The Demonstration of Antibody Binding to Platelet-Associated Antigens in Patients With Immune Thrombocytopenic Purpura

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Platelet destruction in chronic immune thrombocytopenic purpura (ITP) is due either to antibody against platelet-associated antigen(s) that attaches by the antigen-specific Fab portion of the molecule or to platelet-bound immune complexes that bind nonspecifically to a platelet Fc receptor. Since pepsin digestion destroys the Fc fragment, the effect of this agent on platelet binding should allow differentiation between these two mechanisms. Normal serum IgG, aggregated normal serum IgG, and IgG produced in culture by splenic cells from control subjects and ITP patients were radiolabeled and tested for platelet binding before and after pepsin digestion. The binding to target platelets of both aggregated IgG and IgG produced in culture by ITP cells was increased when compared to controls. However, F(ab)₂ fragments from the ITP samples retained their binding ability while those from the aggregated IgG did not. We conclude that these ITP patients produced antibody specific for platelet-associated antigens.

CHRONIC immune thrombocytopenic purpura (ITP) is a syndrome characterized by destructive thrombocytopenia associated with a humoral antiplatelet factor. The results of in vivo challenge studies¹,² and in vitro studies³ show that this antiplatelet factor is either an IgG antibody directed towards a platelet-associated antigen or platelet-bound immune complexes. Present experimental evidence is most consistent with the former hypothesis. In vivo challenge studies show the active factor is a serum globulin¹ and is in the DEAE nonadherent, IgG-rich fraction of ITP serum.² The antiplatelet factor in ITP serum is eluted from DEAE-cellulose by low salt concentrations and is present in the IgG fraction eluted from Sephadex G-200 (Pharmacia, Piscataway, N.J.); in addition, pooled IgG, extracted from the platelets of 4 ITP patients, migrated in the 7S region of a sucrose density gradient.¹ Immunoglobulin G synthesized in culture by washed ITP splenic cells binds to washed target platelets⁴,⁵ and megakaryocytes.⁶ This body of evidence strongly suggests that the antiplatelet factor in patients with ITP is an IgG antibody against a platelet-associated antigen. However, none of these studies completely rules out the possibility of immune complexes as the active agent, particularly if the antigen is of low molecular weight. Recent studies by two groups have shown that circulating immune complexes are readily demonstrable in many patients with ITP,⁹,¹⁰ whether they are involved in the disease pathogenesis is conjectural.¹⁰

If the antiplatelet factor is an IgG antibody against a platelet-associated antigen, as suggested by most studies, it would bind to target platelets by the antigen specific Fab portion of the molecule. Conversely, platelet-bound immune complexes would attach nonspecifically to a platelet Fc receptor.

In the present report, we show that F(ab)₂ fragments of IgG produced in vitro by splenic cells from three ITP patients retain the ability to bind to target platelets. This provides direct evidence that in these patients with ITP the antiplatelet factor is an antibody against a platelet-associated antigen.

MATERIALS AND METHODS

Spleenic cells from 3 patients with ITP and from 3 control subjects (1 patient—hereditary spherocytosis; 2 patients—incidental splenectomy) were cultured as previously described.⁴ Briefly, cultures containing 700 × 10⁶ splenic cells in 350 ml of 20% fetal calf serum in Dulbecco's medium were incubated for 10 days at 37°C on day 5, one half of the media was changed. After harvesting, the cells and cell debris were removed by centrifugation.

The IgG was purified from the culture supernatant fluids and from normal serum in the following manner. A globulin-rich fraction was prepared by 50% ammonium sulfate precipitation and after extensive dialysis against 0.01 M phosphate buffer (pH 8.0), the non-IgG proteins were removed by exposure to DEAE-Sephadex (Pharmacia A-50). The nonadherent IgG was eluted with 0.01 M phosphate buffer, dialyzed against phosphate buffered saline (PBS), pH 7.4, and concentrated by negative pressure dialysis. The IgG concentration was determined by the Fab-anti-Fab method.¹¹ Purity of the IgG samples was documented by radioimmunoelectrophoresis of the labeled IgG against rabbit anti-whole human serum, antihuman IgG and antiwhole bovine serum. In all specimens only one radiolabeled precipitin line was noted using anti-whole human serum. This corresponded in location to the line developed with anti-IgG antisera. No precipitin lines developed using rabbit antihuman serum.

The IgG preparations were radiolabeled using a modification of the lactoperoxidase iodination method.¹² To 10 μg IgG in 5 ml of PBS were added 0.5 ml of sepharose-linked lactoperoxidase (Millipore Corp., Bedford, Mass.), 50 μl of 10⁻³ M KI and 1 mCi²¹¹I (New England Nuclear, Boston, Mass.). After mixing, three 5-μl aliquots of freshly diluted 0.03% hydrogen peroxide were added at 30-sec intervals followed by mixing. The lactoperoxidase beads were

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Supported in part by NIH Grant AM16125.

Submitted January 29, 1980; accepted July 25, 1980.

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0006-4971(80)0606-0000$01.00/0
removed by centrifugation for 5 min at 1500 g and unbound radionuclide was removed by dialysis against PBS.

The F(ab') fragments were prepared by pepsin digestion of the radiolabeled IgG. To an aliquot of radiolabeled IgG containing approximately 10 million protein-bound cpm were added 5 mg of purified normal human serum IgG to serve as carrier protein. When aggregated normal serum IgG was required, the mixture of radiolabeled IgG and carrier IgG was heated for 30 min at 63°C followed by centrifugation at 1500 g for 30 min to remove large aggregates. The precipitate was discarded.

The mixture of radiolabeled and carrier IgG to be digested was dialyzed against 0.1 M acetate buffered saline, pH 4.0. After dialysis, 0.25 mg of pepsin (Cal Biochem, San Diego, Calif.) was added followed by incubation at 37°C for 18 hr. The digestion mixture was centrifuged for 30 min at 1500 g and the supernatant fluid was dialyzed against PBS (pH 7.4). The completeness of the digestion was confirmed by SDS-polyacrylamide gel electrophoresis after reduction of the proteins.

Platelet-specific IgG binding was determined as previously described. Blood from O-positive normal subjects was drawn into ACD-A (5 parts blood to one part ACD-A). After centrifugation at 400 g for 10 min (25°C), the platelet-rich plasma (PRP) was aspirated and the platelet count was determined (Coulter counter, Model A). Triplicate incubations were prepared in 15 ml capped centrifuge tubes under conditions of antigen excess. To each tube were added: 1 ml of PRP and an aliquot of the pre- or post-digestion specimen being tested containing approximately 10^6 normal IgG, which like immune complexes are demonstrable in many patients with immune thrombocytopenia and could be involved in the disease pathogenesis. Although most evidence favors the former possibility, recent studies have shown that circulating immune complexes are demonstrable in many patients with immune thrombocytopenia and could be involved in the disease pathogenesis.

RESULTS

The mean binding to target platelets of radiolabeled serum IgG from 15 normal subjects was 0.038% ± 0.012%; binding of IgG from 6 control spleens was not significantly different and ranged from 0.033 to 0.065. As shown in Table 1, digestion of serum IgG from 3 normal subjects and IgG from 3 control spleens did not affect the percentage of platelet-associated radioactivity.

Incubation of aggregated serum IgG from 2 normal subjects with target platelets resulted in increased binding values of 0.65% and 0.77%. However, after pepsin digestion of these samples, the binding to platelets returned to control levels (Table 1). Increased binding to target platelets of the splenic IgG from the three ITP patients was also noted; platelet-associated values ranged from 0.98% to 1.25% (>3 SD above control values). Pepsin digestion of these specimens resulted in some decrease in platelet binding but the F(ab') fragments from these samples still showed highly significant binding when compared to controls (p < 0.001; Table 1).

DISCUSSION

It is clear that thrombocytopenia in chronic ITP is due to platelet destruction by a circulating antiplatelet factor. This factor is either an IgG antiplatelet antibody directed toward a platelet-associated antigen(s) or platelet-associated IgG immune complexes similar to those described in drug-induced purpura. Although most evidence favors the former possibility, recent studies have shown that circulating immune complexes are demonstrable in many patients with immune thrombocytopenia and could be involved in the disease pathogenesis.

The present studies provide direct support for the presence of an antibody directed towards a platelet-associated antigen in these three patients with ITP since their IgG attached to target platelets via the antigen-specific Fab binding site. Although we noted increased binding to target platelets using aggregated normal serum IgG, which like immune complexes attaches to the membrane by the Fc portion of the molecule, this binding did not occur after digestion of the Fc fragment by pepsin digestion. Conversely, significant platelet binding remained after pepsin digestion of the splenic IgG samples from the ITP patients.

Although these studies show that ITP patients produce antibody against a platelet-associated antigen, they are not useful in determining the nature of
the antigen. Although the clinical and experimental findings in ITP are certainly compatible with an autoimmune process, this need not be the case. It is well known that several native substances adhere tenaciously to the platelet surface (e.g., coagulation factors) and it is likely that foreign molecules may do likewise. An antibody against any of these surface-bound molecules would produce a syndrome compatible with ITP. Identification of the involved antigen(s) is needed before this matter can be settled.

REFERENCES

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