Absence of Platelet-Specific Alloantigens in Glanzmann’s Thrombasthenia


The platelets of 11 patients with Glanzmann’s thrombasthenia and their nearest family members were studied for the expression of the platelet-specific alloantigens of the Zw, Ko and Bak systems. The strength of the expression of the Zw antigen was diminished on the platelets of 3 patients, and the antigen was absent from the platelets of the other 8. The platelets of none of the patients reacted with anti-Zw serum. Therefore, Glanzmann’s thrombasthenia is probably a “Zw-null disease.” The expression of the Zw antigen on the platelets of all the relatives was normal, as indicated on the cytofluorograph. Investigations on the expression of the Ko antigen were complicated by agglutinations of the platelets from genetically Ko-negative thrombasthenic patients with the anti-Ko serum. The Ko antigen was normally expressed. The Bak antigen was absent from the platelets of all thrombasthenic patients and a relatively high percentage of the relatives. A close association between the Glanzmann gene and the Bak(a–) gene is assumed on statistical grounds. Thrombasthenic platelets showed no reaction with EDTA-dependent antibodies, which are reactive with all normal platelets. Owing to immunization by multiple blood and platelet transfusions, serum samples of most patients studied contained HLA antibodies and platelet-specific alloantibodies. However, antibodies directed against the Zw-antigen-bearing glycoproteins were detected in the serum of only one patient and, therefore, seem to be rare.

MATERIALS AND METHODS

Patients

The 11 patients, 5 males and 6 females, were all white. They had the classical clinical features of G.T. and fulfilled the diagnostic criteria: markedly prolonged bleeding time, a normal platelet count, strongly diminished platelet aggregation in response to ADP, epinephrin, collagen and thrombin and the absence of clot retraction. The diagnosis was confirmed in eight patients by SDS-PAG electrophoretic analysis of the platelet-membrane glycoproteins, which showed a marked reduction of GP Iib and Ila (performed by G.J.E. Zonneveld and C.S.P. Jenkins, Wilhelmina Gasthuis, Amsterdam, The Netherlands). Two patients were brothers, the others were unrelated. The age of the patients ranged from 4 to 59 yr. Eight parents, 12 children, and 11 brothers and sisters of the patients in 7 families, were examined. The family members were all healthy and showed no bleeding disorders. The membrane glycoprotein patterns of the platelets from 14 of the family members (also performed by Zonneveld and Jenkins) showed no obvious abnormalities.

Platelet-Specific Antisera

The two anti-Zw sera and the anti-Bak serum were obtained from mothers whose children had neonatal allo-immune thrombocytopenia. The specificity of both anti-Zw sera was confirmed by their reactions with a large panel of platelets from Zw+ and Zw− donors.

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Zw*-typed donors. The anti-Bak* serum was obtained from a mother, who gave birth to a child with severe alloimmune thrombocytopenia. The platelet specific antibodies in this serum appeared to be directed against a new antigen, which we called Bak*. A detailed description will be published elsewhere.\(^5\)

No HLA antibodies were detectable in these sera. In case of ABO incompatibility between serum and platelets tested, the serum was pretreated with AB substance (Biotest-Serum Institut GmbH, Frankfurt/M., West Germany). The anti-Zw* and anti-Bak* were nonagglutinating, noncomplement fixing antibodies of the IgG class detectable in the platelet suspension immunofluorescence test (PSIFT). The anti-Zw*, anti-Ko* and anti-Ko* sera have been described by van der Weerd.\(^{14}\) These three sera contain platelet-specific IgM antibodies, and are specific in the platelet agglutination test, but not in the PSIFT.\(^{19}\)

As negative controls in both test methods for the detection of platelet antibodies, sera were used from young male donors of blood group AB who had never received a blood transfusion. Polyspecific HLA antisera from polytransfused patients were used as positive controls for the immunofluorescence test. We also applied 10 different sera that contained EDTA-dependent platelet specific antibodies. These sera were obtained from patients with pseudo-thrombocytopenia, all patients with spuriously low platelet counts in EDTA blood, and normal counts in citrate or capillary blood.\(^{10}\) The sera appeared to contain agglutinating antibodies, also detectable in the PSIFT, which react with platelets from the patients, and from normal donors, only in the presence of EDTA, and independent of their platelet group Zw or Ko phenotype. A detailed description will be published elsewhere\(^{10}\) (see also references 11 and 12).

**Antiglobulin Reagents**

The following antiglobulin reagents, labeled with fluorescein isothiocyanate (FITC), were used: anti-Ig (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, SH 17-01-F11) in a 1:15 dilution, and anti IgG (Dakopatts, Copenhagen, Denmark, F 1090) in a 1:60 dilution.

**Platelet Suspension Immunofluorescence Test (PSIFT)**

The PSIFT has been described in detail by Borne et al.\(^{13}\) In short, 0.1 ml of a suspension of paraformaldehyde-fixed platelets with a concentration of \(4 \times 10^{11}/\)liter was incubated with 0.1 ml of serum or dilution of serum for 30 min. The anti-Zw* sera were applied at 37°C and the anti-Bak* serum at 20°C, the optimal temperature for this serum. After incubation, the platelets were washed and incubated with FITC-labeled antiglobulin in the dilution described above for 30 min at 20°C. The platelets were washed again, resuspended in glycerol-PBS, mounted on a glass slide and examined with a fluorescence microscope (Leitz Orthoplan with a mercury lamp).

Quantification of the platelet-membrane immunofluorescence was performed with a Cytofluorograph FC 200/FC 4800A (Ortho Instruments, formerly Biophysics Inc.) equipped with a model-2100 distribution analyzer. The method will be described in detail by Helmerhorst et al.\(^{14}\) The relative fluorescence intensity was expressed in micrographs.\(^{15}\)

**Platelet Agglutination Test (PAT)**

The PAT has been described in detail by van der Weerd.\(^{14}\) In short, 0.03 ml of a suspension of unfixed platelets in a concentration of \(4 \times 10^{11}/\)liter was mixed on a nonsilicized Kline plate with 0.06 ml of EDTA-treated serum (9 parts + 1 part of 2% (w/v) Na2EDTA in PBS), covered by a plastic lid, and rotated on a Kline shaker for 30 min at 4°C. The reactions were read microscopically.

**Absorption Experiments With Platelets**

One part of EDTA serum (see PAT) was incubated with one part of packed platelets for 1 hr at 4°C. The mixture was centrifuged, then the supernatant serum was taken off and tested against platelets in the PAT.

**Erythrocyte Antisera**

Specific antisera against the following red-cell antigen systems were used: ABO, Rhesus, P, Kell, Duffy, Kidd, MNS and Lutheran.

**Lymphocyte Immunofluorescence Test (LIFT)**

The LIFT has been described by Décair et al.\(^{18}\)

**Lymphocyte Microcytotoxicity Test (LCT)**

The standard NIH microcytotoxicity test was used. A panel of 150 antisera was used for the HLA-A, -B and -C typing.

**Statistical Evaluation**

Associations were calculated with the Chi-square method.

**RESULTS**

**Typing for Platelet-Specific Alloantigens**

The platelets from 8 of the 11 patients showed absolutely no reaction with the anti-Zw* sera in the PSIFT (Table I). The platelets of the other 3 patients showed some reaction with these sera, but the reactions were much weaker than with platelets from normal homozygous or heterozygous Zw*-positive individuals. This was substantiated by measuring the fluorescence cytofluorometrically, with results as shown in Fig. 1. It is noteworthy that the two brothers showed different expressions of the Zw* antigen on their platelets, one being weakly positive, the other completely negative, with anti-Zw* sera.

The platelets of none of the patients reacted with the anti-Zwb* serum; those of 8 of the 11 patients reacted positively with the anti-Ko* serum, and the

<p>| Table 1. Reaction Patterns of the Platelets of 10 Patients With Glanzmann’s Thrombasthenia and Their Relatives With Platelet-Specific Alloantibodies in Relation to the Phenotype Frequency of the Corresponding Antigens in the Normal Dutch Population |
|----------------------------------------|----------------------|----------------------|----------------------|</p>
<table>
<thead>
<tr>
<th>Antisera</th>
<th>G.T. Patients Number</th>
<th>Relatives Number</th>
<th>Phenotype Frequency in the Dutch Population (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Zw*</td>
<td>3/11 27.3</td>
<td>31/31 100</td>
<td>97.6</td>
</tr>
<tr>
<td>Anti-Zwb</td>
<td>0/11 0</td>
<td>0/31 0</td>
<td>26.8</td>
</tr>
<tr>
<td>Anti-Ko*</td>
<td>8/11 72.7</td>
<td>9/31 29</td>
<td>14.3</td>
</tr>
<tr>
<td>Anti-Koa</td>
<td>11/11 100</td>
<td>31/31 100</td>
<td>99.4</td>
</tr>
<tr>
<td>Anti-Bak*</td>
<td>0/11 0</td>
<td>16/31 52.6</td>
<td>90.8</td>
</tr>
</tbody>
</table>

*Weak reactions.
PLATELET-ANTIGENS IN GLANZMANN'S DISEASE

The platelets of 5 of 6 patients tested did not react in the PSIFT with EDTA-dependent antibodies. The platelets of the sixth patient gave a weakly positive reaction. These reactions were quantitatively similar to the reaction of these platelets with the anti-Zw⁺ sera.

No association between HLA or red-cell antigens and G.T. was established.

Absorption Experiments

In 2 patients, the positive reactions with the anti-Ko⁺ serum could not be explained on a genetic basis, when their families were examined. The Ko genes are inherited as co-dominant factors; thus at least one of the parents of a Ko⁺-positive (Ko[a⁺]) patient must be Ko(a⁺). However, from one Ko(a⁺) patient, both parents were Ko⁺-negative (Ko[a⁻]), while nonpaternity was very unlikely from the distribution of HLA antigens and erythrocyte blood groups. In the family of another Ko(a⁺)-typed patient, the mother was Ko(a⁻), but unfortunately the father was not available for typing. However, among 5 sibs of the patient, no Ko⁺ positives were observed, and his 3 children were Ko(a⁻). Statistical evaluation revealed a very low probability (p = 0.0078) that the positive reaction with the anti-Ko⁺ serum could be genetically explained. Absorption experiments were performed (Table 2) to see whether this irregular positive agglutination with anti-Ko⁺ was caused by the presence of the Ko⁺ antigen on these G.T. platelets.

The anti-Ko⁺ serum was absorbed with platelets of a homozygous Ko(a⁺b⁻) normal donor. After absorption, the serum did not react with the platelets of this donor or with the platelets of the 2 patients mentioned. However, the anti-Ko⁺ serum, after absorption with platelets of a genetically irregular Ko⁺-typed Glanzmann patient, still showed the same strongly positive

| Table 2. Absorption Experiments on the Ko⁺ System in Two Patients With Glanzmann's Thrombasthenia, Who Were Genetically-Irregular, Ko⁺-Positive Typed |
|-----------------|-----------------|-----------------|-----------------|
| Anti-Ko⁺ serum before absorption | +++++ (1/160)* | +++++ (1/640)* |
| Anti-Ko⁺ serum after absorption with platelets of Ko⁺-positive typed normal donor | – | – |
| Anti-Ko⁺ serum after absorption with platelets of Glanzmann patient | + + + (1/160)* | – |

*The greatest dilutions in which antibodies were detectable.
reaction with normal Ko\(^a\)-positive platelets as before absorption, but the reaction with the patient's own platelets had become negative. Thus, the positive reaction of the platelets from two patients with the anti-Ko\(^a\) serum was independent of the presence of the Ko\(^a\) antigen.

**Investigation of the Platelet-Specific Antibodies Detectable in the Serum of Glanzmann Patients**

The sera of the 11 Glanzmann patients, most of them polytransfused, were examined for the presence of alloantibodies against the platelets of 10 random donors of blood group 0, by using the PSIFT. To be able to distinguish between antibodies against HLA antigens, also present on platelets, and platelet-specific alloantibodies, we also performed the LCT and LIFT with the lymphocytes of these donors. If the serum of a patient reacted only with the platelets of a donor and not with the lymphocytes of the same donor, we concluded that platelet-specific antibodies were present in the patient's serum.

In addition, we tested the sera of the patients, using the PAT, against a panel of platelets from 9 donors typed for the platelet-specific alloantigens and from 2 Glanzmann patients, who were completely negative with the anti-Zw\(^a\) sera. In most serum samples, HLA antibodies were detectable. With the PSIFT, platelet-specific antibodies were shown also to be present in some of the samples; these antibodies did not react with all normal donor platelets (Table 3). The specificity of these antibodies could not be determined, because of the difficulty in removing the HLA antibodies by absorption without losing the rather weak, platelet-specific antibodies. Only in one serum were strongly reacting platelet-specific antibodies present in addition to HLA antibodies. This serum also reacted strongly in the PAT with all platelets except thrombasthenic ones. The sera of the other 10 patients reacted neither with platelets from normal donors nor with platelets from thrombasthenic patients in the PAT.

**DISCUSSION**

So far, three bi-allelic platelet-specific allo-antigen systems are known, Zw(P1\(^a\)), Ko and P1\(^b\), all showing a co-dominant inheritance.\(^{19-21}\) Recently, another system, Bak, has been detected, from which only one antigen, Bak\(^a\), can be recognized at present.\(^8\) The phenotype frequency of the platelet-specific alloantigens Zw\(^a\), Zw\(^b\), Ko\(^a\), Ko\(^b\), and Bak\(^a\) in the Dutch population is shown in Table 1.

In accord with the results of Kunicki et al.\(^6\) and Muller et al.,\(^4\) we found a significant association between G.T. and the absence of the Zw\(^a\) antigen, even if we consider the weak reactions obtained with anti-Zw\(^a\) and the platelets of 3 patients to be truly positive \((p < 0.001)\). Moreover, the platelets of none of the patients reacted with anti-Zw\(^b\). The association between the absence of the Zw\(^b\) antigen and G.T. is also significant \((p = 0.32)\). This means that there is no genetical linkage between Zw\(^b\) and this disorder, but that we are actually dealing with Zw-null disease, i.e., no expression of either Zw\(^a\) or Zw\(^b\).

G.T. is transmitted as an autosomal recessive trait.\(^1\) The patients suffering from the disease are genotypically homozygous for G.T., their phenotypically healthy parents, offspring and two-thirds of their healthy siblings are genotypically heterozygous for G.T., and one-third of their healthy siblings are genotypically homozygously normal. The question, therefore, was whether there is a reproducible difference in the quantitative expression of the Zw\(^a\) antigen in the healthy relatives of the patients, which may predict heterozygoscity or carriership for G.T. This was not so. The platelets from all relatives reacted equally well with anti-Zw\(^a\) sera, and of none of them with anti-Zw\(^b\).

Moreover, when anti-Zw\(^a\) binding was measured cyto-fluorometrically, no significant differences were found. However, we also detected no difference in the amounts of Zw\(^a\) on the platelets of normal Zw(a+b−) and normal Zw(a+b+) individuals (unpublished observations). This is in contrast with the report of Shulman et al.,\(^5\) who detected a gene-dose dependency of the expression of Zw\(^a\) on platelets, using the complement-fixation test. Therefore, it could be that our technique is not suitable for the detection of subtle differences in the amount of Zw\(^a\) on platelets of heterozygotes and homozygotes.

Recently, Kunicki et al.\(^6\) have shown that the Zw\(^a\)-antigenic marker is associated with glycoprotein IIIa, deficient in G.T. No obvious quantitative differences in glycoprotein patterns, especially GP IIIa, between platelets from parents or offspring and healthy siblings, were observed. Neither did platelets of normal Zw(a−, b+) donors, show abnormalities in their membrane glycoprotein patterns (Zonneveld and Jenkins' unpublished results), which is in accordance with the observations of Kunicki.\(^6\) These findings together suggest the homozygous presence of a recessive abnormal allele of a structural or a regular gene in

<table>
<thead>
<tr>
<th>Number of Patients</th>
<th>Without Antibodies</th>
<th>HLA Antibodies</th>
<th>Platelet-Specific Antibodies</th>
<th>Number of Donors of Blood Transfusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>&lt;25</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>7</td>
<td>5</td>
<td>&gt;25</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
The Ko-b-antigen phenotype frequency in the Glanzmann patients was in correspondence with the phenotype in the normal Dutch population.

The platelets of none of the patients reacted with anti-Bak b- serum, which gives a positive reaction with the platelets of 90.6% of the normal Dutch population. This means that there is a significant association ($p < 0.001$) between G.T. and negativity for Bak b- . A high percentage of the parents, normal sibs and children of the patients was Bak(a - ), and the segregation of Bak b- in the families was genetically regular. Homozygous Bak (a - ) normal individuals do not have G.T. and there is believed to be no linkage between the Zw and Bak genes. Therefore, the above finding can only be explained by a very close association between the Glanzmann gene(s) and the gene(s) responsible for negativity for Bak b- .

Pegels et al. \cite{ Pegels 1974} recently found that the well-known phenomenon of pseudothrombocytopenia, i.e., platelet agglutination that appears only in vitro in the presence of EDTA, is caused by EDTA-dependent antibodies, that cause a strong immunofluorescence in the PSIFT with all normal human platelets, independent of their phenotype for platelet-specific alloantigens. Platelets of 5 of our patients showed no reaction, those of 1 patient only a weak reaction, with these EDTA-dependent antibodies; the other 5 patients were not tested. This shows that the receptor for these antibodies is present on the glycoproteins IIb and/or IIIa. This is similar to the lack of the receptor for quinine-dependent platelet antibodies on the platelets of patients with the Bernard Soulier syndrome, which is present on GP Ib and Is.

Patients with G.T. often need platelet transfusions and, accordingly, most of our 11 patients studied had received more than 15 blood transfusions from various donors. Because, from nearly all of our patients, the Zw-bearing glycoproteins, present on platelets of normal donors, are missing, immunization against these glycoproteins could be expected to occur often. Degos et al. \cite{ Degos 1973} have reported the presence of an IgG platelet-specific allo-antibody in the serum of a Glanzmann patient that reacted with platelets of 350 normal individuals, but not with the platelets of 8 other thrombocytic patients. Muller et al. \cite{ Muller 1972} reported the presence of such “anti-public” antibodies in the sera of 2 out of 6 patients with G.T. We found such antibodies in the serum of only 1 of the 11 patients. More often did we find weak platelet-specific antibodies that did not react with the platelets of all normal individuals, and HLA antibodies. This could mean that the incidence of immunization against these antigenic determinants is low. Therefore, when Glanzmann patients become refractory to platelet therapy, it is worthwhile to look for normal donors, compatible for HLA and/or

G.T. This gene may be concerned in the synthesis of glycoproteins IIb and IIIa, of which IIIa carries the Zw b- and possibly also the Zw b- -antigenic determinant. Of these two possibilities, the second, i.e., that an abnormal regulator gene operates in this disease, is the more likely. The platelets of thrombasthenic patients showed heterogeneity of the extent of the membrane-protein abnormality and of the expression of Zw b- on their membrane, as Kunicki showed,\cite{ Kunicki 1972} and even in one family, as we observed in this study. If an abnormal structural gene, directly concerned with the synthesis of glycoproteins IIb and IIIa and/or Zw antigens, were responsible for G.T., then it would be improbable, if this gene occurs in a homozygous state, that there were any normal GP IIb - IIIa and any Zw b- on the platelet membrane. In particular, the different expression in one family cannot be explained by abnormal structural genes. Only abnormal gene regulation can explain why the repression of the synthesis of the glycoproteins and Zw antigens is not 100% and may be different in sibs. The Glanzmann patients might then be genotypically Zw-positive, but phenotypically Zw-negative. Their heterozygous relatives, phenotypically Zw(a+, b-), might then be normal homozygous Zw b- Zw b- and not Zw(a- Zw b-). So far, we have not been able to confirm this hypothesis by typing the platelets from a parent of a patient as heterozygous Zw(a+, b+).

It is possible that the Zw antigens are determined by enzymes, the products of the Zw alleles, which transfer the appropriate sugar, determining Zw b- and Zw b+, onto ground substance, glycoprotein IIIa. However, since Kunicki et al.\cite{ Kunicki 1972} showed that the Zw b- antigen is not present on the carbohydrate part of glycoprotein IIIa, but on it its protein part, it is more likely that the structural gene of glycoprotein IIIa (and IIb) and the gene coding for Zw are identical.

Another explanation for the findings in G.T. may be that an abnormal allele of a structural gene is responsible for the formation of an unstable form of the glycoprotein IIIa and IIb.

The platelets of a relatively high number (8/11) of patients reacted positively with the anti-Ko b- serum. The phenotype frequency in the normal Dutch population is 14.9%. However, absorption and elution experiments showed that in at least two patients this reaction was independent from the anti-Ko b- -antibodies. In our anti-Ko b- serum additional antibodies are known to be present giving weak agglutination with normal platelets. Further investigations have to be carried out to explain the strong agglutination of thrombasthenic platelets with these antibodies. A poly-agglutinability, caused by decreased surface charge of the platelets, is unlikely since thrombasthenic platelets show a normal electrophoretic mobility.\cite{ Van Deenen 1969}
CONCLUSION

Glanzmann’s thrombasthenia is a Zw\(\text{null}\) disease. Simple methods to measure the expression of the Zw\(^a\) and Zw\(^b\) antigens on platelets provide a useful marker for the diagnosis of this disease. The gene(s) for G.T. are closely associated to the gene(s) for Bak\(^{(a-)}\). The incidence of immunization against the Zw antigens in thrombasthenic patients is lower than expected.

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REFERENCES

17. de Bruin HG, van der Meulen FW, Aaij C: Quantitation of antibodies bound to cells or particles using flow cytofluorometry, in Flow Cytometry IV. Bergen, Norway, Universitetsforlaget. (In press)
25. Munro HK, von dem Borne AEG Kr, van Leeuwen EF, von Riesz LE, van Loghem JJ, Marguet J, Engelfriet CP: Neonatal allo-immune thrombocytopenia; detection of the responsible antibodies by the platelet immunofluorescence test. Blood. (Submitted for publication)
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