractive approaches to a noncompetitive format of hapten immunoassays. As mentioned above, such an anti-metatype antibody can seldom be generated by the usual antibody production methods; consequently, the information now available concerning the strategy for its production is very poor. Some sort of breakthrough might be necessary for obtaining practical anti-metatype antibodies against any kind of hapten–anti-hapten complex with an acceptable probability. It would be worth investigating the selection of anti-metatype antibodies from a vast library of genetically engineered antibodies (see, for example, Ref. G3).

4.3. Assay Systems Based on a Stable Association of V_H and V_L Domain Peptides in the Presence of the Hapten Molecule

Very recently, a new noncompetitive assay principle called an "open sandwich ELISA" has been reported (S7) (Fig. 15). The assay mechanism could be regarded

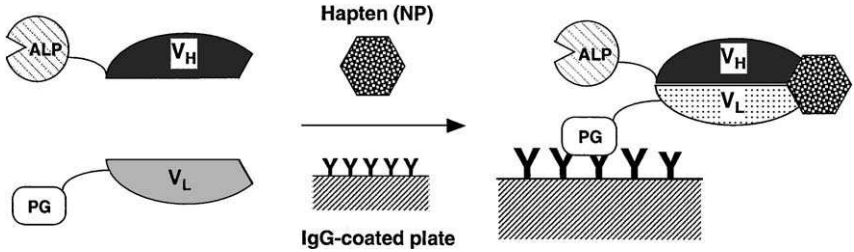

FIG. 15. Schematic representation of the open sandwich enzyme-linked immunosorbent assay of a hapten.

as a variation of principle C (Fig. 4), in which two kinds of fusion proteins were employed, each containing the V domain derived from an antibody toward 4-hydroxy-3-nitrophenyl acetic acid (NP). The V_H domain fused with ALP and the V_L domain fused with protein G (PG) were preferentially associated into the Fv fragment in the presence of the relevant hapten, NP. Thus, the capture of the resulting ternary complex (via its PG moiety) on an IgG-coated microtiter plate generated bound enzyme activity, which increased with an increase of the added hapten. Although this system has not yet afforded practically sensitive dose–response curve for the hapten (micromolar range, so far), it would be of interest to investigate the probability of obtaining a set of the V_H and V_L domains such that they form stable ternary complex via the relevant hapten.

5. Conclusion

It is reasonable that much effort has recently been focused on developing noncompetitive assay for haptens because this important, challenging subject in analytical/clinical chemistry may lead to a breakthrough for hapten analyses with ultrahigh sensitivity and improved specificity and precision. However, no truly universal assay format—one that can be established with acceptable labor and probability and provides dramatically improved sensitivity—has been proposed. Nevertheless, many of the assay procedures reviewed above offer significantly shortened incubation times and their assay formats can feasibly be automated. These advantages of noncompetitive assays, together with the potential for obtaining much higher sensitivity and specificity, should prompt further efforts to develop universal noncompetitive hapten immunoassay methods as a high-throughput diagnostic tool.

An ideal immunoassay format would be a noncompetitive, nonradioisotopic, and homogeneous (washing-free) procedure based on a mechanism in which specific binding of target antigen to antibody paratope triggers signal generation, just as

complement fixation does. From this point of view, a remarkable receptor assay (not immunoassay) of 1α,25-dihydroxyvitamin D_3 (1,25D_3; Fig. 5B) based on reporter gene activation has recently been reported (A1). This assay was performed in a microtiter plate whose microwell contains a mammalian cell line expressing high levels of vitamin D receptor transfected with a luciferase reporter gene under the control of the highly inducible 25-hydroxyvitamin D_3 24-hydroxylase promoter. When 1,25D_3 is added to the well, it binds to the receptor and the resulting complex stimulates the promoter and induces the expression of luciferase. After lysates were prepared (cell washing is necessary at this step), an aliquot was submitted to the measurement of luciferase activity using D-luciferin as a substrate. The resulting bioluminescence increased proportionally with an increase of the added 1,25D_3, in the range of 1 pg (2.4 fmol) to 1 ng.

We expect such an elegant noncompetitive assay principle, which is universally available for various types of haptens providing subfemtomol sensitivity, will be invented in the future.

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DNA GENOTYPING

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1. Overview

Molecular diagnostics technology is rapidly evolving. Completion of the first draft of the human genome has provided an engine for discovery of single-nucleotide polymorphisms (SNPs). Among these are the cSNPs—SNPs in the coding region of genes—which comprise most of the mutations that cause hereditary disease. By the end of 2001, more than 1.5 million SNPs and 90% of human gene sequences were published, offering an unprecedented gaze into the genetic makeup of *Homo sapiens*. From a social and cultural perspective, our newfound

genetic knowledge has brought both promise and foreboding. Although we know that an understanding of genetics will help eradicate hereditary disease and ameliorate suffering, we fear that the misuse of genetics will have odious consequences for us, both as individuals and as cultural beings. While the ethical, legal, and social-issues controversy continues, pharmaceutical and diagnostics developers have sidestepped the argument, using the new knowledge to plan for the future of genetic testing and drug development. Meanwhile, our technical ability to discern point mutations, deletions, duplications, expansions, and insertions has improved markedly.

The purpose of this review is to recount recent technological achievements in DNA genotyping. In the course of doing so, it is appropriate to discuss the social and economic benefits and costs of genetic testing. The conduct of molecular studies for clinical or research purposes can be socially perilous. Mandates and voluntary rules have evolved to make them less so. Clinical molecular genetics must acknowledge a large debt to research. This article does so by recounting the technical details of mutation discovery methods of major import. Molecular testing has become routine for many human mutations characterized only in the past ten years. The end of the genome project has ushered in a new era of molecular diagnostic tools. Pharmacogenomics, a field that studies the relationship between pharmaceutical action and hereditary endowment, is receiving a major boost from our newfound knowledge. New molecular genotyping technologies have made this a propitious moment for geneticists. Resources available for genetic studies can now be conserved because many molecular diagnostic platforms have been standardized, automated, and are beginning to approach a sample-size scaledown near nanotechnology. Predictably accurate and precise genotyping results are expected in any adequately equipped and funded laboratory. Quality assurance and quality control (QA/QC) standards have become more universal. Voluntary guidelines from professional associations often mandate result report content; these are discussed. In sum, it is hoped that this review will offer practitioners of the art a coherent technical discussion of recent progress in the nascent discipline of molecular diagnosis.

1.1. Benefits of Genetic Testing

The modern era of genetic testing dawned in 1961, when Robert Guthrie reported that newborns affected with phenylketonuria could be subjected to an ingenious, rapid, and inexpensive neonatal screening test for phenylalanine hydroxylase deficiency. Earlier observation had established that nutritional intervention (G3), if started sufficiently early, would prevent the onset of what had until then been inexorable, irreversible mental retardation due to high brain phenylalanine levels (C7). Mandatory screening efforts, pioneered in Massachusetts and California, allowed the practice of genetic medicine to begin, portending a reproducible cycle of discovery, diagnosis, treatment, and amelioration of genetic disease (K4).

Through the efforts of Guthrie and other like-minded physicians, including Rimoin (R2, R3, R4), Kazazian, Kaback (B1, C4, F2, K1, K2, K3, K4, K5, M2, R4) Scriver, and Desnick (C6, D1, G2, R6, S1, S2, S3, S4, S5), a model emerged in which some form of "universal genetic screening" within a population could legitimately be performed to reduce genetic disease incidence within that population. This kind of population screening caught on among Europeans, whose national, "socialized" medicine systems could easily support it. Among American academics, this became known as the "state" model of health insurance planning. Meanwhile, a push for genetic testing came in the United States through individual patients, who soon saw that genetic testing could offer the possibility for better pregnancy outcomes and more choice in bringing a pregnancy to term.

1.1.1. *Risk Reduction*

In the late 1970s, efforts to recognize genetic disease early, before it could manifest, took a giant leap forward as a result of work on α-fetoprotein (B4). This protein's appearance was adduced by studies of early mouse development as a marker for correct embryologic development (S6). Rare accidents in neural tube folding gave rise in mouse, as they do in humans, to open tubes, which manifest as ONTDs (open neural tube defects, see Ref. A1). The most common of these is spina bifida, though omphalocoele and anencephaly are also characteristic ONTDs. In the 1970s it became clear that a simple protein immunoassay for α-fetoprotein administered to women between 15 and 20 weeks gestation would allow high early detection rates of ONTDs (H2, N3). This test was offered as a service by many OB/GYN practices and began to allow for genetic screening. Soon thereafter, it was learned that low levels of α-fetoprotein during the same period in pregnancy were associated with Down syndrome births (C3, S7). Because the α-fetoprotein test was inexpensive, easily interpreted, and, coupled with confirmatory post-amniocentesis testing for acetylcholinesterase and amniotic fluid α-fetoprotein, reliably predicted the birth of affected fetuses, it became widely used. Serum α-fetoprotein testing became a model for prenatal genetic diagnosis.

1.1.2. *Diagnosis and Prognosis of Early-Onset Conditions*

Newborn genetic diseases and syndromes can be difficult to diagnose. Because the range of symptoms differs from case to case and because assessment of young, pre-verbal children can be a challenge, pediatric genetics staff and residents often depend on cytogenetic, molecular, and biochemical genetic workups to provide a definitive diagnosis. Prader/Willi Angelman syndrome, spinal muscular atrophy, Duchenne muscular dystrophy, cystic fibrosis, and fragile X syndrome are among the disease etiologies that can be confirmed or refuted through molecular diagnosis. Although treatments are available for cystic fibrosis, the other diseases mentioned are amenable only to psychosocial intervention and some physical and

occupational therapy. In most circumstances, prenatal genetic test results are supplied to affected families during genetic counseling in the hope of preventing the birth of a second child afflicted with the same disorder. In most cases, birth of a child with a hereditary disease is the first clue of a familial predisposition: 80% of offspring with cystic fibrosis are born to parents with no previous family history.

1.1.3. *Value of Knowing*

Patient preference surveys conducted throughout the 1970s and 1980s (P2, P3, P4) reproducibly demonstrated definite value in knowing genetic constitution, whether or not it affects clinical decision making. When clinical decisions are dependent on understanding genetic endowment, hereditary knowledge assumes greater importance. Medical decisions to resort to extraordinary life-saving measures in the nursery are often tempered with certain knowledge of a fatal genetic diagnosis. In less extreme circumstances, families who have already given birth to a child with Down syndrome are known to be more aggressive in seeking prenatal diagnosis than those who have not experienced coping with offspring with a life-long disability.

1.1.4. *Choice (Prenatal Diagnosis)*

It is a truism that without genetic diagnosis, there would be no basis for elective pregnancy termination based on predicted outcome. It is also true that without favorable court rulings, there would be no basis for pregnancy termination based upon a predicted unfavorable outcome. As a consequence of court decisions, application of reliable, accurate methods for predicting pregnancy outcomes, through biochemical, cytogenetic, and molecular technologies, has become the standard of care in clinical medical genetics.

1.1.5. *Treatment Options*

As discussed above, in the case of phenylketonuria, early intervention can make the difference between mental retardation and a near normal life course for a newborn. Congenital adrenal hyperplasia and maple syrup urine disease are two examples of neonatal hereditary disorders where early diagnosis and medical intervention can make the difference between life and death for the newborn. In addition, in a number of genetic diseases, early diagnosis and treatment can help ameliorate symptoms; these include fragile X syndrome, homocystinuria, sickle cell anemia, cystic fibrosis, and many β-thalassemias.

1.2. Clinical Utility

Clinical utility is a measure of the influence that a diagnosis has upon medical management. If there is no change in medical decision as a result of a particular

diagnostic test, we can state that the clinical utility of that test is zero. Clinical utility can also have a "negative" value, in that it may engender more harm than good. When presymptomatic Huntington's disease testing first became available, it caused a controversy in the genetics community: Many professionals feared that it would precipitate suicide as a result of depression among newly diagnosed or anticipating members of affected families. Caution among genetic practitioners is believed to have ameliorated these problems with this medical test. Genetic testing in Huntington's disease would have a net negative clinical utility if it were to result in suicide.

1.2.1. *Do the Clinical Benefits Outweigh the Risks?*

Before genetic counseling, medical geneticists observed that it was unlikely that a couple who had previously conceived a child with a progressive hereditary disease—say, Tay–Sachs, Sandhoff's, or the neurological manifesting forms of Gaucher disease—would ever have another child (N1). Furthermore, before 1991, the cause of a differential burden of mental retardation among boys, though commonly observed, was mysterious. It was not clear why or how families who conceived a disproportionate number of mentally retarded boys became at greater risk through successive generations (S8)—that is, until the molecular phenomenon of "anticipation" was understood, as a resolution to the Sherman paradox (F3). The disproportionate number of mentally retarded boys is now understood to be a consequence of the maternal lengthening of a triplet expansion sequence over generations. Over time, it became clear that the benefits of identifying families at risk of significant hereditary disease outweighed the risks, as needless suffering, both emotional and physiologic, could be avoided through early identification of the causes of a life-long disability and early intervention to minimize the severity of the disease. Cystic fibrosis and sickle cell disease, though perhaps not susceptible to cure, are susceptible to successful intervention: In pediatric patients, sparing use of antibiotics is indicated in each of these maladies. In addition, in the β-thalassemias and sickle cell disease, appropriate use of anti-pain medication combined with hydroxyurea therapy, desoxyferramine chelation, and oxygen treatments has ameliorated symptoms in patients experiencing a crisis (N1).

1.3. Costs

Definitive diagnosis comes at a cost. QA/QC expenses, regulatory expenses, and patent royalty expenses are high, as are supply costs for consumables, reagents, and test kits. Morever, the equipment required for assay and result delivery and accuracy can be expensive. In the sections that follow, an outline of how these costs are calculated is provided and an effort is made to flesh out a difficult area to quantify. Generally, test costs, neglecting administration and overhead, can be

discussed within six categories: (1) direct, (2) labor, (3) overhead, (4) royalties, (5) methodological, and (6) gene-test specific.

1.3.1. *Direct*

Direct costs are those expenditures required directly for the completion of the test procedure itself. This includes the costs of reagents for buffer preparation as well as enzyme and nucleotide purchase; transportation and delivery costs; the costs of consumables used in buffer preparation (e.g., weigh boats, disposable plastic ware); any costs associated with purified water production, autoclaving, or bottling; kit purchases; pipette tip purchases; and purchase of disposable test tubes or washing costs for reusable glass tubes. Some data-processing costs can be direct costs, as can photographic film, paper, or photocopying costs. In short, any items directly required for result production are considered direct costs.

1.3.2. *Labor*

Labor is often the largest of the direct costs for genetic testing. Few technicians or technologists in our current economy are compensated (with fringe benefits) at less than the $15/hour level, and so this is a reasonable basis for calculating labor costs. They can be higher, but are unlikely to be more than 25% lower in any part of the United States. Therefore, labor time is the primary cost component for most molecular genetic assays.

1.3.3. *Overhead*

Molecular diagnostic laboratory procedures require a well maintained facility. Biohazard and toxic chemical wastes must be removed and air circulation in pre-PCR areas should be separate from post-PCR areas. Good lighting, biohazard hoods, and fume hoods, as well as laboratory equipment such as centrifuges, ultra-cold freezers, spectrophotometers and fluorescent plate readers, and automated sequencers, are often required to carry out the procedures described here. As a consequence of complex building code, fire code, and electrical code requirements, architectural and building costs rarely fall below $300 per square foot for a molecular laboratory space. In many institutions, these costs create a conflict between research facility support, which is in part underwritten by the federal government through direct grants or R&D credits, and ongoing clinical laboratory support, which is hosted by institutional endowment. Overhead, because it is such an important component of molecular diagnostic cost, is a hotly debated issue among laboratory managers.

1.3.4. *Royalties*

Intellectual property payments have been among the most rapidly increasing costs for molecular tests in the past few years. Early in the art, there were few

blocking patents. At that time, the direct genomic radioactive RFLP technology (B3) was painstaking, lengthy, and primitive. Shortly after these methods were supplanted by polymerase chain reaction technology (PCR), costs began climbing. PCR, which was developed by Kary Mullis and others at Cetus, was purchased by Roche in a $300 million transaction in 1991 (R1). This large transaction necessitated cost recovery by Roche, which has resulted in a 10–20% royalty being charged to organizations providing molecular diagnostic testing services to patients and physicians. Although the community consensus is that technology was initially worth the licensing cost, additional patent licenses became cost-prohibitive when coupled to the Roche royalty payment. Specifically, in the 1990s, efforts to collect payments for B-cell/T-cell rearrangements by the University of Toronto, working through University Patents; hepatitis C virus (HCV) through Chiron (now Bayer Diagnostics), ligase chain reaction (LCR) through Perkin-Elmer (now Applera's Applied Biosystems), and gap ligase chain reaction (gap LCR) through Abbott; and the amplification refractory mutation detection system (ARMS) through Zeneca Diagnostics, all relied upon piggybacking with diagnostic PCR licenses awarded through Roche Molecular Systems. This has made it very difficult to keep up with the latest diagnostic technology without a considerable cash warchest and significant subsidies to the performing laboratory. As a consequence, there has been a rebellion of sorts in the past several years. As this article goes to press, a serious courtroom battle continues to rage over the validity of the PCR patent itself and patents on Canavan and Gaucher disease testing. Who will win is anybody's guess. But increased pressure to dispute patent costs seems an inextricable future part of molecular genetic diagnostic practice.

1.3.4.1. *Methodological.* Economics guides method selection in mutation detection. In turn, method selection provides a basis for the level of risk reduction or certainty for the genotypes that can be provided to patients and/or clients. Less expensive assay methods, such as homebrew microwell plate reverse allele-specific oligonucleotide (ASO) hybridization or reverse dot blots, often do not provide the same quality of information as more expensive methods such as automated sizing–based PCR quantitative peak examination for deletion and/or duplication analysis or Southern blotting for methylation analysis. Each of these, the inexpensive analytic technology and the more labor-intensive, more highly capitalized methods, have their place in the molecular diagnostics laboratory. Many believe that higher throughput will be achieved through the use of equipment that requires greater capitalization and methods that cost slightly more per sample than manual methods, initially. Highly capitalized equipment requires recovery of initial costs via amortization. Microarray proponents see the future in their platform. MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectrometry adherents see their platform as the logical future of high throughput, and proponents of high-throughput sequencing for mutation

detection see their method as the primary way to provide definitive DNA genotypes. Cost will likely dictate which of these technologies endures and which remain attractive alternatives. In addition, the next few years are likely to see increased understanding of the limitations of each of these methods. Method comparisons will demonstrate the capability of these techniques to identify rare, unpredicted sequences in known mutant-containing regions, deletions, insertions, and duplications.

1.3.4.2. *Gene-Test Specific.* All genetic tests are not created equal. Local nucleotide sequence is very important in deciding how to interrogate aberrant structures. It is not even true that all triplet-expansion disease–based tests are equally informative using the same detection technology. Each triplet expansion is different. Each disease's etiologic cut-off is different, and each gray zone differs. In addition, the melting temperatures of triplet repeats differ from one another, as CGG in fragile X syndrome differs from the CAG repeat of Huntington's disease. Single-nucleotide polymorphisms or point mutations are no exception: Each of these is detected in the context of a local nucleotide background. In addition, the etiologic gene may have more than one point mutation that causes disease. Disease severity often differs based upon which nucleotide pair is mutated. In fact, gene mutation at every expressed locus spans a spectrum of severity that ranges from benign polymorphism to null mutation. This range of expressivity and penetrance must be anticipated before devising a gene test. Otherwise, it will be impossible to provide accurate diagnosis to patients and precise information to physicians and researchers. Two examples may help to illustrate this point. First, mitochondrial mutation giving rise to Leigh's disease starts out as a mixed population of mitochondria, some of which contain one of several Leigh's disease mutations while others contain wild-type mitochondrial DNA. This scenario, which is formally described as heteroplasmy, can evolve over a number of years into a situation where the mutant genome predominates. In devising gene tests for mitochondrial diseases, the appearance of heteroplasmy must be anticipated. Efforts to detect populations of molecules in relatively low abundance must be devised. Another example is provided by the case of the adenomatous polyposis coli (APC) gene mutation, which can lead to colon cancer. In this tumor suppressor gene locus, one nonfunctional allele predisposes such a bearer to colorectal cancer. Yet no simple test for a nonfunctional allele exists. As a consequence, a series of screening tests, such as the protein truncation test (PTT), conformation-specific gel electrophoresis (CSGE), or some similar broad search for mutations at this locus, is necessary before deciding on a likely candidate region for the mutation. All of this is expensive and, in large part, initially uninformative. Some have suggested sequencing as a first step; indeed, one commercial laboratory has recently taken early steps to provide this expensive service for family members at risk for hereditary nonpolyposis colon cancer (HNPCC).

1.4. Regulation

Recent published comments by observers (C5, H3, H4, R5) have suggested that insufficient QA/QC and enforcement of high quality standards is becoming a problem in genetic testing laboratories. In addition, human subjects research has come under increasing scrutiny as a few controversial incidents have been publicized. As a result, there are increasing pressures at the state and federal level to regulate U.S. laboratories. In light of this increased pressure, laboratories have begun extensive self-inspection programs, appointed compliance officers, and begun to adhere to ISO 9001 requirements. These voluntary efforts on the part of the laboratories are aimed at heading off increased regulation from the states, the Food and Drug Administration (FDA) and other federal agencies. For clinical trials and other pharmacogenomic research, Good Clinical Practices guidelines mandate rules similar to those that must be observed in a clinical laboratory. Genetic testing for both research and clinical genetic purposes requires adherence to stringent guidelines for sample accessioning, processing, handling, preservation, testing QA/QC, result formats, permissions, and disposition.

1.4.1. *Current Regulation*

Currently, clinical laboratories must be licensed by states to provide results to physicians within them. Research facilities are subject to the regulation of those who fund them *and,* if engaged in pharmaceutical research, they are subject to the rules of the U.S. FDA, an agency of the Department of Health and Human Services. Because of the Clinical Laboratory Improvement Act of 1988 (CLIA 88), which did not take effect until 1991, all clinical laboratories must employ individuals meeting certain personnel qualifications and must be able to assure the Health Care Finance Administration (HCFA), the Department of Health and Human Services (HHS) agency charged with enforcing the CLIA rules, of a proficiency testing program and of competence testing. These requirements become more stringent each year, as a great many of them are left up to FDA and HCFA laboratory inspectors, who have individual philosophies concerning their enforcement.

1.4.1.1. *CLIA.* The Clinical Laboratory Improvement Acts of 1967 and 1988 have guided the clinical laboratory community since they were first proposed. Several minor scandals, including the licensure of a dog as a clinical laboratory technician in the 1960s, gave rise to a journalistic crusade that ended in congressional action in late 1966. At that time, only M.D. pathologists owned clinical laboratories; moreover, their professional societies had passed rules barring others from owning a clinical laboratory. The U.S. Supreme Court ruled against this practice in the early 1960s, whereupon the American College of Pathologists (ACP) and the U.S. government signed a consent decree forever prohibiting the ACP from preventing citizens other than M.D. pathologists from participating in clinical laboratory practice.

From the time CLIA 88 was passed, it was controversial. Although several levels of laboratory workers, who had earned their qualifications through many years at the laboratory bench, were grandfathered into their positions, newly minted graduates would, after 1991, have to meet stringent educational requirements in order to participate in laboratory supervision or management. Often, these requirements could be met only after years of schooling at salaries that were not sufficiently rewarding to compensate. But, to this day, CLIA 88 remains the law of the land. In New York and California, requirements are even more stringent, as those states established qualifications programs that exceeded the standards of CLIA. Consider, for example, the case of molecular genetics. Even though new certifications have been available for the last few years, they still have no legal standing under CLIA, or under New York State and California law—that is, with the exception of the American Board of Medical Genetics (ABMG) or the American Board of Pathology (ABP) guided examinations in molecular genetics and molecular pathology, which are only now being offered, and these at the Ph.D. level and above.

In addition to setting qualifications for laboratory directors, CLIA 88 also set standards for laboratory performance and mandated that certain QA/QC standards would have to be met to allow laboratories to provide "reportable results."

Perhaps because congress was being so zealous in its rulemaking for the clinical laboratory, that body made an enforcement decision whose passage continues to evoke mixed reactions within the laboratory community. Congress decided that an already overburdened FDA would not be the best agency to enforce the strict requirements of CLIA. Instead, the federal legislature set up a separate body, within the office that administers Medicare payments, the Health Care Finance Administration, in order to police the provisions of CLIA within clinical laboratories. Unfortunately, that office had little expertise, only a bit of inclination, and none of the machinery to enforce CLIA 88 after it was passed; for this reason, congress provided three additional years, until 1991, to enforce the provisions of the act.*

1.4.1.1.1. *CLIAC.* As part of ongoing surveillance of the laboratory industry, the HCFA uncovered a number of billing scandals among commercial clinical laboratories and several universities in the late 1990s. Consequently, Secretary Shalala formed a committee to look into ways in which CLIA 88 might need modification so that HCFA could continue its mission to protect medical laboratory information consumers. This committee, the Clinical Laboratory Improvement Advisory Committee (CLIAC), is sponsored by the Surgeon General and is headquartered at the Centers for Disease Control and Prevention in Atlanta. CLIAC has met several times in Atlanta and elsewhere in the past few years, and come to the conclusion that the primary area in the clinical laboratory requiring additional regulatory attention

* On June 14, 2001, the Secretary of the Department of Health and Human Services announced a name change for HCFA to the Centers for Medicare and Medicaid Services. CLIA enforcement is to continue through the Center for Medicaid and State Operations.

is that of genetic testing. As a consequence, a set of proposed regulations promulgating increased regulation of this class of tests has been published in the Federal Register for public comment.

1.4.1.2. *FDA.* The motive force behind the FDA's involvement in genetic testing dose not arise from the same sources—nor does it prompt the same stridency—as those behind CLIA enforcement. The FDA comes to genetic test regulations through several corridors. First among these is its mission to protect the blood supply. In the past few years molecular virology testing has begun to play an important role in this area. In addition, the FDA has a mission to oversee all pharmaceutical research that bears upon human subjects. This portion of the FDA seeks to understand the genetic research that is being done in the discovery process, as well as the results of clinical trials in genetic terms. This latter mission has given new impetus to the FDA's role in the regulation of genetic testing. In addition, several academics and journalists who cover genetic testing have begun to espouse the belief that regulation in this area is inadequate. As a consequence, Secretary Shalala appointed a special committee to explore this issue. This committee, the Secretary's Advisory Committee on Genetic Testing (SACGT), concluded that increased genetic testing regulation is a necessity. As a consequence, CDRH is soon to be granted enforcement authority over this area.

1.4.1.2.1. *CRDH-CBER MUA.* Interagency agreements within the executive branch are not generally discussed in public. However, as part of continuing public testimony in favor of approval of molecular diagnostic devices, it has emerged that there is an agreement between the Center for Blood Evaluation Research (CBER) and the Center for Radiation, Devices and Health (CDRH), to permit CBER regulatory authority over a variety of viruses that might affect blood transfusions, such as HIV, HCV, and HBV. As part of this agreement, CBER has ceded to CDRH regulatory authority over molecular genetic tests that do not have implications for the safety of the blood supply. It is therefore likely that the division of the FDA that will be mandated authority to enforce regulation promulgated by the SACGT is CDRH.

1.4.1.2.2. *SACGT.* The SACGT was convened after a controversial report by a blue ribbon panel on genetic testing regulation was issued during 1996. In this report, no consensus arose, both minority and majority reports were drafted, though only the minority report was issued as the official recommendation. Many members resigned during the process. A subsequent editorial by N. Holtzman in *Science* suggested that there was insufficient federal regulation in this area. The journalist and social critic, Jeremy Rifkin, directly or indirectly spurred executive action, as he had written a good deal on the subject and provided an important initial impetus for more careful study of public policy. In addition, civil court litigation continues in a case involving a woman who underwent a prophylactic mastectomy after being (mis)informed of her BRCA1 status. Details concerning the laboratory mix-up that led to this unfortunate result are not currently open to the public. Regulations proposed by the SACGT, in conjunction with their consultation and coordination

with CLIAC, will begin a process whereby laboratories will have to keep track of informed consents, as well as provide evidence that they are correctly implementing and interpreting genetic tests. Furthermore, SACGT's proposed regulations are aimed at ensuring the maintenance of strict confidentiality both for genetic test results and for patient samples. Details of the philosophy of regulation proposed by SACGT are illustrated in Fig. 1.

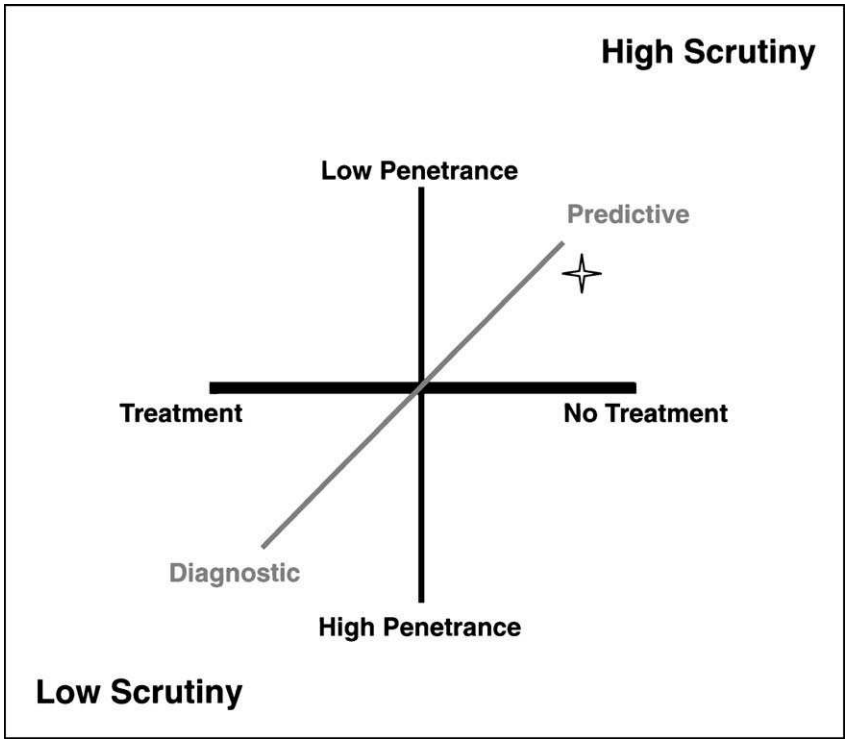

FIG. 1. Regulation levels proposed by the Secretary's Advisory Committee on Genetic Testing. This schematic illustrates one example of an approach to classifying tests using three dimensions. The committee believes that predictive tests require more scrutiny than do diagnostic tests and that tests for weakly penetrant mutations require more assessment than do those for highly penetrant genes. In the committee's view, tests for conditions for which no interventions are available require more review than tests for conditions for which interventions exist. Under these circumstances, a high-scrutiny test would be one that is predictive, detects a mutation that is weakly penetrant, and for which no proven intervention is available. In addition, complex tests, such as linkage analysis for which interpretation is difficult, will require more oversight than a test measuring the presence or absence of a defined mutation. In the suggested scheme, more relaxed rules will guide tests performed solely to detect somatic mutations or to detect genotypic information used exclusively to direct clinical management of symptomatic patients. This figure depicts a predictive test for susceptibility to one hypothetical cancer with low penetrance and no treatment; it therefore falls into a high-scrutiny classification.

1.4.1.3. *State Regulation.* As a result of consumer activism in the 1960s, several states and municipalities, including New York, California, and New York City, sought to regulate laboratory medicine during the 1970s. These localities passed clinical laboratory regulations that were more stringent than those congress endorsed in CLIA 66 or CLIA 88. New York City gave up many of its rights to clinical laboratory licensure in the 1990s, following a dispute with the New York State Department of Health. California took a separate route toward clinical laboratory regulation, insisting that every sample tested in that state be subject to its regulatory codes. Meanwhile, New York took the unusual position that any clinical laboratory test ordered by a New York–licensed physician practicing in that state, whether upon a New York citizen or not, must comply with its codes. Thus, if a sample from New York is sent to a foreign country for analysis, the foreign laboratory must be certified by the State of New York. Laboratories pay licensure and inspection fees to New York for this service. Although the rules mandated by CLIAC and SACGT have not yet become final, New York State has mandated rules on molecular genetic testing for many years. Because the state is so populous, no large reference laboratory can ignore its mandates. Effectively, this means that each laboratory director who accepts New York State samples for genetic testing must hold a Certificate of Qualification in Genetic Testing issued by the State of New York and is subject to prior restraint in the initiation of new genetic tests for New York State patients and physicians. In practice, test methodology and test reporting undergo administrative review before they are advertised to New York State physicians. Other states have been mandating that clinical laboratories operating within their states provide "informed consent" for genetic testing. Florida also requires licensure of laboratories conducting clinical tests on its physicians' samples. However no Florida agent physically travels to inspect these laboratories; nor does the state fine those laboratories or decline to send Medicaid samples when it is unable to certify a receiving laboratory as licensed in Florida.

1.4.1.4. *Non-Governmental Organizations.* Compliance with laboratory regulation is largely voluntary through participation in surveys and challenges that are conducted by the College of American Pathologists (CAP), specifically their Accreditation and Quality Survey program. Participation in this program, which provides a laboratory with objectively scored samples, is a *de facto* requirement for adherence to CLIA standards. Although there are a few other ways to meet HCFA requirements, such as laboratory-to-laboratory sample exchanges and a few other QA programs, the CAP program is considered the surest route to maintaining high standards and licensure. The American College of Medical Genetics (ACMG), a newer organization, has begun cosponsoring many of the challenges issued in molecular genetics within the CAP survey program. ASHG, because it made clinical genetic policy for many years before ABMG or ACMG or the American Board of Genetic Counseling existed, still

participates to some extent in formulating policy or ELSI (Ethical Legal Social Issues questions) on the CAP surveys. Each of these organizations contributes immensely to assuring the public that genetics results are not simply a biased interpretation of life circumstance and laboratory procedure in one isolated laboratory, but rather are being issued under a rigorous standard, which is uniformly observed and adhered to in genetics laboratories throughout the United States.

2. Three Components to Genetic Testing

Recent published statements of the SACGT recognize that genetic testing consists of three distinct phases: (1) the pre-analytic phase, in which samples are secured, demographic information is obtained and recorded, and some specimen preparation may be conducted; (2) the analytic phase, in which validated techniques for genotyping analysis are applied to the samples in question; and (3) the post-analytic phase, in which the results of the validated techniques manifest and are interpreted by a qualified person and these results are merged with demographic information contained in a database to provide an interpreted report. In a clinical laboratory, this third phase is often accompanied by genetic counseling for the patient, family, and ordering physician. In a research laboratory, this third phase, the data analysis, is perhaps the most crucial. It is at this stage that an experimental hypothesis concerning the relation of genetics, environment, and phenotype is ultimately tested and proved or disproved.

TABLE 1
SOME PUBLISHED GUIDELINES FOR GENETIC TESTING[a]

Year	Test	Reference
1987	MSAFP for ONTDs and DS	(1)
1993	Prenatal FISH	(2)
1994	Fragile X Testing	(3)
1995	Stored Genetic Materials	(4)
1995	Apo E Testing in Alzheimer's	(5)
1996	Prader-Willi AND Angelman	(6)
1998	Huntington Disease	(7)

[a]The American Society of Human Genetics, the American Board of Medical Genetics, and the American College of Medical Genetics each formulate a consensus of practice guidelines within their organizations. Although these guidelines are circulated to members and become widely known in professional societies, they are often not published. Current practice guidelines can be found at the American College of Medical Genetics Website. http://www.faseb.org/genetics/acmg/pol-menu.htm.

2.1. Pre-analytic Component

In order to provide complete interpreted results to patients, clinicians, and research groups, demographic and family data must be collected along with patient identifiers. In accordance with state regulations and professional association guidelines, informed consent must be obtained from patients for genetic testing. In this electronic age, laboratory information is generally recorded in a password-protected database within a 21CFR compliant information system. For research and CLIA survey purposes, all abnormal sample identifiers must be recorded in a separate file, gene frequencies must be periodically calculated and recorded on each mutation screened, and a hardcopy accession log (together with a hardcopy log of amended reports and abnormal genotypes discovered) must be present in a clinical laboratory that dispenses results to patients and physicians. For a large laboratory, this is a mountain of administration, which practically requires a staff unto itself.

2.1.1. *Informed Consent*

The Nuremburg trials and the controversies over experiments in two mental hospitals, as well as the Tuskegee syphilis experiments, gave rise to the Belmont Report, whose human rights principles have been used around the world to protect the rights of human subjects in medical experiments. In many quarters, genetic testing is still considered "experimental." The results of any medical test carry a level of uncertainty. Good genetic test results are less uncertain, perhaps, than other medical test results; however, because of incomplete penetrance and the variable expressivity present in many hereditary disorders, genetic tests may carry a level of uncertainty almost equal to that of many other medical tests. For these reasons, genetic tests require patients to agree to testing and to acknowledge being informed that results of these tests will not be absolutely certain. All certainty is relative. And genetic certainty is always articulated in the form of probabilities to patients. Unfortunately, ideas about probability differ from person to person; hence a 5% chance of disease may be large for one person under some circumstances, but may be small for another person under different circumstances. Generally, any post-test probability of disease greater than that of the population at large is considered a significant genetic disease burden.

2.1.2. *Demographic Information*

It is a truism that gene frequencies vary by ethnicity. Polymorphisms are not meaningful unless they can be studied in the context of the ethnic group that harbors them. In the case of risk reduction statistics, for example, it is well known that Jewish Ashkenazim are at 1/27 risk of carrying Tay–Sachs disease. But what is often not understood by the naive observer is that their risk can be reduced to 1/1250 through the prudent application of molecular genetic testing. On the other

hand, if the presenting patient is not of Ashkenazi Jewish origin, or if he or she misunderstands or misrepresents his or her ethnicity, a risk reduction may be provided in error. In such cases, there is a significant possibility that, because the "wrong kind" of test has been administered (a biochemical test for the enzyme hexosaminidase A is much more effective in ruling out heterozygosity in non-Ashkenazim than is the DNA test), a grave error may be committed. The presenting patient may, after all, unknowingly be a carrier of a mutant allele, even though he or she believes s/he has been adequately screened for this eventuality. Similarly, β-thalassemia major is a relatively common autosomal recessive disorder in those of Greek and Italian descent. Screening this population using a common test for sickle cell disease (HbS), even while including the rarer HbC and HbE alleles, may not be sufficient to discern heterozygote status for β-thalassemia. This may explain why significant thalassemias are still often discovered at birth in the U.S. It may also help to clarify why several major medical centers have hemoglobinopathy/thalassemia units devoted, in large part, to patient and physician education and to treatment of the many disease sufferers who slip through the cracks of our genetic surveillance system. Ashkenazim are known to be predisposed to a number of genetic diseases not encountered by other groups, including Tay–Sachs disease, Gaucher's disease, Canavan's disease, Fanconi's anemia, Bloom's syndrome, BRCA-1 mutations, characteristic forms of cystic fibrosis, APC-1 mutations, and familial dysautonomia.

2.1.3. *Family History*

Bayesian probabilities are absolutely reliant upon an accurate family history of the disease in question. Family history affects the prior or *a priori* likelihood that a propositus is a carrier for a genetic disease. Because many hereditary disorders are autosomal recessives and manifest rarely in a sibship, any record of a known hereditary disorder can be important in providing an accurate risk reduction. This can be particularly important in cystic fibrosis, where one of the mutations is often known in affected individuals while the other is often private and uncharacterized. Even an affected first cousin can boost the *a priori* probability in a Caucasian non-Jew from 1/241 to 1/8.

2.2. Analytic Phase

After receipt in the laboratory, samples with alphanumeric identifiers, which correspond to patient information in the accessioning database, are serially subjected to analytic tests. In both a clinical and research laboratory, each of these is run according to a written protocol, with appropriate controls (in parallel with and without the mutation or polymorphism, often amplifying a control tube without DNA), and then through an electrophoretic, fluorescent, luminescent, radiologic,

or colorimetric detection procedure. The purpose of the analytic phase is to reduce the impalpable to the observable by amplifying or otherwise visualizing the mutation or polymorphism in question.

2.2.1. *Properly Identified Patient Specimens*

Patient specimens must meet well-defined criteria before they are subjected to analytic procedures. These include acceptance of anti-coagulated blood—*not* serum or plasma for genomic DNA analysis and *not* whole blood for quantitative tests of viral load in plasma. Also rejected are improperly labeled samples, leaking or broken blood tubes, or significantly hemolyzed samples. Hemin, the major erythrocyte porphorin breakdown product, is a potent inhibitor of reverse transcriptase and many thermostable polymerases. Unfortunately, most DNA extraction techniques are insufficient to remove this substance from mishandled or mis-stored samples. Generally, rules for specimen labeling and handling are aimed at avoiding future problems during the analysis phase. They are commonsense suggestions; but given the complexities of sending test samples across the country or across the world to a reference facility to perform specialized molecular diagnostic testing, commonsense rules can be lost in the shuffle.

2.2.2. *Sample Accessioning*

Accessioning is central to genetic testing. Patient samples must be labeled, and the information on them must be entered into a database. And, quite often, a hardcopy record of sample receipt must be maintained. Because of the need for interpreted reports in a clinical molecular genetics laboratory and the rigorous tests of statistical significance required for genotype–phenotype correlations in a research laboratory, databases must be capable of linking demographic information to laboratory-generated data. Truly, molecular research and diagnosis would not be possible without modern computing capabilities.

2.2.3. *Sample Preparation*

Specimen preparation is an often neglected area for comment in the molecular diagnostics laboratory. Although laboratory robots capable of extracting DNA or RNA from relatively large numbers of samples have recently appeared on the market, the task of making aliquots of whole blood samples from Vacutainer tubes remains manual and burdensome. Furthermore, many of the robots fail to adhere to strict CLIA and/or NYS requirements, as they do not provide hardcopy labels for each tube in an extracted array. Orientation of a 96-well tray becomes critical in such circumstances, and opportunity for sample mix-up abounds. Many laboratories have concluded that specimen preparation robots are not yet ready for prime-time clinical diagnostic applications, while many research laboratories contend that the affordability factor prevents them from applying laboratory robots

to this need. However, the outlook for robotic preparation of DNA and RNA remains bright, as many of the problems mentioned here are being addressed by manufacturers, and are susceptible to resolution in the near future.

2.2.4. *Validated Mutation Detection Techniques*

Mutation detection techniques may not be applied to important clinical specimens or valuable research samples without thorough validation. Purified samples of known genotype are used as controls during the test development process. Once this process is complete and the assay is working and documented, control aliquots are prepared so that some can be run with every analytic batch. Reportable genotyping tests are not a matter of guesswork or happenstance in either the clinical diagnostics lab or the research environment. The National Committee for Clinical Laboratory Standards (NCCLS) publishes a fairly extensive, detailed document that provides the basis for validating clinical laboratory tests. The NCCLS has also produced a number of publications that outline the requirements of a well-documented molecular diagnostics method. One requirement is cross-validation of samples accurately genotyped in another laboratory. Although many research laboratories forgo this level of rigor in developing their assay protocols, genotyping laboratories in a research setting often resort to direct DNA sequencing in order to confirm or refute a particular genotype. Direct sequencing, because it remains arduous and expensive, is not often used as a primary method for genotyping.

2.3. Post-analysis

The results of a molecular genetic test are often not obvious. Experience and a trained eye may be necessary to interpret molecular diagnostic results. Even when a test is explained to a well-trained observer, a wild-type indication at a given locus may have a different meaning for a normal patient than for patient at background risk. For example, a negative cystic fibrosis (CF) carrier screen conducted on a presenting Caucasian Jewish Ashkenazi female patient with no family history of CF still carries a risk of 1/801 that she is an unidentified CF carrier subsequent to testing. This is because most tests cover only about 97% of the mutations found in her ethnic group. There are more than 900 CF mutations currently listed in a database in Toronto, but most CF mutations are “private” and are not routinely subjected to testing. Thus, genetic counseling and the issuance of an interpreted laboratory result aims to take account of all the information available to provide a detailed risk assessment and an interpreted report for each presenting patient.

2.3.1. *A Trained, Qualified Interpretor*

Because of the need to provide statistical expertise and to interpret complex molecular data, molecular diagnosticians cannot approach reporting their results

casually. As a consequence, the genetics profession has instituted postgraduate fellowship and residency programs that train graduates in the interpretation of diagnostic genetic test results and the art of counseling physicians on their meaning. Continual commitment to learning new technology, receptivity to new statistical approaches, and willingness to engage in discussions of ethical, legal, social issues, and diagnostic dilemmas are the hallmarks of a good molecular diagnostician. Each of these is a requirement for completing training in medical genetics. At the end of this process, a difficult board examination is administered, with certification provided upon passage. A geneticist who has been through this kind of thorough training is unlikely to encounter pitfalls in the interpretation of diagnostic genetic test data.

In addition to "book learning," these programs often provide rotation through diagnostic laboratories, to compile "cases"—diagnostic tests with which the trainee is intimately involved, perhaps carrying out parts of the procedure himself/herself, or helping to counsel the patient or physician on some of the results. Providing a molecular diagnosis is an experience that carries with it a great deal of responsibility, and the programs that train experts in this field take their role very seriously.

2.3.2. *Quality Control and Quality Assurance Checks*

One of the primary responsibilities of the director of a molecular genetics testing center is to make certain that appropriate controls are devised for and run with each diagnostic assay. An elaborate system of checks exists in most genotyping laboratories in order to make certain that each released result meets a written QC standard. In the absence of QA/QC, patients, physicians, and researchers cannot rely upon the genotypes being produced.

2.3.3. *Bayesian Statistics and Results*

Bayesian statistics are at the heart of providing an interpreted clinical report and genetic counseling to patients. It is these statistics that provide two different results for a Caucasian non-Jewish woman without a family history and a negative CF test and an Ashkenazi Jewish woman with the same family history and the same results on the same test, as illustrated above. In the case of the Caucasian non-Jew, the no-family-history result for a CF test that covers 90% of the mutations in her ethnic group, the woman is left with a 1/241 probability of being an unidentified CF carrier. However, the Caucasian Jewish woman without a family history is left with a 1/801 probability of being a carrier of the disease because the test covers 97% of the alleles in her ethnic group.

2.3.4. *Confirmation and Exclusion of the Diagnosis*

Excluding a genetic diagnosis can be very complex. Toddlers who exhibit deteriorating motor capabilities often present in genetics clinics subsequent to neurologic

examination for spinal muscular atrophy (SMA). This is often a difficult call. This autosomal recessive disease has several forms, from lethal to less severe. The etiologic lesion is a gene deletion at chromosome 5q31 in a gene called the survival motor neuron (SMN). Unfortunately, SMN is in a very repetitive area within the genome, which is often subject to small deletions. Sadder still, a number of genes that can delete in this region, in close proximity to SMN, do not give rise to spinal muscular atrophy. In fact, in a few cases, individuals with deletions at the suspect locus do not have the disease! There are also diseases that are similar to authentic SMN and can be confused with it. Thus, it is difficult to make a diagnosis of this disease without confirmatory electromyographic data. Needless to say, parents who have had one SMA child are eager to determine the affection status of any new conceptus. When a family has already had one SMA child with a proven deletion, prenatal testing is always offered. But when a family has a disabled child with uncertain diagnosis but suspected SMA, prenatal diagnosis can be difficult. This is especially true after the death of an affected child when no material is available for parallel study in the molecular diagnostics laboratory. This poses a counseling dilemma for professionals: Should a prenatal diagnostic process be begun without certainty that the outcome will be informative? Hopefully, our knowledge of this disease and locus will improve as the genome map of this region approaches completion.

2.3.5. *Communicating Results*

Result triage in a large molecular diagnostics laboratory is important. It matters more, for example, that a fetus is homozygous at the HbS locus than a presenting pregnant patient is discovered to be N370S heterozygous at the Gaucher disease locus. Both patients are deserving of immediate information from their physician, and the standard operating procedures of every clinical genetics laboratory insist that each of these abnormal results be reported as rapidly as possible. However, a pregnancy termination decision has greater urgency than the decision to test a fetus or a father-to-be for a disease that manifests mainly during adulthood (Gaucher).

Communicating genetic testing results requires compassion and some level of empathy for the bearer. There are still great hopes that every hereditary ailment will yield to a therapeutic solution like that offered by insulin or PKU. However, the genome project has informed us that a host of more complex hereditary diseases are not necessarily consequences of one point mutation. Thus it may be difficult to ameliorate the cause of the phenotype simply. Certainly, the leading causes of mortality in America—cancer and cardiovascular disease—are not likely to yield to simple interventions. For this reason, communication of genetic test results must be done nondirectively, in an informed way, with the facts and attendant certainties ready at hand. Otherwise, the physician or patient may be unable to make a decision based upon those results. Clinical molecular diagnostics must hold to a higher quality standard than research work because life-altering decisions hang in the balance. A recent review on high-throughput genotyping has suggested

that an analytic error rate of 0.5% was an adequate specification based upon the techniques being discussed (H1). By several orders of magnitude, this would be an unacceptably high error rate in a clinical molecular diagnostics laboratory today.

3. Pre-analytic Component

Before a sample is accepted into a molecular genotyping laboratory, a number of facts must be known about it: Has the patient or study participant provided informed consent? Who has copies of these documents? If no consent was provided, are the samples anonymous and archived? What tissue will be provided to the laboratory? Is there a history of genetic disease? Is ethnicity provided? Will the laboratory know anything about the patients being tested? Without answers to most or all of these questions, laboratories should not be accepting samples for genotyping: If they do, they might well be violating customs, guidelines, or rules, thereby making it impossible either to publish resulting data or continue to provide the results to physicians and patients.

3.1. Specimen Type

To properly accession and purify nucleic acids for analysis, the receiving laboratory must know the sample type. Both heparin and urine have been reported to inhibit PCR, to the detriment of blood samples containing the former and nearly all samples of the latter. In recent years, extraction technology and amplification chemistry have improved so that each sample type is susceptible to analysis. Proper identification of sample type provides the receiving laboratory with an opportunity to apply appropriate techniques to its extraction and analysis.

3.1.1. *Guthrie Card (Dried Blood Spot)*

Metabolic screening programs collect this sample type from a newborn heelstick, generally within 72 hours of birth. Dried blood spots are amenable to several types of DNA extraction. Through this mechanism, follow-up to a positive sickle cell screen or immunoreactive trypsin assay can be provided by molecular testing for hemoglobinopathies and β-thalassemia or by CFTR mutation testing.

3.1.2. *Buccal Swab*

Paternity testing, testing of institutionalized individuals, and population-level molecular screening are thought to lend themselves to testing via buccal wash or buccal swab technologies. A model program for this sample type was developed by B. Handelin and her collaborators at Integrated Genetics in Framingham, Massachusetts during the late 1980s. And in the 1990s, oral exudates were independently, perfected for Ab-based HIV testing by OraSure, Inc. Neither of

these has yielded the expected bonanza of samples in spite of their ease of collection. Molecular diagnosis generally requires careful professional supervision for administering collection and resulting. Therefore, it is not the mechanism of specimen collection, but the administration of information collection and result delivery that is a medical professional–requiring process. Medical professionals are more comfortable (and DNA yields for analysis are generally higher) with peripheral blood collection than with buccal wash or oral exudate collection. Thus venipuncture remains the preferred sample type for clinical genetic laboratory analysis.

3.1.3. *Peripheral Blood*

This has become the standard sample for analysis in the clinical molecular genetics laboratory, and it is fast becoming the sample of choice in pharmacogenetic studies. Cells derived from these samples can be archived or immortalized (although this is not done in clinical genetics laboratories); moreover, large amounts of DNA, sufficient for Southern blot analysis and many hundreds or even thousands of genotypes, can be secured. Collection specifications still include a prohibition on heparinized blood (largely for historical reasons) as well as clotted or hemolyzed blood samples (owing to their well-documented low DNA yields). An exception is viral load testing, in which plasma, rather than cells, is the subject of nucleic acid purification and testing. In HIV, HCV, hepatitis B virus (HBV), and human cytomegalovirus (HCMV) viral load testing, plasma alone is often subjected to extraction and analysis. In the case of HCMV and human papilloma virus (HPV), cells are often desirable test tissue.

3.1.4. *Chorionic Villus Sampling*

This sample type requires dissection from surrounding maternal tissue and can be contaminated with confounding maternal cells on occasion. As a consequence, subsequent to extraction, chorionic villus samples often require testing to assess the degree of maternal contamination before allowing them to provide a basis for prenatal diagnosis. Polymorphism analysis on a highly variable tandem repeat locus, heterozygous in the maternal tissue, can provide a basis for this assessment. Thus, a reference molecular genetics laboratory must have many accessory techniques at its disposal in order to properly interpret and report genetic results.

3.1.5. *Cultured Amniocytes*

Amniocentesis conducted between 15 and 21 weeks gestation yields cells capable of growth in culture. These can be easily used to extract DNA (or RNA) and subjected to analysis. This tissue is less subject to concern with maternal cell contamination than CVS, but it can still present diagnostic problems, such as confined placental mosaicism. The level of such problems can sometimes be assessed using

informative cytogenetic markers; but occasionally, assessment of mosaicism itself requires the use of molecular tools, such as the tandem repeat markers discussed above.

3.1.6. *Products of Conception*

Products of conception are generally subjected to testing as one means to establish reasons for infertility. Molecular diagnosis of this material generally takes place in conjunction with a detailed cytogenetic characterization. Sex chromosome abnormalities may be carefully examined using molecular diagnostic techniques and a number of markers in the vicinity of SRY, pseudoautosomal regions of the X chromosome, and unique X chromosome markers (such as those in the vicinity of FRAXA, Xq27.3). Occasionally, this procedure can provide an explanation for infertility; but when it does, this type of infertility is often irremediable without the help of donor eggs or sperm.

3.1.7. *Anatomic Pathology Slides or Blocks*

This tissue type is most frequently used for molecular studies of surgically removed or post-mortem tissue, in order to help establish diagnosis. Currently, this tissue type finds broader application in research settings than in clinical settings. In future years, however, it can reasonably be expected to find increasing use in clinical settings in order to help establish staging and accuracy of diagnosis, particularly in solid tumors. Solid tumors have not been amenable to cytogenetic techniques until recent years. But with the advent of molecular cytogenetics, through fluorescent *in Situ* hybridization (FISH) and comparative genomic hybridization (CGH), it has become possible to discern chromosomal breakpoints, fusion genes, and tumor suppressor loci in solid tumors at the frequency previously known in hematologic malignancy. There have been many breakthroughs in cancer treatment since the discovery of the Philadelphia chromosome in 1967, but that discovery largely gave rise to were vast improvements in cancer diagnosis rather than profound changes in the way we treat cancer.* Nevertheless, if cancer diagnosis can be more accurately ascertained, the conviction remains that many cancers will yield to improved treatments. One profound example is the APML/retinoic acid receptor rearrangement discovery. Acute promyelocytic leukemia (APL) is a homogeneous subgroup of acute myelogenous leukemias characterized by phenotypic and genetic markers. APL is associated with a reciprocal translocation t(15; 17), which has been shown to disrupt the α-retinoic acid receptor gene. As a result, chimeric PML/RAR alpha fusion mRNAs are expressed in rearranged cells. APL is the first example of a human malignancy that responds to differentiation therapy. Complete remission can be achieved in up to 90% of manifesting patients by using a differentiation inducer—all-trans retinoic acid (ATRA). Mechanistically, the PML/RAR alpha

* On May 10, 2001, the FDA approved a Novartis drug, Gleevec, a tyrosine kinase inhibitor that specifically targets the BCR/ABL fusion protein.

antagonizes wild-type PML and RXR and could block the differentiation pathways mediated by these two regulators. A variant translocation t(11; 17) has also been discovered in a subset of APL which fuses RAR alpha to a new gene, PLZF on chromosome 11q23. Both PML/RAR alpha and PLZF/RAR alpha display a "dominant negative" effect on the wild-type RAR/RXR. However, t(11; 17) APL patients differ from t(15; 17) APL patients in that they respond poorly to ATRA. Morphologically defined APL cases that do not have PML/RAR alpha generally show no response to ATRA. Recently, it has been shown that PML/RAR alpha can be modulated directly by ATRA. All these data support the idea that PML/RAR alpha is a specific target of ATRA that can overcome the differentiation block imposed by PML/RAR alpha. The ATRA treatment of APL thus further reinforces the concept of differentiation therapy. The presence of these fusion transcripts in APL patients strongly supports the hypothesis that the t(15; 17), and hence PML/RAR alpha, plays a crucial role in the leukemogenesis of this disease.

3.1.8. *Frozen Tissue Samples*

Many research studies are being conducted using frozen tissue, and several clinical laboratories are exploring the acceptance of clinical samples for genetic testing using this specimen type. In accepting samples for RNA testing, whether for quantitation of viral load, for molecular diagnosis of fusion gene transcription by RT-PCR, or for assessement of Prader–Willi syndrome by assessing the SNRPN transcription product assay, a strong case can be made that frozen samples are appropriate.

3.2. Reason for Testing

Genetic test results are complex and must be tailored to their recipients. Diagnostic tests should confirm or exclude a diagnosis or provide an explanation for an equivocal result. If a patient is symptomatic for a condition, the laboratory ought be informed of this fact, so that it may be incorporate this observation into the laboratory report. If a patient is being referred for carrier testing, ethnicity and family history information is generally required to provide a Bayesian risk reduction in the case of a negative test result. The import of positive carrier-study test results to patients or physicians is different from that of positive diagnostic test results. Prenatal test results are generally urgent. These must not be handled in the diagnostic laboratory in the same way that routine diagnostic or carrier test results are handled. In the end, it is not useful for referring physicians to receive risk reduction reports on symptomatic patients referred to confirm a suspected diagnosis, nor is useful to exclude a diagnosis when carrier studies for a pregnant female were the basis for a referral. Physicians should not receive a Bayesian risk reduction probability when a definitive diagnosis on a prenatal sample has been ordered. Prenatal testing is generally ordered as an aid to pregnancy termination decisions. Clarity concerning the disease status of the fetus is required for these

difficult decisions. The at-risk family and the ordering physician deserve no less. In short, clinical molecular genetics must be reported in an orderly fashion.

3.2.1. *Newborn Screening and Special Rules*

Newborn screening attaches special rules to a research or clinical study. Initially, it was thought that public health concerns required that screening be conducted by government mandate, and that permitting people to "opt out" voluntarily would prevent the early detection and amelioration of genetic disorders. As a result, certain mandatory rules for genetic testing were adopted by state legislatures. In most states, any birth is subject to certain mandatory requirements for genetic screening for some conditions. For example, a phenylketonuria screen is required in order to spare children from mental retardation through dietary restriction. Other common screens for newborn diseases include those for PKU, hypothyroidism, and maple syrup urine disease. Many other states have approved an expanded screening program, which allows other metabolites (and some enzymes) to be measured in newborns, in the hope of detecting abnormalities in time for early intervention. Less common genetic diseases—those less easily ameliorated than other, more problematic hereditary newborn illness, such as propionic acidemia and homocystinuria—have traditionally been subject to this extended newborn screening.

3.2.2. *Carrier Test*

Carrier testing is conducted for family planning purposes. Bayesian risk reduction or identification of carrier status in prospective parents is the aim of this type of testing. Often, prospective mothers who have not previously sought the attention of an OB/GYN prior to pregnancy are subjected to this type of genetic testing. If their carrier tests are "screen-positive," rapid efforts are made to identify and test the prospective father; then sometimes, upon amniocentesis, efforts are made to provide a prenatal diagnosis. Without advance knowledge that a test is to be conducted for carrier status, it is not possible to offer clear counseling and patient advocacy.

3.2.2.1. *With Family History.* A family history of hereditary disease changes the course of both testing technology and counseling. Testing methods must take into consideration the mutations present or undiscovered in the presenting patient's family. If a biochemically detected aberrant hexosaminidase A activity or a clinically identified case of cystic fibrosis presents in the patient's family, the presenting patient cannot be treated by the laboratory as if no clinical history is presenting at all. Some Hex A deficiency assays in Ashkenazi Jews require molecular confirmation, as there are pseudodeficiency alleles that can biochemically emulate disease enzymology, yet be benign. On the other hand, patients with a non-Ashkenazi and non–French Canadian heritage cannot easily be confirmed as carriers in a molecular diagnostics laboratory (A2, P1, P5, T2). In a similar exercise of the dependence of molecular technology upon the specific mutation being examined, persons from CF families with identified rare mutations must be tested in laboratories

that can discern those rare mutations. This is common sense; however, there is not yet common agreement on which CF mutations will generally be tested for in molecular diagnostic laboratories.* Thus, while many laboratories check for only a dozen or so mutations, one checks for as many as 86 different mutations. Because of the low population frequencies of many of these mutations, smaller mutation spectrum coverage does not always result in a diminished quality of care to patients presenting at laboratories that check fewer mutations; on occasion, however, quality of care can be negatively affected.

3.2.2.2. *Populations with High Genetic Disease Prevalence.* There are several other categories of demographic information that should be specified in order for a molecular diagnostics laboratory to provide a complete report and inform the patient properly. One such category is the patient's membership in an ethnic group with a high prevalence of hereditary disease. This may be straightforward, as in the case of African Americans presenting for sickle cell testing, or more subtle. There are, for example, more Armenians in Los Angeles than anywhere else except Armenia. As a result, counseling for hereditary disease risks with a high prevalence among Armenians (e.g., familial Mediterranean fever) is indicated among Los Angeles OB/GYNs caring for Armenian patients. Most primary care physicians do not possess detailed knowledge of hereditary disease prevalence among differing ethnic groups. As a consequence, counselors and the laboratory are often called upon to provide advice on appropriate testing. Such advice cannot be provided in the absence of knowledge of presenting patient ethnicity.

3.2.3. *Diagnosis of a Child*

There are some genetic markers that are inappropriately tested in minors. Among these are markers for Huntington's disease and detailed genotyping for apolipoprotein E (ApoE). The latter is performed as a confirmatory test for Alzheimer's disease. The former can provide a presymptomatic indication of the likelihood that the bearer will go on to develop a severe, degenerative neurologic disorder with neuropsychiatric concomitants. Without knowledge of patient age or date of birth, a laboratory cannot determine whether or not it is testing a minor for these illnesses, thereby violating genetics community standards and guidelines. As a consequence, the surest route to appropriate care is for the laboratory to be provided date-of-birth information as it requests it.

3.2.4. *Diagnosis of a Symptomatic Adult*

Reports issued from the laboratory after testing samples from adults suspected of having hereditary disease should be different from those on samples from normal adults presenting for genetic screening. In the former case, a symptomatic patient

*Cystic fibrosis population screening guidelines containing an agreed upon panel of 25 mutations were published in March/April 2001. See Grody *et al.,* Laboratory standards and guidelines for population based cystic fibrosis carrier screening. *Genetics in Medicine* **3,** 149–154 (2001).

with manifest abnormalities usually presents for diagnostic confirmation or rule-out. The goal of the laboratory is to be informative to the patient and his or her physician. Bayesian statistics on carrier frequency would not be meaningful parts of result reporting on this kind of patient. On the other hand, pregnant females presenting for the first time to their attending OB/GYN are often interested in knowing their hereditary disease carrier status. The report issued to a female suspected of carrying a CF gene mutation is very different from a report issued to a presenting sterile male also suspected of carrying a double dose of CF gene mutations. These reports, one of which is aimed at diminishing carrier risk and the other at rule-out of CBAVD (congenital bilateral absence of the vas deferens), read differently and mean completely different things to the attending physician. A diagnosis must be provided in context, and it is appropriate for the laboratory to reiterate that context as it provides its determination.

3.2.5. *Prenatal Diagnosis*

As a practical matter, most cytogenetics laboratories are expert at cell culture, while most molecular laboratories are not as skilled in this art. The livelihood of cytogenetics laboratories derives from its cell culture and dissection skills, while these are not the forte of molecular technology facilities. As a consequence, most prenatal diagnosis involves mutual consideration and cooperation between two different laboratories: one committed to providing a karyotype and the other committed to a definitive prenatal molecular genetic diagnosis. Prenatal diagnoses are specially marked and handled in molecular genetics laboratories. They are often subjected to duplicate parallel analysis, and special care is taken to provide rapid, personal reporting of these results to the ordering physician. To ensure such special handling, most laboratories have procedures that assure special marking and handling of these precious samples.

3.2.6. *Diagnosis Subsequent to Fetal Demise*

Applications of molecular diagnosis to fetal loss studies originated in cytogenetic laboratories for which molecular diagnostics was initially an adjunct. Most early fetal loss can be attributed to chromosome abnormality. Later in pregnancy, a smaller proportion may be due to cytogenetic abnormality but may also be due to Mendelian disorders. Because of this, family history is taken, and suspected hereditary causes of fetal loss are often ruled in or out on the basis of molecular diagnosis. Many molecular diagnostic laboratories have developed detailed protocols for specimen handling and extraction of purified nucleic acids from fetal tissue. These procedures have also found application in forensic cases—those in which newborns have been abandoned or paternity is in question. In these cases, molecular diagnostic laboratories must be informed of the circumstances of acquisition of the tissue they are working with, so that it may be properly handled, tested, and reported.

3.3. Ethnicity Is a Crucial Parameter

Especially in the relatively common Jewish Ashkenzi diseases (Tay–Sachs, Gaucher, Canavan, Fanconi's anemia), the specification of ethnicity is a crucial parameter for molecular diagnostics laboratories. Tay–Sachs has high frequency in French Canadians, as well as in Ashkenazi Jews, but the mutations are different. If the laboratory does not know the proper ethnicity, it cannot determine the proper test. Gaucher disease testing is subject to similar constraints. Only when the laboratory is fully informed can it be expected to perform the correct test. Ethnicity of partners is also important: A pregnant Ashkenazi Jewish female known to be a Tay–Sachs carrier partnered with a French Canadian or a Louisiana Cajun male at high risk for Tay–Sachs offspring should be offered prenatal diagnosis via hexosaminidase A. Genetic laboratories performing molecular testing on these kinds of patients should flag such a couple as high-risk, and offer them appropriate *biochemical* genetic testing. If no ethnicity or familial relationship is specified for such incoming samples, there is a high likelihood of uninformative, incorrect, or seriously deficient result reports.

3.3.1. *Proper Test Choice*

Whether to perform hexosaminidase A testing or a specific DNA diagnostic for Tay–Sachs in a presenting couple is one example of a decision concerning proper test choice. Another of these is sickle cell testing. Many individuals with sickle cell trait and rarer forms of hemoglobinopathy are not properly informed of their genotype, and hence cannot themselves communicate accurate information to the laboratory. Who can blame a person for not knowing the exact scientific name of his or her particular β-thalassemia allele? Yet lack of specific knowledge can have a great effect on which diagnostic test is chosen.

3.3.2. *Appropriate Post-analytic Test Interpretation*

Even negative tests have Bayesian consequences. For a Caucasian non-Jewish individual testing negative for cystic fibrosis, there is a small but certain probability that she possesses a CF allele not tested for. Her revised carrier risk, post-test, may be 1/241 when her prior risk was 1/25, but she still bears a significant risk of being a CF allele carrier. Board-certified genetic counselors and geneticists are trained in the conventions of these statistical methods; they all agree to perform them a certain way. This is the hallmark of certification. Genetics results are, in practice, compromised without the application of Bayesian statistics.

3.3.2.1. *Ethnicity.* In U.S. genetic testing laboratories, ethnicity generally categorizes an individual into one of five or so well characterized groups: Asian, Caucasian, African American, Ashkenazi Jewish, or Hispanic. Genetic statistics sufficient to provide Bayesian interpretations of genetic reports do not exist for other population groups. Members of other population groups presenting for analysis at

a genetic testing Laboratory are provided raw information concerning their tests (e.g. "This patient was negative for all mutations tested"), but cannot be provided with accurate risk reductions owing to lack of statistics.

3.3.2.1.1. *Caucasian.* In the United States, most testing clients are, like the majority of the population, Caucasian non-Jews. This is important for the testing laboratory to know because the Bayesian for carrying a common disease allele, such as that causing cystic fibrosis, differs from that of Ashkenazi Jews, Asians, or African Americans. In addition, Caucasian non-Jews of Northern European descent are more prone to different genetic diseases, such as hemochromatosis, than are individuals of other ethnicities.

3.3.2.1.2. *Non-Jewish.* Because Ashkenazi Jews tend to partner with others from their ethnic group, the mutation spectrum for common hereditary disease is often confined within that population. Mating tendencies cause certain alleles to become more prevalent within the inbred population than in the country as a whole. Therefore, mutation coverage and risk-reduction statistics are specific for a particular ethnicity. As a consequence, an Ashkenazi Jewish individual with a negative CF test by DNA and no family history has a significantly diminished risk of being a carrier (1/801) compared with a Caucasian non-Jew (1/241) with the same assay result.

3.3.2.1.3. *Ashkenazi Jewish (both parents CJA).* Ashkenazi Jews, who are defined as those born of Jews of European origin where both parents were Ashkenazi, have hereditary disease risk characteristics different from those of other groups. Because there has been little outbreeding in the group for at least several hundred years, this group is an effective "bottleneck" for several characteristic genetic lesions.

3.3.2.1.4. *African American.* In addition to sickle cell disease, several β-thalas semias, systemic lupus erythematosius (SLE), and lupus nephritis (LN) are at higher prevalence in the African American community. Allele frequency differences between African American and Caucasian populations are thought to be a cause of the higher prevalence and clinical severity of sarcoidosis among African Americans. In addition, the tailoring of screening tests, such as that for CF, for African Americans has not been as successful as that for Ashkenazi Jews. Whereas a negative screen for 32 relatively common CF mutations allows a presenting Ashkenazi Jew a 98% certainty of not having received a CF allele, presenting African Americans have at best a 69% certainty of not having received a mutant allele, at least in most test panels.

3.3.2.1.5. *Greek and Italian Mediterranean Individuals.* In addition to being susceptible to a number of α- and β-thalassemias, many Greeks and Italians carry an undue genetic burden of glucose-6-phosphate dehydrogenase deficiency, which can result in a nonspherocytic, hemolytic anemia. Other Europeans are not predisposed to hemoglobinopathy at the same high level as Greeks and Italians. As a

result, a good deal of population screening has taken place in the Mediterranean region. Homozygous β-thalassemia has been prevented in this high-risk population by programs based on carrier screening, genetic counseling, and prenatal diagnosis. Genetic counseling is carried out in a nondirective manner following well-established guidelines. The use of extended family screening has magnified the efficacy of the screening program, allowing the identification of the large majority of parents at risk by screening only 13% of the population at child-bearing age. Following counseling, the majority of parents accept prenatal diagnosis. This state-sponsored program has been very effective in identifying at-risk couples and providing informative testing to diminish risk of β-thalassemia births. As a consequence, the rate of thalassemia major births has diminished from 1 : 250 live births to 1 : 4000 in this region.

3.3.2.1.6. *Sephardic Jewish.* Sephardic Jews do not have the same population frequencies of common cystic fibrosis alleles as Ashkenazi Jews or non-Jewish Caucasians. This is believed to be the result of reproductive isolation of these groups during the diaspora. In addition, unique mutations have been found at relatively high frequency in the Tay–Sachs disease (TSD) gene in Moroccan Jews, a subgroup of Sephardic Jews. Three mutations account for the majority of TSD in Sephardim. One of the mutations is an in-frame deletion of one of two adjacent phenylalanine codons at position 304 or 305. Two additional mutations that account for TSD alleles in this population map within exon 5: One is a G→A transition resulting in an Arg170→Gln substitution; the other is a C→G transversion resulting in a nonsense mutation in place of Tyr180. Familial Mediterranean fever occurs mainly in Armenians and Sephardic Jews.

3.3.2.1.7. *Asian.* Asians do not harbor many cystic fibrosis alleles. However, reproductive isolation may explain a higher than expected prevalence of Wilson disease in Asians. Kusuda *et al.* (K7) described the R788L mutation in compound heterozygous state with an in-frame deletion, 3892delGTC, in a Japanese patient and noted the high frequency of this mutation in Asians. Although hemochromatosis exists in the Asian population, it is clearly not a consequence of the same lesion that causes the major form of this disease in the Caucasian population (H5, L2, M3, T3). Efforts to associate Asian hemochromatosis with the C282Y allele have been unsuccessful.

3.3.2.1.8. *Pacific Islanders.* Consistent with the fact that Asians do not harbor many CF alleles, Pacific Islanders do not possess these allels at a high frequency. However, other genetic diseases are frequent in this population. Brody and colleagues (B5) described in Pingelapese people of the Pacific a severe ocular abnormality manifested by horizontal pendular nystagmus, photophobia, amaurosis, colorblindness, and gradually developing cataract. From 4 to 10% of Pingelapese people are blind from infancy. Segregation analysis and equal sex distribution supported the hypothesis of recessive inheritance. The high gene frequency was

attributed to a reduction in the population to about nine surviving males following a typhoon (about 1780), combined with subsequent isolation. The disorder is a form of congenital complete achromatopsia, and has now been mapped to chromosome 8 (S11, W1).

3.3.2.2. *Prevalence Influences Interpretation.* Given that a genetic test result is used as an indication of the presence or absence of the target condition, the simplest and most common situation is that the test itself classifies individuals into just two groups—those with and those without a genetic malady. For instance, in the case of retinoblastoma, a rare form of heritable malignancy with high penetrance, an individual may be either positive or negative for a mutation in the gene. However, in this case, the gene is large and our ability to detect every mutation is less than absolute. In addition, because the disease is rare, inability to locate a mutation in a population that was not subjected to hereditary ascertainment would have little effect on the negative predictive value. That is, for the average person, a negative test for retinoblastoma gene mutations would carry little meaning. However, for a person from a Jewish Ashkenazi family with an *a priori* carrier risk of 1/27, a negative test for mutations in the Tay–Sachs locus, which could rule out 98% of common Jewish Ashkenazi mutations, would be very meaningful ($<$1/1250 chance of being a carrier for TSD). For many genetic diseases in ethnic populations, prevalence data are available. This makes it possible to rapidly confirm or rule out carrier status when the ethnicity is known.

3.3.3. *Specification of Diseases Being Tested for*

Genetic testing is not a panacea. Accurately interpreted results require knowledge of a suspect genetic malady. Although Locus Link lists 1814 human genes for which pathological disorders are described in Online Mendelian Inheritance in Man, the latter source lists 12,379 loci associated with human genetic characteristics. Among these, only a small number are susceptible to rapid and accurate molecular diagnosis. A physician ordering genetic testing has a responsibility to make certain that the test he or she orders is available and will be informative for an at-risk patient.

3.3.4. *Family History and Ethnicity*

Family history influences the degree of risk of an individual for being a genetic disease carrier. In one set of examples commonly provided to genetic counselors in training, first cousins are at 1/4 risk of sharing common hereditary illness, while sib pairs are at 1/2 risk. Effectively, this means that if a brother or sister is affected by cystic fibrosis, the chance that another, apparently unaffected brother or sister carries the gene is, *a priori* 2/3. If a first cousin is affected with cystic fibrosis, the chance that the individual seeking counseling carries one of the disease alleles is 1/4. Only two meioses can eliminate the mutant allele between first cousins,

but just one meiosis can eliminate the mutant allele between sibs. And because both parents are obligate carriers, two of three unaffected offspring are potential carriers.

3.3.5. *Summing up the Pre-analytic Component*

Risk reduction is accomplished through the statement both of prior risk and the risk after testing. Consensus in the medical genetics community has determined that numerical statement of risk is the most succinct and effective way to communicate with potential genetic disease carriers. Therefore, the work product of a molecular genetics laboratory is a detailed letter or report that clearly states the numeric risk to those whose specimens have been tested. This "interpreted report" distinguishes genetic testing from much other medical laboratory testing, in which a normative value can be supplied to the physician-client. In genetics, there is no simple normative value because even a negative genetic test may connote significant risk.

4. Overview of Analytic Phase

4.1. Specimen Accessioning and Sample Preparation

Generally, when a sample arrives in the molecular diagnostics laboratory, it must be reaccessioned, as in all special laboratory areas. Never mind that the testing institution has already established a unique identifier and paper trail for each specimen—Laboratory information systems geared toward providing accurate, timely results for blood glucose levels or blood chemistry profiles are not adequate for the complex pre-analytic information requirements and the post-analytic reports that molecular diagnostics requires. In research settings, there is no effective difference between DNA samples received from an outside laboratory and any other biological material received from outside. Each requires a paper trail of informed consents, IRB approvals, accessioning, unique sample identifiers, and ties to clinical information, if any. Truly, a research genotyping laboratory is only modestly less rigorous than a clinical molecular diagnostics laboratory. Both have ensuring the accuracy of data they produce as their highest objective.

4.2. Running the Analytic Assay

It is a truism that accurate pipettes, proper air circulation, and pure chemicals and active enzymes assure success in any analytic procedure. Molecular diagnosis is no exception to this. In addition, because molecular diagnosis often requires exponential target amplification before detection of a sequence of interest, sequestration of amplified materials (post-PCR) from incoming patient samples and preparation

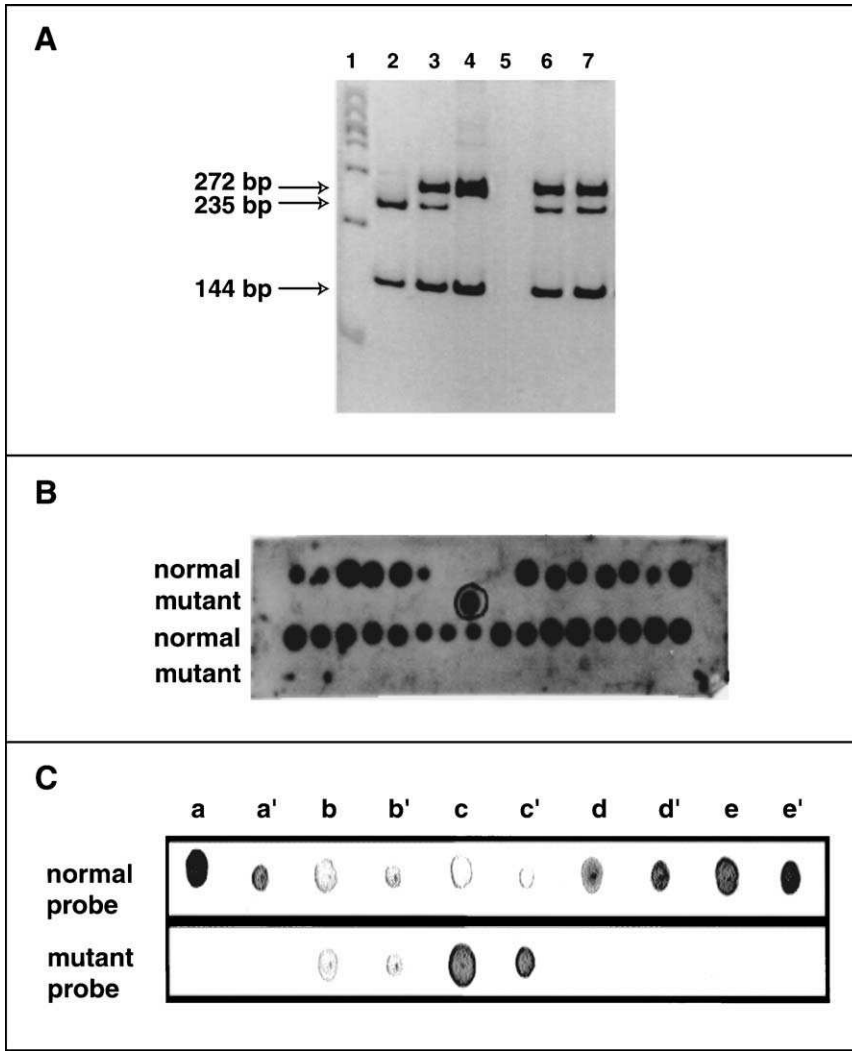

FIG. 2. Three polymerase chain reaction (PCR) mutation visualization methods. (A) Polymerase chain reaction—restriction fragment length polymorphism visualization of the Factor V Leiden mutation. Lane 1 contains a 100-bp ladder; lane 2 contains wild-type human DNA, amplified with primers described in Kowalski, Radu and Gold (K6), and digested with Mnl I; lane 3 contains amplified, digested heterozygous human DNA for the R506Q (Leiden) mutation; lane 4 contains amplified, Mnl I–cleaved, homozygous mutant DNA; lane 5 consists of a negative control reaction, treated simultaneously with all the reagents, but with no human DNA added to the amplification mix; lanes 5 and 6 consist of unknown human samples, assayed in parallel with the controls in lanes 2–5. As can be seen from the photograph, lanes 6 and 7 contain patient DNA heterozygous for Factor V Leiden. (B) A reverse dot blot for hemoglobinopathy and beta-thalassemia determinations. 5′-Amino-link–conjugated

areas has become a necessity. Thus most molecular diagnostics laboratories have been forced to adopt certain PCR-specific Good Laboratory Practices in order to avoid misleading results deriving from contamination of amplicons with incoming specimens. Physical separation of pre-PCR areas and tools from post-PCR areas and tools is one of these precautionary measures. Another is the application of the Amp-Erase Strategy, published first by Thornton (T1), which provides a method to diminish or eliminate amplicon carryover cross-contamination in PCR reactions. There are also a number of analytic assays, which will be discussed in detail.

The analytic phase of molecular genetic testing consists of the application of a variety of techniques, intended to answer the question of whether a mutant allele is present. Probably the oldest of these techniques is the Southern blot, invented by Dr. Edwin Southern in 1975 (S10). This technique is still extensively used for fragile X syndrome testing (G1). Polymerase chain reaction technology, a method for "molecular Xeroxing," made its first appearance in 1987, but was not fully functional until the advent of a commercially available thermostable polymerase and programmable heat blocks. These did not become generally available until 1989–1990. Since that time, PCR has been the mainstay of molecular diagnosis. And, arguably, one version of PCR—one that couples the specificity of the amplification reaction with the specificity of restriction enzymes—is the gold standard. This is the PCR-RFLP (polymerase chain reaction–restriction fragment length polymorphism), which is illustrated in Fig. 2A. In addition to these technologies, many other amplification methods, detection and visualization methods, and varieties of instrumentation have influenced the course of molecular diagnostics. No exhaustive examination of these can be presented here because their development is too rapid. However, this review endeavors to "hit the high points" of

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oligonucleotide probes encoding sequences homologous to wild-type and mutant human beta hemoglobins were robotically spotted in alternating rows onto Immunodyne ABC (Pall Corp., Long Island, New York). Dried spotted strips were hybridized to biotin-labeled, amplified DNA from patients with known mutations. Biotin-containing hybridized fragments were visualized through incubation with an alkaline phosphatase–streptavidin conjugate in combination with a chemiluminescent indicator. Exposure to X-ray film provided the image shown in the figure. In this case, patient DNA homozygous for the sickle cell disease allele (HbS/HbS) was used for the amplification and subsequent hybridization. The circled spot in the middle of the strip represents a strong hybridization signal to the sickle cell probe. Lack of hybridization to the (invisible) spots directly above the sickle cell probe indicates homozygosity for the mutant allele. (C) In the forward dot blot, amplicons are spotted, dried, and incubated with labeled probes under appropriate hybridization conditions. Shown here is a forward dot blot visualization that used patient DNAs amplified in the vicinity of the C282Y mutation indicating hereditary hemochromatosis. Columns are labeled based on the volume of individual amplified patient DNAs spotted; e.g., 2 uL of amplified patient DNA was spotted in column a and 1 uL of that same patient DNA was spotted in column a′. The prime (′) indicates a second spot of the same patient DNA with a lower volume. The first three patients are controls: Patient a is wild type, patient b is heterozygous, and patient c is homozygous mutant at C282Y. Patients d and e were both unknown. They are correctly designated as wild type at C282Y on the basis of this blot.

current technology, at least mentioning some of the emerging technologies for molecular analysis, now making their first appearance in molecular diagnostics laboratories.

4.3. Results First Appear at the End of the Analytic Phase

At the end of the analytic phase, interpretable results are produced. These are most reliably subjected to a two-tiered system of independent data interpretation. In California, where many tests are carried out, a licensed technologist is able to interpret a technical test result. In many large laboratories, one technologist and one board-certified geneticist independently formulate an interpretation of molecular diagnostic results. This provides a system of checks against the release of misinterpreted molecular diagnostic results. After agreement between the two independent readers, interpreted results are released, both electronically and in hard copy, to the ordering physician.

4.3.1. *Interpretations Are Important Parts of the Results*

If a result released from a molecular diagnosis laboratory simply said "band observed at 242 bp" or even "heterozygote at I507C in the cystic fibrosis test," this would be meaningless to a physician-client. This is because the precise methods used to conduct a test differ with each molecular diagnostic laboratory. Unique molecular methods are described as "homebrews" in the vernacular of the clinical laboratory. No uniform testing standards dictate uniform PCR primers such that a finding of a band at 242 bp provides a meaningful and universally interpretable result. Nor would most physicians understand that an I507C heterozygote, an observed polymorphism in the cystic fibrosis gene, has no functional consequences and is included in the panel for technical purposes only (to make certain that no other allele is being overlooked). The subtleties of molecular diagnostics make it impossible to provide a "simple" report, which would say nothing but "normal" or "abnormal" about the patient in question to the physician-client.

5. Specimen Accessioning and Sample Preparation

5.1. Accessioning

Samples should be accessioned into a computerized database, consistent with federal regulations (21CFR). Most rules concerning a compliant database have to do with whether the structure provides for an audit trail, so that anyone who has viewed or written to the database can be traced. Handwritten accessioning procedures are strongly encouraged as well.

5.2. Nature of the Specimen

The specimen will be the basis for the analytic analysis. Is it RNA or DNA? What is the origin of the tissue? Amniocentesis? Was it a spontaneous product of conception? Were anatomic pathology slides or tissue blocks prepared? Are cell lines involved? Are these primary or immortalized? Was a chorionic villus sampling procedure done? Is the sample properly collected peripheral blood? The answers to each of these questions should be noted, and considered part of the validation of a useful nucleic acid extraction method. A molecular diagnostics laboratory should adhere to the highest standards in providing services, and prior validation of applicable nucleic acid extraction procedures is a must to ensure high-quality service.

5.3. Organic Extraction

Although these were among the first nucleic acid extraction procedures used, they are now considered obsolete. The principle relied upon is that charged nucleic acids are water-soluble while lipids are soluble in an organic phase; and because many proteins are amphipathic, they will migrate to the interface in a liquid–liquid extraction. The products of these procedures, both RNA and DNA, remain acceptable for a wide variety of analytic techniques; however, organic methods are inherently dirty. Organic solvents, such as phenol and chloroform, are dangerous and require costly disposal. Moreover, residual organic solvents present in the samples can inhibit enzymatic reactions, so that these must be washed with ethanol or ether (dangerous but effective) and thoroughly dried in air or under vacuum before dissolving in an aqueous solution. This causes a molecular dilemma: When high-molecular-weight genomic DNA is thoroughly dried, it is almost impossible to redissolve it into aqueous solution. So, newer techniques that avoid drying genomic DNA, or do not take it out of solution at all, are now often preferred.

5.4. "Salting-out" Procedures

During the 1990s, these became the most widely used extraction procedures in molecular diagnostics laboratories. Salting-out procedures consistently yield high-quality DNA, which can be restriction-cleaved, mapped, and used as a template for PCR. The yields of DNA are generally large enough that they can be spectrophotometrically quantified. In the procedure, high salt extraction is used after hypotonic lysis is employed to eliminate erythrocytes. The principle underlying the procedure is that high-molecular-weight nucleic acids will condense out of solution under conditions of high salt and a mild organic solvent, such as 60–70% ethanol or 50% propanol. Ordinary salts, such as sodium or potassium chloride, or chaotropic agents, such as guanidium HCL or guanidium isothiocyanate, may

be used. Several companies make kits based upon these kinds of procedures: Two examples are the Gentra Puregene procedure and the manual extraction procedures for the Roche Amplicor product line.

5.5. "Column" Procedures for DNA Extraction

These are methods that rely upon solid supports with a specific affinity for nucleic acids under specified aqueous conditions. Qiagen of Germany has devised a procedure in which glass beads are packed into a column, which is then irrigated with salt solutions containing RNA or DNA. Under low ionic strength conditions, the nucleic acid elutes from the column. Gentra Systems of Minneapolis has developed proprietary columns that bind double-stranded DNA with great affinity, although they do not appear to bind RNA with equally great affinity. Nucleic acids can be eluted from Gentra Generation Capture Columns under low salt conditions to provide high-quality DNA susceptible to analysis using most methods. Several other manufacturers offer nucleic acid purification kits, but in the author's experience, most high-throughput diagnostics laboratories are using those from Gentra Systems.

6. Technology for Analysis I

6.1. Classifying Suspected Mutations by Size

Large chromosomal deletions are known to be the cause of a significant burden of genetic disease. Among these are the so-called contiguous-gene syndromes, including Shprintzen/DiGeorge/velocardiofacial and Prader–Willi/Angelman. These genetic syndromes are often caused by deletions that are too large for easy visualization using current molecular detection technology. As a consequence, they are most often detected in cytogenetic laboratories using conventional Giemsa banding or high-resolution karyotyping, where synchronized cells enable the routine visualization of approximately 1000 bands. Alternatively, such deletions are detected through the use of molecular cytogenetics, using fluorescent *in situ* hybridization (FISH) probes. The contiguous-gene syndromes are caused by relatively large deletions that may be several megabases in length. Smaller deletions, usually consisting of several exons, are observed in large genes, such as the dystrophin locus on the X chromosome and the adenomatous polyposis coli (APC) locus on chromosome 5q. The latter are often screened using denaturing high-performance liquid chromatography (DHPLC), single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis (HET), chemical cleavage analysis (CCM), ribonuclease cleavage (RNAse protection assay), constant denaturing gel electrophoresis (CDGE), conformation-sensitive gel electrophoresis (CSGE), the protein truncation test (PTT), or direct sequencing

of the target. Each of these provides a basis for large gene mutation scanning. Although these procedures may be highly informative, most are tedious and incompatible with high throughput and low cost. Given the requirement in the clinical diagnostic laboratory, and in many research laboratories, for cost-effective genotyping at a relatively high throughput (>500 samples/analysis), a second group of mutation analysis methods has been developed. This set of methods is better adapted to the characterization of known small insertions, deletions, and single base pair changes. These methods include polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP), artificial introduction of restriction sites (AIRS), amplification refractory mutation detection system (ARMS), ligase chain reaction (LCR), oligonucleotide ligation assay (OLA), allele-specific oligonucleotide hybridization (ASOH or ASO), reverse dot blot (RDB), or multiplex allele-specific diagnostic assay (MASDA). Many of these will be discussed in detail in the sections below.

6.2. Scanning Is Helpful for Large Gene Mutation Characterization

For large genes (e.g., the APC locus), scanning methods such as DHPLC, SSCP, DGGE, CDGE, CSGE, or PTT may be helpful to locate the site of a polymorphism. These methods involve amplification of the gene of interest followed by hybridization-based interrogation or a coupled reverse transcription/translation. In five of these six cases, the products are run out on a gel, although each demands a gel of a different sort. Subsequent to electrophoresis, product visualization is accomplished through the use of radioactive tracer on film or by chemiluminescent or fluorometric detection. All six methods allow assessment of allele normalcy by observing electrophoretic or chromatographic mobility.

6.3. Denaturing High-Performance Liquid Chromatography

DHPLC (C2, L3, U1) uses a proprietary matched ion polynucleotide chromatography column devised by Transgenomics, Inc., in combination with an organic solvent and thermal denaturation (rather than an electrophoretic gel) to resolve heteroduplexes containing a suspected polymorphism from a polymorphism-free homoduplex. Homo- and heteroduplexes are visualized through UV absorbance readings of the eluent as it leaves the column.

6.4. Single-Strand Conformation Polymorphism

SSCP (O1) detects single mutations in genes because such mutations alter the mobility of single strands of DNA in native or nondenaturing gels (see Fig. 3A). Labeled PCR primers initiate amplification spanning the sequences of a disease gene at a putative site of mutation. The same region of the wild-type gene is

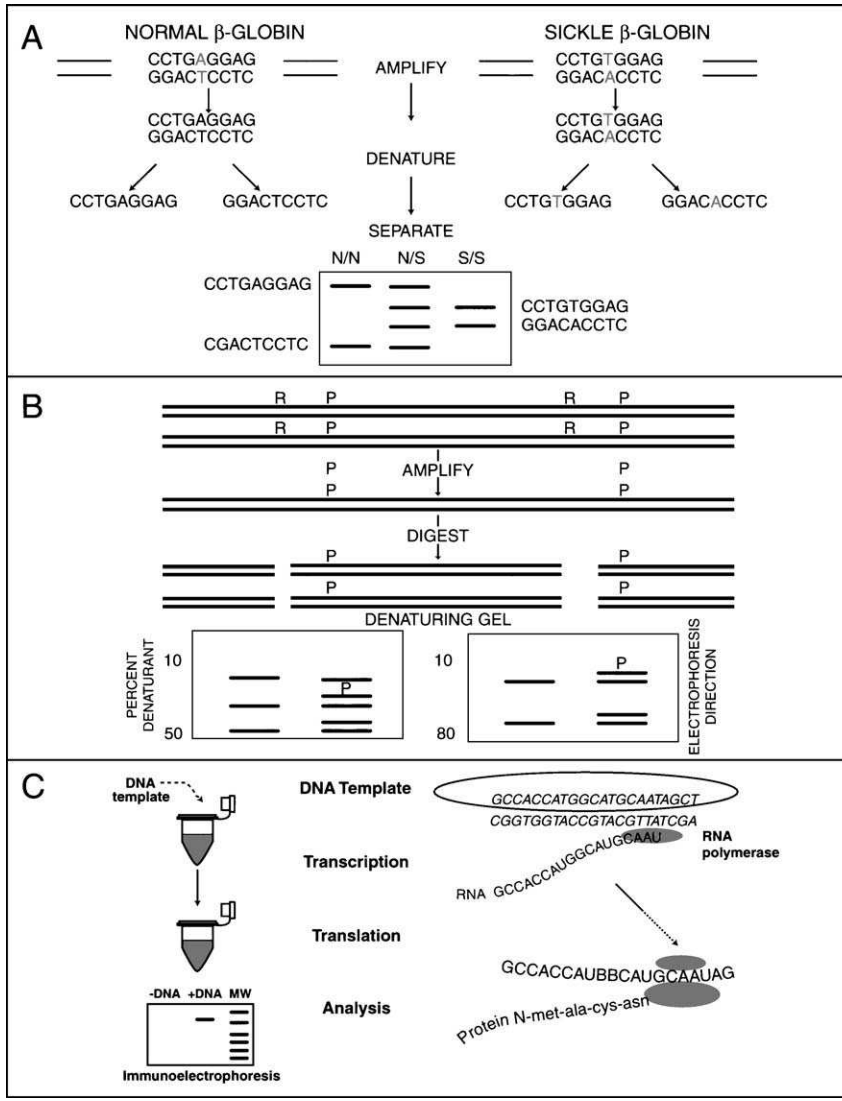

FIG. 3. Three molecular gene mutation detection methods. (A) Schematic of PCR-SSCP. In this PCR-SSCP analysis of normal and sickle cell β-globin genes, the mutation is shown in gray. Specific amplification primers spanning this junction are not shown. Post-amplification PCR products are heat-denatured and subjected to nondenaturing gel electrophoresis. Three possible outcomes of analysis are shown. A homozygous normal or S/S individual will evidence two bands, while a heterozygous individual will evidence four bands after electrophoresis. (B) A schematic of DGGE. In parallel DGGE, two genomic DNA duplexes with polymorphisms (P) are shown. R indicates a restriction enzyme cleavage site. From top to bottom of the panel, the subject DNA is amplified and digested. It is then

also amplified. After amplification, the products are denatured and subjected to nondenaturing polyacrylamide gel electrophoresis. (More recently, it has become possible to dispense with labeled primers and simply incorporate radiolabel into PCR master mixes used with the normal and putative mutant template.) The conformational change between labeled normal and mutant amplification products is observed as a mobility difference upon electrophoresis. Heteroduplex and homoduplex migrate differently. Observed heteroduplex may be explained by the presence of a detected mutation. This observation can then be verified by direct sequencing. This method is believed to detect up to 90% of the mutations present, but is subject to diminished sensitivity changes in regions of high G–C content.

6.5. Denaturing Gradient Gel Electrophoresis

DGGE (B2,C1) takes advantage of the fact that the melting behavior of a stretch of DNA is a function of sequence (Fig. 3B). A characteristic reduction in electrophoretic mobility of a homoduplex or heteroduplex double-stranded DNA occurs as it is electrophoresed through a denaturing gradient. And these characteristic reductions can be scored. DNA sequences differing by a single base pair migrate at different rates along the gel, thereby allowing detection of a polymorphic site. CDGE is a variant of this technique that uses a constant denaturant concentration to achieve the same goal.

6.6. Conformation-Sensitive Gel Electrophoresis

In CSGE , mildly denaturing solvents in an appropriate buffer can accentuate conformational changes produced by single-base mismatches in heteroduplexed DNA. This increases the differences in electrophoretic mobility between heteroduplex and homoduplex.

6.7. RNase Cleavage

Another technique, RNase cleavage (M4), uses the enzyme ribonuclease A to cut RNA–DNA hybrids wherever there is a mismatch between a nucleotide in the RNA

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subjected to electrophoresis on two denaturing gels with differing denaturing gradients. The extra band labeled P indicates the presence of the polymorphism on each gel. Different polymorphisms are optimally visualized using different denaturing conditions. (C) A cartoon illustration of PTT. Human RNA is subjected to reverse transcription and PCR cloning into a plasmid which contains bacteriophage (usually T3, T7, or SP6) promoters. These are used to make RNA which is in turn used as a template for cell-free protein synthesis. At the conclusion of this linked process, newly synthesized protein is subjected to electrophoresis and visualized using an antibody. Properly transcribed and translated protein yields characteristic bands on an electrophoretic gel. Truncated protein species indicate gene mutations.

strand and a corresponding nucleotide in the DNA strand. Using this method, a radioactive RNA probe is produced by using the normal sequence cloned in a vector with a phage RNA polymerase gene. The RNA strand anneals to the test genomic DNA and the mixture is treated with RNase A. If the DNA contains a mutation, the enzyme cuts the RNA strand and two radioactive RNA fragments are detected on a denaturing gel. If the test DNA is normal, a single RNA fragment corresponding to the intact RNA probe is detected. Typically, about 70% of mutations are detected using this method (M4).

6.8. Chemical Cleavage Method

CCM (F1) is based upon a similar principle but uses hydroxylamine and osmium tetroxide to distinguish between mismatched C or T nucleotides, respectively. The position of the mismatch (e.g., the mutation) is defined by sizing on gel electrophoresis after a chemical-mediated cleavage at the reactive position by piperidine.

6.9. Protein Truncation Test

PTT is based on a combination of reverse-transcription polymerase chain reaction (RT-PCR) and linked *in vitro* transcription and translation (Fig. 3C). This combination of procedures can selectively detect translation-terminating or nonsense mutations. Unfortunately, it does not find missense mutations, which may be etiologic, depending upon location.

6.10. Smaller Genes Are Subject to Different Analytic Approaches

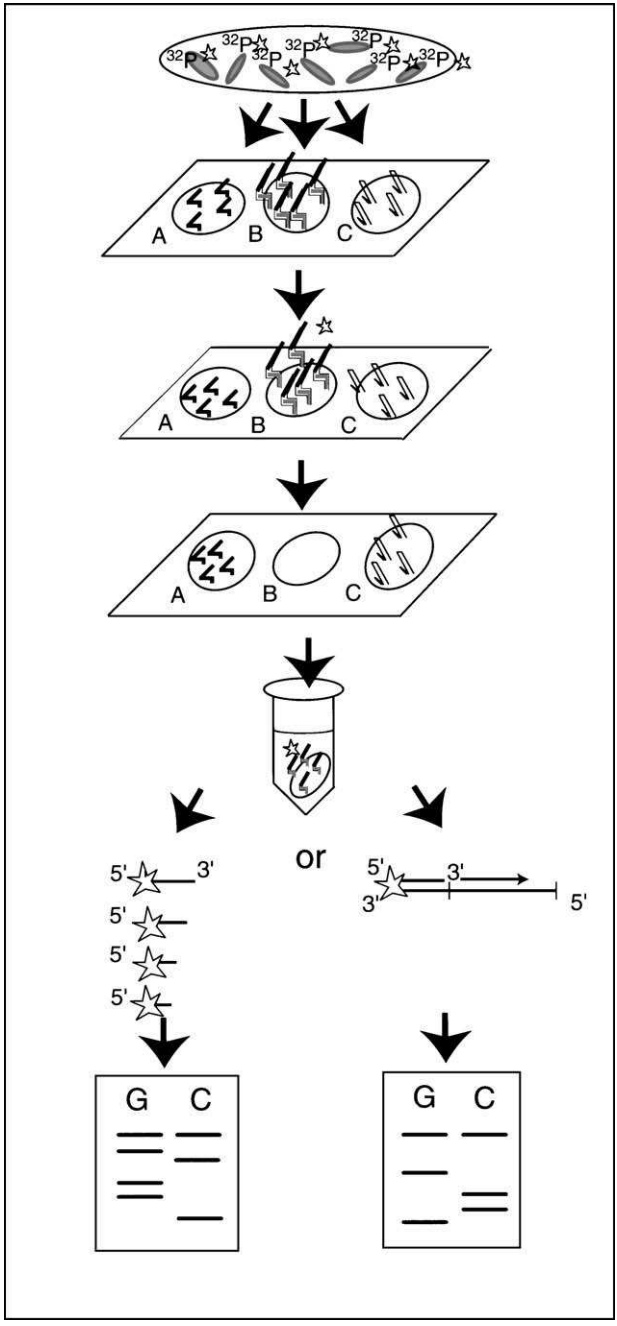
Whereas larger genes require a survey approach to locate mutations, smaller genes require closer analytic scrutiny. Large genes, such as the DMD locus on the X chromosome or the APC locus on chromosome 5q, are more often subject to deletion than are small genes. As a consequence, large genes are analyzed using techniques that detect deletions more easily, such as SSCP or PTT. Small genes are subject to direct analysis via direct sequencing, SSCP, or DHPLC. Each of these techniques provides the location of a mutation, but only direct sequencing can confirm the presence of an implicated mutation. Recently, it has been suggested that direct sequencing is a better gold standard for analysis than PCR-RFLP. However, direct sequencing also has weaknesses. It cannot detect heterozygous deletions unless the site of the deletion is entirely spanned by the region sequenced. In addition, insertion and deletion mutations within a sequenced region are often not easily deconvoluted. These types of mutations can therefore be difficult to characterize via sequence. However, deconvolution software is currently in development.

6.11. "Low Tech"

Relatively low-tech solutions to mutation identification have been adopted in many genetics laboratories; however, this situation is expected to change rapidly in the next few years. Direct sequencing is one alternative, but it remains arduous and expensive, even though 96-well capillary sequencers have become widely available. The problem is that most of the costs of direct sequencing lie in the preparation of the reactions themselves. Initial PCR must be done, followed by primer clean-up and subsequent sequencing reactions. Of course, all of the initial costs discussed for the pre-analytic phase of testing, such as accessioning and specimen preparation, are still required for direct sequence genotype detection. In addition, specialized interpretive and database software and hardware may be required for the storage of large amounts of sequence trace data. This software and hardware can be quite expensive. Moreover, some software issues remain unresolved; for example, algorithms for culling valid heterozygote polymorphisms from trace sequence data are still being tested. All of this suggests that the time has not yet come for the routine reporting of DNA genotypes from sequencing machines.

6.12. PCR-RFLP

Small genes with a high point mutation spectrum (e.g., CF and β-thalassemia) are often characterized using reverse dot blots (RDB) (Fig. 2B) or multiplex allele-specific diagnostic assay (MASDA) (Fig. 4). Each of these methods will be discussed later. In higher throughput genetic tests of a single polymorphism, where one homozygous mutation in a medium-sized gene may give rise to a predisposing condition (e.g., hereditary hemochromatosis, apolipoprotein E genotyping, or medium-chain acyl CoA deficiency), PCR-RFLP is often deployed. PCR-RFLP (Fig. 2A) takes advantage of base-pair changes that create or interrupt nucleic acid palindromes. Mutations at these sites alter the pattern produced by restriction enzyme digestion subsequent to amplification. Digestion-product size alterations, detectable by gel electrophoresis, are created because insertions or deletions within the gene of interest create or remove palindromic restriction sites. Overall, this gel-based genotyping method is straightforward and useful when dealing with fewer than a few hundred samples. As a method, it is labor-intensive, requiring an experienced and skilled technical staff for interpretation. As a result, PCR-RFLP is difficult to apply for large-scale genotyping in clinical trials and high-throughput diagnostic settings. But for historical reasons, PCR-RFLP remains the gold standard for clinical diagnosis of point mutations. New technologies for mutation detection are often compared against it. Recently, however, polymorphisms in the binding site of PCR primers within the hemochromatosis locus have exploded the myth that PCR-RFLP is the most reliable genotyping technique.


32P
A
B
C
or
5'
3'
G
C

6.13. PCR Followed by Hybridization

Two techniques that have provided useful adjuncts to the gold standard where high throughput or low expense is required are allele-specific oligonucleotide hybridization (ASOH) (Fig. 2C) and its opposite, the reverse dot blot (RDB) (Fig. 2B). ASO involves the immobilization and denaturation of PCR products to a membrane, such as nitrocellulose or nylon, informally called "dot blots," followed by hybridization with ASOs. Under empirically devised conditions, an oligonucleotide probe that is fully complementary to one allele will hybridize to that allele only. Similarly, a variant will hydridize only with a specific complementary oligonucleotide probe. The hybridization of the ASO is detected via a linked signal-generating label, such as a radiolabel, fluorophore, or biotin. These are detected, respectively, by autoradiography, fluorescence after the appropriate wavelength UV irradiation, or conjugation to an avidin– or streptavidin–enzyme conjugate that will generate a colorimetric or chemiluminescent signal. Unlike the dot blot, RDB allows the simultaneous analysis of several alleles in a single test. The method is similar to the forward dot blot just described, except that the format is inverted. Preparation for RDB involves immobilizing oligonucleotide ASOs on a membrane. Hybridization is then carried out using a biotinylated or radiolabeled PCR product as the probe. The main advantage with respect to the dot blot is that several probes can be immobilized to detect specific alleles from a single PCR in which several primer pairs were combined.

6.14. Multiplex Allele-Specific Diagnostic Assay

MASDA (S9) (Fig. 4) is a form of ASOH in which radiolabeled dots, formed through hybridization of a labeled oligonucleotide with dot-blotted PCR products, are subjected to electrophoresis after initial visualization on x-ray film. This allows a fingerprint-like identification of specific mutations. This is a forward dot blot strategy, which has permitted one clinical laboratory, Genzyme Genetics in Framingham, Massachusetts, to examine patient DNAs for the presence of 86 cystic fibrosis mutations simultaneously. Unfortunately, it has one drawback: It must use radioactive probes. In the first stage of MASDA technology, amplified patient DNA is immobilized to a sold support and sequentially interrogated with a pool of radioactive ASOs. During hybridization, ASOs corresponding to specific

Fig. 4. Illustration of the multiplex allele specific diagnostic assay. At the top of the panel, radioactive oligonucleotide probes (indicated by stars) are selected by hybridization to amplicons from patient samples affixed to membrane filters. When a putative mutation-bearing allele hybridizes to a radioactive probe, it can be eluted from the filter and subjected to sequencing using chemical or radioactive dideoxy-terminator methods. This permits unequivocal identification of a large number of mutations at a high throughput.

mutations present on the solid support are hybrid-selected. Radiation marks the putative mutation in patient amplicons. The second stage of MASDA follows after a wash step to remove unhybridized probes. Chemical or enzymatic sequencing is performed on the radioactively labeled hybrids. This sequencing reaction provides a confirmation for the presence of mutation.

6.15. Amplification Refractory Mutation Detection System

One limitation of the PCR-RFLP method is the requirement that the polymorphism alter a restriction enzyme site in the target, although there are ways to get around this difficulty (by synthesizing a site into a partially mismatched primer, for instance). ARMS has no such requirement. This is a method in which PCR amplification is conditionally reliant upon an exact match at the 3′ end of a mutant or normal base pair sequence. It is a mismatch form of primer extension, in which nucleotide synthesis does not proceed unless perfect homology exists between the probe and interrogated sequence. ARMS primers will make amplification product if and only if the sequence that matches them is present in the genomic material being tested. Target sequences that differ by a single base can easily be discriminated. The method can be multiplexed, so that several alleles can be simultaneously discerned. Unfortunately, multiplexing conditions can be difficult to optimize. Impurities in extracted nucleic acids can sometimes interfere with the reactions, even when ostensibly equal amounts of genomic DNA have been added to them. This can provide less than robust amplifications that are then difficult to assess on an analytic gel. In addition, multiplexed ARMS can require an expert for analysis, and can be tedious.

6.16. The Roche Patent

In 1991, the Swiss chemical giant F. Hoffman LaRoche bought Cetus and the intellectual property attendant to PCR for approximately $300 millon. Shortly thereafter, Roche adopted a plan to recover its investment through the issuance of patent licenses to laboratories wishing to employ PCR technology for diagnostic (as contrasted with research) purposes. These licenses are administered through an office in Alameda, California, and can be as high as 20% of a diagnostic laboratory's revenue, depending upon the technology employed and reagent purchase agreements. The license fee rarely falls below 9%. These royalties have prompted many laboratories to search for alternatives to PCR technology for mutation identification purposes. Among the alternative methods being used by clinical molecular laboratories for mutation detection purposes are NASBA (TMA), bDNA, OLA and its relatives, Invader[2], strand displacement amplification, and linked linear amplification.

6.16.1. *Branched DNA (bDNA) Detection*

This system has some advantages as a method for the visualization of virus, which are themselves in great abundance and so do not require amplification for

detection (for a detailed review of this system, see Ref. N2). Although the methodology has been applied to genotyping common HIV-1 variants, the manufacturers have found it difficult to provide a reproducible system for genotyping using this strategy. Part of the problem appears to be some batch-to-batch variation in the hybridization or visualization probe characteristics. Several other amplification methods, including NASBA (TMA) and Invader^2, are mentioned below.

6.16.2. *OLA, LDR, LCR, and CAL*

Like ARMS, OLA relies upon hybridization with specific oligonucleotide probes to effectively discriminate between wild-type and variant sequences. Three oligonucleotides are used in this assay: two differently sized allele-specific oligonucleotide probes (one specific for the wild-type allele and one specific for the mutant allele) plus a fluorescent common probe. To perform OLA, the gene fragment containing the polymorphic site is amplified using PCR and then incubated with the probes. In the presence of a thermally stable DNA ligase, fluorescent probe ligation takes place only when the interrogated sequence is perfectly matched with the probe. Since ligation product size corresponds to polymorphic sequence content, electrophoresis on an automated DNA sequencer equipped with a fluorescence detector reveals genotype. By varying combinations of probe dyes and lengths, detection of several polymorphisms in a single reaction can be achieved.

6.16.3. *LCR and Its Variations (Most Require PCR Also)*

Oligonucleotide ligation for the detection of point mutations was first described in 1988 (L1). The assay proceeds on the basis that ligation strongly favors the sealing of nicks created by adjacent probes only if there is a perfect match of oligonucleotides hybridizing around an interrogated site. With T4 ligase, discrimination is fivefold greater when the mismatch is at the 3′ end of the upstream probe than when it is at the 5′-terminal base of the downstream probe. With Taq ligase, optimum allele discrimination is achieved by placing the mismatch at the 3′ end of the upstream probe. Analysis of point mutations is usually achieved by setting up the OLA with two competing allelic probes which are ligated to a common probe only when perfect complementarity is achieved. This technique has been used for discrimination of point mutations in CF, β-globin, α_1-antitrypsin, and 21-hydroxylase. This reaction can be multiplexed, but it is complex, as it requires balancing the T_ms of all the probes so that detectable ligation can be achieved in a variety of constituent nucleotide sequences. Signal balancing under differing salt and temperature conditions is achieved by adjusting the concentrations of each of the oligonucleotide constituents of the ligation reaction. This is termed LDR, for ligation detection reaction. LDR requires a method to resolve different ligation products from each other post-assay: thus, hooks of varying length, prepared through terminal transferase or hexaethylene oxide tails, can be used to vary the length of LDR products, which can be visualized using an automated

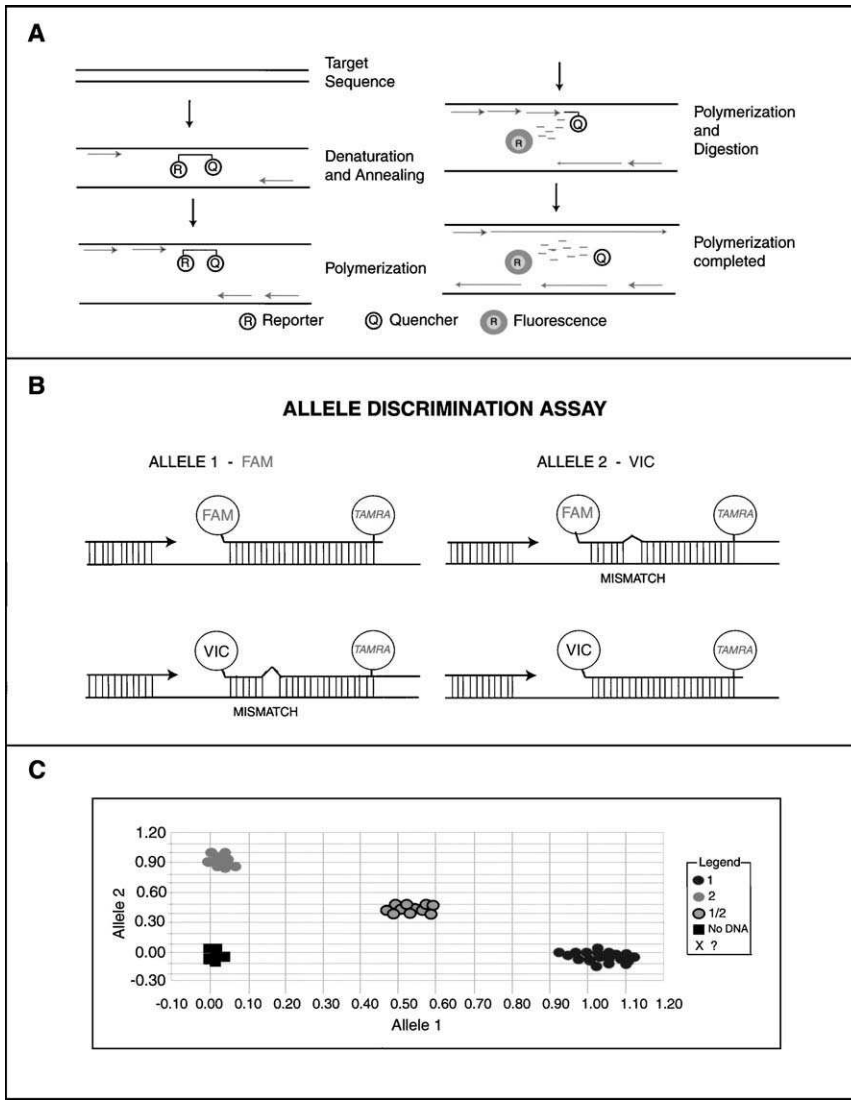


FIG. 5. Illustration of the 5′-nucleotidase (TaqMan) assay for allele discrimination. (A) The allele discrimination assay employs two unlabeled PCR primers and two doubly fluorescent labeled PCR probes for visualization of a mutant allele. The target sequence is initially denatured and amplified in the presence of each of the primers and probes. Increasing polymerization in the presence of a thermostable polymerase which contains a 5′ proofreading function allows cleavage of one fluorescent indicator from an appropriate probe during the cycling reaction. (B) Probes are designed with a fluorescent reporter and a quencher moiety. Amplification reactions are spiked with additional fluorescent quenchers in order to render the reaction initially "dark" to the photomultiplier tube or diode. The probes are designed

electrophoresis device (Ligase chain reaction (LCR) is an extension of LDR that provides exponential amplification. Two pairs of probes (four synthetic oligonucleotides) are used to interrogate both strands of the target DNA simultaneously. The ligation products formed on each strand serve as template strands for subsequent synthesis. In practice, the signal-to-noise ratio of LCR is limited by a small amount of blunt-end ligation of the double-stranded probe which occurs in the absence of any target DNA. The solution to this problem has been successfully addressed by a series of techniques including gap-LCR, a 3′ overhang probe design, and the combination of LCR with PCR, sometimes called CAL (combined amplification and ligation).

6.16.4. *5′-Nucleotidase (TaqMan) Assays*

The 5′-nuclease method is a modified form of PCR in which an amplification is conducted in the presence of a probe labeled with two fluorescent moieties (Fig. 5). The fluorescent moieties on the probe differ in their excitation and emission spectra, such that one quenches the other when in close proximity. The fluorescent molecule selected as the quencher remains attached to the oligonucleotide probe before, during, and after the assay. The reporter is liberated during the PCR extension (as shown in Fig. 5A) and allows measurement of increasing fluorescence throughout the assay, when a homologous sequence is detected. By conducting the PCR in optically clear plastic tubes at the emission wavelength of the reporter, the increasing fluorescence of a positive sample can be visualized during (or shortly after) the test. A small modification of the assay allows two alleles to be discerned in the same sample. Instead of using only one probe, with one fluorescent and one quencher molecule, two different fluorescent molecules—one with fluorescent moiety A and one with fluorescent moiety B, differing by one base pair—can be used simultaneously in the same tube. One of these detects the mutation while the other detects the normal gene sequence (Fig. 5B). Through application of this strategy, it is possible to visualize the presence of homozygous normal, homozygous mutant, or heterozygous samples in a single tube (Fig. 5C). Drawbacks of the technique include the difficulty of optimizing assay conditions, the expense of the complicated synthesis of TaqMan probes, and the cost of the readers required

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so that the reporter dye from each allele differs. In this example, the reporter dye for allele 1 is FAM and the reporter dye for allele 2 is VIC. If allele 1 is properly recognized, FAM will be released, manifesting a green fluorescence. If allele 2 is properly recognized, VIC will be released, allowing an orange fluorescence. Alleles are assigned via cluster analysis as shown in Fig. 5C. In part C, the relative fluorescent intensities of FAM and VIC are compared using ratios. As can be seen from the figure, if no fluorescence results, no allele call can be made. If only FAM fluorescence or only VIC fluorescence is present, one can conclude that the presenting sample contained homozygous allele 1 or homozygous allele 2, respectively. If a heterozygous DNA is present in the assay mixture, an equal distribution of FAM and VIC will be liberated during the reaction.

for visualizing the results. In spite of these disadvantages, this method is quickly becoming adopted for application in high-throughput genotyping environments.

6.16.5. *A Capillary Thermocycler*

A circulating hot air–based capillary thermocycler was developed by Wittwer and his colleagues (S12, W2, W3, W4, W5). As in conventional thermocycling, initial difficulties with sealing tubes gave way to an intelligent system that uses a plastic coupler and screw caps. Thermocycling is very rapid, with a standard 30-cycle reaction completing in less than half an hour. For homogeneous analysis using the 5′-nucleotidase technology, the dynamic range of the laser exciter–diode detector circuit may not be great enough to detect reactions easily quantitated in the PE 7700 instrument. However, the LightCycler offers the unique advantage of being able to produce melting curves of hybridized fluorescent labeled probe annealed to amplified genomic DNA. Using this technology, discerning specific mutations not easily resolved by other technologies becomes straightforward.

7. Technology for Analysis II

7.1. Southern Blots Still Play an Important Role

PCR is not capable of accurately amplifying and allowing visualization of long tracts of GC-rich repeats. And accurate assessment of repeat length is critical for assigning premutation status in carriers of the triplet repeat diseases. Furthermore, in Fragile X families, methylation status assessment can be an important prognostic for severity of the mental impairment. In this assay, probe manufacture now involves PCR or run-off, and the blots can use radioactive, chemiluminescent, or chromogenic visualization. However, in this test, and in the diagnosis of Duchenne/Becker muscular dystrophy carrier status in some putative carrier females, Southern blotting still plays an important diagnostic role.

8. Technology for Analysis III

8.1. Large Gene Mutations Scanning

In a large gene, the ability to scan rapidly for mutations via PCR is constrained by the ability to multiplex the PCR, thereby detecting many potential missense, nonsense, or insertion–deletion mutations simultaneously. Resequencing would appear to be the method of choice, but it is costly, the analysis is demanding, and it has some pitfalls. Therefore, a series of alternative techniques for screening relatively large genes for mutations have become popular. Among these, the protein truncation test (PTT) is offered commercially for mutant alleles in a few genes

that are not susceptible to rapid characterization by any other method. However, each of the other methods mentioned below has worth for large gene mutation scanning.

8.1.1. *SSCP*

Single-strand conformation polymorphism is a technique for locating mutations within large genes by subjecting short sections to amplification, then hybridizing these and subjecting them to nondenaturing electrophoresis (Fig. 3A).

8.1.2. *DGGE*

Denaturing gradient gel electrophoresis takes advantage of the fact that the melting behavior of a stretch of DNA is a function of sequence. A characteristic reduction in electrophoretic mobility occurs as a charged duplex moves through a denaturing gradient (Fig. 3B).

8.1.3. *CSGE*

Conformation-specific gel electrophoresis is a variant of CDGE that uses mildly denaturing solvents in an appropriate buffer to accentuate conformational changes produced by single-base mismatches in heteroduplexed DNA. This increases the differences in electrophoretic mobility between heteroduplex and homoduplex.

8.1.4. *CDGE*

Constant denaturing gel electrophoresis is similar to DGGE, but uses a constantly denaturing gradient to achieve the goal of separating characteristically differently charged molecules on a gel.

8.1.5. *PTT*

The protein truncation test is a way of testing large genes (e.g., NF1) for which an antibody is available. PTT can detect nonsense mutations that are peptide chain terminating. These show up, after reverse transcription/cell-free translation, as shorter-than-normal peptides in an electrophoretic gel (Fig. 3C).

9. Minimal Residual Disease, Viral Load, and Gene Quantitation

9.1. Quantitative PCR

In this procedure, two templates—an experimental one and a control quantitation standard of roughly the same size—compete for a chimeric primer that contains portions homologous to each. After gel electrophoresis, the accumulation of amplified products from the control template is compared with that from the

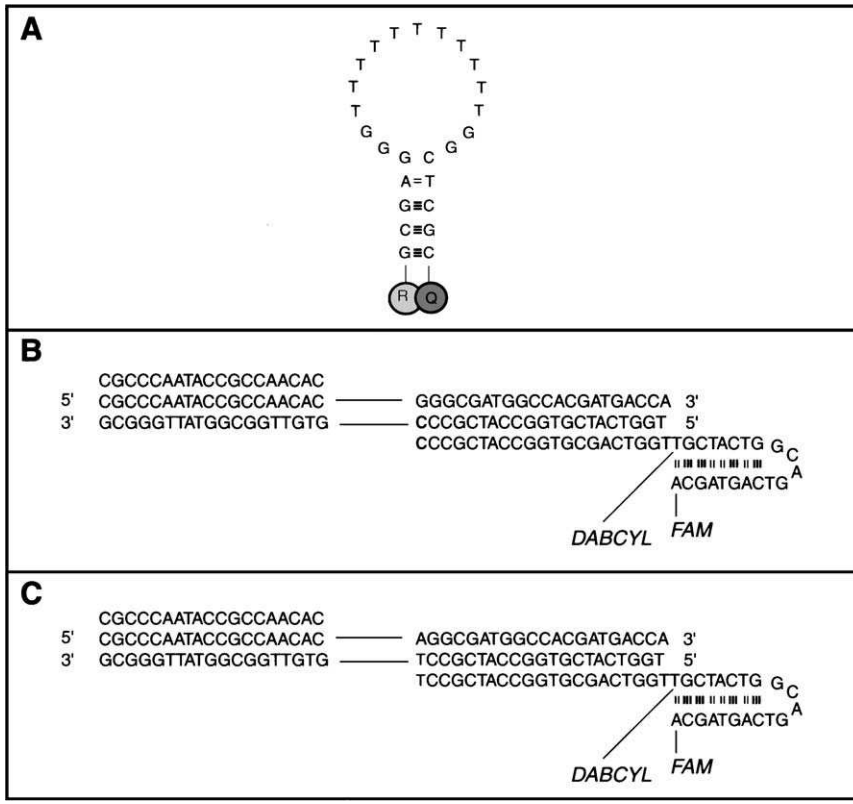

FIG. 6. Schematic mechanism of molecular beacon and Sunrise strategies for amplification product visualization. (A) A schematic representation of a molecular beacon. In a molecular beacon, a fluorophore reporter (R) and quencher (Q) moiety are present on opposite arms of a short oligonucleotide. Secondary structure places the covalently attached quencher and reporter in close proximity, thereby preventing significant initial fluorescence. During amplification, homology to a central loop sequence (in this case GGTTTTTTTTTTGG) sufficiently denatures the quenching structure, so that fluorescence can be emitted. As the central loop sequence can be designed to be any series of nucleotides, molecular beacons can recognize a wide variety of sequences. (B) In Sunrise technology, either a fluorescent moiety or a quencher is placed internal to an oligonucleotide, which itself acts as an amplification primer. By allele-specific amplification, two alleles can be differentiated when tested independently for the presence of amplification product.

experimental template. This allows a precise estimate of the experimental template in the starting mixture. With no experimental template in the starting mixture, only the quantitation standard is amplified. With an abundance of experimental template, virtually no quantitation standard will be amplified. This technique allows calibration of a reverse dot blot. It has proved exact enough to provide for an FDA-approved test for HIV-1 viral load, which is used to monitor the effectiveness of

anti-retroviral therapy in patients all over the world. Quantitative PCR can be carried out in another sense, through rigorous quantitation of amplicons as compared with controls within a reaction. A detailed method for checking for duplications or deletions within the DMD locus on chromosome X has been published by a Canadian group using this technique.

9.2. Colorimetric Microwell Plate

In 1996, after eight years in the planning stage, a quantitative PCR assay for HIV-1 in human plasma was released by Roche Molecular Systems. This test, the result of intensive efforts by John Sninsky and his team at Roche, allowed quantitation of HIV-1 viral load to 400 viral copies (just 200 viral particles, as each HIV-1 virion contains two copies of the virus).

9.3. Rate of Amplification

A slope of increasing fluorescent intensity can be used to quantitate virus particle (or amplified gene) quantities in 5′-nucleotidase assays.

9.4. Other Quantitative Methods

These include Molecular Beacons (Fig. 6A) coupled with NASBA or Sunrise coupled with PCR (Fig. 6B). The Molecular Beacons patent bears striking similarities to the Sunrise patents.

10. Emerging Technologies

10.1. Denaturing High-Performance Liquid Chromatography

DHPLC is a useful, high-throughput method to identify novel polymorphisms and to provide genotyping in a variety of situations. It uses PCR to produce a graph of homoduplex and heteroduplex absorbance as these annealed PCR product moieties elute from a column.

10.2. 5′-Nucleotidase Assays (TaqMan Assays)

The fluorescence energy transfer principle underlies TaqMan genotyping assays. In these, a fluor is separated from a fluorescent quencher to reveal fluorescence characteristic of a particular genotype, based upon the match between the interrogated sequence and a homologous probe (Fig. 5). The homogeneous genotyping assay usually contains one fluor that emits at a particular wavelength for normal and

another that emits at another characteristic wavelength for mutant. However, the TaqMan probe is also capable of accurately quantitating homologous molecules, as well as categorizing SNPs. Quantitation is most often done to measure relative amounts of mRNA in two samples, using real-time RT-PCR. However, amplified genes can also be quantitated using this technology. Large amounts of presenting nucleic acid are characterized either by steeper slopes of ascension to maximum fluorescence or by fluorescence that emerges over a chosen threshold at an early cycle number. When a detection system is accurately calibrated, it is possible to provide for a dynamic range that can accurately measure copy number over several orders of magnitude.

10.3. NASBA or TMA

This technique has many names. It has variously been dubbed self-sustained sequence replication (3SR), transcription-mediated amplification (TMA), and nucleic acid sequence–based amplification (NASBA). Each of these acronyms represents the same basic procedure. The procedure involves (1) reverse transcription of a target sequence; (2) annealing a DNA primer that contains both a transcriptional promoter and a homologous sequence to a target; (3) synthesis of cDNA which incorporates the aforementioned promoter sequence; (4) removal of RNA from the mixture via incubation with Ribonuclease H; (5) transcription from the promoter site; and (5) additional cycles of primer annealing, synthesis of cDNA, removal of RNA from the RNA:DNA hybrid, and synthesis of a second DNA strand. These procedures provide an isothermal but exponential amplification of the target sequence that is roughly as sensitive as PCR. Both Organon Teknika and GenProbe are using varieties of this technology in their respective HIV and HCV detection kits.

10.4. Molecular Beacons

In the method for fluorescence detection first worked out by Fred Kramer and Russell Tyagi, an oligonucleotide probe molecule, dubbed a "beacon," is specifically designed with a central nucleic acid palindrome and derivatized with a fluorescent indicator and a quencher at or near each end (Fig. 6A). When a molecule with homology to the internal palindrome is presented to the beacon, the fluorescent quenching effect produced through interaction of the two energized moieties diminishes. As a result, sequence-specific recognition results in increased fluorescence from the beacon. Following excitation, this emission can be quantified in an appropriate fluorimeter. Molecular beacons have been used to detect a variety of analytes in combination with a variety of amplification methods (M1, T4, T5, V1).

10.5. Sunrise PCR

In Sunrise technology (Fig. 6B), allele-specific primers are designed so that a 3′ mismatch will distinguish between mutant and normal alleles. A quencher and a fluor are incorporated into each of these allele-specific primers. During amplification, the relative proximity of the quencher and fluor changes because of the increasing abundance of sequencees homologous to the region between these two moieties. This allows quenching to diminish and fluorescence to result from the increase in distance between the quencher and fluor. In a more general form of Sunrise, which does not require allele-specific PCR, the sequence at the 5′ end of the Sunrise primers is appended at the 5′ end of any PCR primer. This is dubbed 5′ tailing. Once this sequence is incorporated into the PCR product, the complement serves as the binding site for a "uniprimer." Beginning in the third cycle of PCR, this uniprimer begins to generate a fluorescence. By using the 5′-tailed (unlabeled) primer at about one-tenth the concentration of the labeled uniprimer, most of the PCR product generated at the end of the exponential phase of amplification will contain uniprimer sequence and therefore will be fluorogenic.

10.6. Visible Genetics

Visible Genetics—the Canadian company that gave the world an inexpensive, simplified DNA sequencing device intended for clinical laboratory use—invented a stratified method to locate genetic abnormalities in 1995. This method, developed for locating mutations in the retinoblastoma (Rb) gene, involves multiplexing PCR reactions followed by a sizing to narrow the location of insertion/deletion mutations. Subsequent to sizing, identified regions are sequenced to determine familial mutations. To date, several hundred family-specific mutations in this important cancer-causing gene have been characterized. A distantly related test kit developed by this same manufacturer, and recently submitted for FDA approval, is intended for use in HIV patients experiencing a rebound after a sustained period of successful multidrug therapy. When these patients begin to show renewed symptoms and/or sustained increases in viral load above baseline, sequencing of their virus is recommended. This has therapeutic value in that it can provide a prescribing physician important information on what anti-retroviral drugs may provide the most effective therapeutic course. For this application, a rapid inexpensive DNA sequencing system has been combined with a UNIX-based reference computer platform that provides final, physician-friendly reporting. A large number of HIV mutations have not been rigorously demonstrated to cause anti-retroviral drug resistance; however, this test kit, which provides information understood on the basis of *in vitro* assays, may be important for prescribing physicians.

10.6.1. *Invader*^{2}

Invader^2 is a relatively recent modification of cleavase technology. In it, an oligonucleotide moiety that contains a defined capture sequence hybridizes specifically to an interrogated sequence. The capture sequence is capable of hybridizing with a detector oligonucleotide, called an invader, composed of a capture complementary sequence, a fluorescent quencher, and a fluorescent indicator. The combination of invader and interrogator forms a three-dimensional structure susceptible to cleavage by a proprietary enzyme. This cleavage permits detection of the FRET (fluoresence resonant enegry transfer) probe.

10.7. Microarray Methods. Affymetrix and Hyseq: Resequencing and Sequencing by Hybridization

Hyseq pioneered the use of gantry robots to spot PCR products onto solid supports, probe these using short oligonucleotides, then visualize these arrays subsequent to hybridization. Hyseq has defined and patented this sequencing-by-hybridization (SBH) technology. Identification of previously identified polymorphisms using this technology is referred to as "resequencing" by Hyseq. In patent interference proceedings, Hyseq contends that their SBH concept has been infringed by their competitor, Affymetrix. In the Hyseq protocol, amplified sequences are radiolabeled using 33P-kinase reactions. After hybridization and washing, results are quantified using a phosphorimager, although fluorescent experiments have also been conducted. Alternative platforms developed by Hyseq also include affixation of oligonucleotides onto solid supports by the gantry robot apparatus, followed by interrogation with labeled amplified or cDNA amplified fragments. Affymetrix uses combined solid-phase synthesis, with photolithographic fabrication, to construct high-density oligonucleotide arrays. This is accomplished by using a series of photolithographic masks to define chip exposure sites, followed by stepwise synthetic and blocking reactions. At the end of this process, each probe is located in a predefined position in the array. Individual probe arrays are packaged in injection-molded plastic cartridges, which protect them from the environment and serve as chambers for hybridization.

Once fabricated, Affymetrix arrays are ready for hybridization. Nucleic acid to be analyzed (the target) is isolated, amplified, and labeled with a fluorescent reporter group, usually using terminal transferase. Labeled target is then incubated with the array and stained with fluorescent dye using a proprietary fluidics station and hybridization oven. After hybridization reaction completion, the array is inserted into the scanner, where fluorescence patterns are visualized. The hybridization data are collected as light emitted from the fluorescent reporter groups already incorporated into the target, which are bound at specific sites to the probe array. Probes that most clearly match the target generally produce stronger signals

than those that have mismatches. Since the sequence and position of each probe on the array are known, by complementarity, the identity of the target nucleic acid applied to the probe array can be determined.

10.8. MALDI-TOF Primer Extension

In matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), several synthetic approaches have been used to create short, immobilized single-stranded oligonucleotides attached to a solid support. The qualifying characteristic of these is that they must be able to survive laser irradiation and remain susceptible to rehybridization with newly amplified PCR products. In order to interrogate the genotype of given amplicons, the single-stranded immobilized probes are hybridized with amplified sequences of unknown genotype and incubated with thermosequenase in primer extension reactions. Extended amplified sequences are subjected to MALDI-MS in the presence of a tightly focused laser ($d = 100\ \mu$m). The MS detector allows a precise molecular-weight determination to a resolution within 4–6 daltons. This provides the precision necessary to unambiguously determine extended oligonucleotide sequence, provided the complete extended sequence is under 10 kD.

10.9. Electronic Hybridization and Visualization

On the horizon are two new methods that are at the margin between the microelectronics industry and biotechnology: One is the ability to visualize the results of a molecular test using electronic sensors; the other is the capacity to alter molecular hybridization conditions through the use of electric fields. Each of these is likely to cause a stir among molecular diagnosticians.

Cycling probe technology (CPT) is a nucleic acid detection system based upon signal or probe amplification rather than target amplification as in PCR. It is similar in this respect to bDNA (branched DNA) quantitation technology. CPT relies upon a molar excess of labeled probe that contains a scissile linkage of RNA which may itself act as an electron transfer moiety (ETM) or react in conjunction with an ETM. On hybridization of the probe to the target, the resulting hybrid contains a portion of RNA:DNA. This region of RNA:DNA duplex can be digested by RNAse H, releasing the RNA. This probe cleavage allows one portion to become available for further rounds of hybridization. This portion may specifically hybridize to a single-stranded probe covalently attached via a conductive oligomer to an electrode. This forms a hybridization complex containing a first ETM. Electron transfer between the first ETM and the electrode is then measured. The label is then released and the label detected. Electron transfer accompanying the release can be measured.

Research workers at Nanogen, Inc., in San Diego have demonstrated that controlled electric fields can be used to regulate transport, concentrate, hybridize, and

denature oligonucleotides. They find that discrimination by hybridization among oligonucleotide with varying base compostitions can be achieved through adjustment of the electric field strength on the partner strands. When this approach is used, single base pair mismatch discrimination can be carried out rapidly (<15 sec) and with high resolution.

10.10. No Single Method Is Sufficient to Reliably Type All Single-Nucleotide Polymorphisms

Many research groups are now making efforts to provide a single rapid way to characterize any SNP. At first glance, this appears to be a simple objective. Unfortunately, several groups have found evidence indicating that no one method, short of DNA sequencing, is likely to provide accurate detection for all possible SNPs in a given region of DNA. This is because the proximity of SNPs (several closely clustered) can interfere with the specificity of almost any hybridization probe, including TaqMan probes. In addition, primer extension through GC-rich or repetitive sequences is not foolproof. As a consequence, laboratories wishing to make exhaustive SNP maps in given genomic regions are using at least two genotyping technologies.

11. Post-analytic Component

Upon completion of the analytic test component, a laboratory director can review original data that provides evidence that the assay procedure was run correctly. Customarily, interpreted results are issued under the signature of a clinical laboratory director.

11.1. The Geneticist's Job

Clinical diagnostics laboratories have a solemn responsibility. The results issued by these laboratories provide a basis for life-changing decisions. The interpretation of molecular data discerned in the laboratories must be foolproof. Quality controls must be written into routine test procedures, and quality assurance must be made before any result is released from any test. Basic research laboratories need not meet such stringent QA/QC requirements. However, pharmacogenomics research, a form of clinical molecular diagnostics in which patients may never see the results of studies performed upon them, must adhere to some of the same rigorous controls as a clinical molecular diagnostics laboratory whose results are shared with physician-clients and patients who submit samples for testing.

11.2. Import of Bayesian Statistics

Bayesian statistics are at the heart of genetic counseling. Recently, many referrals for cystic fibrosis testing have come to molecular diagnostics laboratories as a result of abnormal ultrasound reports of meconium ileus in the fetus. For some time it has been clear that there is a relationship between fetal bowel occlusion and cystic fibrosis. Epidemiological studies have established that a fetus in a non-Jewish Caucasian woman has an *a priori* risk of 1/50 (approximately 2%) of carrying a child affected with cystic fibrosis the case of an echogenic bowel. If this woman should test negative, her risk of being a carrier decreases appreciably (to 1/241) while the risk to the fetus also decreases appreciably (1/24,100, if her husband is also CNJ). However, if that mother tests positive for CF (the likelihood of this is 1/25) and the fetus test positive for one mutation (a likelihood of 1/2 if the mother is a carrier), the likelihood that the pregnancy outcome will be a child born with CF can be as high as 1/20 (5%). Some parents consider this a high enough probability to terminate a pregnancy.

11.3. Verbal Communication of Positive Results

Genetic results must be conveyed rapidly, accurately, and directly to the ordering physician by the analytic laboratory. The laboratory director is the titular head and is the only person certain of the results, interpretation, and statistics; it is therefore appropriate that he or she communicate positive results (carrier status found, mutations found) directly. Clinical diagnostics laboratories are strikingly similar to clinical trials labs, where adverse indications for drug testing can now be found on a genetic basis, or soon will be. Research laboratories need to provide genotyping results in a quality-controlled way so that case-controlled or sib-pair and family studies will not be misinterpreted because of poor QA/QC or miscalled genotypes. Each laboratory begins to resemble the other in this respect.

11.4. A Written Verification

In clinical medicine, part of the documentary process is the incorporation of report findings into the patient chart. Verbal communication that a positive result is about to be disclosed must be accompanied by written verification. The father of modern human clinical genetics, Victor McKusick, has recommended that every genetic counseling interview be accompanied by a written report to the patient of results found and consequences for patient, physician, and family. In pharmacogenomic studies, the client is the pharmaceutical company and the principal investigator on the study. The report, though not as individualized as that for study subjects, still needs to be made. Similarly, genotyping research laboratories must do all they can to make correlations of genotype and phenotype for investigators.

Communication of positive results (the finding of a genotype of interest) can make or break a research study. Swift, accurate, assured communication is as crucial for the success of research as it is for the success of clinical medicine.

12. Final Words

Molecular diagnosis is in the midst of a revolution. Characterization of a majority of the point mutations that cause metabolic and childhood hereditary disease is at hand. Deliberations about whether to use this newfound knowledge for prenatal diagnosis are now respected as a matter of personal preference. Assuring the quality of genetic test results has become a matter of public debate, with professional societies and governments beginning to take the regulatory initiative. Experts recognize that technology choices for molecular testing depend upon the particulars of the gene to be tested. Large deletions cannot be assayed using the same technology as point mutations or large triplet base expansions. The analytic phase of genetic testing is now understood to be not the end of the test, but rather the end of the middle portion of testing. In clinical molecular genetics, interpretations are an inextricable part of testing. Risks calculated through the use of Bayesian statistics must be determined and provided to patients, physicians, and counselors at the conclusion of the genetic testing process. In molecular testing for research purposes, data limitations must be addressed: How many sites were tested for mutation within a given gene? Would heterozygous deletions be detected using this genotyping technology? Would sequence changes under an amplification primer be properly visualized? In research as well as in clinical diagnosis, the central role of a well annotated database—one containing easily retrieved, but confidential personal information including ethnicity data, accurate family and medical histories, and diagnostic information—needs to be addressed.

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BIOCHEMICAL HEMATOLOGY OF PLATELETS AND LEUKOCYTES

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1. Introduction

In this chapter we focus on selected aspects of platelet and leukocyte function and dysfunction to illustrate important biochemical pathways. Our approach is not comprehensive, but rather illustrates the complexity of biochemical pathways regulating platelet and leukocyte function in health and disease.

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2. Biochemistry of Normal Platelet Function

Platelets play a key role in hemostasis and thrombosis. Their surface membrane is exquisitely designed for interaction with specific components of the vascular endothelial cell matrix. During hemostasis, circulating platelets rapidly adhere to the exposed vascular subendothelium of damaged blood vessels. Adhesion induces a variety of intracellular biochemical events that lead to platelet aggregation, the secretion of granule contents, and the expression of procoagulant activity to support fluid phase coagulation.

2.1. Platelet Anatomy

Resting platelets exhibit a discoid shape in the circulation. This shape is maintained by the presence of circumferential coils of microtubules arranged just beneath the surface membrane. Upon platelet activation, however, microtubules depolymerize and platelets change their shape to spheres with spiny pseudopodia containing actin filaments.

Unlike its precursor, the megakaryocyte, platelets lack a nucleus and, in turn, the ability to synthesize proteins. Platelets thus represent cytoplasmic fragments of megakaryocytes which contain various constituents in their cytoplasm including mitochondria, glycogen storage granules, alpha granules, dense bodies, lysosomes, and peroxisomes. Following platelet activation, the contents of alpha and dense granules and, to some extent, lysosomal granules can be released and contribute to the growth of the hemostatic platelet plug or pathologic thrombus. Alpha granules are the most numerous cytoplasmic structures and are responsible for platelet staining with Wright–Giemsa stain on peripheral blood smears.

The platelet membrane consists of a unique system of invaginating channels called the open canalicular system. Secretion of platelet granule contents is thought to occur via these channels. Platelets also possess an intracellular system of channels referred to as the dense tubular system. This membrane system is derived from the smooth endoplasmic reticulum of the precursor megakaryocyte, and plays an important role in intracellular calcium fluxes during platelet activation. Indeed, the small (diameter 2–3 μm) resting platelet is equipped with a powerhouse of enzymes, membrane lipids, glycoprotein receptors, and contractile elements, together, these elements orchestrate a sequence of reactions that lead to the formation of platelet plug and the initiation of the coagulation cascade at sites of vascular injury (1).

2.2. Mechanism of Platelet Adhesion and Activation

Glycoprotein receptors on the platelet membrane mediate the adhesion of platelets to damaged blood vessels. The interaction of platelets with von Willebrand

(vW) factor in the vascular subendothelium via platelet membrane glycoprotein Ib (GPIb) is one of the most important events in hemostasis and thrombosis (2, 3). Glycoprotein Ib provides recognition of vWF on the endothelial cell surface and forms a complex with platelet membrane glycoproteins IX and V (GPIb/IX/V complex or CD42). Platelets express approximately 25,000 copies of the GPIb/IX/V complex on their membrane. In addition to vWF, GPIb/IX/V also contains a binding site for thrombin and has been implicated in enhancing the platelet response to low levels of thrombin.

Platelets also interact with a variety of other matrix proteins in the vascular subendothelium, including collagen, thrombospondin, fibronectin, and vitronectin. Platelet membrane integrins play key roles in the recognition of these constituents of the vascular subendothelium. Integrins represent a family of cell surface receptors composed of calcium-dependent heterodimers of alpha (α) and beta (β) subunits. These receptors recognize arginine, glycine, and aspartic acid (RGD) amino acid sequences in their respective ligands, as well as more selective amino acid sequences, there by conferring selectivity to the ligand–integrin interaction (2, 4).

Integrins also have a wide cellular distribution and are involved in cell–cell and cell–matrix interactions. Platelet membrane integrin receptors belong to the beta 1 and beta 3 families of integrins, each having a common beta subunit and a specific alpha subunit. The beta 1 family mediates platelet adhesion by binding to collagen (α_2/β_1), fibronectin (α_5/β_1), and laminin (α_6/β_1) (2, 4), whereas the beta 3 family mediates platelet interactions with fibrinogen, vWF, and fibronectin (α_{IIb}/β_3), as well as vitronectin (α_V/β_3) (2, 5). The beta 3 family of integrins mediates both platelet adhesion and aggregation.

The most abundant member of the platelet beta 3 integrin family is α_{IIb}/β_3, also known as GPIIb/IIIa. Platelets express approximately 40,000–80,000 copies of GPIIb/IIIa on their surface. Although the majority of GPIIb–IIIa is located on the platelet surface membrane, internal pools have been described on the membrane network of the open canalicular system that become expressed upon platelet activation. In addition to mediating platelet aggregation responses in the presence of fibrinogen and vWF, GPIIb/IIIa is also critical for normal clot retraction, a process that consolidates the formed clot at the site of vascular injury. In order for GPIIb/IIIa to bind ligand, it must be activated to a high-affinity configuration (6).

Platelets also express adhesion receptors of the selectin gene family (P-selectin) and of the immunoglobulin gene superfamily (PECAM-1 and ICAM-2) (6). Expression of PECAM-1 and ICAM-2 may play a role in platelet interactions with endothelial cells and leukocytes, respectively.

The selectins are a family of vascular cell surface receptors that are characterized by lectin-like domains at their amino termini, an adjacent epidermal growth factor–like domain, followed by multiple, short consensus repeat units homologous to those of the complement regulatory proteins. Platelet express P-selectin (CD62;

TABLE 1
PLATELET MEMBRANE RECEPTORS

Receptors	Interaction with	Result
1. Glycoprotein		
GPIb/IX/V complex	vW factor Thrombin	Adhesion
GPIV and GPVI	Collagen	Adhesion
2. Integrins		
Beta 1 family		
α_2/β_1	Collagen	Adhesion
α_5/β_1	Fibronectin	Adhesion
α_6/β_1	Laminin	Adhesion
Beta 3 family		
α_{IIb}/β_3 (GPIIb/IIIa)	Fibrinogen	Aggregation
	vW factor	Adhesion
	Fibronectin	
	Vitronectin	
α_V/β_3	Vitronectin	Adhesion
Selectin gene family		
P-Selectin (CD62)	Neutrophils and monocytes on activated platelets	
Immunoglobulin gene superfamily		
PECAM-1	Endothelial cells	
ICAM-2	Leukocytes	

older designations, GMP140 or PADGEM) on the membranes of alpha granules and on the surface membrane following granule secretion. P-Selectin on activated platelets mediates platelet attachment to neutrophils and monocytes (6).

Other platelet membrane glycoproteins that are involved in adhesion reactions are GPIV and GPVI, which play a role in platelet interactions with and activation by collagen (6, 7). GPIV also functions as a receptor for the platelet alpha granule protein thrombospondin (2, 7, 8). Table 1 summarizes the functions of platelet membrane receptors.

Platelet adhesion to vascular subendothelium is accompanied by activation of platelet membrane enzymes such as phospholipase A_2 and C. Phospholipase A_2 activity results in the cleavage of the fatty acid arachidonic acid from the platelet membrane phospholipids. The endothelial cell enzyme fatty acid cyclooxygenase in turn converts arachidonic acid to cyclic prostaglandin endoperoxides—the hydroperoxy PGG_2 and the hydroxy PGH_2. Cyclic endoperoxides are sequentially converted by platelet thromboxane synthetase to thromboxane A_2. Thromboxane A_2 is a strong platelet agonist that activates platelets and initiates the release of platelet-dense granule contents. Adenosine Diphosphate (ADP) is an important platelet-dense granule constituent, which can serve to recruit additional platelets to the site of vascular injury via platelet activation.

Platelets can be activated by a variety of agents including the physiologic agonists ADP, thromboxane A_2, epinephrine, collagen, and thrombin. Platelet activation is generally associated with a change in platelet shape (except for epinephrine-induced platelet activation) from discs to spiny spheres with pseudopodia. Platelet pseudopod formation is dependent on actin polymerization in the activated platelets. The interaction of actin filaments with myosin, mediated by calcium (9), facilitates platelet contractile activity (e.g., clot retraction).

Platelet activation occurs in large part via G protein–coupled agonist receptors and intracellular signaling events that involve activation of phospholipase C (PLC). PLC catalyzes the breakdown of plasma membrane inositol phospholipids, resulting in generation of 1,2-diacylglycerol (DAG) and 1,4,5-inositol triphosphate (IP_3). DAG activates protein kinase C, and IP_3 induces mobilization of calcium from intracellular stores (10).

These signaling events are associated with a conformational change in the platelet integrin glycoprotein IIb/IIIa that allows the receptor to bind to fibrinogen or other RGD-containing ligands with high affinity (11). In addition to arginine–glycine–aspartic acid (RGD), the GPIIb/IIIa complex recognizes specific amino acid sequences present at the carboxy terminus of the fibrinogen gamma chain, lysine–glutamine–alanine–glycine–aspartic acid–valine (KQAGDV). Fibrinogen binding to GPIIb/IIIa is responsible for bridging between adjacent platelets resulting in platelet aggregation (2, 5). Fibrinogen binding to GPIIb/IIIa is generally considered the final common pathway to platelet aggregation. Table 2 provides a list of mediators of platelet activation.

TABLE 2
MEDIATORS OF PLATELET ACTIVATION

Physiologic agonists
ADP
Thromboxane A_2
Epinephrine
Collagen
Thrombin
G protein–coupled agonist receptors
Intracellular signaling events
Activation of phospholipase C
Production of I,2-diacylglycerol (DAG) and 1,4,5-inositol triphosphate (IP_3)
DAG activates protein kinase C
IP_3 mobilizes calcium from intracellular stores
Consequence of intracellular signaling events
Conformational change in GPIIb/IIIa receptor allows binding to fibrinogen through RGD and KQAGDV amino acid sequences
Platelets aggregate

TABLE 3
INHIBITORS OF PLATELET FUNCTION

1. Prostacyclin (PGI) activates adenyl cyclase. Increase in cAMP lowers calcium levels and inhibits platelet activation, aggregation, and secretion of granule contents
2. Nitric oxide
3. Endothelial cell ectoADPase (CD39)

Platelet activation is modulated by a variety of potent endothelial cell inhibitors. Prostacyclin (PGI_2), for example, is produced by endothelial cells when prostaglandin cyclic endoperoxides (PGG_2/PGH_2) are acted upon by the enzyme prostacyclin synthetase. PGI_2 binds to the enzyme adenylate cyclase in the platelet membrane, resulting in the production of cyclic AMP (cAMP) from adenosine triphosphate (ATP). Increases in cAMP lowers platelet cytosolic calcium levels and inhibits platelet activation, aggregation, and secretion of granule contents (2). Other potent inhibitors of platelet function are nitric oxide and the endothelial cell ectoADPase (CD39) (12). Table 3 lists inhibitors of platelet function.

2.3. PLATELET PROCOAGULANT ACTIVITY

The platelet membrane plays an important role in the surface activation of coagulation factors. The platelet membrane phospholipid, phosphatidylserine, which is normally located in the inner leaflet of the platelet membrane, is translocated to the outer surface as platelets are aggregated by strong agonists such as thrombin and collagen. Two major coagulation factor complexes, the tenase complex and the prothrombinase complex, are assembled on this anionic phospholipid, ultimately leading to the generation of thrombin which converts fibrinogen to fibrin and catalyzes the stabilization of the crosslinked fibrin clot (2).

Platelet membrane phosphatidylserine is critical to the formation of the tenase complex since on its surface activated factor VIII ($VIII_a$) generates a high-affinity binding site for activated factor IX (IX_a) in the presence of calcium. Subsequently, this complex activates factor X (2, 13). Platelet membrane phosphatidylserine similarly anchors activated factor V (V_a), favoring the calcium-dependent binding of activated factor X (X_a). The prothrombinase complex is generated on the surface of the anionic platelet membrane phosphatidylserine when factor V_a binds prothrombin. The prothrombinase complex cleaves prothrombin to produce thrombin, which has a multifunctional role (14).

In addition to its procoagulant role, the platelet membrane can also function in anticoagulation by interacting with activated protein C, which inactivates factors V_a and $VIII_a$ (15). Furthermore, platelets may also participate in the regulation of fibrinolysis by binding plasminogen, t-PA, and plasmin (16, 17). Table 4 summarizes the role of platelets in coagulation and anticoagulation.

TABLE 4
PLATELET PROCOAGULANT AND ANTICOAGULANT ACTIVITY

Procoagulant activity
- On surface of platelet membrane phospholipid, phosphatidyl serine forms tenase and prothrombinase complexes
- Tenase complex: $VIII_a + PPL \overset{Ca^{2+}}{\longleftrightarrow} IX_a) \overset{X}{\rightarrow} X_a$
- Prothrombinase complex: $PT \rightarrow V_a + PPL \overset{Ca^{2+}}{\longleftrightarrow} X_a) \overset{PT}{\rightarrow}$ thrombin
  (PPL is platelet phospholipid; PT is prothrombin)

Anticoagulant activity
- Interact with activated protein C, which inactivates V_a and $VIII_a$
- Regulate fibrinolysis by binding plasminogen, t-PA, and plasmin

2.4. MARKERS OF PLATELET ACTIVATION

Several specific markers of platelet activation have been identified. For example, two platelet-specific proteins, platelet factor IV (PF_4) and β-thromboglobulin (βTG), can be released from platelet alpha granules during platelet aggregation (18). Both of these alpha granule proteins can be quantified by specific ELISA assays, and serve as markers of platelet activation and secretion. In addition, CD62 (P-selectin) present in the membrane of platelet alpha granules and CD63 present on the surface of lysosomal membranes can be expressed on the surface membrane of platelets following aggregation and secretion. Both CD62 and CD63 are frequently quantified by flow cytometry using specific antibodies (6). Another marker of platelet activation that is amenable to measurement by flow cytometry is PAC-1 binding. PAC-1 is an IgM anti-GPIIb/IIIa monoclonal antibody that interacts only with the active/high-affinity conformation of GPIIb/IIIa (19).

Flow cytometry with specific anti-platelet monoclonal antibodies is also a powerful method for examining constitutively expressed surface membrane glycoproteins, particularly in patients with suspected congenital deficiencies. Constitutively expressed CD41/61, GPIIb/IIIa, and CD42 (GPIb) have been described previously. Other markers on the surface of the resting platelets include CD36 or GPIV. Although CD41/61 and CD36 expression is increased upon platelet activation, CD42 expression, is downregulated on the surface of the activated platelet (2, 20).

3. Platelet Pathology

3.1. LARGE PLATELETS AND THROMBOCYTOPENIA

3.1.1. *Bernard–Soulier Syndrome*

Bernard–Soulier syndrome (BSS) is a widely studied, albeit rare, disorder whose incidence is approximately less than one in one million. (2, 21, 22). This

autosomal recessive genetic disorder is characterized by the presence of abnormally large platelets seen on peripheral blood smears, mild thrombocytopenia, and prolonged bleeding time. The hallmark of BSS is a defect or congenital deficiency of the platelet glycoprotein Ib/V/IX complex (GPIb/V/IX). GPIb is the receptor for von Willebrand factor (vWF) whereby platelets adhere to the exposed vascular endothelium, and also binds thrombin (2, 21–23).

The GPIb/IX/V complex is composed of four transmembrane polypeptide subunits. GPIb consists of disulfide-linked α and β subunits (GPIbα and GPIbβ). GPIX and GPV are noncovalently associated subunits (21). A short 24–amino acid motif containing highly conserved leucine residues is found in all four polypeptide subunits of the GPIb/IX/V complex (24, 25). These leucine-rich repeats (LRRs) might have a critical role in the assembly of the receptor. Indeed, several mutations found in BSS have been located in the LRRs of the GPIbα and GPIX genes (24). BSS arises from a heterogeneous array of genetic defects leading to decreased or absent glycoprotein expression on the platelet surface. The disorder can be characterized broadly by localization of the defect to the GPIbα gene (chromosome 17ptr-p12), the GPIbβ gene (chromosome 22q11.2), and the GPIX gene (chromosome 3) (24, 26). Several point mutations in the GPIbα coding sequence resulting in BSS have been reported (27, 28). In addition, a dinucleotide deletion in the GPIbα gene resulting in a deficiency of GPIbα on the surface of the platelet membrane was reported in one patient who was homozygous for this deletion mutation (29). Deficiency of GPIbα could also arise from a deletion in a chromosomal region (22q11.2), causing BSS (30). Thus, while many mutations in the GPIbα gene have been reported since the initial report by Nurden and co-workers (31), only five mutations in the GPIX gene, one in the GPIbβ gene, and none in the GPV gene have been reported (31–33). Apparently, the GPV gene is not essential for the expression of a functional GPIb/IX complex (32).

BSS is characterized by a long bleeding time and defective platelet aggregation in response to ristocetin. A variant form of BSS in which the GPIb/V/IX complex is present, but platelets nonetheless demonstrate impairment of ristocetin-induced aggregation (34), has been described. A point mutation in the GPIbα leucine tandem repeat region was noted in this variant form (34).

The severity of thrombocytopenia in BSS is variable, with platelet counts ranging from less than 30 to as much as 200×10^3 μL (<30 to 2000×10^9/L). The platelet lifespan is reduced compared to normal. Clot retraction as well as platelet aggregation in response to agents such as collagen, arachidonic acid, ADP, epinephrine, and thrombin is normal (21, 24). The bleeding time, however, is prolonged. Ristocetin-induced platelet aggregation, as noted earlier, is reduced or absent due to abnormalities in the platelet GPIb/V/X complex (34). The laboratory findings in BSS correlate with molecular defects affecting the expression or function of the platelet GPIb/V/IX complex (21, 35).

In addition, ultrastructural changes have been observed in megakaryocytes of patients with BSS, including an irregularly spaced demarcation membrane system (DMS) and disorganization of microtubules (21, 36). Ultrastructural changes in platelets impart a Swiss-cheese appearance to some of the BSS platelets (37). These changes are strikingly prominent and are reflected in an increase in the number of the surface-connected, dense tubular systems (21, 37). Apparently, normal megakaryocyte maturation and platelet size are dependent on a functional GPIb/IX complex (38). The reduced platelet lifespan and, in turn, the thrombocytopenia seen in BSS may be related to decreased platelet-surface sialic acid expression due to decreased expression of the sialic acid–rich GPIb subunit (39).

3.1.2. *Montreal Platelet Syndrome*

Markedly severe thrombocytopenia with platelet counts in the range of $5–40 \times 10^3/\mu L$ ($5–40 \times 10^9/L$) has been reported in a condition called Montreal platelet syndrome (MPS), which has been reported in three generations of Canadian families (40). In addition, patients have prolonged bleeding time and display spontaneous platelet aggregation with reduced response to thrombin (41) and normal platelet membrane glycoproteins. The calcium-activated neutral proteinase, calpain, which is normally involved in the cleavage of the cytoskeleton proteins, especially the actin-binding protein and talin, may be responsible for spontaneous platelet aggregation by exposure of platelet binding sites to adhesive proteins (41). However, the exact mechanism of the relationship between spontaneous platelet aggregation and severe thrombocytopenia seen in MPS remains to be established.

3.1.3. *Gray Platelet Syndrome*

Lack of alpha granules in platelets results in a condition called gray platelet syndrome (42, 43). Patients have variable degrees of thrombocytopenia, with platelet counts ranging from as low as $20 \times 10^3/\mu L$ ($20 \times 10^9/L$) to normal values. Patients also have a prolonged bleeding time, and large agranular platelets, grayish to gray-blue in appearance, are seen in the peripheral blood smear stained with Wright–Giemsa stain (21). Apparently, there is a defect in megakaryocytes interfering with the ability to package the constituents commonly seen in alpha granules such as thrombospondin, platelet factor 4, vWF, beta thromboglobulin, and fibrinogen (44). Although, dense granule contents such as ADP and serotonin are normal, their release following platelet activation with collagen or thrombin is reduced to varying degrees, suggesting that alpha granules may be involved in modulating the release of dense granule contents (45–48).

3.1.4. *May–Hegglin Anomaly*

Ultrastructural changes and giant platelets are seen in May–Hegglin anomaly. An alteration in the platelet microtubule system affects platelet structure, although platelet aggregation and interaction with vWF is normal. The lack of platelet

spreading and defective pseudopod formation seen in this condition can be explained by the fact that the platelet microtubules play a critical role in maintaining the discoid form of platelets and controlling platelet shape and pseudopod formation (21, 49).

A characteristic of the May–Hegglin anomaly is the presence in platelets (stained with Wright–Giemsa) of neutrophil inclusions called Dohle bodies, which are spindle-shaped and appear bright blue. These inclusions are found in the periphery of the cytoplasm (50). Thrombocytopenia is mild to moderate, with platelet counts in the range of 60–100 $\times 10^3/\mu$L (60–100 $\times 10^9$/L). With some exceptions, most patients have a mild bleeding tendency, with the amount of bleeding dependent on the extent of thrombocytopenia (21). Myocardial infarction due to coronary artery thrombosis has been reported (51). Large platelets may compensate for thrombocytopenia in terms of hemostasis (52).

3.1.5. *Fetchner Syndrome*

This is another condition in which cytoplasmic inclusions are noticeable in platelets on Wright–Giemsa stained peripheral blood smears. Fechtner syndrome is also characterized by renal disease (nephritis) and deafness (53). The cytoplasmic inclusions appear smaller and pale blue in contrast to those seen in the May–Hegglin anomaly. Large platelets are seen in Fechtner syndrome, with thrombocytopenia in the range of 30–90 $\times$ $10^3/\mu$L (30–90 $\times$ 10^9/L) (21). Ultrastructural changes are seen in megakaryocytes characterized by abnormal accumulation of demarcation membranes, leading to ineffective platelet production and resulting thrombocytopenia (54).

Thus, a spectrum of molecular defects in platelets can lead to the production of giant platelets with varying degrees of thrombocytopenia. Platelet glycoprotein complexes such as GPIb/IX complex are crucial in ensuring normal megakaryocyte maturation and regulating platelet size. As noted earlier, the lack of GPIb/IX complex leads to the production of giant platelets in Bernard–Soulier syndrome. In contrast, a deficiency of GPIIb/IIIa complex does not result in the production of giant platelets as noted in Glanzmann's disease (55).

3.1.6. *Other Congenital Platelet Defects*

3.1.6.1. *Glanzmann's Thrombasthenia (GT).* In contrast to BSS, Glanzmann's thrombasthenia is an autosomal recessive bleeding disorder in which platelets fail to aggregate in response to many agonists, including ADP, epinephrine, collagen, arachidonic acid, and thrombin, but respond normally to ristocetin. Clinical manifestations of the disease are due to the absence, reduction, or dysfunction of GPIIb/IIIa. Two types of GT are generally recognized. In Type I GT, no platelet membrane GPIIb/IIIa complexes are detectable, unlike Type II GT, in which markedly reduced levels of GPIIb/IIIa are present (10–20% of normal). In addition GT variants have been described in which GPIIb/IIIa expression is low or

normal but is dysfunctional. A variety of point mutations and deletions associated with both GPIIb and GPIIIa have been associated with GT (26). Patients with GT have normal platelet counts, platelet size, and platelet survival.

3.1.6.2. *Dense Granule Storage Pool Deficiency (SPD).* SPD is defined as a deficiency of dense bodies in megakaryocytes and platelets. SPD is characterized by variable bleeding diathesis, prolonged skin bleeding time, normal platelet morphology when stained by Wright stain, and a normal platelet count. SPD may occur alone or in conjunction with other congenital disorders. The basis of the deficiency of dense granules in humans has not been established (26). The absence of dense granules can be confirmed by electron microscopy. Functional assays such as diminished ATP secretion following platelet aggregation may be used clinically to rule out the diagnosis.

Table 5 lists causes of platelet pathology.

3.2. Therapeutic Approaches to Treat Severe Thrombocytopenia

While platelet transfusion is effective in replacing platelets, high-dose recombinant factor VII_a therapy has recently been used to correct bleeding in patients with severe thrombocytopenia, as well as congenital platelet function defects (56). The mechanism of action of factor VII_a in this setting is under investigation. Conceivably, VII_a may act on platelets in the absence of tissue factor to increase thrombin generation by activating factors IX and X. This production of thrombin on the surface of a few platelets at the site of vessel wall injury may result in production of fibrin, which could recruit additional platelets either directly or indirectly via interaction with vWF. The efficacy of factor VII_a in the treatment of bleeding resulting from intrinsic platelet defects requires further investigation.

TABLE 5
Causes of Platelet Pathology

Large Platelets and Thrombocytopenia
- *Bernard–Soulier syndrome:* Defect or congenital deficiency in GPIb/IX/V complex
- *Montreal platelet syndrome:* Apparent defect in calpain, a calcium-activated proteinase protein that may be responsible for spontaneous platelet aggregation
- *Gray platelet syndrome:* Lack of alpha granules
- *May–Hegglin anomaly:* Alteration in platelet microtubule system; affects platelet structure; platelet cytoplasm contains neutrophil inclusions (Dohle bodies)
- *Fetchner syndrome:* Ultrastructural changes in megakaryocytes; smaller cytoplasmic inclusions in platelets

Other Congenital Platelet Defects
- *Glanzmann's thrombasthenia:* Autosomal recessive bleeding disorder; absence, reduction, or dysfunction of GPIIb/IIIa complex
- *Dense granule storage pool deficiency:* Deficiency of dense granules in megakaryocytes and platelets

3.3. Autoantibodies Directed to Platelet Antigens

In a condition called chronic immune or idiopathic thrombocytopenic purpura (ITP), autoantibodies are produced against platelet surface antigen complexes (57–59). Approximately 75% of patients with ITP present with autoantibodies directed to either the GPIIb/IIIa complex or the GPIb/IX complex on platelets or to both of them (58, 59). Apparently, cation-binding epitopes on GPIIb/IIIa are involved in the binding of autoantibodies in ITP (60). These epitopes may be on an intact GPIIb/IIIa complex or they may reside in antigens close to the calcium-binding sites on GPIIb (61). The primary site of autoantibody production in ITP is the spleen. The presence of antibody in splenectomized patients suggests that the bone marrow is also involved in autoantibody production in ITP (61). Autoantibodies in ITP bind not only platelets but also megakaryocytes, thus affecting both platelet destruction and production (61).

Platelet destruction in ITP occurs when autoantibodies directed against platelets form immune complexes with platelets, which, in turn, bind to the Fc activating receptor (FcγRIII) on macrophages, crosslinking them in the process (62). The crosslinking of FcγRIII on the macrophages elicits within the macrophages activating signals such as the activation of the enzyme phosphatidylinositol 3-kinase (PI3K) which converts phosphatidylinositol biphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 activates macrophages, resulting in their phagocytosing the autoantibody-bound platelet immune complexes.

Intravenous infusion of gammaglobulin (IVIG) is a therapeutic approach to inhibit the platelet destruction of ITP. Apparently, IVIG stimulates macrophages, at least in a murine model of immune thrombocytopenia, to express the inhibitory Fc receptor (FcγRIIB) (63). Since autoantibody-bound platelet immune complexes can bind to both the Fc activating (FcγRIII) and Fc inhibitory (FcγRIIB) macrophage receptors, they can effect the crosslinking of these receptors. The crosslinking of the activating and inhibitory receptors results in the recruitment of an SH-2 containing inositol phosphatase (SHIP), which serves as a signaling molecule that dephosphorylates PIP_3 back to PIP_2. The removal of PIP_3, the molecule that initiates the phagocytosis of autoantibody–platelet immune complexes, results in the cessation of platelet destruction and depletion. Blocking the activating Fc receptor on the monocytes (FcγRIII) with high-affinity anti-receptor antibodies is just as effective in preventing platelet destruction as increasing the number of inhibitory receptors (FcγRIIB); thus, the regulation of phagocytosis by monocytes is apparently controlled by the balance between the activating and inhibitory Fc receptors (63). Fc activating receptor blockage can, in addition, be accomplished by the administration of anti-Rh D antibodies, thus inducing competition between the anti-D–opsonized erythrocytes and autoantibody–platelet immune complexes for binding to FcγRIII (63). Blockage of Fc activating receptor can also be accomplished with the infusion of just the Fc fragment of IVIG. Further

studies are required to clarify the mechanism of action of this therapy, including the role of cytokines and their modulating effects on macrophage signaling.

3.4. Thrombotic Thrombocytopenic Purpura (TTP)

TTP is a disseminated form of thrombotic microangiopathy. Hemolytic uremic syndrome (HUS) is a disease very similar to TTP. Both diseases are associated with thrombocytopenia, microangiopathic hemolytic anemia, and fluctuating neurologic signs and symptoms, as well as renal dysfunction and fever (64). In TTP the presence of platelet aggregates in the microcirculation of the brain has been reported in 50–71% of the cases (65, 66). Whereas the kidney is the primary site of platelet aggregation in HUS, extra-renal sites may also be involved. Likewise, in TTP some degree of renal dysfunction has been described in 50–75% of the thrombotic episodes, making it difficult to make a clearcut distinction between TTP and HUS (65–67).

The pathogenesis of TTP is complex, and possibly involves a variety of mechanisms associated with endothelial cell and platelet activation and injury. Recently, however, the deficiency of vWF cleaving protease activity has been identified as a hallmark of TTP (67, 68). In normal individuals, vWF is secreted from platelets and endothelial cells as variously sized multimers, resulting from the crosslinking of vWF monomers by disulfide bonds into multimers containing a minimum of 2 to a maximum of 50–100 monomer subunits.

Almost all patients with TTP have a deficiency in protease activity. As a result, TTP is associated with the presence of unusually large vWF (uLvWF) multimers in the circulation. These uLvWF multimers can bind to GPIb/IX receptor complex on platelets under conditions of high shear stress in the microcirculation and can also bind to the GPIIb/IIIa receptor on activated platelets, causing aggregation of platelets and subsequent blood vessel occlusion (69).

Excessive release of uLvWF multimers resulting from injury to the renal arterial endothelial cells can also cause platelet aggregation in the renal microvasculature, precipitating the onset of the hemolytic uremic syndrome (67). Interestingly, patients with diagnosed HUS have normal activity of vWF-cleaving proteases, despite the presence of uLvWF multimers in plasma (64). Given the clinical difficulty in distinguishing HUS and TTP, the assay of vWF-cleaving protease activity may be helpful.

3.5. Emerging Concepts of Platelet Activation: Tyrosine Kinase Phosphorylation

In addition to platelet activation by a variety of G protein (guanine nucleotide binding proteins)–coupled surface membrane receptors, including receptors for

thrombin, ADP, epinephrine, and serotonin (10), the engagement of platelet integrins also results in signaling events. These events are largely referred to as outside–in signaling events and involve platelet tyrosine kinase activity and platelet cytoskeletal rearrangements (70). Phosphorylation of protein tyrosine kinases allows their interaction with adaptor molecules such as Grb2, Shc, and Vav, which contain SH2 and SH3 Src homology domains that interact with phosphotyrosine and polyproline sequences, respectively (71).

After the initial binding of ligand to platelet integrin receptors, the protein tyrosine kinase pp72 syk is phosphorylated. Temporally, this is followed by phosphorylation of the major tyrosine kinase in platelets, pp-60src (Src), which accounts for 0.3% of the total protein content of platelets, and appears to be involved in consolidating platelet aggregation (72, 73). Tyrosine phosphorylation of pp125FAK occurs when integrins form complexes analogous to mature focal adhesions. Although tyrosine phosphorylation is emphasized here because it is such a prominent early response to integrin ligation, especially fibrinogen binding to GPIIb/IIIa, a growing list of serine–threonine protein kinases and phosphatases, lipid kinases, adaptor molecules, and other proteins have been identified in focal adhesions or in the membrane skeleton of aggregated platelets (70).

Finally, platelets can be activated by immune complexes following binding of immunoglobulin G (IgG) Fc domains to platelet Fcγ RII. This process involves tyrosine phosphorylation of a motif designated as ITAM (immunoreceptor tyrosine-based motif) found on the cytoplasmic domain of platelet Fcγ RII receptors (73). This may play a role in immune complex diseases, particularly in heparin-induced thrombocytopenia.

4. Biochemical Basis for Neutrophil Function

Briefly, the function of neutrophils is to phagocytose invading pathogens such as bacteria or other foreign material (e.g., a uric acid crystal found in a gouty knee joint). Once pathogenic material has been phagocytosed, it is degraded in lysosomal granules within the neutrophil via lysosomal enzymes such as lysozyme and myeloperoxidase. Energy derived from glycolysis and the pentose monophosphate pathway within the neutrophils is required for phagocytosis and lysosomal fusion. NADP (nicotinamide adenine dinucleotide phosphate) formed in the pentose pathway is regenerated by NADPH oxidase enzyme, producing NAD, the oxidized form of the coenzyme and hydrogen peroxide (H_2O_2). Thus, the energy burst in neutrophils, derived significantly from the pentose monophosphate pathway, results in the production of H_2O_2, which reacts with myeloperoxidase released from the lysosome and, in the presence of halides such as chloride or iodide, effects the killing of bacteria.

TABLE 6
KEY EVENTS IN PHAGOCYTOSIS BY NEUTROPHILS

- Phagocytosed organism fuses with lysosome within the neutrophil.
- Lysosomal enzymes: Lysozyme and myeloperoxidase (MPO) are released.
- Energy burst in the neutrophils derived significantly from the pentose monophosphate pathway generates NADP, NAD, and H_2O_2.
- MPO in the presence of chloride converts H_2O_2 to toxic hypochlorous acid, which in turn is converted to chlorine.
- Lactate produced during glycolysis lowers pH and assists in pathogen killing by lysosomal enzymes optimal at acid pH.

Specifically, in the presence of chloride, myeloperoxidase converts H_2O_2 to toxic hypochlorous acid ($HoCl^-$) or bleach, which in turn is converted to chlorine. Lactate produced during glycolysis results in the lowering of pH, thus assisting further the destruction of the intruding pathogen by lysosomal constituents which is optimal at an acid pH. H_2O_2 can also be generated by other reactions such as the action of superoxide dismutase on superoxide anion, which itself is formed when NADPH oxidase catalyzes the monovalent reduction of oxygen. The regeneration of NADP can also be achieved when H_2O_2 reacts with reduced glutathione and the enzyme glutathione peroxidase to produce oxidized glutathione. The latter can react with an NADPH-linked glutathione reductase to generate NADP. Thus, the continual supply of NADP for the pentose monophosphate pathway, and hence the maintenance of phagocytic efficiency of the neutrophil, is achieved by several enzyme systems within the neutophil (74).

Table 6 recapitulates key events in phagocytosis by neutrophils.

4.1. INITIATION OF THE ACUTE PHASE RESPONSE

Neutrophils utilize blood as a vehicle to travel to the site of infection. They marginate along the capillary and venule walls in the vicinity of target tissue. Neutrophils possess a cell-surface molecule called L-selectin, which recognizes carbohydrate components such as sialyl-Lewis^x, an adhesion molecule on the vascular endothelium. As the neutrophil rolls along the vascular endothelium, the binding of L-selectin on the neutrophil surface to sialyl-Lewis^x on the vascular endothelium arrests its movement (75, 76). The activated neutrophil sheds L-selectin from its surface and replaces it with other cell adhesion molecules such as integrins. During the acute phase response, inflammatory mediators such as bacterial lipopolysaccharide and cytokines, interleukin-1, and tumor necrosis factor α induce the expression of E-selectin on the blood vessel wall. Integrins on the surface of activated neutrophils bind E-selectin on the blood vessel wall, facilitating the entry of the neutrophil into tissue spaces via a process referred to as diapedesis. A variety of inflammatory mediators such as complement components, leukotrienes,

histamine, prostaglandins, and particularly chemokines (a group of chemoattractant cytokines) serve to recruit the activated neutrophil through the vessel wall to the tissue site of infection. The phagocytosis of bacteria at the infected site by activated neutrophils is facilitated by its recognition of complement (C3b) coated bacteria (76).

4.2. Biochemical Defects in Neutrophils

Primary defects in neutrophils expose individuals to recurring infections. These defects may be due to a variety of causes such as neutrophil adhesion defects, defects in phagocyte function, and other causes.

4.2.1. *Neutrophil Adhesion Defects*

4.2.1.1. *Leukocyte Adhesion Deficiency Type 1.* This condition results from loss of β_2 integrin adhesion molecules found on neutrophils. There are three β_2 integrins on neutrophils, and they possess a common β chain known as CD18 but different specific α chains. Defects in CD18 are responsible for the lack of β_2 integrin adhesion molecules on neutrophils (77). Due to this adhesion defect, the binding of neutrophils to the adhesion molecules on endothelial cells is compromised, thus affecting the exit of the neutrophils from blood to the infected tissue site. Hence, the neutrophil count in patients with leukocyte adhesion deficiency type 1, even in the absence of infection, can approximate twice the normal count (78). Aggregation of neutrophils is prevented in this condition (79). Patients are susceptible to recurrent infections of the respiratory, urogenital, and gastrointestinal systems and may suffer from severe periodontitis. Early death in childhood may occur in subjects with undetectable levels of CD18, while those with CD18 levels in the range of 1–10% of normal may survive to 40 years or longer (80).

4.2.1.2. *Leukocyte Adhesion Deficiency Type 2.* Patients with this condition have a defect in the transport or production of the carrier molecule for the carbohydrate L-fucose (guanosine disphosphate L-fucose). The lack of fucose affects the ability of neutrophils to interact with ligands on endothelial cells such as P-selectins and E-selectins (81). Patients are susceptible to recurrent infections similar to those afflicting patients with type 1 leukocyte adhesion deficiency, have periodontal problems, and, in addition, may exhibit growth retardation and neurologic defects. Treatment with oral fucose has been known to be effective in reducing the frequency of infections (80).

4.2.1.3. *Guanine Triphosphatase (GTPase) Deficiency in Neutrophils.* The Ras-related C3 botulinum toxin substrate (Rac2), a predominant GTPase in neutrophils, is critical to the functioning of the actin cytoskeleton. Rac2 deficiency affects chemotaxis of neutrophils toward bacterial targets. This condition was

TABLE 7
NEUTROPHIL ADHESION DEFECTS

Type and effects	Cause
1. Leukocyte adhesion deficiency type 1: Patients are exposed to recurrent infections and teeth problems such as periodontitis.	Loss of β_2 adhesion molecules (CD18) on neutrophils. Affects binding of neutrophils to adhesion molecules on endothelial cells. Exit of neutrophils from blood to tissue site of infection prevented.
2. Leukocyte adhesion deficiency type 2: Same clinical problems as in type 1. In addition, patients have growth retardation and neurologic defects. Treatment with oral fucose reduces frequency of infections.	Defect in transport or production of guanosine diphosphate L-fucose. Without the carbohydrate L-fucose, interaction of neutrophils with P- and E-selectins on endothelial cells is affected.
3. Rac2 deficiency: Patients are subject to recurrent infections.	Rac2 (predominant GTPase in neutrophils critical for functioning of actin cytoskeletion) deficiency affects chemotaxis of neutrophils toward bacterial targets.

reported in a 5-week-old boy with signs typical of leukocyte adhestion deficiency (79, 82).

Table 7 reiterates the causes and effects of neutrophil adhesion defects.

4.2.2. *Defects in Phagocyte Function*

4.2.2.1. *Chronic Granulomatous Disease (CGD).* In this disease there is a defect in any one of the four subunits of neutrophil NADPH oxidase which catalyzes the monovalent reduction of oxygen to superoxide anion, which in turn serves as a substrate for H_2O_2. Hence, in CGD, the neutrophils are deficient in H_2O_2 production, and are defective in destroying invading pathogens. The components of NADPH oxidase are the 91-kD glycoprotein ($gp91^{phox}$) and the 22-kD protein ($p22^{phox}$), both of which are neutrophil membrane–bound and react with the cytoplasmic components of the 47-kD protein ($p47^{phox}$) and the 67-kD protein ($p67^{phox}$). In nearly 70% of patients with CGD, there is an X-linked mutation in the gene for $gp91^{phox}$ (83). A mutation in the gene for the cytoplasmic $p47^{phox}$, which is autosomal recessive, is seen at a lesser frequency compared to the mutation in the gene for $gp91^{phox}$ (83).

Defective phagocytosis by neutrophils in CGD can be demonstrated by the classical nitroblue terazolium test by the absence of dark-blue staining of the cytoplasm, in contrast, normal neutrophils with efficient phagocytic function exhibit dark-blue cytoplasmic staining (84). Flow cytometry is also a powerful tool for the study of CGD (85).

Patients with CGD are susceptible to recurrent infections by catalase-positive microorganisms such as *Staphylococcus aureus*, *Serratia marcescens*, *Aspergillus*

and *Nocardia* species, and *Burkholderia cepacia* (79). These catalase-positive organisms can inactivate their endogenous H_2O_2, and thus are resistant to the CGD H_2O_2-deficient neutrophil attack. In contrast, catalase-negative organisms such as pneumococci and beta hemolytic streptococci, by providing their endogenous H_2O_2 to the CGD-deficient neutrophils, contribute to their own demise. As such, CGD patients rarely have infections with catalase-negative organisms (74).

In addition to infections affecting various organs, patients with CGD are susceptible to excessive formation of granulomas that can obstruct the gastrointestinal and genitourinary tracts. Granulomas resolve upon intravenous corticosteroid therapy (86). Children with CGD resulting from an X-linked mutation have an earlier onset of the disease and are more susceptible to frequent infections and obstructive granulomas than are children with autosomal mutations (79). Mortality rate is also higher in the X-linked form of CGD. Female carriers of X-linked CGD who have pronounced inactivation of the X-linked chromosome are protected from infections by catalase-positive organisms, since these individuals have NADPH oxidase activity at approximately 10% of normal levels and presumably generate some H_2O_2 to effect intracellular killing of pathogens (79).

Treatment with interferon gamma has been especially effective in reducing the incidence of infections in patients with CGD (87). The frequency of life-threatening bacterial infections in patients with CGD is reduced by daily prophylactic treatment with trimethoprimsulfamethoxazole (86).

4.2.3. *Other Causes of Neutrophil Dysfunction*

These include defects in neutrophil granules, mutations, and signaling defects.

4.2.3.1. *Myeloperoxidase Deficiency.* In the presence of halides such as chloride, Myeloperoxidase, a lysosomal enzyme converts H_2O_2 to toxic hypochlorous acid ($HOCl^-$) or bleach, which in turn is converted to chlorine, and thus assists in the killing of bacteria. This enzyme, which is the principal component of primary or azurophilic granules, may be completely missing, or the enzyme may be structurally defective, thus affecting its function (88). Mutations in the gene coding for the enzyme on chromosome 17 have been reported which might affect its transcription or lead to defects in post-transcriptional processing (89). Although the deficiency of myeloperoxidase is the most common inherited disorder of neutrophils, its deficiency is not usually associated with the appearance of clinical disease (79). However, when myeloperoxidase deficiency is found together with diabetes mellitus, such patients are susceptible to disseminated candidiasis (90).

4.2.3.2. *Neutrophil-Specific Granule Deficiency.* In this rare disorder, secondary or specific granules in neutrophils are absent. The defect may arise from a mutation that leads to the loss of function of the transcription factor CCAAT/enhancer binding protein ε (C/EBPε), which is needed for neutrophil response to inflammation (91). Specific granule deficiency affects the migration of neutrophils.

Morphologically the neutrophils display a notched nucleus and are hyposegmented (79). Patients are susceptible to recurrent serious infections of the skin and lungs.

4.2.3.3. *Chediak–Higashi Syndrome.* In this rare disorder, death usually occurs in infancy or childhood as a result of recurrent bacterial infections. Giant granules are found in all cells containing lysosomes. The defect has been attributed to a mutation in the gene LYST which codes for a cytoplasmic protein regulating vacuole formation and function (92). The neutrophils of patients with this syndrome display an abnormal fusion of azurophilic or primary granules with specific or secondary granules. Bacterial killing is compromised since the fusion of the giant lysosomal granules with the phagocytosed bacteria is delayed (93). Chemotaxis of neutrophils is also affected, apparently due to a defect in the microtubules.

Peripheral neuropathy is seen in patients with this syndrome, which may be related to abnormal axonal transport, as a consequence of a defect in microtubules (79). Patients are susceptible to recurrent infections, especially with *Staphylococcus aureus* and beta-hemolytic streptococci.

Table 8 summarizes defects in neutrophil function.

TABLE 8
DEFECTS IN PHAGOCYTE FUNCTION OR NEUTROPHIL GRANULES

Defect	Description
Phagocyte function defects	Neutrophils deficient in H_2O_2
Chronic granulomatous disease (CGD)	Killing of pathogens affected
	Defect due to mutation in neutrophil enzyme NADPH oxidase gene coding for any one of the four subunits of the enzyme
	Patients subject to recurrent infections by catalase-positive organisms and to granuloma formation
	X-linked mutation more severe
Defects in neutrophil granules	Enzyme completely missing or defective, affecting its function
Myeloperoxidase (MPO) deficiency	Mutation in gene coding for MPO on chromosome 17
	Diabetes patients with MPO deficiency subject to *Candida* infections
Neutrophil specific granule deficiency	Specific granules in neutrophils absent
	Mutation affects function of transcription factor needed for neutrophil response
	Migration of neutrophils affected
	Skin and lung infections in affected patients
The Chediak–Higashi Syndrome	Giant granules in lysosomes due to mutation in gene controlling protein regulating vacuole formation
	Fusion of giant lysosomal granules with bacteria delayed
	Chemotaxis of neutrophils affected
	Patients susceptible to staphylococcal infections

4.2.3.4. *Mutations and Signaling Defects: Cyclic Neutropenia.* In this condition, periods of very severe neutropenia, with neutrophil counts of less than 0.2×10^9/L, typically last 3–6 days of every 21-day period. The cycles may be longer, ranging from 14 to 36 days in approximately 30% of patients (94). During periods of cyclic severe neutropenia, patients are susceptible to bacterial infections that could even be fatal. Patients with cyclic neutropenia apparently have a mutation in the neutrophil elastase gene (ELA2). Elastase is released from neutrophils during inflammation and is responsible for destruction of tissue (95). While the link between neutrophil elastase and cyclic neutropenia is not clear, one possibility is defective signaling by granulocyte stimulating factor that could alter the normal control of neutrophil numbers and lead to cyclic neutropenia (96).

4.2.3.5. *Mutations in Signaling Molecules.* Neutrophils are dependent on cascading events that occur during the immune response to invading pathogens. Interleukin-12 (IL-12) is produced by dendritic cells and macrophages when they encounter a foreign pathogen. IL-12 in turn promotes T cells and natural killer cells to produce interferon-γ (IFN-γ). The latter, by binding to INF-γ receptor, activates macrophages and neutrophils to produce tumor necrosis factor α and activate enzyme NADPH oxidase, which in turn initiates reactions that lead to the production of H_2O_2 and to the death of the offending pathogen (97).

Normally, the IFN-γ receptor consists of a ligand-binding chain and a signaling chain. Binding of IFN-γ to the ligand-binding chain of the receptor initiates the signaling process. Mutations have been found in the genes coding for both the ligand binding and the signaling chains of the IFN-γ receptor. Mutations that result in a complete loss of the ligand-binding chain expose children to fatal, atypical mycobacterial infections.

Children do not respond to treatment with high doses of IFN-γ and may present with failure to form granulomas (98). The disease is less severe in patients with a mutation that results in a partial loss of the ligand-binding chain. These patients respond to therapy with high-dose INF-γ and are also able to form granulomes (99).

Since the production of IFN-γ is dependent on the production of IL-12, mutations in the β_1 chain and the signal transducing β_2 chain of the IL-12 receptor, as well as in the P40 subunit of IL-12, can disrupt the IFN-γ–IL-12 axis, and can expose subjects to atypical mycobacterial disease (100, 101). Patients with such mutations, however, respond to therapy with INF-γ (102).

Thus a variety of biochemical defects can compromise the phagocytic function of neutrophils (see Table 8).

5. Lymphocytes and Their Immune Function

Lymphocytes and cells of the myeloid lineage develop from primordial stem cells in the fetal liver and bone marrow. Their development is affected by interaction

with stromal cells such as fibroblasts and cytokines including stem-cell factor and colony-stimulating factors (76).

The B lymphocyte or B cell matures within the bone marrow. The T lymphocyte or T cell migrates to and develops within the thymus. The primitive T cell produced by the bone marrow is processed by the thymus, where only those T cells that can recognize and bind to the peptide sequence presented by the major histocompatability complex (MHC) on the thymic epithelium are selected, while those cells which either do not recognize the MHC peptide or which are strongly reactive are removed (103). This process of positive and negative selection of primitive T cells presented to the thymus eliminates 99% of the cells by apoptosis (programmed cell death), leaving behind barely 1% of the cells to reach maturity. The mature T cell leaving the thymus is equipped with a cell surface receptor containing the marker CD3 together with either CD4 or CD8 as an accessory molecule. (CD stands for "cluster of differentiation," a term that arose from the fact that cell-surface molecules were originally characterized based upon their reactivity to panels or clusters of monoclonal antibodies. The number after CD specifies a particular cell-surface molecule.)

CD4 T cells act as helper T cells and recognize viral or bacterial antigens presented together with the MHC Class II molecules on the surface of an antigen-presenting cell such as a macrophage. This interaction results in CD4 T cells secreting cytokines and initiating a cascade of immune reactions. There are two major types of CD4 T cells: Type 1 (Th1) helper T cells secrete the cytokines, such as interleukin 2 (IL-2) and interferon-γ (IFN-γ); Type 2 (Th2) helper T cells secrete interleukin-4, 5, 6, and 10. The products of Th1 and TH2 helper cells are mutually inhibitory.

IFN-γ produced by Th1 cells in response to IL-12 elaborated by antigen-presenting cells activates macrophages, thus facilitating the killing of the ingested foreign pathogen. Thus, Th1 cells facilitate cell-mediated immunity by activating macrophages and by promoting T cell–mediated cytotoxicity. Th2 cells, however, assist B cells to produce antibodies (104).

CD8 T cells function as cytotoxic cells by recognizing viral peptide antigens presented together with MHC Class I molecule on the surface of the infected cell. CD8 T cells are well equipped to kill the infected cell by injecting enzymes (granzymes) through the infected cell membrane by creating pores with the insertion of molecules called perforins. One of the inserted enzymes can induce apoptosis of the infected cell. Cytotoxic CD8 T cells can also bind to the apoptosis-inducing molecule Fas on target cells by its own ligand (Fas ligand) and effect apoptosis. CD8 T cells can also produce a number of cytokines such as tumor necrosis factor α, lymphotoxin, and IFN-γ (104).

The B cell goes through several stages of development, beginning with the Pro-B cell, followed by the Pre-B cell, which in turn matures into a B cell bearing a mature immunoglobulin-M (IgM) B cell receptor on the cell surface. The population of

these early B cells expresses an adhesion and signaling cell-surface molecule called CD5. As these "CD5" lymphocytes mature, and before they encounter a foreign antigen, they shed the CD5 marker and become B2 cells, which coexpress IgM and IgD molecules on their cell surface. As B2 cells encounter a foreign antigen, they further differentiate, in the presence of co-stimulatory signals, either into plasma cells which produce class-specific antibodies (IgG, IgA, or IgE) or into a memory cell (76).

Antibodies such as IgM or IgG, upon binding to a foreign antigen, make their Fc regions available for complement activation. The classical complement cascade is triggered by IgG or IgM Fc binding to the first component of complement, C1. Through a series of enzymatic reactions, the third component of complement C3 is cleaved. The resulting larger C3b subunit covalently binds to the invading pathogen (opsonization) and allows phagocytosis via specific C3b receptors on neutrophils and macrophages.

In the absence of complement, the foreign pathogen coated with IgG, IgA, or IgE can be phagocytosed via Fc receptors on phagocytic cells. Natural killer cells, macrophages, monocytes, and neutrophils can participate in IgG-mediated antibody-dependent cellular cytotoxicity. In IgE-mediated antibody-dependent cellular cytotoxicity, platelets, macrophages, and eosinophils are involved. When the pathogen is too large for phagocytosis, other mechanisms such as granzymes, perforin, and even reactive oxygen intermediates can effect killing (104).

Table 9 summarizes the major characteristics of lymphocytes.

TABLE 9
CHARACTERISTICS OF LYMPHOCYTES

- *Helper T Lymphocytes (CD4)* recognize viral or bacterial antigens together with MHC class II molecules on surface of antigen presenting cell, such as a macrophage. Interaction leads to cytokine secretion, initiating a cascade of immune reactions.

  Two major types of CD4 cells:

  Type 1 (Th1) secrete IL-2 and IFN-γ.
  Involved in cell-mediated immunity by activating macrophages and promoting T cell–mediated cytotoxicity.
  Type 2 (Th2) secrete IL-4, 5, 6, and 10.
  Assist B cells to produce antibodies. Cytokine products of Th1 and Th2 cells mutually inhibitory.
- *CD8 T cells* function as cytotoxic cells.
  Recognize viral peptide presented with MHC class I molecule on surface of infected cell. Kill by injecting enzymes and also by inducing apoptosis of infected cell.
- *B Lymphocytes (B cells)*
  B1 Cells (early B cells): Bear CD5, a signaling and adhesion molecule.
  B2 Cells: Shed CD5 marker before they meet invading antigen. B2 cells coexpress IgM and IgD on cell surface. When encountering antigen, B2 cells differentiate into plasma cells which produce specific antibodies (IgG, IgA, or IgE) or differentiate into a memory cell.

5.1. Defects in Lymphocytes

Mutations on lymphocytes can compromise immune functions. Mutations in the genes that code for immunoglobulin light or heavy chains or their associated signaling molecules can result in either agammaglobulinemia or hypogammaglobulinemia (105). Thus, only λ light chains are produced if there is a mutation in the κ light chain gene. Circulating B cells are absent if there are mutations affecting the μ heavy chain, the surrogate light chain, Ig α (CD79a), a B cell receptor signaling molecule, and B cell linker adapter protein (BLNK) (105–107). Mutations in the immunoglobulin heavy-chain constant locus genes, such as $\gamma 1$, $\gamma 2$, $\gamma 3$ or $\gamma 4$, E, $\alpha 1$ or $\alpha 2$, can result in deficiencies of individual classes or subclasses of immunoglobulins with antibody function, yet remain normal (108).

Stimulation of B cells by T cells can be affected if there is a T cell defect. Thus in the X-linked hyper-IgM syndrome, the gene that codes for a cell surface molecule on the activated T cells (CD4+ cells), CD154 [also called CD40 ligand since it interacts with its receptors (CD40 on B cells)], is defective (109). Signaling of B cells by T cells is affected if the CD154 protein on the activated T cell cannot interact with its receptor CD40 on B cells. Thus, the B cells are unable to produce immunoglobulins IgG, IgA, and IgE; they can, however, make IgM.

Hyper-IgM syndrome can also arise if there is an intrinsic B cell defect that prevents B cells from switching to production of immunoglobulins other than IgM due to a mutation in the gene that codes for the mRNA-editing enzyme known as activation-induced cytidine deaminase (110).

Mutation in a gene that codes for the Bruton tyrosine kinase, a cytoplasmic protein tyrosine kinase that is necessary for the growth of B cell precursors and their Maturation, results in the absence of circulating B cells in patients with X-linked agammaglobulinemia (111). Mutation in the γ chain of the IL-2 receptor, a chain common to other cell-surface cytokine receptors (IL 4, 7, 9, and 15) results in X-linked severe combined immunodeficiency (T cell–negative, B cell–positive, natural killer cell–negative SCID) (105, 112). This type of SCID was reported to be more common than other causes of SCID (113). T cells in this condition are virtually absent or severely reduced. Although B cells are normal or even increased, their function is affected due to the lack of signaling by T cells. As such, immunoglobulin levels are severely reduced.

During the developmental stages of pro-B cell to pre-B cell and B cell, recombination-activating genes RAG 1 and RAG 2 that code for recombinase enzymes are expressed (76). These genes regulate the rearrangement of T cell–receptor and B cell–receptor genes.

Hence, mutations in RAG 1 or RAG 2 genes can result in absence of functional T and B cells. However, natural killer cells are present (T cell–negative, B cell–negative, and natural killer cell–positive SCID) (114). Not all patients with mutations in RAG 1 or RAG 2 gene have the rearrangement of T cell and

B cell–receptor genes totally compromised. Thus in Omenn's syndrome, where there is a mutation in the RAG 1 or RAG 2 gene, there is a partial rearrangement of receptor genes (115). Omenn's syndrome is characterized by increased IgE levels and eosinophilia due to abnormal activated oligoclonal Th2 helper T cells requiring bone marrow transplantation (105).

Mutation in the adenosine deaminase gene on chromosome 20 can cause severe combined immunodeficiency due to absence of T cells, B cells, and natural killer cells (T cell–negative, B cell–negative, natural killer cell–negative autosomal recessive SCID). The lack of the enzyme adenosine deminase results in the accumulation of adenosine and toxic deoxyadenosine nucleotides. The latter can cause apoptosis of lymphocytes. Lymphocyte counts can be as low as 0.5×10^9/L, affecting primarily T cells which are absent (105). While therapy of missing enzyme has been shown to effect improvement, bone marrow transplantation is the preferred treatment. Milder forms of adenosine deaminase deficiency have been reported (116).

Wiskott–Aldrich syndrome (WAS) is an X-linked syndrome that is characterized by thrombocytopenic purpura with small defective platelets, eczema, and immunodeficiency. The gene located on the short arm of the X chromosome between Xp 11.3 and Xp 11.22, which produces a protein involved in the assembly of actin filaments required for the formation of microvesicles, is defective (117). In most cases, death occurs before 10 years of age due to infection or bleeding. Those surviving longer are susceptible to developing malignancy, the most common cause of death being lymphoma induced by Epstein–Barr virus (EBV) (105). A characteristic finding is the low level or complete absence of antibodies to blood group antigens since subjects with WAS are unable to produce antibodies to polysaccharide antigens. There is a decrease in T lymphocytes, even though the total lymphocyte count is normal. The T lymphocyte response to mitogens *in vitro* is impaired.

Therapy for WAS is directed to reducing bleeding and control of infection. Splenectomy usually restores the platelet count and size. Antibiotic and intravenous immune globulin treatment has been successful in controlling infections. Bone marrow transplantation has been successful in treating a number of patients with WAS (118).

Ataxia telangiectasia is an autosomal recessive disease characterized by neurologic, endocrine, and hepatic abnormalities, as well as a predisposition to malignancy (119). The defect has been traced to a gene on chromosome 11, the ATM gene that codes for a phosphatidylinositol 3-kinase–like protein which is related to the catalytic subunit of DNA-dependent protein kinase. This protein has a role in signal transduction, DNA repair, and control of the cell cycle (120). Affected patients have a defect in cell-mediated immunity. A decrease in serum IgA is seen in a majority of affected patients. IgG2 or total IgG and IgE levels may be decreased, with an increase in IgM. Patients are susceptible to chronic respiratory

infection. As the name of the syndrome indicates, cerebral ataxia and telangiectasia developed in either childhood or adolescence are hallmarks of this syndrome.

Finally, in this brief overview of lymphocyte defects, mention should be made of mutations affecting major histocompatibility-complex (MHC) Class II molecules. These mutations affect a multiprotein transcription factor complex that regulates the expression of MHC Class II molecules (121). Affected patients have undetectable levels of MHC Class II antigens HLA-DP, DQ, and DR on the surface of monocytes and B cells. Lack of these antigen-presenting molecules leads to impaired immune response. Affected individuals have moderate lymphopenia with a severely reduced number of CD4+ T cells and normal or increased numbers of CD8+ T cells. Since MHC molecules in the thymic epithelium play a key role in positive and negative selection of primitive T cells, selection of competent T cells is also affected in the absence of MHC Class II antigens.

Defects in MHC Class II molecules, while exposing affected subjects to a variety of infections, do not result in the severe immunodeficiency seen in patients with SCID. In contrast to mutations affecting MHC Class II molecules, defects in MHC Class I molecules are rare. Mutations affecting MHC Class I molecules are directed to genes on chromosome 6 at the MHC locus that code for peptide-transporter proteins (122). The function of these transporter proteins is to transport the peptide antigens so that a complex with the α chain of MHC Class I molecules and β_2-microglobulin is formed and transported to the surface of the cell.

TABLE 10
CONSEQUENCES OF DEFECTS IN LYMPHOCYTES

1. *Agammaglobulinemia or hypogammaglobulinemia:* Mutations in genes that code for immunoglobulin light or heavy chains or their associated signaling molecules.
2. *X-Linked hyper-IgM syndrome:* Defect in gene coding for CD154 on activated T cell which interacts with receptor on B cells (CD40). B cells make only IgM. Defect also due to mutation affecting switching of IgM to other immunoglobulins.
3. *X-Linked agammaglobulinemia:* Defect in gene coding for Bruton tyrosine kinase responsible for B cell growth and maturation.
4. *X-Linked severe combined immunodeficiency:* Mutation in gene coding for γ chain of IL-2 receptor.
5. *Adenosine deaminase deficiency:* Mutation in chromosome 20 results in T cell–negative, B cell–negative, natural killer cell–negative autosomal recessive severe combined immunodeficiency.
6. *Wiskott–Aldrich syndrome (WAS):* Mutation on X chromosome involved in coding for protein regulating microvesicle formation. Complete absence of antibodies to blood group antigens. Small defective platelets, thrombocytopenia and immunodeficiency.
7. *Ataxia telangiectasia:* Mutation in gene on chromosome 11, which codes for protein involved in signal transduction, DNA repair, and control of cell cycle. Defect in cell-mediated immunity. Cerebral ataxia and telangiectasias are hallmarks.
8. *Mutations affecting MHC Class II molecules:* Mutations affect expression of Class II molecules. Immunodeficiency not as severe as in severe combined immunodeficiency.

Interference in the transport of peptide antigens due to the absence of transporter proteins results in the destruction of the MHC Class I complex in the cytoplasm (123). Immunodeficiency resulting in mutations affecting MHC Class I complex assembly is not severe.

Table 10 is a listing of consequences of defects in lymphocytes.

6. Conclusion

In this overview we have highlighted the normal biochemical functions of platelets, neutrophils, and lymphocytes. We have attempted to relate defects in these cells to derangement in function. With the explosion of knowledge in the chemistry, immunology, and molecular biology of these cells, it is to be hoped that in the near future we will come to a fuller understanding of the basis of hematological disease.

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