Anti-human immunodeficiency virus type 1 antibody complexes on platelets of seropositive thrombocytopenic homosexuals and narcotic addicts

(immunologic thrombocytopenia/antibody-antibody complexes/acquired immunodeficiency syndrome)

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ABSTRACT Patients with human immunodeficiency virus type 1 (HIV-1) infection develop an immunologic thrombocytopenic purpura associated with markedly elevated platelet IgG, IgM, and C3C4 as well as serum complexes determined by the polyethylene glycol (PEG) method. Analysis of their serum PEG-precipitable immune complexes as well as platelet acid eluates revealed the presence of anti-HIV-1 antibody existing as a complex that eluted in the void volume of a Sephadex G-200 gel-filtration column. The complex binds to washed normal platelets, whereas affinity-purified anti-HIV-1 (gp120) antibody does not. HIV-1 antigen or proviral DNA was not detectable in the immune complexes or platelet extracts. However, anti-antibodies directed against anti-HIV-1 antibody were detectable in the immune complexes as well as platelet eluates. Approximately 50% of eluted platelet IgG contained anti-HIV-1 antibody. Thus the markedly elevated platelet immunoglobulin is partly due to the presence of anti-HIV-1 antibody complexes. This may be responsible for the enhanced platelet clearance and thrombocytopenia in patients with acquired immunodeficiency syndrome-related immunologic thrombocytopenia.

Human immunodeficiency virus type 1 (HIV-1)-seropositive homosexuals and narcotic addicts develop a newly described syndrome of immunologic thrombocytopenic purpura (ITP) (1, 2) that is clinically indistinguishable from the classic autoimmune thrombocytopenic purpura (ATP) seen predominantly in females (3). However, their platelets have markedly elevated IgG, IgM, and C3C4 (2, 4) compared to classic ATP patients and their sera contain elevated immune complexes determined by the polyethylene glycol (PEG) method, whereas ATP patients’ sera do not (2, 4). In addition, many of the HIV-1-seropositive thrombocytopenic patients have anti-F(ab)2 antibodies that correlate with their circulating immune complex level (5). It has been proposed that the elevated IgG and C3C4 on their platelets may represent deposition of immune complexes and that this may in part or in whole be responsible for the pathophysiology of the thrombocytopenia (4).

The purpose of this investigation was to determine whether HIV-1 antibody and/or HIV-1 virus is present on these platelets and, if so, whether they exist as a complex. A preliminary report of the investigation has appeared (6).

METHODS

Population. The population studied consisted of 36 HIV-1-seropositive patients with ITP: 15 homosexuals (HSITP) and 21 narcotic addicts (NITP); 10 asymptomatic HIV-1-seropositive subjects with normal hemograms, consisting of 5 homosexuals and 5 narcotic addicts; 15 classic ATP patients; and 14 healthy control subjects.

Non-HIV Viral Antibody. Viral antibody studies were performed for Epstein–Barr virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV), adenoviruses (ADE), and varicella–zoster virus (VZV) by standard immunofluorescent techniques (7).

HIV-1 Antibody. HIV-1 antibody was detected by a solid-phase absorption, ELISA assay employing the Abbott kit, in which the developing antibody was an anti-human IgG coupled to alkaline phosphatase, as well as by an immunoblot procedure employing antigen-nitrocellulose strips supplied by Epitope (Beaverton, OR), in which the developing antibody was an anti-human IgG (H + L) coupled to horseradish peroxidase.

HIV-1 Antigen. HIV-1 antigen was detected by an antigen capture assay kit employing human polyclonal antibodies supplied by Cellular Products (Buffalo, NY) (8).

Platelet-Rich Plasma. Platelet-rich plasma was prepared from EDTA/anticoagulated blood as described (9) for elution and binding experiments or placed on a Sepharose 2B gel-filtration column (Pharmacia) for DNA extraction. The gel-filtration procedure was employed to prepare platelets entirely free of lymphocytes.

Peripheral Blood Mononuclear Cells (PBMCs). PBMCs were prepared from fresh heparinized blood using leukocyte separation medium (Organon Teknika).

HIV-1 Proviral DNA Gene Amplification. The polymerase chain reaction (PCR) method (8, 10–12) was employed to test for the presence of HIV-1 proviral DNA and human β-globin DNA sequences in PBMCs and platelets of eight HIV-1-seropositive patients with immunologic thrombocytopenia to determine whether either or both cell types were infected in vivo and to establish the purity of the platelet preparations. PBMCs were lysed in a sodium dodecyl sulfate (SDS)/proteinase K solution and incubated overnight; DNA was isolated by phenol and chloroform/isoamyl alcohol extraction followed by alcohol precipitation (13). Platelets, devoid of lymphocytes, were similarly treated to precipitate any nonintegrated HIV-1 DNA and mitochondrial DNA that may have been present in these preparations. DNA concentrations were estimated from 260/280-nm absorption.

The PCR was performed on 1 μg of PBMC DNA or the ethanol-precipitable fraction of the purified platelet prepara-

Abbreviations: HIV, human immunodeficiency virus; ATP, autoimmune thrombocytopenic purpura; ITP, immunologic thrombocytopenic purpura; HSITP, homosexual ITP; NITP, narcotic addict ITP; EBV, Epstein–Barr virus; CMV, cytomegalovirus; HSV, herpes simplex virus; ADE, adenoviruses; VZV, varicella–zoster virus; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; VCA, viral cap antigen.

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Table 1. Viral serology of sera and platelet eluates of thrombocytopenic homosexuals (HSITP) and narcotic addicts (NITP)

<table>
<thead>
<tr>
<th>Patient</th>
<th>EBV (VCA)</th>
<th>CMV</th>
<th>HSV</th>
<th>ADE</th>
<th>VZV</th>
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<tr>
<td></td>
<td>S</td>
<td>P</td>
<td>S</td>
<td>P</td>
<td>S</td>
</tr>
<tr>
<td>1</td>
<td>640</td>
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<td>&lt;10</td>
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<td>3</td>
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<td>&lt;5</td>
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<tr>
<td>4</td>
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<td>&lt;5</td>
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<tr>
<td>6</td>
<td>128</td>
<td>&lt;5</td>
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<td>&lt;10</td>
<td>640</td>
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<tr>
<td>7</td>
<td>2,560</td>
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<td>2,560</td>
<td>&lt;10</td>
<td>320</td>
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<td>&lt;5</td>
<td>320</td>
<td>&lt;10</td>
<td>320</td>
</tr>
</tbody>
</table>

S, serum; P, platelets. Patients 1–4, HSITP; patients 5–10, NITP. Reciprocal titers are given. A positive titer represents a dilution of 1:10 or greater.

In a 100-μl aliquot containing 50 mM KCl, 10 mM Tris buffer (pH 8.3), 2 mM MgCl₂, 0.01% gelatin, 0.5 mM (each) deoxyribonucleotide triphosphate, and paired synthetic oligonucleotide primers SK 38 and 39 (at 2 pmol/μl) in an automated heating/cooling block. Primers corresponded to a gag sequence within the HIV-1 isolate HIV SF2 (12, 14). Primers homologous to complementary strands within the human β-globin gene (11) were used as a test for the presence of human genomic DNA (i.e., from contaminating mononuclear cells) in platelet preparations. After initial heat denaturation of the 100-μl aliquot, the sample was cooled to permit hybridization of the oligomers with homologous HIV-1 or β-globin sequences, if present. Synthesis was catalyzed by DNA polymerase from Thermus aquaticus (Cetus) (8, 15). Thirty rounds of amplification were carried out on each sample. The amplified product was detected by hybridization on a nylon membrane substrate with a 32P-end-labeled oligonucleotide (40 bases), SK 19, complementary to a region between the two primers.

Platelet Eluates. Platelet eluates were prepared by acid elution (16). Briefly, 1 × 10⁸ washed platelets (9) were suspended in 200 μl of a modified human Ringer’s solution (9) and acidified by addition of 100 μl of 150 mM H₃PO₄/100 mM NaCl/1.5% bovine serum albumin, pH 2.8, for 10 min, with shaking at 37°C. In some experiments, the platelet suspension was frozen and thawed three times with dry ice/ethanol prior to acidification with similar results. The acidified suspension was centrifuged at 20,000 × g and neutralized with 2.5 M Tris to pH 8.0. Eluted IgG was assayed by immunoassay as described (17).

Platelet Extracts. Platelet extracts were prepared from washed platelets suspended in 1% Triton X-100/0.15 M NaCl/0.01 M Tris buffer, pH 7.4, at a platelet concentration of 5 × 10⁸ per ml, ~1 mg/ml. The suspension was dispersed in a Vortex and centrifuged at 15,000 × g for 3 min, and the supernatant was employed for assay.

Immune Complexes. Immune complexes were prepared from serum by PEG precipitation (5). Precipitates were dissolved in one-fifth the usual volume for platelet incubation experiments (~2-4 mg/ml).

Purified IgG. Purified IgG was prepared by ion-exchange chromatography (5).

Gel Filtration. Gel filtration was performed on Sephadex G-200 as described (4). Fractions were concentrated 10-fold employing a B15 Amicon microconcentrator prior to incubation with platelets (~1 mg/ml).

Binding of Anti-HIV-1 Immune Complexes and/or 7S IgG to Washed Platelets. Washed normal platelets (6 × 10⁸) were incubated with PEG precipitate (2-4 mg/ml), void volume, or 7S IgG fraction (1-2 mg/ml) in a volume of 400 μl in phosphate-buffered saline (PBS) for 3 hr at room temperature; this was followed by overnight incubations at 4°C. The 400-μl suspension was then applied to 4 ml of a 20% sucrose cushion centrifuged at 12,350 × g for 10 min at 4°C to separate bound from unbound ligand. The sucrose was removed and the platelet pellet was eluted with acid, as above.

Affinity-Purified Anti-HIV-1. Affinity-purified anti-HIV-1 was prepared by adsorption of purified IgG to Affi-Gel 10 coupled to recombinant gp120 of the HIV-1 envelope glycoprotein. gp120 was kindly supplied by Scott D. Putney (Repligen, Cambridge, MA). Two milligrams of gp120 was coupled to 1 ml of Affi-Gel 10 as directed by the manufacturer. The gel was incubated with ~20 μg of IgG from 1 ml of HIV-1-positive serum overnight at 4°C. The gel was then washed extensively with five bed volume washes of PBS, followed by five washes of PBS/0.5 M NaCl, followed by five washes of PBS. Anti-HIV-1 antibody was eluted with 0.1 M glycine (pH 2.5), monitored by 280-nm absorption, and immediately neutralized to pH 7.4 with 1 M Tris. One milliliter of serum provided ~200–400 μg of anti-HIV-1 antibody at a titer of ~1:2000.

![FIG. 1. Anti-HIV-1 activity of platelet eluates of thrombocytopenic homosexuals (HSITP), narcotic addicts (NITP), and classic female autoimmune thrombocytopenic patients (ATP). Washed platelets (1 × 10⁸) were eluted with acid at pH 2.8, and the supernatant antibody was neutralized to pH 8.0 and assayed by ELISA utilizing HIV-1-coated beads. The developing antibody was an anti-human IgG (H + L) coupled to horsedarsh peroxidase. OD is given at 405 nm. Arrows indicate mean value for the group.](image-url)
RESULTS

Non-HIV-1 Viral Serology of Sera and Platelet Eluates of HIV-1-Positive Thrombocytopenic Patients. Table 1 demonstrates the presence or absence of anti-EBV [viral capsid antigen (VCA)], -CMV, -HSV, -ADE, and -VZV antibody in the sera and platelet eluates of 4 HSITP and 6 NITP patients. All 10 had evidence for anti-EBV (VCA), CMV, and HSV in their sera but not in their platelet eluates [except for 1 EBV (VCA) eluate measurement, platelets 2]. Similar results were found with anti-ADE in 6 of 10 patients and anti-VZV in 7 of 10 patients.

Anti-HIV-1 Antibody in Platelet Eluates and PEG-Precipitable Immune Complexes of HIV-1-Seropositive Thrombocytopenic Patients. In contrast to the data with other viruses, Fig. 1 demonstrates the presence of anti-HIV-1 antibody in the platelet eluates of 11 of 12 HSITP patients [OD > 1.01 ± 0.14 (mean ± SEM) and 19 of 21 NITP patients (OD > 0.99 ± 0.19)]. Negative results were obtained in 13 of 13 ATP patients (OD = 0.03 ± 0.00) as well as 14 of 14 control subjects (OD = 0.05 ± 0.01) and in 4 of 4 HIV-1-negative homosexuals (OD = 0.17 ± 0.04) (data not shown). Three of the HIV-1-positive samples were confirmed by electrophoretic transfer blot (Western blot) (reacted with gp160/120, p66, gp41, p31, and p24). Eluted anti-HIV-1 antibody correlated with eluted IgG (r = 0.6, P < 0.05, 11 df).

Similar results were found in the immune complexes of 15 of 15 HSITP patients (OD > 1.6 ± 0.14) and 12 of 15 NITP patients (OD > 1.39 ± 0.20). Negative results were obtained in 15 of 15 ATP patients (OD = 0.09 ± 0.01) as well as 14 of 14 control subjects (OD = 0.063 ± 0.02).

Affinity-purified anti-gp120 antibody was employed to construct a standard curve of anti-HIV-1 OD vs. IgG protein concentration, which was linear with a logarithmic-logarithmic plot. Regression analysis revealed a correlation coefficient of r = 0.86, P < 0.001. The specific activity of three different affinity-purified preparations averaged 0.050 OD per ng of IgG. Eluted platelet IgG from patients' platelets was then estimated for percentage of anti-HIV-1 activity. This represented 50% ± 16% for four HSITP patients and 61% ± 3% for four NITP patients. Their serum percent anti-HIV of total serum IgG was 12% ± 5% and 37% ± 4%, respectively. These measurements are based on the assumption that the avidity of other anti-HIV-1 antibodies is not appreciably different from that of anti-gp120. It is supported by the observation that anti-gp120 represents 40% ± 6.4% of total platelet anti-HIV-1 antibody, as determined from the relative binding of platelet eluate IgG to gp120 and virus-absorbed beads (three experiments).

Gel Filtration of Platelet Eluates and Serum Immune Complexes Containing Anti-HIV-1 Antibody. Fig. 2 demonstrates the presence of anti-HIV-1 antibody in the void volume fraction of platelet eluates of HSITP and NITP patients following gel filtration, indicating the presence of anti-HIV-1 antibody as a complex. Thus, neutralization of the acid eluate was sufficient to reassociate any complexes that may have dissociated during this procedure, or the complexes may not have dissociated under these conditions. No anti-HIV-1 activity was demonstrable in the 7S region. Similar results were obtained with two other gel filtrations of HSITP as well as NITP patients (data not shown).

Similar results were obtained with the immune complexes of three HSITP as well as three NITP patients (data not shown).

Binding of Anti-HIV-1 Antibody of PEG-Precipitable Immune Complexes, Void Volume, and 7S IgG Fractions and Affinity-Purified Anti-HIV-1 Antibody to Platelets. Anti-HIV-1 antibody of PEG-purified immune complexes bound to washed normal platelets by saturation-dependent kinetics employing eluted anti-HIV-1 ELISA OD readings as end point (data not shown). PEG-purifiable material containing anti-HIV-1 antibody from four HSITP patients bound to 6 x 10^9 normal platelets with HIV-1 OD values of 0.65 ± 0.17 and 0.83 ± 0.38, respectively, at saturation, whereas six control or six ATP PEG precipitates bound with a HIV-1 OD

Table 2. Binding of anti-HIV-1 antibody of gel-filtration fractions of PEG-purifiable immune complexes to platelets

<table>
<thead>
<tr>
<th>Serum</th>
<th>n</th>
<th>Void volume</th>
<th>7S IgG</th>
<th>Void volume + platelets</th>
<th>7S IgG + platelets</th>
<th>Control 7S IgG + platelets</th>
<th>Buffer + platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSITP</td>
<td>4</td>
<td>&gt;1.468</td>
<td>0.897 ± 0.379</td>
<td>0.505 ± 0.090</td>
<td>0.094 ± 0.017</td>
<td>0.081 ± 0.005</td>
<td>0.040 ± 0.004</td>
</tr>
<tr>
<td>NITP</td>
<td>4</td>
<td>&gt;1.194</td>
<td>0.587 ± 0.258</td>
<td>0.310 ± 0.039</td>
<td>0.101 ± 0.039</td>
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</tr>
</tbody>
</table>

PEG precipitates of HSITP and NITP sera were gel filtered on Sephadex G-200 into void volume and 7S IgG fractions. Washed platelets (6 x 10^9) were resuspended in 400 μl of concentrated void volume or 7S IgG gel-filtration fractions (~1-2 mg/ml) and incubated and processed. The control 7S IgG fraction was obtained from normal serum, similarly gel filtered, and concentrated. Values are expressed as mean ± SEM.
value of 0.03 ± 0.01. Table 2 demonstrates the binding of anti-HIV-1 antibody from gel-filtration fractions of PEG-precipitable immune complexes to washed platelets of four HSITP and four NITP patients. Note binding of the void volume fraction, presumably to platelet Fc receptors, with absent binding of the 7S IgG fraction.

Additional platelet-binding experiments were performed with noncomplexed affinity-purified anti-HIV-1 (gp120) antibody applied to washed platelets on microtiter plates. Three different antibody preparations did not show enhanced binding to normal washed platelets compared to control IgG at 1 mg/ml (data not shown). Similar negative results were obtained with three washed platelet preparations obtained from HIV-1-seropositive patients that had been eluted of platelet-bound IgG when compared to control platelets eluted of IgG (data not shown), ruling out the possibility of HIV-1 antigen incorporation into the platelet membrane.

Measurement of HIV-1 Antigen in Platelets and PEG-Precipitable Immune Complexes. An antigen capture assay was employed to determine the presence of HIV-1 antigen in acid platelet eluates, platelet extracts, and serum immune complexes. Negative results were obtained in 10 of 10 platelet eluates and 7 of 7 platelet extracts of HSITP and NITP patients. Similar results were obtained in 7 of 7 immune complex preparations of HSITP patients and 5 of 7 NITP patients (two NITP patients had slightly elevated ELISA OD values, which were +24% and +34% above the 2 SD range for control subjects).

Measurement of HIV-1 Proviral DNA in Lymphocytes and Platelet Extracts. PBMCs and platelets from eight patients (four HSITP and four NITP) were analyzed. Five of the eight lymphocyte samples were HIV-1 DNA positive by the PCR (Fig. 3). Only one patient’s amplified platelet DNA sample gave indication of HIV-1 sequences. This sample, however, also positive after gene amplification initiated by sequences corresponding to the human β-globin gene. The ratio of HIV-1 to β-globin signals obtained following PCR appeared to be about the same from platelet and PBMC preparations (data not shown). Other patient platelet samples were PCR negative for the globin region, indicating their high degree of purity.

Anti-Anti-HIV-1 Antibody in PEG-Precipitable Immune Complexes and Platelet Eluates. Affinity-purified anti-HIV-1 (gp120) (antibody 1) and anti-anti-HIV-1 (gp120) (antibody 2) obtained from serum of a NITP patient were incubated with each other prior to their addition to nitrocellulose strips containing the HIV-1 antigens to determine whether antibody 2 would inhibit the binding of antibody 1 to HIV-1 antigens (i.e., operate as an anti-idiotype antibody where the paratope and idiotope are the same sites). The binding of antibody 2 was detected by ELISA with an anti-human IgG antibody coupled to peroxidase. "Mirror image" anti-idiotype antibody was not detectable. On the contrary, Fig. 4A demonstrates greater anti-human IgG binding to HIV-1 antigen gp120 when both antibodies are added together (lanes 7 and 8) compared to antibodies added alone (lane 4 for antibody 1; lane 2 for antibody 2) or compared to antibody 1 added together with irrelevant human IgG (lane 5). Similar findings were obtained in one other NITP patient and two HSITP patients. Similar
results were obtained when antibody 2 was obtained from a platelet eluate of a HSITP patient (Fig. 4B) as well as a NITP patient (data not shown). Thus, either antibody 2 enhanced the binding of antibody 1 to HIV-1 antigens by increasing the affinity of antibody 1 for HIV-1 antigens or antibody 2 bound to antibody 1 at sites that do not impede its binding to HIV-1 antigen. In either case, anti-anti-HIV-1 antibody (antibody 2) is demonstrable in the serum as well as on the platelets and could explain the presence of anti-HIV-1 immune complexes in the absence of HIV-1 antigen.

DISCUSSION

HIV-1-seropositive ITP in homosexuals and narcotic addicts is different from classic ATP in that the platelet surface has markedly elevated IgG, C3C4, and IgM (2, 4). Platelet-bound IgG and C3C4 are 3- to 4-fold greater; platelet-bound IgM is 2- to 3-fold greater. In addition, PEG-precipitable serum immune complex levels in the HIV-1-seropositive thrombocytopenic patients are 3- to 7-fold greater than normal and are not elevated in classic ATP patients (2, 4). Thus an attractive explanation for the marked deposition of immunoglobulin and complement on these platelets is the deposition of these circulating immune complexes on platelets, presumably on their Fc receptors. This prompted a careful analysis of the content of their circulating immune complexes as well as platelet membrane eluates. We have recently (5) noted that their PEG-precipitable immune complexes contain numerous antibodies against various viral antigens to which they have been exposed (EBV, CMV, HSV, rubella virus); however, viral antigen was not found. This suggested the possibility of complexes composed of antibody directed against antibody. Further experiments demonstrated the presence of anti-F(ab')2 antibodies in their sera and immune complexes as well as in their platelet eluates (5).

The present report demonstrates the presence of anti-HIV-1 antibody as well as anti-anti-HIV-1 antibody in their serum immune complexes as well as in their platelet eluates. These data support our previous observation on the presence of anti-F(ab')2 antibodies in their serum immune complexes as well as platelet eluates (5). HIV-1 was not found in the platelet eluates or platelet extracts, as determined by an antigen capture technique. Nor was HIV-1 proviral DNA found in the purified platelets themselves, as tested by a gene amplification technique. The latter technique is extremely sensitive for detecting human retroviral sequences present in low copy numbers (10, 12). The concentration of anti-HIV-1 in these eluates was determined from a standard curve prepared from affinity-purified anti-HIV-1 (gp120). This revealed very high concentrations of anti-HIV-1 antibody on platelets, averaging 50% of total platelet IgG. This observation is supported by the positive correlation between platelet eluate IgG and eluted anti-HIV-1 concentration. In addition, other anti-viral antibodies, such as anti-EBV (VCA), CMV, and HSV, were not found in their platelet eluates despite their presence in sera of these patients. Thus the platelet appears to be a reservoir for the deposition of anti-HIV-1 immune complexes composed of anti-anti-HIV-1 as well as anti-HIV-1 antibodies. This is further supported by our inability to demonstrate cross-reactivity between anti-HIV-1 antibody and the platelet membrane. The explanation for this apparent preference of anti-HIV-1 complexes on platelets has not been determined. It is possible that the anti-HIV-1 antibody complexes may be present in greater concentration than other viral antibody complexes. Alternatively, the physical structure of the anti-HIV-1 complexes may have greater affinity for the platelet than other viral complexes. The stoichiometry of the anti-HIV-1 complexes could not be accurately determined. However, it was of interest that the yield of anti-anti-HIV-1 antibody from serum was ~5–10% of the yield of anti-HIV-1 antibody.

We suggest that the markedly elevated platelet-bound IgG, C3C4, and IgM are due in part to the deposition of anti-HIV-1 and anti-anti-HIV-1 antibody complexes. The pathophysiology of this observation remains to be established. However, it is very likely that free Fc or C3b domains of these complexes are capable of binding to their respective receptors on phagocytic cells leading to increased destruction of platelets and/or megakaryocyte damage with resultant thrombocytopenia. This is supported by the successful treatment of the thrombocytopenia in most patients with corticosteroids and/or splenectomy (18).

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