The Molecular Immunology of Human Platelet Proteins

By Thomas J. Kunicki and Peter J. Newman

Our understanding of platelet immunogenicity has closely paralleled the study of platelet membrane glycoproteins (GPs) and their pivotal role as receptors that mediate the hemostatic function of platelets. Indeed, GPs figure prominently as alloantigens, autoantigens, and targets of drug-dependent antibodies. Consequently, the molecular nature of GP epitopes is a major focus of this review. Platelet glycolipids obviously can represent autoantigenic components of the platelet surface. In addition to GP antigens, autoantibodies have also been detected that bind strongly to cytoplasmic or membrane-associated platelet proteins. In comparing the variety of identified platelet antigens, we will try to make a distinction between preexisting (or naturally occurring) antibodies and antibodies that are the direct result of maturation of the antigen-specific immune response. This distinction is important to our understanding of the basis for platelet immunogenicity, but is not always easy to make. The pathogenicity of either type of antibody and its role in the etiology of an immune thrombocytopenia remain characteristics that are difficult to define.

Platelet Membrane Glycoproteins

The initial stage of platelet function, adhesion of platelets to selected extracellular matrix components, such as the proteins fibronectin, collagen, or von Willebrand factor (vWF), is known to be mediated by specific GP receptors. Once platelets have become adherent and are activated, agonists such as ADP or thrombin activate additional membrane GP receptors that mediate platelet-platelet cohesion (aggregation) through the binding of fibrinogen or other adhesive proteins.

The heterodimeric GP receptors IIb-IIIa and Ia-IIa figure prominently among the immunogenic constituents of the platelet membrane and are both members of the family of adhesion receptors known as integrins (Table 1). Human platelets contain at least five integrins (Table 1), including the collagen receptor Ia-IIa (αIβ1), the fibronectin receptor Ic-IIa (αcβ1), the laminin receptor Ic’-IIa (αcβ1), the vitronectin receptor (VnR) αvβ3, and the promiscuous, activation-dependent receptor that is thought to be most responsible for fibrinogen-dependent, platelet cohesion, IIb-IIIa (αIIbβ3).

The plasma membrane heterodimeric protein complex composed of GPIIb and GPIIIa is central to the role of the platelet aggregation response (Fig 1). Whereas the subunits, IIb and IIIa, exist complexed with each other on the platelet surface, they are originally derived from distinct messenger RNA (mRNA) transcripts. Mature IIIa is approximately 90 Kd in size and is synthesized as a single-chain precursor molecule in the endoplasmic reticulum (ER). Fifteen percent of the molecular weight of IIIa is composed of high-mannose carbohydrate residues that are cotranslationally attached in the ER and not modified further in the golgi. IIIa contains five cysteine-rich regions: a unique one at the amino terminus, as well as 4 cysteine-rich repeats located proximal to the membrane.

IIb is a typical α subunit, approximately 145 Kd in size, containing 9 disulfide bonds that are rather evenly spaced throughout its length (Fig 1). IIb is synthesized as a single-chain pro-IIb precursor molecule that later becomes associated with IIIa while still in the membrane of the ER. After complex formation, the Pro–IIb-IIIa complex moves to the Golgi, in which pro-IIb gets cleaved, yielding the mature heavy and light chains that remain linked through a disulfide bridge formed by residues Cys1529–Cys1580. The sugar moieties of IIb also become modified and are processed in the Golgi from high-mannose to complex oligosaccharide chains. There is evidence from endoglycosidase studies that O-linked sugars may also be added to the protein backbone. After maturation of the oligosaccharide chains and cleavage into heavy and light chains, the mature IIb-IIIa complex is finally transported to the cell surface.

The binding of adhesive proteins to IIb-IIIa is mediated, at least in part, by the tripeptide sequence Arg-Gly-Asp (RGD) on the respective adhesive ligands. This sequence is found twice within the Aα chain of fibrinogen and within fibronectin, vWF, vitronectin, and type I collagen. Fibrinogen can also bind to IIb-IIIa via a dodecapeptide HHLG-GAKQAGDV that represents the carboxy-terminal sequence of the γ chain, residues 400-411.

IIb-IIIa is probably the best characterized and most studied of the integrins, and progress has been made toward identifying the recognition sites on IIb-IIIa that are likely to make direct contact with adhesive proteins. Evidence obtained by Santoro and Lawing and D'Souza et al using chemical crosslinking approaches, indicates that RGD peptide recognition sites are associated with both IIb and IIIa, but platelet activation results in a marked increase in binding of RGD peptides to IIIa. This finding argues that there is a preferential recognition of RGD by IIIa. An RGD recognition site(s) on IIIa was localized to an SV8-protease fragment containing the sequence 109-171. That region of IIIa contains a recognition site(s) relevant to the fibrinogen binding capability of the IIb-IIIa complex was confirmed by the finding of Lotus et al that a variant of Glanzmann's thrombasthenia, expressing normal levels of IIb-IIIa and unable to mediate fibrinogen binding was characterized by a single-base substitution in the gene encoding IIIa that results in an Arg to Tyr mutation at...
Table 1. Integrins on Platelets

<table>
<thead>
<tr>
<th>Integrin</th>
<th>Platelet GP</th>
<th>Ligand Specificity</th>
<th>RGD Recognition</th>
</tr>
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<tbody>
<tr>
<td>( \alpha_2 \beta_1 )</td>
<td>Ia-IIIa</td>
<td>Collagen</td>
<td>-</td>
</tr>
<tr>
<td>( \alpha_5 \beta_1 )</td>
<td>Ic-IIIa</td>
<td>Fibronectin</td>
<td>+</td>
</tr>
<tr>
<td>( \alpha_4 \beta_1 )</td>
<td>Ic'-IIIa</td>
<td>Laminin</td>
<td>-</td>
</tr>
<tr>
<td>( \alpha_6 \beta_3 )</td>
<td>Ib-IIIa</td>
<td>Promiscuous (includes fibrinogen, vWF, fibronectin, collagen)</td>
<td>+</td>
</tr>
<tr>
<td>( \alpha_5 \beta_3 )</td>
<td>VnRα-IIIa</td>
<td>Promiscuous (includes vitronectin)</td>
<td>+</td>
</tr>
</tbody>
</table>

residue 119. On the other hand, it has been reported that the dodecapeptide likely associates to a greater degree with IIb. D’Souza et al have recently defined a dodecapeptide-recognition site within the region of IIb bounded by residues 294 to 314.

In addition to the integrins, platelets contain another membrane GP receptor complex (Fig 2) that is both important to hemostasis and relatively immunogenic—IIb-IX, a receptor for vWF that is considered to be most responsible for platelet adhesion to the extracellular matrix in a flowing system under conditions of high shear stress. Mohn et al have localized the sites on the vWF molecule that probably make direct contact with the IIb-IX receptor. In combination, two limited noncontiguous regions of the vWF molecule, each containing 15 amino acid residues, can completely inhibit vWF binding to platelet IIb-IX. The sequences of these residues are Cys474-Pro488 and Leu695-Pro708. These two segments of the vWF molecule, separated by 205 residues, must be maintained in close proximity by disulfide bonding. The pairing of cysteine residues 471-474 and 509-695, established independently by Marti et al, may provide the basis for spatial proximity of these two vWF segments. The corresponding vWF recognition site on IIb has also been identified. Vicente et al, using synthetic peptides, localized one important vWF binding site to amino acids Ser251-Tyr279, a segment that is located within the 45-Kd amino-terminal tryptic fragment of the IIb heavy chain.

The IIb complex is composed of the heavy chain of IIb (IIb\( \alpha \)), disulfide-linked to a light chain (IIb\( \beta \)), and noncovalently associated with two other GPs (IX and V). Iba, IIb\( \beta \), and IX are composed of 610, 181, and 160 amino acids, respectively, in their mature form. All four proteins belong to the leucine-rich GP (LRG) family, and each platelet contains at least 20,000 to 30,000 IIb complexes on its surface. A distinguishing feature of each member of the LRG family is the presence of one or more 24 amino acid “leucine-rich” motifs, from which is derived the family name. Iba has 7 LRG domains, while IIb\( \beta \) and IX each have one. GP V, whose sequence has not yet been fully determined, has at least 1 LRG domain. The functional significance of these LRG domains is not yet known. Three of these GPs, Iba, IIb\( \beta \), and IX, have been cloned and sequenced, and are known to derive from distinct genes located on different chromosomes. In the inherited disorder of platelet function, the Bernard-Soulier syndrome, characterized by a defect in platelet adhesion to the subendothelium, it is notable that all four of these GPs are missing from the platelet surface. The recent preliminary report by Lopez et al that expression of the IIb-IX complex on the cell surface requires normal biosynthesis of at least three components of this complex and a recent report by Modderman et al that IIb-IX are normally complexed with V in the platelet plasma membrane are both consistent with the argument that all four GPs exist in a biologically functional complex at the surface of platelets.
The platelet cytoskeleton, composed mainly of actin filaments, maintains platelet morphology and mediates a variety of contractile events that occur when platelets are stimulated. A subset of actin filaments have been shown to be more closely associated with the platelet membrane, directly or through other proteins that contribute to this membrane-associated actin network. GP Ib-IX binds actin-binding protein (ABP)\(^{35}\); the \(\beta_3\) integrin \(\alpha_{IIb}\beta_3\) becomes associated with the platelet cytoskeleton after platelet activation\(^{46}\); and \(\beta_1\) integrins, such as the fibronectin receptor \(\alpha_{5}\beta_1\), can bind to talin\(^{44}\) and are clustered at points of membrane-cytoskeleton contact at which the cytoplasmic protein vinculin is also localized.\(^{42}\) Given these associations, it is not surprising that the platelet cytoskeleton can affect the expression of platelet antigens in a number of ways. This cytoskeleton association can influence the presentation of surface antigens, while crosslinking of surface antigens can trigger cytoskeleton-mediated intracellular changes.

Binding and crosslinking of human antibodies specific for IIb or IIla has been shown to cause redistribution of these membrane GPs.\(^{43}\) Both alloantibodies and autoantibodies are effective. Fifty percent to 75% of surface IIb-IIla was found to undergo cap formation and to be endocytosed within 5 minutes after the addition of specific human antibodies and an appropriate antihuman Ig secondary antibody. This clustering of IIb-IIla is reminiscent of that induced by ligands such as fibrinogen or RGD peptides.\(^{44}\) Under identical conditions, human antibodies specific for IIb-IX were without effect.

Intermediate filaments are fibrous cytoplasmic structures composed of a variety of proteins, including vimentin, and may play a role in the mechanics of platelet motility.\(^{45}\) Vimentin is a frequently noted target of autoantibodies, particularly those "naturally occurring" antibodies that can be detected in the sera of apparently normal individuals. Interestingly, it has been proposed that vimentin may also be associated with the platelet membrane\(^{45}\) via an interaction with spectrin and ankyrin, both known to be present in platelets.\(^{46,47}\)

### ALLOANTIGENS

The established platelet-specific alloantigen systems are listed in Table 2. Formerly described as \(\text{Pl}^{+} (\text{Zw})\),\(^{40,49}\) Bak (Lek),\(^{50-53}\) Pen (Yuk),\(^{54,55}\) Ko (Sib),\(^{56,57}\) and Br (Hc),\(^{58,59}\) they are now designated according to a uniform alloantigen nomenclature.\(^{60}\) Other alloantigen systems have been tentatively defined, including \(\text{PI}^{+}\),\(^{61}\) Gov,\(^{62}\) and the first private alloantigen system, \(\text{Sr}\).\(^{63}\) The antigen \(\text{Nak}^{+}\),\(^{64}\) originally described as an alloantigen, has subsequently been shown to be an isoantigen present on GPIV of all normal individuals tested.\(^{65}\) Since its initial description, further characterization of \(\text{PI}^{+}\) has not been reported. Two clinically significant syndromes are the direct result of sensitization to platelet-specific alloantigens: neonatal alloimmune thrombocytopenic purpura (NATP) and posttransfusion purpura (PTP). NATP is caused by maternal sensitization to para-

### Table 2. Human Platelet Alloantigens

<table>
<thead>
<tr>
<th>Alloantigens</th>
<th>Synonyms</th>
<th>Phenotype Frequency (%)*</th>
<th>Caucasian</th>
<th>Japanese</th>
<th>GP Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA-1a</td>
<td>Zw*, Pl(^{A1})</td>
<td>97.9</td>
<td>99.9</td>
<td>Ilb</td>
<td></td>
</tr>
<tr>
<td>HPA-1b</td>
<td>Zw*, Pl(^{A2})</td>
<td>26.5</td>
<td>3.7</td>
<td>Ilb</td>
<td></td>
</tr>
<tr>
<td>HPA-2a</td>
<td>Ko(^{c})</td>
<td>99.3</td>
<td>NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPA-2b</td>
<td>Ko(^{c}),Sib(^{+})</td>
<td>14.6</td>
<td>25.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPA-3a</td>
<td>Bak(^{+}),Lek(^{a})</td>
<td>87.7</td>
<td>78.9</td>
<td>IIb</td>
<td></td>
</tr>
<tr>
<td>HPA-3b</td>
<td>Bak(^{b})</td>
<td>64.1</td>
<td>NT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPA-4a</td>
<td>Pen(^{a}),Yuk(^{b})</td>
<td>99.9</td>
<td>99.9</td>
<td>IIa</td>
<td></td>
</tr>
<tr>
<td>HPA-4b</td>
<td>Pen(^{a}),Yuk(^{b})</td>
<td>0.2</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPA-5a</td>
<td>Br*, Zav(^{a})</td>
<td>99.2</td>
<td>NT</td>
<td>la</td>
<td></td>
</tr>
<tr>
<td>HPA-5b</td>
<td>Br*, Zav(^{a}),Hc(^{+})</td>
<td>20.6</td>
<td>NT</td>
<td></td>
<td></td>
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</table>

Abbreviation: NT, not tested.

*Data from von dem Borne and Decary.\(^{23,27}\)

### Table 3. NATP

<table>
<thead>
<tr>
<th>Incidence</th>
<th>Maternal antibodies produced against paternal antigens on fetal platelets.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence: 1 per 3,000 in a retrospective study, 1 per 2,200 births in one prospective study.</td>
<td></td>
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<tr>
<td>Maternal antibodies produced against paternal antigens on fetal platelets.</td>
<td></td>
</tr>
<tr>
<td>Similar to erythroblastosis fetalis, except that 50% of cases occur during first pregnancy.</td>
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<tr>
<td>Most frequently implicated antigen is Pl(^{A1}) (United States/Europe).</td>
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</tr>
<tr>
<td>High risk association with HLA-DRw52a (DR3 locus).</td>
<td></td>
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</table>
Alloantigens Identified on IIIa: PI\(A\) (Zw) and Pen (Yuk)

The PI\(A\) epitopes are associated with IIIa.\(^{21,76-78}\) Using platelet RNA and the Taq I polymerase chain reaction (PCR) technique, Newman et al\(^{79}\) identified a single-base change that encodes a Leu \(\leftrightarrow\) Pro polymorphism at amino acid 33 and correlates precisely with PI\(A\) phenotype (Fig 1). The localization of the PI\(A\) epitope to this region of IIIa was confirmed by Bowditch et al.,\(^{80}\) who screened a \(\lambda\)-gt22 expression library of IIIa cDNA fragments for binding of anti-PI\(A\) alloantibodies. The smallest PI\(A\)-positive clone encoded the first 66 amino-terminal residues of IIIa. Goldberger et al\(^{81}\) used a heterologous expression system to show that the Leu33 form of IIIa reacts with anti-PI\(A\), but not anti-PI\(A^2\) alloantisera, whereas the Pro33 form is recognized only by anti-PI\(A^2\). It thus appears that the PI\(A\) epitopes are expressed at this polymorphic site of IIIa.

According to the disulfide bond pattern proposed by Calvete et al.,\(^{82}\) the Leu33/Pro33 polymorphism would be enclosed within a small 13 amino acid loop formed by the pairing of Cys26 with Cys36. In addition to this small loop, a long-range disulfide bond linking Cys38 and Cys435 has been identified. Residues lying within this 431 amino acid region would form the large disulfide-bonded loop previously identified by Beer and Coller.\(^{83}\) This long-range bond would necessarily bring the amino-terminal region of IIIa, including the small Cys26-Cys38 loop that contains the PI\(A\) polymorphic residue, into immediate proximity with the cysteine-rich region located near the middle of IIIa. Thus, the three-dimensional shape of IIIa in the vicinity of the polymorphism at residue 33 likely influences, and can be influenced by, noncontiguous, nonlinear segments of the polypeptide chain within the cysteine-rich domains. The fact that we do not yet know the precise three-dimensional structure of these regions of IIIa and how they interact may explain the failure, to date, to generate synthetic peptides that mimic the PI\(A\) epitopes. Flug et al\(^{84}\) have shown that small, linear, synthetic peptides surrounding the Leu33 \(\leftrightarrow\) Pro33 polymorphic site are unable to mimic the PI\(A\) determinants. This finding was not unexpected because it had been previously shown that IIIa linearized by 2-mercaptoethanol loses reactivity with anti-PI\(A\) alloantibodies.\(^{76}\) Moreover, murine monoclonal antibodies (MoAbs) raised against linear peptides containing the Leu33 or Pro33 polymorphisms will bind to the corresponding synthetic peptides or to denatured and reduced IIIa of the appropriate phenotype, but fail to bind to native IIIa.\(^{85}\) Thus, although the PI\(A\) epitopes have been localized to the disulfide-bonded loop of IIIa encompassing the polymorphism at residue 33, the structure that confers alloantigenicity to this sequence remains to be discovered.

Anti-PI\(A\) antibodies inhibit clot retraction and platelet aggregation. Platelet aggregation is presumably inhibited because anti-PI\(A\) antibodies block the binding of fibrinogen.\(^{86}\) Aggregation of platelets that are homozygous for the PI\(A\) epitope is completely inhibited by saturation with anti-PI\(A\) antibodies, whereas aggregation of heterozygous platelets can be only partially inhibited by the same or greater amounts of antibody.\(^{87}\) Ryu et al\(^{88}\) have also reported that there is a dose-dependent stimulation versus inhibition of fibrinogen binding induced by anti-PI\(A\). Platelet stimulation occurs when at least 2,000 molecules of antibody are bound per platelet, while saturation of platelets (64,000 molecules per platelet) results in inhibition of fibrinogen binding and aggregation. Similar stimulation/inhibition effects have also been attributed to other platelet inhibitors, particularly disintegrins, such as RGD peptides and certain snake venoms.\(^{89}\)

The Pen (Yuk) alloantigen system has also been localized to the IIIa molecule.\(^{87,90,91}\) Two laboratories\(^{82,93}\) have independently noted an Arg\(^{143}\) to Gln\(^{143}\) polymorphism that correlates with Pen phenotype (Fig 1). Given the proximity of the Pen polymorphism to the RGD binding domain (residues 109-171) of IIIa, it is not surprising that anti-Pen\(a\) antibodies completely inhibit aggregation of Pen\(a^{2}/a^{2}\) homozygous platelets.\(^{87}\)

Because cells other than platelets, such as endothelial cells and fibroblasts, express IIIa as the \(\beta\) subunit of the vitronectin receptor, one would expect to find alloantigens associated with IIIa on these cell types as well. Newman et al\(^{84}\) were the first to report that endothelial cell IIIa carries the PI\(A\) epitopes. This finding was subsequently confirmed by Giltay et al\(^{85}\) and Kawai et al.,\(^{96}\) who presented evidence that endothelial cells do express PI\(A\) but not Bak epitopes; Bak epitopes are found on the platelet-specific integrin \(\alpha\) subunit IIb. Pen epitopes are also found on endothelial cells in culture.\(^{96}\) More recently, both smooth muscle cells and foreskin fibroblasts have been found to also express PI\(A\) epitopes.\(^{97}\) The potential existence in vivo of IIIa alloantigens such as PI\(A\) on cells other than platelets may contribute to the complexity of the clinical picture of alloimmune-mediated thrombocytopenia. At this time, little, if anything, is known about the involvement of tissues other than platelets in these conditions.

**Alloantigens Identified on IIb: Bak (Lek)**

The Bak system has been localized to the heavy chain of IIb.\(^{50,51,58}\) Lyman et al\(^{59}\) have recently identified an Ile/Ser polymorphism at residue 843 of the heavy chain that correlates precisely with the Bak phenotype (Fig 1). Take et al\(^{100}\) have reported that the binding of certain anti-Bak\(a\) antisera to IIb is decreased after desialation of IIb, raising the possibility that glycosylation of IIb may contribute to or influence the expression of the Bak epitopes. This may
alloantigens identified on other platelet GPs: Br(Hc), Sib(Ko), and Nak

The Br (Hc, Zav) platelet-specific alloantigen system is located on Ia. The detection of this system was facilitated by the recent development of a highly sensitive murine monoclonal antibody-based, MoAb immobilization of platelet antigen (MAIPA) assay. The preceding alloantigenic systems, the Br* system is diallelic. Roughly 2,000 copies of Ia are present on the surface of normal platelets, and each Ia molecule expresses a single Br epitope. Ia is distributed on a wide variety of cells, but nothing is currently known about the distribution of the Br antigens. Despite the low copy number of Br antigens on platelets, antibodies specific for Bra can induce PTP associated with significant thrombocytopenia and bleeding.

Two independently reported polymorphisms of the Ia chain, Xa1 and Xa2, have been localized to the heavy chain of GP Ibα (Ibo). Kuikpers et al have found that anti-Ko antibodies bind to the amino-terminal, elastase-sensitive fragment of the Ibo chain, and Murata et al showed that a Thr/Met polymorphism at amino acid 145 is associated with Sibp/Sibβ phenotype (Fig. 2). The Sib antigen system has recently been shown to be identical to the Ko system and reclassified as HPA-2A, although it is possible that the actual epitopes may be influenced by allelic variation in the number of 13 amino acid repeat structures reported to exist in the GPIbα chain.

Recently, Ikeda et al reported another platelet antigen system, Nak*, that was implicated in one patient with refractoriness to HLA-matched platelet transfusions. Tomiyama et al later showed that anti-Nak antibodies react specifically with GPIV, an 88-Kd membrane GP believed to be involved in the interaction of platelets with collagen or thrombospondin. Interestingly, Nak*-negative platelets fail to synthesize biochemically or immunochemically detectable amounts of GPIV, even though their platelets contain mRNA transcripts for this GP. These IV-negative individuals had no platelet functional defects and no hematologic disorders, suggesting that the putative functions of GPIV can be compensated for by other platelet membrane components. Because anti-Nak antibodies do not recognize a molecular variant of the IV molecule, Nak* is more properly classified as an isoantigen rather than an alloantigen.

Kelton et al described another alloantigen system carried by a novel platelet protein with an apparent molecular weight of 150 Kd/175 Kd (nonreduced/reduced). Alloantibodies defining each of two alleles (Govp/ Govp) were detected in two patients who had received multiple platelet transfusions. The phenotypic frequency in the Canadian population of Govp is 81% and of Govp is 74%.

Finally, Kroll et al have described the first case of a "private" alloantigen Sr* that was associated with a case of NATP. The maternal alloantibody binds strongly to the child's and father's platelets, but does not react with platelets from 300 unselected donors or with a panel of donor platelets that represent all known platelet alloantigens. However, the antigen in question was present on platelets of 9 of 20 members in three generations of the paternal family. The Sr* epitope has been localized to the 68-Kd chymotryptic fragment of IIa that has been previously shown to bear PI4 but not Pen epitopes. Like the PI4 alloantigen, Sr* is destroyed by disulfide bond reduction of IIa.

autoantigens

Autoimmune (or idiopathic) thrombocytopenia (ITP or AITP), the most frequently encountered form of immune thrombocytopenia, is caused by the interaction of autoantibodies with platelet or megakaryocyte surface antigens and is characterized by extravascular sequestration of antibody-bound platelets in the spleen and liver. This disorder can be classified as acute or chronic based on the duration of the thrombocytopenia, the chronic form persisting longer than 6 months (Table 5). The acute, self-limiting form occurs predominantly in children, often after a viral illness or immunization, and affects males and females with equal frequency. The chronic form is mainly an adult illness and affects twice as many females as males. Life-threatening bleeding occurs in up to 1% of patients with ITP. The reason that some patients sustain severe hemorrhagic complications and others do not remains unexplained, but because of differences seen in the clinical expression of chronic and acute ITP, it has been theorized that the mechanism of disease for each form is probably different. When one analyzes the isotype of platelet-bound IgG in either primary ITP or secondary immune thrombocytopenia, IgG1 and IgG3 predominate (60% and 61% of samples tested, respectively), while 13% of samples contained IgG2, and 9% contained IgG4.

A number of factors complicate the identification of platelet autoantibodies in ITP. First, serum levels of autoantibody are often very low and frequently undetectable. In
many cases, the only autoantibody that can be detected is tightly associated with the platelet, presumably bound to its antigen. This necessitates the use of platelet eluates as the most reliable source of autoantibody for subsequent identification and characterization. Low levels of serum autoantibody have fostered the development of vastly improved and very sensitive assays for the detection of such serum antibodies. Unfortunately, sensitivity is often inversely related to specificity, and these newer assays are prone to false positive results due to spurious, nonspecific protein interactions. Caution is particularly well-advised against the exclusive use of immunoblot assays to detect less dominant protein antigens as targets for autoantibodies. Reid et al. have very convincingly pointed out that control normal sera often generate antigen profiles that are very similar to those considered to be characteristic of autoimmune disease. Eighty-five percent of normal sera were found to contain IgG that bound to protein bands at 90 to 95 Kd. Less often, positive bands at 100 to 110 Kd, 80 to 85 Kd, 60 to 75 Kd, and 50 to 60 Kd were also seen with normal sera. It is thus important to rigorously distinguish quantitative variations in the patterns produced by normal sera before attributing positive reactions to the presence of true autoantibodies. One should also be aware that antigens expressed on sodium dodecyl sulfate (SDS)-denatured proteins may not be expressed on the same protein in its native state. The argument is often made that true autoantibodies will be associated with clinically definable autoimmune disease and will not be present in apparently normal individuals. On the other hand, it is a perfectly valid argument that autoantibodies identical to those characteristic of patients with clinically severe autoimmune disease may well exist in sera of normal individuals and remain nonpathologic as long as their effector functions are actively controlled or clonal expansion is actively suppressed.

A second complication of the study of autoantibodies in ITP is the normal presence of substantial amounts of internal platelet IgG (on average, 25,000 molecules per platelet) located within the α-granules and presumably internalized by the megakaryocyte. With platelets from normal individuals, no more than 1% of platelet IgG is actually present on the surface. The precise nature of the internal pool of IgG, its clonal makeup, its association (whether specific or otherwise) with other α-granule proteins, and its antigen specificity are just some of the questions that remain to be answered. Obviously, copurification of this internal IgG pool can confound determinations of autoantibody quantity and specificity. Thus, assays that distinguish surface-associated antibodies are likely to provide more relevant information than assays that measure total (internal plus surface) platelet-associated antibodies.

A final complication is that platelets possess surface FcγRII that can readily bind to immune complexes or aggregated IgG. Consequently, platelets can often become saturated with immune complexes, particularly in autoimmune disorders such as ITP. The presence of FcγRII on platelets makes it also more critical to distinguish Fab-mediated antibody binding to platelet antigens from Fc-mediated absorbance of immune complexes.

Despite these pitfalls, significant progress has been made in the identification of autoantigens that are relevant to the pathology of ITP.

**Primary Autoimmune Thrombocytopenia: GPs as Autoantigens**

The dominance of IIb-IIIa. Before 1982, the nature of the majority of platelet autoantibodies in primary ITP remained a mystery. In a pioneering study by van Leeuwen et al., IIb-IIIa was implicated for the first time as a dominant antigen in chronic ITP. IgG eluates of 42 adult patients with chronic ITP were shown to contain antibodies reactive with all normal platelets tested, but 35 of these eluates (91%) did not react with platelets from a patient with Glanzmann’s thrombasthenia that lacked IIb-IIIa. Since this report, it has been confirmed that a majority of the antigenic targets (autoepitopes) implicated in ITP are associated with IIb-IIIa. While most autoantibodies specific for IIb-IIIa apparently induce thrombocytopenia, a minority can induce platelet dysfunction without an increase in platelet clearance. A number of cases of “acquired thrombasthenia” due to autoantibodies against IIb-IIIa have been reported. In one case, platelet dysfunction was observed to fluctuate, without thrombocytopenia, over a period of 8 years, and an IgG1 autoantibody to IIb-IIIa was detected in eluates from the patient’s platelets that was a potent inhibitor of fibrinogen binding to platelets. Another case involved a patient with Hodgkin’s disease who developed chronic ITP. Her platelet count normalized after splenectomy, but severe platelet dysfunction persisted. In this case, an IgG autoantibody could be isolated from the patient’s plasma that bound to IIb-IIIa and inhibited the aggregation of normal platelets. In a third case, IgG autoantibody against IIb-IIIa was detected in plasma and associated with platelets. A unique aspect of this last case is that there was evidence for an internal platelet pool of the patient’s autoantibodies.

The above three cases, although anecdotal, emphasize the need to investigate more closely the question of whether autoantibodies to IIb-IIIa can induce thrombocytopenia or dysfunction or both. One could argue that autoantibodies that lead to platelet clearance and those that induce platelet dysfunction could both be present in individual cases of ITP. Were that the situation, the proportion of each type of autoantibody would be yet another important factor influencing clinical severity of ITP.

Autoantibodies to platelet antigens can also be generated by malignant lymphomas. In two cases, the antibodies in question were found to bind predominantly to IIb-IIIa and to induce not only sporadic thrombocytopenia but also profound abnormalities in IIb-IIIa function, leading to defective platelet aggregation. In one case, radiation treatment of the lymphoma that had localized to the cervical lymph nodes dramatically normalized both platelet count and platelet function.

Two studies have addressed the extent of the autoantigen repertoire on IIb-IIIa by analyzing the competitive binding
between human autoantibodies and murine MoAbs.\textsuperscript{139,140} In the earlier report,\textsuperscript{139} it was found that the binding of one murine MoAb, 3B2, showed decreased reactivity with platelets from 16 ITP patients, presumably because the platelet-associated autoantibodies bound to sites at or close by the 3B2 epitope. This finding would argue for homogeneity of autoepitopes on IIb-IIIa. In the latter study,\textsuperscript{140} the ability of four murine MoAbs specific for IIb-IIIa to block autoantibody binding to heterologous platelets was analyzed. It was found that IgG fractions from six different ITP patients reacted with apparently different epitopes on IIb-IIIa. More recent reports, described below, have directly localized autoantigenic epitopes on IIIa or IIb.

Localization of autoantigens on IIIa. Three recent studies have succeeded in localizing regions of IIIa that contain autoantigenic epitopes.\textsuperscript{118,119,141} Kekomaki et al\textsuperscript{118} determined that a 33-Kd chymotryptic core fragment of IIIa, representing the cysteine-rich region of the molecule and extending from residue 479 to at least residues 636-654, is a frequent target of autoantibodies in ITP (Fig 3). This autoantigenic region was first localized using two exceptionally strong antisera as prototypes. Once the autoantigenic nature of this region of IIIa was determined, the frequency of serum antibodies reactive with this region was determined by enzyme-linked immunosorbent assay (ELISA). Forty-eight percent of patients with chronic ITP (15 of 31) and two of eight patients with acute ITP had serum IgG antibodies that bound to the 33-Kd fragment of IIIa. In three representative cases, it was documented that antibodies that bind to the 33-Kd fragment in the immunoblot assay are identical to those antibodies that bind to purified IIb-IIIa or to IIb-IIIa on the surface of intact platelets. Antibodies of similar specificity were also found in sera of one-third of patients with secondary immune thrombocytopenia or apparent nonimmune thrombocytopenia. It was concluded that this portion of IIIa is a frequent target of autoantibodies in ITP, but that such antibodies may also be detected in cases of thrombocytopenia wherein an autoimmune etiology is not easy to establish or there is another hematologic condition that might independently be associated with thrombocytopenia. These findings corroborate the report of Berchtold et al,\textsuperscript{142} who noted that autoantibodies to IIb-IIIa or Ib-IX could be detected in 15 of 24 patients with disease-related thrombocytopenia. Specifically, 12 patients had lymphoproliferative disease (chronic lymphocytic leukemia [CLL], Hodgkin's disease, or non-Hodgkin's lymphoma) and 12 patients had various autoimmune disorders (systemic lupus erythematosus [SLE], Evan's syndrome, primary biliary cirrhosis [PBC], idiopathic pulmonary fibrosis, Sjogren's syndrome, myasthenia gravis, mixed connective tissue disease, Hashimoto's thyroiditis, or rheumatoid arthritis). Sixty percent of the patients had platelet-bound autoantibodies and one-third had plasma autoantibodies directed at IIb-IIIa or Ib-IX. In one case of SLE and another of myasthenia gravis, adsorption of anti-IIb-IIIa autoantibodies did not affect the levels of autoantibodies reactive with cochlear antigens or acetylcholine receptor, respectively, indicating that the reactivity with IIb-IIIa was not merely a result of crossreactivity of the primary autoantibodies.

Fujisawa et al\textsuperscript{141} used synthetic peptides corresponding to different sequences of IIIa to localize epitopes and determined that autoantibodies in 5 of 13 sera from patients with chronic ITP bound to peptides representing residues 721-744 or 742-762, namely, the carboxy-terminal region of IIIa that is presumed to be located in the cytoplasm of the platelet. It was concluded that the carboxy-terminal cytoplasmic domain of IIIa is important as a target of autoantibodies, but the role of such antibodies in the pathogenesis of the disease remained unclear. In a follow-up study,\textsuperscript{119} it was determined, as one might expect, that epitopes on the cytoplasmic domain of IIIa are "relatively inaccessible" on the surface of intact platelets. Such epitopes were recognized by antibodies in 13 of 21 plasmas tested, while antibodies in all of 26 platelet eluates bound to epitopes in other regions of GPIIb-IIIa. It was not determined whether these "other regions" of IIIa are related to the autoantigenic 33-Kd domain previously identified by Kekomaki et al.\textsuperscript{118} This study emphasizes that the importance of plasma autoantibodies in ITP should be interpreted cautiously because their specificity may be different from those of platelet-bound antibodies.

Human MoAbs are an important tool in the search for GP epitopes that are autoimmunogenic in humans. The first human MoAb specific for a platelet GP was developed by Nugent et al\textsuperscript{143} and derived from a patient with ITP who had produced serum IgG and IgM antibody against IIIa. This monoclonal IgM antibody 5E5 binds to a neoantigen (or a cryptic antigen) associated with IIIa that is expressed on platelets that have either been activated with thrombin or stored for a number of days under blood-banking conditions. The cryptic nature of this neoantigen fits well with one concept of autoimmune, namely, that autoreactive antibodies that recognize cryptic antigens expressed by "aged" or damaged cells are responsible for the clearance of these cells from the circulation through the reticuloendo-
thelial system. These autoantibodies, if they are “naturally occurring,” could play a role in the normal senescence of circulating blood cells.\(^{14}\) If they are otherwise disease-related, they could contribute to premature senescence of these blood cells.

**Localization of autoantigens on IIb.** Autoantibodies reactive with IIb were identified in two patients with chronic ITP.\(^{130}\) and in one of these patients, the antibody was subsequently shown to react with a chymotryptic, 65-Kd, COOH-terminal fragment of the IIb heavy chain (Fig 1).\(^{131}\)

The human monoclonal IgM autoantibody 2E7 is specific for the heavy chain of IIb\(^{145}\) and binds to a contiguous amino acid sequence within residues 231-238 with an immunodominant tryptophan residue at position 235 (Fig 1).\(^{146}\) This is the first time that the precise epitope on IIb or IIIa bound by a human antibody has been identified. One peculiarity of 2E7 specificity is that the antibody binds more strongly to IIb-IIIa in situ in the presence of EDTA. This implies (although direct proof has not yet been obtained) that the epitope recognized by 2E7 is markedly influenced by divalent cations. This is really not surprising because the sequence IIb_{231-238} overlaps the first calcium-binding domain of IIb. One could speculate that 2E7, like the prototype 5E5, recognizes a cryptic epitope that is more available when the divalent cation occupancy of IIb-IIIa is perturbed. It is tempting to speculate that such a condition would be more characteristic of a dysfunctional IIb-IIIa complex. However, we still know very little about the interaction of IIb-IIIa with divalent cations.\(^{147}\) From the standpoint of Ig structure specificity relationships, 2E7 is also intriguing. Both the \(\mu\) chain and \(\kappa\) chain of 2E7 have been cloned and sequenced,\(^{148}\) and each has been found to use V gene segments that are strikingly homologous to other human autoantibodies that bind DNA and carry the 16/6 idiotype.\(^{149}\) Such autoantibodies crossreact with single-stranded or double-stranded DNA, cardioliain, and platelet glycolipid antigens. Unlike members of the 16/6 idiotype family, 2E7 does not bind to DNA or antigens other than the heavy chain of IIb.

EDTA-dependent autoantibodies represent a special category of Igs that are adsorbed by autologous platelets when whole blood is drawn in divalent cation chelators, such as citrate or EDTA.\(^{150,151}\) In one such case of “pseudothrombocytopenia,” an IgM antibody that bound best to platelets in the presence of EDTA and at 4°C was shown to bind to IIb by immunoblot assay and crossed immuno-electrophoresis (CIE).\(^{152}\) The human monoclonal IgM autoantibody 2E7 that binds to the sequence IIb may be a prototype of such antibodies.\(^{146}\) Because these antibodies bind to IIb-IIIa under conditions considered to be nonphysiologic, ie, in the presence of EDTA, their clinical relevance has obviously been questioned. However, the precedent has been established with the murine MoAb PMI.1, which binds to the carboxy-terminal region of the IIb heavy chain, that conformational epitopes that are induced by nonphysiologic stimuli such as EDTA can also be induced by physiologic agonists such as the ligand fibrinogen.\(^{153}\) Consequently, without further evidence to the contrary, one should not underestimate the pathologic potential of IIb-IIIa-specific antibodies such as those that bind best to epitopes induced by the chelation of divalent cations. As another example, heparin can also induce pseudothrombocytopenia.\(^{154-156}\) One could speculate, as did van Vliet et al,\(^{152}\) that under certain circumstances, eg, during heparin anticoagulation, autoantibodies of this kind might bind to platelets in vivo.

Denomme et al\(^{154}\) have characterized additional human MoAbs derived from tonsillar tissue of normal individuals that react with platelet antigens, including IIb. The prototype for this class of autoantibodies, STO 171, does not compete with 2E7 in an antigen-specific ELISA (Denomme G.A. and Kunicki T.J., unpublished observations), suggesting that these “naturally occurring” antibodies recognize distinct epitopes on IIb.

**Autoantigens on other integrins.** IIb-IIIa is not the only integrin implicated as the antigen target for human autoantibodies. Serum IgG autoantibodies specific for glycoprotein IIa (integrin subunit \(\alpha_2\)) were identified in a unique case of autoimmune platelet dysfunction after myasthenia gravis.\(^{155}\) This autoantibody inhibited aggregation of normal platelets induced by collagen or wheat germ agglutinin. This is the first case wherein autoantibodies to IIa were associated with a chronic hemorrhagic disorder, and this study actually provides strong indirect support for a role of the platelet integrin IIa-IIIa (\(\alpha_2\)\(\beta_3\)) in hemostasis in vivo. Integrin-associated autoantigens have also been described on cells other than platelets. Recently, Hartman and Wright\(^{156}\) showed that some patients with autoimmune neutropenia have autoantibodies that bind to the neutrophil adhesion proteins CD11b/CD18, another member of the integrin family. In some cases, these antibodies may interfere with neutrophil function, increasing the risk of infection associated with neutropenia.

**Autoantigens on Ib-IX.** Autoantibodies reactive with epitopes on Ib-IX are the second most frequently encountered autoantibodies in adult chronic ITP.\(^{157}\) In some cases in which autoantibody to Ib-IX was detected, the clinical presentation proved to be particularly severe and refractory to therapy.\(^{158,159}\) One case of “pseudo Bernard-Soulier syndrome” (dysfunction of Ib-IX) was reported to be caused by an autoantibody to Ib.\(^{160}\)

Several human monoclonal autoantibodies specific for the heavy chain of Ib were generated from the lymphocytes of a patient DM who had developed a particularly severe case of ITP that was resistant to therapy.\(^{159}\) Affinity-purified autoantibodies from this patient’s platelets and sera were used to generate rabbit polyclonal and murine monoclonal anti-idiotypic (anti-Id) antibodies that distinguish the Ib-specific DM autoantibodies. Screening of sera from a large number of patients with immune thrombocytopenia of various etiologies and documented serum antibodies reactive with either IIb-IIIa or Ib-IX provided conclusive evidence that the DM Id is restricted to those cases wherein autoantibodies to Ib were present. In fact, all DM Id-positive sera were also positive for anti-Ib autoantibodies, and 18 of 23 sera with anti-Ib autoantibodies (78%) were positive for the DM Id. Interestingly, the DM Id was not present in any of six cases of quinidine-dependent immune
thrombocytopenia characterized by drug-dependent autoantibodies reactive with Ib. Consequently, quinidine-dependent antibodies that bind Ib in the presence of quinidine are likely to be structurally distinct from those autoantibodies to Ib that are not drug-dependent. In an effort to better understand the molecular genetics of the DM Id, the expressed VH genes of five human lymphoblastoid cell lines (LCL) derived from patient DM were sequenced.161 Two LCL secrete anti-Ib and three secrete antibodies that bear the DM Id. The V \text{H} genes used by the three Id-positive antibodies and one of the anti-Ib antibodies belong to the V_{\text{H}4} family, whereas the other anti-Ib antibody belongs to the V_{\text{H}1} family. Compared with germline sequences, all of these VH genes contain multiple base substitutions, suggesting that these LCL represent mature B cells that were selected by virtue of antigen-driven, clonal maturation. This important study by Nugent et al160 lays the groundwork for the development of idiotype-based therapy of ITP and clearly suggests that the repertoire of idotypes expressed by human autoantibodies specific for membrane GPs, such as those of the human platelet, can be narrowly defined and, thus, amenable to study and manipulation.

Antigen identity in chronic versus acute ITP. It had been hoped that the antigenic targets in acute and chronic forms of ITP might be different so that antigen identity might one day be used as an early indicator of clinical outcome. Early comparisons between antigens in acute and chronic ITP raised hopes that this would be the case. In a landmark report by Beardsley et al,127 none of the sera from 8 children with acute ITP could be shown to contain antibody reactive with IIIa, even though IIIa was the dominant antigen bound by antisera from patients with chronic ITP. In a subsequent study, four children with acute varicella-associated thrombocytopenia were found to have autoantibodies reactive with a thrombin-sensitive, 85-Kd GP fitting the description of GPV.162 A possible association of GPV with acute ITP was also proposed by a second study wherein six of seven patients were observed to have antibody of this specificity.163 Subsequent studies have shed more light on the similarities between autoantibody specificity in chronic versus acute ITP, particularly in children.164,165 In the report of Berchtold et al,166 serum IgG autoantibodies specific for IIb-IIIa were detected, by immunobead assay, in 14 of 24 (58.3%) children with chronic ITP. By the same method, autoantibodies to IIb-IIIa were detected in 4 of 15 (26.7%) children with acute ITP. Additional findings were: none of the patients had IgG antibodies specific for Ib-IX; the level of IgG autoantibody to IIb-IIIa was about fourfold higher, on average, in chronic ITP; and, in chronic ITP, there was no correlation between serum IgG autoantibody level and platelet count. These results in chronic childhood ITP were essentially identical to previous findings in adults, in which 56% of patients had autoantibody to IIb-IIIa.128 Along the same lines, Winiarski,165 using an immunoblot assay, found that sera from 13 of 21 children with acute ITP had IgG or IgM antibodies that bound to platelet proteins. Of these 13 sera, 5 bound to IIIa; 1 to Ib; 4 to Ib; 1 to an unidentified 250-Kd protein; and 12 to smaller protein antigens ranging in size from 25 to 52 Kd. Comparing these last two reports, one finds, in the latter report, conspicuous differences: the somewhat higher frequency of antibodies reactive with IIb-IIIa (67%) and the finding of antibodies against Ib. This can be attributed to differences in the methods used to detect antibodies, but may also reflect the fact that Winiarski165 measured both IgG and IgM, whereas Berchtold et al166 only screened for IgG.

Despite this retrospective evidence, a distinction between antigen specificity and an acute versus chronic course in ITP may yet be found in the early stages of the autoimmune response. Clearly, prospective studies aimed at answering this question are still warranted.

Clonal Restriction of Autoantibodies in ITP

Further studies are required to determine the extent to which the production of human autoantibodies to platelet GPs is clonally restricted. If the idiotype repertoire characteristic of autoantibodies against IIb-IIIa is restricted, then the feasibility of using anti-Id to modulate autoimmunization to IIb-IIIa increases. A recent report by Berchtold et al166 provides the best indication that this approach is feasible by showing that intravenous IgG (IVIgG), which is routinely used to reverse acute thrombocytopenia in ITP, may contain anti-Id directed to idiotypes of autoantibody, but not autoantibodies that recognize IIb-IIIa.166 Additional evidence is consistent with the hypothesis that the effect of IVIgG is to downregulate the autoimmune response because synthesis of antiplatelet antibodies has been shown to decrease in certain cases and corrections of impaired suppressor T-cell function have been noted.167-170 Less information is available on the clinical effectiveness of IVIgG in SLE-ITP, but two discrepant reports have appeared. Akashi et al171 reported that in seven patients the response rate after IVIgG was 71%, a value comparable to that observed in patients with primary ITP. On the other hand, Cohen and Li172 found that in three patients, this therapy had limited efficacy.

Other Protein Antigens in Primary ITP

Reports of autoantibodies specific for protein antigens other than IIb-IIIa or Ib-IX have appeared sporadically (Table 6). The increased sensitivity of assays such as the immunoblot assay and immunoprecipitation have permitted the detection of autoantibodies against less prominent protein antigens. Using the immunoblot assay, Lynch and Howe173 analyzed serum antibodies in 23 patients with ITP and 20 normal individuals. None of the normal sera at 1:10 dilution gave reactions above background, whereas 21 sera from ITP patients contained Ig that bound to multiple platelet proteins. Binding to platelet proteins ranging in size from 80 to 95 Kd was most frequently detected, but positive reactions were also often obtained with proteins ranging in size from 96 to 115 Kd and 116 to 145 Kd.

In three cases, autoantibodies that induce platelet aggregation have been described. Sugiyama et al174 identified an IgG autoantibody that recognizes a putative 62-Kd collagen receptor. This antigen is distinct from the 65-Kd protein antigen frequently encountered in cases of SLE (Kunicki T.J., unpublished observations). Pfueller et al175 reported
Table 6. Additional Protein Antigens Recognized by Serum Autoantibodies From Patients With Primary or Secondary ITP

| Primary ITP | Yanabu 1991 |
| 36-Kd surface protein | |
| 55-Kd α-granule protein (p55; PSL) | Barque 1990 |
| 62-Kd collagen receptor | Sugiyama 1987 |
| 80- to 95-Kd proteins | Lynch 1986 |
| 93-Kd surface protein | Pfueler 1990 |
| 106-Kd protein | Honda 1990 |
| Vimentin and desmin | Varon 1990 |
| Vinculin | Tomiyama 1992 |
| SLE | |
| 65-, 80-, and 120-Kd proteins | Howe 1987 |
| | Kaplan 1987 |
| | Jouhikainen 1989 |
| | Kekomäki 1989 |
| Vinculin | Tomiyama 1992 |
| PBC | |
| 70-Kd mitochondrial protein (M2) | Panzer 1990 |

Platelet Protein Autoantigens in ITP Secondary to Other Autoimmune Disorders

**SLE.** SLE with secondary autoimmune thrombocytopenia (SLE-ITP) represents a unique condition in which the platelet autoantigens may be different from those implicated in primary ITP. The immunoblot assay has been the principal method used to identify platelet protein antigens in SLE-ITP. Using this approach, IgG autoantibodies have been detected that react with protein antigens with apparent molecular weights of 65, 80, 108, and 120 Kd.\(^{160-182}\) Howe and Lynch\(^{180}\) were the first to detect antibodies to both the 80- and 120-Kd antigens in 10 of 10 patients with SLE. This antigen-binding pattern was not observed in sera from 20 normal individuals and infrequently in 20 patients with ITP.

We have determined that the 120- and 80-Kd antigens frequently detected by immunoblot assay with sera from thrombocytopenic patients are intact and fragmented vinculin, respectively.\(^{183}\) In contrast to the report of Howe and Lynch,\(^{180}\) we find antibodies reactive with vinculin in 67% of patients with primary ITP or secondary immune thrombocytopenia and in 40% of normal subjects.\(^{180}\) The finding of anti-vinculin antibodies in the sera of normal individuals by immunoblot assay had previously been made by Pfueler et al.\(^{184}\) However, in our hands, significantly higher levels of these antibodies are observed in patients with autoimmune thrombocytopenia, as compared with normal individuals.\(^{183}\)

Additional studies of one of these protein antigens, the 65-Kd protein, have determined that it is a membrane-associated, internal protein with an isoelectric point (pI) of 4.7 to 5.2 that is not recognized by polyclonal rabbit antisera specific for vimentin, a previously identified antigen target of SLE autoantibodies that has similar electrophoretic characteristics.\(^{182}\)

**PBC.** Autoantibodies reactive with IIb-IIIa were detected in a patient who unexpectedly developed thrombocytopenia after a 4-year history of PBC.\(^{186}\) Additional plateletractive antibodies were found that bound to the 70-Kd mitochondrial protein M2, considered a primary antigenic target in about 95% of cases of PBC. Based on these findings, the intriguing hypothesis was put forward that IIb-IIIa and the 70-Kd mitochondrial antigen bear cross-reactive epitopes. Indeed, two short sequences were found to be homologous when human IIIa and human M2 antigens were compared, but neither corresponds to the reported immunodominant epitope of the M2 protein.\(^{187}\) Unfortunately, cross-adsorption studies were not performed, and it has not been established that both antigens are bound by the same population of autoantibodies. The putative immunologic association between the two proteins was not strengthened by the fact that sera from 10 other patients with PBC did not contain antibodies reactive with platelet antigens, and sera from an undisclosed number of patients with ITP known to contain antibodies to IIb-IIIa did not contain antibodies that bind to the M2 protein.

**Human Immunodeficiency Virus (HIV)-Associated ITP**

Immune thrombocytopenia associated with HIV infection occurs predominantly among three groups of individu-
als: patients with hemophilia who use contaminated concentrates of factor VIII,188 narcotic addicts,189 and homosexuals.190 Clinically, HIV-associated ITP is indistinguishable from classical primary ITP, although there is some indication that the immune mechanisms involved in the etiology of ITP among narcotics addicts and homosexuals may be different from those operative in classic primary ITP and HIV-infected hemophilic patients.189-192

Hemophilic patients and classic ITP patients exhibit notable similarities that distinguish them from the other two disorders, namely, one sees an inverse relationship between platelet count and platelet-bound IgG, antiplatelet IgG can be eluted from patients’ platelets, and one does not see a significant increase in circulating immune complexes. Compared with patients with classic ITP, all three groups of HIV-associated patients with immune thrombocytopenia present with significantly higher levels of platelet-bound IgG and complement components C3 and C4.189,190,193,194

While there was an initial report that the antigens involved in HIV-associated ITP might be unique,195 subsequent studies have not substantiated this finding. Using an immunoblot assay, Stricker et al195 found antibodies in the sera of 29 of 30 patients with HIV-related ITP that bound to a 25-Kd platelet protein. They concluded that antibodies specific for HIV antigen(s) crossreact with this unidentified platelet protein. This protein antigen was not characterized further, and the presence of antibody reactive with this protein did not correlate with the presence of thrombocytopenia in these patients. Prevalence of this antibody specificity in HIV-associated ITP has not been established since this initial report. On the other hand, Klaassen et al196 found that autoantibodies both in sera and eluted from the platelets of 16 patients with acquired immunodeficiency syndrome (AIDS) have specificities that are largely the same as those seen in primary chronic ITP, the majority specific for antigens on IIb-IIIa.

Bettaieb et al197 analyzed antiplatelet antibodies in the sera and on the platelets of 68 HIV-infected but AIDS-free patients with ITP. Serum IgG specific for platelet antigens was detected in 72% of these patients. These IgG antibodies did not bind to surface antigens, but did react with intraerythrocytolytic constituents. Platelet-bound antibodies were detected in 75% of patients and isolated in ether eluates. In 44% of the cases, the eluted antibodies were found to bind to normal but not to thrombathaphenetic platelets. By immunoprecipitation, the specificity of autoantibodies in six eluates was determined. In two cases, IIb was the target; in one case, IIIa. In three other cases, reactivity with an unidentified 150-Kd protein was observed. This study confirms the generalization that autoantibody specificity in HIV-ITP is not different from that reported for classical, primary ITP.

Anti-cardiolipin antibodies (aCL) are frequently detected in the sera of HIV-infected patients,196 and a role for aCL in the etiology of the ITP that often accompanies HIV infection has been postulated. This association is based largely on reports that aCL are prevalent in sera of patients with chronic ITP,197 that a correlation is observed between aCL and thrombocytopenia in SLE,200 and that human hybridomas established from peripheral blood lymphocytes of patients with SLE produce MoAbs that bind both to cardiolipin and human platelets.201-203 Conflicting evidence also exists in the literature. For example, Klaassen et al196 noted the important discrepancy that, although aCL were detected in the sera of every patient with AIDS whom they analyzed, aCL were never present in eluates prepared from the platelets of the same patients.

Indeed, while several laboratories argue that thrombocytopenia in HIV-infected persons is a direct result of platelet-specific autoantibodies,193,196,204,205 it has also been proposed that it results from deposition on platelets of immune complexes.206,207 This conclusion is based on the findings that the amount of platelet-associated IgG (PAIgG) in HIV-infected persons with ITP is much higher than that observed in cases of classical primary ITP,290 that there is an increased amount of platelet-bound complement in the case of HIV-infected patients with ITP,206 and that, in eluates of platelets from HIV-infected patients with ITP, not only anti-HIV antibodies but also anti–anti-HIV antibodies (anti-Id antibodies) can be detected.207 Although the level of such complexes was not measured in HIV-infected persons without thrombocytopenia as a control, Karpkin et al207 argued that immune complexes composed of anti-HIV (idiotype) bound by anti-Id antibodies are the cause of Fc receptor-mediated thrombocytopenia in HIV-infected persons. This conclusion is not supported by the study by Klaassen et al, wherein only weak reactivity against HIV antigens was observed in eluates of patient platelets and equivalent weak reactivity was observed in all HIV-infected patients regardless of the presence or degree of thrombocytopenia.

Drug-Dependent Immune Thrombocytopenia: A Unique Form of Autoimmunity

Quinine/quinidine purpura. Although drug-induced thrombocytopenia (DITP) may be a complication of therapy using a variety of drugs, it is most frequently seen in the United States with the administration of quinine and quinidine.208 It has been proposed that the following criteria be met before an individual can be considered to have DITP: (1) the patient is not thrombocytopenic before administration of the drug; (2) thrombocytopenia follows drug ingestion and begins to reverse shortly after cessation of drug; (3) thrombocytopenia does not recur after cessation of drug treatment; and (4) all other causes of thrombocytopenia are ruled out.209

The exact mechanism for platelet clearance is not yet certain. However, cumulative evidence now favors a mechanism whereby the drug induces the expression of a neoantigen on the platelet surface that is recognized by circulating antibodies only in the presence of the drug. The observation that platelets from Bernard-Soulier syndrome (BSS) patients (lacking in Ib-IX and V) failed to lyse in the presence of drug-dependent antibody (ddAb), specific drug, and complement was the first indication that a specific platelet antigen is recognized by ddAb.215 This finding led other laboratories to confirm that purified Ib-IX would
compete for drug plus ddAb and was therefore likely to contain the antigenic epitope in question. Evidence of direct binding of ddAb to Ib-IX was first provided by Chong et al., and Berndt et al. established that the complexes of both Ib and IX are likely required for maximum antigen expression. Whereas epitope(s) on Ib-IX is almost certainly a major antigen in DITP, in a soluble form, has been shown to react with certain ddAb. Continued study of the drug-dependent autoimmune phenomena and their relationship to platelets is warranted with particular attention to a comparison of the clinical significance of drug-dependent autoantibodies that bind to either Ib-IX or IIb-IIIa.

To understand the mechanism whereby drugs like quinine or quinidine induce neoantigen formation, one must first consider the direct effects of these drugs on platelet membrane components, a subject that has not received enough attention by those interested in DITP. Deykin and Hellerstein were probably the first to show that quinidine inhibits in vitro platelet aggregation induced by ADP, collagen, or adrenaline. Lawson et al. showed that quinidine administration will induce the prolongation of the bleeding time without thrombocytopenia. Connellan et al.222 established that the complexes subsequently bind to the platelet surface via the Fc region of the molecule. The weight of recent evidence would suggest that one effect of such perturbations might be the development of neoantigens or the exposure of cryptic antigens.

Chong et al. used a panel of murine MoAbs in competitive binding assays to map the domains on Ib-IX that are bound by drug-dependent antibodies from 12 patients with DITP. The combined data show that one quinidine-ddAb binds to an epitope on the amino-terminal portion of the Ib heavy chain, and five other quinine-ddAb recognize a complex-specific epitope proximal to the membrane-associated region Ib-IX. Each of six quinidine-ddAb contained two specificities, one for the same Ib-IX complex epitope described above and another for IX alone. Additional observations were that ddAb reactive with Ib-IX are more predominant (12 of 12 patients) than those that bind IIIa (3 of 12 patients) and ddAb specifc for Ib-IX are present in titers 10- to 32-fold higher than the corresponding antibodies that bind to IIb-IIIa in the same patient samples. In each case, those antibodies that bound to Ib-IX were distinct from those that recognized IIb-IIIa.

Regions of IIb-IIIa that bind to ddAb have also been further localized by Visentin et al. Of 13 patients' sera samples containing quinidine- or quinidine-ddAb, 10 were reactive with both Ib-IX and IIb-IIIa, 2 reacted with Ib-IX alone, and 1 reacted with IIb-IIIa alone. Again, in those sera in which both specificities were identified, the anti-Ib-IX antibodies were distinct from those that bound to IIb-IIIa. Seven sera containing anti-IIb-IIIa antibodies were further characterized. Three bound only to the IIb-IIIa complex, one bound to Ib alone, and three bound to IIIa alone. Those that recognized IIIa alone were found to bind to epitopes on the major 61-Kd chymotryptic fragment of IIIa that is resistant to deglycosylation with endo-H.

**Heparin-Associated Immune Thrombocytopenia (HAT)**

HAT represents a unique form of platelet clearance, and the detailed mechanisms of platelet destruction remain to be precisely determined. The incidence of thrombocytopenia during intravenous heparin therapy is probably 5%, but estimates from prospective clinical studies range between 0% and 30%. The incidence associated with subcutaneous heparin therapy is much lower.

Chong has proposed a distinction between type I and type II HAT based on clinical findings and mechanisms underlying the pathophysiology of the disease. In type I HAT, the thrombocytopenia is usually mild (>100 × 10^3/μL), often self-limiting even if heparin is continued and probably results from the proaggregatory effects of heparin itself. In type II HAT, thrombocytopenia is more severe (<40 × 10^3/μL), the onset is delayed (7 to 14 days after commencement of heparin administration), and thrombocytopenia gradually regresses upon cessation of heparin, but recurs shortly after reexposure to heparin. Type II HAT is undoubtedly immune-mediated, and its effects can be multiplied by often serious thrombotic complications that are usually distinct from those for which the heparin was initially administered. Approximately 30% of patients with type II HAT and thrombosis die, with an additional 20% developing vascular occlusions that result in gangrene and subsequent amputation. The remainder of this review will focus on immune-mediated or type II HAT.

Heparin-dependent antibodies (HDA) differ from other forms of drug-dependent antibodies in that they are activating, causing not only thrombocytopenia, but heparin-dependent platelet aggregation, thromboxane synthesis, and granule release that can be quantitated by preloading platelets with [14C]serotonin. Two immune mechanisms by which HDA interact with platelets have been proposed. The first immune mechanism is similar to the case for quinine- and quinidine-dependent antibodies, in which the Fab region of the antibody binds to neoantigens formed on the platelet surface as a result of the interaction of heparin with an as yet undefined membrane component. The second immune mechanism is that heparin forms immune complexes with HDA in the plasma, and these complexes subsequently bind to the platelet surface via the Fc region of the molecule. The weight of recent evidence would suggest that the latter of these two scenarios is more likely the case. Kelton et al. showed that the platelet release reaction induced by HDA could be blocked by pretreating platelets with human or goat IgG Fc fragments, and Adelman et al. have shown that the Fab regions of HDA alone are not sufficient to cause platelet activation.
Similar studies have been performed by Chong et al, who showed that purified rabbit IgG and its Fc, but not Fab, fragments markedly inhibited platelet aggregation induced by HDA. Further evidence of Fc receptor involvement in HAT has been provided by a number of laboratories that used the monoclonal anti-Fc receptor antibody, IV.3, to block platelet activation by HDA, further supporting the notion that HAT is an Fc receptor-mediated event that involves both the Fab (for heparin binding) and Fc (for Fc receptor binding) regions of a heparin-IgG immune complex to achieve platelet activation and destruction.

Finally, although MoAbs to Ib-IX can interfere with HDA binding to the platelet surface, this is most probably due to stearic hindrance caused by the close proximity of the FcR to Ib-IX, as two different laboratories have convincingly shown that HDA bind normally to Bernard-Soulier platelets, which lack expression of surface Ib-IX-V.

There are, however, a number of findings that are not consistent with the immune-complex hypothesis. PfueUer and David reported that patient sera can induce platelet aggregation in the absence of heparin provided that priming concentrations of adrenalin are present. They therefore argued the existence of heparin-induced neoantigens on platelets that can subsequently bind antibody in the absence of heparin. Unlike quinine- or quinidine-dependent antibodies, the actual binding of HDA to the platelet surface appears to be of very low affinity, and has been difficult to show. However, Lynch and Howe reported that in an immunoblot assay antibodies from sera of patients with HAT in the presence of heparin bind to three platelet proteins (80, 124, and 180 Kd), none of which are the platelet Fc receptor FcRII (40 Kd). Two other weaknesses of the immune-complex theory are that no one has yet directly demonstrated the existence of the putative heparin-antibody complexes, and that the binding of such soluble complexes to platelet FcRII would be effectively inhibited by normal plasma levels of monomeric IgG. In light of these discrepancies, further studies of the mechanisms underlying the pathophysiology of HAT are clearly warranted.

CONCLUSIONS

A great deal of progress has been made within the last decade in understanding the molecular nature of platelet-specific protein antigens. The cloning and sequencing of platelet integrins and other membrane receptors has enabled us to detect polymorphisms in these important molecules that are alloantigenic. Improved methods for the detection and characterization of autoantigens have led to the localization of epitopes on platelet proteins and GPs that are targets of autoantibodies in chronic and acute ITP, as well as in ITP secondary to other autoimmune disorders. The development of human MoAbs and recombinant human antibodies represents an important technical milestone that will lead to the definition of idiotypes that are associated with pathologic antibodies. This increased knowledge derived from antigen characterization and the definition of idiotype repertoires will likely lead, within the next decade, to the ability to begin to control the immune response against platelets in humans.

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