Identification of the Platelet Alloantigen (PIA1) in Circulating Immune Complexes of Normal Human Sera

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Summary. An analysis of cell membrane material present in circulating immune complexes isolated from normal human sera by precipitation with polyethylene glycol (PEG) has been performed with the platelet alloantigen PIA1 and rhesus antigen used as markers. PEG precipitates obtained from the sera of subjects of known PIA1 and rhesus phenotype were resuspended in buffer and analyzed as representative of circulating immune complexes (CIC). The consumption of anti-PIA1 serum by CIC was determined by immuno-fluorescence and by inhibition of sodium 31chromate release from PIA1-positive target platelets. With these two techniques, PIA1 alloantigen activity was detected in CIC. This finding suggests that at least some of the cell membrane material present in CIC is derived from platelets.

Introduction

Circulating immune complexes (CIC) have been found in a great number of diseases by various techniques [11]. In animal experiments, the infusion of preformed immune complexes or the in vivo occurrence of antigen-antibody complexes are frequently associated with thrombocytopenia [13]. The high frequency of CIC in thrombocytopenic patients [12, 19] suggests that immune complexes may be responsible, in some cases, for the low platelet count and that platelets may contribute to the clearance of immune complexes from the circulation. Previous observations that human platelets can be aggregated by immune complexes [13] and that platelets can react with the first component of complement (C1q) suggest that the immune complex-platelet interaction can occur via platelet receptors specific for C1q. Detailed studies have shown that C1q can react via its globular region with a fragment of IgG and via its collagen-like portion with platelets [17, 18].

The recent observation that cell membrane fragments are associated with polyethylene glycol precipitates prepared from the sera of patients with hematopoietic disease [8] and the potential affinity of platelet-bound C1q for immune complexes [1] prompted us to examine whether such cell fragments could represent remnants of platelet membranes.

In the course of this analysis, the PIA1 alloantigen present on platelets but not on erythrocytes or leukocytes [14] appeared to be a useful antigenic marker for the identification of platelet fragments.

Materials and Methods

1. Sera. Sera were obtained from the blood of healthy volunteers by incubation of whole blood in the absence of anticoagulant for 2 h at 37°C. Sera prepared in this manner were stored at -30°C and never kept for longer than 8 days. CIC were isolated from individual sera or from a pool of sera (eight donors). The sera from 35 PIA1-positive donors and from one PIA1-negative donor were used in platelet immunofluorescence and 51Cr lysis experiments.

CIC were also isolated from the sera of four Rhesus-negative and eight Rhesus-positive donors and tested in hemagglutination assays.

2. PEG Precipitation [6, 7]. A pool of sera diluted with 0.1 M borate buffer pH 8.4 (one volume of serum + 24 volumes buffer) was incubated with a solution of 7% PEG in borate buffer (V/V PEG 6,000 FLUKA).

After 18 h at 4°C, the tubes were centrifuged at 30,000 g for 35 min at 4°C. The pellet was then washed with 3.5% PEG in borate buffer and resuspended in borate-buffered saline. The protein concentration was estimated by measurement of the optical density at 280 nm and expressed in milligrams of equivalent IgG per milliliter. The protein concentration of the CIC used ranged from 32 to 47 mg equivalent IgG per milliliter.

In some control experiments, heat-aggregated IgG or IgM was used as immune aggregates. IgG was obtained by ammonium sulfate (40% w/v) precipitation followed by DEAE cellulose chromatography as previously described [16]. IgM was isolated from the serum of a patient with macroglobulinemia by precipitation in the presence of ammonium sulfate (40% w/v) and gel filtration on a Sepharose 6 B column equilibrated with 0.1 M Tris HCl 1 M NaCl pH 8.

3. Immunofluorescence Experiments. The immunofluorescence technique used was essentially that described by Von dem Borne et al. [4].

Platelet Isolation. Whole blood (9 ml) from normal donors was anticoagulated by addition of 1 ml EDTA (5% Na2 EDTA). Platelet-rich plasma (PRP) was prepared by differential centrifugation and recentrifuged at 2,800 g for 20 min to obtain a platelet pellet. Platelets were then washed three times in EDTA-phosphate-buffered saline (PBS) (0.009 M Na2 EDTA, 0.0264 M Na3 HPO4, 0.140 M NaCl, pH 6.8, 7.0). Washed platelets were fixed by resuspension in 3 ml 1% (w/v) glutaraldehyde.
paraformaldehyde in PBS. Fixed platelet suspensions were then freed of paraformaldehyde by repeated centrifugation in EDTA-PBS.

**Platelet-Specific Antisera.** Serum containing antibodies specific for the P1A1 alloantigen was obtained from a patient with post-transfusion purpura. The specificity of antibodies present in this serum was determined by application of three different techniques: the platelet radioactive Coombs test [15], the antiglobulin consumption test [3], and an indirect immunofluorescence assay [4]. In all tests performed this serum gave a positive reaction with P1A1-positive platelets, but failed to react with P1A1-negative platelets.

The serum contained HLA antibodies against Aw 23, Aw 24, and B12. This anti-HLA activity was extremely weak and not detectable in diluted serum.

**Platelet Immunofluorescence Test.** Fluorescein isothiocyanate-labelled antiglobulin specific for IgG (Organon Teknika) was used in immunofluorescence assays. A suspension of fixed platelets (0.1 ml; 300 ± 50 × 10⁶ platelets) was incubated with 0.1 ml serum at various dilutions. Before the test, the anti-P1A1 serum (0.1 ml) was incubated with CIC (10–35 mg/ml) or with phosphate buffer for 1 h at 37°C in a final volume of 0.2 ml. The mixture was then centrifuged for 15 min at 1,500 g and the resulting supernatant (0.1 ml) added to the suspension of fixed platelets in plastic tubes. The mixture was incubated for 30 min at 37°C. The platelets were then washed three times with EDTA-PBS. Washed fixed platelets were then incubated with 0.1 ml of a suitable dilution of FITC-labelled globulin for 30 min at room temperature in the dark.

Following additional washes in PBS to remove non-bound FITC-globulin, the platelets were examined on glass slides with a Zeiss microscope equipped with vertical illumination.

### 5. Hemagglutination Assay

Human erythrocytes of Rhesus-negative (D−) and Rhesus-positive (D+) types were collected on sodium citrate (3.8%) and washed extensively in saline. Washed erythrocytes (2 × 10⁶/ml) were incubated with anti-D serum (Ortho Diagnostics, Inc.) for 1 h at 37°C, extensively washed, and finally incubated for 1 h at room temperature with a rabbit antihuman IgG serum (Ortho Diagnostics, Inc.). Agglutination was recorded with a light microscope [5].

CIC obtained from eight D+ donors (32 mg/ml) and CIC from four D− donors (33 mg/ml) were incubated for 1 h at 37°C with various dilutions of anti-D antisera (1/1–1/8) (0.1 ml CIC +0.2 ml antisera). After centrifugation for 15 min at 15,000 g, supernatants were tested for the presence of unabsorbed anti-D in the above hemagglutination assay.

### Results

#### 1. Immunofluorescence (Table 1)

Unabsorbed anti-P1A1 serum gave positive results at a dilution of 1/16–1/8. The inhibition of the reaction of anti-P1A1 serum with P1A1-positive platelets was proportional to the amount of CIC incubated with the anti-P1A1 before the immunofluorescence test.

For example, when anti-P1A1 serum was incubated in the presence of 1, 2, or 3.5 mg CIC prepared from a P1A1-positive individual, the highest dilutions of the absorbed serum giving positive fluorescence were 1/4, 1/2, and 1/1, respectively.

Under identical experimental conditions CIC prepared from the serum of a P1A1-negative individual did not inhibit fluorescence. Heat-aggregated IgG and IgM also had no effect.

#### 2. 51-Chromium Lysis (Fig. 1)

Anti-P1A1 serum induced 89% of the maximum platelet lysis. Prior incubation of antisera with CIC inhibited lysis, the inhibition being proportional to the amount of CIC added: 100% inhibition with 940 μg; 50% inhibition with 470 μg. The results shown in Fig. 1 represent the mean values from three experiments. The variation was about 5% for the two highest concentrations of CIC and 11% for the two lowest.

### Table 1. Immunofluorescence assays

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Immunofluorescence (highest dilution giving a positive result)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-P1A1 + Buffer</td>
<td>1/16–1/8</td>
</tr>
<tr>
<td>Anti-P1A1 + CIC P1A1-positive (10 mg/ml)*</td>
<td>1/4</td>
</tr>
<tr>
<td>Anti-P1A1 + CIC P1A1-positive (15 mg/ml)**</td>
<td>1/4–1/2</td>
</tr>
<tr>
<td>Anti-P1A1 + CIC P1A1-positive (20 mg/ml)**</td>
<td>1/2</td>
</tr>
<tr>
<td>Anti-P1A1 + CIC P1A1-positive (35 mg/ml)**</td>
<td>1/1</td>
</tr>
<tr>
<td>Anti-P1A1 + CIC P1A1-negative (35 mg/ml)*</td>
<td>1/16–1/8</td>
</tr>
<tr>
<td>Anti-P1A1 + Agg. IgG (20 mg/ml)</td>
<td>1/16–1/8</td>
</tr>
<tr>
<td>Anti-P1A1 + Agg. IgG (18 mg/ml)</td>
<td>1/16–1/8</td>
</tr>
</tbody>
</table>

* CIC were isolated from individual sera (*) or from a pool of sera (**) from P1A1-positive or P1A1-negative donors. Anti-P1A1 (0.1 ml) and CIC (0.1 ml) were incubated for 1 h at 37°C and tested with P1A1 + platelets. The experiments with 20 and 35 mg/ml of test sera were performed on three occasions; those with 15 mg/ml, on two occasions.