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CONTENTS

Tumor Markers: Reclassification and New Approaches to Evaluation

KAISER J. AZIZ

Branched DNA Signal Amplification for Direct Quantitation of Nucleic Acid Sequences in Clinical Specimens

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PREFACE

The completion of this volume marks the happy milestone for me of a 15-year editorial association with Academic Press. It is appropriate, therefore, to acknowledge the professionalism of the staff and management, as well as their manifold contributions to the success of these volumes. It also marks my embarkation on new and more challenging professional pursuits. These ultimately will allow more opportunity to continue to perform the editorial tasks associated with *Advances in Clinical Chemistry,* as well as other educational and scientific activities.

The contents of Volume 33 again demonstrate the commitment of all those involved to serve an evolving and increasingly multidisciplinary scientific discipline. Included are chapters titled Endogenous Mediators in Sepsis and Septic Shock; Current Concepts of Coagulation and Fibrinolysis; Red Blood Cell Enzymes and Their Clinical Utility; Tumor Markers: Recent Developments and New Approaches to Evaluation; and Branched DNA Signal Amplification for Direct Quantitation of Nucleic Acid Sequences in Clinical Specimens. Future volumes are planned to continue to expand the clinical and basic sciences encompassed. To adequately cover new and emerging aspects of the science and practice of clinical chemistry, future volumes will be published on a two volume per three year basis. This production schedule will allow the scientific experts, editors, and publisher to produce an even higher quality product. Monographs are being considered, with a view to providing a multifaceted and complete overview of the core and satellite sciences constituting this field. The Editorial Board is being reformulated to meet the challenges of clinical chemistry in the new millenium. Two regional associate editors will be added to the editorial team. One will be based initially in Europe and the second in Asia. The composition of the Editorial Board itself will continually be partially reconfigured to reflect the great diversity of talent, knowledge, and perspectives of an international scientific community.

I would like to take this occasion to thank the editors of this volume, as well as the authors. It was a pleasure working with each of them. There is considerable work in assembling each of these books, but it is more than compensated by the collegiality of all concerned as well as the amount of true education the experience provides. Most especially, I thank my wife, Joanne, for her continuing patience and support in these efforts.

HERBERT E. SPIEGEL

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RED BLOOD CELL ENZYMES AND THEIR CLINICAL APPLICATION

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1. Introduction

Mature red blood cells do not have nuclei, mitochondria, or microsomes; therefore red blood cell function is supported through the most primitive and universal pathway. Glucose, the main metabolic substrate of red blood cells, is metabolized via two major pathways; the Embden–Meyerhof glycolytic pathway and the hexose monophosphate pathway (Fig. 1). Under normal circumstances, about 90% of the glucose entering the red blood cell is metabolized by the glycolytic pathway and 10% by the hexose monophosphate pathway.

The glycolytic pathway is the only pathway of ATP synthesis in the mature cell. For every mole of glucose consumed, 2 moles of ATP are generated. Important functions of red blood cell ATP include active transport of sodium and potassium, maintenance of low intracellular calcium levels, phosphorylation of membrane protein, and sustenance of glycolysis itself. Glycolysis is also the major source of red blood cell reduced nicotinamide–adenine dinucleotide (NADH), an essential cofactor for NADH cytochrome b_5 reductase, which catalyzes the conversion of methemoglobin to functional hemoglobin. At the step of phosphoglycerate kinase, energy generation is bypassed by the Rapoport–Luebering cycle, as a result of which 2,3-diphosphoglycerate (2,3-DPG) is formed. 2,3-DPG has an important role in regulating the oxygen affinity of hemoglobin and also provides a reservoir of triose. In the 11 glycolytic enzymes, at least 7 enzyme abnormalities associated with hereditary nonspherocytic hemolytic anemia have been reported. Under such circumstances, hemolytic anemia results from decreased viability of the red blood cell.

The most important product of the hexose monophosphate pathway is reduced nicotinamide–adenine dinucleotide phosphate (NADPH). Another important function of this pathway is to provide ribose for nucleic acid synthesis. In the red blood cell, NADPH is a major reducing agent and serves as a cofactor in the reduction of oxidized glutathione, thereby protecting the cell against oxidative attack. In the syndromes associated with dysfunction of the hexose monophosphate pathway and glutathione metabolism and synthesis, oxidative denaturation of hemoglobin is the major contributor to the hemolytic process.

Deficiencies of enzymes involved in glycolysis, the hexose monophosphate pathway, the closely related glutathione metabolism and synthesis, and nucleotide metabolism have emerged as causes of hereditary nonspherocytic hemolytic anemias (Table 1) (F10, F11, M27). Some enzyme deficiencies, such as diphosphoglycerate mutase deficiency, lactate dehydrogenase deficiency, and NADH cy-

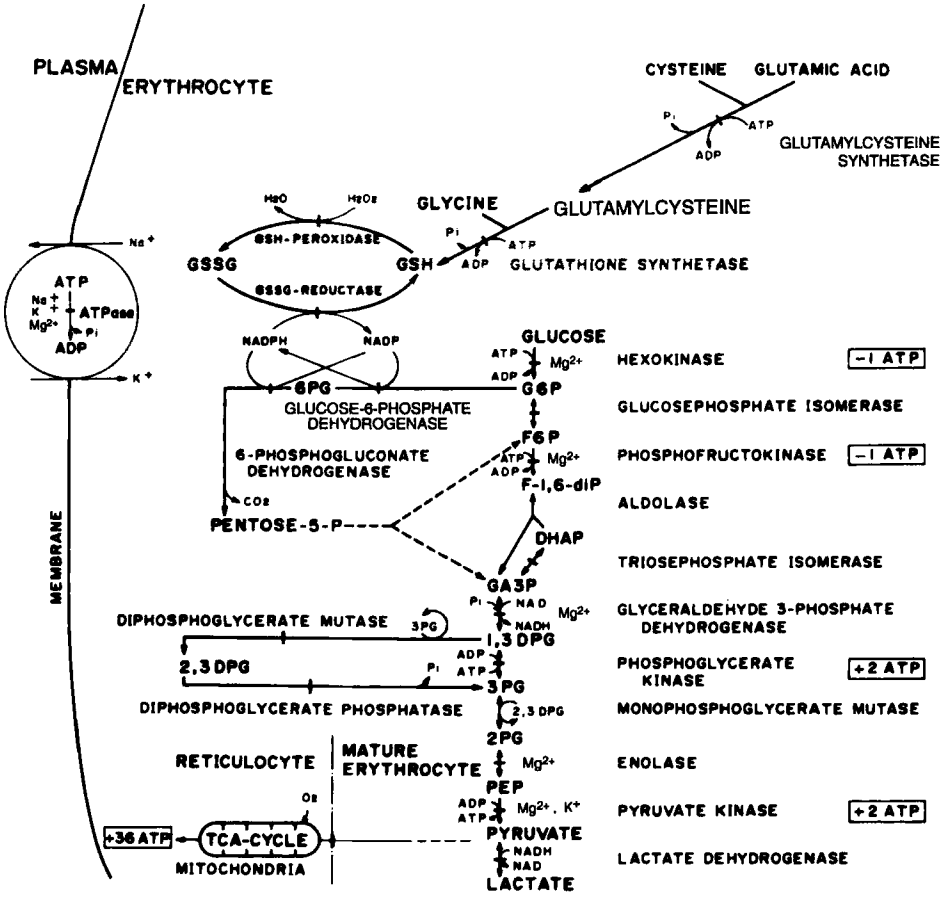


FIG. 1. Major pathway of energy metabolism in mature red blood cells and reticulocytes. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F-1,6-diP, fructose-1,6-diphosphate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde-3-phosphate; 1,3 DPG, 1,3-diphosphoglycerate; 2,3 DPG, 2,3-diphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; 6PG, 6-phosphoglycerate; GSH, reduced glutathione; GSSG, oxidized glutathione; ATP, adenosine triphosphate; ADP, adenosine diphosphate; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; ATPase, adenosine triphosphatase; Pi, inorganic phosphate.

tochrome b_5 deficiency, do not show an apparent shortening of the red blood cell life span.

Since the discovery of glucose-6-phosphate dehydrogenase deficiency (C3) and

TABLE 1
RED BLOOD CELL ENZYME ANOMALIES ASSOCIATED WITH HEREDITARY HEMOLYTIC ANEMIA

Red blood cell enzyme anomalies	Mode of inheritance
Embden–Meyerhof pathway	
Hexokinase	Autosomal recessive
Glucose phosphate isomerase	Autosomal recessive
Phosphofructokinase	Autosomal recessive
Aldolase	Autosomal recessive
Triose phosphate isomerase	Autosomal recessive
Phosphoglycerate kinase	X-linked
Pyruvate kinase	Autosomal recessive
Hexose monophosphate pathway and glutathione metabolism and synthesis	
Glucose-6-phosphate dehydrogenase	X-linked
Glutathione reductase	Autosomal recessive
Glutathione peroxidase	Autosomal recessive
Glutamylcysteine synthetase	Autosomal recessive
Glutathione synthetase	Autosomal recessive
Nucleotide metabolism	
Adenylate kinase	Autosomal recessive
Pyrimidine 5′-nucleotidase	Autosomal recessive
Adenosine deaminase (overproduction)	Autosomal dominant

pyruvate kinase deficiency (V1), erythroenzymopathies associated with hereditary hemolytic anemia have been extensively investigated. Kinetic and electrophoretic studies have shown that most erythroenzymopathies are caused by the production of a mutant enzyme. Although single amino acid substitutions have been identified in some variant enzymes by studies of the enzyme protein, it has been difficult to purify and to characterize the patient's enzymes because of the low protein content in the red blood cells. Genomic DNA or complementary DNA (cDNA) for most of the enzymes causing hereditary hemolytic anemia has been isolated using the technique of molecular biology (F11, M28). This has set the stage for rapid advances in understanding the molecular basis of erythroenzymopathies. The abnormalities are mostly missense mutations. Nonsense mutation, gene deletion, gene insertion, and splicing mutation have also been found in several variant enzymes (Fig. 2).

It is possible to diagnose nonhematologic hereditary disorders by measuring red blood cell enzyme activities if the activity of the enzyme in red blood cells and the target organ(s) is under the same genetic control. Examples of these disorders include three types of galactosemia, the porphyrias, and prolidase deficiency. Among the immunodeficiency syndromes, adenosine deaminase deficiency and

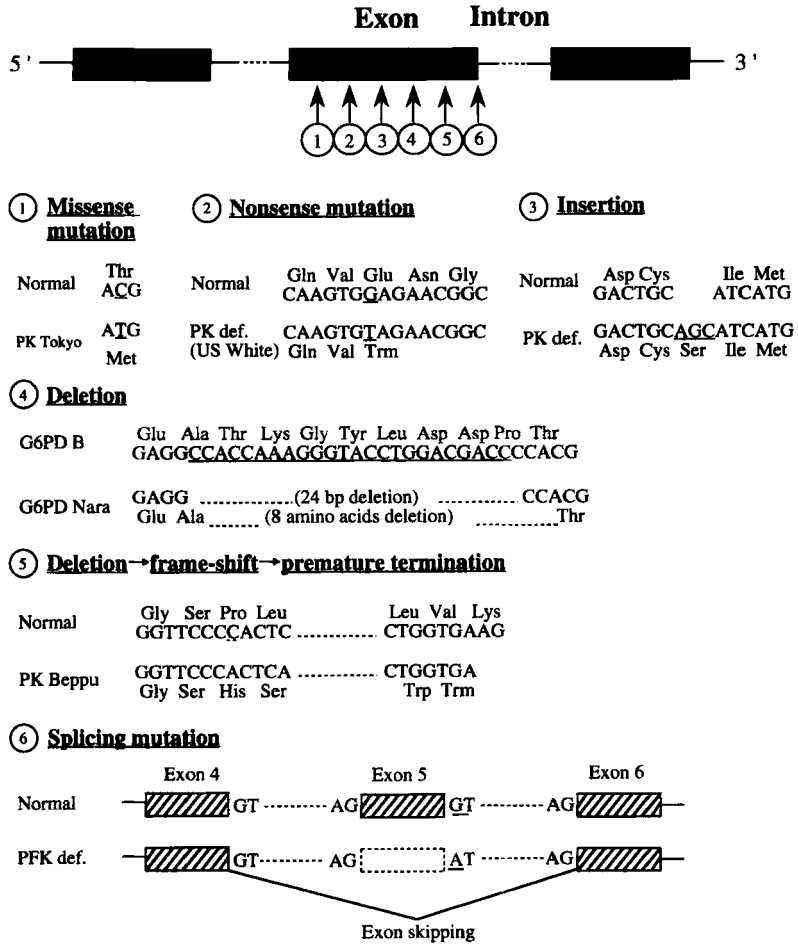


FIG. 2. Representative mutations causing erythroenzymopathies. PK, pyruvate kinase; G6PD, glucose-6-phosphate dehydrogenase; PFK, phosphofructokinase.

purine nucleoside phosphorylase deficiency can be detected by studying red blood cells. In the metabolism of purines, hypoxanthine–guanine phosphoribosyltransferase deficiency and adenine phosphoribosyltransferase deficiency can also be diagnosed by measuring the enzyme activity in red blood cells. Acatalasemia is the first red blood cell enzyme deficiency to have been discovered in Japan (T1). Certain types of renal tubular acidosis are due to carbonic anhydrase deficiency. The lack of red blood cell cholinesterase (J2, S15) and AMP deaminase (O2) appears not to have any clinical consequences and is entirely asymptomatic.

2. Structure and Function of Major Red Blood Cell Enzymes

2.1. Hexokinase

The initial step in glycolysis, in which glucose is catalytically phosphorylated to glucose-6-phosphate by hexokinase (Hx), is critical to human red blood cell metabolism. Hx catalyzes one of the rate-limiting steps of the glycolytic pathway. Among all the red blood cell glycolytic enzymes, Hx has the lowest catalytic activity and is the most age-dependent enzyme. It has been estimated that the mature red blood cells may have no more than 2 to 3% of the Hx activity originally presented in the reticulocyte (V2). Hx has three isozymes (Hx I, II, and III). In general, the type I isozyme is expressed in the brain, red blood cell, and kidney; type II in muscle and adipocytes; and type III in cell nuclei. Isozymes I, II, and III are similar in that they consist of a single polypeptide chain of 100 kilodaltons (kDa). The Hx in red blood cells is mainly type I, and the mature red blood cells contain small amount of Hx III, which is not found in the fetal red blood cell. Although Hx I and Hx II are both expressed in skeletal muscle and adipose tissue, Hx II is the predominant isoform in these tissues. Catalytic activity of Hx II is increased by insulin, whereas that of Hx I is unaffected. These properties contrast with those of a fourth type of Hx found in liver and pancreas, called type IV or more commonly, "glucokinase (GK)." This enzyme is similar to the Hx found in yeast, consisting of a single polypeptide chain of 50 kDa, and differs from the other mammalian Hx because of its low affinity for glucose, lack of inhibition by glucose-6-phosphate, and kinetic cooperativity with glucose. The most important physiological regulators of GK gene expression are insulin and glucagon.

cDNAs encoding human Hx I (N8), Hx II (P14), Hx III (F14), and GK (N9) have been isolated and their genes localized to human chromosome bands 10q22 (M7), 2p13.1 (L5), 5q35.2 (F15), and 7p13 (N9), respectively. Analysis of cDNAs encoding mammalian type I–III isozymes showed that they consist of a tandem arrangement of two highly homologous polypeptides, the amino acid sequences of which are very similar to those of the 50-kDa yeast Hx or GK. In rat, the structure of genomic DNA of Hx II consisted of a duplication of the genomic DNA of GK with the same intron–exon structures (K22). Therefore, it has been considered that the 100-kDa Hx evolved by gene duplication encoding an ancestral Hx similar to yeast Hx and GK.

2.2. Glucose Phosphate Isomerase

Glucose phosphate isomerase (GPI) catalyzes the reversible interconversion of glucose-6-phosphate and fructose-6-phosphate. GPI plays an essential role in carbohydrate metabolism in all cells of the body. The substrates of this enzyme, fruc-

tose-6-phosphate and glucose-6-phosphate, are intermediates in glycolysis and gluconeogenesis, as well as intermediates in the hexose monophosphate pathway.

In humans, the structural gene locus is on chromosome 19 (M17), and the gene spans over 40 kilobases (kb) including 18 exons and 17 introns (W2, X2). Neuroleukin, a protein that acts as both a neurotrophic factor and a lymphokine, has been isolated from mouse salivary glands (G7), and subsequently the primary structure of neuroleukin was found to be identical to that of GPI by comparison of the cDNA sequences (C7, F1). The cDNA sequence encodes 558 amino acid residues. The enzyme consists of two identical subunits with a molecular weight of approximately 63,000 and neuroleukin is active as a monomer.

2.3. Phosphofructokinase

Phosphofructokinase (PFK) is a key regulatory enzyme of glycolysis that catalyzes the conversion of fructose-6-phosphate to fructose-1,6-diphosphate. The active PFK enzyme is a homo- or heterotetrameric enzyme with a molecular weight of 340,000. Three types of subunits, muscle type (M), liver type (L), and fibroblast (F) or platelet (P) type, exist in human tissues. Human muscle and liver PFKs consist of homotetramers (M_4 and L_4), whereas red blood cell PFK consists of five tetramers (M_4, M_3L, M_2L_2, ML_3, and L_4). Each isoform is unique with respect to affinity for the substrate fructose-6-phosphate and ATP and modulation by effectors such as citrate, ATP, cAMP, and fructose-2,6-diphosphate. M-type PFK has greater affinity for fructose-6-phosphate than the other isozymes. AMP and fructose-2,6-diphosphate facilitate fructose-6-phosphate binding mainly of L-type PFK, whereas P-type PFK has intermediate properties.

The genes for PFK-M, PFK-L, and PFK-P isoforms have been cloned (E3, L9, N1). The human PFK-M gene is a single-copy gene that spans ~30 kb of genomic DNA and contains 24 exons. The coding region encompasses 2340 bp; the polypeptide encoded by the gene comprises 780 amino acids and has a predicted molecular mass of 85 kDa. The respective genes have been assigned to different chromosomes: *PFKM* to chromosome 12q13 (H21), *PFKL* to chromosome 21q (V13), and *PFKP* to chromosome 10p (M30).

2.4. Aldolase

The hexose phosphate, fructose-1,6-diphosphate, is split by aldolase into two triose phosphates: glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Aldolase consists of four 40-kDa subunits. Three tissue-specific forms exist in human tissues; aldolase A (ubiquitous and very active in the muscle), aldolase B (liver, kidney, and small intestine), and aldolase C (specific to the brain). These three isozymes have nearly the same molecular size but differ in substrate specificity,

kinetic and immunological properties, and tissue distribution. The aldolase in red blood cells is type A.

The nucleotide sequences of human aldolase A and B cDNA and the genomic structure of these genes were determined (M11, M32, R6, S2, S3). Both aldolase A and aldolase B have 363 amino acid residues with highly conserved amino acid and nucleotide sequences, indicating that both arose from a common ancestral gene. The single-copy genes, A, B, C, and a pseudogene map to chromosomes 16, 9, 17, and 10, respectively (T15).

2.5. Triose Phosphate Isomerase

Triose phosphate isomerase (TPI) catalyzes the interconversion of glyceraldehyde-3-phosphate and dihydoxyacetone phosphate and has an important role in glycolysis, gluconeogenesis, fatty acid synthesis, and the hexose monophosphate pathway. Red blood cell TPI activity measured *in vitro* is approximately 1000 times that of Hx, the least active glycolytic enzyme. TPI is a dimer of identical subunits, each of molecular weight 27,000, and does not utilize cofactors or metal ions. Posttranslational modification of one or both subunits may occur by deamidination, resulting in multiple forms of the enzymes and creating a complex multibanded pattern on electrophoresis.

The human TPI gene spans 3.5 kb of DNA located on the short arm of chromosome 12 (12p13) and comprises seven exons encoding a 1.2-kb messenger RNA (mRNA) that is translated into a 248-amino-acid protein (B35, M10).

2.6. Diphosphoglycerate Mutase

Diphosphoglycerate mutase (DPGM) in the Rapoport-Luebering cycle is a multifunctional enzyme that catalyzes the synthesis and the degradation of 2,3-DPG. In humans, DPGM activity is detected only in red blood cells, and 2,3-DPG exists at a high concentration in these cells. 2,3-DPG binds to the β chains of the deoxy form of hemoglobin (Hb) at the ratio of one molecule per Hb tetramer ($\alpha_2\beta_2$), stabilizing this conformation and thus decreasing its oxygen affinity and increasing the oxygen delivery to the tissues in the physiologic range of Po_2.

The main function of DPGM resides in its synthase activity, and DPGM also possesses a phosphatase activity, commonly referred to as diphosphoglycerate phosphatase (DPGP). DPGM and DPGP activities are performed by a single molecule. A third enzymatic activity is identical to another glycolytic enzyme, monophosphoglycerate mutase (MPGM), although at a much lower level than MPGM in red blood cells. There are two isozymes of MPGM; a muscle-specific form (MPGM-M) and a non–muscle-specific form (MPGM-B) found in liver, kidney, brain, and red blood cells. The cDNAs for these enzymes have been cloned (J4, J5, S4, S12). The DPGM cDNA encodes a protein of 258 amino acid residues.

MPGM-M and MPGM-B have 254 and 253 amino acids, respectively. Both amino acid and cDNA sequence studies have shown that DPGM and MPGM-B are highly homologous but are encoded by two different structural genes. The gene for DPGM maps to chromosome 7 (B2) and that for MPGM-B to chromosome 10 (J6). The genes encoding these two enzymes probably arose by gene duplication and subsequent recombination.

2.7. Phosphoglycerate Kinase

Phosphoglycerate kinase (PGK) is a key enzyme for ATP generation in the glycolytic pathway and catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. The PGK reaction is bypassed by the Rapoport–Luebering cycle. PGK has two isozymes, PGK-1 and PGK-2. PGK-1 is the ubiquitous enzyme that is expressed in all somatic cells and is encoded by a single structural gene on the X chromosome q13 (W7).

Normal human PGK1 has been completely sequenced from the purified protein (H22). Subsequently, the cDNA sequence and genomic organization for PGK-1 were elucidated (M19, M20). The gene spans 23 kb and contains 10 introns. The coding region is 1254 bp in length. PGK-1 consists of 416 amino acid residues, and the monomeric enzyme of about 48 kDa is catalytically active. The three-dimensional structure of the horse muscle enzyme had been determined by X-ray crystallography (B1). That of the human enzyme remains unknown, but there are only 14 amino acid differences between human and horse PGK, suggesting close structural similarity (Fig. 3). PGK2 is an autosomal gene expressed in a tissue-specific manner exclusively in the late stages of spermatogenesis (M16). The gene locus of PGK2 has been assigned to chromosome 19 (G3).

2.8. Pyruvate Kinase

Pyruvate kinase (PK) is one of the three postulated rate-controlling enzymes of glycolysis. The high-energy phosphate of phosphoenolpyruvate is transferred to ADP by this enzyme, which requires for its activity both monovalent and divalent cations. Enolpyruvate formed in this reaction is converted spontaneously to the keto form of pyruvate with the synthesis of one ATP molecule. PK has four isozymes in mammals; M_1, M_2, L, and R. The M_2 type, which is considered to be the prototype, is the only form detected in early fetal tissues and is expressed in many adult tissues. This form is progressively replaced by the M_1 type in the skeletal muscle, heart, and brain; by the L type in the liver; and by the R type in red blood cells during development or differentiation (M26). The M_1 and M_2 isozymes display Michaelis–Menten kinetics with respect to phosphoenolpyruvate. The M_1 isozyme is not affected by fructose-1,6-diphosphate (F-1,6-DP) and the M_2 is allosterically activated by this compound. Type L and R exhibit cooperatively in

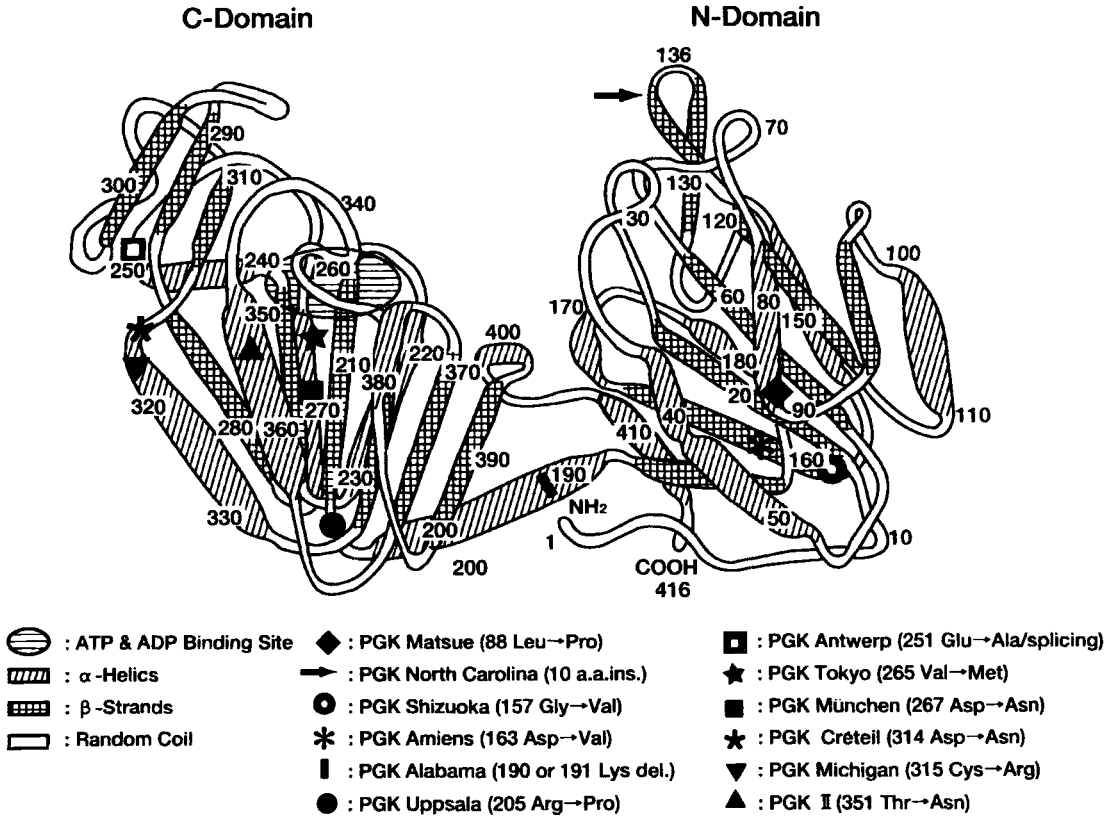


FIG. 3. Three-dimensional model of human phosphoglycerate kinase based on the structure of horse enzyme. Positions of the molecular abnormalities of variant enzymes are also shown.

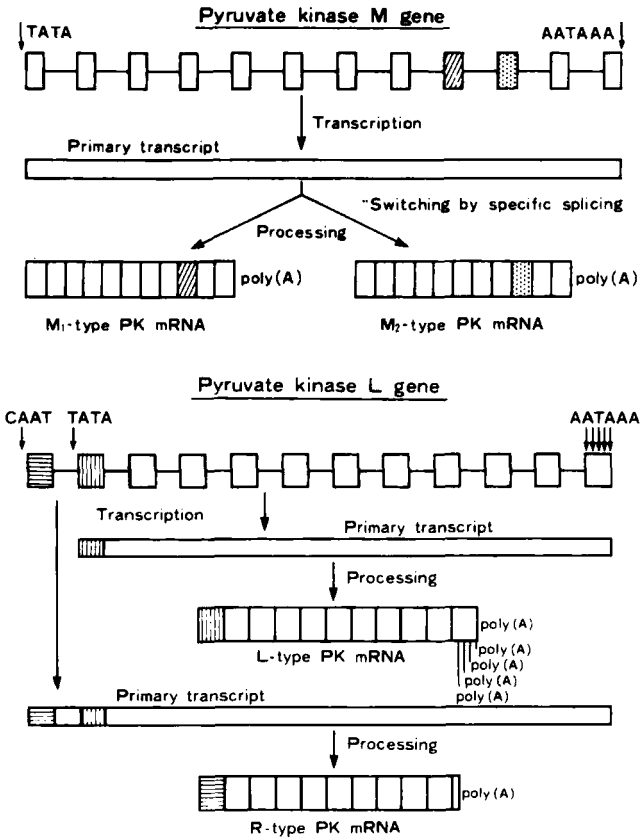


FIG. 4. Schematic representation of expression of the rat pyruvate kinase (PK) gene. Exons specific to each isozyme are indicated by marked boxes. The exons common to M_1- and M_2-type PK and common to L- and R-type PK are shown by open boxes. CAAT, CAT box; TATA, TATA box; AATAAA, polyadenylation signal.

their kinetics towards phosphoenolpyruvate, and both are allosterically activated by F-1,6-DP. Kinetic, electrophoretic, and immunological properties suggest that both L and R types differ from M_1 and M_2 types and that these two kinds of isozymes are under the control of different genes.

Pioneer studies of the rat PK genes have been done by Noguchi *et al.* (Fig. 4) (N10, N11, T4). Subsequently, we have cloned human L- and R-type PK cDNAs and the structural gene for these isozymes, the L-PK gene (K4, K6, T10). The cDNA sequences for M_1- and M_2-type PK and the genomic organization for PK-M have also been elucidated (T5, T11). The human L-PK gene is organized in 12 exons over 9.5 kb, and the first and second exons are specifically transcribed to

the R- and L-type PK mRNA. The 5′-flanking region upstream from the first exon has two CAC boxes and four GATA motifs within 250 bp from the translation initiation codon. The full-length R-type PK cDNA was 2060 bp long and encoded 574 amino acids, the same number as that of rat R-type PK. Compared with human L-type PK, R-type PK was 31 amino acids longer at the amino terminus. The human M-type PK gene is approximately 32 kb and consists of 12 exons and 11 introns. Exons 9 and 10 contain sequences specific to the M_1 and M_2 types, respectively, indicating that the human isozymes are also produced from the same gene by alternative splicing as in the case of the rat PK-M gene. The 5′-flanking region of the gene contains putative Sp1 binding sites but no TATA box or CAAT box. Human M_2-type PK cDNA contained the 109-bp 5′-untranslated region, the 1593-bp coding region, and the 585-bp 3′-untranslated region and encoded 530 amino acid residues. *In situ* hybridization using the cloned cDNA probe disclosed that the genes for human L- and M-type PKs are located on chromosome 1q21 and 15q22, respectively (S7, T11).

Three-dimensional structures of *Escherichia coli* and cat muscle PK had been refined. These studies disclose the essential residues that determine the relative orientations of domains and the precise nature of intersubunit contacts (A3, M15).

2.9. Glucose-6-phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD), an NADP-dependent enzyme, is the initial and rate-limiting enzyme of the hexose monophosphate pathway and catalyzes the dehydrogenase of glucose-6-phosphate to 6-phosphogluconate. The next oxidative step is catalyzed by 6-phosphogluconate dehydrogenase (6-PGD), which also requires NADP as a hydrogen acceptor, to give the pentose, ribulose-5-phosphate. Ribulose-5-phosphate is converted back to the main stream of glycolysis by transketolase and transaldolase. NADPH, provided from the hexose monophosphate pathway, reduces oxidized glutathione (GSSG) to reduced glutathione (GSH), catalyzed by glutathione reductase. In turn, GSH removes oxidants, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), from the red blood cell by the reaction catalyzed by glutathione peroxidase. This reaction is important because the accumulation of oxidants may decrease the life span of the red blood cell by increasing the rate of oxidation of protein, that is, hemoglobin, red blood cell membrane, and enzyme protein. A G6PD knockout mouse is quite sensitive to H_2O_2 and to the sulfhydryl group oxidizing agent, indicating that this enzyme has a major role in the defense against oxidative stress (P3).

Human G6PD had been purified and characterized (Y1), and the structure of the cDNA and genomic clone has also been identified (M12, P11, T6). The monomer of G6PD consists of 515 amino acids including the initial methionine residue. Only the tetrameric or dimeric forms composed of a single type subunit are catalytically active. In human red blood cells, the dimers are the predominant form. The en-

zyme has tightly bound NADP that cannot be easily removed by dialysis. The three-dimensional structure of G6PD from the bacterium *Leuconostoc mesenteroides* was determined (R9), and thereafter a model of the human enzyme was proposed by using the structure of this bacterial enzyme (N3). The regions of substrate and coenzyme binding sites and the dimer interface have been identified, and this study enables us to discuss the structure–function relationships of the mutant enzymes. The gene for G6PD maps to the region Xq28 on the X chromosome (F3).

2.10. Adenylate Kinase

Adenylate kinase (AK) is a ubiquitous monomeric enzyme that catalyzes the interconversion of AMP, ADP, and ATP. This interconversion of the adenine nucleotides seems to be of particular importance in regulating the equilibrium of adenine nucleotides in tissues, especially in red blood cells. AK has three isozymes (AK 1, 2, and 3). AK 1 is present in the cytosol of skeletal muscle, brain, and red blood cells, and AK 2 is found in the intermembrane space of mitochondria of liver, kidney, spleen, and heart. AK 3, also called GTP:AMP phosphotransferase, exists in the mitochondrial matrix of liver and heart.

The genes for AK 1 and AK 3 have been assigned to different regions of chromosome 9, whereas the gene for AK 2 is localized to chromosome 1 (A2). The human AK 1 gene has been isolated and has been shown to be 12 kb pairs long and split into seven exons (M13). It consists of 194 amino acid residues. A cDNA clone encoding human AK 2A has been isolated (L4). The deduced gene product of human AK 2A is composed of 239 amino acids with a molecular mass of 26 kDa.

2.11. Pyrimidine 5′-Nucleotidase

Pyrimidine 5′-nucleotidase (P5N) is a unique enzyme that was recognized from studies of families with relatively common hemolytic disorders. The enzyme catalyzes the hydrolytic dephosphorylation of pyrimidine 5′-nucleotides but not purine nucleotides. The role of this enzyme is to eliminate RNA and DNA degradation products from the cytosol during erythroid maturation by conversion of nucleotide monophosphates to diffusible nucleosides. P5N is inhibited by lead, and its activity is considered to be a good indicator of lead exposure (P1).

P5N has two isozymes, P5N-I (pyrimidine nucleotidase) and P5N-II (deoxyribonucleotidase) (H6, P2). P5N-I is active principally with pyrimidine substrates at an optimal neutral pH; P5N-II activity occurs with both purine and pyrimidine substrates and was maximal with deoxy analogues at an acidic pH optimum. This enzyme was partially purified from human red blood cells and had a molecular weight of 28,000 (T19). The primary structures of both isozymes have not been

determined because of their extremely low protein contents in red blood cells and the difficulty of the protein sequence. The structural gene locus for P5N-II is on chromosome 17 (W8).

2.12. Adenosine Deaminase

Adenosine deaminase (ADA) is an amino hydrolase that catalyzes the deamination of adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively. High activity of ADA is seen in thymus and other lymphoid tissues. ADA has been shown in many different physical forms. A small form of the enzyme predominates in the spleen, stomach, and red blood cells, whereas the large form predominates in the kidney, liver, and skin fibroblasts. The small form of the catalytic subunit can be converted to the large form by complexing with a protein termed binding protein or complexing protein.

The structure and sequence of the catalytic moiety have been determined (O6, V6, W6). The enzyme consists of 362 amino acids and 40,638 daltons of the protein predicted by the cDNA sequence. The ADA gene spans 32 kb and consists of 12 exons. The apparent promoter region of the gene lacks the TATA and CAAT sequences often found in eukaryotic promoters and is extremely G/C rich. The location of the ADA gene is on chromosome 20q12-q13.11 (J1).

3. Hereditary Hemolytic Anemia Associated with Red Blood Cell Enzyme Deficiency

3.1. General Aspects

Symptoms and signs of most red blood cell enzyme abnormalities may be limited to the manifestations of hemolysis or, if the enzymopathies disturb other tissue metabolism, may involve other organ dysfunction. A severe neurologic disorder is accompanied in triose phosphate isomerase (TPI) deficiency and phosphoglycerate kinase (PGK) deficiency; myopathy in glucose phosphate isomerase (GPI) deficiency, phosphofructokinase deficiency, and PGK deficiency; mental retardation in GPI deficiency, aldolase deficiency, TPI deficiency, and PGK deficiency; and granulocyte dysfunction and cataracts in glucose-6-phosphate dehydrogenase deficiency. Chronic ulcerations of the legs are a peculiar and relatively uncommon complication of enzymopathies. Expansion of the erythroid bone marrow may lead to skeletal abnormalities in severely affected patients during active phases of growth and development.

The major clinical features of hemolysis include anemia, jaundice, splenomegaly, and cholelithiasis. Anemia is normochromic in most cases. Macrocytosis and polychromatophilia are seen in patients with marked reticulocytosis. Red

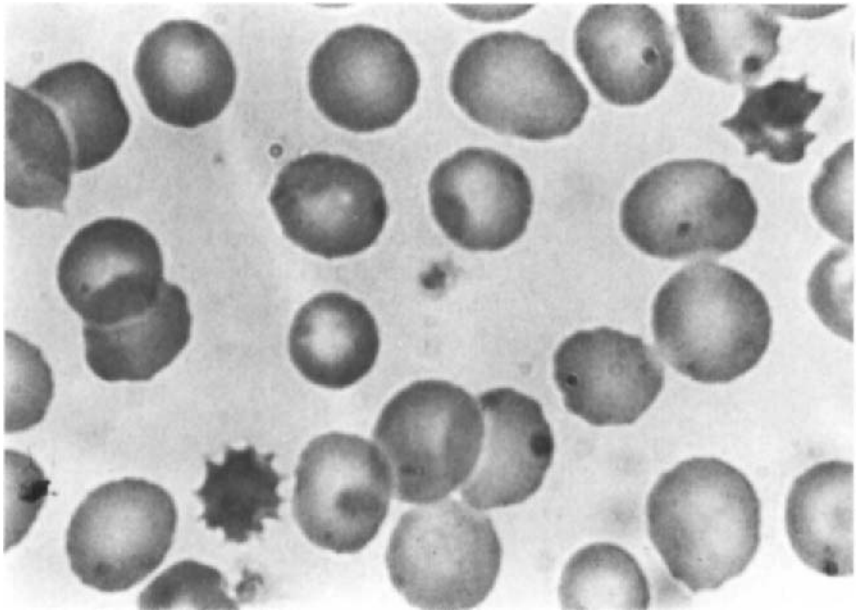


FIG. 5. Spiculed red blood cells (echinocyte) in peripheral blood smear of a splenectomized patient with pyruvate kinase deficiency.

blood cell morphology is usually unremarkable. In pyruvate kinase (PK) deficiency, spiculed red blood cells (echinocytes) are noted, especially after splenectomy (Fig. 5). Basophilic stippling of the red blood cells is the hallmark of pyrimidine 5′-nucleotidase deficiency (Fig. 6). Laboratory signs of accelerated red blood cell destruction are reticulocytosis, erythroid hyperplasia of bone marrow, decreased red blood cell life span, increased serum unconjugated bilirubin, increased rate of urobilinogen excretion, and increased serum lactate dehydrogenase activity. In addition, hemoglobinemia, hemoglobinuria, hemosidenuria, and decreased haptoglobin are observed in case with intravascular hemolysis.

Definitive diagnosis of erythroenzymopathies depends upon quantitative assay of enzyme activity (B14, B16, M26). It is important to measure the enzyme activities after the complete elimination of leukocytes and platelets (B16), because the enzyme activity in leukocytes may be normal if red blood cells and leukocytes are under separate genetic control. Mutant enzymes vary in their *in vitro* properties, and the characterization of such properties has led to the understanding of the genetics and pathogenesis of the shortened red blood cell life span (B11, M23). Measurement of glycolytic intermediates and adenine nucleotides provides confirmation of the *in vivo* significance of enzyme function (B16, M21, M26). Accumulation of proximal and depletion of distal intermediates are the usual findings

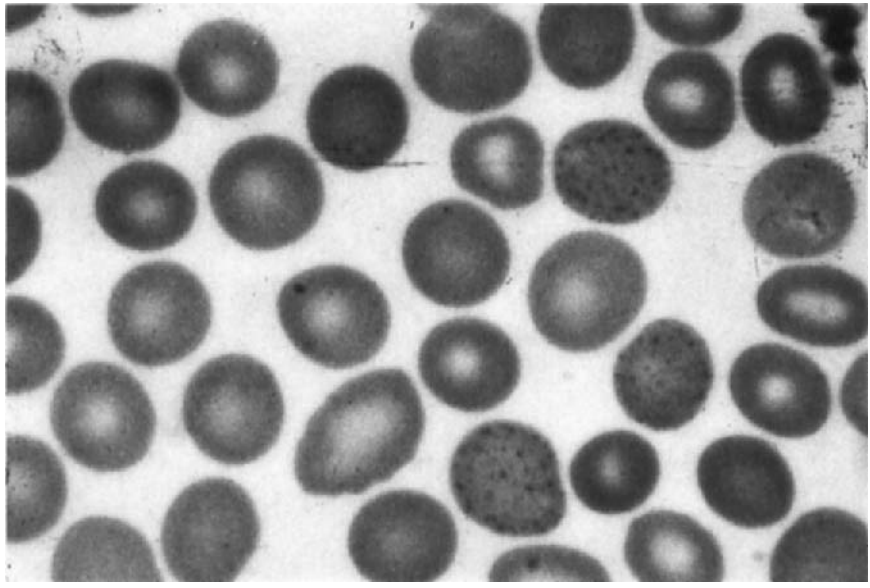


FIG. 6. Basophilic stippling in peripheral blood smear of a patient with pyrimidine 5′-nucleotidase deficiency.

and give rise to a characteristic transition or crossover pattern at the step of the abnormal enzyme.

There is no specific therapy for hereditary hemolytic anemia associated with red blood cell enzyme deficiency. A patient having an acute attack of hemolysis should be treated by appropriate fluid infusion to relieve shock and to maintain urinary output. Red blood cell transfusion may be employed when the anemia is severe with rapid progression. For patients with chronic hemolysis, folic acid may sometimes be useful to prevent megaloblastic crisis. Splenectomy may provide relief in patients with deficiencies of glycolytic enzymes. In PK deficiency, 2–3 g/dl elevations of hemoglobin level are expected after splenectomy. In enzyme deficiencies of the hexose monophosphate pathway and glutathione metabolism and synthesis, further exposure to any possible drugs or other etiologic agent must be avoided if they are considered to be the cause of hemolysis.

3.2. DEFECTS IN THE EMBDEN–MEYERHOF PATHWAY

3.2.1. *Hexokinase Deficiency*

Hexokinase (Hx) deficiency in red blood cells is a rare disease in which the predominant clinical effect is chronic nonspherocytic hemolytic anemia. After the

first case reported by Valentine *et al.* (V2), 15 unrelated families were described (F11). Most cases show only hemolysis. Some patients manifested associated disorders such as multiple malformation, latent diabetes mellitus, and psychomotor retardation. Thirteen of these patients exhibited altered electrophoretic and/or kinetic properties that suggest a structural gene mutation. The molecular defect has been determined in a compound heterozygous case named "Hx Melzo" (B31). One allele has the 96-bp deletion within amino acids 162 to 193, and the other allele shows a single base substitution from T to C at position 1667 that causes the amino acid change from Leu to Ser at 529.

Mutations in GK (Hx IV) causes maturity-onset diabetes of the young (MODY), a form of non–insulin-dependent diabetes mellitus (NIDDM) characterized by onset before 25 years of age and an autosomal dominant inheritance (P12). This suggests that the mutations in other forms of Hx may also contribute to the development of NIDDM. Among them, Hx II is a particularly attractive candidate, although this isozyme is not expressed in red blood cells. Hx II has been analyzed extensively in the muscle of prediabetic insulin-resistant individuals. But studies have shown that Hx II mutation alone is unlikely to have a significant role in the development of peripheral insulin resistance and NIDDM (L6).

3.2.2. *Glucose Phosphate Isomerase Deficiency*

Glucose phosphate isomerase (GPI) deficiency is the fourth most common hereditary enzyme defect of red blood cells, following glucose-6-phosphate dehydrogenase, pyruvate kinase, and pyrimidine 5′-nucleotidase deficiencies. After the first report by Baughan *et al.* (B10), more than 40 unrelated families were described (F11). It is inherited in an autosomal recessive manner, and about half of the affected individuals are thought to be homozygous and the other half appear to be compound heterozygotes. Although this enzyme is considered to be expressed in virtually all tissues, clinical manifestations are limited to hemolysis with a few exceptions. Only two patients were mentally retarded, and one stored excess glycogen in an enlarged liver.

To date, 15 GPI variants have been analyzed at the molecular level, and 16 missense mutations, 1 nonsense mutation, and 1 splicing mutation due to a four-nucleotide deletion have been reported (Fig. 7) (B9, F13, K14, W1, X1). The GPI gene mutations were heterogeneous, although most GPI variants had common biochemical characteristics such as heat instability and normal kinetic properties. We have determined the molecular abnormalities of four homozygous variants, GPI Matsumoto, GPI Iwate, GPI Narita, and GPI Fukuoka (K14). GPI Narita has a homozygous mutation from A to G at position 1028 (343 Gln to Arg), and the same mutation was reported in an Italian patient, GPI Moscone (B9). The substituted Gln is adjacent to the reported active site residue, 341 Asp. Homozygous missense mutations, C to T at position 14 (5 Thr to Ile) and C to T at position 671 (224 Thr to Met) have been identified in GPI Matsumoto and GPI Iwate, respectively. GPI

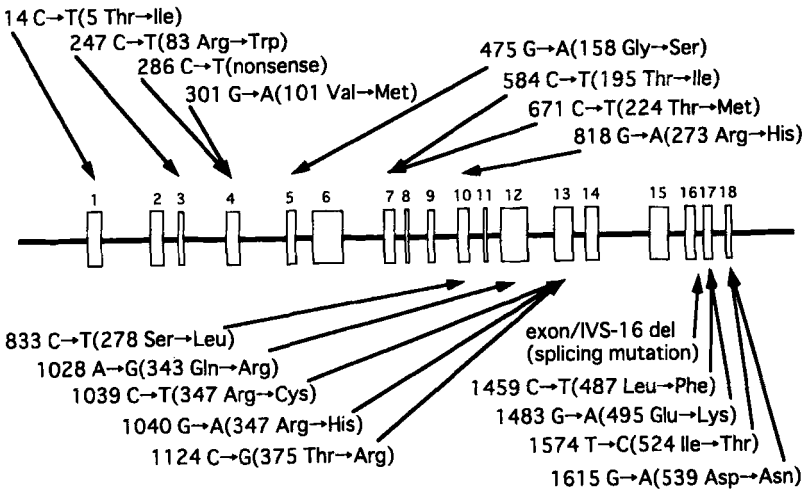


FIG. 7. Mutations in glucose phosphate isomerase gene.

Fukuoka was found to be homozygous for the 1615 G to A (539 Asp to Asn) mutation. This mutation occurred at relatively conserved amino acid residues and caused an alteration in hydrophobicity. Recently, we examined the structure–function relationship of these variants using the recombinant protein (F14). Although all of the four variants were found to be heat labile, the residual GPI activity seems to reflect clinical severity, such as the degree of anemia and episodes of hemolytic crisis. GPI Matsumoto, associated with severe anemia and hemolytic crisis, was extremely unstable, and GPI Iwate, which is associated with compensated hemolytic anemia, showed moderate heat instability. Affinity for substrate, fructose-6-phosphate, was slightly decreased in GPI Narita and GPI Fukuoka, which were associated with moderate anemia and hemolytic crisis.

3.2.3. *Phosphofructokinase Deficiency*

Phosphofructokinase (PFK) deficiency is associated with a heterogenous group of clinical symptoms characterized by myopathy and/or hemolysis or an asymptomatic state. Since the first report involving myopathy and hemolytic anemia described by Tarui *et al.* (T14), over 34 unrelated families with PFK deficiency have been reported (F11). According to the results of biochemical and immunological studies, clinical symptoms are considered to depend on the nature of defective isozymes. Muscle PFK deficiency (Tarui disease; glycogenosis type VII) is an inherited disorder characterized by exercise intolerance, cramps, and myoglobinuria with signs of hemolytic anemia and hyperuricemia.

Studies have led to the identification of 14 alleles associated with PFK deficiency. Eight missense mutations, one nonsense mutation, one frameshift muta-

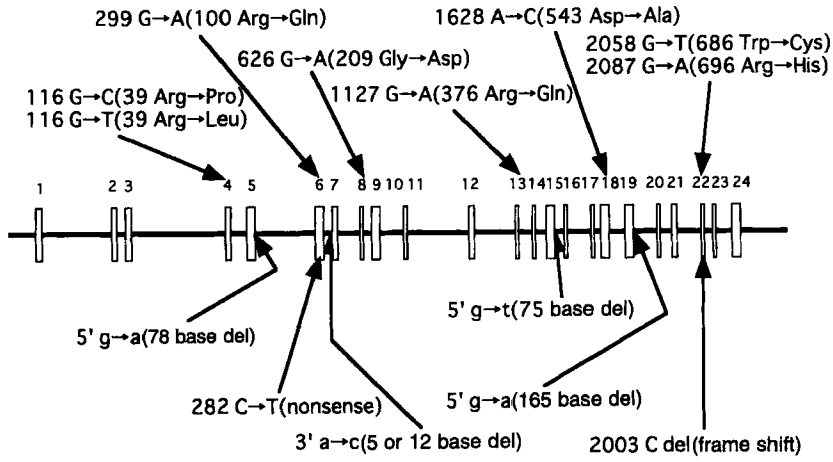


FIG. 8. Mutations in muscle phosphofructokinase gene.

tion, and four splicing mutations have been reported (Fig. 8) (H1, H2, M27, N2, R2, R3, S13, T23, V8). In two Japanese patients with typical clinical manifestations of Tarui disease, 5′-splice junction mutations resulting in splicing to a cryptic site within an exon or exon skipping were identified (H1, N2). These mutations led to in-frame deletions, causing a severe deficiency of the PFK-M isozyme. PFK-M deficiency appears to be prevalent among people of Ashkenazi Jewish descent. The predominant mutation in this group is a splicing defect at the 5′ donor site of intron 5, resulting in an in-frame deletion of exon 5 sequence in the transcript (R1). The second, less frequent, mutation is a deletion of a C nucleotide at position 2003 in exon 22 (S13). The deletion results in a frameshift, introducing a stop codon 47 nucleotides downstream, and would predict generation of a truncated protein with 16 amino acids of incorrect sequence at the COOH terminus. Four other forms of mutations, including splicing defects due to a 3′-splice junction mutation (T23), a nucleotide deletion resulting in a frameshift and premature termination (S13), nonsense mutation (V8), as well as missense mutations (H2, R2, R3, S13, T23), have been identified.

A naturally occurring animal model of PFK-M deficiency has been reported in English springer spaniels. Molecular analysis of this canine PFK-M deficiency disclosed that the enzyme deficiency was caused by a nonsense mutation in the penultimate exon of the PFK-M gene, leading to rapid degradation of a truncated (40 amino acid residues) and therefore unstable enzyme protein (S18).

3.2.4. *Aldolase Deficiency*

Deficiency of aldolase B, although this isozyme is not expressed in red blood cells, is responsible for hereditary fructose intolerance, an autosomal recessive dis-

ease characterized by hypoglycemia and clotting disorders upon fructose feeding. At present, 21 mutations have been reported; 15 of these are missense mutations, 4 nonsense mutations, and 2 splicing mutations. Two large deletions, 2 four-base deletions, a single-base deletion, and a seven-base deletion/one-base insertion have also been found (T16).

On the other hand, a deficiency of aldolase A is a rare cause of hereditary hemolytic anemia. Only three families with aldolase A deficiency have been reported. In the first case, hereditary nonspherocytic hemolytic anemia, many dysmorphic features and mental and growth retardation were observed (B13). The second family had only hemolysis but no signs of myopathy (M24). The third case had both hemolytic anemia and predominantly myopathic symptoms (K25).

Nucleotide analysis of the second family revealed the substitution of a single nucleotide from A to G at position 386 within the coding region (K19). As a result, the 128th amino acid, Asp, was replaced by Gly. The mutated enzyme expressed in *E. coli* was thermolabile, as was the enzyme isolated from red blood cells of the patient. This demonstrated that a single base substitution was responsible for the pathogenesis of this disorder. The third patient carried a new homozygous mutation (619 G to A) in which the negatively charged Glu is changed to the positively charged Lys at residue 206 (K25). In this patient, the extent of impairment of the main subunit interface of the aldolase tetramer probably exceeds the capacity of transcriptional factors to compensate for the muscular enzyme deficiency and may accompany the myopathy.

3.2.5. *Triose Phosphate Isomerase Deficiency*

Hereditary triose phosphate isomerase (TPI) deficiency is an autosomal recessive disorder that has the most severe clinical manifestations of the erythroenzymopathies, including hemolytic anemia, neurological dysfunction, sudden cardiac death, and increased susceptibility to infection. Since the first description by Schneider *et al.* (S10), more than 25 unrelated families have been reported (F11). Cases of decreased TPI activities associated with cat cry syndrome and pancytopenia were reported, whereas the correlation between TPI deficiency and these disorders was not clear. Although the degree of anemia is variable, most patients require blood transfusions. Neurological involvement, such as paraparesis, weakness, and hypotonia, is progressive in most cases. No specific therapy is available for the neuropathic manifestations of the disease, and most severely affected children fail to survive beyond the age of 5 years.

Nine mutations of the TPI gene have now been described. A guanine-to-cytidine transversion in codon 315 had been determined in several unrelated individuals homozygous for TPI deficiency (D1, P6, S9). This substitution results in a thermolabile protein having an Asp in the place of the 104th amino acid, Glu. First-trimester prenatal diagnosis of this mutation was done by chorionic villus DNA analysis in two unrelated families (A6). Thereafter, two homozygotes with missense mutation (N6, P10) and six compound heterozygotes with missense muta-

tion (A5, W3), missense mutation/nonsense mutation (C9, D2), and missense mutation/decreased mRNA (C5) have been clarified.

3.2.6. *Phosphoglycerate Kinase Deficiency*

Hereditary deficiency of phosphoglycerate kinase (PGK) is associated with hereditary hemolytic anemia and often with central nervous system dysfunction and/or myopathy. The first case, reported by Kraus *et al.* (K24), is a heterozygous female, and the results are not so clear. The second family, reported by Valentine *et al.* (V3), is a large Chinese family, whose pedigree study indicates that PGK deficiency is compatible with X-linked inheritance. To date, 22 families have been reported (O4, T25, Y3). Nine of these have manifested both symptoms; five have shown only hemolysis; seven have shown the central nervous system dysfunction and/or myopathy but without hemolysis; and one case, PGK München, is without clinical symptoms (F5). PGK II is an electrophoretic variant found in New Guinea populations (Y2). Red blood cell enzyme activity, specific activity, and the kinetic properties of this polymorphic variant are normal.

At present, the structural abnormalities of 12 mutants, PGK Matsue (M2), PGK North Carolina (T24), PGK Shizuoka (F12), PGK Amiens (C12, T26), PGK Alabama (Y3), PGK Uppsala (F7), PGK Antwerp (O4), PGK Tokyo (F8), PGK München (F5), PGK Créteil (C12), PGK Michigan (M3), and PGK II (Y2), have been elucidated (Fig. 3). Single amino acid substitutions have been identified in PGK Matsue, PGK Shizuoka, PGK Amiens, PGK Uppsala, PGK Tokyo, PGK München, PGK Créteil, PGK Michigan, and PGK II. A guanine-to-adenine substitution at the 5′ end of intron 4 was determined in PGK North Carolina. Activation of a cryptic splice site within intron 4 causes a 30-bp insertion into the transcript, resulting in the insertion of 10 additional amino acids. PGK Alabama revealed a 3-bp deletion in exon 7, inducing the deletion of one of the tandem Lys residues existing at amino acid 190–191. The mutation in PGK Antwerp is a single base substitution (A to C) just adjacent to the 3′ end of exon 7. This mutation should produce two kinds of mRNA. The major component has a missense mutation (Glu to Ala at position 251) with normal splicing, and the minor one contains the 5′ region (52-bp) of intron 7 due to the abnormal splicing. An in-frame termination codon exists in the minor mRNA, and the COOH-terminal half should be deleted in the translation product. Recently, the structural and functional consequences of PGK Uppsala were examined using the corresponding mutant in yeast PGK (T21). The most significant difference when compared with the wild-type enzyme was observed to be a decrease in stability; the kinetic parameters of the mutant were not found to be greatly affected, the catalytic constant being lowered by only 10–20%.

3.2.7. *Pyruvate Kinase Deficiency*

Deficiency of pyruvate kinase (PK) is the most common and well-characterized enzymatic deficiency involving the glycolytic pathway and causing hereditary he-

molytic anemia. Nearly 400 cases of PK deficiency have been reported since the first description by Valentine *et al.* (V1). Although this disorder has been reported from around the world, most cases of PK deficiency have been found in persons of Northern European origin. An autosomal recessive mode of inheritance has been observed in most family studies. Clinical symptoms are seen in the homozygous or doubly heterozygous state. Anemia is moderate to severe, and in some severe PK deficiency cases exchange transfusions are required during the neonatal period. Death may result in early infancy without effective treatments including transfusion or splenectomy, as described in the Amish cases (K10). As a rule, hemolytic anemia and jaundice are observed in infancy or childhood with mild to moderate splenomegaly. The chronic hemolytic process may be exacerbated by infection. After the first decade of life, gallstones are detected with high frequency. Red blood cell morphological abnormalities are not a prominent feature in PK deficiency. The red blood cell is normochromic with a slight anisocytosis and poikilocytosis. Echinocytes may be seen occasionally before splenectomy, but they increase in number and may become conspicuous after splenectomy (Fig. 5). Serum indirect bilirubin is moderately increased, and haptoglobin is decreased or absent.

The diagnosis of PK deficiency depends on the determination of quantitative enzyme activity or qualitative abnormalities of the enzyme. In 1979, the International Committee for Standardization in Haematology (ICSH) established methods for the biochemical characterization of red blood cell PK variants (M22). Since the establishment of these methods, many PK-deficient cases have been characterized, including 13 cases of homozygous PK deficiency. Residual red blood cell PK activity is not usually associated with phenotypic severity,whereas enzymatic characteristics such as decreased substrate affinity, thermal instability, or impaired response to the allosteric activator fructose-1,6-diphosphate (F-1,6-DP) correspond to a more severe phenotype.

To date, 83 mutations in the L-PK gene associated with hereditary hemolytic anemia have been analyzed at the molecular level, and 58 missense mutations, 5 nonsense mutations, 10 deletions, 5 insertions, and 5 splicing mutations have been identified (Fig. 9) (B7, B8, B28, M27). We have analyzed PK genes responsible for hereditary hemolytic anemia in Japanese, American, and Chinese homozygous PK variants by cDNA or genomic DNA cloning (K4, K5, K7, K9, K10, K11, K12). Among 13 families, seven distinct missense mutations, a one-base deletion, and a splicing mutation have been identified (Fig. 10). These studies revealed that the biochemical parameters of the variant enzyme such as the Michaelis constant for phosphoenolpyruvate or the allosteric activation by F-1,6-DP correlate with the expected effects of the missense mutation on the PK subunit. It is considered that the variant enzymes with these missense mutations may change the conformation of the active site or the tetramer formation of PK subunits, resulting in a drastic loss of activity. A point mutation in the 5′-donor site of intron 7 of the human PK-

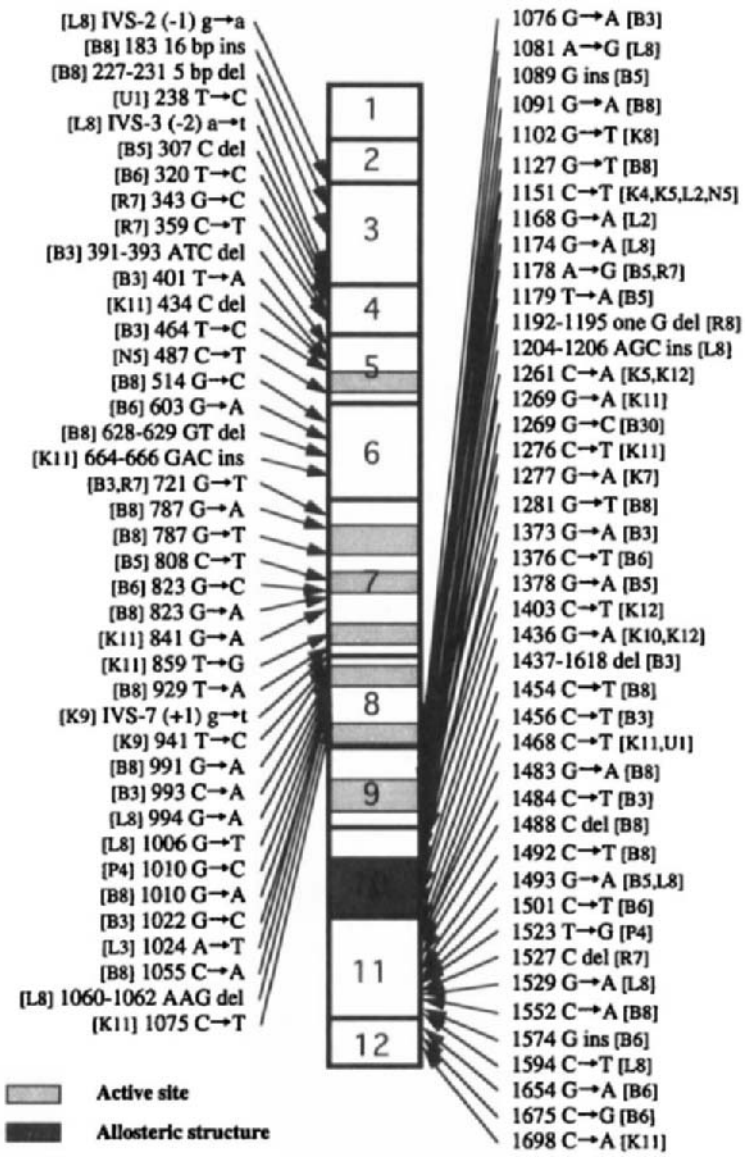
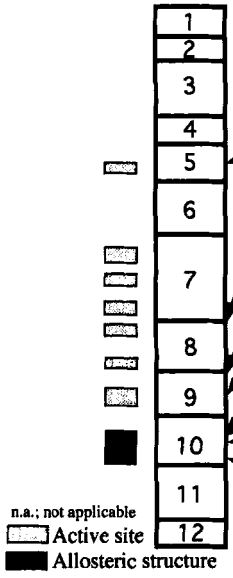


FIG. 9. Mutations in L-type pyruvate kinase gene. [ ] shows the references. ins; insertion, del; deletion.

L gene was identified in PK Kowloon, Nepalese nonidentical twin girls who have been transfusion dependent (K9). The +1 position of intron 7 was replaced from GT to TT, resulting in retention of intron 7 in the R-PK mRNA. Consequently, the



PK variant	DNA defect	Substrate Affinity	F-1,6-DP Activation
PK Beppu	434 C→del	n.a.	n.a.
PK Kowloon	IVS-7 (+1) g→t	n.a.	n.a.
PK Hong Kong	941 T→C	-	-
PK Tokyo	1151 C→T	↓	normal
PK Nagasaki		↓	normal
PK Fukushima	1261 C→A	↓	↓
PK Maebashi		↓	↓
PK Sendai		↓	↓
PK Naniwa	1276 C→T	↓	↓
PK Sapporo	1277 G→A	↓	↓
PK Hadano	1403 C→T	-	-
PK Amish	1436 G→A	normal	↓ ↓
PK Shinshu		normal	↓ ↓

FIG. 10. Molecular and biochemical abnormalities of homozygous pyruvate kinase deficiency discovered in our laboratory.

translational product may lack one third of the COOH-terminal portion of the R-PK, because premature termination would occur in the region encoded by the intron 7 sequence. Although the PK isozyme switches from M_2 to R type during normal red blood cell maturation, R-PK has rarely been observed in hemolysates in some cases of severe PK deficiency. In PK Beppu (K11), one of the most severe PK variants, the M_2-type PK persists in mature red blood cells and in the liver. We found that the variant was homozygous with a one-base deletion (434 C del) of the L-PK gene, resulting in a frameshift and premature termination of translation. The truncated R-PK subunit lacks about two-thirds of the COOH-terminal portion and has no catalytic activity. The affected patient may survive by means of compensatory M_2-PK expression.

We discovered a mouse with PK deficiency, splenomegaly, and hemolytic anemia from an inbred colony of the CBA strain (M29). The red blood cell PK activity was about 16.2% of the normal control value, and zymograms revealed that the isozyme expressed in the mutant red blood cells was the M_2-type PK. A homozygous missense mutation was identified in the cDNA sequence of the mutant, causing a single amino acid substitution near the substrate binding site of PK (K13). This is the first model mouse of PK deficiency whose genetic basis has been characterized at the molecular level. This mouse is considered to be a useful experimental model of gene therapy for PK deficiency.

3.3. Defects in the Hexose Monophosphate Pathway and Glutathione Metabolism and Synthesis

3.3.1. *Glucose-6-Phosphate Dehydrogenase Deficiency*

It had been recognized since the 19th century that certain oxidant drugs, such as primaquine, produced an acute hemolytic crisis in some susceptible individuals. Beginning in 1952, systematic studies were done in the United States to determine the cause of this type of drug sensitivity. Cross-transfusion studies with ^{51}Cr-labeled red blood cells indicated that primaquine sensitivity was due to an intrinsic abnormality of the red blood cell (D5). Thereafter, the content of reduced glutathione was found to be lower in primaquine-sensitive red blood cells than in normal cells (B12). Finally, deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD) was identified by Carson *et al.* in 1956 (C3).

Hereditary deficiency of G6PD is one of the most common genetic disorders, more than 400 million people being affected worldwide. The incidence of this disorder is approximately 20% in African Bantu males, 12% in American black males, and 8% in Brazilian blacks. A high prevalence of G6PD deficiency is also seen in the people of the Mediterranean basin, East Indians, Orientals, and Filipinos. Northern European and Japanese people rarely have this enzyme deficiency. The incidence in Japanese people is considered to be 0.1%. Because of its high prevalence in populations in which malaria is endemic, the geographic distribution is considered to be due to a selective advantage of G6PD deficiency against malaria infection.

G6PD deficiency is caused by the production of variant enzymes with abnormal properties. Each variant causes various degrees of enzyme deficiency and they are associated with hemolytic anemia with a range of clinical severity, from chronic hemolytic anemia and drug-induced acute hemolysis to being completely asymptomatic. In 1967, a committee of the World Health Organization proposed standard procedures for characterizing variants using parameters such as enzyme activity, electrophoretic mobility, the K_m for glucose-6-phosphate (G6P) and NADP, utilization of substrate analogues, heat stability, and pH optimum (B11). This made it possible to compare the properties of variants identified in different laboratories. The relationship between enzymatic properties of G6PD variants and clinical severity is somewhat ambiguous. A low inhibition constant (K_i) for NADPH, increased K_m for G6P, and decreased heat stability have been considered to be important causative factors of chronic hemolytic anemia.

Up to now, 101 different mutations have been identified (Fig. 11) (B29, H18). Most of the variant enzymes are produced by one or two missense mutations in the structural gene. G6PD Vancouver is caused by three nucleotide substitutions (M4). Although nucleotide deletions or nonsense mutations are common molecular abnormalities that may cause a variety of genetic disorders, they are rare in G6PD deficiency cases. Nucleotide deletions have been found in only five variants

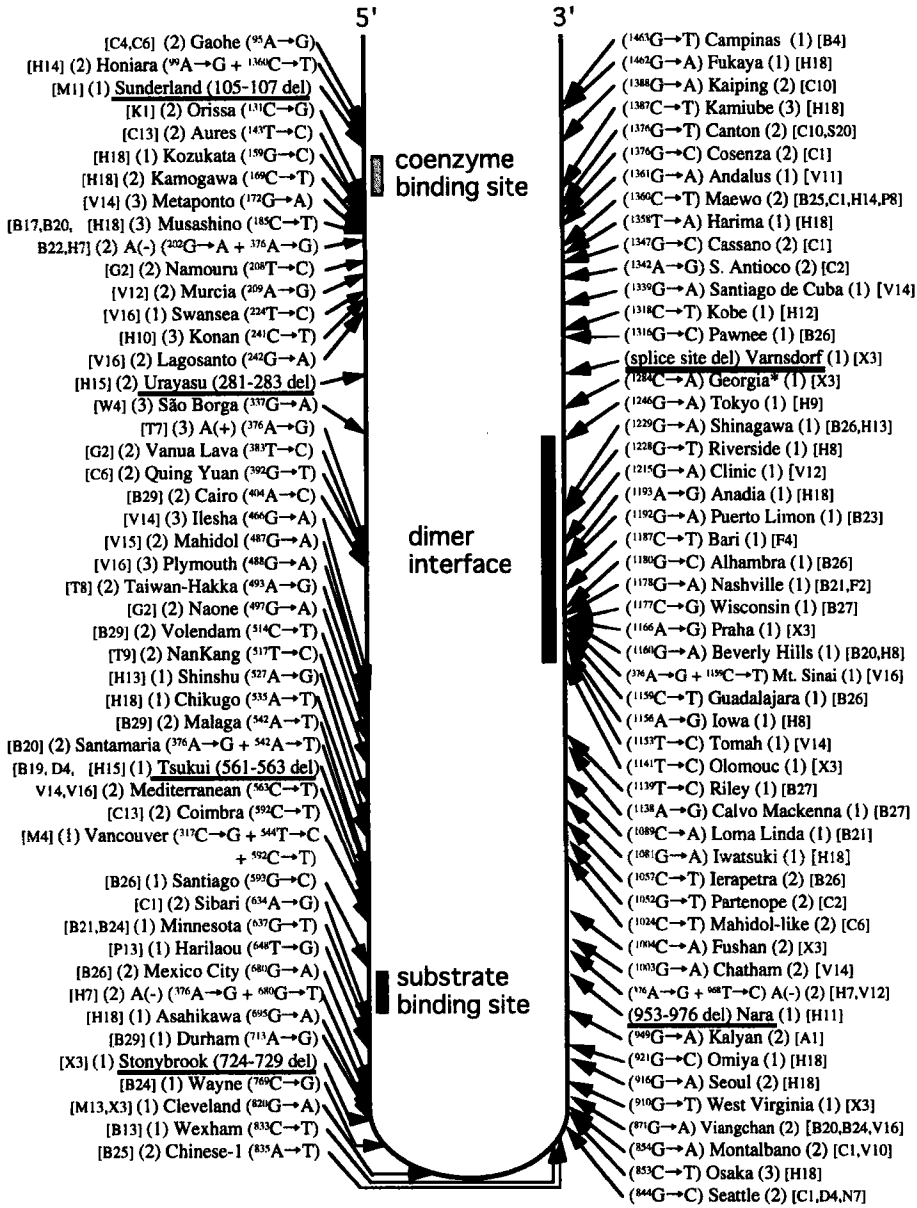


FIG. 11. Mutations in glucose-6-phosphate dehydrogenase deficiency. [ ] shows the references. (1) chronic hemolytic anemia; (2) acute hemolysis; (3) asymptomatic variant. ——, deletion; ═══, splicing mutation; *, heterozygote of nonsense mutation.

(G6PD Sunderland, G6PD Urayasu, G6PD Tsukui, G6PD Stonybrook, and G6PD Nara), and each causes deletion of not more than eight amino acid residues (H11, H15, M1, X3). The low frequency of amino acid deletion as a cause of G6PD deficiency might imply that severe tissue dysfunction usually associated with such drastic structural aberration is presumably lethal unless the involved region is functionally insignificant. A nonsense mutation was identified in a Filipino G6PD-deficient heterozygote, whereas the hemizygous state of this mutation has not been discovered. A mutation of a 3′ acceptor splice site at the COOH terminal has been reported, but details of the splicing error were unknown because of the unavailability of mRNA analysis (X3).

Molecular analysis of G6PD variants combined with standard characterization has provided several interesting findings regarding the structure–function relationship of the enzyme. Amino acid substitutions of the substrate and NADP binding sites are very rare, as shown in Fig. 11. The mutations of these sites are considered to be lethal. It is interesting that most variants associated with chronic hemolysis are clustered surrounding the site of the dimer interface. This might indicate that the dimer formation is closely related to the important function of the active enzyme.

3.3.2. *Glutathione Reductase Deficiency*

Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH provided from the hexose monophosphate pathway. GR, a ubiquitous flavoenzyme, maintains a high value of two for the GSH/GSSG ratio in the red blood cells. 1,3-Bis(2-chloroethyl)-nitrosourea (BCNU) selectively inhibits cellular GR. GR is composed of two identical subunits, each of molecular mass 50 kDa (S8). The three-dimensional structure and mechanism of catalysis have been established for human GR (K17).

Since the first report of decreased GR activity by Löhr and Waller (L10), several cases of GR deficiency have been reported. GR deficiency is a relatively common feature of disorders that are compounded by suboptimal nutrition and are associated with a variety of hematological disorders. The poorly defined clinical effects of the putative deficiency and the unconvincing nature of the family studies led to the suggestion that GR deficiency was a secondary manifestation of a poorly understood basic disorder. In fact. GR activity in the hemolysates of riboflavin-deficient humans was activated by an addition of a small amount of flavine adenine dinucleotide (FAD). Furthermore, administration of riboflavin restored the GR level of the red blood cells of the secondary deficient individuals to normal within a few days. Genetically determined GR deficiency has been reported in three cases by Loos *et al.* (L11). They were offspring of a consanguineous marriage. Complete GR deficiency was not affected by the administration of FAD *in vitro* and riboflavin *in vivo*. Clinically, this deficiency was manifested by hemolytic crisis after eating fava beans. The amount of GSH in the red blood cells

was normal, but severely diminished glutathione stability during incubation with acetylphenylhydrazine was observed. The precise molecular defect of GR deficiency has not been elucidated.

3.3.3. *Glutathione Peroxidase Deficiency*

Glutathione peroxidase (GSH-Px) catalyzes the destruction of hydrogen peroxide (H_2O_2) by GSH, protecting membrane lipids and hemoglobin against oxidative damage by H_2O_2. The enzyme is a homotetramer, with each subunit containing one atom of selenium. The cDNA and genomic sequence of human GSH-Px have been reported (I1, S21), and the structural gene locus is on chromosome 3 (O1).

Necheles *et al.* (N4) first reported a genetically determined homozygous GSH-Px deficiency associated with neonatal jaundice and mild hemolysis. Spontaneous recovery from hemolysis was noted 3 months after birth. Thereafter, several cases with GSH-Px deficiency were reported. Newborn infants exhibit significantly lower red blood cell GSH-Px activity and serum selenium concentrations than adult control subjects, and a significantly positive correlation between selenium concentration and GSH-Px activity has been observed. Furthermore, the addition of selenium stimulates, both *in vivo* and *in vitro,* the GSH-Px activity. The neonatal red blood cell GSH-Px deficiency may be partially due to insufficient availability of selenium during pregnancy (P9). Therefore, the diagnosis of GSH-Px deficiency in newborn infants must be made carefully.

3.3.4. *Glutamylcysteine Synthetase Deficiency*

Glutathione, a simple tripeptide, is synthesized in two steps catalyzed by glutamylcysteine synthetase (GC-S) and glutathione synthetase (GSH-S) from glutamic acid, cysteine, and glycine. One molecule of ATP is broken down to ADP and phosphate for each peptide bond generated. The first of these enzymes, GC-S, catalyzes in the rate-limiting reaction in GSH biosynthesis and consists of two subunits, a heavy catalytic subunit with a molecular mass of 73 kDa and a light regulatory subunit with a molecular mass of 28 kDa (M18). cDNA clones for these subunits have been isolated (G5, G6). The human genes that encode the light and heavy subunits of GC-S are assigned to chromosomes 1p21 and 6, respectively (S16, S17).

Deficiency of GC-S is extremely rare; only five cases from four unrelated families have been reported so far (B18, H17, K23). This enzyme deficiency appears to be inherited as an autosomal recessive and has been clearly associated with a moderate chronic hemolytic anemia and a marked decrement of red blood cell GSH. Spinocerebellar degeneration and aminoaciduria were present in both homozygous siblings in the first family, whereas no neurologic deficit was noted in the other three families.

3.3.5. *Glutathione Synthetase Deficiency*

In the second step, GSH-S catalyzes the synthesis of GSH from γ-glutamylcysteine and glycine in the presence of ATP. A cDNA encoding human GSH-S has been cloned, and the deduced protein consists 474 amino acids with a subunit molecular weight of 52,352 (G1). Active enzyme is considered to be a homodimer.

GSH-S deficiency is a more frequent cause of GSH deficiency (H17), and more than 20 families with this enzyme deficiency have been reported since the first report by Oort *et al.* (O5). There are two distinct types of GSH-S deficiency with different clinical pictures. In the red blood cell type, the enzyme defect is limited to red blood cells and the only clinical presentation is mild hemolysis. In the generalized type, the deficiency is also found in tissues other than red blood cells, and the patients show not only chronic hemolytic anemia but also metabolic acidosis with marked 5-oxoprolinuria and neurologic manifestations including mental retardation. The precise mechanism of these two different phenotypes remains to be elucidated, because the existence of tissue-specific isozymes is not clear. Seven mutations at the GSH-S locus on six alleles—four missense mutations, two deletions, and one splice site mutation—have been identified (S14).

3.4. Defects in Nucleotide Metabolism

3.4.1. *Adenylate Kinase Deficiency*

Red blood cell adenylate kinase (AK) deficiency is a rare genetic disorder. So far six families have been reported (B15, B33, L1, M25, S23, T18), five of which were associated with chronic nonspherocytic hemolytic anemia. In two black siblings with undetectable red blood cell AK activity, one had hemolytic anemia but the other did not. Structural analysis in our case showed a single nucleotide substitution (cytidine to thymine) in an allele which resulted in a change of Arg to Trp at the 128th amino acid residue (M14). By introducing the same amino acid substitution by site-directed mutagenesis into chicken AK 1, the enzymatic properties were examined. The mutant chicken AK 1 expressed in *E. coli* showed reduced catalytic activity as well as decreased solubility and a change in affinity for phosphocellulose. Therefore, this substitution was considered to be the cause of the enzyme deficiency.

3.4.2. *Pyrimidine 5′-Nucleotidase Deficiency*

Pyrimidine 5′-nucleotidase (P5N) deficiency appears to be the third most common cause of hereditary nonspherocytic hemolytic anemia after G6PD and PK deficiencies. To date, more than 42 cases have been reported worldwide (F11) since the first report by Valentine *et al.* (V4). This syndrome is characterized by hemolytic anemia, pronounced basophilic stippling of red blood cells (Fig. 6), and a

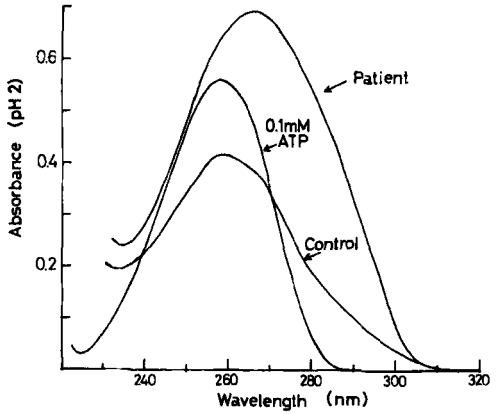


FIG. 12. Absorption spectra of perchloric acid extracts of whole blood from normal subject and a patient with pyrimidine 5'-nucleotides (P5N) deficiency. Absorption peak shift occurs in P5N deficiency, reflecting intracellular accumulation of pyrimidine nucleotides.

marked increase in both red blood cell GSH and pyrimidine-containing nucleotides. Basophilic stippling of the red blood cells is an important and useful exception to the usual lack of distinguishing morphologic abnormalities in erythroenzymopathies. In normal red blood cells, adenine nucleotides form 96% of the nucleotide pool, but more than 50% of the nucleotide pool consists of pyrimidine nucleotides in P5N-deficient cells. Spectroscopic examination of the perchloric acid extract of red blood cells shows that the position of the absorption maximum is shifted from 260 nm in normal cells to 270 nm in the deficient cells (Fig. 12). The maximum at 260 nm corresponds to that of adenine nucleotides. The shift to 270 nm indicates the presence of abnormal nucleotide compositions and suggests that a major part of the abnormal nucleotide pool consists of cytidine nucleotides that have a maximum peak at 280 nm.

Electrophoretic and kinetic studies of the patient's enzyme have been reported in several cases (F10). Most of them showed decreased substrate affinity and abnormal electrophoretic mobility. The main cause of P5N deficiency is considered to be an abnormality of P5N-I, probably arising from a structural gene mutation (H6). The precise molecular defect has not been clarified, because the normal gene for P5N-I has not been isolated.

3.4.3. *Overproduction of Adenosine Deaminase*

Markedly increased adenosine deaminase (ADA) activity in red blood cells develops into hereditary hemolytic anemia. The mode of inheritance is autosomal dominant. Only four families have been reported so far, including our two (K3,

M22, P7, V5). The defect appears to be tissue specific, because ADA activity in leukocytes and skin fibroblasts is normal. Red blood cell ADAs from normal subjects and from a patient were purified by using antibody affinity chromatography in the second kindred (F6). There were no differences in the enzymatic and chemical properties between the ADAs from these two sources. The rate of ADA synthesis in erythroid colony cells cultured from the patient's bone marrow cells was 11-fold greater than that from the normal subjects (F9). The accumulation of structurally normal ADA in the patient seems to be due to its increased synthesis in the precursors of red blood cells. Western blotting of partially purified ADA from the red blood cells of the fourth case revealed an increased amount in the patient's red blood cells (K3). No gene amplification or gene rearrangement was found by Southern blot analysis. We constructed a genomic DNA library and obtained three clones containing the 5′-promoter region of the ADA gene. The 2.2-kb ADA promoter fragment of these clones was fused to the chloramphenicol acetyltransferase (CAT) gene, transfected into the human erythroid cell line K 562, and assayed for CAT activity. One of the clones, pADOP 2 cat, expressed about 2.6 times higher CAT activity than clones carrying the normal ADA promoter fused to the CAT gene in K 562, but such enhancement was not seen in the human nonerythroid cell lines HL 60 and Raji. From these results, it is most likely, although not conclusive, that the 5′-promoter fragment of the ADA gene of the patient was responsible for the cell-specific enhancement of protein synthesis. Thereafter, increased TAAA repeats located at the tail end of an *Alu* repeat approximately 1.1 kb upstream of the ADA gene were identified in affected individuals (C8). This cis-acting mutation might cause the overexpression of ADA in red blood cells.

4. Hereditary Nonhemolytic Blood Disorders Associated with Red Blood Cell Enzyme Deficiency

4.1. Diphosphoglycerate Mutase Deficiency

Although some cases with partial deficiency of diphosphoglycerate mutase (DPGM) activity and a moderate erythrocytosis had been reported, most of them were considered to be heterozygotes. A complete deficiency of DPGM associated with a moderate erythrocytosis was discovered in a man of French origin (R4). DPGM activity was undetectable in red blood cells, as was that of diphosphoglycerate phosphatase. The 2,3-DPG level was below 3% of normal values. A low level of 2,3-DPG resulted in increased oxygen affinity of hemoglobin and a compensatory elevation of red blood cell mass with erythrocytosis, but there was no hemolysis. Sequence studies of this case indicated heterozygosity and cytidine-to-thymine substitution at nucleotide 413 and another heterozygosity with deletion of cytidine at nucleotide 205 or 206 (L7). Therefore, the complete enzyme defi-

ciency results from a genetic compound with one allele coding for a missense mutation (Arg to Cys at 89) and the other bearing a frameshift mutation.

MPGM-M deficiency (glycogenosis type X) has been reported in 13 patients, although this isozyme deficiency cannot be diagnosed by assay of red blood cell enzyme activity. Clinical manifestations of this enzyme deficiency included exercise intolerance, myalgia, cramps after intense exertion, and recurrent myoglobinuria but not hematological abnormalities (T20). Molecular genetic analysis disclosed that the enzyme deficiency was due to the missense mutations or the nonsense mutation.

4.2. Lactate Dehydrogenase Deficiency

Lactate dehydrogenase (LDH) catalyzes the interconversion of lactate and pyruvate with nicotinamide adenine dinucleotide as coenzyme. In mammals, LDH-A (M, muscle), LDH-B (H, heart), and LDH-C (testis) polypeptide chains are encoded by three different genes. The genes for these isozymes have been cloned (C11, S1, T2, T3, T22). Analysis of both human LDH-A and LDH-B genes has shown that their protein-coding sequences are interrupted by six introns at homologous positions. LDH-B contains 333 amino acid residues, and LDH-A possesses 331 residues with two deletions located at positions 18 and 332 of the LDH-B sequence. These appear to have originated from an ancestral gene during the course of evolution. The human gene for LDH-B is located on chromosome 12, whereas LDH-A and LDH-C are closely linked on chromosome 11.

Hereditary deficiency of LDH-B was first reported by Kitamura *et al.* in 1970 (K21). Since then, this enzyme deficiency has been discovered in at least five families in Japan. There were no clinical symptoms in these cases. On the other hand, LDH-A deficiency was associated with an exertional rhabdomyolysis and myoglobinuria after severe exercise (K15). One Japanese and one Italian with LDH-A deficiency showed the typical skin rash. To date, nine LDH-A variants have been analyzed at the molecular level, and four missense mutations, one nonsense mutation, one frameshift mutation due to a single base insertion, and three gene deletions have been elucidated (K16, M5). Missense mutations have also been identified in LDH-B deficiency (M6).

4.3. NADH Cytochrome b_5 Reductase Deficiency

The NADH-dependent methemoglobin reductase system (NADH methemoglobin ferrocyanide reductase, NADH diaphorase, or NADH cytochrome b_5 reductase) is the most important one for the conversion of methemoglobin to functional, oxygen-binding hemoglobin. Methemoglobin reduction needs a hemoprotein, cytochrome b_5, and the electron flow of the NADH-methemoglobin reductase system is NADH cytochrome b_5 reductase–cytochrome b_5–methemoglobin.

Two forms of this enzyme are known, a membrane-bound form mainly found in microsomes of all cells and a soluble form present in red blood cells. Structurally, the soluble form with 275 amino acid residues lacks a hydrophobic segment at the NH_2 terminus which is present in the membrane-bound enzyme with 300 amino acid residues. Both isoforms are produced by a single gene on chromosome 22 (T17, Y4).

Hereditary methemoglobinemia is classified into three types: a red blood cell type (type I), a generalized type (type II), and a blood cell type (type III). Enzyme deficiency of type I is limited to red blood cells, and these patients show only the diffuse, persistent, slate-gray cyanosis not associated with cardiac or pulmonary disease. In type II, the enzyme deficiency occurs in all cells, and patients of this type have a severe neurological disorder with mental retardation that predisposes them to early death. Patients with type III show symptoms similar to those of patients with type I. The precise nature of type III is not clear, but decreased enzyme activity is observed in all cells (M9). It is considered that uncomplicated hereditary methemoglobinemia without neurological involvement arises from a defect limited to the soluble cytochrome b_5 reductase and that a combined deficiency of both the cytosolic and the microsomal cytochrome b_5 reductase occurs in subjects with mental retardation. Up to now, three missense mutations in type I and three missense mutations, two nonsense mutations, two in-frame 3-bp deletions, and one splicing mutation in type II have been identified (M3, M8, M31).

5. Hereditary Nonhematologic Disorders That Can Be Diagnosed by the Determination of Red Blood Cell Enzyme Activity

5.1. Enzyme Deficiencies Associated with Immunological Disorders

5.1.1. *Adenosine Deaminase Deficiency*

Low levels or absence of adenosine deaminase (ADA) is associated with one form of severe combined immunodeficiency disease (SCID) characterized by B- and T-lymphocyte dysfunction due to toxic effects of deoxyadenosine (H19). Most patients present as infants with failure to thrive, repeated infections, severe lymphopenia, and defective cellular and humoral immunity. Disease severity is correlated with the degree of deoxyadenosine nucleotide pool expansion and inactivation of *S*-adenosylhomocysteine hydrolase in red blood cells. Up to now, more than 40 mutations have been identified (A4, H20, S5, S6). The majority of the basic molecular defects underlying ADA deficiency of all clinical phenotypes are missense mutations. Nonsense mutations, deletions ranging from very large to single nucleotides, and splicing mutations have also been reported. It is likely that severe

ADA deficiency is most commonly associated with these mutations of the ADA structural gene that result in either unstable or inactive enzyme protein. Immune reconstitution would be achieved by enzyme replacement therapy with polyethylene glycol–modified bovine ADA (PEG-ADA), alone or in combination with gene therapy (H3).

5.1.2. *Purine Nucleoside Phosphorylase Deficiency*

Purine nucleoside phosphorylase (PNP) deficiency engenders a combined immunodeficiency and neurologic abnormalities and is usually fatal in childhood (G4). Patients with PNP deficiency have profound lymphopenia and a small thymus with poorly formed Hassall corpuscles. Lymphocyte enumeration shows markedly decreased numbers of T cells and T-cell subsets, with normal percentages of B cells. Point mutations and a splicing mutation have been identified in some PNP-deficient patients (H4).

5.2. Enzyme Deficiencies in the Metabolism of Purine

5.2.1. *Lesch–Nyhan Syndrome*

The Lesch-Nyhan syndrome is an inherited disorder associated with a virtually complete deficiency of hypoxanthine–guanine phosphoribosyltransferase (HPRT) and is inherited as an X-linked recessive. This syndrome is characterized clinically by the excessive production of uric acid and certain characteristic neurologic features, such as self-mutilation, choreoathetosis, spasticity, and mental retardation. Partial HPRT deficiency is associated with increased *de novo* purine synthesis and hyperuricemia, which results in nephrolithiasis and gouty arthritis. The HPRT gene is located on the long arm of the X chromosome and consists of nine exons and eight introns spanning 44 kb (P5). This gene is transcribed to produce an mRNA of 1.6 kb, which contains a protein encoding region of 654 nucleotides (J3). The genetic lesions that result in HPRT deficiency are heterogeneous. A missense mutation, nonsense mutation, gene deletion and insertion, and duplication of exon have been described (S11).

5.2.2. *Adenine Phosphoribosyltransferase Deficiency*

Adenine phosphoribosyltransferase (APRT) deficiency is an inherited disorder of purine metabolism and is inherited in an autosomal recessive manner (K18, V7). This enzyme deficiency results in an inability to salvage the purine base adenine, which is oxidized via the 8-hydroxy intermediate by xanthine oxidase to 2,8-dihydroxyadenine (2,8-DHA). This produces crystalluria and the possible formation of kidney stones due to the excretion of excessive amounts of this insoluble purine. Type I, with virtually undetectable enzyme activity, found predominantly in Caucasians, is found in homozygotes or compound heterozygotes for null alleles. Type II, with significant APRT activity, found only in Japan, is related to a missense mu-

tation at 136 from ATG to ACG (*APRT*J*) (H5). Among Japanese, the most common mutant allele (about 70%) is *APRT*J*, and about 20% of the mutant alleles involve nonsense mutations at codon 98 from TGG to TGA (K2).

5.3. Prolidase Deficiency

Prolidase is a ubiquitous enzyme that splits dipeptides with a prolyl residue in the COOH-terminal position. This enzyme plays a critical role in the recovery of imino acids from endogenous collagen turnover as well as from exogenous dietary proteins. Human prolidase is a homodimer with a subunit of 54,300 Da and the gene locus is on the short arm of chromosome 19 (E2). The gene for human prolidase is over 130 kb long and consists of 15 exons (T12). Prolidase deficiency is an autosomal recessive trait with a characteristic clinical syndrome that includes chronic dermatitis, mental retardation, and recurrent infections and is associated with massive imidodipeptiduria. More than 28 cases have been reported so far. The molecular basis of prolidase deficiency in Japanese patients has been found to be the gene deletion (T12, T13).

5.4. Acatalasemia

Acatalasemia is a rare hereditary deficiency of tissue catalase and is inherited as an autosomal recessive trait (O3). This enzyme deficiency was discovered in 1948 by Takahara and Miyamoto (T1). Two different types of acatalasemia can be distinguished clinically and biochemically. The severe form, Japanese-type acatalasemia, is characterized by nearly total loss of catalase activity in the red blood cells and is often associated with an ulcerating lesion of the oral cavity. The asymptomatic Swiss-type acatalasemia is characterized by residual catalase activity with aberrant biochemical properties. In four unrelated families with Japanese-type acatalasemia, a splicing mutation due to a G-to-A transition at the fifth nucleotide in intron 4 was elucidated (K20, W5). We have also determined a single base deletion resulting in the frameshift and premature translational termination in the Japanese patient (H16).

5.5. Galactosemia

Galactosemia is a hereditary disorder associated with a cellular deficiency of galactokinase, galactose-1-phosphate uridyltransferase, or uridine diphosphate galactose-4-epimerase. These enzyme deficiencies are transmitted by autosomal recessive inheritance. The clinical manifestation in galactokinase deficiency is milder and is mainly manifested only by cataracts. Cloning of the galactokinase cDNA and identification of mutants have been done (S19). In transferase and epimerase deficiency, galactose ingestion is characterized by inanition, failure to

thrive, vomiting, liver disease, cataracts, and mental retardation. More than 32 mutations of the human galactose-1-phosphate uridyltransferase gene have been elucidated (E1).

5.6. Porphyrias

The porphyrias are inherited or acquired disorders in which the activities of the enzyme of the heme biosynthetic pathway are deficient. Eight enzymes are involved in the synthesis of heme, and, with the exception of the first enzyme, δ-aminolevulinic acid (ALA) synthetase, an enzymatic defect at each step of heme synthesis accompanies each form of porphyria. Among them, deficiencies of uroporphyrinogen III cosynthetase, ALA dehydratase, porphobilinogen deaminase, and uroporphyrinogen decarboxylase can be diagnosed by measuring the red blood cell enzyme activities. Congenital erythropoietic porphyria is due to a deficiency of uroporphyrinogen III cosynthetase and is characterized by marked skin photosensitivity. ALA dehydratase deficiency is the rare form of porphyria and involves neurologic symptoms without skin photosensitivity. Acute intermittent porphyria due to a deficiency of porphobilinogen deaminase is the most common autosomal dominant form of acute hepatic porphyria and is characterized by attacks of abdominal pain, neurological disturbances, and psychiatric symptoms. So far, 19 different mutations including single base substitution, single base deletion, single base insertion, nonsense mutation, and abnormal splicing have been reported (D3). Uroporphyrinogen decarboxylase deficiency causes porphyria cutanea tarda, which is the most common form of porphyria. Patients with this enzyme deficiency have mild to severe photosensitivity and often have overt liver disease.

5.7. Carbonic Anhydrase Deficiency

Carbonic anhydrase (CA) exists in three known soluble forms in humans. All three isozymes (CA I, CA II, and CA III) are monomeric, zinc metalloenzymes with a molecular weight of approximately 29,000. The enzymes catalyze the reaction for the reversible hydration of CO_2. The CA I deficiency is known to cause renal tubular acidosis and nerve deafness. Deficiency of CA II produces osteopetrosis, renal tubular acidosis, and cerebral calcification. More than 40 CA II–deficient patients with a wide variety of ethnic origins have been reported. Both syndromes are autosomal recessive disorders. Enzymatic confirmation can be made by quantitating the CA I and CA II levels in red blood cells. Normally, CA I and CA II each contribute about 50% of the total activity, and the CA I activity is completely abolished by the addition of sodium iodide in the assay system (S22). The cDNA and genomic DNA for human CA I and II have been isolated and sequenced (B34, M33, V9). Structural gene mutations, such as missense mutation, nonsense

mutation, gene deletion, and splicing mutation, at the CA II locus on chromosome 8 have been identified (R5).

6. Summary

Red blood cell enzyme activities are measured mainly to diagnose hereditary nonspherocytic hemolytic anemia associated with enzyme anomalies. At least 15 enzyme anomalies associated with hereditary hemolytic anemia have been reported. Some nonhematologic diseases can also be diagnosed by the measurement of red blood cell enzyme activities in the case in which enzymes of red blood cells and the other organs are under the same genetic control.

Progress in molecular biology has provided a new perspective. Techniques such as the polymerase chain reaction and single-strand conformation polymorphism analysis have greatly facilitated the molecular analysis of erythroenzymopathies. These studies have clarified the correlation between the functional and structural abnormalities of the variant enzymes. In general, the mutations that induce an alteration of substrate binding site and/or enzyme instability might result in markedly altered enzyme properties and severe clinical symptoms.

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ENDOGENOUS MEDIATORS IN SEPSIS AND SEPTIC SHOCK

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1. Introduction

1.1. Pathogenesis of Sepsis

The incidence of septic shock has increased progressively during the past 50 years, so that it is now the most common cause of death in intensive care units (P4). Mortality from septic shock remains 30–60% despite considerable advances in

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supportive care (B52, P3, W11). The relative lack of effective pharmacologic interventions highlights the complex pathophysiologic events involved in sepsis (P3). The principal pathophysiologic abnormalities in sepsis include peripheral vasodilatation, increased capillary permeability, and organ dysfunction (B34, S12). The early circulatory changes are increases in heart rate, cardiac index, and oxygen delivery in order to maintain oxygen consumption by increasing the supply of oxygen and its substrates. The increased oxygen consumption indicates increased metabolic activity as a compensation for antecedent tissue hypoxia resulting from maldistribution of the microcirculatory flow (S12).

The clinical manifestations of sepsis are the result of an excessive host response to an (usually bacterial) infection (B34). Although host defense mechanisms are essentially beneficial and designed to localize and neutralize invading microorganisms, remove dead and damaged cells, and repair tissue damage, excessive activation may be detrimental. A wide array of activated cells as well as inflammatory mediators are involved in the pathogenesis of sepsis, and the complex interaction of these mediators can be seen as a cascade (Fig. 1) (B37, H11). Much of our understanding of the role of inflammatory mediators is based on results from studies in several animal models of sepsis and from studies in humans subjected to low-dose endotoxin. However, an increasing number of studies in patients with severe sepsis have also contributed to our understanding of the pathogenetic mechanisms related to the release and activation of mediator systems. There is abundant evidence that proinflammatory cytokines are released into the circulation and that several cells, including neutrophils, monocytes, macrophages, platelets, and endothelial cells, become activated. In addition, plasma protein cascade systems such as the complement, coagulation, fibrinolytic, and contact systems are activated, and lipid mediators such as eicosanoids and platelet-activating factor, as well as oxygen and nitrogen radicals, are produced and released (B26). The stress hormone response is also part of the general host response. Interestingly, it has now been documented that anti-inflammatory cytokines (e.g., interleukin-10), soluble cytokine receptors (e.g., sTNF-R), and acute phase proteins are released as well, which seem to have a regulatory function and may limit or blunt the (general) inflammatory response (B38). Apparently, in the presence of the excessive general inflammatory reaction observed in severe sepsis, these endogenous regulatory systems have failed. Although our knowledge of the pathophysiology has increased markedly in recent years, our understanding of the precise role of individual mediator systems is extremely limited. In particular, their role in the pathogenesis of organ dysfunction or failure and circulatory shock is unclear and needs to be eludicated (B7). What is clear is that an extremely complex network of activated cells and mediators, as well as toxic products, is operative in patients with sepsis. There is no doubt that cytokines have a pivotal role in this network. Measurement of plasma levels and evaluations of correlations with clinically relevant variables ia a necessary and the essential first step in assessing their possible significance (T5).

There is increasing evidence that activation of the vascular endothelium by various agonists (e.g., tumor necrosis factor and interleukin-1) is also central to the pathological response to sepsis (B43, H27)

1.2. Definitions

To develop a more uniform set of definitions, a consensus conference (B39) proposed that sepsis and similar disorders be called *systemic inflammatory response syndrome* (SIRS). The consensus conference made another key decision, to restrict the use of the word *sepsis* to cases in which infection is documented, because SIRS can be caused not only by bacterial, viral, fungal , or other infections but also by 'sterile' insults such as multiple trauma, severe burns, hemorrhagic shock, acute pancreatitis, and other life-threatening conditions (B39). These definitions are presented in Table 1 and are useful clinically and experimentally, because they allow us to identify patients and to assess the outcome of treatment in a uniform fashion. Nonetheless, sepsis, the sepsis syndrome, and septic shock are not discrete entities; rather these terms delineate increasingly severe stages of the same disease probably resulting from the same pathophysiologic processes (B33). It has become clear that the clinical syndrome of septic shock is the result of endogenous mediators secreted by the injured host. Other than initiating the production of inflammatory mediators, the infecting microorganism plays a minor role. It has been recommended that the term septic syndrome should no longer be used, because it has been applied to a variety of inflammatory states (B39, D5). As Table 1 points out, sepsis requires the presence of hyper- or hypothermia, tachycardia, and tachypnea. However, many patients with confirmed bacteremia do not fulfill these criteria, and reliance on "traditional" criteria of sepsis may not be sufficient either. Bacteremia has been a traditional and prognostic tool, although the exact role of bacteremia as an independent risk factor is also in doubt (B39). In addition, bacteremia was found in less than half of the patients in recent studies of clinical sepsis (B52) and is uncommon in trauma patients.

There are various "*severity of illness*" *scoring systems* for sepsis and trauma (R11). Severity scoring can be used, in conjunction with other risk factors, to anticipate and evaluate outcomes, such as hospital mortality rate. The most widely used system is the Acute Physiology, Age, Chronic Health Evaluation II (APACHE II) classification system (K12). The APACHE III was developed to more accurately predict hospital mortality for critically ill hospitalized adults (K13). It provides objective probability estimates for critically ill hospitalized patients treated in intensive care units (ICUs). For critically ill posttrauma patients with sepsis or SIRS, another system for physiologic quantitative classification and severity stratification of the host defense response was described recently (R11). However, this Physiologic State Severity Classification (PSSC) has yet not been applied routinely in ICU setting.

TABLE I
DEFINITIONS

Systemic inflammatory response syndrome: The systemic inflammatory response to a wide variety of severe clinical insults. The response is manifested by two or more of the following conditions:
Temperature >38°C or <36°C
Heart rate >90 beats/min
Respiratory rate>20 breaths/min or $Paco_2$ <4.3kPa
WBC >12,000 cells/mm^3, <4000 cells/mm^3, or >10% immature (band) forms

Sepsis: The systemic response to infection. This systemic response is manifested by two or more of the following conditions as a result of infection:
Temperature >38°C or <36°C
Heart rate >90 beats/min
Respiratory rate >20 breaths/min or $Paco_2$ <4.3 kPa
WBC >12,000 cells/mm^3, <4000 cells/mm^3, or >10% immature (band) forms

Severe sepsis: Sepsis associated with organ dysfunction, hypoperfusion, or hypotension. Hypoperfusion and perfusion abnormalities may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status.

Septic shock: Sepsis with hypotension (a systolic blood pressure of <90 mm Hg or a reduction of <40 mm Hg from baseline), despite adequate fluid resuscitation, along with the presence of perfusion abnormalities as seen by severe sepsis. Patients who are receiving inotropic or vasopressor agents may not be hypotensive at the time that perfusion abnormalities are measured.

Multiple organ dysfunction syndrome: Presence of altered organ dysfunction in an acutely ill patient such that homeostasis cannot be maintained without intervention.

2. Sepsis and Cytokines: Current Status

2.1. Proinflammatory Cytokines: TNF, Interleukin-1, Interleukin-8

Mediators of the cytokine class are assumed to exert pathophysiological influences in septic and critically ill patients (B27). Among the cytokines exhibiting significant proinflammatory characteristics, tumor necrosis factor-α (TNF), interleukin-1 (IL-1), and interleukin-8 (IL-8) appear to be the most promising candidate mediators promoting both acute septic responses and multiple organ failure (Table 2) (P11). The availability of techniques for assessing the systemic appearance of cytokines by radioimmunoassay or enzyme-linked immunosorbent assay (ELISA) determinations has generated numerous observations in critically ill patients. These assays have still to achieve the real-time efficiency necessary for use in clinical practice. Especially, there is the problem of assay standardization. The sensitivity and specificity of these assays vary widely and a consensus standard for cytokine detection has yet to be achieved. Furthermore, there is evidence that because of the dynamic turnover of such circulating mediators, given the short half-lives and episodic production, their detection is possible only during a tran-

TABLE 2

PRO- AND ANTI-INFLAMMATORY CYTOKINES AND CIRCULATING NATURAL ANTAGONISTS

Cytokine (mass in kDa)	Source	Functions
Proinflammatory		
IL-1 (17.5)	Monocyte/macrophage, lymphocyte, neutrophil, endothelium, fibroblast keratinocyte	Activation of T cells, B cells, natural killer cells, osteoblasts, and endothelium. Induces fever, sleep, anorexia, ACTH release, hepatic acute phase protein synthesis and HSPs. Leads to myocardial depression, hypercoagulability, hypotension/shock, and death. Simulates production of TNF, IL-6, and IL-8 and stress hormone release. Suppression of cytochrome P-450, thyroglobulin, and lipoprotein synthesis. Procoagulant activity. Antiviral activity.
TNF (17.5)	Monocyte/macrophage, lymphocyte, neutrophil, endothelium, fibroblast, keratinocyte	Activation of T and B cells, natural killer cells, neutrophils, and osteoblasts. Stimulation of endothelial cells to release chemotactic proteins, NO and PGI_2. Tumoricidal activity. Induces fever, sleep, hepatic acute phase protein synthesis, catabolism, ACTH release. Lead to myocardial depression, hypotension/shock, hypercoagulability, and death. Stimulates production of IL-1, IL-6, IL-8, IFN-γ, and H_2O_2. Suppression of cytochrome P-450, thyroglobulin, and lipoprotein lipase. Induces complement activation, release of eicosanoids, including PAF. Procoagulant activity.
IL-8 (8)	Monocyte/macrophage, lymphocyte, fibroblast, endothelium, keratinocyte	Recruitment and activation of neutrophils, chemotactic for lymphocytes, angiogenesis.
Antiinflammatory		
IL-10 (35)	T cell, fibroblast	Suppression of B- and T-cell proliferation. Inhibition of LPS-induced monocyte IL-1, IL-8, and TNF production. Induction of IL-1ra. Suppression of free radical release and NO-dependent microbicidal activity of macrophages.
IL-6 (21–28)	Monocyte/macrophage, T cell endothelium, fibroblast, keratinocyte	Induction of fever and the hepatic acute phase response. Stimulates cortisol production. Decreases IL-1 and TNF production. Participates in activation of B and T cells, facilitates Ig production by B cells. Induction of granulocyte-macrophage colony-stimulating factor, stimulation of hematopoietic progenitors.
IL-1ra (17.5)	Monocyte/macrophage, fibroblast	Specifically inhibits IL-1 effects, including SIRS and sepsis in animal models and humans. Attenuation of coagulation, fibrinolytic, and complement systems, levels of PAF and neutrophil elastase.
sTNF-R (30–40)	Unknown, but monocyte/macrophage and neutrophil likely	Specifically inhibits TNF effects, including SIRS and sepsis.

sient period. A considerable degree of cytokine activity may exist without detection of circulating forms, which is further complicated by a polymorphism, as documented for TNF and IL-1 (S36, W12).

2.1.1. *Tumor Necrosis Factor (TNF)*

Human TNF is produced as a prohormone of 233 amino acids and is processsed to a 157-residue mature protein of 17 kDa by cleavage of a 76-residue signal peptide (T12). Overproduction of TNF is considered to be important for the pathogenesis of shock and tissue injury during SIRS. It is therefore important to understand the factors that regulate its production. Monocytes and macrophages are the main producers of TNF, but T cells, natural killer (NK) cells, and neutrophils can also secrete TNF (T12). Many infectious or inflammatory stimuli are capable of triggering TNF biosynthesis, including bacterial endotoxin or lipopolysaccharide (LPS) and other products of bacteria, parasites, and yeasts. This process is tigthly regulated. Within the macrophage (the principal source of TNF) regulation operates at transcriptional, posttranscriptional, and translational levels. In response to LPS, TNF gene transcription is enhanced threefold (B24). Glucocorticoid hormones, if administered before activation, strongly inhibit TNF synthesis by inhibiting the quantity of TNF messenger RNA (mRNA) and by preventing its translation (B21). Once the cells are activated by LPS, no inhibiting effects of corticosteroids on TNF synthesis occur. TNF synthesis can also be suppressed by IL-4 (K4), IL-6, pentoxifylline (S5), and platelet-activating factor (PAF) antagonists (T7). Production of TNF can be enhanced by interferon-γ (IFN-γ) (P10, K15), IL-1, and reactive oxygen species, which are ubiquitously present during inflammation (C14). In addition, several endogenous factors that determine TNF responsiveness have been identified. The interindividual differences in TNF secretory ability are caused by differences in genetic control (different TNF genotypes)(S36), and genetic factors considerably influence production of TNF (and IL-10) (W12). The hypophysis–adrenal axis also affects the TNF response; TNF release is enhanced in the absence of endogenous steroids (Z8).

TNF is produced and secreted by activated cells within minutes following contact with LPS, reaching peak levels at 90–120 minutes after the admnistration of *Escherichia coli* endotoxin in human volunteers (M27). Van Deventer *et al.* (D15) could not detect serum TNF levels during experimental endotoxemia. Even during continuous intravenous administration of recombinant TNF (rTNF), serum TNF rapidly becomes undetectable (M27). It has been proposed that circulating soluble TNF receptors (sTNF-Rs) may be an important down-regulating mechanism (G10).

TNF activates inflammatory functions of various immune cells, not only as a direct effector but also as part of the cytokine network (synergism with IL-1, IFN-γ, and LPS), as shown in Table 2 (K11). TNF interacts with the complement system and induces the additional release of eicosanoids. TNF has many effects on

the endothelium: it stimulates the production of vasodilating substances such as prostaglandin I_2 (PGI_2) and nitric oxide. In addition, TNF enables endothelial cells to express procoagulant activity and to reduce fibrinolytic potential, contributing to a shift from anticoagulant activity to a procoagulant state (P15). TNF induces several metabolic abnormalities (D4). TNF inhibits lipid uptake in adipose tissue and enhances lipogenesis in the liver. TNF causes an efflux of amino acids from skeletal muscle and decreases proteolysis in the liver, resulting in a loss of total body nitrogen (F12). TNF administration leads to a decline in blood glucose levels. TNF produces a variety of neuroendocrine effects, including stimulation of the anterior pituitary, adrenal cortical, and pancreatic secretion and sympatic activation (see also Sect. 5) linking the immune and endocrine systems (D4, H21). In rats, TNF administration caused hemoconcentration, lactic acidosis, biphasic fluctuations in blood glucose concentrations, hypotension, and death, usually caused by respiratory arrest (T11). The pathophysiology and pathology seen at autopsy of patients with septic shock were reproduced in animals given rTNF (T11). rTNF admnistrated to humans caused hemodynamic and metabolic derangements that closely resemble the responses to endotoxemia (C14). The physiological significance of TNF effects is not fully understood. Possibly, this cytokine is beneficial at a local level and deleterious when present systemically in large quantities.

Several clinical studies have demonstrated the major involvement of TNF in the pathogenesis of shock and tissue injury during SIRS and sepsis:

1. Intravenous administration of rTNF induces a disease state that closely resembles septic shock accompananied by tissue damage (M28, T12). TNF induces fever, leukocyte aggregation, hypotension, stress hormone release, lung edema, and hemorrhagic necrosis of various organs (T12).
2. The cytokine is released early and in large amounts in response to bacterial stimuli in experimental situations (M16, M28). Several studies have reported significantly elevated levels of TNF in septic patients (C3, C12, D1, D32, E7, G4, G22, M10, M18, W2). However, considerable discrepancies in plasma levels have been reported in these patients. For example, plasma TNF levels of 10–100 pg/ml found in only 25% of septic patients tested (D6) are in contrast to the plasma TNF levels of 100–5000 pg/ml found in 100% of patients in another study (D1). DeGroote *et al.* (G12) detected circulating TNF in only 16% of patients with gram-negative sepsis. Waage *et al.* (W2) demonstrated TNF in 23% of patients with meningococceal disease.
3. Pretreatment with monoclonal anti-TNF antibodies prevents mortality (B23, M32) and organ damage (M16) in experimental sepsis. In clinical studies using anti-TNF antibodies, however, the overall benefit of this treatment showed encouraging but no evident results (L22). Recently, the INTERSEPT study suggests a possible role for anti-TNF antibody as an adjunctive therapy, but with no reduction of mortality (C21). There is no plain cause–effect relation between TNF re-

lease and the development of septic shock. If TNF blocking agents are given together with or after elicitation of disease, such intervention is not successful or even enhances mortality (E2). Further, rTNF can also protect the host against lethal bacterial infection (C25).

The different methods of plasma TNF measurements may have contributed to the different results in the literature. Engelberts *et al.* (E12) described an accurate sandwich ELISA for the measurement of biologically active TNF. They also showed that false-positive and false-negative results can be a consequence of improper handling of blood samples. This can be prevented by using an EDTA-containing system and separating blood cells from plasma within 15 minutes after blood collection. Data from Marecaux *et al.* (M10) indicate a large variability in TNF (and also IL-6) levels, which limits their prognostic significance in patients with septic shock. In addition, TNF would have obvious limitations because large variations may be expected in the same patient depending on the timing of blood sampling. Some immunoassays also detect biologically inactive TNF–TNF receptor complexes or even give a positive signal in the presence of free soluble TNF receptors. Studies using these assays should be reevaluated (E12).

2.1.2. *Interleukin-1*

Two forms of interleukin-1 exist (IL-1α and IL-1β); they bind to the same cellular receptor and exert essentially identical biological effects (Table 2). The synthesis and release of IL-1 from macrophages and other cells are initiated by microorganisms, endo- or exotoxins from a variety of bacteria, C5a, TNF, IL-1 itself, or tissue injury. Il-1 acts like an endogenous adjuvant, serving as a cofactor during lymphocyte activation, primarily by inducing the synthesis of other cytokines and the activation of resting T cells (D18). IL-1 is, like TNF, capable of inducing a septic shock–like state in a rabbit model, especially resulting in lung damage (D19). IL-1 often acts in a synergistic fashion with TNF. The potential benefit of IL-1 to the host is in promoting antibacterial resistance, whose mechanism is unknown.

The complexity of measuring IL-1 in blood specimens has been discussed as a possible reason for the lower incidence of IL-1 detection during sepsis in humans (C6). In normal volunteers given an intravenous dose of *E. coli* endotoxin, a rise in IL-1 levels was noted, with peak levels reached within 2–3 hours after challenge (C5). The detection of circulating IL-1 in patients with sepsis has been noted in up to 37% of patients (B31, C2, C3, C5, C11, G10) and such results may be influenced by the assay methods used. In a study by Damas *et al.* (D1), IL-1 serum levels were only slightly elevated in septic shock and could not be correlated with severity and/or mortality. However, Waage *et al.* (W3) demonstrated that IL-1 was detected only in serum from patients with septic shock who also had high levels of IL-6, TNF, and LPS and fatal outcomes. They mention that combinations of LPS

and cytokines may be particularly potent in producing lethal septic shock. Interestingly, it has been showed that tissue concentrations of IL-1 are markedly higher than they are in the plasma from animals and patients with hemorrhagic pneumonia or adult respiratory distress syndrome (ARDS). The appearance of IL-1 in the circulation may result from excessive tissue production that distributes into the plasma compartment.

IL-1 receptor antagonists (IL-1ra) are able to block the activity of IL-1 both *in vitro* and *in vivo* (see also Sect. 2.3). A 10–100-fold excess of IL1ra is required to inhibit 50% of IL-1–induced responses *in vitro* and in animal models; this is consistent with the presence of a large excess of IL-1 receptors compared with those that need to be occupied in order to trigger a biological response to IL-1. Protective effects of IL-1ra have been shown in animals with sepsis caused by gram-negative and gram-positive organisms (O6). The dramatic reduction in hypotension in these studies is probably due to blockade of IL-1–induced NO synthesis (M4). Any coincident reduction in the production of IL-1 and TNF may also play a role in reducing progression of the shock state. Interestingly, IL-1 receptor blockade markedly attenuated activation of coagulation and/or fibrinolysis in septic patients (B31).

In conclusion, IL-1 (or TNF) given intravenously can reproduce the elements of sepsis, and blocking the activities of IL-1 can abrogate the septic state. IL-1 acts synergistically with TNF in producing septic physiology, implicating both as crucial triggers in the initiation of sepsis.

2.1.3. *Interleukin-8*

Interleukin-8 (IL-8) is a small cytokine (8 kDa) with the function of neutrophilic chemotaxis and activation, as well as angiogenesis (Table 2). Some studies show an important role for this cytokine in neutrophilic recruitment and activation at sites of inflammation, resulting in amplification of the local inflammatory response as well as tissue damage. IL-8 is secreted by many cell types, including fibroblasts, endothelium, and peripheral blood mononuclear cells. IL-8 secretagogues include endotoxin, IL-1, and TNF (Z2). *In vitro*, significant amounts of this cytokine are produced in response to minute concentrations of these stimulants. At 1–10 pg/ml, IL-1 or TNF can induce significant IL-8 production in human endothelial and fibroblast cell lines. IL-8 levels increase during intravenous administration of IL-1 in primates (Z1) and are also elevated during human sepsis (H8, B9). Patients with clinical signs of shock had higher levels of IL-8 than normotensive patients (H8). Large individual variations were observed. Friedland *et al.* (F19) demonstrated elevated IL-8 levels in 8 of 18 patients with sepsis or localized *Pseudomonas pseudomallei* infection. In patients with meningococcal disease, detectable levels of IL-8 were found in 28 of 62 patients (H12). In patients with fever and neutropenia due to hematologic malignancies and chemotherapy, IL-8 levels were consistently elevated, with a rapid decline in patients responding

to antibiotics (E10). Miller *et al.* (M29) detected high concentations of IL-8 in pulmonary edema fluid, coupled with relatively low plasma IL-8 levels, in patients with sepsis-induced ARDS. This suggests that the lung is the primary source of IL-8 in the alveolar edema fluid in these patients. IL-1ra treatment had no effect on circulating levels of IL-8 in baboons with sepsis (F9) and humans (B31). No data are available on the role of IL-1 in the induction of IL-8 in patients with clinical sepsis.

2.2. Anti-Inflammatory Cytokines: Interleukin-6 and Interleukin-10

The previous section stated that a severe proinflammatory reaction contributes to the onset and continuation of sepsis. However, a massive compensatory anti-inflammatory reaction appears to be important in the pathophysiology of sepsis (B38). Anti-inflammatory agents such as IL-4, IL-6, IL-10, IL-11, IL-13, soluble TNF and IL-1 receptors, and IL-1 receptor antagonists are released to control the inflammation and to restore homeostasis (B37). If systemic levels of proinflammatory mediators are high enough, patients develop the clinical signs of sepsis. At times, a mixed response with both pro- and anti-inflammatory components prevails (K15). High systemic levels of anti-inflammatory mediators result in anergy and/or immune suppression with alterations in lymphocyte activity. If the balance is restored by these opposing forces, patients may survive.

2.2.1. *Interleukin-6*

Interleukin-6 (IL-6) is a small polypeptide with a molecular mass of 26 kDa (see Table 2). IL-6 can be induced in various cell types, including fibroblasts, macrophages/monocytes, epithelial cells, T cells, B cells, and diverse tumor cells (L4). TNF, IL-1, and LPS can stimulate IL-6 gene expression in macrophages/monocytes and fibroblasts. *In vivo* studies showed that systemic administration of TNF, LPS, and IL-1 was followed by a rapid induction of circulating IL-6 (B49, J2). Also, endothelin (ET) at concentrations observed pathophysiologically may trigger production of IL-6 (M17).

IL-6 is a pleiotropic cytokine exerting multiple biologic activities: induction of B-cell differentation, activation of T cells, induction of acute phase proteins (synergistically acting with IL-1 and TNF), inhibition of TNF production, inhibition of cell growth, and induction of adrenocorticotropic hormone (ACTH) synthesis and consequently cortisol (L4). IL-6 and IL-1 have overlapping activities. The interaction with other cytokines acts at two levels: IL-1 and TNF promote IL-6 production. IL-6 inhibits TNF synthesis and therefore makes part of a negative regulatory loop controlling TNF production (see Fig. 6). It has been shown that IL-6 has a protective effect against LPS toxicity by blocking LPS-induced TNF release both *in vitro* and *in vivo* (S9, A2) IL-6 is thought to be a mediator of the acute phase response, acting on hepatocytes both alone and in combination with TNF and IL-

1. Studies also suggested that IL-6 causes endothelial cell dysfunction and decrease of prostacyclin production. Soluble IL-6 receptors seem to play a modulating and enhancing role in IL-6 activity (F18). The incidence of detection appears to be less influenced by the method of assay, as either ELISA or bioassay techniques yield consistent results, with a high correlation between these techniques. IL-6 values may be more constant and endocrine-like than those values of TNF and IL-1.

It has been demonstrated (W3) that IL-6 and IL-1 are released into the serum during the initial phase of meningococcal septic shock. High serum levels have also been found in patients with other forms of gram-negative septic shock (C4, H9) or intra-abdominal sepsis (H12) and have been associated with severity of illness (D2, G4, K16, R12) or fatal outcome. (C11, W3). IL-6 levels remain very high until clinical recovery (G4, H12), but a larger study showed a peak concentration of IL-6 near the onset of shock and a rapid decrease to undetectable levels within 24 hours in most patients (C2). In most studies overlapping values were noted in the survivors and nonsurvivors (M10). However, a persistent increase has been correlated with poor outcome. In intra-abdominal sepsis, nonsurvivors had low levels of IL-6 (and TNF) (H12). By far the majority of studies detected the highest levels of IL-6 at the time of diagnosis or admission and showed a consistent decrease over time, nearly always irrespective of outcome (T5).

The complete role of IL-6 in septic shock is not fully understood. It may act as an alarm hormone, reflecting the magnitude and persistence of cellular damage. It suppresses the production of TNF and IL-1 release and increases serum cortisol levels. On the other hand, IL-6 is associated with fever and severity of illness. Whether IL-6 is just a "mirror of disease" reflecting the biological effects of LPS-induced TNF and IL-1 or whether it is directly responsible for the pathological changes seen in human septic shock remains to be investigated. Interestingly, a recent study showed high IL-6 concentrations together with below normal soluble IL-6 receptor concentrations during acute meningococcal infections (F18). Anti–IL-6 antibodies caused higher levels of TNF in *E. coli*–infected mice, supporting the notion that IL-6 acts as a counterregulator. However, mortality in the treated mice was lower (S32).

In conclusion, there is no doubt that IL-6 is produced massively and released to the circulation during sepsis. However, the significance of IL-6 levels and the therapeutic role of anti–IL-6 are still unclear.

2.2.2. *Interleukin-10*

Interleukin-10 (IL-10) affects antigen presentation capacity but also interferes with many other functions of monocytes and macrophages (Table 2) (F8). *In vitro,* IL-10 is a potent inhibitor of cytokine production, including production of TNF, IL-1, IL-6, and IL-8 by LPS-activated monocytes/macrophages (F8). It also inhibits tissue factor–dependent procoagulant activity induced by LPS in human

monocytes (P20). IL-10 is able to suppress free radical release and NO-dependent microbicidal activity of macrophages against various pathogens (G6). Interestingly, IL-10 does not inhibit the *in vitro* production of potent macrophage-derived anti-inflammatory mediators such as transforming growth factor-β (TGF-β) and even induces their release (W1). NK cells appear to be another target for the anti-inflammatory properties of IL-10. Data have shown that IL-10 inhibits the IL-2–induced IFN-γ production by NK cells *in vitro* (H29).

In endotoxinemic mice IL-10 treatment inhibits proinflammatory cytokine release and leads to a reduction in LPS toxicity (G8, H28). IL-10 does not influence the LPS-induced production of IL-6 *in vivo* (M9). This suggests that IL-10 could differentially regulate TNF and IL-6 production by macrophages *in vivo,* in contrast to data obtained *in vitro* (W1), or that cell types other than macrophages are a major source of IL-6 *in vivo* and are resistant to IL-10 (S23).

LPS induces the release of IL-10 in mice (D31). Interestingly, circulating IL-10 was detected 90 minutes after LPS injection and was still present at 6 hours. These data show that IL-10 is released at the same time as TNF and IFN-γ, suggesting that their production could be regulated by IL-10 during endotoxemia. In addition, Marchant *et al.* (M9) showed that anti–IL-10 pretreatment resulted in increased production of both TNF and IFN-γ after LPS injection. Furthermore, this increased cytokine release was associated with higher mortality. It seems, therefore, that IL-10 produced during endotoxemia is an important protective mechanism against LPS toxicity.

There are few clinical data regarding IL-10 in human septicemia and septic shock. Marchant *et al.* (M8) found high IL-10 levels in 22 of 48 patients with normotensive sepsis (46%) and in 17 of 21 patients with septic shock (81%). Patients with septic shock had higher IL-10 levels, peaking during the first 48 hours and remaining detectable for 3–5 days after admission. Possibly, the intensity of the anti-inflammatory response of IL-10 is related to the importance of macrophage activation (E14). In addition, IL-10 was associated with the development of sepsis in patients with severe trauma (S19).

Chlorpromazine (CPZ) and pentoxifylline (PTX) were shown to inhibit TNF release and improve survival during murine endotoxemia (G1). CPZ (M25) and epinephrine (P16) pretreatment markedly up-regulated IL-10 production induced by LPS, a phenomenon also observed with cyclosporine (D1). PTX pretreatment did not affect LPS-induced IL-10 release. Thus, TNF and IL-10 can be differentially regulated during murine endotoxemia. The sustained or even increased production of IL-10 could play a role in the protective effects of these drugs against LPS toxicity *in vivo.*

2.3. Soluble TNF and IL-1 Receptors, IL-1 Receptor Antagonists

It has been proposed that the presence of endogenously derived TNF and IL-1 antagonist molecules in the circulation may serve, at least in part, as a surrogate

marker for the antecedent activity of proinflammatory cytokine species (D14, G16). Soluble IL-1RII, which represents the extracellular domain of the membrane-bound IL-1RII formed by proteolytic cleavage, inhibits IL-1 activity by competing with the cellular IL-1 type I receptor for IL-1 binding (G11). The IL-1 receptor antagonist (IL-1ra) is a cytokine that competitively inhibits IL-1 by binding to the type 1 IL-1 receptor without agonist activity. They represent two distinct endogenous mechanisms by which the body modulates exaggerated levels of IL-1 in times of inflammatory challenge. sTNF-Rs, which represent the extramembranous part of the cellular TNF receptor that has been shed, block the effects of TNF to a great extent. Shedding of the receptor occurs by proteolytic cleavage induced by binding of TNF to its receptor. The consequence of shedding is that overstimulation of the target cell is prevented. To this end, the concept that natural cytokine antagonist levels may reflect the relative magnitude of preceding inflammatory insult is supported by observed incremental increases in their circulating levels along a spectrum from mild endotoxemia (F9, F10, Z1) to accidental injury (C20) to severe infection (R13).

Several problems limit the interpretation of sTNF-R and IL-1ra levels, including the specificity of currently available assays for free receptor or receptor–ligand complexes (E13). In addition, acute or chronic organ dysfunction may significantly alter the clearance of both sTNF-R and IL-1ra, and the potential impact of established immunological dysfunction upon their production has not been clarified.

In critically ill patients who ultimately survived an episode of severe bacterial infection, the levels of sTNF-R noted at initial diagnosis were similar to those achieved in response to mild endotoxin challenge in normal subjects (R13). However, patients who succumbed to the sequelae of sepsis exhibited higher levels of sTNF-R both at the time of diagnosis and for the duration of their hospital course. The initially elevated levels could not be ascribed to organ dysfunction, as the degree of organ failure was similar to that observed in survivors. By contrast, the initial levels of IL-1ra were not increased over those achieved during controlled endotoxemia. IL-1ra could not discriminate between patients who survived and those who ultimately expired. Follow-up levels of sTNF-R have been reported in a few studies (G10, R13). In children with severe meningococcal disease, a significant decline in levels of sTNF-RII during 6 hours following admission was observed in survivors, whereas levels did not change in non-survivors (G10). In critically ill patients with sepsis, levels of sTNF-RI remained consistently elevated throughout an extended period of time and were significantly higher in nonsurvivors (R13). Gardlund *et al.* (G4) found high IL-1ra levels in all septic patients, with significant correlation with APACHE II scores. The molar excess of IL-1ra to IL-1 was >2000-fold in 11 of the 13 patients. Similarly to sTNF-R levels, IL-1ra levels decrease slowly, reflecting either slow clearance or ongoing production. Van Deuren (D13) showed differential expression of IL-1 and IL-1ra, suggesting a mechanism of protection against overstimulation by the proinflammatory effects of IL-1. Pri-

utt *et al.* (P21) found both elevated plasma IL-1ra and soluble IL-1RII concentrations in patients with sepsis syndrome. Shedding of the IL-1RII and increased release of IL-1ra may play important roles in down-regulating the frequency with which local IL-1 production leads to a systemic inflammatory response.

2.4. Heat Shock Proteins

Heat shock proteins (HSPs) are synthesized by cells in response to an increase in temperature, as well to various other stressful stimuli. Their main function is to ensure intracellular protein homeostasis, thus preserving the cells' viability in the presence of aggression. Current evidence points to a protective role for HSPs in several aspects of critical disease, such as ischemia–reperfusion, ARDS, and multiple organ failure. The increase of a few degrees Celsius above the normal environmental temperature of cells leads to the heat shock response: 1) rapid expression of heat shock genes, 2) suppression of normal protein synthesis, and 3) the ability of cells to survive a second and otherwise lethal heat challenge (thermotolerance).

HSPs are part of a larger family of proteins known as molecular chaperones, whose task is to maintain the conformational and structural integrity of intracellular proteins (J9). Many of the inducers of the stress response exert their action on HSP gene induction via the generation of reactive oxygen species (ROS). Heat shock can induce protection against oxidative stress, and exposure to oxidants can be followed by thermotolerance (D27). The possible mechanisms by which HSPs might protect cells from oxidative damage are promotion of free radical scavenging, prevention or repair of DNA damage, inhibition of ROS production, and inhibition of phospholipase A_2. Also, HSP can modulate nitric oxide production in stressed cells (D16).

2.4.1. *Ischemia–Reperfusion*

Ischemia–reperfusion is thought to mediate the severe organ dysfunction witnessed after shock or cardiac arrest. HSPs could play a major role in the defence against ischemia–reperfusion injury. Many *in vivo* experimental models of ischemia and/or ischemia–reperfusion have demonstrated HSP induction.

2.4.2. *ARDS*

ROS are an important determinant of acute lung injury in situations such as ARDS, irradiation, or hyperoxia. In ARDS, activated phagocytic cells recruited to the lungs can initiate or amplify lung damage through their ability to release large amounts of ROS (B46). Direct exposure of cultured endothelial cels to endotoxin can generate a threefold increase in superoxide anion production (B47). Clinical studies have demonstrated high amounts of H_2O_2 in expired breath (K8, K48) and increased catalase activity in serum of ARDS patients (L6). Furthermore, in-

creased levels of serum antioxidants seem to be predictive of the subsequent development of ARDS in sepsis (L5, L6). However, studies performed so far on the use of antioxidants in ARDS failed to show any benefit (J6). Oxidative stress is an inducer of HSP induction. The prior induction of HSPs in a model of acute lung injury due to phospholipase A_2 has been shown to reduce lung damage and mortality (V6). Alveolar macrophages from patients with ARDS exhibit increased expression of HSP 70 (P14). Exogenously administered HSPs are capable of exerting protective effects on endothelial cells exposed to ROS (J8). However, the clinical application of enhancing HPS synthesis should be explored further.

2.4.3. *Multiple Organ Dysfunction Syndrome*

Multiple organ dysfunction syndrome (MODS) results from the perpetuation or amplification of a generalized host inflammatory response to an initial triggering event such as shock or sepsis. HSPs might represent part of a protective response against the cellular toxicity of the inflammatory process leading to MODS. There are many links between oxdative stress and HSP expression, partly mediated by proinflammatory cytokine activity (W17). The induction of HSPs leads to inhibition of arachidonate metabolism and cell damage (J1). There is an indication of the presence of a certain hierarchy in cellular protein synthesis (C1), and heat shock decreases the rate of constitutive protein synthesis and impaired protein secretion (M3). These studies indicate that, when hepatocytes are submitted to shock, a heat shock response is induced that can, at least under certain conditions, take precedence over the acute phase response (S13). Hepatocytes could at times respond in excess to stress by shutting down acute phase proteins, thus prioritizing *self-preservation* over organism preservation. A shortage of acute phase proteins and an unchecked inflammatory response leading to MODS might thus ensue. Whether the preferential expression of HSPs merely reflects the severity of cellular injury, rather than an excessive response to stress, is unclear. Finally, HSPs could possibly, through molecular mimicry, amplify the immune and inflammatory response (Y3).

3. Vasoactive Agents

The endothelium has many diverse functions that enable it to participate in inflammatory reactions (H27). These include modulation of vascular tone, and hence control of local blood flow; changes in structure that allow leakage of fluids and plasma proteins into extravascular tissues; local accumulation and subsequent extravasation into tissues of leukocytes; and synthesis of surface molecules and soluble factors involved in leukocyte activation (B43). The endothelial cells themselves can modulate vascular tone by the release of vasoactive substances such as prostacyclin, nitric oxide (NO), ET. Endothelium-derived vasoactive substances

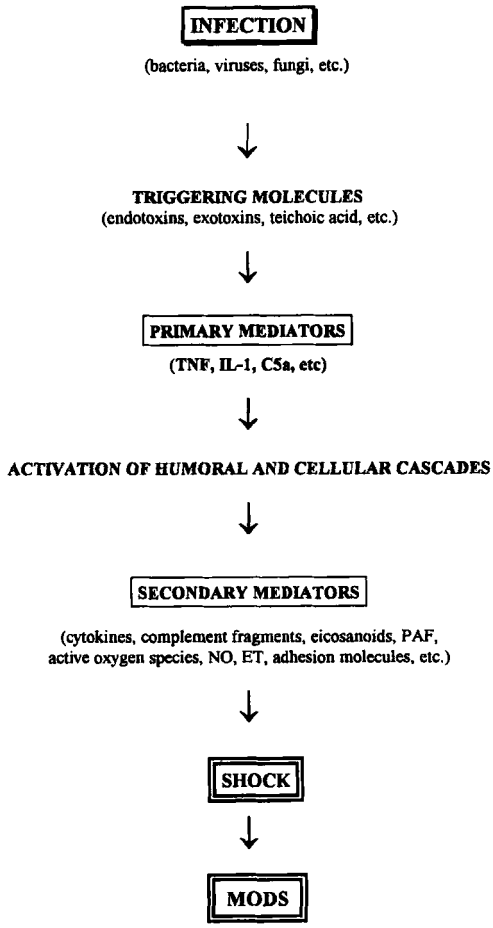

FIG. 1. Main steps in the pathogenesis of sepsis, leading to septic shock and/or multiple organ dysfunction syndrome (MODS).

are intimately involved in the pathophysiology of circulatory abnormalities and insufficient tissue perfusion during sepsis. Local endothelial injury may result in the release of substances that may initiate or sustain derangements of microcirculatory hemodynamics, volume homeostasis, and blood pressure (H25).

Substances released by the heart as atrial natriuretic peptide (ANP) have also offered a new dimension when looking at regulators of circulation (see Sect. 5.2.3) (Y2).

3.1. Endothelin

Endothelin is a 21-amino-acid vasoconstrictor belonging to a class of peptides that consists of three structurally closely related peptides called ET-1, ET-2, and ET-3 (D26, Y1). ET-1, which is the best studied, is derived from a 203-amino-acid precursor known as preproendothelin, which is cleaved after translation by specific endopeptidases to form a 38-amino-acid peptide, proendothelin or big ET. Big ET is then converted to active ET by a putative endothelin-converting enzyme. The major source of ET is the vascular endothelium, but ET is also produced by monocytes/macrophages (E3, M17) in response to a variety of stimuli, including circulating catecholamines, TNF, IL-1, and IL-6 (in various types of cell cultures). Localization studies of ET binding sites showed receptors not only in the cardiovascular system but also in the lung, kidney, adrenal gland, brain, spinal cord, gastrointestinal tract, liver, and spleen (L9, L10, M4). Also, it was reported that ET mRNA was widely expressed in human tissue. Two endothelin receptor subtypes have been cloned and expressed so far. The endothelin-A receptor has been localized to vascular smooth muscle cells, but not to the endothelium, and has a high affinity to ET-1 (C26). The ET-B receptor is expressed on the endothelium and has equal affinity for all three endothelins.

ETs are polyfunctional cytokines with vasoconstrictor, secretory, growth factor, and angiogenic roles in multiple organs and tissues. ET is also a neuroendocrine stimulator and a leukocyte activator. The effects of ET are mostly in the paracrine environment, because plasma ET concentrations are one-hundredth to one-thousandth of the concentrations shown to have effect on organ cells and tissues *in vitro*. *In vivo* studies have demonstrated that at pharmacological concentrations ET is a extremely potent coronary, renal, and systemic vasoconstrictor in association with a decrease in heart rate and cardiac output, which may decrease sodium excretion and activate the renin–aldosterone–angiotensinogen system (RAAS) (G14, L9, L10). Different effects of high- and low-dose ET infusion on blood pressure, pressure responsiveness, and baroreflex sensitivity suggest the complex actions of ET (M13, N1). The interaction with endogenous vasodilators, such as ANP and NO, may also regulate local vascular tone (M31). ET has been reported to enhance directly or indirectly the release of ANP (O7), and increased ANP release may act to preserve the effects of ET on cardiovascular and renal functions (G14, L11).

ET measured in the plasma appears to be a "spillover" of the ET that is (locally) released by the endothelium. It is known that low levels of ET (ranging from 0.25 to 5.0 pg/ml) are normally present in the circulation (B8, P12). These levels are increased by 3–10 times in patients with renal failure (S30), diabetes (V3), hypertension, burns (H32), myocardial infarction, primary pulmonary hypertension (N8), and cardiogenic shock (L1, L9, L10, P1, W8). ET is assumed to be released

by pathophysiological states that are characterized by a limitation of cardiac output or hypotension.

Endotoxin stimulated ET release *in vitro* and *in vivo* (M38, M37, M45, P8). This increase is probably caused by release or by increased production of injured and activated endothelium and macrophages (E3). A number of clinical studies showed increased plasma ET levels during sepsis and septic shock (B8, E8, E9, P12, S4, T1, V7, W10). We found a significant correlation of ET with the presence of shock and severity of illness (B8). We also observed a prolonged elevation of ET during 6 consecutive days, which poses the question of whether this is the result of stimulated continuous synthesis or a reflection of impaired elimination of ET (B8). Most studies also showed higher values of ET in nonsurvivors, indicating a prognostic significance of ET and reflecting more pronounced endothelial damage in these patients (E8, E9, V7). In clinical studies of human sepsis and low-cardiac-output states, ET values increased, but vascular resistance was low, not high (P12, V7). Therefore, there is a significant discrepancy between the effects of ET in healthy animals and the hemodynamic effects observed in septic patients. Clinical studies also showed that increased ET production may contribute to the development of organ failure, including ARDS (S4) and disseminated intravascular coagulation (A10). ET causes renal dysfunction in sepsis primarily by evoking severe reductions in renal blood flow and glomerular filtration rate (K14, Z4). However, these studies did not demonstrate whether the source of the ET was poor tissue perfusion, ischemic or injured endothelium, or monocytes/macrophages. Possibly, it is an endotoxin stimulus rather than hypoperfusion that triggers the production of endothelin or the conversion of big ET by ET-converting enzymes (L20). This finding is a very important differentiation as we try to determine whether anti-ET strategies might be useful in sepsis. Renal vasomotor tone increases, not decreases, in sepsis and this increase may be mediated by increased endogenous ET leading to selective intrarenal vasoconstriction (K14, Z4). The renal vasculature is up to 100 times more sensitive to ET than the peripheral vessels and 10 times more sensitive than the coronary vessels. Thus, anti-ET strategies might have clinical utility in preserving renal function in septic patients.

Further, there may be a therapeutic focus on monocyte activation when we consider the monocyte source as a major one. In addition, it can be hypothesized that, during septic shock, a low systemic vascular resistance is the result of an overall preponderance of endogenous vasodilating (NO and ANP) over vasoconstrictive substances, such as ET, but that regionally, microvascular injury and endothelial dysfunction result in an imbalance toward the latter (B32, E9). ET and NO have mutually antagonistic effects. NO synthesis is promoted by ET and NO is said to suppress ET synthesis. Interestingly, ET and NO, which have opposing actvities, reach high levels almost simultaneously. ET was reported to stimulate ANP from rat myocytes *in vitro* and *in vivo* (F9, F21, S33). ET was reported to stimulate the release of cathecholamines and renin. Further, the chemotactic and proinflamma-

tory eicosanoids leukotriene B_4 and 5-HETE, in a reciprocal manner, stimulate ET secretion by endothelial cells and possibly take part in the formation of local vasoregulatory and proinflammatory feedback loops.

In conclusion, ET is a polyfunctional cytokine that affects monocytes as well as vascular smooth muscle cells, anterior pituitary cells, and renal mesangial cells. In the biologic interface between ischemic or injured endothelium and monocytes, neutrophils, or lymphocytes, ET may play a significant role.

3.2. Nitric Oxide

Nitric oxide is an unusual intercellular messenger (M35, S27). It is the smallest known bioactive molecule, composed of one atom of nitrogen and oxygen. NO is an uncharged molecule with an unpaired electron, so it can diffuse freely across membranes. With an unpaired electron, it is called a radical molecule, it is highly reactive (having a half-live of 2 to 30 seconds), and it has high water and lipid solubility. After transmitting a signal, NO decays spontaneously into nitrate. NO is made by NO synthase in an unusual reaction that converts arginine and oxygen into citrulline and NO. The constitutive NO synthase (cNOS) isoforms in neuronal or endothelial cells are always present and inactive until there is an increase in intracellular calcium, which, after forming a complex with calmodulin, binds to and activates NOS (F13). In contrast, the (calcium-independent) inducible NO synthase (iNOS) isoform is normally absent from macrophages and hepatocytes, but when these cells are activated by certain cytokines (IFN-γ, TNF, LPS), an inducible NO synthase enzyme is produced and, once produced, always synthesizes large amounts of NO. IL-4 and IL-10 (alone and synergistically) are the key suppressors of iNOS activity, produced by the Th1 and Th2 subsets of $CD4^+$ T cells, respectively (F2). Glucocorticoids also inhibit the induction but not the activity of iNOS.

NO diffuses out of the cell that generates it and into target cells, where it interacts with specific molecular targets such as iron, contained in certain proteins as a heme group or as an iron–sulfur complex, to nucleic acids and radical molecules (e.g., superoxide anion) (L16, F2). The endothelial L-arginine NO pathway is activated by shear stress exerted by the circulating blood as well as by receptor-mediated mechanisms activated by agonists such as acetylcholine, bradykinin, substance P, histamine, and platelet-derived products (M34). NO has very potent vasodilator effects; it mediates vasodilatation, attenuates vasoconstriction, and regulates basal pulmonary vascular tone. NO inhibits platelet adhesion and aggregation (and synergizes with prostacyclin). Furthermore, platelets themselves generate NO, which acts as a negative feedback mechanism to inhibit platelet activation. NO inhibits leukocyte activation and suppresses antigen-presenting cell activity and T-cell proliferation in a negative feedback fashion. NO has an antiproliferative effect on vascular smooth muscle cells. It exerts a negative

chronotropic and inotropic effect on the heart (L16). NO automatically regulates blood flow in response to local changes in some regions of the vasculature (B6). Ischemia and reperfusion cause vasodilatation only in the affected tissue, a response that is mediated by NO, which also leads to generation of reactive oxygen species. In large quantities, NO can kill almost any nearby cell (N4). It kills or inhibits the growth of many pathogens, including bacteria, fungi, and parasites. Possibly, it blocks viral replication as well. Abundance evidence indicates that NO is a neurotransmitter in the entire nervous system, involved in many functions (S27, F2).

3.2.1. *NO and Vascular Hyporeactivity in Sepsis*

Several *in vitro* observations indicate that NO mediates the vascular hyporesponsiveness to pressors in sepsis ("vasoplegia"). NO synthesis by various cell lines is enhanced after endotoxin exposure (R4, S2). Inhibition of NO synthase reversed the endotoxin-induced vascular hyporeactivity, independently of the presence of endothelium. Induction of NO synthase has been demonstrated only after 3–4 hours of endotoxemia and hence may well contribute to the delayed hyporeactivity to adrenoreceptor agonists (J10). The immediate vascular hyporeactivity is probably caused by an enhanced formation of NO due to activation of endothelial cNOS. At later stages of endotoxic shock, there seems to be an isoform switch, or down-regulation of cNOS and up-regulation of iNOS (P7, S39). This indicates a loss of the constitutive enzyme (T10). Both phases are reversed by NOS inhibitors, whereas dexamethasone prevents only the delayed hypotensive phase. Evidence for massive generation of NO via the L-arginine pathway as a cause of the reduced sensitivity to pressor agents in patients with septic shock was found in a study investigating resected mesenteric arteries and assessing their functional status (T14). TNF and IL-1 also reduced vascular responsiveness to vasoconstrictors *in vitro*. They induce the expression of iNOS by LPS in various cell types, such as the vascular smooth muscle cell (R4), but do not affect the immediate hypotension caused by LPS, as at least 30–60 minutes are required for their increase after LPS injection (S40).

3.2.2. *NO and Myocardial Contractility in Sepsis*

It has been proposed that NO mediates the myocardial depression associated with sepsis (F6, L14). NO synthesis induced by endotoxin blunts beta-adrenergic responsiveness (B2). *In vivo,* the use of NO synthase inhibitors led to conflicting results (M26), with a general decreased cardiac output and oxygen delivery being observed. NO synthase inhibition improved left ventricular contractility in endotoxemic pigs but also increased ventricular afterloads, which ultimately is detrimental to cardiac function (H20). Possible sources of NO in the heart may be the vascular cells, the endothelial cells, and the cardiac myocytes (P6).

3.2.3. *NO and Experimental Septic Shock*

NO_x levels are increased in plasma and urine of septic animals. Many nonselective NO synthase inhibitors (e.g., L-NMMA) are used in several models with experimental induced sepsis (S40). In most studies it was shown that the cardiovascular abnormalities associated with sepsis were reversed, increasing blood pressure and systemic vascular resistance (F7, K9, M26, N5), together with a improvement in renal function (B42, H24). Also, selective inhibition of iNOS prolonged survival in septic rats (A7).

3.2.4. *NO and Clinical Sepsis*

There is evidence to implicate the release of NO in the hemodynamic changes of clinical sepsis (F6, L14). Biochemical evidence includes elevated levels of cGMP and nitrites and nitrates, the metabolites of NO (E8, O1, S11). Interestingly, in pediatric sepsis, an association was found between cytokine activation (IL-6 versus IL-10), nitric oxide (increased plasma nitrite or nitrate), and organ failure (D29) Administration of L-NNA resulted in hemodynamic improvement in severe sepsis (L14). Methylene blue, which inhibits soluble guanylate cyclase, can increase vascular tone in septic shock, but the clinical benefit is unclear. The effects of NO synthase inhibitors are strongly dose dependent, with different effects on hemodynamics and mortality. The constitutive production of NO contributes to the maintainance of blood pressure, tissue perfusion, and platelet function homeostasis (at a early stage), whereas iNOS is involved in the hypotension and tissue damage resulting from septic shock. Accordingly, selective inhibition of iNOS (e.g., aminoguadine) may result in more beneficial effects.

In conclusion, despite the beneficial effects of various nonselective NOS inhibitors in several animal sepsis models and septic humans, there are few data indicating improvement in organ failure and/or survival. Inhibition of NO synthesis has been claimed to be beneficial by opposing the decrease in systemic vascular resistance. However, this was invariably accompanied by a decrease in cardiac output. Although NO may function as a deleterious mediator in sepsis, this potent signaling molecule may play a beneficial role by limiting adherence of neutrophils and platelets to the endothelium, promoting the integrity of the microcirculation.

4. Hemostasis in Sepsis

Septic shock is frequently complicated by massive activation of the coagulation system. This can occur concomitantly with biphasic change in the fibrinolytic system, involving both activation and inhibition of plasminogen activation. The net result of the altered hemostatic state in sepsis is widespread microvascular trombosis. The early events leading to these disturbances are incompletely understood,

although changes in the functional properties of the vascular endothelium seem to be of major importance. In this section we describe the different aspects of altered hemostasis and the role of the endothelium in sepsis.

4.1. The Role of Plasma Cascade Systems in Sepsis

4.1.1. *The Coagulation System*

Pathological findings frequently observed in organs of patients who have died of sepsis include disseminated intravascular coagulation (DIC), manifested as diffuse thrombotic occlusions in the entire microvascular system, associated with alterations in the hemostatic mechanism and clinical signs of hemorrhagic diathesis. Many observations indicate that DIC contributes to the major symptoms of the systemic inflammatory response syndrome (SIRS), which frequently complicate sepsis (H1, H2, H3, T6).

Activation of the coagulation system may start after activation of the contact factors (intrinsic system) or after release of tissue factor (extrinsic system).

For many years the initial activation of the hemostatic mechanism in DIC was considered to reside in the *intrinsic coagulation system.* This system, starting with the contact system, consists of the zymogens factor XII (Hageman factor), prekallikrein (PK; Fletcher factor), and a nonenzymatic cofactor, high-molecular-weight kininogen (HMK). The intrinsic pathway is activated as a consequence of contact between plasma proteins and the subendothelium. These interactions result in the conversion of factor XII (F-XII) to an active serine protease, F-XIIa. F-XIIa activates coagulation factor XI to F-XIa, which in turn converts F-IX to F-IXa. F-IXa complexes with F-VIII in the presence of Ca^{2+} ions and platelet factor 3 (pf-3), resulting in F-VIIIa. This complex converts F-X, in the presence of Ca^{2+} ions and pf-3, to F-Xa. From this step on the intrinsic and extrinsic coagulation cascades follow the same pathway to form prothombinase and end up in fibrin formation (see Fig. 2). In sepsis, substances released by microorganisms, notably LPSs, can activate the contact system, resulting in the occurrence of DIC. The decrease in F-XII levels in septic patients and the formation of F-XII–C1 inhibitor complex and of kallikrein–C1 inhibitor complex provided arguments for the hypothesis that the activation of the contact system leads to the syndrome of DIC in sepsis (D28). F-XII is the protein that initiates the activation of the contact system, and it has been observed that inhibition of F-XII in septic baboons attenuates clinical symptoms mediated by contact activation (J3, J4).

At present, the activation of the *extrinsic coagulation system* is considered to be of more importance in the initiation of DIC than the activation of the contact system (L12, C13). The activation of the extrinsic system starts with the release of tissue factor (TF) from endothelial cells. TF is a macromolecule, composed of a protein and a lipid fraction, that is synthesized by endothelial cells and monocytes. TF

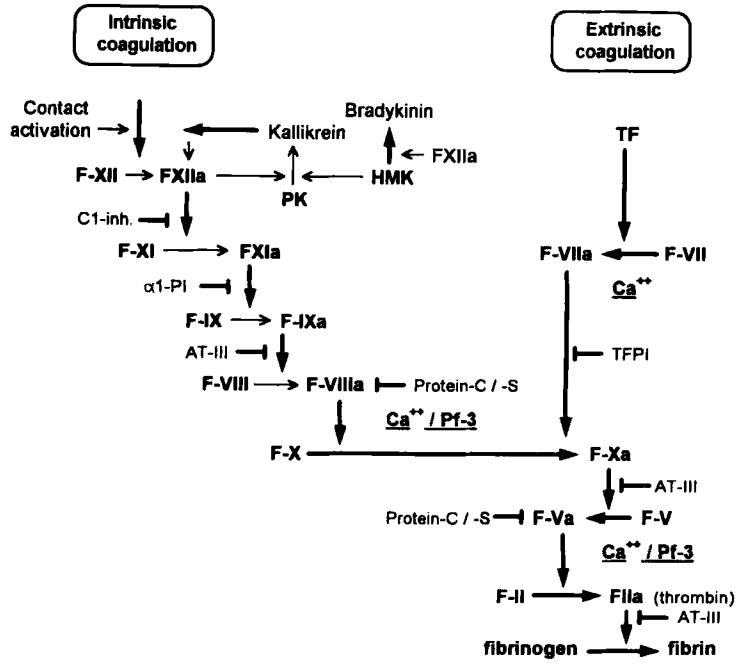

FIG. 2. The intrinsic and extinsic cascade coagulation systems. →, activation; ⊣, inhibition; HMK, high molecular kininogen; C1-inh., complement-1 esterase inhibitor; AT-III, antithrombin III; α1-PI, alpha-1 protein inhibitor; pf-3, platelet factor 3.

is released into the plasma in response to inflammatory agents and to antigen–antibody complexes. The infectious, inflammatory agents include endotoxin and cytokines, such as TNF (C24). When TF is released into plasma it binds in the presence of Ca^{2+} ions with F-VII, which complex activates F-X to F-Xa, at which the extrinsic coagulation joins the intrinsic system in a common pathway ending up in fibrin formation (Fig. 2). Different studies have demonstrated the importance of the extrinsic pathway in the pathophysiology of sepsis. In chimpanzees submitted to endotoxin, previous administration of anti-TF or anti–F-VII monoclonal antibodies provided protection against the occurrence of lethal DIC (B25).

There are various *inhibitors* within the coagulation system that counterregulate activation of the coagulation cascade. Among them, antithrombin III (AT-III) and protein C (PC) are the most important (S1). AT-III binds in the presence of heparin the activated factors F-IXa, F-Xa, and F-IIa (thrombin). PC is activated by a complex formed between thrombin and thrombomodulin, a surface protein of endothelial cells. Once activated, PC in the presence of protein S (PS) specifically degrades activated factors F-Va and F-VIIIa. PC decreases in the course of sepsis in relation to the severity of the condition (L15). Experimental studies have

demonstrated that administration of activated PC (PCa–PS complex) protects animals from the lethal effects of bacteria in the circulation (F14).

4.1.2. *The Contact System*

As shown in Fig. 2, the proteins involved in contact activation of the intrinsic coagulation pathway consist of F-XII (formerly known as Hageman factor), prekallikrein and the nonenzymatic cofactor high-molecular-weight kininogen. HMK has no protease activity but acts as a cofactor. In plasma, PK circulates as a complex bound to HMK (PK–HMK complex). Activation of the contact system starts with binding of F-XII to an activating surface or agent. Upon binding, F-XII undergoes a conformational change and evolves to activated F-XIIa. F-XIIa binds to the PK–HMK complex and converts the PK to kallikrein, which in turn cleaves and further activates FXII by its limited proteolytic potential to F-XIIa (C22, C23, H4, W4). Kallikrein leads to neutrophil degranulation and release of elastase. The reciprocal activation of F-XII and PK amplifies the contact cascade tremendously, once it is activated. FXIIa also cleaves HMK, which results in the formation of bradykinin, which at very low concentrations induces vasodilatation and increases vascular permeability.

There are two classes of inhibitors of the contact system:

1. Inhibitors that interfere with the binding of F-XII and HMK to activating agents or surfaces. Various proteins, including complement factor C1q, platelet factor 4, collagen types III, IV, V and β_2-glycoprotein-I, are able to prevent binding of F-XII to activating agents.
2. Inhibitors that block the protease activity of F-XIIa and kallikrein. Plasma contains two protease inhibitors that regulate contact activation: C1 inhibitor (C1-Inh) and α_2-macroglobulin (α_2-M).

C1-Inh belongs to a superfamily of serine protease inhibitors (serpins) and is a major inhibitor of F-XIIa and kallikrein. It is also an inhibitor of activated complement factors C1q, C1r, and C1s. C1-Inh thus regulates the activation of two important plasma cascade systems. Proteases induce a conformational change in the plasma protein α_2-M, which results in entrapment of the protease into the α_2-M "cage" (B4). *In vivo,* α_2-M acts as a second inhibitor of kallikrein.

Bacterial products such as lipopolysaccharides (endotoxins) and cytokines (IL-2) are able to activate the contact system *in vitro* and *in vivo* (D9, H4, H7, M41). Immediately after severe trauma or after surgical intervention and particularly during sepsis, a reduction of plasma contact system proteins has been found (C10, K1, N9). Gel filtration studies of plasma demonstrated that plasma PK after activation becomes complexed with α_2-M and C1-Inh (W4). These complexes are rapidly eliminated from the circulation *in vivo.* In experimental studies in which pulmonary insufficiency was induced in dogs, a significant reduction of plasma kallikrein inhibitors was observed together with reduced HMK. Analysis of the relation be-

tween hypotension and circulating inflammatory parameters in 48 patients with sepsis revealed that F-XII and PK levels correlate with the mean arterial pressure (H5, N9). *Bradykinin* has been implicated in the pathogenesis of septic shock because of its ability to induce arteriolar dilatation and venular constriction and to lower blood pressure (D9). After experimental infusion of *E. coli* endotoxin in volunteers, as well during clinical sepsis, a decrease of PK and HMK and a simultaneous increase of kallikrein–kinin–α_2-macroglobulin complexes and FXIIα–C1-Inh complex were noted (N10, S6). These observations provide circumstantial evidence that the contact system is activated during sepsis (D9, H4). Nevertheless, prove of contact system activation during sepsis is still lacking, because F-XIIα– and kallikrein–C1-Inh complexes have never been demonstrated in the circulating blood, presumably due to rapid clearance of these activation products. It remains to be established whether inhibition of F-XII in patients with sepsis prevents the development of complications or reduces the mortality rate.

4.1.3. *The Fibrinolytic System*

Activation of the fibrinolytic system has been recognized in experimental models and in most clinical observations following sepsis. Activation of fibrinolysis results in the activation of plasminogen to the active protease plasmin. Plasmin is generated from plasminogen by plasminogen activators (PAs), including the serine protease urokinase (u-PA) and the tissue plasminogen activator (t-PA). Both u-PA and t-PA are released as proenzymes. t-PA is responsible for the *extrinsic fibrinolytic pathway.* Pro-t-PA is present in endothelial cells, from which it is released by various stimuli such as exercise, ischemia, venous occlusion, acidosis, presense of thrombin, and bradykinin. The *intrinsic fibrinolytic pathway* involves activation of pro-u-PA, which circulates in the blood and is bound to various cells expressing receptors for u-PA, such as endothelial, liver and kidney cells. Plasmin binds firmly to fibrin, which makes it highly thrombus specific. The main function of plasmin is the proteolytic degradation of fibrin to fibrin degradation products (FDPs) during clot dissolution (Fig. 3). Plasma protease inhibitors regulate and control the activation of the fibrinolytic system at several levels (K17). Plasminogen activator inhibitors (PAIs) inhibit both the extrinsic and intrinsic plasminogen activators and their precursors. Two types of PAIs have been recognized: PAI-1, which is released from platelets, and PAI-2, released by monocytes and macrophages. Septic patients with MODS show higher levels of PAI-1 than septic patients without organ damage. It has been suggested that suppression of the fibrinolytic system contributes to the imbalance between coagulation and fibrinolysis and that this leads to the onset of MODS (K7). The main inhibitors of plasmin are α_2-M and α_2-antiplasmin (α_2-AP) circulating in the plasma, which prevent the degradation of fibrinogen and other proteins.

Following trauma, sepsis, or endotoxemia, a marked decrease in t-PA and increases in PAIs, more pronounced in nonsurvivors, have been noted (H20). An-

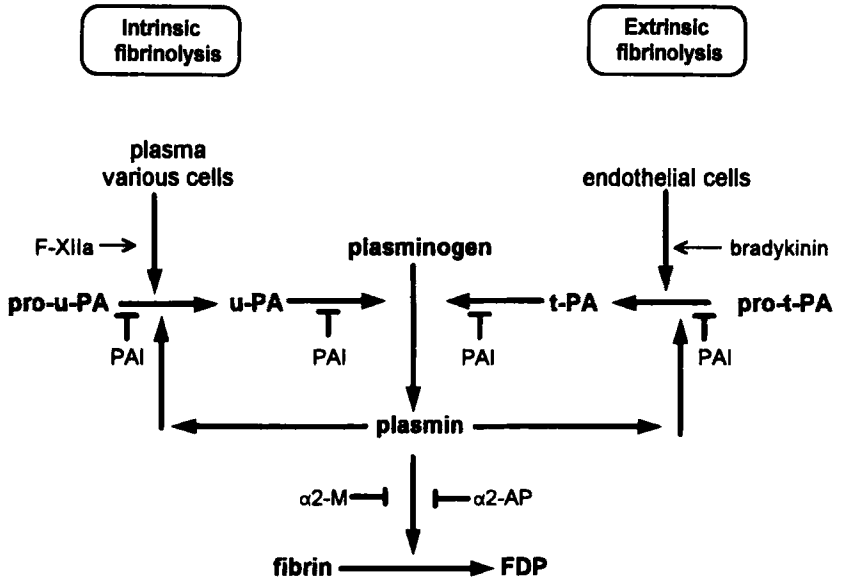

FIG. 3. The intrinsic and extrinsic cascade fibrinolytic systems. →, activation; ⊣, inhibition; t-PA, tissue plasminogen activator; PAI, plasminogen activator inhibitor; α2-M, α_2-macroglobulin; α2-AP, α_2-antiplasmin.

tiplasmin levels have been found to be increased in patients with severe trauma. In baboons it was observed that α_2-M is inactivated during sepsis and that this inactivation is more pronounced in animals that received a lethal dose of *E. coli* (B30). Breakdown of fibrin resulting in FDPs has been associated with increased capillary permeability and formation of pulmonary edema. Elevated concentrations of cross-linked fibrin degradation products in plasma, consistent with increased fibrinolytic activity, have been observed in all patients with gram-negative bacteremia (D7). In conclusion, there is convincing evidence that activation of the fibrinolytic system is a constant phenomenon after trauma or sepsis and that the grade of activation is related to the severity of the organ dysfunction (M15, K7).

With respect to both the coagulation and fibrinolytic cascade systems, in 28 patients who developed septic shock a relation was found between lowered plasma levels of F-XII and antithrombin III and elevated levels of PAI-1 and thrombin–antithrombin III complexes at the diagnosis of sepsis and the severity of disease, expressed according to the APACHE II scoring system (L7). Nevertheless, administration of inhibitors of coagulation or enhancement of fibrinolysis did not improve the outcome in patients with sepsis (B35).

4.1.4. *The Complement System*

The complement cascade may become activated via two pathways: the classical pathway or the alternative pathway.

The *classical pathway* can become activated by immune complexes, bacteria, viruses, and F-XIIa. Binding occurs to the complement C1q, a part of complement factor 1 (C1). This initiates a cascade of activations, first of C1r, C1s, then of C4. This C4 activates C2, after which C3 becomes activated. Activated C3 initiates a cascade of activations, which are in common with the alternative pathway and which end up in activated C5–9, a "membrane attack complex" that lyses the target.

The *alternative pathway* may become activated by lipopolysaccharides, endotoxin (sepsis), virus, fungi, immunoglobulin A–antigen (IgA-Ag) immunocomplexes, and foreign material. These activate C3, after which the common pathway of complement activation takes place (Fig. 4). There are also a number of inhibitors that regulate and control complement activation. The most important are the C1-esterase inhibitor (C1-Inh) and the membrane attack complex inhibitor factor (MACIF; CD59). In sepsis a relative deficiency of C1-Inh has been reported. Administration of C1-Inh to patients with septic shock attenuates complement acti-

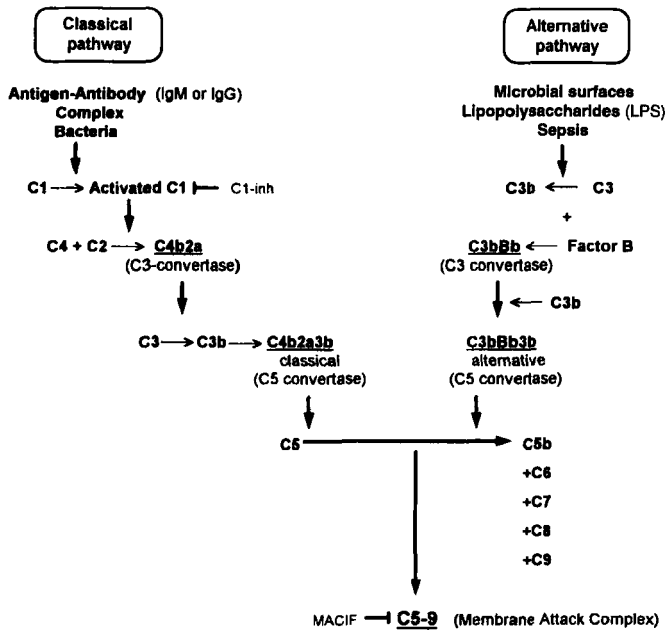

FIG. 4. The classical and alternative pathway cascade of complement activation. →, activation; C1-inh., C1-esterase inhibitor; MACIF, membrane attack complex inhibiting factor (=CD59).

vation (H6). Whether this therapy may reduce morbidity or mortality of SIRS, ARDS, MODS, or vascular leak syndrome (VLS) has still to be established.

4.1.4.1. *Vascular Effects of Complement Activation.* During complement activation a number of complement fragments (anaphylatoxins), which are polypeptides with inflammatory properties, are released. The anaphylatoxins C3a and C5a induce smooth muscle contraction and enhance vascular permeability (H31). The most pronounced activation of complement with the formation of anaphylatoxins and terminal C5–9 complexes has been observed in septic shock (B29, B30, P2). Studies indicate that there is a relation between high concentrations of anaphylatoxins and C5–9 complexes and the development of ARDS or MODS in patients with sepsis (H10).

4.1.4.2. *Cellular Effects of Complement Activation.* C5a is chemotactic for neutrophils, activates their oxidative metabolism, and induces secretion of lysosomal enzymes from the granulocytes and macrophages. C5a may also induce the production of cytokines and prostaglandins (H19, S14).

4.1.4.3. *Interaction with Other Cascade Systems.* Interactions between the complement system, the kinin, and the coagulation and fibrinolytic systems have repeatedly been reported (S37, P19). Activation of one system induces activation of the other systems. The reciprocal activation of the various cascade systems may have an important role in the pathogenesis of ARDS and MODS as complications of sepsis. Nevertheless, until now no convincing prophylactic or therapeutic effects of intervention in the complement cascade system on the severity of septic complications have been reported.

4.2. The Role of the Endothelium in Sepsis

4.2.1. *Endothelial Cells as Regulators of Coagulation*

One of the major functions of the endothelium is communication between the circulating blood and blood cells and the underlying organs,in which it acts as a doorkeeper in blood cell margination and extravasation (B43). This function of the endothelial cells is orchestrated by cytokines and inflammatory substances and is expressed by the endothelial cell synthesis of mediators that modulate the defense mechanisms. Endothelial cells become stimulated in response to various agonists such as thrombin, oxygen radicals, and complement complexes. Activation of endothelial cells occurs under the influence of cytokines and lipopolysaccharides. The most important cellular target of endotoxin or LPS is the vascular endothelium. Endothelial cell activation results in cytokine expression (IL-8), pro- and anticoagulant activities, immunologic functions, expression of adherence molecules (ELAM-1, VCAM-1), release of thrombomodulin (TM), and increased leukocyte adhesiveness (C8, G5, I3). During the hyperdynamic phase of septic shock a substantial decrease of the peripheral vascular resistance occurs. The underlying mechanism may include increased synthesis of the endothelium-derived vasodi-

lating factor (NO) and/or increased reactivity to this substance. Simultaneously, the microvascular permeability is altered, leading to a generalized vascular leakage with formation of protein-rich edema. These phenomena result from the damaging effect of bacterial toxins and are modulated by mediators of inflammation and activation products of the plasma cascade systems. Endothelial cells can be activated by TNF and IL-1 to express leukocyte adhesion molecules, such as ICAM, E-selectin, and VCAM (P13, R2). Simultaneously, a modulation occurs in the endothelial cell production of prostacyclin, NO, tissue factor, and various cytokines (D10, W9). The endothelium has not merely a passive function in the septic syndrome but plays an active role by production and release of mediators, attraction of leukocytes, and activation and inhibition of the plasmatic cascade systems. TNF is known to interact with specific endothelial receptors and to alter endothelial coagulant and anticoagulant properties (N7). After appropriate stimulation, endothelial cells produce both antithrombotic and procoagulant factors (B50).

The *antithrombotic factors* produced by endothelial cells are thrombomodulin (TM) and protein S (PS), components of the vitamin K–dependent protein C (PC) anticoagulant pathway, inhibiting F-Va–F-VIIIa (E15); tissue plasminogen activator (tPA), responsible for fibrinolysis (N2, L18); and the lipoprotein-associated coagulation inhibitor (LACI), which inhibits F-VIIa–TF complex and F-Xa (B51).

The *procoagulant factors* produced by endothelial cells are the coagulation factors von Willebrand factor (WF), F-V, F-VIII, tissue factor (TF), and plasminogen activator inhibitor (PAI), which blocks the activators u-PA and t-PA and counteracts fibrinolysis (G21, F16). It has been shown that under the influence of complement activation (C9), in response to endotoxin *in vitro* (C24), in experimental *E. coli* sepsis in baboons (D30), and after stimulation with TNF (A1, N6), endothelial cells up-regulate the expression of TF, down-regulate TM and inhibit the production of t-PA and PAF. Thus, the balance may shift in the procoagulant direction with a large excess of PAI-1.

4.2.1.1. *Thrombomodulin.* In patients with SIRS and DIC due to sepsis the serum soluble TM level was higher than in nonseptic and non-DIC patients (A9, B32, G3, I1, K7). In experimental ARDS in rats induced by LPS, administration of soluble recombinant TM inhibited the occurrence of intravascular coagulation and prevented the increase in pulmonary vascular permeability (U1).

4.2.1.2. *Protein C.* PC is a vitamin K–dependent protein that, after activation by thrombin complexes with thrombomodulin at the endothelial surface, develops anticoagulant activity by inactivating F-Va and F-VIIIa. Free circulating PS functions as a cofactor and potentiates the PC activity. The free level of PS depends on the level of the C4b binding protein (C4bBP). The levels of PC and PS and their functional activity are significantly decreased in patients with sepsis and increased levels of plasma C4bBP inhibit PS. These marked reductions may be factors contributing to the development of thromboembolic complications, which often com-

plicate the sepsis (S20). From experimental studies on the inflammatory–coagulant axis in a baboon model of *E. coli* sepsis it appeared 1) that the endothelium is the primary target of endotoxin; 2) that the endothelial cells in response express the antithrombotic factors TM; and PC and activate the TF–inhibitor pathway (F-Xa/ATIII) by production of glycosaminoglycans; and 3) that when these anticoagulant mechanisms are overridden by inflammatory events, the endothelium changes its anticoagulant phenotype and mounts a fibrinolytic attack through release of t-PA on its luminal side (T3).

4.2.1.3. *Platelet-Activating Factor.* PAF was originally known as a factor, derived from antigen-stimulated IgE-sentitized basophils, that causes platelets to aggregate. Its chemical structure is acetyl glycerol ether phosphocholine (AGEPC). In addition to platelet stimulation, PAF causes increased vascular permeability, leukocyte aggregation and adhesion, chemotaxis, and a number of systemic hemodynamic changes, especially hypotension and myocardial depression. PAF is therefore, along with arachidonic acid metabolites, another phospholipid-derived mediator of inflammation. PAF is produced by a variety of cells, such as polymorphonuclear cells, monocytes, macrophages, endothelial cells, and platelets. It has been shown that TNF up-regulates the production of TF, t-PA inhibitor (PAI), and PAF. Also, treatment with PAF has been shown to increase cytokine production (TNF, IL-1). In addition, PAF plays a central role not only in amplification but also in down-regulation of inflammatory mediator release. There is substantial evidence that LPS releases PAF in a great variety of *in vitro* and *in vivo* animal models. It modulates membrane-dependent processes that play a role in homeostasis, contributes to coagulation dysfunction, and stimulates arachidonic acid metabolism (thromboxane A_2, TXA_2) (O9). Much of what is known of PAF biology has come from the discovery and use of purified PAF and selective PAF antagonists (K18, T7). Plasma PAF concentration is elevated in patients with septic ARDS compared with patients with sepsis alone (F7). It has been shown that infusion of PAF reproduces the host response to endotoxemia and induces neutrophil activation, plasma extravasation, and shock (A5, K10, M2, O9). The effects of PAF and LPS are very similar, but the concentration of PAF that induces shock is usually about 500- to 1000-fold less than that of LPS.

4.2.2. *Therapeutic Approaches to Attenuate Sepsis*

The effects of corticosteroids, cyclooxygenase blockers, leukotriene blockers, PAF antagonists, anti-TNF antibodies, oxygen radical scavengers, opiate antagonists, antihistamines, and calcium channel blockers in endotoxic shock were reviewed in 1990 (H17). In this section studies on this subject that have been published during the last few years are summarized.

4.2.2.1. *Antithrombin III.* AT-III administration holds some promise because it inhibits a number of activated coagulation factors: F-XIa, F-IXa, F-Xa, and thrombin. There are, however, no data to support the use of heparin. Although a

reduction in the duration of DIC has been reported after treatment of septic patients with AT-III concentrates, none of the studies was able to document a statistically significant reduction in mortality (F14, M6).

4.2.2.2. *Plasminogen Activators.* PAs may prove helpful in increasing fibrinolysis; however, plasminogen activators may be most effective in conjunction with hirudin or synthetic hirudin analogues.

4.2.2.3. *Protein C and Protein S.* PC and PS may inhibit thrombin formation and complex with PAI, thereby promoting fibrinolysis. During sepsis, PC activity is significantly reduced, either by consumption or by TM down-regulation, while increased levels of C1bBP inhibit PS. Infusion of activated PC and PS protected animals from the lethal effect of bacteria. Administration of PC and PS concentrates should be studied carefully in septic patients before their use is recommended (F14).

4.2.2.4. *Complement 1 Inhibitor.* C1-Inh is reduced in sepsis. Substitution with C1-Inh concentration has been safely performed and preliminary results are consistent with a beneficial effect on hypotension in patients with septic shock. Whether this therapy may reduce mortality has still to be established (H6).

4.2.2.5. *PAF Antagonists.* PAF antagonists are now manufactured by a number of pharmaceutical companies studying the beneficial effects in human disease. A exhaustive list of PAF antagonists mentioned in the literature includes Abbott-84768, BN-52021, CL-184,005, E-5880, Ro-24–4736, TCV-309, and WEB-2086. Studies of the therapeutic potential of these substances in various shock conditions have concentrated on entirely new aspects of PAF and PAF antagonists in cerebral, pulmonary, myocardial, and intestinal ischemia. Experimental animal studies suggest that PAF antagonists appear to be effective in cases of severe endotoxin shock, possibly by suppressing LPS-induced TNF generation and protecting against the release of TXA_2. Alternatively, PAF antagonism may promote the beneficial feedback loop termed *down-regulation*, because they have relative little effect on PGI_2 release, being responsible for cAMP generation, an important factor in the down-regulation of inflammatory mediator release (K6, A12). PAF antagonists inhibit monocyte TNF production but have no effect on IL-6 production (E13). PAF antagonists inhibit vascular leakage; attenuate hypotension, DIC, and MODS; and increase the survival rate in animals (M36, B35, D8, D22, K5, K6, K10, K18, O8). In patients with septic shock PAF antagonists alleviated thrombocytopenia (O6). The PAF antagonist Ro-24–4736, given to healthy volunteers 18 hours before administration of endotoxin intravenously, alleviated rigors and myalgias (T7). Some observations suggest that PAF antagonists may have therapeutic value not only in septic shock but also in anaphylactic reactions (H23).

4.2.2.6. *Cyclooxygenase Inhibitors.* The synthesis of prostaglandin and thromboxane has been linked with multiple organ failure in animals and humans with sepsis. Bernard *et al.* (B18) reported the results of a large trial of the cyclooxygenase inhibitor ibuprofen in patients with sepsis. Treatment for 48 hours with

ibuprofen lowered temperature, heart rate, oxygen comsumption, and levels of lactid acid, but it did not decrease the incidence of organ failure or mortality at 30 days. However, the treatment period was very short, considering the fact that septic patients continue to have inflammation for many days or weeks (W7).

4.2.2.7. *Monoclonal Antibodies.* *HA-1A* is a human monoclonal IgM antibody that binds to LPS and lipid A. The initial phase 3 trial demonstrated no overall benifit of HA-1A compared with placebo (Z5). However, HA-1A appeared to afford significant protection to a subgroup of patients with gram-negative bacteremia (Z5). A second large-scale trial documented a lack of overall clinical benefit of HA-1A (W11).

In chimpanzees, administration of Fab fragments of a monoclonal *anti–F-VII* antibody preceding an endotoxin bolus injection effectively blocked the activation of the coagulation pathway (B25). Administration of monoclonal *anti–IL-6* under the same experimental conditions attenuated the activation of coagulation, while the fibrinolytic system remained unaltered. However, administration of monoclonal *anti-TNF* enhanced the tendency to microvascular thrombosis (P17,18). Monoclonal *anti-TF* antibodies administered to baboons as a pretreatment attenuated coagulopathy after induction of *E. coli* sepsis in these animals (T4). Primates pretreated with *anti-C5a* antibodies before infusion of *E. coli* developed less hypotension and had better survival rates than untreated animals, who developed ARDS and septic shock with a mortality rate of 75% (S35, Z6). No favorable treatment results have been published yet with one of these treatment modalities given to humans.

Despite improvements in our understanding of the pahological events leading to sepsis, adjunctive therapy has not yet altered the course of this catastrophic illness. Because of the complex nature of the inflammatory pathways involved in sepsis, it may be dificult to establish that the inhibition of any one pathway alone is protective (B37, M6). The logical progression of research would be to combine different therapeutic agents (L22), as single therapies targeting unique pathways may easily fail (W11). Furthermore, the cytokines released in sepsis are rapidly deployed and hit their cellular targets quickly. Thus, immunotherapies, if they are to be effective, will have to be given early or perhaps administered prophylactically to patients identified to be at high risk.

5. Hormonal Regulation

More than a century ago Claude Bernard speculated that the "milieu interne" must be maintained to preserve life (B17). Later, Walter Cannon indicated that physical disturbances could elicit a coordinative response of the organism to keep "homeostasis" (C7). The stress concept of Hans Selye noted that these stimuli that disturbed the physical integrity of the organism resulted in a general adaptation

TABLE 3
HORMONAL CHANGES DURING SEPSIS AND SEPTIC SHOCK

Hormone	Acute phase[a]	Catabolic phase	Key references
Stress hormones			
HPA axis			
CRH	↑	?	T9, R8
ACTH	↑	↓	V4
Cortisol	↑↑	↑	S7, V4
CBG	↓	↓↓	B28, S10
DHEA(S)	↓	↓↓	B5, W5
Catecholamines	↑	N	F16
Prolactin	↑	?	B48
GH	↑	↓	R16
IGF-1	↓	↓	B16, V9
Insulin	↓	↑	F16
Glucagon	↑	↑	V9
Neuropeptides			
Nedorphins/enkephalins	↑	↑	H16
Neuropeptide Y	↑	↑	A8
Substance P	↓	↓	A8
CGRP	↑	↑	A8
Procalcitonin	↑	↑↑	A11, G19
Hormones of water and electrolyte homeostasis			
RAA axis			
Renin	↑	↑	F4, Z7
Angiotensin II	↑	↑	F4
Aldosterone	↑	↓	F4, R1
Vasopressin	↑	↑	R14
Atrial natriuretic peptide	↑	↑↑	B8, G13
Hormones of the pituitary–thyroid axis			
TSH	↓	N	B41, C15
TT_4	N	↓	D23
TT_3	↓	↓	H18
rT_3	↑	↑	W18
FT_4	N	N	E5
FT_3	N	N	S25
DIT	↑	↑↑	M19
TBG	?	?	?
Hormones of the reproductive axis			
LH–FSH	↓	↓	W20
Testosterone	↓	↓	S17
Estradial	↑	↑	B14, C19
Estrone	↑	↑	L21

[a]↓, low; ↓↓, very low; N, normal; ↑, high; ↑↑, very high; ?, questionable or no data available.

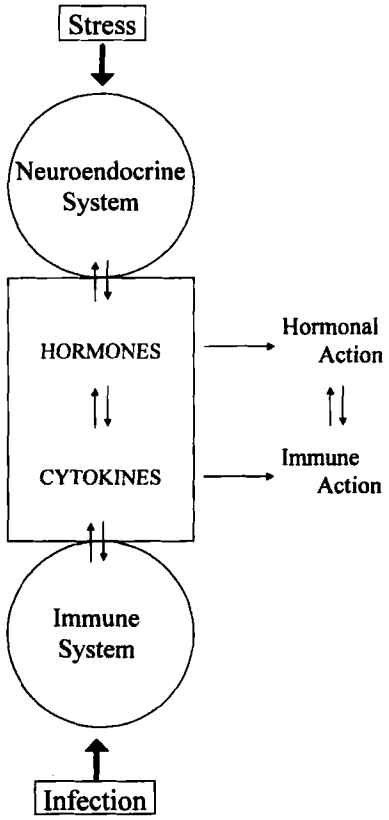

FIG. 5. Interactions between the neuroendocrine and immune systems occurring during an inflammatory and/or stress reaction.

syndrome (S16). These changes are associated with increased activity of the hypothalamic–pituitary–adrenal (HPA) axis and may have survival value in preparing the body for "fight or flight." Sepsis represents a threat to homeostasis and is related to a systemic inflammatory host response to an inciting event. In general terms, sepsis is a severe stress stimulus that disturbes the milieu interne and induces homeostatic responses specific to the stimulus and generalized responses when the disturbances are severe. The sepsis-induced hormonal responses are part of the well-orchestrated defense mechanisms of the three major central systems of the individual: the nervous system, the endocrine system, and the immune system (T9, B22) (Fig. 5). Sepsis induces acute changes in endocrine functions that are generally adaptive and provide optimal conditions for the fight, including metabolic changes, optimal intravascular volume, and perfusion pressure. This acute

phase lasts typically about 12–24 hours, depending on the severity of the infection, and is characterized by an appropriate (teleologically speaking) hormonal reaction: the mobilization of fuel stores of the organism, together with apparent restraints on their utilization (Table 3). However, there are limits to the ability of the humoral system to compensate adequately. If the critical illness is prolonged or the defense response is not sufficient, the hormonal response can contribute to a worsened clinical status. This "catabolic phase" (F17) lasts for days and is characterized by inappropriate hormonal responses, resulting in a chronic increase in metabolic rate and a breakdown of body tissue (Table 3). The exact course of the hormonal responses to sepsis or SIRS is not predictable and is linked more to the severity than to the time course of the infection.

5.1. Stress Hormones

5.1.1. *Hypothalamic–Pituitary–Adrenal Axis*

The HPA axis and the sympathoadrenal system are the key players in the homeostatic response to infection during the acute phase (Fig. 6). Serum *cortisol* levels are generally elevated in patients with sepsis and septic shock (S7, S21, S29, V4). This adrenocortical activation is due not only to pituitary *ACTH* release but also results partly from a decreased cortisol extraction rate (M20), a decrease in blood level of *corticosteroid-binding globulin* (*CBG*), and a decreased binding capacity of cortisol to CBG (B28, F3, H12, H30, M21). It has been known for a long time that bacterial endotoxins can stimulate the pituitary–adrenal axis (D12, M21). Bacterial endotoxins are often used in animal models to study the hormonal mechanisms of sepsis, but in patients with intestinal damage or with massive gram-negative bacteria–induced infections, endotoxins may reach the blood stream, resulting in septic shock (M12, M33). The role of endotoxins is demonstrated by the fact that peripheral administration of endotoxins to animals and humans results in a septic syndrome that is very similar to the events observed after infection with gram-negative bacteria (M12, R3). The acute responses of the HPA axis to peripheral administration of endotoxin are mediated by the central affect: endotoxin induced *corticotropin-releasing hormone* (*CRH*) release from hypothalamic neurons (R10). Accordingly, morphine or pentobarbital treatment or passive immunization with monoclonal antibody to CRH can prevent the endotoxin-induced HPA activation (O3, R9). On the other hand, the late responses seen after high endotoxin doses are due to peripheral effects. High-affinity endotoxin receptors have been demonstrated on macrophages and lymphocytes (M42). Proinflammatory cytokines, including TNF, IL-1, and IL-6, are produced in response to endotoxins by these cells (B22, D25, F20, G12, R10), and elevated concentrations of these cytokines have been found in plasma of patients with the early phase of septic shock (C12, D25, G4, G12). All of these cytokines are known to induce activation of the HPA axis (R7, W13) and mutually affect each other's production (D21, L13, S18). TNF can

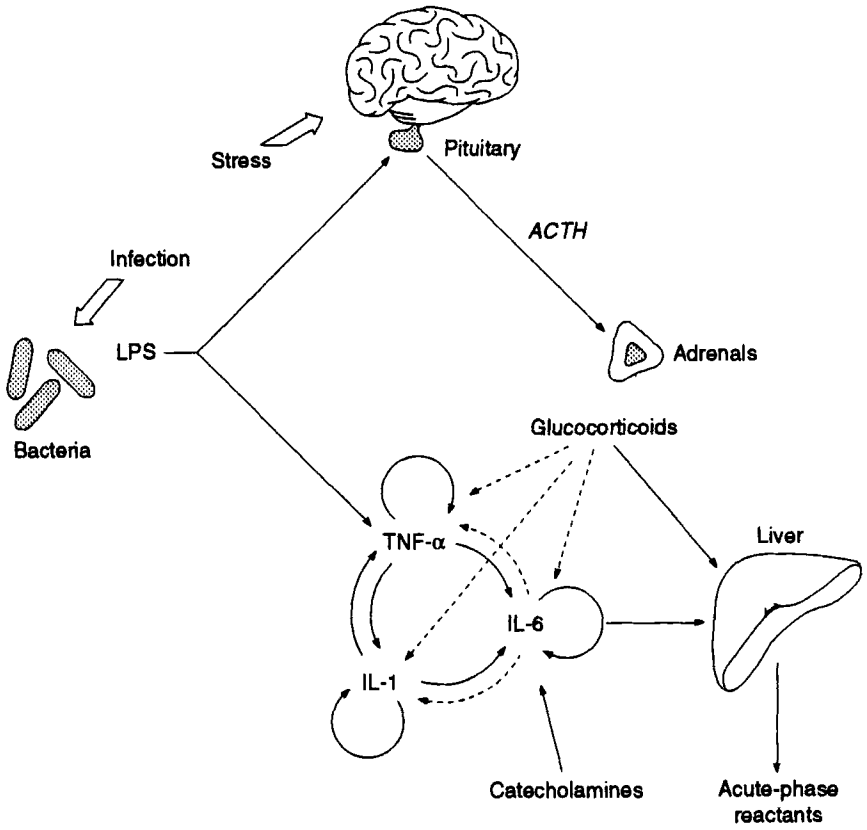

FIG. 6. Interaction among the hypothalamic–pituitary–adrenal axis and the inflammatory cytokines during an inflammatory and/or a stress reaction. →, stimulation; ⊣, inhibition.

induce IL-1 production, and both can provoke the production of IL-6 (D21, L13). On the other hand, IL-6 can down-regulate TNF production (A2, S32), so the interpretation of these results is complicated. Injection of bacterial LPS into normal volunteers increased plasma TNF levels within 90 minutes, followed by increases in plasma ACTH and cortisol levels (I2). However, despite the development of highly sensitive immunoassays, it is still controversial whether TNF, IL-1, and IL-6 exist in normal plasma. It is known, however, that various kinds of infections could induce cytokine production, but the production of these cytokines in peripheral tissue is very low. In addition, cytokines may be produced in peripheral blood as a result of sampling. Therefore, the demonstration of various cytokines in blood does not necessarely prove their role in the activation of the HPA axis. To elucidate the role of endogenous cytokines, cytokine antibodies or inhibitors have

been used, and it was found that TNF-α is the main cytokine in initiating the endocrine and metabolic responses to sepsis (B23, T13).

In critically ill septic patients the HPA function does not follow a simple activation response but demonstrates a biphasic process, suggesting a complex interplay between the different cytokines. Typically, there is a prompt, dramatic, and sustained increase in both cortisol and ACTH concentrations in blood. This activation is accompanied by a loss of circadian rhythms, ACTH pulsatility, and the feedback sensitivity of the pituitary gland. The ability of the HPA system to respond to CRH or ACTH stimulation is also disturbed in this first stage, which is characterized by a convential hyperactivation of the adrenocortical system. However, during the second phase, the high plasma cortisol level is accompanied by paradoxically low ACTH levels. Several possible mechanisms can be considered to explain this paradoxical second phase in HPA adaptation during sepsis, but tissue damage and/or an inflammation-induced immune reaction seems to be the most important (V4). We cannot exclude, however, that vasoactive peptides such as vasopressin, ET, and ANP are partly responsible for the adrenal activation in this stage. The degree of cortisol elevation correlates with the degree of homeostatic disturbances and is inversely correlated with the survival rate (R5, S28).

It has been suggested that the critical illness-induced changes in the free fraction of the serum cortisol concentration are even more pronounced than the observed increase in the plasma concentration of total cortisol (H14, B28). The CBG concentration and the CBG binding affinity for cortisol are both decreased in patients with severe illness (M21, B28). The mechanisms that regulate plasma concentrations of CBG are poorly understood. One possibility is that circulating glucocorticoids modulate the CBG level. This hypothesis is supported by observations that show that adrenalectomy increases CBG and that glucocorticoid treatment decreases plasma concentrations of CBG (F3, H13, H30, S10). Another possibility is that certain stressors can decrease CBG concentrations via indirect mechanisms such as increased CBG clearance or decreased liver synthesis of CBG (F11). In septic patients these changes in the free/bound cortisol ratio could be even more pronounced because CBG is degraded by neutrophil elastase (H15, B28). Elastase cleaves the CBG molecule, causing it to release bound cortisol and thereby increasing the amount of free cortisol at the site of the inflammation (H15). The serum cortisol binding capacity was found to be significantly lower in patients with evidence of a recent inflammatory response (B28). Patients with septic shock showed the most pronounced reduction, but low values of serum cortisol binding capacity were not restricted to the shock state.

Dehydroepiandrosterone (*DHEA*) and *dehydroepiandrosterone sulfate* (*DHEAS*) are the most abundant steroids secreted by the adrenal cortex under pituitary ACTH control (B5). Very little plasma DHEA(S) appears to be of testicular or ovarian origin. Physiologically, the concentration of DHEA(S) in the blood oscillates coincidentally with cortisol, consistent with the response of adrenal

DHEA(S) secretion to ACTH stimulation, but there is no feedback control of DHEA(S) release at the hypothalamus–pituitary level. The mechanism of action of DHEA(S) is poorly known and may include partial transformation into sex steroids, increase of bioavailiable insulin-like growth factor 1 (IGF-1), and effects on neurotransmitter receptors. The serum DHEA(S) level is a highly specific parameter of the individual hormonal milieu and a potent modulator of the immune response (E1). The basal serum concentration and the ACTH-induced response in DHEA(S) decrease gradually with advancing age, unlike that of cortisol secretion, which is maintained. The same discrepancy between serum concentrations of DHEA(S) and cortisol has been found in the acute phase of severe illness, indicating a defect in ACTH-stimulated DHEA(S) reserve in serious illness (B5, B15, L8, L21, P5). A remarkable correlation between serum DHEA(S) concentration and the expressed feeling of well-being has been observed in an epidemiologic study (B20). In addition, low DHEA(S) concentrations have been measured in blood when people are in poor general condition because of stress or coincident with immunological disturbances (B5). DHEA(S) has been shown to reduce lethality after burn injury or endotoxin administration in animal experiments (A6, D3). DHEA(S) levels were significantly lower 60 minutes after endotoxin administration; however, exogenous DHEA(S) administration failed to blunt the associated septic symptoms and pulmonary failure (S15).

5.1.2. *Catecholamines*

The adrenal catecholamines *epinephrine* and *norepinephrine* are also classical and well-recognized stress hormones and are central to the "fight or flight" response. Epinephrine is secreted from the adrenal medulla in direct response to increased sympathetic tone; however, the majority of the circulating norepinephrine is released from the sympathetic nerve endings. Measurements of plasma concentrations of catecholamines have limited value as an index of sympathetic nervous system activity because of their extremely short half-life, because most norepinephrine is taken up by pre- and postsynaptic neurons and only some gains access to the plasma. But the response of both epinephrine and norepinephrine is logarithmically related to the severity of the illness, considering the fact that very high levels of epinephrine, in particular, are found in critically ill patients (F16). Catecholamines support blood pressure, heart rate, myocardial contractility, cardiac output, respiration, and bronchial tone. They also redirect blood flow toward muscle and away from the skin, viscera, and kidney. In addition, epinephrine is an important catabolic hormone, inducing lipolysis, glycogenolysis, and gluconeogenesis. Epinephrine is a first-line defense against hypoglycemia via inhibition of insulin release and stimulation of glucagon release. The sympathoadrenal response to severe stress situations such as sepsis is marked, rapid, and transient and plays an important role in the early metabolic changes during the acute phase of sepsis (F17) (Fig. 7).

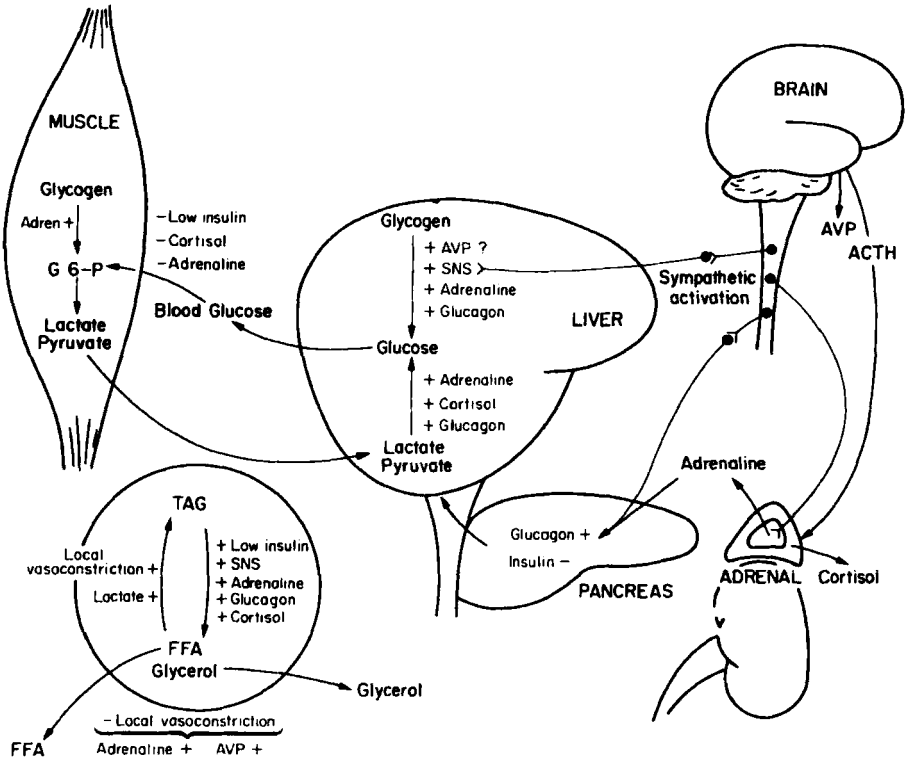

FIG. 7. Neurohumoral control of the metabolic changes during the acute phase of septic shock. AVP, vasopressin; SNS, sympathetic nervous system; TAG, triacylglycerol; FFA, free faty acid. (Reproduced with permission from Clin. Endocrinol. K. N. Frayn, **24**, 577–599, 1986, by copyright permission of Blackwell Science Ltd., Oxford, UK.)

5.1.3. *Other Stress Hormones*

In addition to the classical stress hormones already reviewed, several other hormones are augmented in response to stress. Stress-induced *prolactin* release is one of the most frequently studied examples. There is no doubt about the causal relationship between stress and increased pituitary prolactin release, but the biological meaning is much less clear (G2). This phylogenetically old hormone has been shown to have more than 85 different functions in all vertebrate species. However, besides its role in the induction of maternal lactogenesis, the physiological importance of prolactin is at present not fully established. Experimental and clinical evidence supports the view that prolactin is also an immunoregulating hormone (M44, R18). Prolactin receptors are present on human T and B lymphocytes (R18), and T lymphocytes depend on prolactin for maintenance of immunocompetence (B19). In addition, it has been shown that prolactin is able to influence the devel-

opment of inflammatory processes in animal experiments (M22). The proinflammatory effect of prolactin and the anti-inflammatory effect of bromocriptine were clearly demonstrated in animal models (B19, M22). In critically ill patients prolactin concentrations have been shown to be increased in both men and women with disruption of the circadian rhythm (B48). The sepsis-induced changes in prolactin secretion are disturbed by dopamine administration. Dopamine has been used for more than 20 years as an important inotropic drug in treatment of patients with septic shock, and according to the general view it appeared to improve short-term survival in states of shock (G15). On the other hand, specific membrane-bound dopamine receptors of the D2 subtype have been identified in the anterior pituitary and in the median eminence of the hypothalamus, both located outside the blood–brain barrier. Consequently, dopamine, as the physiological prolactin-inhibiting factor, after systemic injection can influence the pituitary secretion of prolactin (B10, B13). In critically ill patients dopamine infusion induced hypoprolactinemia, which has indirect effects on cellular immunity (D17). Accordingly, dopamine infusion may provoke or aggravate the susceptibility to infectious complications in critically ill patients during the second, catabolic phase of sepsis (Z3, B12).

Growth hormone (*GH*) is a 191-amino-acid polypeptide with anabolic, lipolytic, and immune-stimulating properties. As with other anterior pituitary hormones, its secretion is under hypothalamic neurohumoral control and occurs in a pulsatile fashion with diurnal variation. The basal GH level is elevated and the pulse frequency is decreased in sepsis syndrome (R16). The blunted GH secretory pattern appears during prolonged critical illness and the low GH level is associated with increased mortality (R17). In these patients a GH response to growth hormone–releasing hormone (GHRH) stimulation and an exaggerated GH response to GH-releasing peptide 2 (GHRP-2) are present (B16, G18). GH action is reflected by the serum concentration of IGF-1, which is generated by the liver and bound to specific binding proteins (IGFBPs). In septic patients, serum IGF-I and IGFBP-3 concentrations are decreased and correlate well with conventional nutritional indices such as nitrogen balance (R17, V9). The amplitude of secretory GH pulses is reduced and serum concentrations of insulin are elevated if high-calorie nutrition is provided, as commonly used in standard intensive care. *Insulin* concentrations are inappropriately high for the plasma glucose concentrations. Despite hyperglycemia, *glucagon* levels are elevated in sepsis, because of the increased release of catecholamines (R12, V9). In sepsis, glucose intolerance and insulin resistance may in part result from production of cytokines and glucocorticoids (F17). Because of the anabolic potential, the therapeutic use of GH with appropriate nutritional support has been advocated by some authors to attenuate the protein catabolism in patients suffering from sepsis (V8). A GH dose of 0.1 mg/kg/day resulted in a significant decrease of urea generation and excretion of potassium (V8). It has been shown that both basal and pulsatile GH secretions are moderately increased

by continuous infusion of GHRH, substantially increased by GHRP-2 alone, and dramatically increased by the combination of both (B16). IGF-1 levels are significantly increased within 24 hours during these treatments, which opens perspectives for the acute treatment of the catabolic state present in septic patients (B16).

There are experimental observations that *neuropeptides* may play a role in the pathogenesis of septic shock. *Endogenous opioid peptides* are a well-known family of hormonal, neurotransmitter, and leukocyte-derived peptides (H16). They are involved in the down-regulation of stress-induced HPA and sympathetic axis activation and are probably important mediators in the overall response to endotoxin (H16). Animal studies of septic shock showed a beneficial hemodynamic response to administration of the opioid antagonist naloxone in septic shock (F1). It has been suggested that central endorphin receptor blockade increases adrenal catecholamine release, based on the observation that naloxone administration was associated with a rise in serum adrenaline levels in animals (H25). However, studies of patients with septic shock intended to test the short-term effects of bolus administration of naloxone have failed to show a consistent improvement of hemodynamic features (B40, D11, G20). In a placebo-controlled double-blind study, continuous infusion of nalaxone resulted in improvement in hemodynamic status in a small population of patients suffering from septic shock. A significant reduction in vasopressor–inotrope requirements and a significant fall in heart rate, together with improvement in stroke volume but without any reduction in cardiac output, have been observed in septic shock patients receiving opiate antagonist infusion. It has been shown that serum concentrations of β-endorphin and methionine enkephalin have a mortality-predictive value in septic patients (B11).

The endogenous release of the potent vasoconstrictor *neuropeptide Y* (*NPY*) is increased during sepsis and the highest levels are detected in patients with shock (A8). NPY is a 36-amino-acid peptide belonging to the pancreatic polypeptide family of neuroendocrine peptides (T2). It is one of the most abundant peptides present in the brain and is widely expressed by neurons in the central and peripheral nervous systems as well as the adrenal medulla (A3). NPY coexists with norepinephrine in peripheral sympathetic nerves and is released together with norepinephrine (L19, W14). NPY causes direct vasoconstriction of cerebral, coronary, and mesenteric arteries and also potentiates norepinephrine-induced vasoconstriction in these arterial beds (T8). It appears that vasoconstriction caused by NPY does not counterbalance the vasodilatator effects of *substance P* in patients with sepsis. The properties of vasodilatation and smooth muscle contraction of substance P are well known (I4), but because of the morphological distribution and the neuroendocrine effects a possible stress hormone function for substance P was also advocated (J7). Substance P, which is a potent vasodilatator agent and has an innervation pathway similar to that of NPY, shows a low plasma concentration in septic patients with and without shock (A8).

Calcitonin and other peptide products of the calcitonin gene are known to be el-

evated not only in patients with medullary thyroid carcinoma (S34) but also in those with several systemic diseases including systemic infections (C4, M5, S22). At present, four genes with nucleotide sequence homologies corresponding to calcitonin are known and called the "calcitonin gene family" (W16). These human CALC I–IV genes do not all produce the peptide hormone calcitonin. In nervous tissue of the central and peripheral nerve systems a neuropeptide called *calcitonin gene–related peptide* (*CGRP*) is the main product of CALC-I transcription. CGRP is a vasoactive neuropeptide with a wide physiological effect. CGRP is a neurotransmitter or neuromodulator in the central and peripheral nervous systems (M14, M39). It is a potent vasodilatator and has positive chronotropic and inotropic effects on hearts in humans (M40). CGRP is released into the blood stream from the sensory afferent nerves scattered throughout most arterial beds (B44). Functionally, it turned out to be a powerful vasodilatator. In both animal and human studies, infused synthetic CGRP has been shown to be the most potent vasodilatator and hypotensive agent yet tested (O5).

It has been shown that CGRP is released into the circulation during the development of human sepsis and septic shock (A8). Plasma CGRP levels correlated with the APACHE II score as well as with cardiac index and systemic vascular resistence index. There is also a relationship between the initial plasma CGRP levels and the severity of the disease at the time of admission to the ICU. Plasma CGRP levels are related to the hemodynamic changes seen early in septic shock.

On the other hand, there is overwhelming evidence that another important product of CALC-I gene transcription, *procalcitonin,* is a diagnostic indicator of bacterial infections with systemic reactions (A11). In healthy individuals hormonally active calcitonin is produced and secreted by the C cells of the thyroid gland. Protein synthesis of calcitonin starts with the translation of a 141-amino-acid precursor protein (preprocalcitonin) after transcription of the CALC-I gene in the C cells (M43). By specific proteolysis procalcitonin and calcitonin are cleaved intracellularly. Procalcitonin is a 116-amino-acid protein, is a very stable protein *ex vivo,* and is not degraded to hormonally active calcitonin in plasma. The plasma concentration of the prohormon procalcitonin in healthy individuals is very low (pg/ml range). However, endotoxin injection in normal subjects induces release of this prohormone, resulting in plasma procalcitonin concentrations of 4–6 ng/ml but without detectable plasma concentrations of calcitonin (D2). Accordingly, during severe bacterial infections high plasma concentrations of procalcitonin are found, also without a significant change in the plasma calcitonin concentration (A4, A11, G19). In septic patients plasma concentrations of procalcitonin ranging from 1 ng/ml to above 1 μg/ml are found (G19). Serum concentrations seem to be correlated with the severity of microbial invasion and therefore can be used as a monitoring parameter in critically ill septic patients (A11). This bacterial infection–induced procalcitonin release is most likely not from the C cells of the thyroid but from the neuroendocrine cells of the lung or the intestine. High plasma concen-

trations are found not only in sepsis but also in MODS and in patients with prolonged circulatory failure. In contrast, the release of the prohormon is not stimulated by viral infections or by chronic nonbacterial inflammation and allergic reaction. Accordingly, it can be used for the differential diagnosis of bacterial versus nonbacterial inflammation (A4). Procalcitonin could be a promising marker for patients with infectious diseases, but further studies are needed to elucidate the possible physiological and/or pathological role of this peptide. Its endocrine effect and the place of procalcitonin induction in the cytokine cascade that occurs in sepsis remain to be investigated.

5.2. Fluid and Electrolyte Homeostasis

Regulation of volume and water balance is of critical importance to survival in sepsis; therefore it is not surprising that multiple mechanisms exist to maintain normovolemia and adequate blood pressure.

5.2.1. *Vasopressin*

Vasopressin is a peptide hormone produced by the hypothalamus and secreted by the posterior pituitary in response to stimulation. Normal stimuli for vasopressin release are hyperosmolarity and hypovolemia, with thresholds for secretion of greater than 280 mOsm/kg and greater than 20% plasma volume depletion. A number of other stimuli, such as pain, nausea, epinephrine, and numerous drugs, induce release of vasopressin. Vasopressin release is inhibited by volume expansion, ethanol, and norepinephrine. The physiological effect of vasopressin is to promote free water clearence by altering the permeability of the renal collecting duct to water. In addition, it has a direct vasoconstrictor effect. Consequently, vasopressin results in water retention and volume restoration. In patients with septic shock, vasopressin is appropriately secreted in response to hypovolemia and to elevated serum osmolarity (R14).

5.2.2. *Renin–Angiotensin–Aldosterone Axis*

Renin is a glycoprotein hormone produced by the renal juxtaglomerular cells (JGCs) in response to volume depletion and low blood pressure (Fig. 8). Its primary action is to cleave angiotensinogen into the decapeptide angiotensin I. In the pulmonary circulation, angiotensin-converting enzyme removes two more amino acids from angiotensin I to produce angiotensin II, which is the active hormone with a direct strong vasoconstrictor effect and an effect on the adrenal cortex to stimulate aldosterone production. Aldosterone is the key regulator of the potassium balance and induces sodium and water retention (Fig. 8). In critical illness, the appropriate response of the RAA axis is that hypotension and volume depletion induce renin release from the JGCs. The high concentration of renin in peripheral blood triggers, via angiotensin II, aldosterone secretion by the adrenal, resulting

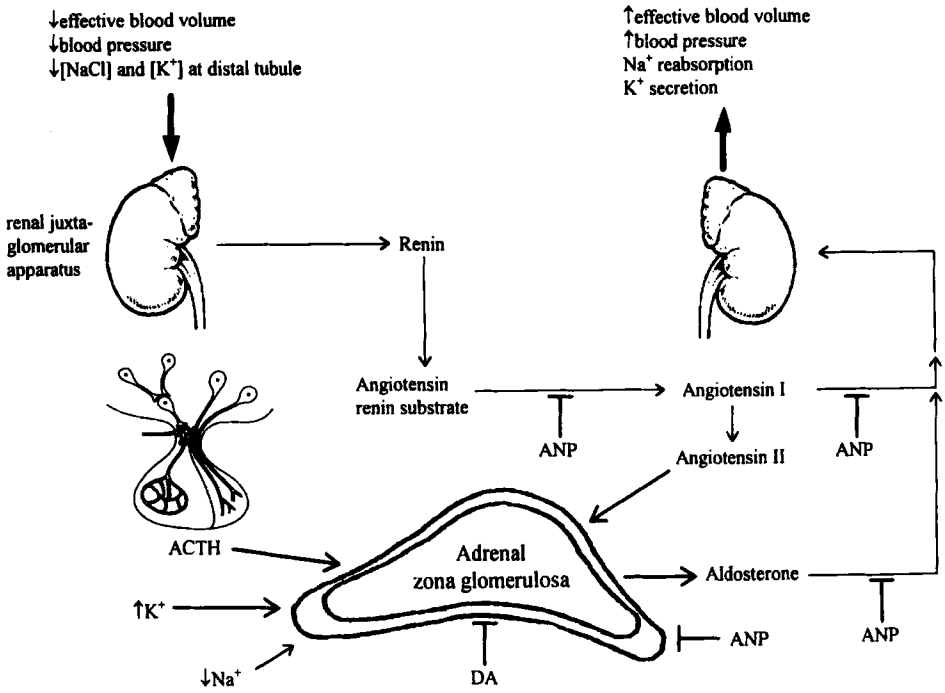

FIG. 8. Regulation of renin–angiotensin–aldosterone axis. →, stimulation; ⊣, inhibition.

in hyperreninemic hyperaldosteronism. The critical illness as stressor-induced pituitary ACTH hypersecretion also induces secondary hyperaldosteronism via ACTH, independent of volume status (R14).

However, a syndrome of elevated plasma renin activity accompanied by inappropiately low aldosterone levels has been identified in some seriously ill patients (F5, Z7). This entity has been called hyperreninemic hypoaldosteronism and occurs in about 20% of critically ill patients. Because the appropriate response to high renin is enhanced aldosterone secretion, a normal aldosterone level is considered inappropiate. A diffuse impairment of the zona glomerulosa is present; however, mineralocorticoid insufficiency is not the clinical feature of these patients. This syndrome is characterized by normokalemia, appropriate hypercortisolemia, normal metabolic clearance of aldosterone, and normal production of angiotensinogen II (R1). Hyperreninemic hypoaldesteronism seems to be a syndrome presenting after the acute phase, in fact in the same phase in which an inappropriate reaction of the HPA axis, with a paradoxically low ACTH level, is also present (V4). It has been demonstrated that such patients exhibit subnormal responses to ACTH stimulation but can respond appropriately to dopamine block-

ing agents (R1). This syndrome is more common in patients who have been critically ill for over a week, and when it is suspected, adrenocortical insufficiency should be ruled out by measuring serum cortisol levels and by testing adrenocortisol reserve functions (O2). Its presence has been associated with increased severity of the underlying diseases and with increased mortality due to the septic shock. The exact cause of this hyperreninemic hypoaldosteronism is uncertain, but a role of atrial natriuretic peptide was suggested earlier (E6, N3, R1). In light of the evidence for decreased adrenal androgen secretion in critically ill patients (P5), this dissociation may be the result of a relative shift in the metabolism of adrenal pregnenolone in septic patients away from mineralocorticoids and adrenal androgens and toward glucocorticoids. This suggests that the dissociation of renin and aldosterone secretions may represent an adrenal adaptation to severe illness and be part of the overall neuroendocrine response to systemic illness after the acute reaction.

5.2.3. *Atrial Natriuretic Peptide*

Atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) are members of a family of so-called natriuretic peptides, synthesized predominantly in the cardiac atrium, ventricle, and vascular endothelial cells, respectively (G13, Y2). ANP is a 28-amino-acid polypeptide hormone released into the circulation in response to atrial stretch (L3). ANP acts (Fig. 8) on the kidney to increase sodium excretion and glomerular filtration rate (GFR), to antagonize renal vasoconstriction, and to inhibit renin secretion (M1). In the cardiovascular system, ANP antagonizes vasoconstriction and shifts fluid from the intravascular to the interstitial compartment (G14). In the adrenal cortex, ANP is a powerful inhibitor of aldosterone synthesis (E6, N3). At the hypothalamic level, ANP inhibits vasopressin secretion (S3). It has been shown that some of the effects of ANP are mediated via a newly discovered hormone, called *adrenomedullin,* controlling fluid and electrolyte homeostasis (S8). The diuretic and blood pressure–lowering effect of ANP may be partially due to adrenomedullin (V5).

Relatively few data are available on the response of ANP to endotoxemia or septic shock. In an ovine model, a 13-fold increase in blood ANP concentration has been found 2 hours after endotoxin administration in a dose of 1.5 μg/kg body weight (L17). The ANP level remained elevated during the first 6 hours and was associated with marked diuresis and natriuresis and with decreased cardiac output and increased peripheral resistence (L17). In human studies, a significantly higher ANP blood level was observed in ARDS (E4) and in patients with acute respiratory failure associated with sepsis (M30). In a longitudinal study, we found that plasma ANP levels were increased in patients with sepsis, but the ANP levels showed no relation to the severity of disease or to the presence of shock (B8).

ANP works to oppose the function of the RAA axis via inhibiting the secretion and effects of renin, the effects of angiotensin II, and the adrenal secretion of al-

dosterone (L3). Accordingly, it is a potential candidate that may play a causal role in the alteration of the water and electrolyte homeostasis in sepsis. In this construction, the RAA axis primarily defends sodium balance and blood pressure, with ANP having an increasing counterinfluence in situations involving high blood pressure or sodium surfeit. Further studies are needed to clarify the significance of ANP in the pathophysiology of septic shock.

5.3. Pituitary–Thyroid Axis

Thyroid hormone metabolism is commonly affected by critical illness, which results in characteristic abnormalities of thyroid function (testing) known as nonthyroidal illness (NTI) or euthyroid sick syndrome (ESS) (C15, D23, W18). The term nonthyroidal illness syndrome (NTIS) has also been advocated and suggests functional abnormalities of the pituitary–thyroid axis (C15, W18). The effects of infectious diseases on thyroid function tests can be classified as the low triiodothyronine (T_3), normal thyroxine (T_4), and low T_3/T_4 syndromes. During the first phase of the infection both T_4 and T_3 decreased, reflecting decreased secretion of pituitary thyroid-stimulating hormone (TSH), decreased thyroidal secretion, accelerated T_4 disappearance, and the inhibition of hormone to transport protein (D23). Therapy of the infection is associated with resumption of TSH release and a progressive rise in serum T_4/T_3 levels. A decreased total T_3 level with normal total T_4 is the most common thyroid abnormality during sepsis. The TSH level is usually normal, reflecting the euthyroid status of these patients (B41), but it is generally accepted that evaluation of thyroid function in patients with NTI is difficult. The cause of the alterations is probably multifactorial, but they may also be the result of accelerated conversion of T_4 to rT_3 and conversion of T_3 to 3,3′-T_2. These reactions markedly lower the circulating level of T_3, resulting in the low T_3 syndrome. The pathogenesis of this syndrome is thought to involve cytokines such as TNF-α secreted by inflammatory cells, which inhibit type 1,5′-deiodinase, accelerating inner ring deiodination of T_4 (C16, D23, D24, P15). The low T_3 syndrome is accompanied by elevated levels of rT_3, due to the deficient activity of 5′-monodeiodinase (R6). This finding is useful for differentiating this syndrome from secondary hypothyroidism in which rT_3 is also low. The true serum concentration of free T_4 (FT_4) in patients with low T_3 syndrome is controversial and seems to depend on the assay method used (C15, D23, D24, D34, S25, S26). Early studies have documented decreased FT_4 concentrations by equilibrium dialysis (M23, S24) and ultrafiltration (W6) and found normal (K3, S38) or increased (D23, D24) FT_4 concentrations using the same techniques. The results are even more conflicting when the FT_4 concentration is measured with analog methods (E5). The differences in reported FT_4 concentrations in critically ill patients may be attributed to discrepancies in selection of patients and/or methodology. Furthermore, some of these patients possess a circulating inhibitor of T_4 binding to a serum pro-

tein (C17, D23, D24), which can interfere in the FT_4 measurements. With use of equilibrium dialysis of minimally diluted sera followed by radioimmunoassay (RIA) of the dialysate for FT_4, there is a general agreement now that the FT_4 concentration is normal or even above the normal level in patients with low T_3 syndrome (C15, H8, W19). True hypothyroidism can be ruled out by the normal total thyroxine TT_4, FT_4, and TSH and the elevated rT_3.

Patients with low T_3/T_4 syndrome are usually much sicker and the mortality rate is higher in this group. TT_4 levels decline progressively with increasing severity of illness and may serve as a predictor of clinical outcome. In the majority of these patients serum concentrations of TSH and FT_4 are normal and those of rT_3 are elevated. FT_4 concentrations measured by equilibrium dialysis proved to be normal in the phase of decreased TT_4. Clinically, these patients are still euthyroid, and supplemental thyroid hormones have not improved mortality (B45). It has been suggested that free fatty acid present in the serum of these patients blocks the T_4 binding more than T_3 binding (C17). Nonesterified fatty acids (NEFAs), mainly oleic acid, are nondialyzable inhibitors of thyroid hormone binding suggested to be present in the plasma of some nonthyroid ill patients and thus exhibit interference in the measurement of FT_4, depending on the method used (E5). Others, however, could find no evidence for the presence of these inhibitors (D23, M24). In addition, it has been shown that in septic patients an alternative mechanism might exist to explain the low T_4 concentration measured during severe infections. Early *in vitro* studies showed that phagocytosing human leukocytes metabolize T_4 by peroxidase-mediated cleavage of its diphenyl ether yielding diiodotyrosine (DIT) (B53). When an RIA was used to measure DIT in 125 severely ill intensive care patients, displaying the typical thyroid hormone alteration of NTI, only the patients whose clinical course was complicated by severe bacterial infections showed significantly higher DIT serum concentrations (M19). Serial measurements revealed a close temporal connection between the infection phase and increased DIT levels, suggesting that the increased phagocytotic activity of leukocytes in sepsis causes a rise in extrathyroidal DIT formation by ether link cleavage of T_4. Accordingly, DIT might be a relatively specific serum parameter for the presence and the course of severe bacterial inflammation in patients with nonthyroidal disorders, in contrast to rT_3 as a general marker of nonthyroidal illness (M19). Measurement of DIT as a general infection marker could be useful for intensive care patients suspected of having altered thyroid functions.

In view of the frequent and often chronic use of dopamine infusion in critical care medicine, an iatrogenic effect of dopamine concerning thyroid function in septic patients has been also suggested (K2, B12). Dopamine infusion induces or aggravates the low T_3 syndrome in these patients through direct inhibition of TSH release and through effects on thyroid hormone conversion, resulting in a low FT_4 concentration. Other medications such as glucocorticoids may potentiate the effect of dopamine on thyroid functions. Dopamine and glucocorticoids both de-

crease pituitary TSH secretion, decrease the biological activity of TSH, and diminish the thyroidal response to TSH. T_3 is an endogenous inotropic factor and it is required for protein synthesis, for fuel utilization by muscle, and for growth hormone responsiveness. Consequently, an iatrogenic decrease of circulating T_3 perpetuates the catabolic state of critical illness. In addition, the low T_3 level may play a role in other problems present in septic shock, such as diminished cognitive status with lethargy, somnolence, or depression; glucose intolerance; and insulin resistance (B12). On the other hand, agents frequently used in intensive care units such as heparin, amiodarone, or iodinated radiocontrast agents cause iatrogenic alterations in thyroid function tests (F4, J5, K2).

We can conclude that several abnormalities of circulating thyroid hormones occur in critically ill septic patients. The most common abnormality is a decrease in serum TT_3 concentration together with an increase in the free fractions of T_3 and T_4 due to the decrease in serum concentration of binding proteins, the presence of endogenous binding inhibitors and exogenous drugs, or some combination of these factors. The total serum T_4 concentration can be normal or low, but there is an inverse correlation between serum TT_4 concentration and the severity of illness and mortality rate. However, the relationship between the circulating and intracellular hormone concentrations and the patient's thyroid status at the tissue level produces a practical diagnostic problem in the intensive care setting and probably results in the greatest number of inaccurate test results (H18, H26, C15).

5.4. Reproductive Axis

Profound alterations in the function of the reproductive axis occur in response to physiologic stress of many types. The observation that stress has a disruptive effect on reproductive function in animals and in humans can be explained by the CRH-induced inhibition of gonadotropin secretion (B3, O4, R8). As we described earlier, stressful situations are characterized by activation of the HPA axis (see Sect. 5.1.1.). It has been demonstrated that CRH acts on the suprapituitary site via increased endogenous opioid tone to inhibit gonadotropin-releasing hormone (GnRH) secretion (G9). It is well known that endogenous opiates inhibit GnRH secretion (V2); consequently, infusion of the opiate antagonist naloxone reversed the inhibition of gonadotropin secretion occurring during CRH infusion or during stress in humans and animals (B3, P9). Accordingly, the compromised reproductive function occurring during stress is secondary to inhibition of gonadotropin secretion induced by endogenous opioids secreted in response to endogenous CRH.

Patients who are critically ill develop temporary hypogonadotropic hypogonadism regardless of the nature of the illness (B14, G7, S17, S31, W20). These patients had a normal response to releasing hormone (GnRH) stimulation, and the hypogonadotropism also occurs in the presence of nonfunctionating gonads. Thus, the gonadotrope appears to be fully responsive during acute illness but is not be-

ing stimulated appropriately. These observations suggest a central, hypothalamic and/or suprahypothalamic origin for these alterations. Generally, it is manifested by low testosterone levels, and estradiol levels in young women fall into the postmenopausal range (L8, L21, P5, W5, W20). The low testosterone concentrations are not accompanied by corresponding increases in the free fraction and are not due to reduced sex hormone binding capacity (L21, S31). Low levels of blood testosterone have been found in patients with sepsis or with septic shock (C19, F15, L21). Testosterone is the most important of the endogenous anabolic steroids. In men, a decrease of testosterone availability results in a negative nitrogen balance, which can be restored by testosterone administration. Negative nitrogen balance is a common problem in critically ill patients and is not easily corrected by parenteral nutrition or GH supplementation (W15). This catabolism probably has several etiologies, but androgenes may theoretically serve as a useful adjunct to parenteral nutrition or GH therapy of catabolism in patients with severe illness.

The changes in blood concentration of estrogens in patients with sepsis are more complicated. In contrast to the low androgen levels, estrogens have been found to be increased in animals (C18) and in humans (C19, F15, L21) with sepsis, whereas serum gonadotropin levels were decreased. The major source of estrogens in men and in postmenopausal women is conversion by aromatization of testosterone to estradiol and androstenedione to estrone in muscle and adipose tissue. An increase in aromatase activity is the main mechanism that might explain the high concentration of estrogens during sepsis. The experimental observation that an endotoxin injection–induced increase in estrogens is absent in animals treated with aromatase inhibitors supports this view (C18). A significant increase in estrogen levels was observed in patients with sepsis and septic shock, either males or females (C19, F15). The correlation between the estrogen levels and outcome is not clear, and further studies are needed to document the potential consequences of increased estrogen secretion in septic patients.

6. The Interplay of Mediators in Sepsis

For many years, the general assumption was that microorganisms produced toxic substances that, upon entrance into the circulation, cause hypotension, decreased perfusion, acidosis, and death (W11). There is evidence that the systemic response to invading organisms is independent of the type of organism and that the host-dependent response is more important (D5, M11). Sepsis and SIRS are characterized by excessive production of inflammatory mediators and excessive activation of inflammatory cells, resulting in metabolic anarchy; the body's defense mechanisms are overwhelmed and it can no longer control its own inflammatory response (B38). The main consequences of this uncontrolled inflammatory response are involvement of many organs, the onset of shock, and the development

of multiple organ dysfunction syndrome (L2). The host septic response is extremely complex: a) there is a cascade involving more than 100 mediators, b) these mediators have overlapping biological effects, c) SIRS also evokes activation of endogenous regulators of that response, d) the main actions of mediators take place in the local microenvironment in an autocrine or paracrine manner, e) measurement of both triggers and mediators is technically difficult, and f) plasma levels of mediators do not correlate clearly with definable clinical processes (M11).

1. The *induction phase of SIRS* may be initiated by various microorganisms or by noninfectious causes such as trauma or burn injury. Gram-negative sepsis can serve as a model, with *LPS or endotoxin* to be considered as the most important exogenous mediator. LPS binds to, and can be ingested by, phagocytic cells, endothelial cells, and platelets. This interaction leads to the release of various categories of endogenous mediators. In the presence of certain cytokines, LPS can also trigger autolysis of cells, which may add to already existing tissue damage. On the other hand, LPS can also activate the complement system and the blood clotting cascade (Hageman factor of the intrinsic pathway and F-VII of the extrinsic pathway) (H22).

Activated complement augments permeability of the endothelium, causes immune adherence, helps peptidoglycans and endotoxins to activate platelets, induces production of tissue factor by leukocytes and endothelial cells, induces IL-1 production in monocytes, and potentiates neutrophils in inflicting oxidative and proteolytic injury to endothelia. Disturbances in the blood clotting system can lead to DIC, which frequently accompanies sepsis. Factors involved are a) tissue factor, produced by polymorphonuclear leukocytes (PMNs) and endothelial cells, under control of cytokines, b) activation of Hageman factor, and c) disturbed balance in pro- and anticoagulant activities of endothelial cells, with a shift toward the procoagulant direction with a large excess of PAI-1, mediated by TNF and IL-1.

2. In a second phase *cytokine synthesis and production* start (B38, D5, D14). In general, cytokines are not stored as preformed molecules and their synthess is initiated by new gene transcription or translation of preformed RNA. Cytokine genes are permanently inactivated in many cell types, so these genes are "accessible" in a limited number of tissues. Posttranscriptional control of cytokine biosynthesis is the most prominent method of regulation. At this level small amounts of cytokines, often undetectable, are released into the circulation. This cytokine response is directed toward defense of the local environment and probably not pathologic or abnormal. Macrophages and platelets are recruited, and production of growth factors is stimulated. An acute phase response may be initiated, which is controlled by a diminution of the proinflammatory mediators and a simultaneous increase of endogenous antagonists (e.g., IL-1ra, sTNF). This complex network of mediators is aimed at restoring homeostasis. When this fails, phase 3 sets in, leading to SIRS or sepsis.

3. *The cytokine cascade.* A massive reaction begins with destructive rather than protective effects of cytokines. The integrity of capillary walls is destroyed and cytokines spill out and into end organs, producing additional sites of damage. It is unclear why the control of the initial cytokine response is lost (B37). The balance between pro- and anti-inflammatory cytokines may be disturbed (L2). Also, these cytokines closely integrate with neuroendocrine hormones in an immunoregulatory feedback, as the activity of the HPA axis is increased in sepsis (H2) TNF, also called the "king of cytokines," plays a primary role in sepsis in close relation with IL-1, both induced by endotoxin. TNF augments the procoagulant properties of endothelial cell surfaces: TNF induces PAF, TF, PAI-1, and ELAM-1 synthesis and reduces thrombomodulin production. TNF targets the neutrophil, leading to production of oxygen-derived radicals, induction of increased adherence, and disruptive potential for the endothelium. TNF is a potent inducer of other cytokines: IL-1, IL-6, and IL-8 (E11). Aside from its actions on cells, it also acts as a catalyst for humoral systems, such as the complement cascade, the clotting system, and the kallikein–kinin system. Negative feedback occurs with endogenous antagonists such as sTNFr. IL-1 also shifts the balance to a prothrombotic state by increasing TF and PAI-1 and by decreasing thrombomodulin (D20). These effects all contribute to DIC. IL-1 can be stimulated not only by endotoxin but also by thrombin, TNF, and IL-1 itself. IL-1 activates the production of TNF, IL-6, and IL-8. IL-1 augments leukocyte adhesiveness to endothelial cells and induces prostacyclin synthesis. It also triggers negative feedback mechanisms via the acute phase protein production of hepatocytes, in concert with TNF, IL-6, and corticosteroids. The main paracrine function of IL-8 is the attraction (recruitment and activation) of leukocytes to local sites of microbial infection, which can lead to tissue injury. IL-8 appears to be activated by TNF, LPS, and IL-1 and is produced mainly by activated macrophages. Peak levels in sepsis parallel those of IL-6.

The counterinflammatory cytokines comprise IL-6 and IL-10 (M8). The exact role of IL-6 in sepsis is uncertain, and the appearence of IL-6 in plasma may be related directly to TNF and IL-1 production. IL-6 is the principal hepatocyte-stimulating factor, responsible for the induction of acute phase proteins such as protease inhibitors and C-reactive protein, which play a role in activation of the classical complement pathway, activation of macrophages, modulation of neutrophil activity, and triggering of platelet aggregation and degranulation (B26). IL-6 may inhibit TNF, thereby exerting anti-inflammatory effects (E11). IL-6 regulates production of ACTH and hence of corticoids, which have an overall immunosuppressive and anti-inflammatory effect. IL-10 also has predominant counterinflammatory actions. *In vitro,* IL-10 can inhibit TNF, IL-1, IL-6, and IL-8. IL-10 is a potent macrophage-deactivating factor and possibly prevents LPS lethality by controlling TNF and IFN-γ secretion (M7, M8). In this complex cytokine network, several endogenous circulating antogonists exist. Soluble cytokine receptors have been described for TNF, IL-1, and IL-6. Endotoxin leads to release of excessive

amounts of sTNF-R and IL-1ra into the circulation, with different kinetics compared with TNF and IL-1, respectively (D13). Their exact role is uncertain, but by forming complexes (e.g., TNF–sTNF-R), they may serve as a slow-release reservoir (TNF), which could prolong the inflammatory response.

4. *Secondary mediators and end products causing cellular damage.* Normally, blood flow is effectively regulated to match the tissue's metabolic need. In the critically ill, physiologic compensatory responses aim at the maintenance of overall circulatory function and integrity. Abnormal distribution of flow is an important factor in the development of organ dysfunction in sepsis, with several components involved at the central, regional, and microregional levels (H25). The endothelium plays an important role in this last phase of sepsis or septic shock, with evidence for increased procoagulant and inflammatory activity (B43, V1). Endothelium is an active barrier that enhances or limits the vascular entry of substances by secreting many molecules active in the regulation of vascular tone, coagulation, and permeability (renin, ET, prostacyclin, NO, PGE_2, active amines, etc.). Cytokines lead to increased expression of adhesion molecules (e.g., ELAM-1) on the endothelial cells and neutrophils, resulting in increased migration and maintainance of activated cells in injured tissues. Many substances are released, including arachidonic acid metabolites, free oxygen radicals, NO, and ET. PAF interacts with cytokines and hematologic growth factors to amplify or down-regulate mediator release. The balance between vasoconstriction (catecholamines, ET) and vasodilatation (NO, ANP, prostacyclin) may be disturbed, leading to microregional circulatory abnormalities such as local tissue blood maldistribution (H25, B1). Endothelium-derived local substances are in a very complex interplay with central mechanisms such as the autonomous nervous system. Therefore, correlation of one of the (endocrinologic) regulators with hemodynamic alterations is very difficult. At least, changes of substances responsible for guaranteeing sufficient circulation are significantly altered in sepsis (B32). Cathecholamines stimulate ANP secretion, by which negative effects on the microcirculation of epinephrine may be compensated. NO can also down-regulate the adrenoreceptor system. Adrenergic responsiveness diminishes during sepsis, leading to loss of control of the microcirculation. Both renin and vasopressin can potentiate the vasoconstricting effects of cathecholamines. ANP antagonizes the vascular effects of vasopressin and the RAAS and also acts on cathecholamine synthesis. ET induces ANP release and was reported to stimulate cathecholamines and renin. Endothelial injury causes increased capillary permeability with subsequent edema formation, which is, next to vasodilatation, a characteristic abnormality in sepsis. Some complement activation products, in particular the anaphylatoxins, directly increase vasopermeability (H5).

Thus, inflammatory mediators of humoral and cellular origin are largely implicated in the development of sepsis and SIRS. These mediators, together with the inflammatory cells themselves, activate and damage the endothelial cells, which

leads to dysfunction of the endothelium and production of specific endothelial mediators. Induced expression of adhesive molecules aggravates PMN adhesion to the endothelium, a main consequence of which is increased vasopermeability and extension of the acute inflammatory reaction, leading to multiple organ failure. Mechanisms for down-regulating this inflammatory reaction exist: natural inflammation inhibitors and some mediators themselves have a negative feedback on the inflammatory reaction. The production of inflammatory cytokines (TNF, IL-1, IL-6, IL-8) by activated phagocytes and endothelial cells can be regulated by other cytokines (IL-2, IL-4, or IL-10) or by inflammatory agents secreted by the phagocytes themselves, such as PGE_2. Cytokines induce the liver to produce acute phase proteins, some of which are antiproteases active against both complement and coagulation proteases and active enzymes released by activated PMNs. The reactive oxygen species and free radicals released by PMNs are neutralized by antioxidant enzymes (superoxide dismutase, catalase) (G17).

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CURRENT CONCEPTS OF COAGULATION AND FIBRINOLYSIS

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1. Introduction

Blood is essential for the sustenance of life. It transports oxygen and nutrients to the tissue. Hence, the first priority when a blood vessel breaks is to arrest the bleeding by forming a coagulum. Once the blood vessel has been repaired, the mesh of fibrin in the coagulum has to be dissolved to permit blood flow. This will result in the resumption of the delivery of oxygen and essential nutrients to the tissues. Thus coagulation and the lysis of the fibrin clot mediated by fibrinolysis can be pictured as two players riding a seesaw, with an exquisite balance between the two being maintained by circulating procoagulants and anticoagulants. When the balance is tilted toward clot formation the thrombotic episode is initiated. In contrast, excessive activation of the fibrinolytic system leads to bleeding.

Over the past decade there has been an explosion of knowledge on the mechanisms of both coagulation and fibrinolysis that has contributed to our appreciation of the basic concepts of these two pathways.

This review will attempt to explore our current understanding of both coagulation and fibrinolytic systems, their clinical impact, and variables affecting the laboratory assessment of thrombotic and bleeding disorders.

2. Basic Concepts of Coagulation

2.1. Role of Platelets

The first step toward the formation of a coagulum is the adhesion of platelets to the exposed endothelial cell surface of the broken vessel. The adhesion of platelets to the surface of the broken vessel is mediated by glycoprotein receptors on the platelet membrane. Thus, a complex of platelet membrane glycoprotein Ib receptor with glycoprotein IX and glycoprotein V (GPIb/IX/V complex or CD42) binds to von Willebrand (vW) factor on the exposed endothelial cell surface of the broken blood vessel during the process of platelet adhesion (1).

In addition to vW factor, other ligands are recognized by specific platelet membrane receptors during the process of platelet adhesion to the exposed surface of the damaged blood vessel. Thus, glycoprotein IV recognizes the ligands collagen and thrombospondin on the endothelial cell surface (2, 3). A family of receptors on the platelet membrane called integrins are also involved in binding to molecules on the endothelial surface of the exposed blood vessel. These integrins are heterodimeric molecules with alpha (α) and beta (β) subunits (4).

Integrins themselves are found on nearly all cells and mediate several physiological responses, such as cell–cell and cell–matrix interactions. Three families of integrins, each family with a common beta subunit in combination with distinct alpha subunits, have been recognized. The beta 1 family, also called very late lymphocyte-activation antigen or VLA, has receptors mediating extracellular matrix interactions with molecules such as collagen, laminin, and fibronectin. Naturally, platelets contain many of the receptors of the beta 1 family.

Thus, while glycoprotein Ia/IIa ($\alpha_2\beta_1$) binds to collagen, glycoprotein Ic/IIa ($\alpha_5\beta_1$) binds to fibronectin (5). The other integrin belonging to the beta 1 family that is involved in platelet adhesion is $\alpha_6\beta_1$, which binds laminin.

The beta 2 family has receptors on leukocytes (also called LeuCAM) and mediates inflammatory and immune recognition functions.

The beta 3 family, known as cytoadhesins, includes receptors found on platelets and other cell types such as $\alpha_v\beta_3$, which interacts with vitronection on the exposed endothelial cell surface of the ruptured blood vessel (6). The vitronectin receptor ($\alpha_v\beta_3$), where the subscript v stands for vitronectin, also recognizes ligands such as fibrinogen, von Willebrand factor, and fibronectin, all of which are recognized by another member of the beta 3 family, the glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) receptor found on platelets and megakaryocytes.

In the intact blood vessel, ligands involved in adhesion to platelets, such as collagen, fibronectin, and von Willebrand factor, are sequestered in the subendothelium, thus preventing access to platelet adhesive receptors. Table 1 summarizes the functions of platelet membrane integrin receptors.

Adhesion of platelets to ligands such as collagen on the subendothelial matrix

TABLE 1
PLATELET MEMBRANE INTEGRIN RECEPTORS

Integrin receptor family	Ligand recognized	Result
Beta 1 family		
$\alpha_2\beta_1$ (GPIa/IIa)	Collagen	Adhesion
$\alpha_5\beta_1$ (GPIc/IIa)	Fibronectin	Adhesion
$\alpha_6\beta_1$	Laminin	Adhesion
Beta 3 family		
$\alpha_{IIb}\beta_3$ (GPIIb/IIIa)	Fibrinogen	Aggregation
	Von Willebrand factor	
	Fibronectin	
	Vitronectin	
$\alpha_v\beta_3$	Vitronectin	Adhesion
	Von Willebrand factor	
	Fibrinogen	
	Fibronectin	

activates platelet membrane lipases (phospholipase A_2 or C), resulting in the release of arachidonic acid from the platelet membrane. Arachidonic acid is converted by the endothelial cell fatty acid cyclooxygenase enzyme to prostaglandin cyclic endoperoxides (PGG_2, the hydroxperoxy, and PGH_2, hydroxy, compounds). Thromboxane synthetase within the platelets converts cyclic endoperoxides to thromboxane A_2, which in turn causes release of adenosine diphosphate (ADP) from the platelet dense or β granules. ADP promotes aggregation of platelets. The activation of platelets triggers a change in its shape and induces conformational changes in the integrin glycoprotein IIb/IIIa receptor ($\alpha_{IIb}\beta_3$) so that it binds readily to fibrinogen. In effect, fibrinogen through specific amino acid sequences such as arginine–glycine–aspartic acid (RGD) or lysine–glutamine–alanine–glycine–aspartic acid–valine (KQAGDV) binds to GPIIb/IIIa receptors bridging adjacent platelets and promoting platelet aggregation (5). The binding of fibrinogen to platelet GPIIb/IIIa receptors initiates a series of biochemical events beginning with the activation of phospholipase C through interaction of thrombin with its receptor on platelets and signal transduction through guanosine triphosphate (GTP)–binding regulatory protein or G proteins. Second messengers such as diacylglycerol (DAG) and inositol triphosphate (IP_3) are produced by cleavage of platelet membrane phosphatidylinositols (phosphatidylinositol biphosphate). IP_3 causes cellular uptake of calcium. DAG together with calcium activates protein kinase C, which is needed for the phosphorylation of myosin light chain and change in platelet conformation, with the net effect being the exposure of additional GPIIb/IIIa receptors on adjacent platelets for fibrinogen binding (7, 8). This domino effect ensures the formation of the platelet aggregate. The importance of GPIIb/IIIa receptors to the process of platelet aggregation is evidenced by the presence of close to 50,000 of these receptors on a single platelet (5). It would almost seem that the platelets were designed just for clotting! Release of contents of platelet dense or β granules such as ADP and serotonin together with other agonists such as thrombin and thromboxane A_2 leads to activation of additional platelets, formation of a platelet plug, and initiation of coagulation. Among the various agonists thrombin is the most potent platelet activator on platelets. Figure 1 summarizes steps in platelet activation. In the endothelial cell the enzyme prostacyclin synthetase converts prostaglandin cyclic endoperoxides (PGG_2–PGH_2) to prostacyclin (PGI), which in turn can stimulate the enzyme adenyl cyclase within the platelets, resulting in an increase in platelet cyclic AMP (cAMP) produced from ATP (adenosine triphosphate). Increase in cAMP inhibits release of contents of platelet granules and thus prevents platelets from aggregating.

2.2. Activation of the Coagulation Cascade

Within the intact blood vessel, coagulation factors circulate as inactive zymogens. The formation of a platelet plug to arrest bleeding from a ruptured blood ves-

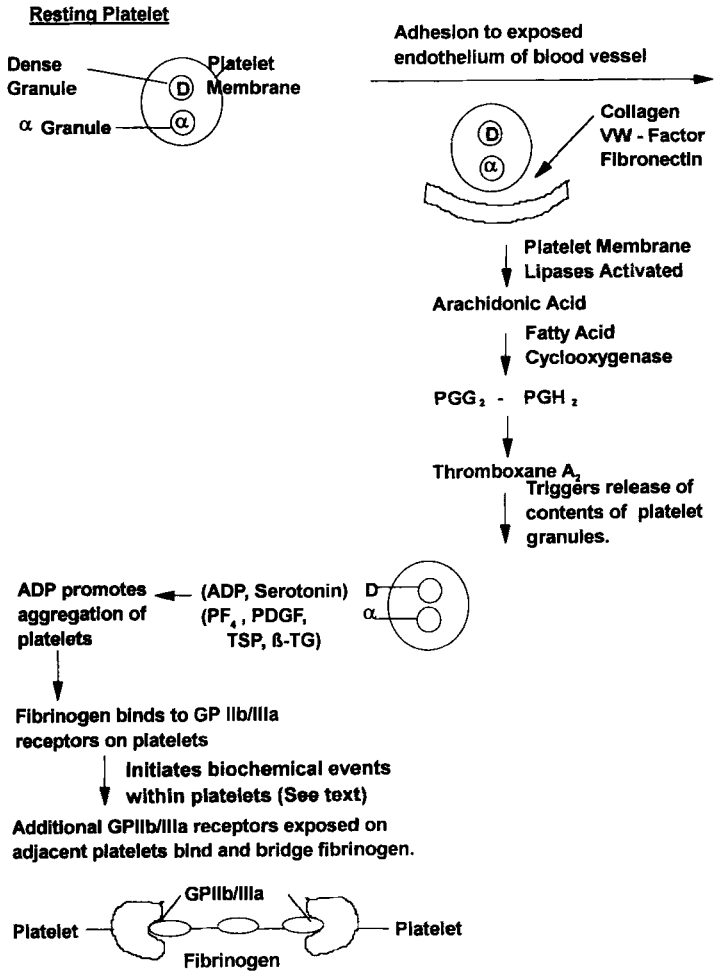

FIG. 1. Steps in platelet activation.

sel provides a surface for activation of coagulation factors. Normally, the negatively charged phospholipid phosphatidylserine is localized in the inner leaflet of the cell membrane. The influx of calcium that results upon platelet activation is apparently responsible for the translocation of phosphatidylserine to the outer surface of the platelet membrane (9). This anionic phospholipid is important for the assembly of two major coagulation factor complexes, the tenase and the prothrombinase complex, which lead to thrombin generation, and the subsequent conversion of fibrinogen to fibrin and the stabilization of the cross-linked fibrin clot.

By providing a negatively charged surface on the aggregated platelet plug, thrombin that is ultimately generated is efficiently localized to the area of the disrupted blood vessel it is designed to repair (9, 10). The traditional view that coagulation was being effected by two separate pathways, the intrinsic and the extrinsic, has undergone revision. Our current understanding is that the tissue factor pathway is the primary pathway in initiating blood coagulation. Tissue factor is an integral membrane glycoprotein expressed on the surface of a variety of activated or disrupted cells, including the subendothelial fibroblast-like cells lining the blood vessel (11). It can bind either factor VII or VIIa with similar affinity. Factor VIIa itself is formed when factor VII is cleaved by activated factor X (Xa) at Arg-152/Ile-153 (12). It is a soluble plasma protease that functions as a catalytic subunit. Tissue factor, in contrast, can be regarded as an essential regulatory subunit.

The activity of factor VIIa is enhanced astronomically (10 millionfold) upon binding to tissue factor. The VII or VIIa–tissue factor complex activates factors IX and X and autoactivates factor VII. Although the activity of the tissue factor–factor VII complex is expressed without the presence of the negatively charged phosphatidylserine, the activity can be enhanced by its presence (9).

As mentioned earlier, the negatively charged phospholipid surface is essential for the formation of the tenase and prothrombinase complexes. The tenase complex is generated when activated factor VIII (VIIIa) interacts with the surface of the anionic phospholipid, phosphatidylserine, to generate in the presence of ionic calcium a high-affinity binding site for activated factor IX (IXa). This complex converts factor X quickly to its activated form (10). The formation of the prothrombinase complex is facilitated with the binding of activated factor V (Va) to the surface of the negatively charged platelet phosphatidylserine, which in turn favors the binding of activated factor X (Xa) in the presence of ionic calcium. Factor V also binds to prothrombin, thus confining it to the site of the assembly of the prothrombinase complex. Thrombin is generated when prothrombin is cleaved by the prothrombinase complex. Figure 2 diagrammatically depicts the formation of the tenase and prothrombinase complexes.

Along with thrombin, a small fragment called prothrombin fragment 1.2 (PF1.2) is released from prothrombin, whose clinical significance we shall discuss later. Small amounts of thrombin can activate factors V, VIII, and even XI to produce a burst of thrombin generation. Thrombin activation of factor XI is critical for preventing fibrin clots from undergoing fibrinolysis (13). Indeed, whereas a deficiency of either factor XII or other proteins of the contact system such as prekallikrein or high-molecular-weight kininogen does not result in bleeding, individuals deficient in factor XI are disposed to bleeding from tissues such as the urinary tract, nose, oral cavity, or tonsils that are subjected to localized increased fibrinolytic activity (14). Thus, although contact factors (factor XII and other as-

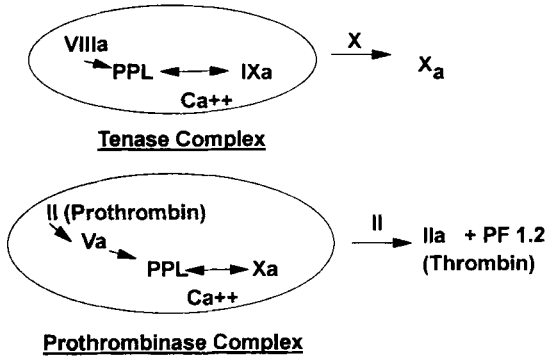

FIG. 2. Generation of tenase and prothrombin complexes. PPL represents the anionic phospholipid surface provided by the platelets (platelet phospholipid). Cleavage of prothrombin by the prothrombinase complex results in the formation of thrombin and the release of a small fragment called prothrombin fragment 1.2 (PF1.2).

sociated proteins) are critical for the clotting of blood *in vitro* in a test tube, they are less significant in the initiation of clotting *in vivo.*

Thrombin converts fibrinogen into fibrin monomers, which in turn are cross-linked when thrombin activates the enzyme factor XIII. Fibrinogen itself is uniquely structurally suited to form the polymerized network when acted upon by thrombin. It is a disulfide-linked molecule that contains two Aα, two Bβ, and two gamma (γ) chains. Indeed, the six polypeptide chains are held together by 29 disulfide bonds. It is a 340-kDA molecule. Thrombin acts on the Aα chain at position 16 to generate fibrinopeptide A (FPA) and the α chain. It also cleaves the Bβ chain at position 14 to generate fibrinopeptide B (FPB) and the β chain. Figure 3 illustrates the fibrinogen molecule and the formation of FPA and FPB. The cleavage of FPA permits end-to-end fibrin polymerization. The cleavage of FPB, in contrast, occurs at a considerably slower rate (15). The removal of FPB facilitates side-to-side polymerization of the end-to-end linked fibrin monomers. Thrombin targets just 2 of the arginine–glycine bonds out of 181 arginine–lysine peptide bonds in effecting the removal of FPA and FPB. Both the highly electropositive fibrinogen recognition exosite on thrombin and the active site (apolar binding site near the catalytic site) contribute to the specificity of thrombin's cleavage of FPA and FPB (16). Figure 4 depicts the sites on the thrombin molecule involved in the interaction with fibrinogen. Subsequent to the removal of FPA and FPB, cross-linking of the monomers occurs through the activation of factor XIII (XIIIa), a transglutaminase enzyme, resulting in the formation of a firm fibrin clot. A molecule of ammonia is released in this process. The catalyzation of the cross-linking of the α and

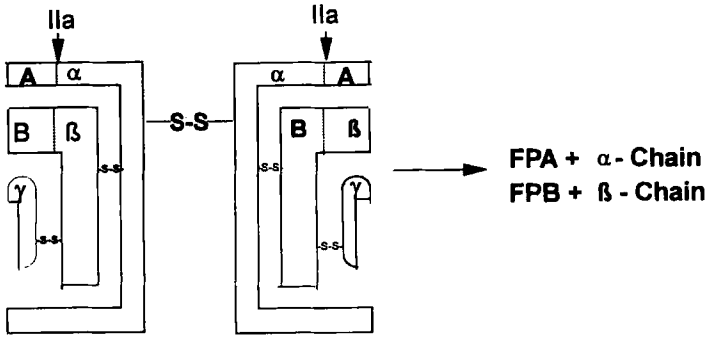

FIG. 3. Fibrinogen molecule with its two A α, two B β, and two γ chains. Thrombin (IIa) acts on the A α chain to generate fibrinopeptide A (FPA) and the α-chain. It also cleaves the B β chain to generate fibrinopeptide B (FPB) and the β-chain. S–S represents disulfide bonds. Altogether, 29 disulfide bonds hold together the six polypeptide chains that make up the fibrinogen molecule.

γ chains of fibrin by factor XIII results in the formation of high-molecular-weight α-polymers and γ-dimers. The resulting fibrin clot is rendered resistant to the fibrinolytic action of the enzyme plasmin, due to the fact that factor XIIIa also cross-links the α_2-plasmin inhibitor (α-PI) to the fibrin α-chains (17). Figure 5 depicts the coagulation pathway as currently understood.

2.3. CONTRIBUTION OF LEUKOCYTES TO COAGULATION

When a blood vessel breaks, the body literally sounds an alarm and recruits leukocytes to the affected site. Monocyte-macrophages express procoagulant activity, enabling coagulation to be effected on their surface (18). In addition, various agonists such as phorbol esters, prostaglandins, endotoxin, and complement can elicit procoagulant activity in monocytes (19). Coagulation can also be elicited on the surface of neutrophils and lymphocytes (20, 21).

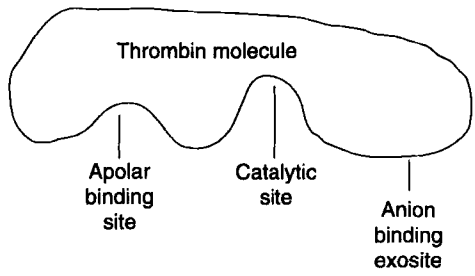

FIG. 4. Sites on the thrombin molecule involved in the interaction with fibrinogen.

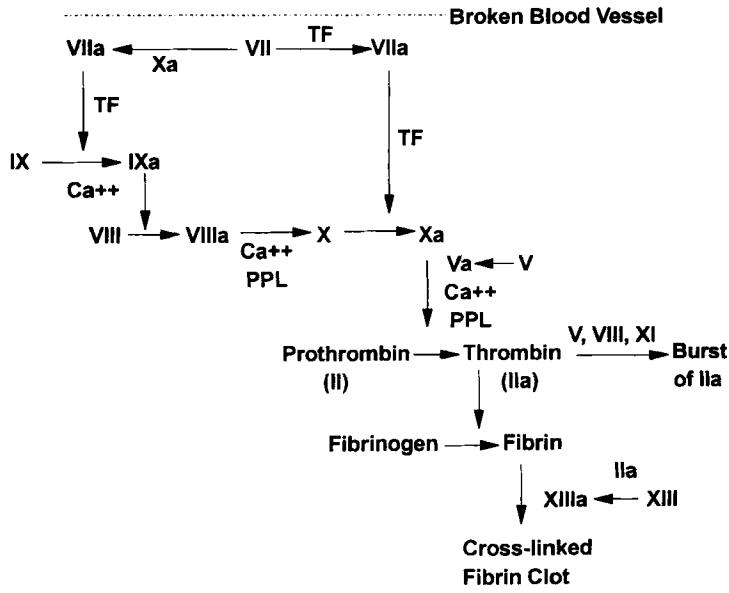

FIG. 5. Schematic representation of coagulation pathway as currently understood. TF, tissue factor; PPL, platelet phospholipid.

2.4. INHIBITORS OF COAGULATION

Circulating serine proteinase inhibitors (SERPINs) such as antithrombin III (ATIII), heparin cofactor II, and proteinase nexins 1 and 2 function as endogenous inhibitors of coagulation, thus serving to regulate the process. ATIII inhibition of thrombin requires binding of heparin with more than 18 saccharide chains to both ATIII and thrombin in order to bring the two molecules closer together in a ternary complex (22). In contrast, inhibition of factor Xa by ATIII requires a conformational change at the active site upon binding to heparin, which can even be mediated by a pentasaccharide chain of heparin (23).

Heparin cofactor II, when activated by binding to glycosaminoglycans (dermatan sulfate, heparins, and heparin), inhibits thrombin (24). The 43-kDa serpin, proteinase nexin 1, possesses 30% sequence homology with ATIII and can be activated by binding to heparin to inhibit several serine proteinases including thrombin (25). Proteinase nexin 2 is found within the platelet α-granule and is released when platelets are activated (26). It is able to inhibit factor XIa.

Tissue factor pathway inhibitor (TFPI), a 42-kDa protein with three Kunitz domains, is a potent inhibitor of coagulation. It inhibits tissue factor–factor VIIa complex upon binding to the active site of Kunitz domain one. Factor Xa is inhibited upon binding to the active site of the second Kunitz domain of TFPI (27).

A major portion of TFPI is bound to the endothelial surface and can be released from the cells by heparin (28). Platelets contain very small amounts of TFPI, which are released when platelets are activated (29). Figure 6 illustrates the mechanism of inhibition by TFPI.

3. Basic Concepts of Fibrinolysis

The binding of thrombin to a receptor protein called thrombomodulin on the endothelial cell membrane of the exposed blood vessel abolishes its procoagulant activity (15). Instead, it activates protein C, which in turn binds to protein S on nearby platelet and endothelial cell membranes to inactivate coagulation factors V and VIII. The protein C–protein S complex also activates tissue plasminogen activator (t-Pa), which in turn converts plasminogen to plasmin. Both the fibrin clot and the residual fibrinogen monomers are converted to smaller fragments by the enzyme plasmin. These fibrinogen–fibrin degradation products (FDP–fdp) are called X, Y, D, and E. Fragments X and Y are further cleaved by plasmin to produce two D fragments and one E fragment.

A type I transmembrane protein called endothelial cell protein C receptor (EPCR), which is expressed at high levels exclusively on a subset of endothelial cells, has also been identified. EPCR has a role in the protein C pathway (30). EPCR binds to both protein C and activated protein C (APC) with equal affinity. Activation of protein C presumably requires interaction of the protein C–EPCR complex with the thrombin–thrombomodulin complex. APC that is formed as a result of this interaction is reversibly bound to EPCR until it dissociates to react subsequently with protein S. The APC–protein S complex inactivates activated factor V (Va).

Although the fibrinolytic pathway is activated when thrombin binds to thrombomodulin, the thrombin–thrombomodulin complex, in addition to activating protein C (APC), activates a fibrinolysis inhibitor called the thrombin-activatable fibrinolysis inhibitor ($TAFI_a$). Thus plasmin generation and, in turn, fibrinolysis are

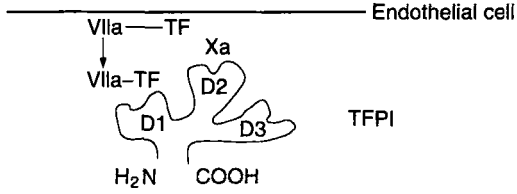

FIG. 6. Mechanism of inhibition of tissue factor pathway inhibitor (TFPI). Kunitz domain 1 (D1) inhibits TF–VIIa complex. Domain 2 (D2) inhibits Xa.

modulated by $TAFI_a$ (31). Figure 7 summarizes our current concept of the protein C pathway.

Plasminogen is synthesized by the liver. Its nominal concentration in plasma is approximately 20 mg/dl. For fibrinolysis to occur efficiently, plasminogen that is referred to as glutamic plasminogen (Glu-plasminogen) has to undergo a change in conformation. This occurs when Glu-plasminogen is activated by activators (t-PA, urokinase) to Glu-plasmin. Glu-plasmin in turn hydrolyzes the Lys-77/Lys-78 bond in the Glu-plasminogen molecule to convert the latter to lysine plasminogen (Lys-plasminogen). Apparently, Lys-plasminogen has a conformation more readily accessible than that of Glu-plasminogen to the plasminogen activators to convert it to active plasmin (32). The actual conversion of Glu-plasminogen to Lys-plasminogen is effected when the former interacts with fibrin monomers that have polymerized and have become cross-linked through fragment D domains. This interaction, although it is moderately weak, is stronger than the binding of Glu-plasminogen to fibrinogen. Both high- and low-affinity lysine binding sites on Glu-plasminogen are responsible for binding to polymerized and cross-linked fibrin. The fragment E domain of fibrin apparently interacts with the high-affinity lysine binding site of Glu-plasminogen. However, when one of the weaker lysine binding sites on Glu-plasminogen interacts with the fibrin fragment D domain, the conformation of Glu-plasminogen is modified to appear more like that of Lys-plasminogen. As a result of this conformational change, Glu-plasminogen is activated by plasminogen activator to Glu-plasmin. The latter in turn transforms free or fibrin-bound Glu-plasminogen to Lys-plasminogen (33).

The lysine binding sites on free Lys-plasminogen or free Lys-plasmin are susceptible to inhibition by α_2 plasmin, the primary inhibitor of plasmin, because these sites are not protected by interaction with fibrin. However, when Lys-plasminogen is tightly bound to the fragment E domain, it is rapidly activated by the

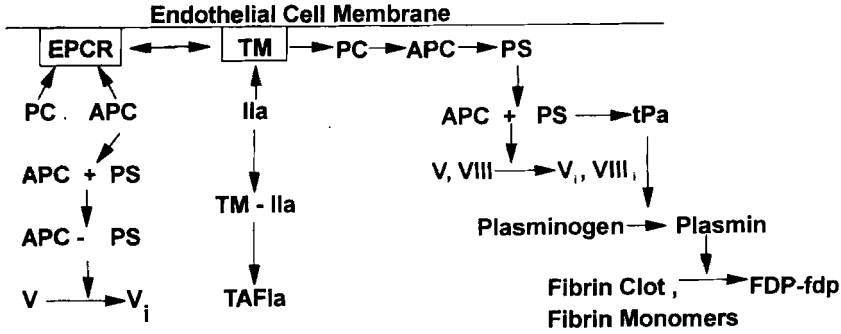

FIG. 7. Our current understanding of the protein C pathway. V_i and $VIII_i$ represent inactivated factors V and VIII, respectively. For an explanation of other abbreviations see text.

plasminogen activator urokinase to active plasmin, thereby shielding it from autolysis (33). The fibrinolytic action of plasmin is accomplished with the formation of various complexes consisting of D-dimers, fragment D, and fragment E. Thus D-dimers represent the lysis product of cross-linked fibrin. Figure 8 summarizes the changes that occur in the conformation of the Glu-plasminogen molecule that lead to the generation of active plasmin.

It should be noted that the fragments resulting from the action of plasmin have biological activity (34). For instance, fragment X, like the fibrinogen molecule, can effect ADP-induced platelet aggregation and can also be acted upon by thrombin to produce a clot. Coagulation as determined by the thrombin clotting time assay can be inhibited by fragments D and Y. Fragments D and E can stimulate fibrinogen synthesis by the liver (35). Figure 9 summarizes steps in the formation of fibrinogen–fibrin degradation products.

3.1. Fibrinolysis Activators

Tissue-type plasminogen activators (t-PAs) and urokinase-type plasminogen activators are two of the well-recognized extrinsic plasminogen activators. The cleavage of the Arg-560/Val-561 bond (Arg560/Val561) of plasminogen by either of these two types of activators results in the activation of plasminogen.

The 68-kDa t-PA molecule, although synthesized as a single chain, undergoes modification to result in A and B polypeptide chains connected by a single disulfide bridge (36). This modification is mediated by factor Xa, tissue kallikrein, and directly on the surface of a thrombus by plasmin (37). The affinity of the A chain

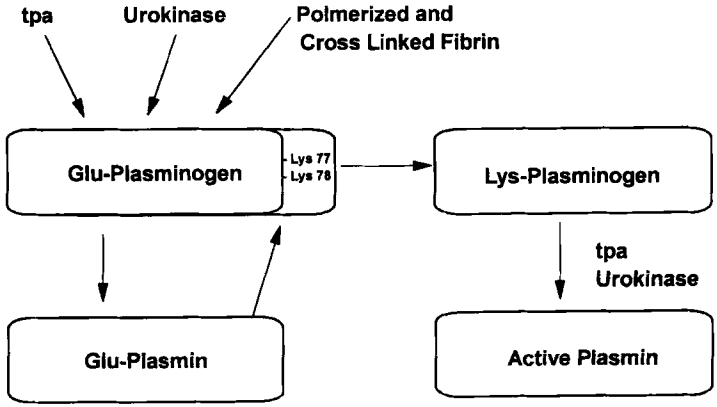

Fig. 8. Conformational changes in the Glu-plasminogen molecule leading to the generation of active plasmin.

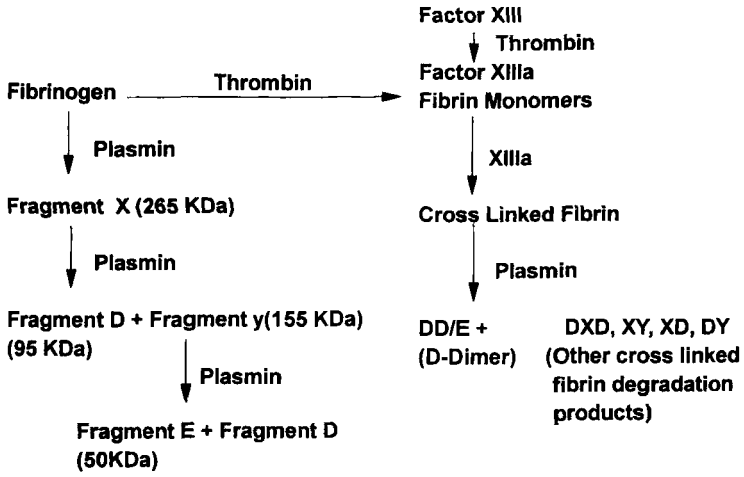

FIG. 9. Steps in the formation of fibrinogen–fibrin degradation products. The approximate molecular masses of fibrinogen degradation products in kilodaltons (KDa) are indicated.

of t-PA for fibrin is due to the presence of lysine binding sites on that chain. The active site of t-PA is located in the B chain. The binding of t-PA to fibrin followed by binding to plasminogen to form a ternary complex is a device to activate plasminogen selectively at the site of the thrombus, thus avoiding the activation of circulating free plasminogen, because in the absence of fibrin, t-PA is a poor activator of Glu-plasminogen. The release of t-PA by the vascular endothelium is mediated by a wide range of physiological stimuli varying from exercise and venous stasis to vasoactive substances such as desamino-8-D-arginine vasopressin (DDAVP), vasopressin (AVP), and catecholamines, to list a few (36, 37).

The urokinase-type plasminogen activator (u-PA or UK) is secreted mainly, although not exclusively, by the renal parenchymal cells. It is first synthesized as a 54-kDa single chain precursor molecule called prourokinase (scu-PA or pro-UK). The cleavage of the Lys-158/Ile-159 bond by factor XII, kallikrein, or plasmin results in the formation of the active form of the molecule called high-molecular-weight urokinase (HMW-UK). The HMW-UK has A and B chains connected by a disulfide bridge. The HMW-UK is further degraded by uroplasmin, among other enzymes in urine, to a 32-kDa low-molecular-weight form (LMW-UK).

The fibrinolytic activity of scu-PA is more efficient than that of either LMW-UK or HMW-UK. Apparently, fibrin within a clot neutralizes an inhibitor in plasma that normally hinders binding of scu-PA to plasminogen, thereby facilitating binding of scu-PA to Glu-plasminogen and its resultant activation (37, 38). A cell surface receptor for scu-PA has been implicated in the activation of plasminogen and the internalization and degradation of u-PA complexed to inhibitors (39).

In addition to the two types of endogenous extrinsic plasminogen activators such as t-PA and u-PA, other endogenous intrinsic activators of plasminogen are present. An example of such an activator is the coagulation factor XIIa, which can activate plasminogen either directly or indirectly through the activation of prekallikrein or factor XI, resulting in plasmin generation and fibrinolysis (37). Thus the so-called contact system consisting of proteins high-molecular-weight kininogen (HK), prekallikrein, and factor XII actually have a role in initiating fibrinolysis, in contrast to the traditionally held view that they were required to initiate coagulation (40). Binding of prekallikrein to HK on the endothelial cell surface results in its conversion to kallikrein. The latter can convert single-chain urokinase to two-chain urokinase, resulting in a 4.3-fold increase in the activation of plasminogen (40).

An exogenous plasminogen activator that has been used in clinical trials as a fibrinolytic agent is the 53-kDa single-chain polypeptide called streptokinase (SK). It forms a complex with plasminogen on an equimolar basis. The resulting 156-kDa streptokinase–plasminogen complex (plg–SK) converts Glu-plasminogen to Glu-plasmin (41).

3.2. Fibrinolysis Inhibitors

Specific inhibitors are present in plasma for the inactivation of plasmin and plasminogen activators. α_2-Antiplasmin and α_2-macroglobulin are inhibitors of plasmin. Several types of plasminogen activator inhibitors (PA inhibitors) are present (42). The endothelial cell–type plasminogen activator inhibitor 1 (PAI-1) is synthesized by endothelial cells and hepatocytes. It is also located within the α-granule of platelets. Upon release by endothelial cells or hepatocytes, PAI-1 is deposited in the cell substratum attached to the extracellular matrix, where it is apparently stabilized by complex formation with vitronectin (37, 43). Virtually more than 95% of circulating t-PA is complexed with PAI-1. A large interindividual variation ranging from 0.0 to 1.3 nM is seen in the plasma concentration of PAI-1 in healthy individuals (42). PAI-1 can inhibit both single- and two-chain t-PA and two-chain u-PA.

Plasminogen activator inhibitor type 2 (PAI-2) is present in human placenta and monocytes (37, 42). The single-chain inhibitor molecule (sPAI-2) inhibits both two-chain u-PA and two-chain t-PA. Its inhibition is 10 times greater for two-chain u-PA compared with two-chain t-PA and it inhibits single-chain t-PA only minimally (37). Plasma concentrations of PAI-2 up to 2 μM have been observed in the third trimester of pregnancy (42).

Other plasminogen activator inhibitors are PAI-3, which is believed to be identical to the activated protein C inhibitor, and proteinase nexin 1, found in the renal epithelial cells, cytosol of fibroblasts, and cardiac myocytes (37, 42, 44, 45).

Kallikrein, which activates prourokinase, is inhibited by complement I^1 esterase inhibitor ($C^1$1-INH).

4. Anticoagulant Therapy

4.1. Conventional Heparin

Heparin has been used as an anticoagulant for a variety of clinical conditions ranging from the treatment of myocardial infarction to venous thrombosis (46, 47). It does not cross the placenta. Hence, it lends itself for use as an anticoagulant in pregnancy (48). Because of its mode of clearance from plasma by receptor-mediated uptake by macrophages and endothelial cells and also by the kidney, a linear relationship between the dose used in the therapeutic range and the anticoagulant effect cannot be predicted (49). Conventional or unfractionated heparin has a molecular mass ranging from 3 to 30 kDa with a mean of 14 kDa (49). In addition to its anticoagulant effect, it has a wide range of biological effects including binding to human monocytes, platelet factor 4 (PF4) and other chemokines, and histidine-rich glycoprotein (50, 51). Not only is the bioavailability of conventional heparin decreased by interactions with substances such as PF4 but also its size is a hindrance to its efficient absorption when administered subcutaneously. The interaction of PF4 with heparin can also result in a low platelet count (heparin-Induced thrombocytopenia). The bioavailability of heparin is very high when 30,000 units of heparin is administered by continuous intravenous infusion (49).

4.2. Low-Molecular-Weight Heparins

The limitations of conventional heparin have led to the introduction of low-molecular-weight heparin fractions ranging in size from 1 to 10 kDa with a mean of 4 to 5 kDa (49). These low-molecular-weight heparins (LMWHs) are prepared from conventional heparin either by chemical methods ranging from treatment with nitrous acid to peroxidative cleavage or by enzymatic methods such as the use of heparinase. Because only 25–50% of the various LMWHs contain more than 18 saccharide units, they act primarily by binding to ATIII through the pentasaccharide sequence and inhibit factor Xa, with less effect on thrombin. In contrast, unfractionated heparin with more than 18 saccharide units has equal anti-Xa and antithrombin activity (49).

The increased bioavailability of LMWHs together with their limited interactions with platelets and other plasma proteins lends itself to once-a-day dosing, in contrast to two or more injections needed with conventional heparin. Thus, unlike conventional heparin, which needs frequent monitoring with the activated partial

thromboplastin time (APTT) assay, LMWH levels do not require daily monitoring (52). LMWHs have been proved to be effective in the treatment of deep venous thrombosis (53).

4.3. Oral Anticoagulant Therapy

4-Hydroxycoumarin compounds are vitamin K antagonists. Vitamin K is required for the conversion of glutamyl to the γ-carboxyglutamyl group (γ-carboxylation) in vitamin K–dependent clotting factors such as factors II (prothrombin), VII, IX, and X and the natural anticoagulant proteins C and S. The reduced form of vitamin K, KH_2 a hydroquinone, is involved in the catalysis of the carboxylase enzyme in the presence of carbon dioxide and molecular oxygen. In the initial step of the reaction, KH_2 is oxidized to vitamin K epoxide. Two other enzymes, vitamin K epoxide reductase and vitamin K quinone reductase, are implicated in the continual supply of KH_2 to maintain the γ-carboxylation reaction. Vitamin K epoxide reductase regenerates vitamin K from vitamin K epoxide. Vitamin K (the quinone form) is reduced by vitamin K quinone reductase to regenerate KH_2 required for the γ-carboxylation reaction. γ-Carboxylation is a prerequisite for calcium binding of vitamin K–dependent coagulation proteins and subsequent anchoring to the platelet phospholipid surface (54).

4-Hydroxycoumarin compounds such as warfarin sodium (Coumadin), phenprocoumon, and acenocoumarol function by inhibiting vitamin K epoxide reductase and possibly also vitamin K quinone reductase (55). An anticoagulant effect is achieved by the inhibition of vitamin K–dependent coagulation factors. Oral anticoagulant therapy, monitored by the prothrombin time (which is sensitive to deficiency of factor II, VII, and X), has been widely used for the treatment of a wide range of clinical conditions ranging from deep vein thrombosis to myocardial infarction (56). Because the oral anticoagulant drugs cross the placenta, they should not be used during the first trimester of pregnancy and, if possible, should be avoided through the duration of pregnancy (56).

The risk of bleeding is related to the intensity of oral anticoagulant therapy, which can be reduced by decreasing the dose of the oral anticoagulant. Serious bleeding is encountered with a combination of high-dose aspirin at more than 1 g/day and high-intensity warfarin therapy (56). The inhibitory effect of aspirin on platelet function contributes to the risk of bleeding.

The optimal therapeutic range for oral anticoagulant therapy as determined by prothrombin time is expressed in terms of the international normalized ratio (INR). The INR is the prothrombin time ratio (patient's prothrombin time divided by the mean of the normal prothrombin time) that would have been obtained had a World Health Organization reference thromboplastin preparation been used to determine the prothrombin time. The sensitivity of the various reagents (thromboplastins) used to determine the prothrombin time is related to the international sensitivity

index (ISI), which is a measure of the slope of the line (c) when INR on a log scale is plotted versus the patient's prothrombin time ratio (PT ratio) obtained with a specific thromboplastin. Thus INR = (PT ratio)^c, where c = ISI. Reagents with a lower ISI are more sensitive. Maintenance of less intense oral anticoagulant therapy is indicated by an INR range of 2.0 to 3.0, whereas a more intense therapeutic regimen corresponds to an INR range of 2.5 to 3.5 (56).

4.4. Thrombin Inhibitors

The most potent thrombin inhibitor is hirudin, originally isolated from the salivary glands of the medicinal leech *Hirudo medicinalis.* Its inhibition constant is in the femtomolar (10^{-15} M) range (57). It is a 65-amino-acid tyrosine-sulfated single-chain polypeptide. Recombinant hirudin differs from native hirudin by the absence of the sulfate group on tyrosine 63 (Tyr-63) and is referred to as desulfato hirudin. The loss of this sulfate group reduces the thrombin inhibitory potency by 10-fold.

Both the amino and carboxy terminal regions of hirudin are involved in the interaction with thrombin. The amino terminal region of hirudin binds to the apolar binding site of thrombin. The highly acidic carboxy terminal region of hirudin reacts with the anion binding exosite on thrombin that is needed for the binding of thrombin to fibrinogen. The interaction of hirudin with thrombin is a two-step process. Initially, an ionic interaction takes place that is diffusion controlled. Subsequently, the thrombin–hirudin complex rearranges itself to effect tight binding (58). Indeed, hirudin has been visualized as wrapping itself around the thrombin molecule, thus blocking the various sites responsible for the multiple functions of thrombin (59).

The coupling of hirudin to polyethylene glycol (PEG) increases its half-life. PEG–hirudin is also less susceptible to proteolytic degradation (60).

Carboxy terminal portions of hirudin corresponding to amino acids 53 to 64 have been synthesized to bind the anion-binding exosite of thrombin. One such compound, called Hirugen, was originally designed to inhibit fibrinogen binding to thrombin, leaving the catalytic site free for interaction with ATIII either alone or in the presence of heparin (58).

Bivalent inhibitors of thrombin have been synthesized to bind the anion-binding exosite and active (catalytic) site of thrombin simultaneously. By coupling the carboxy terminal fragment of hirudin to a tripeptide (D-Phe-Pro-Arg) by including a spacer molecule, both the anion exosite and the catalytic site are blocked. An example of such a molecule is Hirulog, which has 20 amino acids and has a K_i of 2 nM (61). Its ability to block the active site has been questioned, since thrombin has been shown to cleave the Arg–Pro bond of Hirulog slowly *in vivo* (58). In addition to hirudin and hirudin-like compounds, three other classes of site-directed thrombin inhibitors deserve mention.

Synthetic heterocyclic and modified amino acid derivatives have been grouped in a class of thrombin inhibitors called peptidomimetics. An example of such a compound is argatroban, with a molecular mass of 532 Da. It blocks thrombin's active catalytic site by binding to the adjacent apolar binding site. This selective reversible inhibitor of thrombin has a K_i of 19 nM and blocks thrombin's role in coagulation and fibrinolysis (62).

A group of peptide derivatives such as peptide arginals and boronic acid peptide derivatives belong to another class of reversible thrombin inhibitors. One such inhibitor is PPACK (D-Phe-Pro-Arg chloromethyl ketone), which functions as a powerful irreversible thrombin inhibitor by alkylating the histidine residue at the catalytic site of thrombin (58). It, however, is unstable in neutral solution, as it undergoes cyclization and inactivation. However, the D-methyl derivative of D-Phe-Pro-Arg-H (D-Mephe-Pro-Arg-H) called efegatran, with a molecular mass of 515 Da, is a stable selective reversible inhibitor of thrombin with a K_i of approximately 100 nM. The basic amino terminus in this compound is responsible for promoting the specificity toward thrombin (63).

DNA- and RNA-derived oligonucleotides (either double- or single-stranded DNA or single-stranded RNA) can bind and inhibit thrombin. These oligonucleotides are referred to as aptamers (64). Some of the RNA-based aptamers have a high affinity for thrombin (65). Of special interest is a heterogeneous compound called defibrotide, which consists of several single-stranded DNA fragments with a molecular mass ranging from 2000 to 30,000 Da. Its antithrombotic effects may apparently be related to its ability to increase cAMP levels and the release of tissue factor pathway inhibitor (TFPI), thus interacting with platelets and endothelial cells (66, 67). Table 2 presents a list of thrombin inhibitors together with their properties.

Direct thrombin inhibitors such as hirudin, Hirulog, the peptide aldehyde efegatran, and peptidomimetic compound argatroban have undergone clinical trials. Their application in the prevention and treatment of deep vein thrombosis contin-

TABLE 2
THROMBIN INHIBITORS

Inhibitor	Approximate molecular mass (Da)	Inhibition constant (K_i)
Hirudin	7000	Femtomolar range
Hirulog (bivalent inhibitor)	20 amino acids	2 nM
Argatroban (peptidomimetics)	532	19 nM
Efegatran (peptide arginals)	515	100 nM
Defibrotide (aptamers: DNA-or RNA-derived nucleotides)	Single-stranded DNA fragments ranging from 2000 to 30,000	nM range

ues to be examined. Their obvious application in heparin-compromised patients with heparin-induced thrombocytopenia rests on the outcome of several clinical trials that are in progress.

4.5. Platelet Inhibitors

The widely used platelet inhibitor aspirin or acetylsalicylic acid, by acetylating the enzyme cyclooxygenase, inhibits platelet function by preventing the formation of thromboxane A_2 and the synthesis of prostaglandin I_2 (PGI_2) (68). Aspirin has been used in combination with other antiplatelet agents such as ticlopidine, which inhibits ADP-induced platelet aggregation (69).

A short-acting platelet inhibitor called dipyridamole functions by maintaining a high level of cAMP within the platelets by inhibiting the enzyme phosphodiesterase, which would otherwise degrade cAMP. It also raises the adenosine concentration in plasma by decreasing its cellular uptake and degradation (70).

Prostacyclin and its analogues also function by increasing the level of platelet cAMP, presumably by activation of the enzyme adenyl cyclase. A chemically stable analogue of prostacyclin called Iloprost has been effective in preventing consumption of platelets (71).

Drugs targeting specific sites of platelet activation have been developed. Drugs that act as glycoprotein IIB/IIIa receptor antagonists target the RGD (arginine–glycine–aspartic acid) recognition site involved in the binding of fibrinogen. Several compounds, including monoclonal antibodies, have been developed to target GPIIb/IIIa receptors, and thus effectively prevent platelet aggregation (72).

Drugs that target other sites of platelet action include thromboxane synthetase inhibitors, serotonin or 5-hydroxytryptamine (5-HT_2) receptor blockers, and thromboxane A_2 receptor blockers, in addition to cyclooxygenase inhibitors and prostaglandin analogues.

5. Clinical Aspects

5.1. Molecular Defects

In recent years the molecular and genetic basis for coagulation abnormalities has been extensively studied. The congenital deficiency of the platelet glycoprotein Ib/V/IX complex (GPIb/V/IX), which is a receptor for von Willebrand factor (vWF) and thrombin, has been implicated in the Bernard–Soulier syndrome (BSS). The characteristics of this rare autosomal recessive genetic disorder are the presence of abnormally large platelets noted on peripheral blood smears, prolonged bleeding time, and thrombocytopenia. Mutations in the glycoprotein Ibα

coding sequence have been reported in BSS (73, 74). In one patient a dinucleotide deletion in the GPIbα gene resulted in deficiency of GPIα on the surface of the platelet membrane (75). This patient was homozygous for the deletion mutation. Heterozygous mutations with one allele showing an insertion of a single base and another allele accounting for a deletion of a single base were reported in another BSS patient, resulting in deficiency of GPIbα (75). Mutations in the GPIX gene contributing to the absence of GPIX have been reported (76). A variant form of BSS has been described in which GPIb/V/IX complex is present, with impairment, however, in the aggregation of platelets in response to ristocetin (77). In this variant form of BSS, a point mutation in the platelet GPIbα leucine tandem repeat region has been reported (77). Deletion in a chromosomal region resulting in a deficiency of GPIbβ has been reported in a BSS patient (78). Thus, the sequential assembly to the platelet surface membrane of GPIbβ, GPIX, GPIbα, and GPV to produce the GPIb/V/IX complex can be compromised by a range of molecular defects in BSS (75).

A number of abnormalities in the fibrinogen molecule have been described in the literature and are referred as dysfibrinogenemias. A mutation from arginine to cysteine at residue 14 of the Bβ chain has been noted in fibrinogen Chirstchurch II and fibrinogen Seattle. As a result of this mutation, the release of fibrinopeptide B (FPB) by thrombin is impaired and thrombin and reptilase clotting times are prolonged (79). The deletion of the Bβ 9–72 region results in a molecule called fibrinogen New York I, which is also characterized by prolonged thrombin and reptilase times (80). An abnormal fibrinogen called fibrinogen Fukuoka II is characterized by the substitution of glycine for cysteine at residue 15 of the Bβ chain (79). The release of FPB by thrombin is impaired in this molecule with the abolition of fibrin monomer repolymerization under physiological conditions. Thrombin and reptilase clotting times were also prolonged in fibrinogen Fukuoka II. In contrast, similar substitution of glycine for cysteine at residue 15 of the Bβ chain (as in fibrinogen Fukuoka II) reported in fibrinogen Ise resulted in a prolonged thrombin time but a normal reptilase time (81). This discrepancy between fibrinogen Fukuoka II and fibrinogen Ise is not clear, but presumably additional mutations may be involved in the fibrinogen Ise molecule (79).

Mutations in the antithrombin (AT) gene have been the basis of AT deficiency. In type I AT deficiency the level of circulating protein molecule and activity are reduced to nearly 50% of normal. Molecular defects in the AT gene resulting in gene deletions at specific DNA sequences may be the basis for type I AT deficiency predisposing such patients to thrombosis (82).

Eighty distinct mutations in type I AT deficiency, ranging from single nucleotide substitutions, to deletion or insertion of a small number of nucleotides of 22 base pairs or less, to major deletions of either a part of or the entire AT gene, have been recognized (83).

Type II AT deficiency comprises mutations that affect the interaction of AT with

its target protease enzymes (83). AT mutations can also compromise its binding efficiency to heparin (83).

Two point mutations in the prothrombin gene have been described in a patient with a severe bleeding tendency (84). By far the most widely described molecular defect is in the factor V molecule. Replacement at nucleotide 1690 of guanine by adenine in the factor V gene results in a change from arginine to glycine at position 506. This mutation has been referred to as factor V Leiden (85). This mutated factor V is resistant to inactivation by activated protein C (APC). APC resistance is rare among Asians and absent from native Africans, Australians, and Americans, but its incidence in the European population is relatively high (85, 86). APC resistance has been noted in 21 to 60% of thrombotic patients as well as 3 to 10% of healthy European subjects. Data on patients who are homozygous for factor V Leiden mutation indicate that they have far less severe thrombotic complications than patients with mutations responsible for homozygous protein C and protein S abnormality (87). One explanation is that the factor V Leiden mutation affects only one of the three cleavage sites of activated factor V (Va). Thus, in the absence of cleavage at position 506, factor V inactivation by protein C is not abolished but is 10-fold less compared with normal factor V. Furthermore, in contrast to patients with protein C or protein S abnormalities, in patients with factor V Leiden mutation, the inactivation of factor VIII is unimpaired (87).

A G (guanine) to A (adenine) transition in position 20210 of the prothrombin gene (prothrombin 20210 A allele) has been described that leads to increased prothrombin concentrations and, in turn, increased risk for venous thrombosis (88). This G-to-A transition that occurs in the 3′ untranslated region of the gene may not be the only reason for increased prothrombin concentrations because apparently only 25% of individuals with prothrombin concentrations greater than 115% carry the prothrombin 20210 A allele (88).

More than 160 different mutations have been described for the protein C gene, which has nine exons ranging in size from 53 to 587 base pairs, separated by 8 introns ranging in size from 92 to 2668 base pairs (89). These mutations can result in a defective or even absent protein C molecule. Mutations that lead to reduced amounts of protein C molecule without accompanying evidence for an abonormal protein C molecule in the circulation are characterized as causing type I deficiency. Type II deficiency is represented by mutations that lead to the production of more or less normal amounts of defective protein C molecule (89).

Homozygous protein C deficiency can cause life-threatening thrombotic syndromes immediately after birth.

Individuals with heterozygous protein C deficiency are seven times more likely to be afflicted with venous thrombosis than normal individuals. A combination of protein C deficiency with a mutation in the factor V gene (factor V Leiden) carries a much greater risk for venous thrombosis than the presence of only one of these conditions (89).

The active protein S gene (*PROS1*) has 15 exons. Approximately 70 different mutations have been identified in the protein S gene (*PROS1*) (90). Screening of consecutive patients with low protein S levels with unexplained thrombosis uncovered a mutation in the protein S gene in 70% of cases (type I deficiency) (91). Whereas mutations in patients with type I deficiency were distributed throughout the coding sequence of the protein S gene, patients with type III protein S deficiency had mutations confined to a particular domain (sex hormone–binding globulin homologous domain) within exons XII, XIII, and XIV (91). Of the protein S gene mutations associated with type III deficiency, 82% involve a single mutation substituting serine 460 for proline resulting in abnormal binding of the altered protein S molecule to the complement C4b-binding protein and low free protein S levels (91). However, half of the patients who had the serine 460 to proline mutation in the protein S gene and unexplained thrombosis also had a defect in either the factor V gene (factor V Leiden) or the protein C gene, suggesting that a combination of genetic alterations may be operative in triggering the thrombotic episode (91).

Without going into the molecular defects in other coagulation factors, suffice it to say that our current understanding of mechanisms leading to the clinical expression of thrombosis and bleeding has been enhanced by the knowledge of such mutations.

5.2. Laboratory assessment of Hemostatic Activation

Sensitive molecular markers can be used for the assessment of activation of coagulation that has occurred *in vivo*. The activation peptides released from the zymogen molecules in the coagulation cascade have a relatively longer half-life than the corresponding enzymes. Hence, the measurement of these markers allows an assessment of *in vivo* activation of coagulation. The measurement of prothrombin fragment 1.2 (PF 1.2) formed when prothrombin is converted to thrombin by the action of factor Xa during the activation of coagulation is a measure of thrombin generation in vivo (92). PF 1.2 provides an assesssment of the extent of ongoing thrombosis. Thrombin can complex with antithrombin-III. The measurement of the thrombin–ATIII complex (TAT) provides an assessment of thrombotic disorders in which the level of TAT would be expected to be increased (92). In contrast, the measurement of fibrinopeptide A, which is formed when free thrombin acts on fibrinogen to generate fibrin, is useful in uncovering hypercoagulable states in which increased thrombin generation is not necessarily associated with increased thrombin activity (92). Deficiency of AT-III, protein C, and protein S predisposes to hypercoagulability.

The measurement of plasminogen activator inhibitor-1 (PAI-1), which complexes with tissue plasminogen activator (t-PA) and thus affects the ability of the latter to activate fibrinolysis, is useful in the assessment of fibrinolytic disorders (93). The complex formed by the fibrinolytic enzyme plasmin with its inhibitor

α_2-antiplasmin (PAP) is useful in the assessment of fibrinolytic activation, with increased levels seen not only in patients with primary fibrinolysis but also in those who have disseminated intravascular coagulation (DIC) (93).

Measurement of a peptide released by the action of plasmin on both cross-linked and non–cross-linked fibrin, which is called Bβ 15–42, is useful for the assessment of fibrinolytic activity (94).

By far the most widely measured marker of hemostatic activation is D-dimer, which is a product formed by the action of plasmin on cross-linked fibrin (95). D-dimer levels in plasma are generally elevated in DIC. The consumption of platelets and coagulation proteins as a result of thrombin generation leads to the deposition of fibrin thrombi at multiple organ sites. This triggers fibrinolysis with an increase in the formation of fibrin degradation products, which can cause bleeding at multiple sites. Because DIC can have a variety of causes and may coexist with systemic fibrinolysis, such as in pulmonary embolism or deep vein thrombosis, the d-Dimer test is not specific for DIC (95).

Release of markers bound to the endothelial cell such as thrombomodulin is indicative of vascular damage. Increased levels of soluble thrombomodulin in plasma are diagnostic (93). Other endothelium-derived markers such as 6-keto-prostaglandin $F_{1\alpha}$, which is a metabolite of prostacyclin, are useful in the assessment of endothelial function, with lower levels indicative of inability to synthesize this marker due to defective or damaged endothelium through plaque formation (93).

In thrombotic episodes accompanied by activation of complement, C_1-esterase inhibitor is consumed by binding to the C_1-esterase that is formed, leading to an increase in the C_1-esterase inhibitor complex (93).

The macrophage-derived tumor necrosis factor level in plasma is elevated in thrombotic conditions associated with malignancy (93). Likewise, mast cell-derived platelet-activating factor levels in plasma are increased in thrombotic conditions accompanied by mast cell activation (93).

A specific immunoassay for measuring two-chain factor VII_a levels in plasma has been developed to identify activation of factor VII in patients with acute coronary syndromes suchs as myocardial infarction and unstable angina (12). Because regulation of factor VII_a is believed to be mediated by tissue factor pathway inhibitor (TFPI), its measurement is also useful in assessing thombotic and cardiovasular disorders. Because TFPI is released by heparin, its measurement is also useful in assessing the efficacy of heparin and endothelial cell function (93).

5.3. Antiphospholipid Antibodies

Antiphospholipid antibodies include lupus anticoagulants (LAs) and anticardiolipin (aCL) antibodies. Lupus anticoagulants are immunoglobulins that are characterized by their ability to inhibit phospholipid-dependent coagulation assays. In contrast, aCL antibodies are measured in an enzyme-linked immunosorbent assay

(ELISA) in which the antibodies bind to a target lipid, which is usually cardiolipin. Platelet antibodies directed to phosphatidylserine and phosphatidylinositol are referred to as anticardiolipin antibodies and their presence is characterized as anticardiolipin antibody–thrombosis syndrome (96).

Whereas β2 glycoprotein 1 (β2-GPI) is the target of anticardiolipin antibodies, prothrombin is the antigen for most lupus anticoagulants. Both these antibodies are risk factors for both venous and arterial thrombosis. In addition, complications such as thrombocytopenia and recurrent miscarriages are manifestations of the so-called antiphospholipid syndrome (97).

A phenomenon that is induced *in vitro* upon collection of blood in ethylenediaminetetraacetic acid (EDTA) is due to the presence of antibodies in blood that are reactive to platelets. These antibodies become reactive to antigens such as the glycoprotein IIb/IIIa complex that is hidden within the platelet membrane. EDTA exposes these antigens upon chelating calcium. The exposed platelet antigen becomes modified at low temperature. Hence, this EDTA-induced phenomenon causes platelet antibodies to agglutinate platelets at low and room temperature but not at 37°C, since the glycoprotein IIb/IIIa complex is dissociated at the higher temperature. This *in vitro* phenomenon is called EDTA-induced pseudothrombocytopenia (98).

5.4. The Study of Platelet Function by Flow Cytometry

Whereas the *in vivo* activation of platelets can be evaluated by the measurement of contents of platelet granules released during activation, such as platelet factor 4 and β-2 thromboglobulin, by immunoassay, flow cytometry permits the measurement of changes in cell surface markers. In clinical conditions such as acute myocardial infarction, stroke, and peripheral vascular disease, measurement of platelet activation by flow cytometry can provide useful information, as well as platelet defects and monitoring of treatment with GPIIb-IIIa receptor antagonists (99). Platelet activation can be studied using as little as 2 μl of whole blood. The following platelet activation markers are examples of markers that can be studied by flow cytometry (99).

1. CD41/61 representing the GPIIb-IIIa complex, which is a receptor for fibrinogen, von Willebrand factor, fibronectin, and vitronectin. This complex is essential for platelet aggregation.
2. CD62P or P-selectin, which is a component of the platelet α granule membrane of resting platelets. It is expressed on the platelet surface membrane only after α granule secretion. P-selectin mediates the adhesion of activated platelets to neutrophils and monocytes.
3. CD36 or GP IV is a marker that is expressed on both resting and activated platelets. Labeled antibodies can be used that exhibit increased binding to activated platelets.

4. CD42 represents the GPIb-IX-V complex, which is a receptor for von Willebrand factor that is critical for the adhesion of platelets to damaged blood vessels. This marker is down-regulated on activation of platelets.

Of the four markers just mentioned, the expression of P-selectin on the activated platelets is the most widely studied. However, CD62P may not be the ideal marker for detection of circulating degranulated platelets, because they may rapidly lose their surface P-selectin but yet may continue to function. The decrease in the platelet surface expression of the GPIb-IX-V complex (CD42) perhaps represents a more sensitive marker of *in vivo* platelet activation. Apparently platelet activation related to strenuous exercise is more readily detected using CD42 than other markers (99).

6. Nonanalytical Variables Affecting the Laboratory Evaluation of Hemostasis

Many of the coagulation factors measured by global coagulation tests have limited stability, and the time and temperature of storage of sample will affect their measurements. Concepts of analyte stability and half-life in plasma extend to markers measured by immunoassay. Markers of platelet activation are affected by artifactual activation *in vitro* upon collection of the blood specimen. This section will highlight some of the nonanalytical variables that, if uncontrolled, can lead to spurious results and thus affect the interpretation of laboratory data.

6.1. Preanalytical Variables Affecting Global Coagulation Tests

Preanalytical variables that affect global tests for coagulation such as prothrombin time (PT) and activated partial thromboplastin time (APTT) include the choice and concentration of anticoagulant, anticoagulant-to-blood ratio, pH, concentration of divalent cations, hematocrit, and storage temperature, to mention a few.

As to the choice of anticoagulant, citrate is preferred to oxalate, because factor V is more stable in citrate than in oxalate. In addition, citrate rapidly complexes with calcium, forming a soluble complex, in contrast to the slow formation of the insoluble complex of calcium with oxalate (100).

Two concentrations of citrate have been routinely used as anticoagulant for tests such as PT and APTT (either 0.129 or 0.105 M). The effective molarity depends on whether the dihydrate or the anhydrous citrate salt was used in preparation of the citrate solution. A 3.2% solution of sodium citrate prepared using the dihydrate salt is 0.105 M. However, the molarity of a 3.2% solution of sodium citrate prepared with the anhydrous salt is 0.124 M. Similarly, a 3.8% solution of sodium cit-

rate prepared with the dihydrate salt is 0.129 M, whereas a 3.8% solution of anhydrous sodium citrate is 0.147 M (100).

The concentrations of citrate (0.105 or 0.129 M) cannot be interchanged by a laboratory that is reporting results of prothrombin time in INR units for patients who are receiving oral anticoagulant therapy. INR values are generally higher when a responsive PT reagent is used, such as a recombinant thromboplastin that is similar in sensitivity to World Health Organization thromboplastin (ISI = 1) (101). Using responsive PT reagents, the differences in INR between the two concentrations of citrate can vary from 0.7 to 2.7 INR units (101).

Errors will also be introduced in the calculation of INR when a laboratory that routinely uses 0.105 M citrate occasionally also analyzes a specimen collected with 0.129 M citrate but uses the mean of the normal range obtained with the former concentration in the calculation of the PT ratio (101).

For global coagulation tests (PT and APTT) a 1:9 ratio of anticoagulant (sodium citrate, either 0.129 or 0.105 M) to blood is traditionally used. However, if less blood is collected than the nominal volume required to maintain a 1:9 ratio, the effective concentration of citrate increases, thus seriously affecting the APTT result. For instance at a 1:7 ratio of anticoagulant to blood there is a significant increase in the APTT result compared with the result obtained using the nominal anticoagulant-to-blood ratio of 1:9. The effect of the anticoagulant-to-blood ratio on PT is noticeable only when the ratio reaches 1:4.5, which occurs when the blood collection tube is filled to just less than half of its nominal volume (100).

The pH of blood is a variable, and loss of carbon dioxide and the resultant change in pH can be avoided by leaving the stopper of the blood collection tube in place during processing steps such as centrifugation and storage.

The biphasic effect of divalent cations such as calcium on APTT is well recognized. Thus, whereas the addition of 0.025 M calcium chloride to citrated plasma has no effect on the APTT result, both higher and lower calcium chloride concentrations such as 0.065 or 0.004 M can artifactually elevate the APTT result (100).

Spurious results can also be obtained as a result of zinc ion contamination, with lower levels (10 mg/L) abolishing the effect of heparin on the APTT result by producing a normal APTT result for a heparinized sample. At higher zinc levels (100 mg/L) the APTT results obtained for both heparinized and nonheparinized samples are increased, thus confounding the clinical picture (100). Zinc ion contamination between 30 and 100 mg/L can also artifactually increase the PT value (100).

The increase in hematocrit on decreasing the plasma compartment has an effect of concentrating citrate and thus effectively increasing its concentration in plasma. The extra citrate present in the plasma compartment will complex with calcium added during PT and APTT measurements, thereby artifactually elevating both PT and APTT, because of insufficient calcium. This effect can be minimized by adjusting the citrate concentration in accordance with the hematocrit value by us-

ing empirical formulas or by using a higher dilution of anticoagulant to blood, such as a 1:19 ratio (102).

The storage temperature of a specimen influences the results of coagulation tests. Generally, when plasma is stored in contact with cells and maintained at 4°C for up to 7 hours, the PT is not artifactually shortened (103). However, beyond 7 hours factor VII is activated, thereby shortening the PT (104). At room temperature (25°C), provided the specimen container is well stoppered, the PT has been shown to be stable for up to 48 hours (104). Even freezing plasma at −20°C and at −70°C did not activate factor VII. Both PT and APTT results were shown to be stable in plasma frozen at −20°C for 10 days and at −70°C for 21 days (104).

Factors II, VII, and X are stable in plasma maintained under refrigeration for up to 6 hours. Plasma refrigerated for 6 hours and subsequently frozen at −20°C and at −70°C showed no deterioration in the levels of these factors for up to 14 days. Factor V was stable for 6 hours when plasma was stored at 4°C. However, 20% of the activity of factor V was lost in plasma stored frozen at −20°C for over 7 days (104). Even in samples stored frozen at −70°C, 10% of the activity of factor V was lost after 7 days (104).

The least stable factor is factor VIII, with 10% of activity lost in 4 hours when plasma is stored at 4°C. Even in plasma stored frozen at −20°C for 3 days, 20% of the factor VIII activity is lost (104).

Some residual platelets still remain in blood centrifuged for 15 minutes at the high speeds attainable in ordinary laboratory centrifuges (1800 × *g*), since the thrombin clotting time in plasma frozen for 48 hours and thawed to room temperature prior to testing is shortened by 10% (104). However, in blood centrifuged at 11,000 × *g* there is very little platelet contamination. Tests such as the PT, APTT, ATIII, fibrinogen, D-dimer, and dilute Russell viper venom test, the last named test used for the detection of lupus anticoagulants, were reported to be unaffected by centrifugation at such high speeds (105).

6.2. Minimizing *in Vitro* Platelet Activation

Measurement of one of the many constituents within the platelet α-granule which is released upon activation of platelets can be used to assess *in vivo* platelet activation. When platelets are activated the contents of α granules such as platelet factor IV (PF_4), beta thromboglobulin (βTG), platelet-derived growth factor (PDGF), thrombospondin (TSP), fibronectin, fibrinogen, albumin, and factor VIII–related von Willebrand factor polymers (VIII:vWF) are released. As noted previously, platelet activation also results in the release of contents of platelet dense granules such as ADP and serotonin (8).

Various additive mixtures have been proposed for inclusion in blood collection tubes to prevent *in vitro* platelet activation. One formulation involves the use of a mixture of acid-citrate-dextrose (ACD, 1:5 dilution), 30 μM acetylsalicylic acid

(aspirin), and 1 μM prostaglandin E_1 (PGE_1). The rationale is that aspirin, by acetylating and inhibiting the fatty acid cyclooxygenase enzyme in platelets, will inhibit release of contents of the platelet α-granules and ADP-induced platelet aggregation by preventing the formation of thromboxane A_2 (106). However, because of the stability problems associated with PGE_1 and the need to use ethanol to dissolve aspirin and PGE_1, an alternative inhibitor of platelet aggregation and release has found application. This additive mixture is called CTAD (citrate, theophylline, adenosine, and dipyridamole) (107). The rationale for the utilization of CTAD is based on the maintenance of increased intracellular levels of cAMP, which is a potent inhibitor of platelet aggregation. Adenosine activates the enzyme adenyl cyclase leading to increased levels of cAMP. Whereas red blood cells are a source of free adenosine formed from adenine nucleotides, they also avidly take up the added adenosine, as a result of which the level of plasma adenosine is low. The uptake of adenosine by the red cell is inhibited by dipyridamole, thus permitting increased activation of adenyl cyclase by adenosine, resulting in increased cAMP levels, which, in turn, prevents platelet aggregation and release. The degradation of cAMP by the enzyme phosphodiesterase is inhibited by the theophylline and to a certain extent by the dipyridamole present in the CTAD mixture. Citrate, of course, by chelating calcium, functions as an anticoagulant (108).

The CTAD additive mixture has found application in the monitoring of heparin therapy by either the chromogenic substrate assay or the APTT and in the measurement of platelet markers such as P-selectin (CD62) by flow cytometry (108, 109).

In vitro platelet activation is dependent on the anticoagulant that is used for blood collection. In one study it was demonstrated that PF_4 levels in platelet-poor plasma isolated after incubation without any stimuli for 1 hour at 37°C were as follows: conventional heparin, 1180 ng/ml; hirudin, 469 ng/ml; citrate, 440 ng/ml; and EDTA, 217 ng/ml (110). EDTA appears to suppress platelet degranulation. PF_4 levels obtained with a low-molecular-weight heparin preparation called Fragmin were, however, comparable to those obtained with hirudin (110).

6.3. Preanalytical Variables Affecting Assessment of Fibrinolysis

Measurement of nonspecific fibrinogen–fibrin degradation products may be problematic in patients receiving heparin therapy. The conversion of residual fibrinogen to fibrin by thrombin (used together with soybean trypsin, a plasmin inhibitor for blood collection) will be slow in the presence of heparin, thus yielding spuriously high FDP values due to the remaining unconverted fibrinogen (111). This problem can be circumvented by incorporation of snake venom, also known as reptilase, which can rapidly convert any residual fibrinogen to fibrin even in the presence of heparin (111).

A plasmin inhibitor such as aprotinin used for blood collection, while effective in inhibiting activation of plasminogen by urokinase, is ineffective against the ac-

tivation of plasminogen *in vitro* by recombinant tissue plasminogen activator (rt-PA) (112). Hence, a thrombin inhibitor such as PPACK (D-phenylalanine-proline-arginine-chloromethyl ketone), together with an anticoagulant such as EDTA or citrate, is useful if rt-PA therapy is monitored by measurement of FDP (113). As PPACK is unstable in neutral pH, the blood specimen should be kept cold (4°C), centrifuged promptly to obtain plasma, and processed without delay or kept frozen until ready for analysis.

Some physiological variables influence the measurement of fibrinolytic activators and inhibitors. For instance, both t-PA and plasminogen activator inhibitor 1 (PAI-1) levels in plasma are subject to diurnal variation in a 12-hour period. Even in samples taken at the same time of day the coefficient of variation (CV) of measured PAI levels range from 8 to 143%! To account for this diurnal variation, blood samples spaced over several time intervals during a 24-hour period should be collected. Consumption of alcohol induces the PAI level in plasma. The half-life of t-PA is 360 seconds. However, in the presence of trauma or inflammation, when the PAI-1 level is expected to be elevated 10-fold, the half-life of t-PA is reduced to 36 seconds (114).

Smoking causes an acute increase in t-PA levels. Hence, subjects should refrain from smoking for at least an hour before collection of blood (114).

Measurement of free t-PA in plasma presents challenges in terms of preventing t-PA from complexing to PAI-1 released from platelets after blood collection. To dissociate any preformed t-PA–PAI-1 complex, the anticoagulant pH has to be close to 3.0. Even if blood is collected with an acidic anticoagulant, the blood pH will rise because of the powerful buffering action of hemoglobin. Thus, the pH of plasma has to be adjusted to 3.0 in order to dissociate the t-PA–PAI-I complex (115).

Molecular methods used to uncover mutations are subject to several variables. The anticoagulants used for blood collection can affect digestion with restriction enzymes and amplification reactions. The type of detergent used in cell lysis can affect amplification of DNA by inhibiting the DNA-amplifying enzyme such as the taq polymerase used in the polymerase chain reaction (116). The control of contamination is crucial in ensuring the quality of results obtained by molecular analysis (117).

Finally, with the use of direct thrombin inhibitors such as hirudin for anticoagulant therapy, laboratory tests such as APTT may not be sensitive enough to follow therapy.

An enzyme purified from venom from the snake *Echis carinatus* (Ecarin) can convert prothrombin to meizothrombin. The latter can to a certain extent convert fibrinogen to fibrin and activate platelets. Since hirudin inhibits meizothrombin as soon as it is formed, only after all the hirudin has complexed with meizothrombin can the additional meizothrombin generated convert fibrinogen to fibrin, resulting in clotting of the sample. Thus the Ecarin clotting time can be used to follow ther-

apy with direct thrombin inhibitors (118). Figure 10 illustrates the Ecarin-mediated formation of meizothrombin from prothrombin.

7. Strategy for the Systematic Examination of Thrombophilia

We will conclude this chapter on a personal note, sharing with you a scheme that has been instituted at the Kyushu University Hospital (119).

After clinical assessment of thrombosis based on clinical history and imaging techniques such as computed tomography, magnetic resonance imaging, scintillation analysis, and angiography, a series of basic laboratory tests are performed. These include platelet counts, prothrombin time, thrombotest, α_2-plasmin inhibitor activity, euglobulin lysis time, fibrin and fibrinogen degradation products, thrombin-antithrombin III complex, and α_2-plasmin inhibitor–plasmin complex. These basic laboratory tests are intended to exclude severe liver dysfunction, disseminated intravascular coagulation, and vitamin K deficiency as causes and also to evaluate antithrombotic therapy.

The initial laboratory diagnosis is arrived at after measuring the ATIII activity, anticoagulant activities of protein C and protein S, plasminogen, and fibrinogen and heparin cofactor II activity and detecting lupus anticoagulants. Plasma concentrations of aberrant factors are confirmed immunologically. In addition, the concentration of C4b-binding protein, protein C amidolytic activity, and progressive ATIII activity are determined. After a diagnosis of thrombophilia is made, genetic analysis is performed to uncover mutations in protein S, ATIII, protein C, and plasminogen genes to distinguish between inherited and acquired thrombophilia. When this scheme was used for 115 patients with venous, arterial, and small ves-

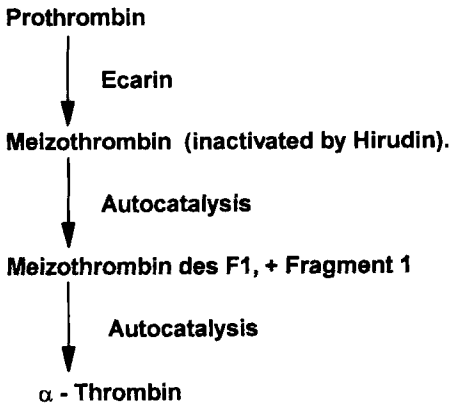

FIG. 10. Ecarin-mediated formation of meizothrombin from prothrombin.

sel thrombosis, approximately 40% of patients were shown to have some defect in the regulatory system of coagulation. Twenty-three of the 115 patients had decreased protein S activity, emphasizing the important role of protein S in the pathogenesis of thrombosis in the Japanese population. Decreased activity of any factor in the regulatory system of coagulation could be an etiological cause that induces thrombosis irrespective of whether the decreased activities of ATIII, protein C, protein S, and plasminogen were inherited or acquired. Thus the systematic assessment of thrombophilia was facilitated by employing this comprehensive scheme of laboratory tests (119).

8. Conclusion

Our concepts of coagulation and fibrinolysis are still evolving. The increasing use of molecular methods in the laboratory has uncovered new mutations and has facilitated the understanding of the genetic basis of hemostatic defects. With an increasing armamentarium of laboratory tests for studying defects in coagulation and fibrinolysis, the importance of recognizing nonanalytical variables that could affect the quality of laboratory results and, in turn, the interpretation of laboratory data should not be underestimated.

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TUMOR MARKERS: RECLASSIFICATION AND NEW APPROACHES TO EVALUATION

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1. Introduction

Tumor markers are tumor-associated analytes that are used in patients with known malignancy (carcinoma, sarcoma, lymphoma, etc., which have been diagnosed and confirmed by biopsy). Tumor marker *in vitro* diagnostic (IVD) tests are

*The views expressed herein are those of the author and do not necessarily reflect the views of the U.S. Food and Drug Administration.

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intended to detect or measure various analytes associated with tumors. A variety of tumor markers are proposed for three major intended uses: screening, diagnosis, and monitoring. For each intended use, performance characteristics need to be well established. The value of a marker depends heavily on two predominant performance parameters—sensitivity and specificity. These parameters must be established with respect to the intended clinical use of the marker. Therefore, it is important to balance the analytical/clinical sensitivity and the resultant claims for the test. The Food and Drug Administration (FDA) review and evaluation of a tumor marker test focuses on the intended use and the clinical utility of the marker. The sponsor must prove all specific claims. The data must support well-designed scientific and statistical protocols and clinical studies (1–3).

Tumor markers are technically defined as substances that can be measured in body fluids or tissue to diagnose presence of cancer, to predict prognosis, and to monitor therapy. These substances may be cellular or bound to cell surface membranes. They may be identified in intact cells by immunohistopathology or flow cytometry, or they may be released into the circulation and measured by a variety of testing systems. The clinical utility of tumor markers requires simultaneous availability of both the marker test and the treatment modalities. Also, the different tumor marker tests available can give different results for a variety of reasons, including poor calibration because of lack of common purified and certified calibrator materials, different antibody production and specifications, robustness features of immunoassays such as human antimouse antibodies (HAMAs), hook effects, and variations in reference ranges quoted in the literature. At the same time, tumor markers are the fastest changing area in the clinical laboratory specialty area and oncology. This chapter provides an overview of the current state of tumor marker testing for monitoring purposes and what to expect in the future.

2. Background

The FDA of the U.S. Department of Health and Human Services (DHHS) administers the regulatory controls for the Food, Drug, and Cosmetic Act of 1906 and the 1976 and 1990 amendments, which provide approval for commercial distribution of safe and effective medical devices. The 1976 amendments directed the FDA to regulate medical devices under control levels that are necessary to ensure safety and effectiveness. In order to achieve this task, the Medical Device Law under the amendments required the FDA to issue regulations placing all medical devices on the market at that time into one of three regulatory classes:

- Class I—General controls
- Class II—Special controls
- Class III—Premarket approval

Under the Medical Device Law, the FDA was directed to obtain a recommendation from an advisory panel to place each IVD product into one of the three categories (classes) depending on the regulatory control needed to provide reasonable assurance of safety and effectiveness (Table 1). In 1982, the final ruling, published under classification regulations 21 Code of Federal Regulations (CFR) (Part 866, Subpart G), contained Tumor-Associated Antigen Immunological Test Systems. Generally, all new tumor markers were placed in class III because of the advisory panel's concern about the highly serious consequences of false negatives or false positives for cancer patients. Tumor markers placed in class III needed to undergo extensive premarket review and evaluation. The regulation requiring the PMA is in section 515(h) of the Medical Device Law. For example, each manufacturer that made a new tumor marker testing system (i.e., carcinoembryonic antigen, cancer antigen 125) submitted a premarket approval application (PMA), which provided testing data to support their claim that their device is safe and effective (3).

The Safe Medical Devices Act of 1990, a major revision to the 1976 amendments, among other revised requirements provided two major mechanisms for bringing an IVD medical device to market: premarket notification and premarket approval. The act is administered by the FDA's Center for Devices and Radiological Health, of which the Division of Clinical Laboratory Devices (DCLD) is a part. The premarket notification process is used for devices that can be classified

TABLE 1
CLASSIFICATION OF DEVICES

Category	Levels of Control
Class I	General: Devices require the lowest level of regulation. These devices are subject to "general control" requirements, which include manufacturer registration, device listing, and Good Manufacturing Practices. Examples of class I IVD devices are immunoelectrophoresis and immunonephlometric devices intended for general-purpose use.
Class II	Special: Devices for which general control alone is insufficient to provide reasonable assurance of safety and effectiveness and for which sufficient information exists to establish special controls to provide this assurance. These devices require special controls in addition to general controls (e.g., guidelines, performance standards, postmarket surveillance). Until a special control is established by regulation, only general controls apply. Examples of class II IVD devices are C-reactive protein, rheumatoid factor, complement components, and tumor marker tests for monitoring purposes.
Class III	PMA: Devices in this class require the highest level of regulation, because the general and special controls are insufficient to provide reasonable assurance of safety and effectiveness. This must be demonstrated by laboratory testing and clinical studies. Examples of class III IVD devices are automated PAP cytology screening devices.

or reclassified as class I or II and can be compared to a legally marketed predicate device (substantial equivalence). The premarket approval process is used for brand-new devices that are in class III and can be evaluated for their proposed new clinical use. This chapter focuses on the premarket review and evaluation of tumor markers that are being reclassified in the class II category.

3. Reclassification

From a background perspective, reclassification of the tumor-associated antigen immunoassay system is governed by section 520(1) of the act, 21 U.S.C. 306j. Tumor-Associated Antigen Immunoassay Systems have been regulated as class III devices because, prior to the amendments, they were subject to an approved "new drug" application submitted under section 505(b) of the Device Amendment Act. Devices subject to approved new drug applications prior to the Medical Device Amendments of 1976 are known as "transitional devices" and are automatically placed in class III to ensure continuity of regulation. This classification has been modified to down-regulate some tumor markers to class II. In 1973, the FDA regulated Tumor-Associated Antigen Test Systems as licensed biologicals. Subsequently, the Medical Devices Amendments of 1976 designated "Tumor Markers as Transitional Devices" and placed them by statute in class III. This action was based on concerns that the clinical application of these markers was, as yet, unsubstantiated. Since 1976, the FDA has approved several specific types of serum tumor markers, such as carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), prostate-specific antigen (PSA), CA-125 (residual epithelial ovarian cancer), and soluble interleukin-2 (IL-2) receptor. A petition to reclassify these tumor markers was filed in 1995, proposing that all tumor-associated antigen tests used for monitoring be placed in class II. A review of the basic and clinical aspects of these devices indicated that the use of special controls could support adequate assurance of safety and effectiveness for these types of devices. For example, these tests can be placed into several broad categories:

- Oncofetal proteins, such as CEA and AFP
- Organ-specific antigens, such as PSA
- Monoclonal antibody–defined antigens, such as tumor-associated glycoproteins, CA-125 and CA 15-3
- Enzymes, such as prostate acid phosphatase

At the FDA, a major effort is under way to streamline the process by which medical devices are reviewed and evaluated. The FDA has made significant progress in its quest to provide review of medical devices in a more timely manner. In fact, the agency has completely cleared its backlog of 510(k) and PMA applications for medical devices and is now turning its attention to quicker, more efficient reviews

and reengineering of its device evaluation programs. As mentioned before, the Medical Devices Amendments of 1976 designated tumor marker tests as investigational new drugs (Transitional Devices), which were placed in class III. In 1995, a reclassification petition was filed with the FDA requesting that these devices be down-classified into class II. Data and information were presented to the FDA demonstrating that these devices, when intended for monitoring patients for recurrence of disease or for response to therapy, could be reclassified as class II devices. The FDA's Immunology Device Advisory Panel held an open public hearing in December 1995 and unanimously recommended that Tumor-Associated Antigen Test Systems intended for monitoring be considered as class II medical devices. The panel's recommendation was based on a guidance document and the existence of voluntary standards from the National Committee for Clinical Laboratory Standards (NCCLS) that can be used as special controls for these devices. It was further recommended that these documents serve as guidelines for the type of data and information that is needed for the FDA to review and evaluate 510(k) submissions for these devices, and together they serve as a special control for the reclassification. Therefore, the reclassification of these devices into class II now allows sponsors to submit premarket notification 510(k)s to the FDA instead of more complex and involved PMAs. It is anticipated that this guidance document will be revised to accommodate advances in science and medicine, the development of additional voluntary documents and standards, and the cumulative experience of both the FDA and sponsors with these submissions. The significance of these process changes will be presented in this article.

This generic reclassified device category is intended for use in clinical laboratories as an IVD test for the qualitative or quantitative measurement of tumor-associated antigen levels in serum, plasma, or other body fluids measured or detected by immunoassay technologies. This generic device category does not include tissue receptor assays, immunohistochemical stains, or direct tests for oncogenes of other genetic markers with a predisposition to development of certain cancers. Measurement of tumor-associated analyte levels in various body fluids can help in the monitoring of certain cancers. Monitoring the serum tumor marker is defined as assessing the progression of tumor growth or assessing the response of a tumor-associated analyte to therapy. This includes the serial measurement in patients with histologically confirmed diagnosis who are undergoing therapy for residual or advanced disease. Increased tumor marker concentrations may be indicative of progressive disease, whereas decreasing concentrations are often indicative of response to therapy, and constant serum tumor marker levels are associated with stable disease. Monitoring is further defined as serial measurements used as an aid in the detection of recurrent or residual disease in patients following primary curative treatment. Sustained elevations in marker analyte concentrations are suggestive of residual disease, whereas increasing marker analyte concentrations are indicative of recurring disease. Recent reclassification of tumor marker tests used in monitoring patients has created a

renewed interest and new developments for cancer diagnosis. It is expected that under reclassification, the 510(k) process can facilitate the availability of many new tumor markers in the market in a timely and cost-effective manner and contribute to improved care of patients (4).

Reclassification of serum tumor markers has accelerated the clearance process for the availability of these tests. The regulatory challenge is to ensure adequate evaluation of new tumor markers without any regulatory burdens or barriers to the introduction of useful tests in the future. Tumor markers are used to detect disease recurrence or to monitor tumor burden during therapy. The clinical utility of each marker relates to the responsiveness of the cancer to treatment. Current major concerns providing suggestions for 510(k) submissions regarding tumor marker tests employing immunochemistry methodology are based on guidelines that suggest that sponsors validate the assay cutoff in terms of minimal performance characteristics required to establish the effectiveness of the test. Data on the performance characteristics should include evaluations of sensitivity and specificity, linear range, and reproducibility and repeatability studies. Comparison studies between the sponsor's test and another legally marketed test system is helpful for evaluation purposes. Tumor markers intended for use in screening for the early detection or diagnosis of cancer in either the general population or a high-risk population or for disease staging were not addressed during these reclassification deliberations. The tumor markers reclassified in this category are considered to be important aids in the management of cancer patients. These tests are considered to be adjunctive in nature, providing a part of the broad picture of clinical information available to the health care providers (5–7, 11, 15, 17).

The special controls designated to be used for this generic class II category are guidance documents for the submission of 510(k) notifications to the FDA and voluntary standards for test performance promulgated by the NCCLS. This guidance document designated as a special control provides the review and evaluation criteria that describe the data necessary for a 510(k) submission to the FDA. The document also points out suggestions for the nonclinical laboratory performance studies and the design, conduct, and analysis of appropriate clinical studies in support of applications of these tests. The NCCLS documents designated as part of special controls are EP5-T2, Evaluation of Precision Performance of Clinical Chemistry Devices; EP7-P, Interference Testing in Clinical Chemistry; and EP9-P, Method Comparison and Bias Estimation Using Patient Samples. These standards/guidelines provide evaluative techniques to ensure the accurate performance of these tests. Any additional voluntary standards and/or guidelines, developed by either NCCLS or an equally capable voluntary organization that the FDA believes to be applicable in the evaluation of future tumor markers, could be considered as special controls and listed in further revisions of the guidance document. These guidelines could be considered as horizontal standard guidelines for the evaluation of tumor marker tests (Table 2).

TABLE 2
NEW 510(K) PARADIGM APPLICABLE TO TUMOR MARKERS

510(k) Major Modifications—Reliance on Design Controls and Select Device Modifications	Abbreviated 510(k)—Use of "Special Controls" and "Declarations of Conformity" with Standards
Device Modifications	*Special Controls and Declarations of Conformity with Standards*
Sponsor modifies own legally marketed class II device and determines a 510(k) is required	FDA/industry guidance
Modification does not affect intended use/indications for use or technology	National voluntary standards organization guidelines (e.g., NCCLS)
	Horizonal
Sponsor assesses modifications in accordance with design controls (21 CFR 820.30)	EP5 precision performance
	EP6 evaluation of linearity (reportable range)
510(k) submitted with "Declaration of Conformity" to design controls	EP7 interference testing
	EP9 method comparison and bias estimation using patient samples
Results of risk analysis	
Verification and/or validation required (including methods or tests used)	EP14 evaluation matrix effects
	EP17 estimation of detection limits
Design outputs/formal reviews	
	Vertical
Design controls to ensure input requirements are appropriate to the intended use and user needs	Purification and characterization of PSA and PSA-ACT for use as primary standards

4. The Premarket Evaluation Process

The premarket notification application, 510(k), is reviewed by the FDA scientific staff. This evaluation takes into consideration tumor-associated analytes, test requirements, medical usefulness of the test system for a particular clinical claim, and its application (i.e., monitoring or treatment follow-up). The FDA determines the appropriate performance requirements for each tumor analyte category. The agency's staff considers factors, such as consequences of a false positive or false negative, and the importance or impact of an absolute versus a significant change in the results or values of the tumor marker tests. The performance criteria (parameters) of a particular tumor marker test are compared with those of previously

reviewed tumor markers within that category or in new tumor marker applications. Generally, the device's performance parameters are compared with established referenced medical procedures and studies (3, 15).

The establishment of performance criteria for a given tumor marker test is not a simple process because accuracy and precision are unique for each type of analyte and its application. Establishing methodological limits for accuracy, precision, sensitivity, and specificity often requires standard reference materials, quality control materials, comparative studies, and actual clinical specimens. Accuracy and precision must be measured over the analyte reportable range for which the device is intended to be used. Sensitivity and specificity must be considered with respect to the intended clinical use of the device. Also, the indications for use should be carefully considered in the design of the study protocol. The indications for class II should be to monitor residual tumor after surgery (or radiation), the recurrence of tumor, or response to therapy. A 510(k) must provide clear evidence that the device is accurate, safe, effective, and substantially equivalent to a device legally marketed in the United States.

The FDA evaluates each tumor marker test against its own labeling claims as to how well it performs and compares it with other cleared and marketed devices identified in the 510(k) submission. The intended use claim for monitoring must be supported by valid scientific and clinical data. Labeling a tumor marker test for a particular claim can mean that the testing system may have to show supporting data for that claim. For example, a tumor marker proposed for a monitoring claim will require laboratory and clinical data to support that claim (4, 5).

The evaluation of tumor marker tests is based on criteria ranging from testing performance requirements to medical usefulness of the product as applicable to care of patients and treatment follow-up. A tumor marker may have some value when used in a particular clinical setting, where history of the patient's disease or a physical examination follow-up is also helpful in the utility of the marker. The value of a marker in a given setting depends heavily on two predominant performance characteristics—sensitivity and specificity. These parameters must be established with respect to the intended clinical use of the marker. The value of the marker in a particular situation also depends on the effectiveness of therapy for the malignancy. However, it is imperative to recognize that the evaluation of a tumor marker should relate to the intended clinical setting. Advances in technology have made ultrasensitive tests possible. It is important for a sponsor to balance the analytical and clinical sensitivity and specificity of the test and the resultant outcome of the test. This publication provides conceptual and application guidance and clarification on what information is necessary before the FDA can clear a device for marketing. The FDA can make more informed decisions based on a uniform database. It is hoped that this will lead to more reliable, reproducible, and standardized commercial tests (11, 15, 17).

5. Substantial Equivalence

The basic concept behind 510(k) clearance is that the device being reviewed is very much like others already being sold in the United States. For FDA processing, a determination of whether a device is considered old or new is made on the basis of whether it was in the commercial marketplace or traceable to the commercial marketplace at the time of passage of the Medical Device Amendments of 1976. Two working terms must be considered when arriving at such a conclusion—"substantial equivalence" and "predicate device." The 510(k) notification must show that the device is substantially equivalent to one or more predicate devices legally marketed in the United States. A predicate device is often one that was legally marketed on or before passage of the Medical Device Amendments to the Food, Drug, and Cosmetic (FD&C) Act on May 28, 1976. However, a number of predicate devices were first marketed after that date. They have been declared by the agency to be substantially equivalent after submission of a 510(k) application. New versions of old devices are handled through the 510(k) process. The review objective is to establish that the new product is "substantially equivalent" to its predicate.

Substantial equivalence for new serum tumor markers can be established by directly comparing two existing serum tumor markers of the same intended use (in the case of "me-too" markers) and/or by recognized IVD reference procedures as well as other non-IVD procedures (in the case of new analyte tumor markers). The type of data and information required for tumor marker 510(k)s depends on the intended use, technological features of the new device, and type of claims made by the sponsor. In the FDA review process, a finding of substantial equivalence requires either of two major conditions to be met. Substantial equivalence is likely to be determined if the device being reviewed is found to have the same intended use and the same technological characteristics as the predicate device. The device can have different technological characteristics. For example, it may be made up of different materials, have a different design, use a different energy source, or even rely on different principles of operation and still be substantially equivalent to a predicate device—but only if it has the same intended use and the sponsor can demonstrate that it is as safe and effective as the predicate device (Table 3).

6. A New 510(k) Paradigm

A new 510(k) paradigm provides guidance for sponsors of 510(k)s to consider and use national and international standards and a mechanism for declaration of conformity assessment criteria for these standards. It is expected that many domestic and international standards will address, in part or full, certain aspects of

TABLE 3
COMPARISON OF METHODS[a]

Characteristic	Tumor marker X (CA 27.29)	Tumor marker Y (CA 15-3)
Intended use/ndica-itions for use	The assay values in human serum and plasma (EDTA) are intended for the management of breast cancer patients. Serial testing for patient CA 27.29 assay values should be used in conjunction with other clinical methods for monitoring breast cancer.	The assay values in human serum and plasma (EDTA) are intended for the management of breast cancer patients. Serial testing for patient CA 15-3 assay values should be used in conjunction with other clinical methods for monitoring breast cancer.
Antigen measured	Mucins encoded by the human *MUC1* gene	Mucins encoded by the human *MUC1* gene
Monoclonal antibodies	B27.29	DF3 and 115D8
Assay principle	Radioimmunoassay (RIA)	Microparticle enzyme immunoassay (MEIA)
Detection of immuno-complex	Antigen in sample competes with antigen-coated tube for binding by iodine-125–labeled antibody.	Antigen in sample is bound by antibody-coated micro-particles and enzyme-labeled antibody.
Standard curve	7 days curve storage	14 days curve storage
Specimen type	Serum/EDTA plasma	Serum/EDTA plasma
Sample volume	25 μL for a single test	105 μL for a single test
Assay time	Approximately 3 hours	Approximately 50–60 tests/hour
Assay temperature	Room temperature	34 ± 0.5°C
Instrumentation	Gamma counter	ABC analyzer
Assay range	0–200 U/ml	0–250 U/ml

[a]Clinical correlation studies between CA 27.29 levels and CA 15-3 levels typically yield correlation coefficients of >0.95. It has been suggested that the epitope mapping studies reflect that the CA 27.29 antigen is essentially the same breast cancer–associated mucinous antigen detected by the two methods.

safety and effectiveness of IVD medical devices. This chapter has presented tumor markers as examples in which the use of voluntary guidelines or standards is helpful in establishing the substantial equivalence of new tumor markers as compared with legally marketed predicate markers. Thus, this type of assessment approach can serve as a surrogate for comparative information to show that the new marker is as safe and effective as the predicate via the areas covered by the standards (Table 2). The new paradigm gives device manufacturers some optional ap-

proaches to obtaining marketing clearances for the class II devices while maintaining the traditional (routine) evaluations for devices that require extensive proof of equivalence to predicate devices.

One of the alternative approaches under the 510(k) paradigm is "Special 510(k): Device Modifications." This approach utilizes certain aspects of the Quality System Regulations (Quality System Requirements for Good Manufacturing Practices). The other alternative is the "Abbreviated 510(k)." This approach utilizes special controls in which standards or voluntary guidelines can facilitate 510(k) review and expedite evaluation.

6.1. Special 510(k): Device Modification

The Safe Medical Devices Act of 1990 (SMDA) amended section 520(f) of the act, providing the agency with the authority to issue regulations requiring preproduction design controls. Under this regulation, the FDA may require the methods used in, and the facilities and controls used for, the manufacturing process, preproduction design validation (including a process to assess the performance of a device), packing, storage, and installation of a device conforming with current good manufacturing practice, in order to ensure that the medical product will be safe and effective. This portion of the law was based on findings that a significant proportion of device recalls were attributed to faulty design. The FDA has revised its current good manufacturing practice requirements to include preproduction design controls that device manufacturers must follow when initially designing devices or when making subsequent modification to those designs (21 CFR 820.30).

Effective June 1, 1997, manufacturers of class II and certain reserved class I devices had to follow design control procedures for their devices, including device modifications. Product modifications that could significantly affect the devices' safety and effectiveness are subject to 510(k) requirements under 21 CFR 807, as well as design control requirements under 21 CFR 820.30. In accordance with the Quality System Regulations, manufacturers must have a systematic set of requirements and activities for the management of design and development, including documentation of design inputs, risk analysis, design output, test procedures, verification and validation procedures, and documentation of formal design reviews (Fig. 1). In this process, the manufacturer must ensure that design input requirements are appropriate so that the device will meet its intended use and the needs of the population of patients. The manufacturer must also establish and maintain procedures for defining and documenting design output in terms that allow an adequate evaluation of conformance to design input requirements. Thus, manufacturers may need to refine their device design requirements as verification and validation results are obtained. The design specifications that result from this process are the design outputs that form the basis for the Device Master Record (DMR), which is subject to inspection by FDA personnel.

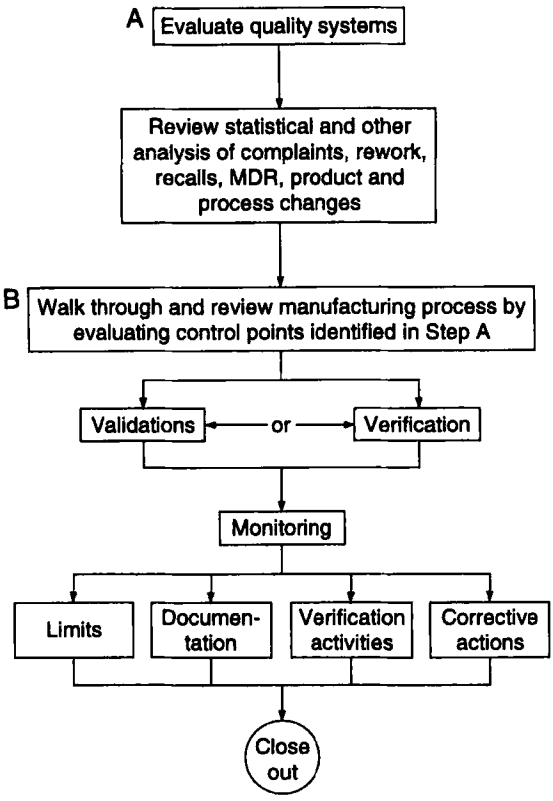

FIG. 1. Quality systems inspections.

Because design controls are now in effect and require the conduct of verification and validation studies of a type that have traditionally been included in 510(k) submissions, it is expected that the test results generated pursuant to the new design control requirements will be sufficient to contribute to substantial equivalence decisions. With the design controls in place, it is expected that it may be appropriate, in certain situations, not to conduct a detailed review of the data normally required in traditional 510(k)s. It is believed that the FDA would rely on the existence of certain data generated during design control activities. Therefore, under this proposal, conformance with design controls could be used as an alternative to some of the traditional data required to demonstrate substantial equivalence for certain device modifications.

Under this proposal, a manufacturer would use the FDA guidance entitled "Deciding When to Submit a 510(k) for a Change to an Existing Device," and

this would help to determine whether a device modification could be implemented without going through a new or extensive data-driven 510(k). In this proposal, if a new 510(k) is needed for the modification, but it does not affect the intended use of the device or the scientific technology principle of the device, conformance with design controls could contribute to clearing the application for commercial distribution. Under this proposal, a manufacturer who intends to modify a legally marketed class II device would conduct the necessary verification and validation activities (as determined by a risk analysis under Quality System Regulations) to demonstrate that the design outputs of the modified device meet the design input requirements. Once the manufacturer has ensured the satisfactory completion of this process through design review, it is conceived that a "Special 510(k): Device Modification" could be submitted. Although the overall content of the 510(k) (21 CFR 807.87) would remain the same, this type of submission could reference a previously cleared 510(k) and refer a manufacturer to a "Declaration of Conformity" to design control requirements, focusing on the essentials of the application (Table 3). It is conceived that under this proposal, a manufacturer could have the option of using a third party's expertise to assess conformance with design controls. In this case, the third party would perform a conformance assessment for the device manufacturer and provide the manufacturer with a statement to this effect. The marketing application would then include both the statement from the third party and a declaration of conformity attested by the manufacturer.

6.2. Abbreviated 510(k)

The SMDA introduced the concept of special controls to ensure the safety and effectiveness of class II devices. Special controls are defined by the statute as those controls, such as performance standards, postmarket surveillance, patient registries, development and dissemination of guidelines, recommendations, and the appropriate actions, that provide reasonable assurance of the device's safety and effectiveness. In reclassifying tumor markers from class III to class II, considerable effort has been expended to develop the concept of a Special Control Guidance Document (SCGD). The tumor marker SCGD was developed in accordance with the FDA's Good Guidance Practices in collaborative efforts between the American Association for Clinical Chemistry (AACC) and the industry experts. In addition to the tumor marker SCGD, there are ongoing efforts to recognize horizontal and vertical standards that could be useful for class II IVD devices and future tumor markers. These standards (NCCLS Guidelines) were cited in the SCGD for applications as "special controls" that address specific concerns associated with multiple/generic tumor marker categories. NCCLS EP5, EP6, EP7, and EP9 are examples of such standards (Table 2). They have broad applicability to many IVD medical devices including tumor markers. It is expected that the FDA's recog-

nition of these standards (NCCLS Guidelines), combined with modified review procedures, could streamline the review of many 510(k)s.

Under this proposal, device manufacturers could choose to use horizontal standards for class II devices when an SCGD exists or when the FDA has recognized an individual special control such as relevant vertical standards (e.g., prostate-specific antigen). In addition to the required elements of a 510(k) (21 CFR 807.87), these applications could include summary information that describes how "special controls" have been used to address the risks associated with the device type and a declaration of conformity with any relevant recognized standard(s), as applicable. As expected, a declaration of conformity with a standard would provide a summary of a manufacturer's efforts to conform with the recognized standard and would outline any deviations specifying:

- Any elements of the standard that are applicable to the particular device;
- Any standard or part of a family of standards, including collateral and/or particular parts applicable to the device;
- Any deviations from the standards; and
- Any deviations or differences between the tested device and the device to be marketed with adequate supporting justifications.

In this proposal, a manufacturer could also have the option of using a third party to assess conformance with the recognized standard. The third party could perform an assessment of conformance with the standard and provide the manufacturer with a statement to this effect, and the 510(k) application could then include the statement as well as summary on declaration of conformity. The abbreviated 510(k) submissions may compete with routine 510(k)s, and it is anticipated that their review and processing will be more efficient and timely than those of routine (traditional) submissions, which tend to be intensively data driven (Fig. 2).

7. Comparison Studies

The performance of the device can be established by comparison with any legally marketed medical device with the same intended use, and/or by other studies, to determine the operating characteristics of the device. All claims for substantial equivalence and specific performance characteristics of the device must be supported by appropriate data analysis and conclusions (e.g., charts, scattergrams, histograms). In order to demonstrate clinical utility as an aid in monitoring, evaluations of new tumor marker analytes should demonstrate that the marker is a significant indicator of changing clinical status. This may be demonstrated by testing a suitable sample of patients and evaluating the testing power of the marker against, or in conjunction with, other known clinical diagnostic variables such as age, sex, disease stage, recurrence, remission, and other conditions including pri-

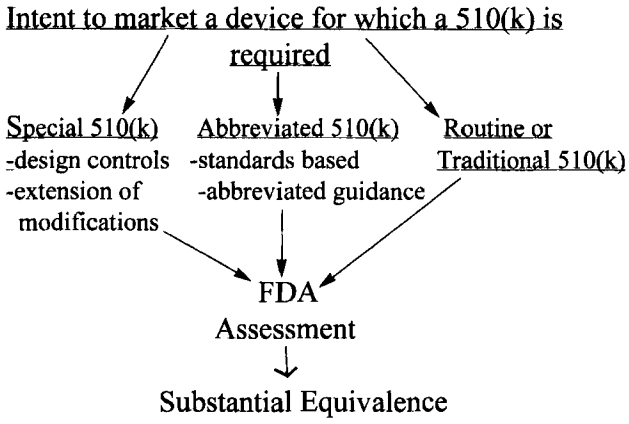

FIG. 2. New 510(k) paradigm.

or treatment regimens. Appropriate statistical analysis is always helpful, such as logistic or discriminate regression analysis, to perceive the distinction in terms of clinical sensitivity, clinical specificity, and predictive power of positive and negative results. Other approaches can be used such as Cox regression or logistic regression, which can be useful for measuring relative risk of recurrence associated with a positive test as compared with a negative test result. A recognized reference method may also be used for comparison purposes in order to establish a proposed device's performance characteristics. This comparison is very significant where there are wide differences in methodology and/or technology between the new device and the legally marketed device. Quantitative test systems should include, at a minimum, an evaluation of systematic and random errors in comparison with a legally marketed or reference methodology. The comparison may be directly between the two devices and/or indirectly with the new and old test systems when compared with a reference method.

Linear regression analysis may be used when comparing two tests with substantially similar linear performance parameters. Estimated slope and intercept along with the 95 percent confidence intervals can be presented. Where comparing two tests, or when one of the tests does not show linear performance indicators, or where use of linear regression appears to be inappropriate, other statistical techniques such as concordance measurements or McNemar's test may be appropriate. Linear regression analysis can be helpful when comparing results obtained using known positive tumor marker samples free from interfering substances. In such comparisons, use of low and high levels of tumor markers covering the dynamic range is desirable. The correlation studies should cover all serum specimens of patients with benign, healthy, and malignant diseases. These samples can be an-

alyzed with the new and old tests side by side, on the same day or for the same run. It is recommended that both tests (new and old) should be performed according to the specifications and instructions of the manufacturer according to the labelings or manuals (12, 17, 19, 23).

8. Tumor Marker 510(k) Principles and Applications

The 510(k) notifications for tumor markers involve new analyte or same intended use (indications for use) in comparison with marker tests already on the market. The focus of these reviews and evaluations involves analytical performance characteristics and/or other scientific data to demonstrate that the new or modified device is substantially equivalent to the predicate. Generally, review of most of these 510(k) submissions is based on analysis of the fundamental analytical principles if IVD tests, including accuracy, precision, sensitivity, specificity, interpretations, and use of results in the monitoring of patients. As mentioned before, the 510(k) application must show that the device is substantially equivalent to one or more predicate tumor marker tests legally marketed in the United States. In a typical tumor marker 510(k), the sponsor describes the device analyte, device methodology or technology, and its intended indications of use for monitoring cancer treatment patients. The sponsor compares and contrasts the review device with similar legally marketed devices with supporting comparable data or, in the case of new marker analytes or modified technologies (new matrices and new monitoring ranges), data to demonstrate that the sponsor has considered the effect of the change on safety and effectiveness and has changed the labeling accordingly (Table 3).

The analytical sensitivity of a marker assay is defined as the minimal concentration of marker analyte that has a high probability of being detected. The analytical sensitivity levels may be different for each tumor analyte category and/or technology. This variance may be due to the inherent capability of the state-of-the art technology as applied to the type of marker. Generally, the sensitivity comparison within a marker category should meet NCCLS guideline criteria. For tumor markers, the cutoff concentration is a chosen concentration that distinguishes positive from negative specimens. Validation of the marker test near the chosen cutoff concentration is of critical importance in evaluating and labeling new marker tests. This is generally demonstrated using a statistically valid number of "negative and positive" specimens equally distributed around the cutoff concentration (the NCCLS guidelines recommend that such specimens be within 25 percent of the cutoff). The sponsors establish this type of capability by running marker reference materials with the test system in determining positive or negative results at or near a selected cutoff concentration. In addition, clinical samples covering the entire range of the proposed test are tested to support the robustness of the selected or proposed cutoff (3).

Analytical specificity of a marker test refers to the ability of the methodology or technology to identify and distinguish a specific marker analyte(s) from other substances in the specimen matrix. This specificity can be a function of one or all of the processes associated with the device technology, such as marker analyte isolation, separation, and detection. The antibodies used in immunoassays may differ substantially in their reactivity to various analytes and metabolites. Some of the cross-reactivities encountered in the testing of tumor markers are commonly prescribed therapeutic drugs, antineoplastics, antibiotics, and common over-the-counter drugs. Typically, in a marker 510(k), the sponsor provides a summary of sensitivity and specificity comparison data under actual conditions of testing and description of protocols used to generate the comparison data. The data included in the notification demonstrate that the proposed marker test is substantially equivalent to another marker test in detecting the analyte within established sensitivity and specificity criteria. Therefore, the comparative assessment of a marker test in a given setting depends heavily on two predominant performance characteristics—sensitivity and specificity. These parameters must be established with respect to the clinical use of the test. In some instances, the impact of a particular test also depends on the effectiveness of therapy for the malignancy. Advances in technology have made ultrasensitive tests possible; therefore, it is important for a sponsor to balance the analytical and clinical sensitivity and specificity of the tests and the resultant claims for the test (4, 5).

9. Tumor Marker Monitoring

The tumor markers that were down-classified from class III to II are those that are intended only for monitoring. Measurement of tumor-associated analyte levels may aid in the monitoring of patients for disease progression or response to therapy or for the detection of residual or recurrent disease. Monitoring is defined as assessing disease progression, recurrence, or response to therapy. This includes the serial measurement of tumor-associated analytes in patients with a histologically confirmed diagnosis who have residual or advanced disease. Tumor marker tests, like drug monitoring tests, have been useful for monitoring patients with a variety of cancers. The change in concentration of serum marker reflects a change in tumor burden and allows therapeutic intervention for the patient. An increase of the marker value in a patient during remission may signal a recurrence of tumor. A decrease of the marker to an undetectable level after surgical removal of the tumor may indicate the complete success of surgery. Unfortunately, most markers are not specific for malignancy, because elevation in a marker value is also observed in benign conditions. Therefore, some of these markers are not very useful in screening for cancer in asymptomatic patients. Recent scientific literature indicates that mortality from all cancers in the United States is declining for the first

time in this century. For many new tumor markers, clinicians need to know what kinds of benefits they offer, for instance, improved overall survival, disease-free survival, quality of life, and cost-effectiveness. Although studies have addressed issues of cost-effectiveness, experts state that tumor markers can potentially hold down health care costs while improving care of patients, which is the primary goal of the managed care process (6, 7, 11, 34).

10. The Role of Receiver Operating Characteristic (ROC) Curves in the Evaluation of Tumor Markers

A new tumor marker is evaluated using the same criteria used for many diagnostic tests (i.e., sensitivity, specificity, and accuracy). The diagnostic sensitivity and specificity are best represented by a receiver operating characteristic (ROC) curve. The ROC curve is constructed with the true-positive rate versus false-positive rate at various decision levels. As a test improves in its diagnostic performance, it shifts upward and to the left as the true-positive rate increases and the false-positive rate decreases.

The comparison of the effectiveness of different tumor markers (old versus new) for a given malignancy should be carried out using split samples from both patients and healthy control subjects (Table 4). Several different decision levels could be used to establish an interpretive scale instead of using a single point. How to establish the decision levels is described in the following:

TABLE 4
ESSENTIALS OF TUMOR MARKER 510(K) APPLICATIONS

1. Medical significance, indications for use, and analytic goals for the test.
2. Description of the disease as it relates to the type of demographics intended for (e.g., sex, age).
3. Historical perspectives of test technologies used for the measurement of tumor-associated analyte levels.
4. Description on statistical applications and clinical interpretation of test results by providing interpretive algorithm for appropriate test follow-up (e.g., an upward trend in test levels in successive time periods or a test value exceeding a specific cutoff level).
5. Clinical and analytical interpretation of false-negative and false-positive results.
6. Background description of the clinical utility of the test.
7. Advantages and limitations of the proposed tumor marker technology as compared with other available clinical methodologies.
8. Description of types of specimen(s) or body fluid matrices used by the proposed tumor marker test system(s).
9. For new markers, provide pertinent scientific and clinical literature that relates to the proposed analyte for specific cancer(s) to be monitored.

1. The normal positive/negative cutoff points calculated on the basis of the marker levels found in healthy controls.
2. The decision level calculated on the basis of the marker levels found in patients with benign disease—any level above this value should indicate the presence or probability of disease that is not necessarily the target malignancy.
3. These decision levels calculated on the basis of marker levels found in patients with confirmed advanced malignancies should indicate a very high probability of the presence of malignancy.

11. Clinical Aspects and Applications of Tumor Markers

Researchers have developed a multitude of new markers, and it is considered that these new markers will have greater clinical utility than markers of the past. These research studies show that we can expect these markers to bring about a change in therapy of patients or quality of life. As these new markers become available for some of the most common forms of cancer, the clinical laboratories will play an important role in diagnosing and treating malignancies and standardizing marker tests to achieve comparable results that contribute to the patients' medical care and quality of life.

11.1. Prostate Cancer

Prostate cancer is the leading type of cancer being diagnosed currently in the United States. The number of diagnosed prostate cancer cases continues to increase with the availability of better and sensitive tests. By measuring the amount of a protein known as prostate-specific antigen (PSA) in the blood, the tests may unmask a cancerous prostate earlier than the onset of symptoms. PSA is now recognized as an important tumor marker for prostate cancer and is utilized for its detection and monitoring.

Increased diagnosis of prostate cancer is attributed in part to the increased utilization of PSA testing. In fact, the American Cancer Society (ACS) now recommends measurement of PSA in addition to digital rectal examinations (DREs) in men over 50 years of age. Early detection of clinically localized prostate cancer can potentially result in a cure with radical prostatectomy or other treatments. PSA tests are used to monitor therapeutic efficacy and detect recurrent disease in patients with prostate cancer.

PSA determination can be used to investigate the necessity for a prostate biopsy. PSA is considered a useful analyte in the diagnosis and management of prostate cancer; however, increased serum concentrations of PSA are also seen in patients without cancer of the prostate (e.g., patients with bacterial prostatitis or benign

prostatic hypertrophy, BPH). Studies have shown that PSA complexes with other proteins in the blood, including α_1-antichymotrypsin (A1 ACT) and α_2-macroglobulin (A2 MG). Lower ratios of free PSA (uncomplexed PSA) to the PSA–A1 ACT complex have been reported in patients with prostate cancer. Recent studies have shown that clinicians can differentiate patients with prostatic carcinoma from those with benign disease by evaluating these ratios (free PSA/PSA–A1 ACT).

Patients must be monitored to assess their response to treatment and to detect recurrent diseases. PSA as a specific marker for prostate cancer is most useful in monitoring patients who have been treated with radical prostatectomy, radiation therapy, or endocrine therapy. The concentration of PSA falls to undetectable levels following a radical prostatectomy because all prostate tissue has been removed. Generally, PSA is measured at periodic intervals. In studies, the extent of disease at the time of surgery correlated well with the postoperative PSA concentration. A significant measurable PSA concentration after prostatectomy indicates that residual tumor may be present. PSA concentrations decline gradually after radiation therapy (36).

The dimensions of the prostate can be estimated by transrectal ultrasonography and the prostate volume can be calculated from these dimensions. For any given PSA concentration, prostate glands with BPH are typically much larger than those with prostate cancer because the cancerous prostate releases much more PSA into the serum than those with BPH for any given volume of tissue. By calculating the PSA density (defined as the PSA concentration divided by the prostate gland volume), elevated PSA concentrations are corrected for large gland volumes due to BPH.

There is considerable overlap in PSA concentrations between prostate cancer patients and BPH patients because both groups may have elevated PSA levels. Studies have suggested that monitoring pre- and postoperative PSA concentrations is useful in determining the extent of tumor removal; therefore, it is useful as a marker for monitoring patients after radical prostatectomy for the recurrence of prostate cancer. Serum levels of PSA are used along with the DRE and the ultrasound-guided biopsy in the management of prostate cancer patients. However, measurement of PSA concentration, in combination with DRE, is the most widely accepted method for detecting prostate cancer (12, 23, 36).

It has been reported that PSA exists in multiple isoforms in serum: free PSA (30-kDa protease) and complexed PSA (100-kDa complex between PSA and A1 ACT). While the PSA in the prostate is in the free form, when the PSA enters the blood stream, the majority binds to A1 ACT. Recent studies have shown that PSA in serum occurs in two molecular forms, free (f-PSA) and bound; both PSAs gave equal detectable signals ("equimolar"). Most of the PSA in serum is complexed with either A1 ACT or A2 MG. Different proportions of free and complex isoforms have been detected in the sera of prostate cancer and BPH patients. The fraction

of f-PSA was shown to be substantially smaller in patients with untreated prostate cancer than in patients with BPH. Therefore, combined measurements of f-PSA and total PSA (t-PSA) may provide a better differential diagnosis between BPH and prostate cancer. Some studies have shown that the f-PSA/t-PSA ratio is helpful in the differential diagnosis of BPH and prostate cancer (39).

About 90 percent of the PSA in serum exists as PSA–ACT complex, and 10 percent is free. Some of the current immunoassays for PSA detect these two forms of PSA differently, which results in significant differences in the molar response of the assays of the two forms. Some immunoassays detect both free and complexed forms of PSA, making it essential to develop immunoassays for PSA to recognize these forms specifically. It has been noted that the term "equimolar" means that both free and bound PSA gave equal signals. If the antibodies used in the test do not give equal responses, variability could occur among different PSA immunoassays. It is also suggested that the measurement of different forms of PSA could be used to discriminate better between BPH and prostate cancer. Other studies indicated that concentrations of complexed PSA were higher in patients with prostate cancer than in patients with benign disease or in normal individuals. Specific assays for various forms of PSA have been developed, and the determination of the ratio of complexed or free PSA to total PSA has been shown to improve the specificity for prostate cancer because the number of false-positive results due to BPH will be reduced.

Differences in the relative proportion of f-PSA and PSA–ACT can affect the result obtained for t-PSA because of the differences in the nature of calibration and the molar response, sensitivity, and specificity of antibodies used in various immunoassays. The efficiency of these immunoassays has been evaluated by several investigators. Because the proportion of free and complexed PSA varies in benign and malignant diseases, these immunoassays measure one form or the other, giving rise to different results for different patient groups. It is very important that data from clinical studies support the proposed intended uses of these assays, since as many as 5 percent of men with a negative free PSA test (free PSA values >25 percent) will have cancer and not be recommended for biopsy. Therefore, a goal for standardization is to detect total and free PSA accurately in equimolar fractions.

Currently, there are several assays for the measurement of PSA. All of them contain monoclonal or polyclonal antibodies labeled with enzymatic, fluorometric, or radioactive markers. These assays have shown significant variations within the same patient specimens. These variations may result from differences in antibody specificity, reaction kinetics, calibration, or the system's sensitivity. Studies have shown that only free PSA and PSA–ACT show immunological reactivity in these assays. Also, reaction kinetics can influence the molar ratio. Some of these assays with shorter incubation times may specifically bind the free PSA molecule (which is a lower weight form of PSA). In the equimolar assays, changing the incubation

time from hours to minutes would result in a nonequimolar response because of the increased binding of free PSA in comparison with complexed PSA. Other assay features such as pH and temperature may also affect the molar response (9, 39).

The current PSA decision range is 4 to 10 ng/ml, and a PSA level from 10 to 25 ng/ml is considered to be a diagnostic gray zone. If the PSA level is below 4 ng/ml, the patient is considered normal. If the PSA level is 10 ng/ml or above, patients are treated aggressively using additional tests and procedures, and in some situations a biopsy of the prostate is performed. For PSA ranges of 4 to 10 ng/ml, the patient is monitored more frequently. PSA ranges (t-PSA) of 4 to 10 ng/ml have been extensively studied to attempt to improve the low specificity of PSA in distinguishing benign conditions of the prostate from prostate cancer. It has been reported that quantifying t-PSA in a patient's serum in the 4 to 10 ng/ml range and calculating the proportion of free PSA provided the ability to distinguish benign histological conditions of the prostate from prostate cancer. Studies have suggested that the f-PSA/t-PSA ratio may be helpful for differential diagnosis of BPH and prostate cancer in the diagnostic gray area of 4 to 25 ng/ml t-PSA (12, 23, 27).

Reports show that quantifying t-PSA in a patient's serum in the 4 to 10 ng/ml range and calculating the ratio of f-PSA to t-PSA enhanced the ability to distinguish benign histological conditions of the prostate from prostate cancer while still retaining high sensitivity for detecting cancer. These studies reported that, within the 4 to 10 ng/ml t-PSA data set, the subset of patients whose f-PSA/t-PSA was 25 percent were diagnosed with benign disease only. Thirty percent of all patients with benign conditions whose t-PSA values lay between 4 and 10 ng/ml fell in this category. Researchers have proposed that by increasing specificity in the diagnostic gray zone, the percentage of patients requiring biopsy to detect cancer may be reduced. More than 20 percent of men with prostate cancer have serum PSA levels below the usual upper normal reference level of 4 ng/ml, and cancer may be detected within 3 to 5 years in up to 20 percent of men with total PSA levels between 2.6 and 4 ng/ml. It has been suggested that consideration be given to measuring the f-PSA in men with t-PSA of 2.6 to 4 ng/ml, with biopsy recommended when the f-PSA is <27 percent of total PSA. An appreciable prevalence of detectable prostate cancer (22 percent) can be found in men with a total PSA of 2.6 to 4 ng/ml, and measuring the free PSA may reduce unnecessary biopsies in those with a normal prostate examination (8, 9, 22).

Such factors as age and race and their relevant medical significance should be used to detect PSA concentrations in the 0 to 4 ng/ml range. PSA age-related, specific-reference ranges have been reported in the literature, and it is suggested that both age and race should be considered when interpreting PSA results. The following factors should also be considered when interpreting the PSA results:

- Calculation of the percentage of free PSA compared with the total PSA;
- Use of age-specific reference ranges;

- Calculation of the serum PSA concentration per unit volume of prostate gland (PSA density);
- Monitoring the change in serum PSA concentration over time (PSA rate, PSA velocity, or rising PSA values); and
- Use of computerized neural network–derived indices to enhance sensitivity and specificity (ProstAsure Index).

Factors such as assay variations, age, and prostate gland size are known to affect cutoff values. Also, free to total PSA cutoffs are influenced by the sensitivity and specificity values chosen, the reflex range for total PSA used, differences in free PSA assays, and differences in populations studied. Different PSA values are considered due to differences in cutoffs in different assays. Studies have shown that the comparison of a chemiluminescent free PSA showed a 25 percent difference in values. These types of variations suggest a need for standardization (9, 29).

Ultrasensitive assays for PSA contribute to the earlier detection of prostate cancer relapse and (or) residual disease in prostatectomized patients as well as the more timely evaluation of response to current therapies. PSA determinations can be useful in detecting metastatic or persistent disease in patients following surgical or medical treatment of prostate cancer. Persistent elevation of PSA following treatment, or an increase in the pretreatment PSA concentrations, is indicative of recurrent or residual disease. Hence, PSA is widely accepted as an aid in the management of prostate cancer patients, and serum levels are most useful when sequential values are obtained and monitored over time. After complete removal of the prostate gland (radical prostatectomy), PSA levels should become very low or undetectable. A rise of the serum PSA level in prostatectomy patients indicates residual prostate tissue, recurrence, or metastasis of the disease (13, 16, 24, 36).

Prostatic tumor volume contributes to the levels of serum PSA as well as pathological staging, with higher PSA concentrations associated with advanced staging. In the majority of men, PSA concentrations at the time of surgery were found to be <4 ng/ml (organ confined). Half of those with PSA concentrations >10 ng/ml had capsular penetration, whereas those with PSA values >50 ng/ml showed positive pelvic lymph nodes.

The application of PSA measurements for clinical monitoring of prostatic carcinoma requires fine tuning of PSA assays. One important aspect of this tuning is to have well-defined standards (primary calibrators). Calibrators or primary reference materials consisting of PSA complexed with ACT have been prepared and are available to sponsors of commercial immunoassays. As a result of this, some sponsors have studied calibration stability and have shown that calibration did not change within 14 to 90 days. Primary references of 90 percent PSA-ACT and 10 percent f-PSA have been shown to minimize differences in PSA measurements between different assays [NCCLS Document—Primary Reference Preparations Used to Standardize Calibration of Immunochemical Assays for Serum Prostate

Specific Antigen (PSA): Approved Guideline, 1996]. This guideline could be considered as a vertical standard guideline or vertical probe for the evaluation of PSA tests (Table 2).

A general objective in immunoassay standardization is that the standard reference material should resemble as closely as possible the analyte that is being measured. This means that for serum assays, the calibrator should comprise a mixture of free PSA and PSA–ACT and such calibrator material should contain assigned and validated portions of f-PSA and PSA–ACT to show good agreement or a well-defined bias between different assays designed for f-PSA/t-PSA ratio determinations. Thus, well-defined PSA primary reference material can be useful for calibration of equimolar response assays for total and free PSA. These primary reference materials can be used to assign values to serum-based commercial secondary reference materials or calibrators for PSA testing. Methods for PSA purification described in the NCCLS approved guidelines can be helpful in producing secondary PSA reference materials of comparable quality (10, 26, 35).

11.2. Breast Cancer

Breast cancer has a very high incidence in women. Breast cancer frequently develops into (or is) a systemic disease. The best way to reduce risk due to breast cancer is early tumor detection and surgical removal. The increase in detection rate of breast tumors that are <1 cm in diameter over the past 10 years can be mostly attributed to improvements in the sensitivity and utilization of mammography. The main utility of CA 15-3 in breast cancer is to monitor patients after mastectomy (14).

Generally, a CA 15-3 cutoff of 25 U/ml is used to detect stage I breast cancer. In higher stages, the sensitivity is reported to be much better, which makes it a good test of tumor burden. CA 15-3 is reported to be elevated in other disease conditions such as liver disease (particularly chronic hepatitis, cirrhosis, and carcinoma), some inflammatory conditions (sarcoidosis, tuberculosis, systemic erythematosus), and other carcinoma (lung and ovary). For this reason, positive CA 15-3 results should be interpreted with caution (20, 21).

Generally, changes in the tumor marker concentration reflect changes in tumor burden. It has been reported that an increase in the tumor marker concentration is associated with tumor recurrences. An increase of 25 percent or more can be due to tumor progression. Similarly, a decrease could be due to tumor regression or response to therapy (18, 28, 32).

CA 15-3 serum tumor marker is intended to detect disease recurrence in stage II and stage III breast cancer patients. It has been reported that CA 15-3, together with other suitable markers, is preferred in measuring the effect of applied hormonal therapy or chemotherapy in metastatic disease. Studies have indicated that CA 15-3 assay values are frequently elevated in patients with breast cancer. These

studies have suggested that the CA 15-3 assay may be of clinical value for monitoring the response of patients undergoing therapy because increasing and decreasing values correlated with disease progression and regression, respectively (Table 5). Elevations of CA 15-3 assay values have been reported in individuals with nonmalignant conditions such as cirrhosis, hepatitis, autoimmune disorders, and benign diseases of the ovary and breast. CA 15-3 assay values are not elevated in most normal individuals. As with CEA, one of the limitations of the use of CA 15-3 is that it is not often elevated with minimal disease. For instance, CA 15-3 levels are elevated in only about 9 percent of patients with stage I disease. It appears that CA 15-3 is not a cancer-specific antigen (it is elevated in roughly 5 percent of healthy individuals) and is found much more frequently in patients with some benign diseases, especially of the liver. Thus, it seems that CA 15-3 has very little utility in screening the general population for breast cancer. Studies have shown that although up to 75 percent of patients develop elevated CA 15-3 levels at the time of recurrence, the lead time provided by using this is usually no more than a few months (6).

At present, there is no reference method available. There is tremendous need to develop a purified reference material to standardize the assays.

11.3. Ovarian Cancer

CA 125 is a mucin-like glycoprotein antigenic determinant expressed on the surface of coelomic epithelium and human ovarian carcinoma cells; however, it does not appear to be specific for ovarian cancer because elevated levels have been reported in breast and colorectal cancers. Studies have shown increased CA 125 levels in patients with ovarian cancer, whereas decreased CA 125 levels in chemotherapy are associated with improved possibility for survival. Some studies have shown failure of CA 125 levels to return to normal after chemotherapy, indicating

TABLE 5
Comparative Indices for the Evaluation of Diagnostic Testing

Characteristic	CA 27.29 (%)	CA 15-3 (%)
Sensitivity	51	30
Specificity	90	98
Efficiency	71	61
Positive predictive value	84	95
Negative predictive value	66	53

Note. Adapted from Abbate, I., *et al., J Tumor Marker Oncol.* **8,** 69–72 (1993).

the presence of residual tumor or treatment failure. Therefore, an increase in CA 125 levels during chemotherapy can be due to the presence of progressive disease, and a rise after the return to baseline would indicate recurrence. Even though an elevated CA 125 level is correlated with the presence of epithelial or ovarian cancer, normal values have been reported in patients with recurrence of residual disease at second-look laparotomies or subsequent laparoscopies for ovarian cancer. Second-look procedures in ovarian cancer are performed because of reasonably greater survival rates associated with therapeutic responses (3).

CA 125 is a widely used cancer marker for monitoring treatment responses and detecting disease recurrences in patients with ovarian cancer. Generally, the CA 125 cutoff value of 35 U/ml is used for the mean value in normal women. It has been shown that values above and below this cutoff correlate reasonably well with the regression or progression of disease (6, 7).

Evaluation of CA 125 tests has shown moderate sensitivity, higher specificity, and predictive values in ovarian cancer patients when determining the presence of an intraperitoneal tumor or future occurrence at the time of second-look procedures. Studies have shown that the CA 125 level obtained at the time of a second-look procedure correlates reasonably well with the size of the tumor. As mentioned before, the predictive value of a marker depends on the prevalence of a particular type of malignancy in the intended population. Thus, evaluating a marker's diagnostic potential must be based on prevalence in a well-defined group for results to be generally applicable (i.e., prevalence of ovarian cancer in women with pelvic masses).

Periodic testing for CA 125 in women suspected of having ovarian cancer is recommended. Biopsy is the definitive test; however, women older than 40 should have a cancer-related physical checkup each year. CA 125 is relatively more sensitive in low-stage (stage I or II) ovarian cancer than CA 15-3 in breast cancer, but because of the relatively low prevalence of ovarian cancer, it is not recommended for screening use. Studies have shown that serum CA-125 is elevated more than 35 U/ml in approximately 80 percent of women with carcinoma of the ovary, 26 percent of women with benign ovarian tumors, and 66 percent of women with non-neoplastic conditions such as pregnancy, menstruation, acute salpingitis, adenomyosis, endometriosis, cirrhosis, or inflammation of the peritoneum and uterine fibroids. The most significant use of CA 125 is in the evaluation of patients for recurrent diseases such as after oophorectomy. The major clinical use of the CA-125 marker has been to follow the course of disease in patients known to have ovarian cancer or primary carcinoma of the peritonium (30, 31).

Ninety-five percent patients monitored for recurrence show CA 125 concentrations greater than 35 U/ml and residual ovarian carcinoma. However, a negative result is not conclusive because half of the patients with negative results have microscopic residual carcinoma. Therefore, it is essential to have a second-look procedure in order to rule out residual carcinoma. Postsurgical monitoring of patients

for recurrent ovarian carcinoma is achieved by combining second-look operations and CA-125 monitoring (31).

Treatment involves surgical removal of one or both ovaries, the uterus, and fallopian tubes. Radiation is also commonly employed, which may consist of placing a radioactive liquid in the abdomen. Sometimes chemotherapy is also used along with CA 125 monitoring in response to therapy.

11.4. Colon Cancer

Carcinoembryonic antigen (CEA) is the most frequently used marker in the monitoring of a number of different tumor types including gastrointestinal tract, lung, and breast cancer. CEA has been detected in high concentrations in the plasma of adults with carcinomas of the colorectum. The incidence of CEA elevations varies with the stage of the tumor (Dukes stage A, B, C, and D colorectal neoplasms). It is uncommon for CEA levels to remain elevated after ablative surgery in patients who are clinically free of residual tumor. Measurement of both pre- and postsurgical CEA levels is recommended for determining a baseline for monitoring patients for tumor recurrence and therapeutic efficacy. Therefore, it is assumed that the CEA-producing tumor has recurred when the CEA values reach a certain threshold. CEA values less than 10 ng/ml indicate a better prognosis in patients when compared with preoperative values greater than 10 ng/ml (25).

Recently, a panel of the American Society of Clinical Oncology (ASCO) evaluated several serum and tissue markers for colorectal and breast cancer to develop clinical practice guidelines for their use. For colorectal cancer, it was recommended that CEA be drawn preoperatively and monitored postoperatively in patients who would be eligible for primary resection and for resection of hepatic metastases. It was also suggested that CEA can be used to identify ineffective treatment in patients undergoing chemotherapy for metastatic disease. However, the CEA marker is applied predominantly for control of patients suffering from colon cancer and cancers of the gastrointestinal tract. Second-look surgery should be performed before the CEA level exceeds 11 ng/ml, because patients with less than this level have shown the best 5-year disease-free survival rate. Generally, high CEA concentrations are frequently found in cases of colorectal adenocarcinoma. Slight to moderate CEA elevations (rarely above 10 ng/ml) occur in 20 to 50 percent of benign diseases of the intestine, the pancreas, the liver, and the lungs (e.g., liver cirrhosis, chronic hepatitis, pancreatitis, ulcerative colitis, Crohn's disease, and emphysema). Smokers also have elevated CEA values. The main indication for CEA determination is the follow-up and therapy management of colorectal carcinoma (6, 7, 25).

CEA is a prototypic tumor marker. It is neither organ specific nor tumor specific. CEA concentrations are elevated (greater than 3.0 ng/ml) in the sera of patients with colorectal, gastric, pancreatic, hepatocellular, and biliary carcinoma. The

CEA levels are elevated in benign diseases such as inflammatory bowel disease, chronic gastritis and peptic ulcer, cirrhosis, and hepatitis. CEA testing is recommended primarily to monitor patients after surgery for recurrent colorectal carcinoma. Twenty percent of colorectal carcinomas do not express CEA; therefore, immunohistochemical methods are recommended to identify the negative cases. If a 5.0 ng/ml cutoff is used as the detection criterion, approximately 60 to 90 percent of the clinical cases will be detected for recurrences 2 to 10 months prior to clinical symptoms (37, 38).

11.5. Bladder Cancer

Bladder tumor–associated antigen (BTA), a human complement factor H, is produced by bladder cancer cells (men two to three times as often as women). Cancer cells are sometimes seen in urine samples by microscope cytoscopy (examination of the bladder with an instrument inserted into the urethra), which can reveal abnormal areas. Biopsy is needed to confirm the diagnosis. Early stage cancer confined to the bladder wall can often be removed with a cytoscope. If several tumors are present, they are removed by infusing the bladder with a solution containing bacteria able to stimulate the immune system.

Chemotherapeutic drugs may also be put directly into the bladder to lower the risk of recurrence. If the cancer cannot be easily removed, radiation (from an external source or from a radioisotope placed in the bladder) may be needed. If the cancer has spread through the bladder wall, the bladder may be removed, and chemotherapy may be needed after metastasis.

The most common BTA test is an immunoassay-based assay that uses monoclonal antibodies to detect the presence of bladder tumor–associated antigen in urine. In clinical studies, the BTA test was compared with cytoscopy-voided urine for the detection of recurrent bladder cancer. The sensitivity of BTA appeared su-

TABLE 6
Comparison between Bladder Tumor–Associated Antigen (BTA) and Cytology

	Sensitivity (%)	
Stage/grade	BTA	Cytology
Ta/1	9	0
Ta/2	71	21
Ta/3	100	60
Carcinoma *in situ*	80	100
Overall	65	32

Note. Adapted from Sarosdy, M. F., *et al., J Urol.* **154,** 379–384 (1995).

perior to that of conventional cytology (Table 6). These studies showed that differences are most significant in early stages and for early grade tumors (e.g., Ta/1, Ta/2, and Ta/3). The overall sensitivity was 65 percent for BTA versus 32 percent for the conventional cytology test (33).

12. Conclusion

This chapter has presented an overview of the reclassification, processing, and evaluation of new tumor markers. The clinical significance and analytical goals of particular markers are presented. The utility of a marker in a given setting depends on two predominant performance characteristics—sensitivity and specificity. These parameters must be established with respect to the intended use of the marker. The value of the marker in a particular situation also depends on the effectiveness of therapy for the malignancy. Recent developments promise new and more specific tumor markers. Several tumor markers are already available. In the future, new markers are anticipated that may greatly expand the range of usefulness in cancer diagnosis and monitoring. Cancer diagnostic tests provide an example of the current explosion of technology in the IVD industry. These tests will provide us with new and better tools but require appropriate oversight to ensure a positive impact on patients and public health.

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BRANCHED DNA SIGNAL AMPLIFICATION FOR DIRECT QUANTITATION OF NUCLEIC ACID SEQUENCES IN CLINICAL SPECIMENS

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1. Introduction

Detecting the presence of a specific nucleic acid sequence may be sufficient for some applications, but there is an increasing demand for quantitation of the

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number of copies of a given nucleic acid sequence. Although the initial driving force behind this demand has been viral load testing for chronic viral diseases such as hepatitis C and the acquired immunodeficiency syndrome (AIDS), gene quantitation has value in other research and diagnostic applications. The branched DNA (bDNA) signal amplification system, developed by Chiron Corporation, Emeryville, California, is one of several methods that are currently available for quantitation of nucleic acid sequences in clinical specimens. This chapter will attempt to provide an overview of basic concepts, principles, and applications of bDNA.

2. Branched DNA

2.1. Overview

The bDNA signal amplification system is a solid-phase, sandwich hybridization assay incorporating multiple sets of synthetic oligonucleotide probes and several simultaneous hybridization steps (Fig. 1). Multiple target-specific probes (five to nine, depending on the assay), termed capture extenders, are used to capture the target nucleic acid (DNA or RNA) onto the surface of a microtiter well plate. A second set of target-specific probes (18–39, depending on the assay), termed label extenders, hybridize to the target and, in the first-generation assays, also serve as binding sites for the synthetic bDNA amplifier molecules. The amplifier molecules each have 15 identical arms, each of which can bind three alkaline phosphatase–labeled probes. As many as 3000 enzyme-labeled probes can be hybridized to each target molecule in this manner. Detection of the bound labeled probes is achieved by incubating the complex with an enzyme-triggerable chemiluminescent substrate, dioxetane, and measuring the light emission. Because the number of target molecules is not altered, the resulting signal is directly proportional to the concentration of the target nucleic acid. The quantity of the target in the sample is determined from a standard curve.

In the second- and third-generation bDNA assays, a preamplifier molecule is used to increase further the number of labeled probes that can be bound to the target (Fig. 2). The label extender probes are designed such that two probes must be bound to adjacent regions of the target for efficient hybridization to the preamplifier molecule to occur. Each preamplifier molecule can bind up to eight amplifier molecules. In the second-generation bDNA assay for human immunodeficiency virus type 1 (HIV-1) RNA, each captured RNA molecule may bind as many as 10,080 separate alkaline phosphatase–labeled probes (Kern *et al.*, 1996). The lowest concentration of HIV-1 RNA that could be distinguished from the negative control was 390 copies/ml in this assay.

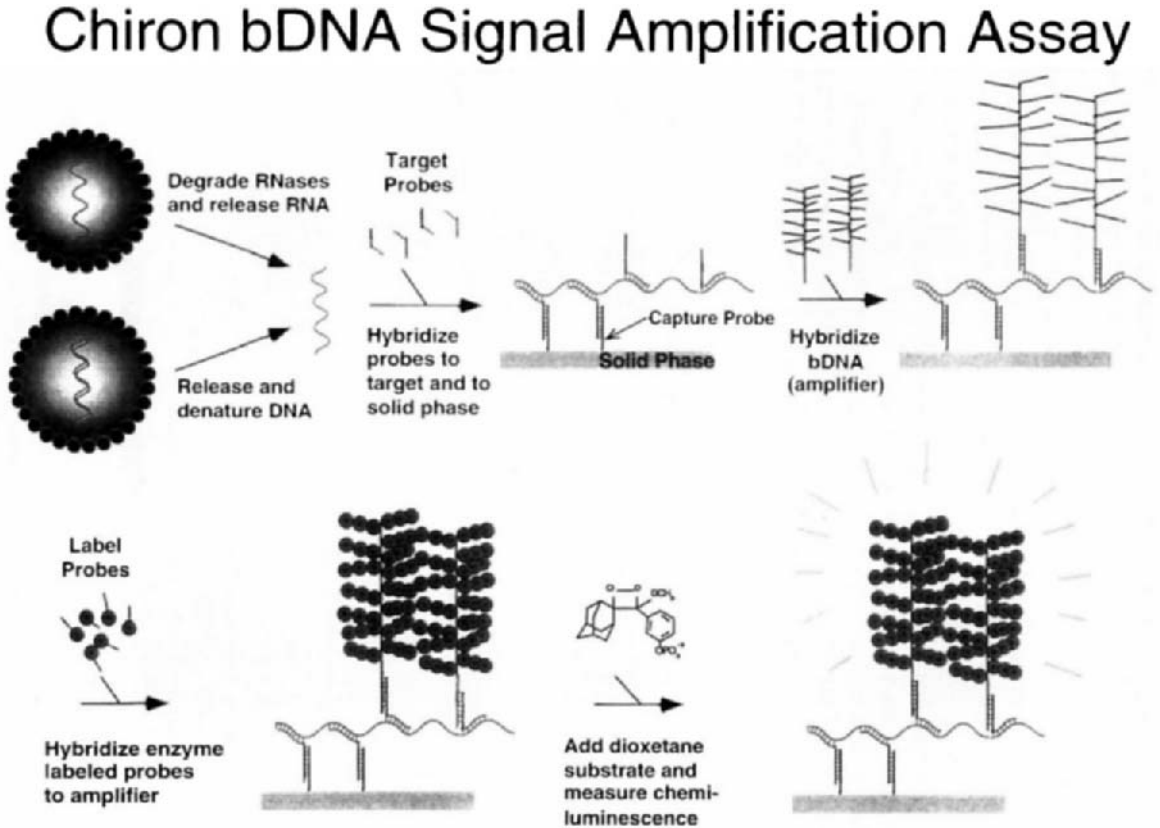


FIG. 1. Diagram of the first-generation bDNA signal amplification assay.

2.2. Sample Collection and Preparation

Serum or plasma is the specimen used in most clinical applications of bDNA. Blood should be collected in sterile tubes and, for plasma specimens, EDTA is the preferred anticoagulant, but acid-citrate-dextrose and heparin are acceptable. The minimum volume required ranges between 0.2 and 2 ml, depending on the application. Unlike many target amplification strategies, nucleic acid extraction and purification are not required for bDNA assays. Nucleic acids are liberated from the virions and denatured by addition of a simple lysis reagent to the sample. The lysis reagent contains stabilized proteinase K and 0.05% sodium azide and 0.05% ProClin as preservatives. An untracentrifugation step (25,000 × *g*), prior to addition of the lysis reagent, is used to concentrate the virions from 1 ml of plasma in the HIV-1 bDNA assay.

Nucleic acid extraction protocols using guanidine hydrochloride, sodium sarcosyl, and ethanol have been developed to quantify viral RNA by bDNA in lymph node tissue, liver tissue, and peripheral blood monocytes (Wilber and Urdea, 1995).

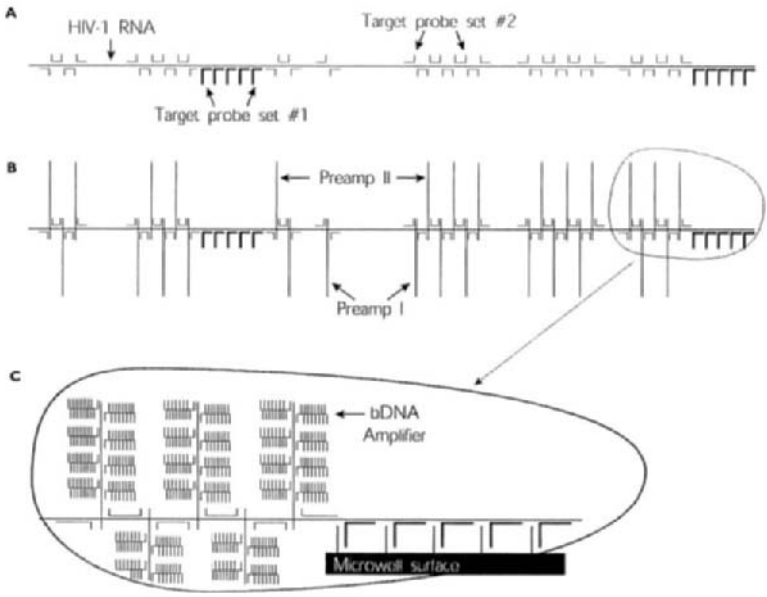


FIG. 2. Schematic representation of the second-generation HIV-1 bDNA signal amplification assay. (A) Target probes hybridize to unique 33-base sequences at different positions along the conserved region of HIV-1 *pol* gene. Target probe set 1 mediates capture of the HIV-1 RNA on the microwell surface, whereas target probe set 2 mediates preamplifier binding. (B) Neighboring target probes are bridged by preamplifier molecules (preamp I and preamp II). (C) Enhancement of the signal is accomplished by the binding of up to eight bDNA amplifier molecules to each preamplifier anof 45 alkaline phosphatase–labeled probes to each bDNA amplifier molecule. (From Kern *et al.*, 1996.)

Because bDNA assays are designed to be quantitative, it is important that the physiological level of virus in the sample be preserved. RNA targets are very susceptible to degradation by the high levels of RNase present in blood. Serum or plasma should be separated from the cells within 4 hours of collection and then, ideally, stored at −70°C until tested (Cuypers *et al.*, 1992; Davis, 1994). Short-term (≤7 days) storage at 4°C of serum or plasma is acceptable. Repeated freezing and thawing of the samples should be avoided to prevent target degradation.

2.3. Target Probes

Probes that mediate capture of the target nucleic acid are termed capture extenders. These probes are approximately 50 bases, one portion (20 to 40 bases) of which is complementary to the target, while the second portion (approximately 20 bases) binds the probe–target complex to a capture probe that is coupled to the surface of a microtiter plate well.

An alkylamine linker is incorporated into either the 5′ or 3′ end of the capture probe and the probe is activated with ethylene glycol bis-succinimidylsuccinate. The activated capture probe is bound to polystyrene microtiter wells precoated with poly(Lys-HBr, Phe). Nearly all of the solid-phase probe coupled to the polystyrene by this procedure is available for hybridization (Running and Urdea, 1990).

Probes that hybridize to the target and also to either preamplifier or amplifier molecules are termed label extenders. The locations of the capture and label extender probes used in the hepatitis B virus (HBV), hepatitis C virus (HCV), and HIV-1 assays are shown in Figs. 3, 4, and 5, respectively. All target probes are designed to hybridize to the most conserved regions of the genomes. For HBV, the

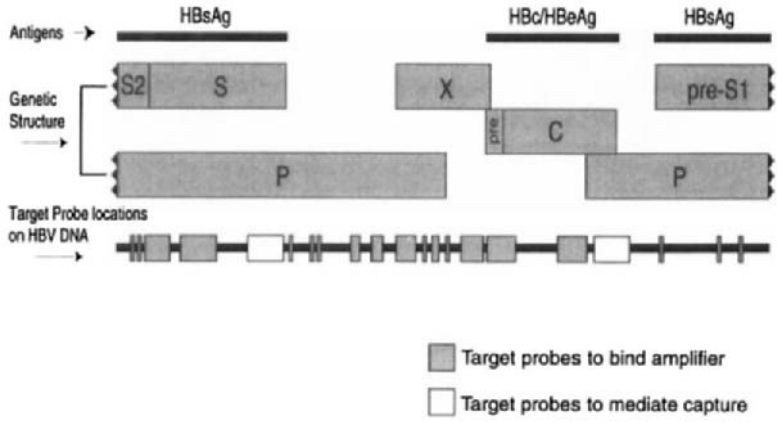


FIG. 3. Location of the probes in the HBV DNA bDNA assay.

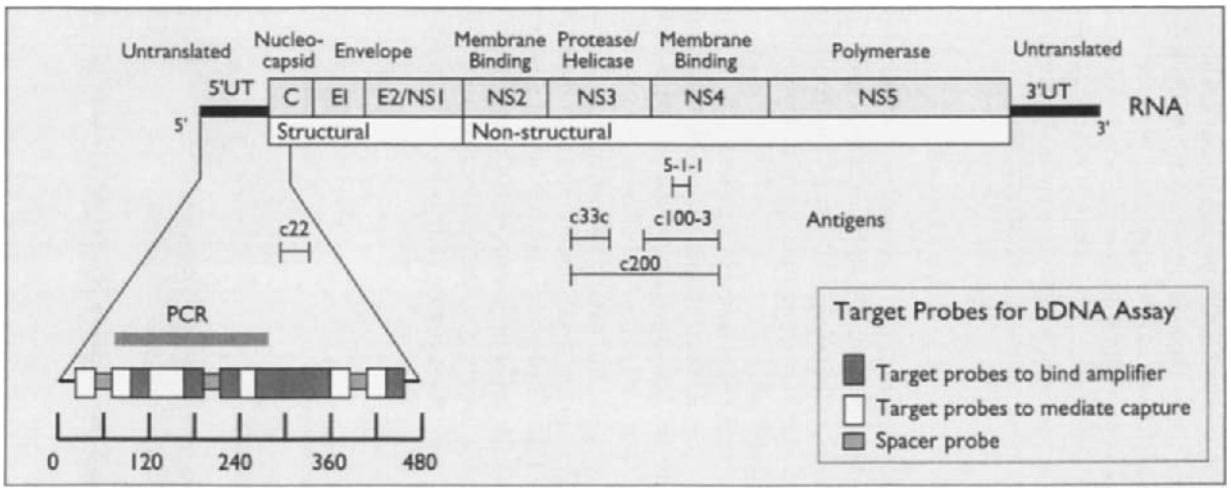


FIG. 4. Location of the probes in the HCV RNA bDNA 2.0 assay.

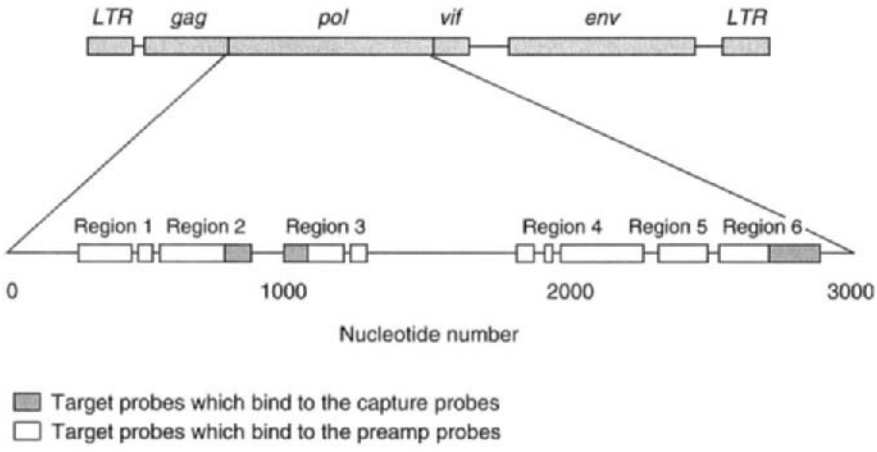


FIG. 5. Location of the probes in the HIV RNA bDNA assay.

probes are located in conserved regions scattered throughout the genome; for HCV, in the 5′ untranslated region; and for HIV-1, in the *pol* gene. Multiple capture and label extender probes are used in each application. The second version HIV-1 assay uses a total of 49 different probes to recognize the HIV-1 sequence. Each probe includes 33 bases, which are complementary to sequences in the *pol* gene. These probes were designed after analyzing the *pol* sequences from 18 isolates of HIV-1 representing the major subtypes, A–E. The use of multiple probes selected from conserved regions makes the bDNA approach particularly well suited for detection and quantitation of targets with known sequence heterogeneity, as is the case with RNA viruses (e.g., HIV-1 and HCV).

2.4. bDNA AMPLIFIERS

The bDNA amplifier molecule is the key to Chiron's signal amplification method. Initially, bDNA amplifiers were synthesized from oligodeoxyribonucleotides that contained three equally distributed N^4-alkylamino cytidines, coupled to one another through bifunctional amine reactive cross-linking agents (Urdea *et al.*, 1987). As few as 60,000 copies of the HBV genome could be detected using these polymers and enzyme-labeled probes. However, construction of larger multimers by this cross-linking method led to only small incremental improvements in signal amplification. Molecular modeling indicated that the internal oligonucleotides located within the nearly spherical cross-linked structure were inaccessible to the large enzyme-labeled probes. The investigators reasoned that the synthesis of linear primary sequences with branching secondary sequences would greatly facilitate binding of the labeled probe by increasing access.

The incorporation of branching monomers (BMs) during the chemical synthesis of oligonucleotides proved to be a more useful method for making effective bDNA amplifiers (Horn and Urdea, 1989). BMs are phophoramidite reagents containing at least two protected hydroxyl functions. BMs are synthesized from 4-(1,2,4-triazolo)-1-(β-D-*O*-dimethoxytrityl-3-*O*-*t*-butyldimethylsiyl-2-dexyribofuranosyl)-5-methyl-2(1*H*)-pyrimidinone by triazole displacement with 6-aminohexanol. Subsequent primary hydroxyl protection followed by phosphitylation yields the derivatives BM1 and BM2. Both primary hydoxyl groups are protected by dimethoxytrityl groups in BM1, whereas in BM2 the N^4-(hydoxylhexyl) function is protected by a levulinyl group and the 5′-hydroxyl function is protected by a dimethoxytrityl group. Two schemes have been used for synthesis of bDNA molecules. The first employs simultaneous deprotection of both hydroxyl functions of BM1 that leads to formation of "fork" structures. The second uses selective removal of the levulinate group from BM2 that leads to formation of "comb" structures. The comb architecture offers a more open network that permits complete access of the secondary sites.

In general, a primary linear fragment is synthesized with multiple appropriately spaced BMs. Several simultaneous secondary syntheses are then conducted from the branch points. bDNAs containing several hundred nucleotides have been prepared in this manner. However, the synthesis of the large bDNA molecules used in the current assays involves a combination of solid-phase chemistry and enzymatic ligation methods (Urdea, 1993). The bDNA amplifier consists of 1068 nucleotides and contains a maximum of 45 enzyme-labeled probe binding sites. It is constructed by synthesizing a molecule with 15 identical branches of 168 nucleotides each, which is then combined with a complementary linker that is in turn complementary to a branch extension or "arm" containing 60 bases, each of which has three binding sites for alkaline phosphatase–labeled probes. The amplifiers are assembled by treatment with T4 DNA ligase and then analyzed by capillary electrophoresis. On average, 11–13 of the possible 15 arms are incorporated into each bDNA molecule.

2.5. Preamplifier

In the second-generation bDNA assay for HIV-1 RNA, preamplifier molecules and unique target probe sets are included in an effort both to reduce the background level and to increase the number of amplifier molecules used to generate the signal (Kern *et al.*, 1996). Two preamplifier molecules, preamp I and preamp II, are used. Preamp I consists of 237 bases and is constructed by enzymatic ligation of three oligomers of 86, 79, and 73 bases by using synthetic linkers. Preamp II consists of 239 bases and was constructed in a manner identical to that used for preamp I except that an 88-base oligomer was substituted for the 86-base oligomer. Hybridization and ligation of the oligomers are carried out overnight with T4 ligase under standard conditions

and full-length products are purified by 10% denaturing polyacrylamide gel electrophoresis (PAGE). The two preamplifier molecules contain the same repeat sequence and differ only in the sequences hybridized to the target probes.

Each preamplifier contains a site for hybridization with sequential overhang sequences of target probe set 2 and eight sites for hybridization with the amplifier molecules (Fig. 2). The overhang sequences of target probe set 2 are 15 or 16 bases in length and individually cannot efficiently bind to the preamplifiers. However, when the overhang sequences of two target probes are adjacent, the T_m increases, thereby stabilizing the hybridization of the preamplifiers. The preamplifier used in the second-generation HIV-1 RNA assay is an equimolar mixture of the two preamplifier molecules.

2.6. Labeled Probe

Alkaline phosphatase–labeled probes are synthesized so that 18 bases are complementary to sequences on the arms of the bDNA. Three hybridization sites are located on each branch for a total binding capacity of 45 labeled probes per bDNA molecule. The alkaline phosphatase catalyzes the dephosphorylation of chemiluminescent substrate, dioxetane (Lumi-Phos Plus, Lumigen, Detroit, MI). The intensity of the light emission is measured with a plate luminometer as relative luminescent units.

2.7. Probes with Nonnatural Bases

The molecular sensitivities of the first and second generations of the bDNA assays were limited by nonspecific hybridization between the amplification probes and other nucleic acids. Short regions of hybridization between any of the probes constituting the amplification system, (preamplifier, amplifier, and labeled probe) and any nontarget nucleic acid sequence leads to amplification of the background signal. Capture probes, capture extenders, and sample nucleic acid are all sources of this background hybridization (Collins *et al.*, 1997).

In order to reduce the hybridization potential to all nontarget nucleic acids, the nonnatural bases isocytidine (isoC) and isoguanosine (isoG) were incorporated into the amplification probes in the third-generation assays (system 8 bDNA). IsoC and isoG are among the six base pairs capable of forming Watson and Crick base pairs joined by mutually exclusive hydrogen-bonding schemes incorporating three hydrogen bonds (Piccirilli *et al.*, 1990; Switzer *et al.*, 1993). Although isoC and isoG can be incorporated into duplex DNA and RNA by DNA and RNA polymerases, these potential extra letters in the genetic alphabet are not found in natural oligonucleotides. IsoC and isoG bases pair with each other but not with any of the four naturally occurring bases. Sequences containing isoC–isoG base pairs are $-2°C$ more stable per base than their C–G congeners. Procedures for prepar-

ing derivatives of these isobases suitable for incorporation into DNA using an automated DNA synthesizer have been described (Switzer *et al.*, 1993).

In the system 8 bDNA assay for HIV-1, approximately every fourth nucleotide of the preamplifier, amplifier, and alkaline phosphatase–labeled probe was either isoC or isoG, which attenuates the hybridization of these molecules to natural sequences (Collins *et al.*, 1997). The use of isoC- and isoG-containing probes in the system 8 bDNA assays increased the target-specific amplification without a concomitant increase in the background from nontarget sequences, thereby greatly enhancing the sensitivity to a detection limit of approximately 50 molecules/ml. A prototype system 8 bDNA assay for HCV RNA in plasma is claimed to have a detection limit of 200 molecules/ml (Chiron, unpublished data).

2.8. Molecular Standards

The accuracy of any quantitative assay depends on the use of standards that have been thoroughly characterized by accepted and independent methods. Without careful preparation of standards, the reported values for samples will be systematically higher or lower than the true value. Chiron has devoted considerable effort to the development of "gold standard" preparations of RNA from HIV-1 and HCV and DNA from HBV for use in the bDNA assays. These standards have been made available to the U.S. Food and Drug Administration and the World Health Organization.

The preparation and characterization of RNA standards for use in bDNA assay for HCV were recently described by Collins and coworkers (Collins *et al.*, 1995). *In vitro* transcripts from cDNA clones containing the probe binding regions were prepared and purified by phenol extraction after gel electrophoresis or column chromatography. Aliquots of the transcripts were digested to nucleosides and phosphate and quantified by phosphate analysis against the U.S. National Institute of Standards and Technology phosphate standard. The quantitation was checked by optical density (OD_{260}), hyperchromicity, and isotopic tracer analysis. RNA standards were assigned to quality level 1 or 2 or were rejected based on the criteria for % full-length, % correct sequence, and accurate quantitation by phosphate, hyperchromicity, and OD_{260}. Quality level 2 RNA standards are suitable for the bDNA assays. Quality level 2 standards must be >50% full length, >99% correct sequence, and quantitate within 20% by the different chemical methods.

The standard RNAs were used to test the ability of the bDNA assay to quantify accurately target RNAs regardless of size or slight sequence variation. Standard RNA preparations of 1.3, 2.2, and 3.2 kb showed no detectable effect on quantitation. The quantitation of standard transcripts prepared from clones of HCV subtype 1a and 3a differed by a factor of 1.6-fold with one probe design and were indistinguishable with another probe design. These two 475-mer transcripts differed at 30 positions.

Three different lots of a standard 3.2-kb HCV RNA were serially diluted and quantified over a thousandfold range. The average signal per attomole of target varied by less than 20% among the three lots.

The reference standards are used to quantitate the standards that are employed in the kits to generate the standard curves. The kit standards are recombinant single-stranded DNA molecules that are added to either negative serum or plasma at known concentrations. Because the standard curve is not constructed with reference standards, Chiron initially chose to use the term "equivalent" to describe the units of nucleic acid quantitation in clinical samples. An equivalent was defined as the amount of nucleic acid in a clinical sample that gave a signal equal to one molecule of the reference standard nucleic acid. The term "copy" rather than "equivalent" is used to describe the units of nucleic acid quantitation in the HIV-1 bDNA assay. The terms are now used interchangeably.

2.9. Assay Requirements

Each bDNA kit contains sufficient reagents and materials to perform one 96-well assay consisting of 4 standards, 2 controls, and 42 patient specimens tested in duplicate. The kit may also be used to assay two half-plates consisting of 4 standards, 2 controls, and 18 patient specimens tested in duplicate. The enzyme-linked immunosorbent assay (ELISA)–like format of procedure requires general-purpose equipment found in most molecular diagnostic laboratories and equipment specifically designed by Chiron for use in bDNA assays. The general-purpose equipment includes precision pipettes, a 12-channel pipette, serological pipettes, transfer pipettes, a vortex mixer, a heat block, a water bath, a microcentrifuge, a vacuum wash system with an eight-well aspirator, and a class II biological safety cabinet. The sample preparation protocol for the HIV-1 assay also requires a refrigerated centrifuge with RCF of 23,500 $\times$ g and a 45° fixed-angle rotor capable of accommodating at least twenty-four 1.5-ml tubes.

Chiron provides a microwell plate heater, a luminometer, and data management software. The plate heater is specially designed to provide precise control of the hybridization temperature (0$\pm$0.5°C) and to distribute heat evenly throughout the microwell plate. The luminometer maintains a temperature of 37°C and accommodates the 96-well plates. The data management software runs on an IBM PC or compatible computer with a minimum of 80386, 16-Mhz microprocessor, 2 Mb of RAM, monitor, mouse, compatible printer, MS DOS (version 5.0 or greater), and Windows (version 3.1 or greater).

Recently, Chiron introduced the System 340 platform that automates incubation, washing, reading, data processing, and report generation. It is anticipated that automation will dramatically reduce operator-to-operator differences while decreasing labor requirements. In one laboratory the average coefficient of variation was reduced 43% and the hands-on labor was reduced 39% with the automated

platform for the HIV-1 RNA 2.0 assay (Chiron, unpublished data). Future improvements in reproducibility and operational efficiency await the development of a system for automated sample preparation.

2.10. Data Analysis

Light emission from the chemiluminescent substrate is directly proportional to the amount of the target nucleic acid in the sample, and the results are recorded as relative luminescence units (RLUs). All samples, standards, and controls are run in duplicate, and the mean RLU is used in data analysis. The percent coefficient of variation (%CV) for duplicate RLU for controls and samples must be within the recommended limit for that assay for the results to be valid. For example, negative samples must have a CV of <30% and positive samples <20% in the HCV assay.

A standard curve is defined by light emission from the standards containing known concentrations of recombinant bacteriophage. A quadratic equation is used to fit the curve to the RLU of the four standards. A maximum of two points from different standards may be eliminated by the data management software in order to achieve the best curve fit. The concentration of the target nucleic acid in the sample is determined from this standard curve. An example of the output from the data management software for the second-generation HCV assay is shown in Fig. 6.

The assay controls include the four standards (A to D) and two controls. Depending on the application, the controls may be low and high positives or a positive and a negative. The RLU for the standards must be in rank order and the r^2 value for the curve fit must be ≥ 0.985. In addition, the concentration of the target nucleic acid in the low and high controls must be within limits, and the %CV of the RLU must be ≤ 25:% for the run to be considered valid. If a negative control is included, it must quantify below the lower limit of the assay. Any samples with mean RLU $\geq$ the lower limit of the assay with %CV higher than the recommended limit must be retested. The data management software automatically checks these quality control criteria.

3. Signal versus Target Amplification Systems

Both target and signal amplification systems have been successfully employed to detect and quantitate specific nucleic acid sequences in clinical specimens. Polymerase chain reaction (PCR), nucleic acid sequence–based amplification (NASBA), transcription-mediated amplification (TMA), strand displacement amplification (SDA), and ligase chain reaction (LCR) are all examples of enzyme-mediated, target amplification strategies that are capable of producing billions of

CHIRON HCV RNA 2.0 ASSAY — Software Version 3.43 RUO

Operator: C. THURMOND — ID List File: 101497.DML

Lot Number: MMB995 — Expiration Date: 012798

Data File: 971014C1.DMS — Collected: 10/14/97 10:41

Relative Luminescence

	1	2	3	4	5	6	7	8	9	10	11	12
A	172.9	189.2	22.17	22.29	2.531	2.612	0.755	0.750	2.904	2.477	77.90	83.31
B	15.69	20.08	1.533	1.538	20.35	15.32	7.584	6.823	12.45	12.88	1.803	1.554
C	9.447	9.268	6.672	6.013	5.921	5.662	6.450	6.186	4.275	4.221	6.299	5.797
D	32.78	36.24	3.665	4.799	1.964	1.959	2.332	2.342	6.515	6.823	26.86	30.23
E	7.859	7.908	9.204	9.787	5.284	4.815	9.376	8.318	12.11	13.58	2.618	3.001
F	2.045	1.884	43.89	44.75	4.458	4.534	0.858	0.901	13.98	13.87	8.664	8.901
G	1.538	1.403	2.823	3.039	45.35	41.27	15.69	15.44	2.126	2.116	1.695	1.441
H	1.538	1.570	5.722	6.801	6.882	6.272	0.350	0.663	0.966	1.058	2.369	2.272

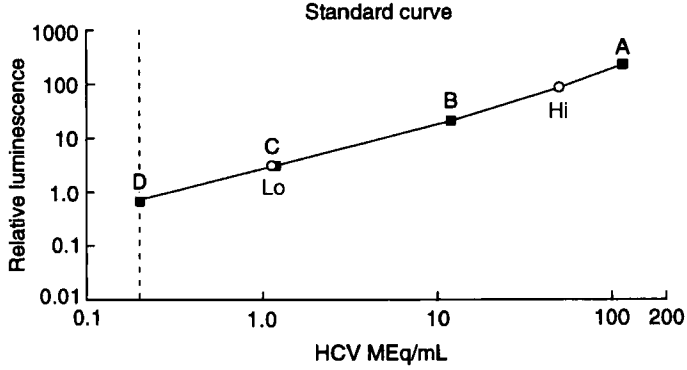
Standards and Controls

Loc	ID	MEq/mL	Log Avg RL	%CV
A1-2	Std A	120	180.9	6.4
A3-4	Std B	12	22.23	0.4
A5-6	Std C	1.2	2.571	2.2
A7-8	Std D	0.2	0.752	0.5
A9-10	Lo-Ctl	1.3	2.682	11.2
A11-12	Hi-Ctl	56	80.56	4.7

Comments

Control Results

	MEq/mL
Lo-Ctl	1.118
Hi-Ctl	51.69



Diagnostics: 0.999 0.390 <0.001 0.796 <0.001 0.052 0.029

FIG. 6. Example of the output from the data management software for the second-generation HCV bDNA assay.

copies of a nucleic acid target (Compton, 1991; Guatelli *et al.*, 1990; Saiki *et al.*, 1988; Walker *et al.*, 1992; Wu and Wallace, 1989). Because these methods produce large amounts of amplified product, relatively insensitive means can be used for product detection. A major challenge in developing quantitative target ampli-

fication systems has been in establishing the relationship between the initial amount of the target sequence in the specimen and the amount of amplified product (Clementi *et al.,* 1993; Piatak *et al.,* 1993).

The total amplification achieved by PCR is described by the expression, $(1 + E)^n$, where E is the average per-cycle efficiency and n is the total number of cycles. The amount of target sequence and the variable presence of inhibitors in clinical specimens influence both the efficiency and the kinetics of amplification. As seen in the preceding expression, small differences in the efficiency of amplification are exponentially compounded and lead to very large and unpredictable differences in product yield. The situation is even more complicated when the target is RNA. PCR must be preceded by reverse transcription to produce complementary DNA (cDNA), and the efficiency of this process is another variable that may influence product yield.

Competitive PCR (cPCR) has emerged as the best strategy for controlling the sample-to-sample variability of PCR. In cPCR different templates of similar lengths and with the same primer binding sequences are coamplified in the same tube. This ensures identical thermodynamics and amplification efficiency for both templates. The amount of one of the templates must be known and, after amplification, products of both templates must be distinguishable and separately quantifiable.

Because the templates compete for amplification and, in the case of reverse transcription PCR (RT–PCR), also for reverse transcription, any variable affecting amplification has the same effect on both. Thus, the ratio of PCR products reflects the ratio of the initial amounts of the two templates as demonstrated by the function $C/W = C^i\ (1+E)^n / W^i(1+E)^n$, where C and W are the amounts of competitor and wild-type product, respectively, and C^i and W^i are the initial amounts of competitor and wild-type template, respectively, (Clementi *et al.,* 1993). From this linear relationship, it could be concluded that a single concentration of competitor could be sufficient for quantitating unknown amounts of wild-type templates. However, in practice, the precise analysis of two template species in very different amounts has proved difficult and cPCRs using three to four competitor concentrations within the expected range of wild-type template concentrations are usually performed. In a recent study of different standardization concepts in quantitative RT-PCR assays, coamplification on a single concentration of a competitor with wild-type template was comparable to using multiple competitor concentrations and was much easier to perform (Haberhausen *et al.,* 1998).

In bDNA the number of target molecules is not altered. The signal of direct hybridization rather than the nucleic acid sequence itself is amplified and thus is directly proportional to the amount of target sequence present in the clinical sample. Both RNA and DNA sequences can be measured directly in clinical specimens, and there is no need to transcribe RNA into cDNA as there is with PCR.

bDNA is a nonenzymatic process and is less prone to sample-to-sample variation that is a concern with the enzymatically mediated target amplification sys-

tems. Because enzyme inhibitors are not a concern in bDNA assays, the cumbersome sample preparation steps often used to remove inhibitors are not required. Also, the efficiency of nucleic acid purification schemes used in target amplification assays introduces another source of variability.

A major limitation of all target amplification systems is false-positive reactions resulting from carryover contamination of negative sample with amplified product. The tremendous numbers of target molecules produced in each reaction can be difficult to contain. Physical separation of pre- and postamplification activities, unidirectional work flow, plugged pipette tips, physical containment, and enzymatic or chemical methods for amplicon inactivation are all used to limit false positives with PCR and other target amplification strategies. None of these practices are necessary with bDNA because the target molecules are not amplified.

False-positive results with bDNA have been observed with proficiency testing specimens for HIV-1 in the College of American Pathologists HIV-1 viral load survey and HCV in the viral quality control program administered by the Netherlands Red Cross. The reason for the false-positive results with these proficiency testing specimens is not known but may be sample matrix effects. The extent to which this problem occurs with clinical samples has not been determined. However, both the HIV-1 and HCV bDNA assays were designed to have a false-positive rate of $\leq 5\%$.

Target amplification systems generally have greater analytical and clinical sensitivity than bDNA. However, the use of the preamplifier molecules and probes containing isobases has lowered the limits of detection and quantitation to levels that rival PCR in some applications (Collins *et al.,* 1997; Kern *et al.,* 1996). The sensitivity of bDNA is ultimately limited by the signal-to-noise ratio. The principal component of the noise in bDNA is nonspecific hybridization between the amplification sequences and other nucleic acids. The use of amplifier probes containing isobases has effectively reduced the noise due to nonspecific hybridization. In principle, this should allow the use of preamplifiers and larger amplifiers or the use of multiple layers of amplification to further enhance sensitivity, because the signal can be augmented without concomitant amplification of noise.

To test this hypothesis, 5 attomoles of three different isoC- and isoG-containing targets were hybridized to capture probes on microtiter wells (Collins *et al.,* 1997). The first target was detected by hybridization with the alkaline phosphatase probe; the second target was detected by hybridization with amplifier followed by alkaline phosphatase probe; and the third target was detected by hybridization with preamplifier, followed by amplifier, followed by alkaline phosphatase probe. The signal was measured by dioxetane chemiluminescence in RLU. The noise was the RLU associated with no oligonucleotide target. The signal-to-noise ratios were 5.5, 19.6, and 154.3 for the first, second, and third targets, respectively.

PCR and related target amplification systems typically employ a single pair of primers. Each primer is usually 20 to 40 bases in length and anneals to the complementary sequence on the target nucleic acid to initiate the amplification reac-

tion. Mismatches between the primer and target sequences can lead to failure to amplify the target or to inefficient amplification, depending on the number and position of the mismatches. PCR primers must be carefully selected from highly conserved regions to ensure equal amplification of all genotypes.

bDNA employs as many as 49 different target-specific probes to recognize the target sequence in clinical samples. Each probe includes approximately 30 bases that are complementary to target sequence. In theory, bDNA should be less susceptible than PCR to errors resulting from target sequence heterogeneity. In practice this has been demonstrated for two viral RNA targets, HCV and HIV-1 (Dunne and Crowe, 1997; Hawkins *et al.*, 1997).

In summary, bDNA has a number of distinct theoretical and practical advantages over target amplification systems for direct quantitation of specific nucleic acid sequences. The following sections review the specific clinical and research applications of this technology.

4. Infectious Disease Applications

4.1. Hepatitis B Virus

Despite the availability of an effective vaccine, HBV infections remain a major global public health problem. About 5% of patients become chronic carriers of hepatitis B surface antigen (HBsAg) after acute infection, and there are an estimated 300 million HBsAg carriers worldwide (Lau and Wright, 1993). Chronic HBsAg carriers can be divided into two groups: those with low-level viral replication and normal liver function tests and those with active viral replication and progressive liver disease that may progress to cirrhosis and death (Hoofnagle *et al.*, 1987; Kaneko *et al.*, 1990).

Many assays are used for diagnosis and management of HBV infections. The presence of HBsAg in serum or plasma indicates HBV infection but does not provide information on the replicative activity of the virus. The secretory version of the HBV core protein, the e antigen (HBeAg), has served as a marker for active viral replication. In the treatment of chronic hepatitis B, the presence or absence of HBeAg is an indicator of a high or low replicative state of the virus, respectively. However, HBV precore mutants, which do not produce HBeAg irrespective of their replicative state, have been described (Carmen and Thomas, 1992).

The level of HBV DNA in serum or plasma probably better reflects the replicative activity of HBV. Several assays for the quantitation of HBV DNA are commercially available. In the Genostics assay (Abbott Laboratories), an ^{125}I-labeled probe binds to single-stranded HBV DNA in solution, followed by separation of free probe and hybrids using Sepharose chromatography (Kuhn *et al.*, 1988). The

radioactivity in the column eluate is measured in a gamma counter. A cutoff value above which a sample is considered reactive is calculated from three negative and two positive controls (HBV DNA content 103 pg/ml or 3×10^7 copies/ml). A quantitative result in pg/ml is calculated by a simple formula that relates the sample counts to the negative and positive control counts. The concentration HBV DNA in the positive control standard is determined indirectly by plaque assays.

In the Hybrid-Capture assay (Digene), a full-length RNA probe is hybridized to denatured HBV DNA in solution and the hybrids are captured on the surface of a tube coated with anti DNA:RNA hybrid antibody. The bound hybrids are reacted with antihybrid antibody labeled with alkaline phosphatase. A chemiluminescent substrate is converted to a luminescent compound by the bound alkaline phosphatase. Light emission is measured in a luminometer and the concentration of HBV DNA, in pg/ml, is determined from a standard curve. The concentrations of the standards are determined spectrometrically (A_{260nm}/A_{280nm}).

The Quantiplex HBV DNA assay (Chiron) represents a first-generation bDNA assay (Hendricks *et al.,* 1995). It employs a total of 48 target probes, 9 of which mediate capture of HBV DNA to the microwells and 39 mediate binding of the amplifier molecules. The target probes are complementary to approximately 45% of the minus-sense strand of the HBV genome (Fig. 3). The standard curve is 7×10^5 to 4.5×10^9 HBV DNA equivalents/ml. One picogram of double-stranded HBV DNA equals 2.85×10^5 DNA equivalents. The primary HBV standard used for quantitation of assay standards is the entire HBV genome, subtype *adw2*, which was purified from a recombinant plasmid and quantitated by total phosphate determination, diphenylamine analysis, absorbance (A_{260nm}) of intact DNA, and absorbance (A_{260nm}) of deoxyribonucleosides released by complete digestion of DNA with calf intestinal phosphatase and snake venom phosphodiesterase (Collins *et al.,* 1995; Urdea, 1992).

The bDNA assay exhibited nearly a four-log dynamic range of quantitation and an analytical sensitivity of approximately 1×10^5 equivalents/ml (Hendricks *et al.,* 1995). At a quantitation limit of 7×10^5 equivalents/ml, the assay had a specificity of 99.7% when sera from 987 healthy blood donors were tested. The interassay CV ranged from 10 to 15% when performed by novice users with different sets of reagents. HBV DNA was detected in 94% of HBeAg-positive specimens from chronically infected patients from the United States and in 100% of HBeAg-positive specimens from chronically infected patients from Japan. HBV DNA was detected less frequently in HBeAg-negative specimens. HBV DNA was detected in 27% and 31% of HBeAg-negative specimens from patients in the United States and Japan, respectively.

Several comparisons of bDNA with other methods for quantitation of HBV DNA have been published (Butterworth *et al.,* 1996; Hwang *et al.,* 1996; Kapke *et al.,* 1997; Pawlotsky *et al.,* 1997; Zaaijer *et al.,* 1994). Although these comparisons have been difficult owing to the use of different standards and units of mea-

surement as well as differences between assays in dynamic ranges and quantification limits, the studies are consistent in their findings.

The analytical sensitivities of the different quantitation methods have been compared using serial dilutions of patients' specimens (Butterworth *et al.*, 1996) and the Eurohep HBV DNA standards (Zaaijer *et al.*, 1994). In both cases, bDNA was shown to be about 10-fold more sensitive than the liquid hybridization (Abbott) and the hybrid capture (Digene) assays. Using the Eurohep HBV standards, the detection limits were 2.5×10^6 genomes/ml for bDNA and 2.5×10^7 genomes/ml for both liquid hybridization (LH) and hybrid capture (HC) assays.

Clinical sensitivity of the three assays has been assessed in several different settings. In an evaluation employing 109 randomly selected HBsAg-positive sera, HBV DNA was detected in 39% by bDNA and in 28% by LH (Zaaijer *et al.*, 1994). Both assays detected HBV DNA more frequently in the 30 sera that were also HBeAg positive (bDNA, 73%; LH 67%) than in the 79 sera that were HBeAg negative (bDNA, 25%; LH, 13%). Hwang *et al.* (1996) compared the sensitivity of bDNA and LH using 114 serial specimens obtained from 13 patients with chronic active hepatitis B who had received 600 mg of ribavirin daily for 4 weeks. bDNA detected HBV DNA in 94% and LH in 77% of the sera. Only two of seven sera were below the quantitation limit of the bDNA assay and were found to contain HBV DNA by a sensitive, qualitative PCR assay. When the data were stratified by bDNA result, LH detected HBV DNA in 95% of the sera with $\geq 10^7$ genome equivalents/ml and in only 17% of the sera with $\leq 10^7$ genome equivalents/ml. The authors also examined the effect of ribavirin therapy on serum HBV DNA levels measured by bDNA. The geometric mean level of HBV DNA decreased from 227 million equivalents (Meq)/ml at the start of therapy to 182 Meq/ml at the end of therapy and finally to 15 Meq/ml 8 weeks after treatment. Four patients lost serum HbeAg, and two patients lost serum DNA by both bDNA and PCR 8 weeks after therapy.

Analysis of paired assay results below and above each assay's limit of quantitation (LOQ) indicated that a significantly larger proportion of observations were below the LH LOQ but above the bDNA LOQ, indicating that bDNA was more sensitive than LH (Kapke *et al.*, 1997). In another study comparing bDNA and HC for quantitation of HBV DNA in 300 HBsAg-positive sera, 15% more positives were detected with bDNA (Pawlotsky *et al.*, 1997). However, HBV DNA was detected by a qualitative PCR in 50% of the specimens with values below the bDNA LOQ. A large percentage, 49%, of these bDNA-negative, PCR-positive patients had no clinical or biological evidence of disease.

Clearly, bDNA is more sensitive than LH, HC, and in-house membrane hybridization assays, but its relatively high LOQ is not optimal for detecting lower titers associated with active liver disease. There have been no differences in clinical specificity among the HBV DNA quantitation methods reported.

The correlation, agreement, and precision of results of the different commer-

cially available assays have also been examined in several studies. The results of the three assays correlated (r values 0.70 to 0.96) but the agreement was poor (Butterworth *et al.*, 1996; Hwang *et al.*, 1996; Kapke *et al.*, 1997; Pawlotsky *et al.*, 1997; Zaaijer *et al.*, 1994). The bDNA results were consistently higher than LH and HC results. The ratios of results for bDNA to LH ranged from 6 to 40 and for bDNA to HC from 0.4 to 115. Kapke *et al.* (1997) developed the following equation for conversion of LH values to bDNA values, which maximized the linear correlation: $\text{Meq/ml} = 5.82 \times (\text{pg/ml})^{1.24}$. The poor agreement between the assays is attributable to differences in the technology and in the HBV DNA standards employed. The accuracy of each assay cannot be assessed in the absence of a commonly accepted gold standard HBV DNA preparation. The HBV bDNA assay was more precise than the other commercially available assays with reported CVs ranging from 5 to 20%.

Monitoring levels of HBV DNA in serum may be useful for identifying individuals most likely to respond to antiviral therapy, evaluating the efficacy of therapy, and following the viral load after therapy (Hendricks *et al.*, 1995). During therapy the clinician can monitor viral load, perhaps by measuring the absolute reduction in or the kinetics of decrease in viral load. The enhanced sensitivity of bDNA is clinically relevant because its use will identify more patients with actively replicating HBV and these patients are candidates for antiviral therapy. Clearly, target amplification methods for HBV DNA are more sensitive than bDNA with analytical detection limits of 0.4 to 4 fg/ml. However, it is not clear how ultrasensitive detection methods will be useful in management of chronic hepatitis B, with reports of long-term persistence of low levels of HBV DNA in individuals who show clinical and serologic resolution of disease and infection (Carreno *et al.*, 1992; Kuhn *et al.*, 1988; Mason *et al.*, 1992).

4.2. Hepatitis C Virus

For more than 20 years, researchers sought the virus or viruses that were thought to be the cause of non-A, non-B (NANB) hepatitis. Attempts to identify a virus by conventional immunological and virological methods failed. In 1989, a group from the Centers for Disease Control and the Chiron Corporation cloned a portion of the HCV genome from the blood of a chimpanzee inoculated with the serum of a patient with NANB hepatitis (Choo *et al.*, 1989). HCV now recognized as the major cause of what used to known as NANB hepatitis. Nearly 4 million Americans are infected with HCV, and it is responsible for an estimated 8000 deaths annually. Hepatitis C is now the leading reason for liver transplantation in the United States.

HCV is a single-stranded RNA virus of the Flaviviridae family. It has a 9.4-kb positive-sense genome encoding a polyprotein precursor of 3011 amino acids. Individual isolates of HCV consist of closely related yet heterogeneous populations

of viral genomes referred to as quasispecies (Martell *et al.*, 1992). Phylogenetic analysis of nucleotide sequences from different HCV isolates from around the world established six major genotypes and many more subtypes (Simmonds *et al.*, 1994). The extensive genetic heterogeneity of HCV has important diagnostic and clinical implications.

Interferon-α is currently the only agent of proven clinical efficacy in the treatment of hepatitis C; however, only 10 to 51% of patients enrolled in clinical trials showed a sustained improvement (Davis *et al.*, 1989; Di Bisceglie *et al.*, 1989). Current interferon-α therapy is, typically, 3 million units, thrice weekly, given subcutaneously for 12 months. Both HCV genotype and pretreatment viral load have been shown to influence the response to interferon (Lau *et al.*, 1993; Yoshioka *et al.*, 1992).

The practicing clinician has a number of different tests available to aid in the evaluation of patients with suspected hepatitis C. These include measurement of alanine aminotransferase (ALT) levels, liver biopsy, serological tests (ELISA and recombinant immunoblot assay), and molecular methods for detection and quantitation of HCV RNA.

The quantitation of HCV RNA in serum may be important in predicting and monitoring response to antiviral therapy (Davis, 1994). A variety of methods have been used to quantitate HCV RNA, including endpoint dilution RT-PCR, competitive PCR, multicyclic RT-PCR, nucleic acid sequence–based amplification, RT-PCR with a single internal quantitation standard, and bDNA (Chazouilleres *et al.*, 1994; Detmer *et al.*, 1996; Ishiyama *et al.*, 1992; Kaneko *et al.*, 1992; Klevits *et al.*, 1991; Kobayashi *et al.*, 1993; Miskovsky *et al.*, 1996).

The current bDNA assay for HCV RNA (Quantiplex HCV RNA 2.0) is a second-generation assay that was redesigned to provide accurate quantitation of all HCV genotypes. Although the first-generation assay included multiple probes designed to include HCV sequence diversity, the hybridization efficiency and quantification varied among the HCV genotypes such that version 1.0 underestimated genotype 2 by a factor of 3 and type 3 by a factor of 2 (Collins *et al.*, 1995; Lau *et al.*, 1995).

The version 2.0 assay uses a different set of probes designed to hybridize to genotypes 1 to 6 with equal efficacy (Fig. 4). The new probe set not only enhanced the efficiency of binding to genotypic variants but also lowered the LOQ from 3.5×10^5 to 2×10^5 HCV RNA equivalents/ml (Detmer *et al.*, 1996). The version 2.0 assay displayed almost a 600-fold dynamic range up to 1.2×10^8 RNA equivalents/ml. The LOQ was set at 2×10^5 to ensure a specificity of ≥95%. The assay was reproducible, with a mean CV of 14% for replicates of low-, middle-, and high-titer sera. Serial dilutions of quality level 1 RNA transcripts (Collins *et al.*, 1995) representing 5′ untranslated and core sequences from each of the six major genotypes were tested in the version 2.0 assay and the maximum variance observed was a 1.5-fold difference in quantification between genotypes 3a and 6a.

The sample preparation is minimal, and as many as 42 patient specimens can be tested in duplicate with one kit.

Hawkins *et al.* (1997) examined the accuracy of four different methods for the quantitation of HCV RNA in plasma using samples from individuals infected with different genotypes 1, 2, and 3 and using RNA transcripts of predetermined concentrations. The methods employed were bDNA versions 1.0 and 2.0, a commercially available quantitative RT-PCR (HCV Monitor, Roche) (Miskovsky *et al.*, 1996), and an in-house method based on limiting dilution RT-PCR with nested primers from the highly conserved 5′ noncoding region. Replicate testing revealed that highly reproducible results were obtained with both bDNA 1.0 and 2.0, with $\log_{10}$ variances of 0.102 and 0.052, respectively. Greater variability was observed in the results of the RT-PCR methods with $\log_{10}$ variances of 0.184 and 0.316 for the in-house and Monitor assays, respectively. Significant differences in the efficiency of detection of genotypes 1, 2, and 3 were observed for bDNA 1.0 and Monitor assays, whereas bDNA 2.0 and limiting dilution RT-PCR were able to quantitate the different genotypes with similar efficiencies. By quantitating RNA transcripts of different genotypes, the sensitivities of the Monitor assay for sequences of type 2 and 3 transcripts were estimated to be 11% and 8% of those achieved with type 1. The genotype sensitivities of bDNA 2.0 for type 2 and 3 transcripts were 50% and 68% of those achieved with type 1.

When correction factors were applied to Monitor results to adjust for the different efficiencies of genotype quantitation, the observed differences in virus load between patients' specimens with different genotypes became insignificant. This approach has also been used to resolve the apparent genotype differences in virus load observed with bDNA 1.0 (Lau *et al.*, 1995). These results suggest that many of the previous studies evaluating the effect of genotype and virus load on the response to interferon using the Monitor and bDNA 1.0 require reinterpretation.

Jacob *et al.* (1997) compared the relative sensitivities of bDNA 1.0 and 2.0 with that of the Monitor assay for detection of HCV RNA in 174 serum samples from 53 patients with chronic hepatitis C. The sensitivities of bDNA 2.0 and Monitor were similar at 91% and 92%, respectively, and both were more sensitive than bDNA 1.0 ($p < .001$). Both assays detected HCV RNA in all 11 patients with genotypes 2a, 2b, or 3a, whereas only 45% were detected in bDNA 1.0. Major differences in quantitation between bDNA 2.0 and Monitor were noted on a given specimen, with the Monitor result an average of 41-fold lower (range 0 to 703-fold) than those obtained with bDNA 2.0. Plots of values obtained with the Monitor and bDNA 2.0 assays revealed a persistent bias. This bias became larger as the RNA concentration increased, indicating a possible saturation effect in Monitor. These results indicate that both methods can be used to detect HCV RNA in patients infected with the genotypes most commonly found in the United States. The bDNA 2.0 may offer advantages over the Monitor assay for quantifying high-level viremia.

bDNA has also been used to quantitate HCV RNA in the liver of patients with chronic hepatitis C (Idrovo *et al.,* 1996). Frozen liver biopsy specimens were weighed and then rapidly homogenized in 8 M guanidine HCl. RNA in the homogenized tissue was precipitated with one-half volume of ethanol. After the precipitate was washed with 70% ethanol, it was resolubilized in the bDNA sample diluent and processed in the same manner as serum samples. The final results were normalized on the basis of weight and expressed as HCV RNA genome equivalents per gram of tissue. There was a strong correlation between RNA levels in the right and left lobes of the liver as well as a strong correlation between RNA levels in the liver and serum. However, there was no significant correlation between severity of hepatic histology (Knodell's index) and levels of HCV RNA in serum and liver among patients with chronic active hepatitis.

Quantitative HCV RNA assays are useful tools for investigators in studying the natural history, pathogenesis, progression, and treatment of hepatitis C. However, the role of these assays in the management of individual patients has not been established. The National Institutes of Health convened a consensus development conference on the management of hepatitis C on March 24, 1997 (Internet http://consensus.nih.gov). The expert panel recommended that interferon therapy should not be withheld from patients on the basis of HCV RNA levels, mode of acquisition, risk group, HIV status, or HCV genotype. The efficacy of interferon-α therapy is currently defined biochemically as normalization of serum ALT and virologically as the loss of serum RNA. These two parameters are assessed at two time points: at the end of treatment and 6 months post treatment. Nonresponders to interferon can be identified early by assessing the serum ALT level and presence of serum RNA after 3 months of therapy. If the ALT level remains abnormal and HCV RNA remains detectable in the serum, then therapy should be stopped, as further treatment with interferon is unlikely to produce a response. The failure of bDNA to detect low levels of viremia limit its use in assessing the efficacy of interferon therapy. Determination of viral load may become more clinically relevant as effective alternative therapies become available.

4.3. Hepatitis G Virus/GB Virus C

Viruses associated with hepatitis in humans were identified independently in two laboratories and designated hepatitis G virus (HGV) and GB virus C (GBV-C) (Linnen *et al.,* 1996; Simons *et al.,* 1996). The viruses show a high degree of sequence homology (84%) and are referred to collectively as HGV/GBV-C. HGV/GBV-C is a positive-sense RNA virus of approximately 9300 nucleotides that encodes a single polyprotein. The virus is distantly related to HCV and its genetic organization is similar to that of members of the Flaviviridae family. Parenteral transmission of HGV/GBV-C has been documented, but the clinical implications of infection and its disease associations are not clearly defined.

Although as many as 20% of patients with chronic hepatitis C may be coinfected with HGV/GBV-C, coinfection does not appear to influence the severity of liver disease or response to interferon-α therapy (Martinot *et al.*, 1997).

A prototype bDNA assay was developed for quantification of HGV/GBV-C RNA in serum (Pessoa *et al.*, 1997). The assay employed target probes based on the relatively conserved sequence in the 5′ untranslated region of the HGV/GBV-C genome. Preamplifier molecules and incorporation of isoC and isoG into the sequences common to bDNA assays were used to enhance the analytical sensitivity. The provisional limit of detection was 32,500 genome equivalents/ml based on dilutions of a 700-nucleotide synthetic HGV/GBV-C RNA transcript. The run-to-run variance of the assay was $<15\%$.

The bDNA assay was used to study the clinical impact of coinfection and the effects of interferon-α and ribavirin therapy of HGV/GBV-C RNA levels in patients chronically infected with both HGV/GBV-C and HCV (Lau *et al.*, 1997; Martinot *et al.*, 1997; McHutchinson *et al.*, 1997). There were no differences in the clinical, biochemical, or histological features of the coinfected patients compared with those infected with HCV alone. Interferon-α treatment caused a marked but usually transient reduction in serum levels of HGV/GBV-C RNA, and ribavirin caused a modest reduction of viral load of 0.5 to 1 $\log_{10}$.

HGV/GBV-C infection was present in 22% of patients with end-stage cryptogenic cirrhosis undergoing liver transplantation (Pessoa *et al.*, 1997). Serum HGV/GBV-C RNA levels were measured by bDNA and were not correlated with severity of liver disease as judged by histological score. Patients with HGV infection were similar in their clinical and histological features to those without HGV infection, raising questions regarding the etiologic importance of this virus in cryptogenic liver disease.

4.4. Human Immunodeficiency Virus Type 1

The quantitation of HIV-1 RNA in plasma has provided insights into the natural history of HIV-1 infection and expedited the development of antiretroviral drugs. The plasma HIV-1 RNA level, a direct measure of viral burden, is the best available marker of HIV-1 infection according to the following criteria. Levels of plasma HIV-1 RNA are associated with disease stage and progression, have low biological variability, and are strongly correlated with other known prognostic markers (Coombs *et al.*, 1996; Mellors *et al.*, 1995, 1996; O'Brien *et al.*, 1996). Changes in plasma HIV-1 RNA levels can be used to assess the activity of antiretroviral agents (Saag *et al.*, 1996). Plasma HIV-1 RNA levels decrease in response to effective antiretroviral therapy and increase upon selection and proliferation of resistant virus or cessation of drug therapy. The measurement of plasma HIV-1 RNA levels has quickly become an accepted clinical practice.

Currently, there are three commercially available assays for quantitation of HIV-

1 RNA: the AMPLICOR Monitor test (Roche), NASBA-QT (Organon-Teknika), and Quantiplex version 2.0 (Chiron). These assays differ in their sample volume requirements, sample preparation, methods of amplification, methods of detection, throughput, turnaround time, and cost (Caliendo, 1997). Only the Monitor assay has been approved by the U.S. Food and Drug Administration for *in vitro* diagnostic use, but a variety of assays have been used in clinical research and in multicenter clinical trials of antiretroviral drugs.

The bDNA 2.0 assay was developed by modifying the first-generation assay described by Pachl *et al.,* (1995) to reduce the background level and increase the number of bDNA amplifier molecules used to generate the signal (Kern *et al.,* 1996). The background noise in the assay was reduced by using shorter overhang sequences for the capture extenders (target probe set 1), which reduced the T_m for hybridization of target probe set 1 to HIV-1 RNA by approximately 12°C. By relying on the concatenation of nearby probes hybridized to the target to increase the T_m, nonspecific hybridization was diminished and background noise was reduced. The cruciform design of the label extenders (target probe set 2) further decreased the background level. The overhang sequences of target probe set 2 are 15 to 16 bases in length and individually cannot efficiently bind to the preamplifier. However, when the overhang sequences of two target probes are adjacent, the T_m increases and stabilizes the hybridization of the preamplifier (Fig. 2).

The sensitivity of the assay was increased by the addition of preamplifier molecules that require the specific alignment of oligonucleotide sequences and contain eight bDNA amplifier hybridization sites, as described in Sect. 2.5. The probes in target probe set 2 can bind up to 28 preamplifier molecules per target molecule. Each preamplifier molecule can bind up to eight amplifier molecules and each amplifier molecule contains 15 branches, each of which has 15 branches, each of which can bind three alkaline phosphatase/labeled probes. At the end of the hybridization steps, as many as 10,080 labeled probes may be bound to each captured HIV-1 RNA molecule. These changes resulted in at least a 20-fold increase in sensitivity over the first-generation assay. The quantification limit of the second-generation bDNA assay for HIV-1 RNA is 500 copies/ml.

The second-generation assay retained many of the desirable characteristics of the first-generation assay, including accuracy, linearity over a wide range of values, and reproducibility (Kern *et al.,* 1996). The effect of genotypic variation on HIV-1 RNA quantitation was assessed by testing serial dilutions of *pol* gene sequence transcripts represent HIV-1 subtypes A to F. All six genotypes were quantified equally, with a maximum observed variance of 1.4-fold difference in quantification between subtypes B and E. The assay showed a linear response over a dynamic range of 390 to 1.6 million copies/ml using serial fourfold dilutions of a patient specimen ($r^2 = 0.986$). The reproducibility was assessed by testing replicates of specimen panels covering a 2 $\log_{10}$ range of HIV-1 RNA concentrations in 30 separate assay runs by five different operators. The overall assay precision

(%CV) ranged from 17 to 39%. The %CV was highest near the quantification limit of the assay. The results from the first- and second-generation assays were highly correlated ($r = 0.96$), allowing meaningful comparisons of virus concentrations in specimens tested with either assay.

A number of different assays for the quantitation of HIV-1 RNA have been used in multicenter clinical trials of antiretroviral drugs. The need to ensure comparability of the HIV-1 RNA data within and among trials obtained from different test sites by the various methods prompted two large multicenter evaluations of these methods. In 1994, a multicenter evaluation of a variety of in-house and commercially developed assays, including bDNA version 1.0, showed that several of the procedures were sufficiently reproducible that an empirical fourfold change could be viewed as statistically significant (Lin *et al.*, 1994). Comparison of the individual assay standards with a common set of standards showed disagreement with the nominal HIV-1 copy number value assigned by electron microscopic viral particle count. Because systematic differences between methods were also observed, it was unclear whether a common set of standards would be useful in aligning the differences measurements of HIV-1 RNA. HIV-1 RNA levels correlated with proviral DNA and were inversely correlated with $CD4^+$ cell counts. HIV-1 RNA assays were more positive than plasma viremia measured by culture and p24 antigen assays.

The AIDS Clinical Trials Group virology laboratory quality assurance program for quantitation of HIV-1 RNA demonstrated that 65% of the laboratories, using different commercial and in-house assays, could attain a level of intraassay precision (standard deviation ≤ 0.15 log) to detect reliably a fivefold difference in HIV-1 RNA copy number (Yen-Lieberman *et al.*, 1996). Laboratories using bDNA versions 1.0 and 2.0 reported the lowest intraassay standard deviations of 0.08 and 0.06 $\log_{10}$, respectively. The fitted regression lines of estimated RNA concentrations on nominal RNA concentration indicated that the differences between laboratories that used the same kit were generally greater than the differences among population-average regressions for the kits themselves. They suggested that the use of a common set of standards across clinical trial protocols would allow cross-protocol comparisons. However, the experience with patients' samples indicates that the variations among assay values for individual patients are not consistent (Coste *et al.*, 1996; Nolte *et al.*, 1998; Revets *et al.*, 1996; Schuurman *et al.*, 1996).

The version 1.0 bDNA assay was compared with the Monitor and NASBA HIV-1 RNA assays in three clinical evaluations (Coste *et al.*, 1996; Revets *et al.*, 1996; Schuurman *et al.*, 1996). Coste *et al.* (1996) found that the sensitivity of the bDNA assay was lower (68.3%) than that of both the Monitor (93.3%) and NASBA (100%) assays for detection of HIV-1 RNA among 60 plasma specimens. When results with specimens for which the RNA levels were higher than the lower quantitation limit of each method were analyzed, the mean levels by Monitor, bDNA, and NASBA were 5.38 ± 0.52, 5.03 ± 0.55, and 5.39 ± 0.53 $\log_{10}$ copies/ml, re-

spectively. The mean bDNA value differed significantly ($p < .01$) from both the Monitor and NASBA mean values. In contrast, using smaller numbers of clinical specimens, Revets *et al.* (1996) showed no significant differences among the assays in sensitivity and Shuurman *et al.* (1996) reported that there was no significant difference in the mean RNA levels determined by the three assays. The precision of the three assays was similar in all of these evaluations, with the bDNA assay being the most precise. Both studies that examined longitudinal specimens from patients receiving antiretroviral therapy found that the changes measured in response to therapy were comparable (Revets *et al.*, 1996; Schuurman *et al.*, 1996).

The genetic diversity of HIV-1 affects the quantitation of HIV-1 RNA by the various techniques differently. Although HIV-1 subtype B predominates in North America and Europe, AIDS is a global epidemic and greater subtype diversity exists in other parts of the world. HIV-1 subtype A is not detected by Monitor and subtype G is not detected by NASBA; however, all subtypes are quantitated with similar efficiency by bDNA (Coste *et al.*, 1996; Dunne and Crowe, 1997; Nolte *et al.*, 1998). Also, Monitor appears to be less efficient than bDNA in quantitating subtypes E and F and is affected more by interstrain genetic variability within these subtypes. The copy number detected by Monitor may be more than 1 $\log_{10}$ lower than the copy number detected by bDNA for some subtype E and F strains.

The performance characteristics of the bDNA 2.0 assay were compared with the Monitor assay in a clinical evaluation using dilutions of a stock virus suspension, a subtype panel, and plasma specimens from 64 HIV-1–infected individuals (Nolte *et al.*, 1998). Plots of HIV RNA copies/ml versus nominal virus particles/ml demonstrated that both assays were linear over their stated dynamic ranges, but comparison of the slopes of the linear regression lines suggested that Monitor had greater proportional systematic error. The between-run CVs of the assays were similar. HIV-1 subtypes B, C, and D were quantitated with similar efficiencies by bDNA and Monitor; however, Monitor was less efficient in quantitating subtypes A, E, and F. The sensitivities of the two assays for detection of HIV-1 RNA in clinical specimens were similar (bDNA, 83%; Monitor, 86%). The Monitor copy number values were consistently greater than the bDNA values, with population means of 142,419 and 67,580 copies/ml, respectively ($p < .01$). The correlation of the results of the two assays was high ($r = 0.91$) but the agreement was poor, with a mean difference in $\log_{10}$ copies/ml $\pm$ 2 standard deviations of 0.45 ± 0.61. The differences between the values obtained with the two methods were not consistent between patients. Because the limits of agreement (mean difference in $\log_{10}$ copies/ml $\pm$ 2 standard deviations) between the methods exceeded what is generally considered a biologically relevant change in plasma HIV-1 RNA levels (>0.5 $\log_{10}$), the assays should probably not be used interchangeably to monitor the effects of antiretroviral therapy.

The introduction of HIV-1 protease inhibitors has driven viral loads to levels below the 500 copies/ml detection limit of the bDNA 2.0 assay. The background

noise due to nonspecific hybridization of assay components resulted in a limited signal-to-noise ratio of approximately 1.8 at the lower quantitation limit of this assay. The prototype third-generation assay incorporates isoC and isoG in the label extender, preamplifier, amplifier, and alkaline phosphatase–labeled probes to minimize nonspecific hybridization, and a 14- rather than 8-site preamplifier molecule further enhances the signal generation capacity (Collins *et al.*, 1997). These changes resulted in an eight-fold increase in the signal-to-noise ratio over the previous generation of the assay. It was designed to have a sensitivity of 50 copies/ml, a specificity of >95%, a broad dynamic range of 50 to 500,000 copies/ml, and equal quantitation of all HIV-1 subtypes. The bDNA version 3.0 was also designed for testing in singlet, which will reduce the sample volume requirement from 2 to 1 ml. To date, no independent evaluations of the bDNA 3.0 assay have been published.

HIV-1 RNA levels have quickly become part of the routine management of patients infected with HIV-1. Recommendations for the appropriate use and interpretation of HIV-1 viral load assays in clinical practice have been developed, but a thorough discussion of these recommendations is beyond the scope of this review (Saag *et al.*, 1996; Centers for Disease Control and Prevention, 1998).

4.5. Cytomegalovirus

Cytomegalovirus (CMV) is an important cause of morbidity and mortality in organ transplant recipients and in HIV-infected individuals. The development of safe and effective antiviral therapy for CMV infections led to the search for methods for the timely detection of this slowly growing virus. Detection of CMV in the blood is the best marker for identifying the patients most at risk for developing symptomatic disease and for disease diagnosis. Rapid culture techniques, pp65 antigen staining, and detection of CMV DNA or mRNA by PCR have all been used to detect CMV in leukocytes (Gleaves *et al.*, 1985; Jiwa *et al.*, 1989; Van der Bij *et al.*, 1988) CMV DNA has also been detected in plasma using PCR (Nolte *et al.*, 1995). Because CMV DNA can be detected in the blood of latently infected, asymptomatic individuals, its mere presence does not always indicate active disease. Quantitative CMV DNA assays may be required to realize the full prognostic and diagnostic value of this marker.

A bDNA assay for the direct quantitation of CMV DNA in blood has been described (Chernoff *et al.*, 1997). The assay required the harvest of peripheral blood mononuclear cells by a dextran settling procedure and preparation of cell pellets of $2–6 \times 10^6$ cells. The cell pellets were lysed under denaturing conditions to release the CMV DNA. The assay employed 43 target probes: 9 to mediate capture of the CMV DNA and 34 to mediate binding of the amplifier molecules to the CMV DNA. Each target probe contained a 33-base sequence that hybridizes to either the *gB* or *UL56* region of the CMV genome. These regions were selected be-

cause of the high degree of sequence conservation among different CMV isolates. Four assay standards were prepared by serial dilution of linearized plasmid DNA containing one copy of the *gB* and *UL56* genes to 5.6×10^6, 3.5×10^5, 4.4×10^4, and 4.4×10^3 copies of CMV DNA per well. The assay also included a positive and a negative control prepared for infected and uninfected fibroblast cells, respectively. The results are expressed as CMV DNA copies per 10^6 cells.

The assay displayed a linear response over the nearly 3 $\log_{10}$ range of the standard curve. The specificity of the assay was evaluated by testing 99 leukocyte specimens obtained for healthy CMV-seronegative and -seropositive blood donors and found to be 96%. The clinical sensitivity of the bDNA assay for CMV was compared with leukocyte culture using 211 specimens from seropositive subjects. The bDNA assay detected 95% of the culture-positive specimens. CMV DNA was also detected by bDNA in 14% of the culture-negative specimens. It in unlikely that these additional positives by bDNA were false positives because 90% were from patients either receiving specific antiviral therapy or later diagnosed with CMV-related disease. The bDNA assay appears to more sensitive than standard culture methods for detection of CMV in leukocytes. The CVs for overall assay precision ranged from 14.4 to 46.2%. The assay was sufficiently precise to discern 1.5- to 5.0-fold changes in CMV DNA levels. A number of different microorganisms and drugs that may be found in the blood of immunocompromised patients were tested in the bDNA to assess whether these substances would affect the signal generated in the assay, and no effects were found.

The potential clinical utility of the bDNA assay in AIDS patients was demonstrated by monitoring CMV DNA levels in the blood of a patient undergoing gancilovir therapy (Chernoff *et al.*, 1997), in semen specimens from patients being treated with the antiviral nucleotide analogue cidofovir (Lalezari *et al.*, 1995), and in cerebrospinal fluid from patients with CMV neurologic infections (Flood *et al.*, 1997). These studies demonstrate that bDNA can be used to measure CMV DNA in various clinical specimens. To date, no comparisons of bDNA with other strategies for detection of CMV DNA in clinical specimens have been published.

4.6. *Trypanosoma brucei* spp.

Human sleeping sickness is caused by the hemoflagellates *Trypanosoma brucei gambiense* in West Africa and *T. brucei rhodesiense* in East Africa. Sleeping sickness is a major public health problem for both humans and cattle in many African countries. African typanosomiasis is an acute and chronic disease with hemolymphatic involvement in the early stages and invasion of the central nervous system in the later stages, resulting in death if not treated (Bales, 1991).

The clinical manifestations of trypanosomasis are variable and nonspecific. Diagnosis depends on the demonstration of typanosomes in body fluids or tissues. Demonstration of typanosomes in the blood is problematic because of the typically

low numbers of parasites and the wavelike fluctuations in parasitemia. The large repertoire of antigenically variable surface glycoproteins of *T. brucei* are expressed one at a time and routinely switched by the parasite to avoid the host immune response. This hampers the development of immunodiagnostic tests. Molecular methods such as PCR and bDNA may hold promise for the detection of trypanosomes in blood.

Indeed, a bDNA assay for diagnosis of African trypanosomiasis was developed and compared with buffy coat microscopy for detection of *T. brucei* in human blood samples (Harris *et al.*, 1996). Two repetitive DNA sequences found only in the *T. brucei* complex, a 177-bp satellite repeat and the ribosomal mobile element, were selected as targets in the bDNA assay. The assay used the standard bDNA components: capture probes, target probes, amplifier molecules, and alkaline phosphatase–labeled probes. Various blood fractions and sample preparation methods were examined. Ultimately, buffy coat samples resulted in the highest sensitivity. Although typanosomes do not infect leukocytes, they cosediment with them.

The limit of detection of the assay was estimated to be 200 parasites/ml of blood. The detection limit is well within the range of sensitivity needed to diagnosis trypanosomiasis, as the parasitemia may vary from 5000 to 1,500,000 parasites/ml (Vickerman, 1974). The bDNA assay was compared with buffy coat microscopy for detection of *T. brucei* in 56 blood samples (36 buffy coat positive and 20 buffy coat negative by microscopy). There was complete concordance between the results of the two tests in terms of identifying specimens as positive of negative. However, the numbers of parasites observed by microscopy were lower overall than those calculated with the bDNA assay. The authors suggested that the excess of leukocytes in the buffy coat could interfere with the microscopic detection of typanosomes, resulting in lower apparent parasitemia than the true value.

The authors also demonstrated a low-technology method for recording the chemiluminescent signal of the bDNA assay. The light emission was recorded on black-and-white film Polaroid film using a handheld camera. The bDNA assay could be applicable to field situations because of the stability of the reagents and the ability to record the data without the use of sophisticated equipment.

5. Quantitation of Messenger RNA

Interferon-γ (IFN-γ) mRNA levels were measured in unstimulated peripheral blood mononuclear cell (PBMC) and purified cell populations, using a bDNA assay, to characterize the cell types that contribute to the *in vivo* increase in IFN-γ gene expression seen in HIV infection (Breen *et al.*, 1997). IFN-γ is a cytokine that can be produced by multiple cell types and is considered to enhance cellular responses by activation of monocytes and macrophages. It is one of the type 1 cy-

tokines that is considered to contribute to effective cell-mediated immunity. IFN-γ drives the production of neopterin, an activation marker detectable in serum or plasma that is elevated in HIV-1 infection and serves as a predictor of disease progression (Fahey *et al.*, 1990; Melmed *et al.*, 1989). IFN-γ itself is difficult to measure in serum, so its production is often assessed by examining mRNA expression in cells or measuring IFN-γ protein secreted into cell culture supernatants.

Cellular mRNA was obtained from cell pellets containing known numbers of cells after homogenization in 8 M guanidinium HCl and precipitation in 50% ethanol at −20°C overnight. The IFN-γ mRNA bDNA assays were performed directly on the RNA solutions, without further purification, reverse transcription, or amplification of the target sequence. The assay used first-generation bDNA technology. IFN-γ mRNA results were expressed as relative light units per 10^6 cells.

PBMC and $CD8^+$ T cells from HIV-1–seropositive subjects showed a 2.5-fold increase in mean IFN-γ RNA levels over the mean for HIV-1–seronegative subjects. Within individual subjects, $CD8^+$ T cells showed the highest IFN-γ expression regardless of the HIV-1 status, suggesting that HIV-1 infection enhances IFN-γ gene expression in $CD8^+$ T cells rather than inducing a shift to and/or increasing expression of the IFN-γ mRNA in other cell types. HIV-1–infected subjects with increased PBMC IFN-γ mRNA had elevated plasma levels of HIV-1 RNA, neopterin, and β_2-microglobulin. No differences in IFN-γ mRNA levels were seen among HIV-1–infected subjects stratified by $CD4^+$ T cell number. The authors concluded that increased IFN-γ may result from or be a contributing factor to increased viral load.

A bDNA assay for quantitation of mouse insulin II mRNA was developed to study the regulation of insulin preRNA splicing by glucose (Wang *et al.*, 1997). Although most species have a single gene, in mice and rats a duplication has resulted in a second copy. The ancestral and duplicated genes are termed insulin II and insulin I, respectively. Different hybridization conditions allowed DNA and RNA target sequences to be distinguished. The samples were harvested in a neutral buffer with sodium dodecyl sulfate and proteinase K to ensure that the DNA remained double stranded and did not hybridize with the probes and that the RNA is protected from degradation. The assay was both sensitive and highly specific. Mouse insulin II mRNA was detected from a single beta-cell, whereas 1 million non–insulin-producing alpha-cells gave no signal. The limit of detection was approximately 10^4 insulin mRNA molecules as determined with *in vitro* transcripts from mouse insulin II cDNA. By using intron and exon sequences, probes were designed for bDNA assays that distinguished the various spliced and partially spliced insulin preRNAs from mature insulin mRNA.

Insulin mRNA splicing rates were estimated from the rate of disappearance of the insulin preRNA signal from beta-cells treated with actinomycin D to block transcription. The two introns in mouse insulin II are not spliced with the same ef-

ficiency: intron 2 is spliced out more efficiently than intron 1. As a result, some mRNA-retaining intron 1 enters the cytoplasm and accounts for approximately 2 to 10% of the insulin mRNA in the cell. When islets cells grown in high concentrations of glucose were shifted to low-glucose medium, the levels of insulin preRNA and the rate of splicing fell significantly. The authors concluded that glucose stimulates insulin gene transcription and insulin preRNA splicing.

The studies described here demonstrate that bDNA can be used to quantitate mRNA at physiologic levels and that the technology can also be used effectively in cell biology as well as infectious diseases. The bDNA assays do not have the sensitivity of RT-PCR assays that have been described for the same purposes, but bDNA may be better suited for truly quantitative mRNA measurements, since it does not require reverse transcription or amplification of the target sequences.

6. Summary

In this chapter I have reviewed the development of bDNA as a method for quantitation of nucleic acid targets and the application of this technology to the study of infectious diseases and cell biology. The ability to quantify viral nucleic acids in clinical specimens has led to a better understanding of the pathogenesis of chronic viral infections such as HIV-1, HCV, and HBV. The information provided by these methods can also be important in the management of patients with these infections. The prognostic value of a single baseline HIV-1 RNA level rivals that surgical staging procedures for cancer, which are among the most powerfully predictive tests in medicine (Mellors *et al.*, 1996). These methods have been used to assess rapidly the effects of antiviral therapy, which has both expedited the development of antiviral drugs and improved the management of patients with HIV-1 and HCV infections.

bDNA has several characteristics that distinguish it from the quantitative target amplification systems, including better tolerance of target sequence variability, more direct measurement of target, simpler sample preparation, and less sample-to-sample variation. However, the first- and second-generation bDNA assays lacked sensitivity compared with the target amplifications systems. The changes incorporated into the third-generation assays have effectively increased the signal-to-noise ratio to such a high level that the analytical sensitivity of system 8 bDNA approaches that of PCR. In theory, bDNA can be made even more sensitive by increasing both the sample volume and the signal-to-noise ratio. Nonspecific hybridization can be further reduced by finding more effective blockers for the solid phase or by redesigning the amplifier molecule or the solid phase itself. The increased sensitivity may create new applications for the technology in filter and *in situ* hybridization assays.

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INDEX

A

D

E

F

G

H

I

J

K

T