Presence of Cross-Reactive Antibody Between Human Immunodeficiency Virus (HIV) and Platelet Glycoproteins in HIV-Related Immune Thrombocytopenic Purpura

By Ali Bettaieb, Patricia Fromont, Fawzia Louache, Eric Oksenhendler, William Vainchenker, Najib Duédari, and Philippe Bierling

We previously reported the presence in platelet eluates of autoantibodies directed against epitopes of the platelet glycoprotein (GP)IIb/IIa complex in acquired immunodeficiency syndrome (AIDS)-free human immunodeficiency virus (HIV)-infected patients with immunologic thrombocytopenic purpura (ITP). We investigated whether HIV antibodies recognized platelet membrane antigens to determine whether the virus might be directly or indirectly responsible for the thrombocytopenia in this context. Direct eluates of platelets from 25 patients with HIV-related ITP contained IgG reacting with HIV-GP160/120 and also, in 45% of patients, detectable antigens to determine whether the antiplatelet antibodies, immunochemically characterized as serum HIV-GP160/120 antibodies could be absorbed on and eluted from platelets from normal non–HIV-infected healthy donors and infected patients. In contrast, GP160/120 antibodies present in the serum of nonthrombocytopenic HIV-infected patients were not absorbable on normal platelets in most patients, suggesting a pathogenic role in HIV-related ITP. We performed detailed studies of a patient with the highest titer of both HIV-GP160/120 and GPIIb/IIa antibodies in direct and indirect platelet eluates. No antibody binding to GPIIb/IIa-deficient Glanzmann thrombasthenic platelets was detected. Furthermore, binding/elution experiments conducted with insoluble recombinant GP160 (expressed in baculovirus) and purified platelet GPIIb/IIa demonstrated that the patient’s IgG bound specifically, through the F(ab’)2 portion, to a common epitope of HIV-GP160/120 and platelet GPIIb/IIa. This common epitope was present on a recombinant GP160 expressed in baculovirus but absent from another recombinant GP160 expressed in vaccinia virus, suggesting that the cross-reactivity is dependent on the glycosylation or conformational structure of the GP. We conclude that molecular mimicry between HIV-GP160/120 and platelet GPIIb/IIa may explain some of the pathogenic role in HIV-infected patients.

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DIOPATHIC thrombocytopenic purpura (ITP) is a frequent hematologic disorder characterized by immunologic platelet destruction. ITP most often results from the presence of platelet autoantibodies directed mainly against epitopes localized on the glycoprotein (GP)IIb/IIa complex. A syndrome similar to ITP frequently occurs in HIV-infected patients regardless of the risk group to which they belong, eg, homosexual men, intravenous drug abusers, and hemophiliacs. Immunologically mediated platelet destruction has been clearly demonstrated in such patients, but the precise mechanism of the thrombocytopenia has not yet been established. Two main hypotheses have been advanced. The first hypothesis implicates nonspecific deposition of circulating immune complexes on the platelet surface with subsequent clearance of the opsonized platelets. This hypothesis is supported by the presence of anti-HIV antibodies and the absence of detectable HIV antigens on the platelet surface. Certain evidence indicates that anti-F(ab’)2 antibodies directed against HIV antibodies (antiidiotypic antibodies) might play a role in the formation of such immune complexes. The second hypothesis, supported by the findings of two independent teams of investigators, is based on the presence of specific platelet antibodies. Thus, von dem Borne et al reported the presence of platelet autoantibodies in most HIV-infected patients with or without thrombocytopenia and concluded that the nature of these autoantibodies was not different from that of the autoantibodies observed in classic ITP. We demonstrated that ITP in HIV-infected AIDS-free patients results, at least in some patients, from the presence of platelet autoantibodies directed mainly against epitopes localized on platelet GPIIb/IIa. To reconcile the two hypotheses, we investigated the possible involvement of molecular mimicry between HIV and platelet antigens. Our results, showing cross-reactivity of HIV antibodies with platelet GPIIb/IIa, could explain some cases of ITP in HIV-infected patients.

PATIENTS AND METHODS

Patients. Blood samples were obtained from HIV-infected patients with (n = 28) or without (n = 11) ITP. The presence of HIV antibodies was demonstrated by enzyme-linked immunosorbent assay (ELISA) and Western blotting analysis in the serum of all subjects. The patients with ITP belonged to the following risk groups: drug addicts (n = 18), homosexual men (n = 7), and transfusion recipients (n = 3). We previously reported immunohematologic findings in 22 of these 28 patients, including the three whose platelet eluates precipitated GPIIb/IIa. Among the nonthrombocytopenic patients, five were homosexual men, four were drug addicts, and two were female sexual partners of seropositive men. The patients with ITP satisfied the following diagnostic criteria: isolated thrombocytopenia with a platelet count less than 50 x 10⁹/μL, an increased number of megakaryocytes in an otherwise normal bone marrow (BM) aspirate, absence of Centers for Disease Control (CDC)-defined AIDS and absence of other causes of peripheral thrombocytopenia (eg, thrombotic thrombocy-
topenic purpura, disseminated intravascular coagulation, hypersplenism). The clinical characteristics of most of the patients with HIV-related ITP have been described elsewhere. Patients Bou, born in 1960, was a drug addict from 1978 to 1983. HIV seropositivity (CDC stage IV/C2) and ITP were diagnosed in 1988. Treatment with zidovudine was started in August 1988 and the platelet count increased to near-normal levels (142 x 10^9/L). Pulmonary tuberculosis, diagnosed in January 1990, resolved with specific treatment.

Platelet autoantibody determination. Platelet-associated IgG (PAIgG) was detected according to the method described by von dem Borne et al.3 Samples (sera, eluates or F(ab')2 fragments) were tested for antplatelet membrane activity by means of an indirect immuno-fluorescence test (PIHT) on paraformaldehyde-dead-treated platelets22 with fluorescein-conjugated F(ab')2 antihuman (γ) IgG or antihuman light chains ( Dakopatts, Copenhagen, Denmark).

In some cases, sera and eluates were also studied in immunoprecipitation experiments. Platelets 1 x 10^11 were surface-labeled with 125Iodine using the lactoperoxidase catalyzed method.23 The platelets were subsequently solubilized in 1 mL buffer A [containing Tris-HCl 20 mmol/L NaCl 150 mmol/L (Tris-buffered saline-TBS), Triton X-100 1%, EDTA 5 mmol/L, phenylmethylsulfonyl fluoride (PMSF) 2 mmol/L, and 0.5 μg/mL leupeptin] for 30 minutes at 4°C, and the lysate was centrifuged for 30 minutes at 17,600g at 4°C. Fifty microliters of lysate were then incubated overnight at 4°C with 50 μL serum, 100 μL eluate, or 10 μL purified monoclonal antibody (MoAb). Two hundred microliters of a 10% suspension of protein A-Sepharose (Pharmacia, Paris, France) were added to the lysis and incubated for 1 hour at 4°C. The beads were then washed five times with buffer A, two times with buffer B supplemented with 0.2 mol/L NaCl, and two times with buffer C supplemented with 0.35 mol/L NaCl. Fifty microliters of 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol were added to each pellet and, after boiling for 5 minutes, the solutions were analyzed in a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel according to the method of Laemmli.24 Gels were then fixed, dried, and autoradiographed.

HIV antibody determination. HIV antibodies were detected using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Abbott Laboratories, Paris, France) and by Western blot immunoassay (Du Pont de Nemours, Les Ulis, or Diagnostics Pasteur, Marnes-La-Coquette, France). The ELISA kit contained mainly GP41 and GP24 recombinant-HIV proteins, Western blot assays were performed with patient sera. Results were interpreted only if the last platelet washing sample was negative. Negative controls included direct eluates of platelets from normal individuals and HIV-negative patients with ITP.

Binding and elution experiments performed with eluates, sera, and F(ab')2 fragments on recombinant GP160 and purified platelets. Two recombinant GP160s (rGP160) of the HIV1 isolate (rGP160-1, rGP160-2), supplied by Drs M. Girard and M. Kaczorek (Pasteur Merieux, Val de Reuil, France) and by Repligen (Cambridge, MA), respectively were studied. rGP160-1 is secreted into the culture medium of baby hamster kidney (BHK 21) cells infected by the recombinant vaccinia virus VV 1163.28 Amino acid sequencing has shown that rGP160-1 lacks 22 amino acids at position 696 to 717.28 rGP160-2 (derived from the IIIB isolate of HIV1) is expressed as a full-length protein in insect cells by using a baculovirus expression vector and is neither processed nor secreted.29,30 Four hundred micrograms of the rGP160 was coupled to 0.5 mL Affi-gel 10 according to the manufacturer's directives (BioRad, Paris, France).
Germain en Laye, France), and then dialyzed against TBS containing 0.1% Triton-X 100. Further purification was performed with insoluble anti-GPIIIa MoAb (SZ21) (Immunotech, Marseille, France) coupled to a column of Affi-gel 10 (1 mg antibody/1 mL Affi-gel 10). GPIIb/IIIa complex was eluted from the column with 0.1 mol/L glycine, 0.1 mol/L NaCl, and 0.1% Triton-X 100, pH 2.8, and the eluted fraction was immediately neutralized with 1 mol/L Tris. The purified GPIIb/IIIa was greater than 90% homogeneous as analyzed by SDS-PAGE. The only slight contaminant observed was the fibrinogen that was probably copurified with the GPIIb/ IIIa. In addition, in an ELISA, the GPIIb/IIIa preparation failed to react with HLA class I, CD42b, CDw49b, and CD62 MoAbs demonstrating that this material was free of the other major platelet GPs. Five hundred micrograms of purified GPIIb/IIIa was coupled to 0.5 mL Affi-gel 10 according to the manufacturer's directives (BioRad, Paris, France).

Absorption experiments with eluates, sera or F(ab')2 fragments were performed using Affi-gel 10 coupled to rGP160 or to purified GPIIb/IIIa. After several washes, absorbed materials were eluted by addition of an aqueous buffer containing 150 mmol/L H3PO4/100 mmol/L NaCl/1.5% BSA, pH 2.8, for 10 minutes at 4°C. The acidified suspension was centrifuged and then neutralized with 1 mol/L Tris. Eluted materials were tested for antiplatelet and anti-HIV activity in comparison with the last GP160 or GPIIb/IIIa Affi-gel column washing samples. Results were interpreted only if the last platelet washing sample was negative.

RESULTS

Platelet antobody determination. In accordance with a previous report,18 PAIgG levels were increased in 20 (80%) of the 25 patients with HIV-related ITP studied. For 9 of these 20 patients (45%), the direct eluate contained platelet antibodies that recognized in the PIIFT all the platelet panel except platelets from ABO compatible type I GT patients. Five of these positive eluates, including the three reported in our previous work,18 precipitated platelet GPIIb/IIIa (partially illustrated in Fig 3). In contrast, a weak increase in PAIgG was observed in only 2 of the 11 nonthrombocytopenic patients (18%) and was never elutable. Likewise, sera of 25 of the 28 thrombocytopenic but only 3 of the 11 nonthrombocytopenic patients were positive in the PIIFT.

Presence of HIV antibodies on platelets from HIV-infected thrombocytopenic patients. Direct platelet eluates from HIV-infected thrombocytopenic (n = 25) and nonthrombocytopenic patients (n = 11) were tested using ELISA and Western blot procedures for the presence of HIV1 antibodies (Fig 1). In the ELISA, 21 of the 25 eluates (84%) from thrombocytopenic patients were positive, whereas only 3 of the 11 eluates (27%) from nonthrombocytopenic patients were weakly positive. Furthermore, all 25 direct eluates from HIV-infected ITP patients were positive in the HIV Western blot immunoassay, whereas only four of the nonthrombocytopenic patients were positive (including two who were very weakly positive), and seven were negative. As shown in Fig 1, all 29 eluates positive in HIV-Western blot recognized HIV GP160/120, and 12 also recognized other proteins encoded by the Gag or Pol genes (p17, p24, p31, p53, p68).

Viral antigens on platelets of HIV-infected ITP patients. Immunoblotting and immunoprecipitation procedures with anti-HIV MoAbs were used to detect the presence of HIV antigens on HIV-infected ITP patient platelet extracts. Negative results were obtained for all four patients studied. Similar negative results were obtained with three other platelet samples in the indirect immunofluorescence test. These results do not strictly eliminate the presence of HIV antigens on the platelet membrane in these patients,

![Fig 1. Analysis of direct and indirect platelet eluates by means of HIV-Western blot immunoassay. Direct and indirect platelet eluates were obtained by ether elution from HIV-infected patient platelets and from normal platelets first incubated with patient sera, respectively, and were analyzed by commercially available HIV1-Western blot kits (Dupont or Diagnostics-Pasteur). According to the manufacturer's instructions, the markers (left) correspond to the major HIV proteins. Positive control (1), and representative aspects of immunoblots obtained with direct eluates from HIV-infected ITP patients (2-5). Only anti-GP160/120 was observed in 14 of 25 eluates (6,7). Positive immunoblots obtained with direct eluates from HIV-infected nonthrombocytopenic patients. In these cases, negative results were obtained in 7 of 11 eluates (8-10). Pattern of immunoblots obtained with indirect eluates from HIV-infected ITP patients. Only anti-GP160/120 was observed in 12 of 14 indirect eluates (11). The weakly positive immunoblots were obtained with indirect eluates from HIV-infected nonthrombocytopenic patients. Negative results were obtained in six of the eight nonthrombocytopenic patients studied. Direct and indirect eluates of patient Bou (2 and 8, respectively). All direct and indirect eluates were used at a dilution of 1:40 in these experiments.]
however, owing to the limited sensitivity of the methods used.

**Binding of serum HIV antibodies to normal platelets.** Because HIV antibodies were present but HIV antigens were not detectable on the membrane of platelets from the HIV-infected ITP patients, we determined whether HIV antibodies could bind to platelets from uninfected healthy blood donors. Indirect platelet eluates were thus obtained by ether elution after incubation of patient sera with normal platelets. As shown in Fig 1, all 14 indirect platelet eluates obtained with serum from HIV-infected ITP patients were positive in the HIV-Western blot immunoprecipitation assay (Fig 1, strips 2 and 8, Table 1). To determine if two distinct antibodies were present in these eluates, we performed absorption/elution experiments with ABO-compatible platelets from two GT patients. Neither the anti-HIV nor antiplatelet activity present in the patients’ sera or in the direct or indirect eluates were able to bind to these GT platelets. These results, contrasting with those obtained in binding/elution experiments with normal healthy blood-donor platelets (described above), suggested that both patient Bou HIV and platelet antibodies bound to GPIIb/IIIa (Fig 2 and Table 1).

The presence of cross-reactive antibody between platelet GPIIb/IIIa and HIV GP160 in patient Bou serum and the anti-GPIIb/IIIa specificity of Bou platelet antibody was confirmed by binding/elution experiments with patient Bou serum on insoluble purified GPIIb/IIIa. As shown in Table 1, the antibodies eluted from purified immobilized GPIIb/IIIa recognized healthy blood-donor ABO-compatible platelets in the PIIFT and HIV-GP160 in the HIV-Western blot immunoprecipitation assay. The anti-GPIIb/IIIa specificity of the platelet antibody was confirmed by an immunoprecipitation procedure, because, when incubated with 125I-labeled platelets, the eluate obtained from purified immobilized GPIIb/IIIa incubated with patient serum (as well as with direct platelet eluate) precipitated a band of 97 Kd in reducing conditions which comigrated with the GPIIIa precipitated by an PLA1 antibody or a GPIIIa MoAb (SZ21) (Fig 3). Controls of these absorption-elution experiments included serum of a nonthrombocytopenic HIV-infected patient (that did not recognize the immobilized GPIIIa) and polyclonal GPIIb/IIIa antibodies from an HIV-negative GT patient. (The eluate obtained from the immobilized GPIIb/IIIa column after incubation with this latter serum contained only GPIIb/IIIa antibody).

To determine whether binding of the cross-reactive platelet antibody to normal platelets or to purified GPIIb/IIIa was specific, the same experiments were performed with the F(ab’)2 fragment of purified serum Bou IgG. As shown in Fig 2, similar results to those observed with patient serum were obtained with this material. Thus, both the platelet and HIV GP160 antibody activities present in patient Bou F(ab’)2 fragments were capable of binding to normal platelets.

### Table 1. Characterization of Patient Bou IgG Platelet Antibody

<table>
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<tr>
<th>Characteristic</th>
<th>Patients' Sera</th>
<th>Antibodies Eluted From Normal Platelets</th>
<th>Antibodies Eluted From Normal Platelets After Incubation With Patients' Sera</th>
<th>Antibodies Eluted From Purified GPIIb/IIIa After Incubation With Patients' Sera</th>
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<tbody>
<tr>
<td>IF on normal platelets</td>
<td>++ to ++++</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>IF on GT platelets</td>
<td>- to +</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>Platelet immunoprecipitation</td>
<td>Negative</td>
<td>GPIIa</td>
<td>NG</td>
<td>GPIIa</td>
</tr>
<tr>
<td>HIV-ELISA (cutoff = 0.3 OD)</td>
<td>&gt; 2</td>
<td>1.54 ± 0.30</td>
<td>1.29 ± 0.44</td>
<td>ND</td>
</tr>
<tr>
<td>HIV-Western blot assay</td>
<td>All HIV-proteins and glycoproteins</td>
<td>HIV-GP160/120</td>
<td>HIV-GP160/120</td>
<td>HIV-GP160/120</td>
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Platelet membrane fluorescence was graded as negative (−), weakly positive (+), or positive (++ to ++++) in comparison with results obtained using a negative control (serum from a non-transfused healthy male blood group AB donor), and a positive control (pool of sera from multitransfused immunized patients).
normal healthy donor platelets but not to GT platelets. Likewise, binding/elution experiments with this material on insoluble purified GPIIb/IIIa demonstrated that GPIIb/IIIa antibody was entirely absorbed on the column and that the GPIIb/IIIa column eluate contained HIV GP160 antibody (data not shown). Furthermore, in these experiments, F(ab')2 binding could be demonstrated only with an anti-light chain but not with an antiglobulin directed against the Fc portion of the \( \gamma \) chain, ruling out binding by residual IgG contaminating the F(ab')2 preparation. Overall, these results demonstrated the presence in patient Bou of GP160/120 antibodies able to bind specifically to platelet GPIIb/IIIa.

**Binding of the cross-reactive antibody to r-GP160 of the HIV-1 envelope GP.** To characterize the cross-reactive epitope between HIV-GP160/120 and platelet GPIIb/IIIa, several experiments were conducted (Table 2). Direct Bou platelet eluate did not recognize the cell membrane of HIV-1--infected cell lines (U937-HTLVIII and CEM-Bru), whereas intracytoplasmic staining was observed when infected cells were permeabilized with methanol and Triton X-100, suggesting that the eluate contained HIV antibodies probably directed against the native immature GP160. Binding and elution experiments were performed with patient Bou platelet eluates on the two immobilized r-GP160s of the HIV envelope. Nonabsorbed and eluted materials from the column were tested for anti-HIV and antiplatelet activities. As shown in Table 2, neither the antiplatelet nor anti-HIV activities present in the Bou eluate bound to r-GP160-1, whereas they bound almost completely to r-GP160-2. The same results were obtained with the patient Bou serum F(ab')2 fragments. These results suggest either that the cross-reactive epitope recognized was located on the deleted portion of r-GP160-1 (present on r-GP160-2) or that it is dependent on the glycosylation or conformational structure of the GP.

**DISCUSSION**

Immunologically mediated platelet destruction has been clearly demonstrated in a proportion of HIV-infected patients.\(^{6,7,9,10,12,13,32}\) The responsibility of immune complex deposition or platelet autoantibody binding in this process is controversial, however. We and other investigators have reported the presence of autoantibodies directed against platelet GPIIb/IIIa and/or other platelet membrane GPs in HIV-infected ITP patients.\(^{16-18}\) On the other hand,
Karpatin et al suggested that both autoantibody fixation and nonspecific immune complex deposition may be responsible for the thrombocytopenia.

The present findings concerning 28 HIV-infected ITP patients confirm our previous results. IgG with antiplatelet activity was observed in the serum of 89% of the patients, PAIgG levels were increased in 80%, and 9 of 20 direct platelet eluates contained platelet antibodies sometimes immunochemically characterized as anti-GPIIb/IIIa. In addition, we noted that in one of these patients (patient Bou), serum antiplatelet IgG specifically bound to normal platelets and purified immobilized GPIIb/IIIa. This binding was not explained by the presence of HIV antigens on the platelet membrane because viral antigens were not detectable in various immunologic tests and especially because the anti-HIV activity also involved will be of importance because it will have to be taken into account in treatment of such patients and in production of vaccines based on HIV envelope components.

Therefore, we clearly demonstrated in patient Bou the presence of a platelet-bindable antibody that specifically recognizes an epitope (not precisely identified) shared by HIV GP160/120 and platelet GPIIb/IIIa, suggesting that homologies exist between the two GPs. Perhaps only small numbers of HIV-infected patients develop such antibodies at a titer sufficient to lead to platelet destruction. Alternatively, HIV antibodies may also occasionally bind to undetectable viral antigens on the platelet membrane, because we and other investigators have demonstrated the presence of HIV transcripts and, occasionally, HIV proteins in megakaryocytes from HIV-infected thrombocytopenic patients. However, the presence of both GP160 and GPIIb/IIIa antibodies in at least 5 of the 20 direct platelet eluates studied argues for the frequent responsibility of cross-reactive antibodies in the immune platelet destruction observed in HIV-infected patients. Finally, if the hypothesis of cross-reactivity is confirmed, the antigenic structure involved will be of importance because it will have to be taken into account in treatment of such patients and in production of vaccines based on HIV envelope components.

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<table>
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<tr>
<th>Table 2. Binding of Patient Bou Cross-Reactive Antibody to r-GP160s of the HIV1 Envelope</th>
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<tbody>
<tr>
<td>Assay</td>
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<tr>
<td>PIFA</td>
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<td>HIV-ELISA (cutoff = 0,28)</td>
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<td>HIV-Western blot</td>
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PIFT, platelet indirect immunofluorescence test on normal platelets. Platelet membrane fluorescence was graded as negative (–), weakly positive (+), or positive (+ to ++++) in comparison with results obtained using negative control (serum from a nontransfused healthy male blood group AB donor), and a positive control (pool of sera from multitransfused immunized patients). Direct eluate, antibodies eluted from patient Bou’s own platelets; indirect eluate, antibodies eluted from normal platelets preincubated with patient Bou serum.

*r-GP160 (1) and (2), two different recombinant GP160 of the HIV1 envelope (described in the Materials and Methods section).
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Presence of cross-reactive antibody between human immunodeficiency virus (HIV) and platelet glycoproteins in HIV-related immune thrombocytopenic purpura

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