Pseudothrombocytopenia: An Immunologic Study on Platelet Antibodies Dependent on Ethylene Diamine Tetra-Acetate


Antibodies specifically reacting with platelets only in the presence of EDTA, by the platelet immunofluorescence test, were found in the serum of 20 patients with pseudo-thrombocytopenia due to in vitro EDTA-dependent platelet agglutination. These antibodies reacted optimally at 0-4°C. In 19 patients, IgG antibodies were detected; in 8 patients, IgM or IgA antibodies were also found. In one patient, only IgM antibodies were found. In 14 patients, the IgG antibodies were IgG1, but IgG2, IgG3, and IgG4 antibodies were also seen in 7 patients. The reaction of platelets with the antibodies was detectable in the presence of Na2EDTA, the K2, Ca, and Mg salts of EDTA, and K2EGTA. F(ab')2 or F(ab') fragments of the IgG antibodies reacted as strongly as the intact antibodies, indicating that the reaction is dependent on the antibody-combining site. The EDTA-dependent antibodies did not show platelet-group specificity. However, platelets from patients with Glanzmann disease did not react with the antibodies.

MATERIALS AND METHODS

Patients

Three patients from our hospital with known pseudothrombocytopenia were studied extensively. The platelet count was low (<70 x 10^9/liter) in EDTA-anticoagulated blood as found in an electronic particle counter, and large agglutinates were seen in smears from EDTA blood. In capillary blood, obtained by finger puncture, the platelet count was normal and agglutinates were absent. The sera of 17 patients in other hospitals were examined. In 7 cases, the diagnosis of pseudothrombocytopenia had been made; in 10 cases, idiopathic thrombocytopenia had been erroneously diagnosed.

Sera

Sera from the patients was heat-inactivated for 30 min at 56°C before use. Heat-inactivated pooled sera from healthy random nonimmunized AB donors were used as a negative control.

Serum containing anti-Zw* antibodies and a serum that contained several HLA antibodies were used as positive controls.

Blood

Blood anticoagulated with EDTA (one part of 5%, w/v, Na2EDTA and 9 parts of blood) and citrate (one part of 3.8%, w/v, trisodium citrate, 2H2O, and 9 parts of blood) from the patients and from healthy random donors of blood group 0 were used for the preparation of platelet suspensions, as described by von dem Borne et al.1 In this report, platelets from blood anticoagulated with EDTA will simply be referred to as EDTA platelets, and platelets from blood anticoagulated with citrate as citrate platelets.

Antiglobulin Reagents

For the immunofluorescence test (IFT) on platelets, we used polyclonal sheep anti-human Ig labeled with fluorescein isothiocyanate (FITC) (code number SH 17-01-F08), specific rabbit anti-human IgG labeled with FITC (KH 16-103-F4), rabbit anti-human IgG1 (KH 161-51-A1), anti-IgG2 (KH 162-05-A3), anti-IgG3 (KH 163-45-A1), and anti-IgG4 (KH 164-46-A1), in conjunction with horse anti-rabbit Ig labeled with FITC (PK 17-2-F4), all products from our laboratory. Specific rabbit anti-human IgG, IgM, and IgA labeled with FITC from Dakopatts (Copenhagen, Denmark) were also used. For the IFT on lymphocytes, we used specific rabbit anti-human IgG labeled with FITC (KH 16-103-F4), and for the IFT on granulocytes, F(ab')2, fragments of polyspecific sheep anti-human antimmunoglobulin labeled with FITC (S26-H17-1-F3), also products from our laboratory. The optimal dilution of the antiglobulin reagents was determined by titration with various dilutions in saline of antibody-containing sera and normal AB sera. The optimal dilution was the dilution that gave maximal fluorescence with the antibody-containing sera, but no fluorescence with the normal AB sera.

Methods

The immunofluorescence test on lymphocytes (LIFT) and on granulocytes (GIFT) was performed as described by Décaray et al.17 and Verheugt et al.9 and the platelet agglutination test as described by van der Weerd.10 The direct and indirect platelet suspension immunofluorescence tests on paraformaldehyde-fixed cells (PSIFT) were performed as described by von dem Borne et al.1 Platelets were prepared by differential centrifugation from the EDTA blood of the donor. The platelets were washed 3 times in a Na2EDTA-PBS solution, pH 7.4. The platelets were fixed with 1% paraformaldehyde in PBS for 5 min at room temperature, washed again twice, and resuspended in Na2EDTA-PBS solution to a final platelet concentration of 1.5 x 10^9/ml. Platelet suspension (0.1 ml) and serum or eluate (0.1 ml) from the platelets were mixed. After incubation for 30 min at 20°C, the platelets were washed three times, and 0.1 ml FITC-labeled antiglobulin reagent, diluted in...
PBS, was added. This mixture was incubated for 30 min at 20°C. The platelets were washed again twice and resuspended in a mixture of glycerol and PBS. This mixture was examined under the fluorescence microscope. The patients' platelets were prepared in the same way, but incubated directly with the FITC-labeled antiglobulin. In some experiments, the incubations with the serum were performed at 37°C, 4°C, or 0°C. After incubations at 4°C or 0°C, the washings and the incubations with the antiglobulin were also done at 4°C or 0°C.

When platelets from citrate blood were used, the platelets were washed and resuspended in PBS (NaCl 0.82%, w/v, Na2HPO4 0.16%, w/v, NaH2PO4 0.02% w/v).

Absorption and Elution Experiments

Equal volumes of packed platelets (obtained from EDTA or citrate blood) and serum were thoroughly mixed, incubated at 4°C or 20°C for 30 min, and centrifuged at 1700 g for 15 min. The supernatant fluid was removed and the procedure was repeated twice, each time with a fresh volume of packed platelets. The absorbed fluid was finally centrifuged at 15,000 g for 15 min and frozen at -20°C until use.

The remaining platelet pellets were combined and washed 3 times with EDTA-PBS or PBS at 4°C. The platelets were then resuspended in 1 volume of PBS and 2 volumes of ether. After being vigorously shaken, the mixture was incubated for 30 min at 37°C. It was then centrifuged for 10 min at 1700 g, and the ether was removed. The eluate was taken from the tube by a Pasteur pipette and stored at -20°C until use.

Preparation of F(ab')2,G and F(ab')2,G fragments of EDTA-Dependent Platelet Antibodies

Patients' sera containing IgG EDTA-dependent antibodies were fractionated by DEAE cellulose column chromatography in a modified procedure of Levy et al. This modification included both the starting buffer (0.02 M NaH2PO4, pH 6.3) and the gradient buffer. The latter consisted of the starting buffer to which 0.25 M NaCl was added, and it was used in a linear gradient.

The eluate obtained with the starting buffer comprised pure IgG. The IgG was digested with pepsin to F(ab')2 fragments according to Nisonoff et al. The F(ab')2 fragments were isolated by Sephacryl-S200 gel filtration. F(ab')2 fragments were then reduced with 0.01 M L-cysteine HCl during 18 hr at room temperature. The free SH groups were blocked with 0.03 M N-ethylmalimide during 6 hr at 4°C, and dialyzed against PBS during 16 hr at 4°C. Under those circumstances, a complete conversion of F(ab')2 to F(ab') fragments was obtained, as verified by analytical Sephacryl-S200 gel filtration.

Determination of the Immunochemical Nature of the Antibodies

By applying FITC-labeled specific anti-human IgG, IgM, IgA, A, and G as well as unlabeled anti-IgG1, anti-IgG2, anti-IgG3, and anti-IgG4, in conjunction with FITC-labeled horse anti-rabbit Ig, the immunochemical nature of the antibodies was determined by the PSIFT.

Table 1. Results of the Initial Investigations of the Patients' Platelets and Sera in the Immunofluorescence Test

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Patients' Sera x Autologous Cells</th>
<th>Patient's Sera x Donor Cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Platelets</td>
<td>Lymphocytes</td>
</tr>
<tr>
<td>1. Ma</td>
<td>+ +/++/++</td>
<td>--</td>
</tr>
<tr>
<td>2. St</td>
<td>+ ++/++</td>
<td>--</td>
</tr>
<tr>
<td>3. Ve</td>
<td>+ ++/++</td>
<td>--</td>
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</tbody>
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RESULTS

Serologic Characterization

The results obtained with sera from the three original patients with pseudothrombocytopenia investigated in the PSIFT and the IFT on lymphocytes and granulocytes, at 20°C, are summarized in Table 1. The sera gave a positive reaction, both with the patients' own platelets and with donor platelets; whereas no reaction was seen with donor granulocytes or donor lymphocytes, even in the presence of EDTA. When the indirect PSIFT was carried out with citrate platelets that had been washed and resuspended in PBS, no reaction occurred in two sera. In one serum (no. 3), citrate platelets also gave a positive reaction, although it was weaker. This latter reaction was stronger at 4°C. This result indicated that in this serum, in addition to EDTA-dependent antibodies, EDTA-independent cold antibodies against platelets were present. These EDTA-independent antibodies also reacted with platelets from heparinized blood, but the EDTA-dependent antibodies did not when no EDTA was added.

To prove the presence of separate cold antibodies, absorption and elution experiments were carried out. The sera from all three patients were absorbed with EDTA platelets or citrate platelets at 4°C; and from the absorbing platelets, ether eluates were prepared. The results are shown in Table 2. The sera absorbed with EDTA platelets showed no reactivity in the PSIFT against EDTA platelets. The reaction of the absorbed serum of patient Ve (no. 3) with citrate platelets also became negative. However, when this serum was absorbed with citrate platelets, a positive reaction against EDTA platelets remained, whereas the reaction against citrate platelets had disappeared. Eluates from the EDTA platelets showed a positive reaction with EDTA platelets, but not with citrate platelets in the first two patients. With the third serum, the eluates from both EDTA and citrate platelets showed a positive reaction with EDTA platelets as well as with citrate platelets.

EDTA Dependency of the Reaction

The two sera were studied that contained only EDTA-dependent antibodies. The sera gave a positive
Table 2. Absorption and Elution Experiments With the Sera of the Original Patients

<table>
<thead>
<tr>
<th>Sera nos. 1 and 2</th>
<th>Indirect Immunofluorescence Test</th>
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<tr>
<td></td>
<td>EDTA Donor Platelets</td>
</tr>
<tr>
<td>Unabsorbed</td>
<td>++ +/+ + + + +</td>
</tr>
<tr>
<td>Absorbed with EDTA</td>
<td>-</td>
</tr>
<tr>
<td>Eluate from EDTA</td>
<td>++ / + + + + +</td>
</tr>
<tr>
<td>Serum no. 3</td>
<td>Indirect Immunofluorescence Test</td>
</tr>
<tr>
<td></td>
<td>EDTA Donor Platelets</td>
</tr>
<tr>
<td>Unabsorbed</td>
<td>+ + + +</td>
</tr>
<tr>
<td>Absorbed with EDTA</td>
<td>-</td>
</tr>
<tr>
<td>Eluate from EDTA</td>
<td>++ + + + + +</td>
</tr>
<tr>
<td>Absorbed with citrate platelets</td>
<td>-</td>
</tr>
<tr>
<td>Eluate from citrate platelets</td>
<td>+</td>
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</table>

reaction, with citrate platelets, when Na₂EDTA was added simultaneously. The minimal concentration for a positive reaction was 0.3 mM Na₂EDTA, a concentration that is 20 times lower than that needed to prevent clotting. However, when the EDTA solution was added after incubation with the serum and one washing, the reaction was negative. When EDTA platelets were washed with PBS, the reaction became negative after at least 4 washings. It became positive again upon addition of EDTA.

The reaction was independent of the cation composition of the EDTA. Not only Na₂EDTA, but also K₂EDTA, gave a positive reaction. Also, when CaCl₂ or MgCl₂, in equivalent amounts, were added to the Na₂EDTA solution, the reaction remained positive. Moreover, when instead of Na₂EDTA, the analogue K₂EGTA was added, a positive reaction was also found.

The Reactivity of F(ab')₂ and F(ab') Fragment

F(ab')₂ and F(ab') fragments of the purified IgG fractions of the first two patients were tested in the immunofluorescence test in the presence of EDTA. Both fragments, when added in amounts equivalent to that of the original IgG fraction, gave reactions of comparable strength.

Serologic Findings in the Additional Patients

In the investigation of over 500 patients whose blood had been sent to our laboratory for serologic investigation and in whom the diagnosis of ITP had been made by the physician, we found EDTA-dependent antibodies in 10 samples when the patients’ sera were tested with EDTA platelets and with citrate platelets. When the platelet count was repeated in these patients using capillary blood, it was normal in all. The serologic data obtained from these 10 patients, together with those obtained from the original 3, and those in a further 7 patients with pseudothrombocytopenia, are depicted in Table 3.

In two further patients, there were, in addition to EDTA-dependent antibodies, EDTA-independent platelet cold antibodies. All patients with cold antibodies had a normal platelet count both in citrate blood and in capillary blood.

Temperature Dependence of the EDTA Antibodies

The EDTA agglutination phenomenon takes place best at temperatures below 37°C. The cold reactivity of the EDTA-dependent antibodies was confirmed in the PSIFT, as shown in Fig. 1. In this technique, the highest titer was found at 0–4°C (0 and 4°C were not different).

Immunochromatographic and Serologic Properties of the EDTA-Dependent Antibodies

The immunochromatographic nature of the EDTA-dependent antibodies was studied in the PSIFT with monospecific immunoglobulin reagents. In 19 of 20 sera, the antibodies were IgG; in 7, IgM antibodies were also present, and in one, IgA antibodies were present. In one serum, only IgM antibodies were found.

The subclass of the IgG antibodies was determined (Table 4). The subclass IgG1 was most often found (14 of 19), sometimes combined with IgG2 (2 of 14) or IgG3 (1 of 14). IgG2 (1 of 19) or IgG3 (2 of 19) alone were rarely seen. In one serum, we were not able to determine the subclass type with certainty.

In 16 of 17 sera, the light-chain type of the EDTA-dependent antibodies was found to be κ, in two, λ.

Table 3. Serologic Data of All Patients

<table>
<thead>
<tr>
<th>Clinical Diagnosis</th>
<th>Number of Patients</th>
<th>Positive Indirect Immunofluorescence</th>
<th>Platelet Count x 10⁹/Liter</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>EDTA Platelets</td>
<td>Citrate Platelets</td>
</tr>
<tr>
<td>Pseudothrombocytopenia</td>
<td>10</td>
<td>10/10</td>
<td>3/10*</td>
</tr>
<tr>
<td>Idiopathic thrombocytopenia</td>
<td>10</td>
<td>10/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

*Due to EDTA-independent platelet cold antibodies.
light-chain antibodies were also found. Antibodies with λ light chains alone were seen in one serum. In two sera, the light-chain type of the antibodies could not be determined; in one it was not tested.

In 18 sera, the titer in both the immunofluorescence and the platelet agglutination tests was determined. In only 5 sera, the antibodies were demonstrable in the PAT and in a much lower titer (1:1–1:2), whereas the titer in the immunofluorescence test was 1:1–1:128.

**Blood Group Specificity**

All the sera were also tested in the PSIFT with platelets from donors with different Zw and Ko genotypes. Platelets from donors homozygous or heterozygous for Zw*, Zwβ, Koα, and Koβ reacted equally well. However, platelets from patients ($n = 5$) with Glanzmann disease did not react with the EDTA-dependent antibodies.

**DISCUSSION**

Several investigators have reported that a spuriously low platelet count in EDTA-anticoagulated blood can be caused by EDTA-dependent platelet agglutinins present in the plasma. These agglutinins were found to be immunoglobulins that could be IgG and/or IgM.

By using the platelet suspension immunofluorescence test (PSIFT), we characterized the responsible antibodies in more detail. We found that the antibody binding to platelets is strictly EDTA-dependent, occurs even at an EDTA concentration as low as 0.3 mM, independent of the kationic composition of the EDTA solution and also with EGTA.

The antibodies appeared to belong to all major immunoglobulin classes, all IgG subclasses, and both light-chain types, but were mostly IgG1, κ. With IgG antibodies we could demonstrate that the reaction also takes place with F(ab')2 fragments and F(ab') fragments of the antibodies and thus likely via the antigen-combining sites. This rules out a reaction mediated via platelet Fc receptors.

The EDTA-dependent antibodies reacted with platelets from normal donors, independents of their platelet group, but not with platelets from patients with Glanzmann disease. Thus, the membrane glycoproteins IIb and/or IIa, which are missing in this disease, seem to be involved in the EDTA-dependent antibody reaction.

It is unlikely that the EDTA-dependent platelet antibodies are directed against EDTA itself. They may be cross-reacting antibodies directed against a commonly occurring natural chemical substance related to EDTA and EGTA or they may be directed against a hidden antigenic determinant of platelet membrane glycoproteins IIb and/or IIa which are only exposed in the presence of EDTA.

EDTA-dependent platelet antibodies were also detected in several patients who were thought to have idiopathic thrombocytopenia. In some of these patients, corticosteroid therapy had been instituted and one patient had been splenectomized. None of these patients had clinically manifest hemorrhagic symptoms, and a normal platelet count was obtained from capillary blood. This illustrates the importance of excluding the diagnosis of pseudothrombocytopenia in patients in whom a low platelet count is found in routine testing. EDTA-dependent platelet antibodies may be a source of confusion in testing for platelet antibodies. When platelets from EDTA blood are used to detect platelet allo- or autoantibodies using tests.

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**Table 4. EDTA-Dependent Platelet Antibodies—Reaction Patterns With Specific Anti-IgG Subclass Reagents: Results of the PSIFT in 18 Patients**

<table>
<thead>
<tr>
<th>Anti-IgG1</th>
<th>Anti-IgG2</th>
<th>Anti-IgG3</th>
<th>Anti-IgG4</th>
<th>Number</th>
</tr>
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<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11</td>
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<tr>
<td>+</td>
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<td>+</td>
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<td>1</td>
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based on agglutination or binding of immunoglobulins to platelets, these antibodies may give a falsely positive result.

Although, in all patients, platelet agglutination leading to spuriously low platelet counts occurred at room temperature in native whole blood anticoagulated in EDTA, the standard agglutination test for platelet antibodies was often negative. This discrepancy may be explained by the conditions under which this test is performed. In the standard agglutination test, the platelet concentration is much higher than in native blood, the serum to be tested is diluted and the platelets are continuously agitated during incubation with the serum.

The finding that EDTA-dependent platelet antibodies do not react with platelets of patients with Glanzmann disease may be used in the diagnosis of this disease.

REFERENCES

Pseudothrombocytopenia: an immunologic study on platelet antibodies dependent on ethylene diamine tetra-acetate

JG Pegels, EC Bruynes, CP Engelfriet and AE von dem Borne