The Human Platelet Alloantigens, PI^{A1} and PI^{A2}, Are Associated with a Leucine[33]/Proline[33] Amino Acid Polymorphism in Membrane Glycoprotein IIIa, and Are Distinguishable by DNA Typing

Peter J. Newman,*‡ Rebecca S. Derbes,* and Richard H. Aster*§
**The Blood Center of Southeastern Wisconsin, Milwaukee, Wisconsin 53233; ‡Department of Anatomy and Cellular Biology, and §Departments of Medicine and Pathology, The Medical College of Wisconsin, Milwaukee, Wisconsin 53233*

Abstract

The human platelet alloantigens, PI^{A1} and PI^{A2}, comprise a diallelic antigen system located on a component of the platelet fibrinogen receptor, membrane glycoprotein (GP) IIIa. Of the known platelet alloantigens, PI^{A1}, which is carried by 98% of the caucasian population, appears to be the alloantigen that most often provokes neonatal alloimmune thrombocytopenic purpura and posttransfusion purpura. The structural features of the GPIIIa molecule responsible for its antigenicity are as yet unknown. Using the polymerase chain reaction (PcR), we amplified the NH_2-terminal region of platelet GPIIIa mRNA derived from PI^{A1} and PI^{A2} homozygous individuals. Nucleotide sequence analysis of selected amplified cDNA products revealed a C ↔ T polymorphism at base 196 that created a unique Nci I restriction enzyme cleavage site in the PI^{A2}, but not the PI^{A1} form of GPIIIa cDNA. Subsequent restriction enzyme analysis of cDNAs generated by PcR from 10 $PI^{A1/A1}$, 5 $PI^{A2/A2}$, and 3 $PI^{A1/A2}$ individuals showed that Nci I digestion permitted clear discrimination between the PI^{A1} and PI^{A2} alleles of GPIIIa. All $PI^{A2/A2}$ individuals studied contain a C at base 196, whereas PI^{A1} homozygotes have a T at this position. This single base change results in a leucine/proline polymorphism at amino acid 33 from the NH_2-terminus, and is likely to impart significant differences in the secondary structures of these two allelic forms of the GPIIIa molecule. The ability to perform DNA-typing analysis for PI^{A} phenotype may have a number of useful clinical applications, including fetal testing and determination of the phenotype of severely thrombocytopenic individuals.

Introduction

The human platelet membrane glycoprotein (GP)[1] IIb-IIIa complex mediates platelet aggregation by acting as the functional receptor for fibrinogen on the platelet surface (1). In addition to this physiological role, both GPIIb and GPIIIa are known to bear a number of clinically important alloantigenic determinants that are responsible for eliciting the immune response in two well-described clinical syndromes, posttransfusion purpura (PTP) and neonatal alloimmune thrombocytopenic purpura (NATP) (2, 3). The alloantigen system most frequently implicated in these disorders is PI^{A}. There are two serologically defined allelic forms of the PI^{A} alloantigen, PI^{A1} and PI^{A2}, both of which have been localized to the GPIIIa molecule (2). The gene frequencies for these two alleles have been calculated to be 85% A1:15% A2 (4), based upon the observation that PI^{A2} homozygous individuals represent only 2% of the caucasian population. Since 98% of the population carries the PI^{A1} antigen, PI^{A2} homozygotes are at risk of producing anti-PI^{A1} antibodies against paternally inherited PI^{A1} antigens present on fetal platelets, and are most likely to develop PTP after blood transfusion.

Molecular definition of platelet alloantigenic determinants would contribute significantly to the understanding of the morphological features of platelet membrane glycoproteins that are responsible for eliciting an alloimmune response. Carbohydrate residues have been shown not to contribute significantly to the formation of the immunogenic PI^{A1} determinant (5), but determination of the amino acid sequence variation(s) that are presumably responsible for forming the relevant epitopes has not yet been possible, due largely to the formidable task of obtaining protein sequence information of both PI^{A1} and PI^{A2} forms of the 100-kD GPIIIa molecule. The complete amino acid sequence of GPIIIa has recently been deduced from the nucleotide sequences of both endothelial cell (6) and human erythroleukemia (HEL) cell (7, 8) GPIIIa cDNA clones, however it is likely that each of these clones represent the PI^{A1} rather than the PI^{A2} allele, owing to the relative abundance of PI^{A1} in the human gene pool.

Recently, we described a new approach for examining platelet-specific mRNA sequences from single individuals (9). We demonstrated that the RNA derived from the platelets present in 50 ml of whole blood can be converted to cDNA and then enzymatically amplified using the polymerase chain reaction (PcR) to produce microgram quantities of platelet-specific cDNA. By isolating and amplifying mRNA from a number of individuals of known PI^{A} allotype, it should be possible to examine whether phenotype-specific nucleotide sequence variations exist in the GPIIIa gene. In this report, we present the first molecular description of a polymorphism associated with a human platelet alloantigen, and show that the PI^{A1} allele can be distinguished from PI^{A2} by differential restriction endonuclease digestion of amplified platelet mRNA.

Address reprint requests to Dr. Newman, The Blood Center of Southeastern Wisconsin, 1701 West Wisconsin Avenue, Milwaukee, WI 53233.

Received for publication 24 October 1988 and in revised form 17 January 1989.

1. *Abbreviations used in this paper:* GP, glycoprotein; HEL, human erythroleukemia; NATP, neonatal alloimmune thrombocytopenic purpura; PcR, polymerase chain reaction; PTP, posttransfusion purpura.

J. Clin. Invest.

0021-9738/89/05/1778/04 $2.00
Volume 83, May 1989, 1778–1781

Methods

Serological determination of PI^A phenotype. The PI^A phenotype of 18 different individuals was determined using a standard antigen-capture ELISA assay (10, 11). 10 homozygous PI^{A1} individuals, 5 homozygous PI^{A2} individuals, and 3 heterozygotes for the PI^A allotype, all of whom had been unambiguously identified using well-characterized anti-PI^{A1} and anti-PI^{A2} antisera, were used throughout this study.

Amplification of platelet mRNA. Human platelet mRNA was prepared from anticoagulated whole blood as described previously (9). Two pairs of primers were constructed; an outer pair (primers 1 and 3), and an internally nested pair (primers 2 and 4), and used to amplify a region of the GPIIIa mRNA molecule that encodes the amino terminal 103 amino acids of mature glycoprotein IIIa. Bases 1–400 of the GPIIIa mRNA molecule are known to be encoded by at least three different exons that are broken by introns comprised of > 10 kb of sequence (personal communication from Drs. Gilbert C. White II and Susan Gidwitz, University of North Carolina-Chapel Hill and Ann B. Zimrin and Mortimer Poncz, University of Pennsylvania, Philadelphia), permitting ready distinction between PcR products that might be inadvertently derived from genomic DNA, rather than mRNA sequences. Primer 1 (5′-CGCGGGAGGCGGACGAGATGCG-3′) corresponds to the RNA strand from bases 4–25 of the published nucleotide sequence (6). Primer 2 (5′-GACTCGAGACTGTGCTGGCGCTG-3′) corresponds to bases 56–71 of the RNA strand, with an additional 7 bases encompassing an Xho I restriction enzyme recognition site incorporated onto the 5′-end to facilitate subsequent subcloning into plasmid vectors. The two anti-sense oligonucleotides were primer 3 (5′-CGCACTTGGATGGAGAAATTC-3′), which corresponds to nucleotides 412–392, and primer 4 (5′-CCGGATCCTTGGATGGAGAAATTC-3′), which corresponds to bases 408–392 plus an additional 7 bases that contain a Bam HI site. Primer 3 was used to prime first strand cDNA synthesis, using platelet mRNA as a template, in a total volume of 50 μl as previously described (9). All ensuing PcR reactions were performed in a programmable DNA thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT). The first 10 rounds of PcR were performed using primers 1 and 3 in a total volume of 100 μl using a regimen consisting of denaturing nucleic acid strands at 94°C for 90 s, annealing primers at 37°C for 2 min, and primer extending with Taq polymerase (Perkin-Elmer Cetus Corp.) at 72°C for 3.5 min. After the fifth thermal cycle, the primer annealing temperature was increased to 42°C. After the tenth cycle, the first primer pair was removed by centrifuge-driven dialysis of the PcR reaction mixture into a nearly identical buffer using Centricon 30 microconcentrators (Amicon Corp., Danvers, MA). The second reaction mix was identical to the first, except that internally nested primers 2 and 4 were used in place of primers 1 and 3. After oligonucleotide exchange, the reaction volume was again brought to 100 μl, including 2.5 U of fresh Taq polymerase, and PcR continued for an additional 21 thermal cycles. Primer annealing was performed at 42°C for rounds 11–15; 47°C for rounds 16–20, and 55°C for rounds 21–31. We have found that these conditions maximize specificity and yield for amplification of cDNA (unpublished observations). The presence of the additional bases used to form the restriction enzyme sites at the 5′ ends of primers 2 and 4 had no detrimental effect on the quantity of specific DNA produced during the PcR.

Analysis of amplified cDNAs. Selected amplified cDNAs were subcloned into the plasmid vector pGEM-7Zf (Promega Biotech, Madison, WI), and subjected to nucleotide sequence analysis. Dideoxy sequencing was performed using the modified T7 phage DNA polymerase, Sequenase (United States Biochemicals, Cleveland, OH), according to the manufacturer's directions. Most PcR reaction products were directly exchanged into sterile water using Centricon 30 microconcentrators, and then digested with Nci I (purchased from either New England Biolabs, Beverly, MA, or Bethesda Research Laboratories, Gaithersburg, MD). Restriction digests were analyzed on 1.5% agarose gels. Computer analyses of protein and nucleic acid sequences were performed using the program PC/GENE (Intelligenetics Inc., Mountain View, CA) operating on an IBM PC/AT-compatible microcomputer.

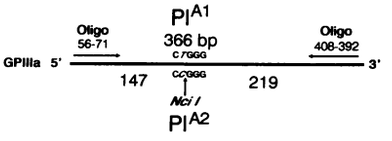

Figure 1. Diagramatic representation of the NH_2-terminal region of the GPIIIa mRNA molecule. The locations of the two oligonucleotide primers used for the PcR are shown, as well as the polymorphic sequence at base 196 of the amplified cDNA.

Results

Nucleotide sequence analysis of GPIIIa from a PI^{A2} homozygous individual. The nucleotide sequence of GPIIIa has been independently determined in three different laboratories (6–8). Except for a single silent polymorphism in the codon for Val^{355}, no interlaboratory sequence differences exist within the coding region for the mature GPIIIa molecule. Since the PI^{A1} phenotype is present in 98% of the population, it is likely that each of the three previously published sequences were derived from clones encoding the PI^{A1} form of GPIIIa. To examine the amino terminal region of the PI^{A2} allele, we used the polymerase chain reaction to amplify a 366-bases region near the 5′ end of platelet GPIIIa mRNA. Our amplification strategy used two sets of oligonucleotide primers, one nested internally to the first. The first 10 thermal cycles of the PcR amplified bases 4–412 ~ 1,000-fold, and provided a sufficient quantity of GPIIIa-specific cDNA to permit more stringent conditions to be used in subsequent rounds. The remaining thermal cycles amplified bases 56–408 using an internally nested primer pair, which are graphically depicted in the top portion of Fig. 1. This region encodes the first 103 amino acids of the mature GPIIIa protein, as well as a majority of the signal peptide. Using this protocol, we produced microgram amounts of the expected 366 bp cDNA from a number of individuals of known PI^A phenotype. After subcloning into the plasmid pGEM-7Zf, the complete nucleotide sequence of bases 79–408 from one PI^{A2} homozygous individual was determined on both strands, and found to be identical to the three previously reported sequences for GPIIIa, except at base 196 (sequence numbering according to reference 6), which had a deoxycytosine (C) in place of a deoxythymidine (T). This single base change (shown in Fig. 2) results in substitution of a Pro for Leu at amino acid residue 33 of the mature GPIIIa molecule, and is likely to impart significant secondary structural differences.

Restriction enzyme analysis of GPIIIa allotypes. Computer analysis of the GPIIIa sequence from bases 56–408 revealed that substitution of a C for a T at base 196 would create a recognition site for the restriction enzyme Nci I, which cleaves at 5′-*CCGGG*-3′ but not 5′-*CTGGG*-3′ sequences. To determine whether the T → C substitution found in the PI^{A2} individual studied above was related to PI^A allotype, or merely represented an artifact generated in vitro during either the reverse transcriptase or Taq polymerase reactions, or an unrelated polymorphism of GPIIIa, platelet RNA was prepared from a total of 18 individuals of known PI^A phenotype, and then amplified using PcR to yield the same 366-bp product. As

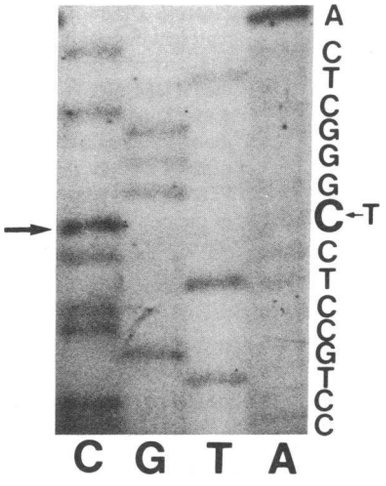

Figure 2. DNA sequence analysis of amplified GPIIIa cDNA derived from a PI^{A2} homozygous individual. The 366-bp PcR product was subcloned into the plasmid pGEM-7Zf, and sequenced on both strands using primers corresponding to the SP6 and T7 RNA polymerase promoter sequences. A segment of the autoradiograph, encompassing bases 188–203, is shown. The DNA sequence for the presumed PI^{A1} allele of GPIIIa has been previously reported, and is printed on the right in normal size lettering for reference. The single base substitution of a C for a T at base 196 is indicated with an arrow.

illustrated in Fig. 1, Nci I should not be able to cleave the 366-bp cDNA from a PI^{A1} homozygous individual, whereas two fragments of 147 and 219 bp would be generated in a PI^{A2} homozygote. Fig. 3 shows that, as anticipated, the 366 bp cDNA amplified from PI^{A1} homozygous individuals was not cleaved by Nci I, whereas the predicted restriction fragments were obtained from $PI^{A2/A2}$-derived cDNA. Serologically determined heterozygotes for the PI^{A} phenotype yielded both uncut 366 bp cDNA and the 219- and 147-bp restriction fragments, as expected. To date, we have successfully used this DNA typing procedure to predict the phenotype of 10 $PI^{A1/A1}$, 5 $PI^{A2/A2}$, and 3 $PI^{A1/A2}$ individuals ($P = 3.8 \times 10^{-6}$).

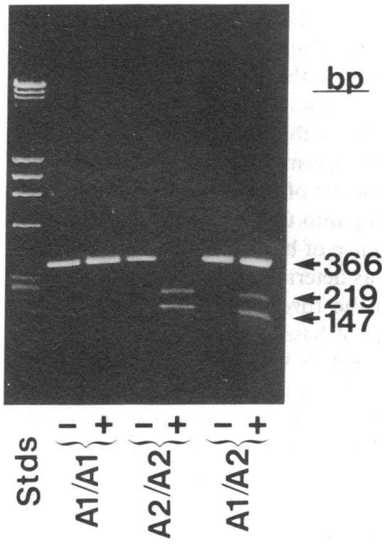

Figure 3. Analysis of PI^{A} phenotype by restriction endonuclease digestion. Bases 56–408 were enzymatically amplified from platelet RNA from 18 individuals of known PI^{A} phenotype. The resulting PcR products, both before (−) and after (+) digestion with the restriction enzyme Nci I, were analyzed on 1.5% agarose gels stained with ethidium bromide. The results from three representative individuals, one of each possible phenotype, are shown on this gel. cDNA derived from PI^{A1} homozygous individuals contain the sequence 5′-CTGGG-3′, and is not cleaved by the enzyme, whereas cDNA amplified from PI^{A2} homozygotes yielded two fragments of the expected sizes (see Fig. 1), confirming that the base pair polymorphism at nucleotide 196 segregates with PI^{A} phenotype. Heterozygotes for the allele show three bands, corresponding to undigested (PI^{A1}-derived) and cleaved (PI^{A2}-derived) GPIIIa cDNA. Though not readily visible in this figure, faint bands corresponding to nonspecifically amplified PcR products or to minute quantities of contaminating cDNA were occasionally visible in the gel lanes.

Discussion

The PI^{A1} antigen commonly stimulates the alloimmune response associated with two syndromes, neonatal alloimmune thrombocytopenia and posttransfusion purpura. The PI^{A1} and PI^{A2} antigenic determinants are carried on platelet membrane GPIIIa, and have been further localized to a 17–23-kD fragment of this molecule (5, 12). Our ability to directly compare the amino acid sequence of GPIIIa derived from PI^{A2} versus PI^{A1} individuals was made possible by: (*a*) the availability of the complete nucleotide sequence of GPIIIa cDNA (6–8) and (*b*) the application of gene amplification techniques to the study of phenotype-specific platelet mRNA sequences (9). Based upon the published sequence of GPIIIa cDNA, we constructed oligonucleotide primer pairs that allowed us to enzymatically amplify a segment of the GPIIIa mRNA molecule that encodes the amino-terminal 103 amino acids of the mature glycoprotein. Examination of the nucleotide sequence derived from one PI^{A2} homozygous individual revealed a C ↔ T polymorphism at base 196. This single nucleotide substitution fortuitously results in the creation of a unique restriction enzyme cleavage site. Utilizing the ability of the enzyme Nci I to discriminate between these two polymorphic sequences, we analyzed the phenotypes of 17 additional individuals, and were able to demonstrate that the PI^{A2} form of GPIIIa mRNA contains the codon *CC*G (Pro^{33}) in place of the *CU*G (Leu^{33} present in the PI^{A1} allele. As such, these findings represent the first molecular genetic characterization of a polymorphism associated with a platelet-specific alloantigen system, and provide compelling evidence that the PI^{A} phenotype is a genetically determined, inherent property of the GPIIIa molecule, rather than a posttranslationally acquired epitope that becomes associated with this membrane glycoprotein.

Although the PI^{A} phenotype is clearly associated with a Leu/Pro polymorphism at amino acid 33, these studies do not directly address the issue of which amino acids form the actual antigenic epitope. There are a number of possible ways that this residue might participate in the formation of the PI^{A1}/PI^{A2} antigenic determinant. Leu/Pro^{33} could be physically located at the antigenic site, in which case this residue would constitute an integral part of the structure of the epitope. It is also possible that the presence (PI^{A2}) or absence (PI^{A1}) of Pro^{33} alters the secondary structure of the polypeptide chain, causing the actual antigenic determinant to be formed at another site. A third possibility, though less likely, is that the Leu/Pro^{33} polymorphism is simply coinherited with an as yet unidentified additional polymorphic region of GPIIIa. It should be possible to test these models by producing phenotype-specific synthetic peptides encompassing amino acid 33, which can be examined for their ability to directly bind anti-PI^{A1} or anti-PI^{A2} antibodies, or to inhibit the binding of these alloantibodies to platelets of the appropriate phenotype. Similarly, antipeptide antibodies can be produced and examined for their ability to react selectively with platelets of known PI^{A1} and PI^{A2} phenotype. One potential obstacle in using peptides to examine PI^{A} epitopes is that they are often incapable of mimicking complex conformationally dependent determinants. Thus, an alternative strategy might involve the production of cell lines trans-

fected with GPIIIa molecules differing only at amino acid 33. Serological evaluation of such "phenotype-specific" transfectants may ultimately be more useful in defining the molecular nature of these alloantigenic determinants. In any event, it is clear that more work needs to be done in order to localize the epitopes on the native GPIIIa molecule to which Pl^A-specific antibodies bind.

Studies are currently in progress to determine whether other regions of the GPIIIa molecule contain polymorphisms that correlate with Pl^A phenotype. Zimrin et al. (7) and Rosa et al. (8) have each noted a potential silent C ↔ A polymorphism at base 1163, which is the third base in the codon for Val^{355}, and Burk et al. (13) have recently shown by analysis of genomic Southern blots that a Taq I polymorphism exists within the GPIIIa gene. Our inspection of the sequence surrounding base 1163 reveals that the polymorphic sequence 5′-T*C*GA-3′, but not 5′-T*A*GA-3′ represents a Taq I site, and is therefore probably identical to the Taq I polymorphism detected by Burk et al. using Southern analysis. This Taq I site is present in ~ 47% of the population (13), and is therefore unlikely to segregate with either the Pl^{A1} or Pl^{A2} allotype, which have gene frequencies of 0.85 and 0.15, respectively.

The ability to determine Pl^A phenotype by DNA typing may have several useful clinical applications. Current serologically based methods involve the use of human alloantisera, which in addition to being consumable, can vary widely with respect to titer and often contain contaminating anti-HLA antibodies. The PcR-based procedure, on the other hand, can readily be adapted such that genomic DNA, rather than platelet mRNA, is used as the source of the nucleotide sequence to be amplified. Lench et al. (14) have recently reported that sufficient DNA for gene analysis by PcR can be isolated from buccal epithelial cells obtained by mouthwash. One could envision applying this noninvasive method to the analysis of platelet phenotypes in a clinical setting, where the only other materials needed would be two primers, the restriction enzyme Nci I, and access to a thermal cycler. An obvious advantage over serologically based evaluation of platelet allotype would be that platelets themselves would not be needed to perform the analysis. Genomic DNA typing for platelet phenotype might be of greatest utility for testing severely thrombocytopenic patients with PTP or NATP, in performing fetal testing in situations where a high-risk for developing NATP is suspected, and in phenotyping individuals who are reluctant to donate blood.

Finally, we anticipate that the strategy employed in this investigation could readily be adapted to define polymorphisms in other alloantigen system, such as those of erythrocytes and granulocytes, that have thus far escaped detailed characterization using more classical biochemical and serological approaches.

Acknowledgments

We thank Dr. Jack Gorski of The Blood Center of Southeastern Wisconsin for his scientific counsel, and for critically reading the manuscript. We are also grateful to the Platelet Antibody Laboratory of The Blood Center of Southeastern Wisconsin for performing serological evaluation of platelet phenotype.

This work was supported by grants from the National Institutes of Health HL-38166 to Dr. Newman and HL-13629 to Dr. Aster.

References

1. Phillips, D. R., I. F. Charo, L. P. Parise, and L. A. Fitzgerald. 1988. The platelet membrane glycoprotein IIb-IIIa complex. *Blood.* 71:831–843.

2. Kunicki, T. J., and P. J. Newman. 1986. The biochemistry of platelet-specific antigens. *In* Current Studies in Hematology and Blood Transfusion. A. Hassig, editor. S. Karger, Basel. 18–32.

3. Aster, R. H. 1984. Clinical significance of platelet-specific antigens and antibodies. *In* Advances in Immunobiology and Bone Marrow Transplantation. J. McCullough and G. Sandler, editors. Alan R. Liss, New York. 103–118.

4. Shulman, N. R., R. H. Aster, A. Leitner, and M. C. Hiller. 1961. Immunoreactions involving platelets. V. Post-transfusion purpura due to a complement-fixing antigen. A proposed mechanism for thrombocytopenia and its relevance in "autoimmunity". *J. Clin. Invest.* 40:1597–1620.

5. Newman, P. J., L. S. Martin, M. A. Knipp, and R. A. Kahn. 1985. Studies on the nature of the human platelet alloantigen, Pl^{A1}. Localization to a 17,000-dalton polypeptide. *Mol. Immunol.* 22:719–729.

6. Fitzgerald, L. A., B. Steiner, S. C. Rall, Jr., S-S. Lo, and D. R. Phillips. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. Identity with platelet glycoprotein IIIa and similarity to integrin. *J. Biol. Chem.* 262:3936–3939.

7. Zimrin, A. B., R. Eisman, G. Vilaire, E. Schwartz, J. S. Bennett, and M. Poncz. 1988. Structure of platelet glycoprotein IIIa. A common subunit for two different membrane receptors. *J. Clin. Invest.* 81:1470–1475.

8. Rosa, J-P., P. F. Bray, O. Gayet, G. I. Johnston, R. G. Cook, K. W. Jackson, M. A. Shuman, and R. P. McEver. 1988. Cloning of glycoprotein IIIa cDNA from human erythroleukemia cells and localization of the gene to chromosome 17. *Blood.* 72:593–600.

9. Newman, P. J., J. Gorski, G. C. White II, S. Gidwitz, C. J. Cretney, and R. H. Aster. 1988. Enzymatic amplification of platelet-specific messenger RNA using the polymerase chain reaction. *J. Clin. Invest.* 82:739–743.

10. Woods, V. L., E. H. Oh, D. Mason, and R. McMillan. 1984. Autoantibodies against the platelet glycoprotein IIb/IIIa complex in patients with chronic ITP. *Blood.* 63:368–375.

11. Furihata, K., D. J. Nugent, A. Bissonette, R. H. Aster, and T. J. Kunicki. 1987. On the association of the platelet-specific alloantigen, Pen^a with glycoprotein IIIa. Evidence for heterogeneity of glycoprotein IIIa. *J. Clin. Invest.* 80:1624–1630.

12. Van Der Shoot, C. E., M. Wester, A. E. G. Kr. von Dem Borne, and H. G. Huisman. 1986. Characterization of platelet-specific alloantigens by immunoblotting: localization of Zw and Bak antigens. *Br. J. Haematol.* 64:715–723.

13. Burk, C., C. Ingram, M. Weiner, E. F. Rappaport, E. Schwartz, and M. Poncz. 1988. A Taq I polymorphism for the human platelet glycoprotein IIIa gene (GPIIIa). *Nucleic Acids Res.* 16:7216.

14. Lench, N., P. Stanier, and R. Williamson. 1988. A simple non-invasive way to obtain DNA for gene analysis. *Lancet.* i:1356–1358.