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Prevalence of Antiphospholipid and Antiplatelet Antibodies in Human Immunodeficiency Virus (HIV)-Infected Chilean Patients

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Antiphospholipid (aPL) and antiplatelet (aPlt) antibodies, found in patients with autoimmune diseases, are also detected in infectious diseases. The purpose of this study was to examine the prevalence of these antibodies in HIV patients and to evaluate an association of these antibodies with thrombocytopenia and/or thrombosis. Sixty-three HIV-seropositive patients and 52 normal controls were studied. Anticardiolipin (aCL), anti-β_2 glycoprotein I (anti-β_2GPI), and antiprothrombin (aPT) antibodies were determined and the lupus anticoagulant (LA) test was performed. Antiplatelet antibodies (aPlt) were also determined. Seven out of 63 (12.7%) HIV patients were positive for aCL, four of 63 (6.3%) for anti-β_2GPI, and five of 63 (7.9%) for aPT. No patients studied were LA positive. Six out of 63 (9.5%) patients were positive for aPlt. One of them showed weak reactivity for GPIb-IX. The platelet count of patients ($202 \pm 63 \times 10^3$ platelets/μL) was significantly lower than in the controls ($343 \pm 6 \times 10^3$ platelets/μL) ($P < 0.001$). There was no correlation between the presence of aPL and/or aPlt and thrombocytopenia. Of the HIV-infected patients, 22.2% presented aPL and 9.4% aPlt antibodies. In this study, the presence of aPL and aPlt antibodies was not associated with the development of thrombosis and/or thrombocytopenia. J. Clin. Lab. Anal. 17:209–215, 2003.

Key words: HIV infection; antiphospholipid antibodies; antiplatelet antibodies; anticardiolipin antibodies

INTRODUCTION

Antiphospholipid antibodies (aPL) are a heterogeneous group of antibodies that have been associated with thrombosis, recurrent fetal loss, and thrombocytopenia (1,2) in patients with antiphospholipid syndrome (APS) and/or systemic lupus erythematosus (SLE). Similarly, the presence of antiplatelet antibodies (aPlt) specific for platelet surface glycoproteins (i.e., GPIIb-IIIa, GPIb-IX, and GPIa-IIa) has been associated with thrombocytopenia primarily in patients with idiopathic immune thrombocytopenia and also in APS or other connective tissue diseases. It has also been associated with drugs and infections, particularly of a viral origin (3–5).

aPL antibodies are autoantibodies directed against anionic phospholipids or protein-phospholipid complexes (6). They are measured by solid-phase immunoassays as anticardiolipin (aCL) (7) or as an activity which prolongs phospholipid-dependent coagulation assays, the so-called lupus anticoagulants (LA) (8). Anticardiolipin ELISA is not only positive in patients with APS but also in a variety of disorders, including connective tissue diseases and infectious diseases such as

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syphilis (9), Q fever (10), lyme disease (11), mycoplasma (12), tuberculosis (13), leprosy (14), etc. Furthermore, aPL antibodies have also been reported in viral infections: HIV (15), cytomegalovirus (16), varicella zoster virus (17), hepatitis C (18), and Epstein-Barr (19).

In HIV-infected patients the prevalence of aCL has been reported from 8–75% (9,20–22), but generally their presence is not associated with thrombotic events (22–25). The prevalence of aPlt in HIV-infected patients is 6.5–50% (26,27). Thrombosis (28,29) and thrombocytopenia (30) have been reported occasionally in HIV-infected patients. This may have important implications in the management and treatment of affected patients. This study addresses this question by determining aPL by different assays and antiplatelet antibodies and the correlation with thrombosis and/or thrombocytopenia in a group of HIV patients.

PATIENTS AND METHODS

Patients and Controls

Sixty-three confirmed HIV-infected patients from the Regional Hospital of Talca, Chile, and the Clinical Hospital of the University of Chile were studied. The mean age of the patients was 37 $\pm$ 12 yr. There were 48 males (76.2%) and 15 females (23.8%). Twenty-eight patients (44.4%) acquired the disease through homosexual contact, 30 (50.8%) by heterosexual contact, two (3.2%) were bisexual, and one (1.6%) was hemophilic. Thirteen out of 30 patients (43.3%) had hemoglobin $<$13 g/dL; in 10 out of 33 (30.3%) the leukocyte count was $<$5000/μL; and in 15 out of 35 (42.9%) the CD4+lymphocyte was $<$200 μL (normal value: 600–1200 μL). Twenty-two out of 40 HIV-patients (35.0%) were asymptomatic; three out of 40 (7.5%) were symptomatic; and 23 out of 40 (57.5%) had AIDS. Twelve out of 25 patients (48.0%) were treated with Isoniazide and/or Cotrimoxazol; six out of 25 (24.0%) received lamivudine (3TC) and zidovudine (AZT) for treatment; and seven out of 25 received Isoniazide and/or Cotrimoxazol and 3TC and AZT.

The normal control group included 52 healthy blood donors (35 $\pm$ 9.3 yr; males 66% and females 34%) that attended the Blood Bank of the Regional Hospital of Talca.

Methods

Ten milliliters of blood were collected by venipuncture. Two milliliters were anticoagulated with EDTA (1 mg/mL) for platelet studies, 2.5 mL were anticoagulated with sodium citrate 3.2% in a ratio of 9:1 blood:anticoagulant for detection of lupus anticoagulant activity (LA), and 5.5 mL were used to obtain serum for all the ELISA determinations. Serum samples were separated and stored at -70°C until testing.

Platelet count

Platelets were counted in EDTA-anticoagulated blood by using a hemocytometer and phase contrast condenser by microscopy. Thrombocytopenia was defined as a platelet count less than 140×10^3 platelets/μL.

aCL ELISA

For the determination of aCL (IgG, IgM, and IgA), an ELISA assay was used as described (31–33). Briefly, nonirradiated microtiter plates (Maxisorp 469914-A, NUNC, Naperville, IL) were coated with 50 μL of Cardiolipin (CL) at a concentration of 50 μg/mL in 70% ethanol. The plates were air-dried overnight at 4°C. After washing with phosphate-buffered saline (PBS) solution 0.01 M (pH = 7.4), plates were blocked with 10% fetal bovine serum (FBS) (Gibco BRL, Gibco, Auckland, Scotland) in PBS (FBS-PBS) for 1 hr at room temperature (RT) (22–25°C). After washing with PBS, 50 μL of serum samples (1:50 dilution in FBS-PBS) normal controls and positive controls were added in duplicate. To perform aCL ELISA, standards were included to express the results in GPL, MPL, and APL units (Antiphospholipid Standardization Laboratory, Morehouse School of Medicine, Atlanta, GA). After incubation for 1 hr at RT, plates were washed with PBS and 50 μL of alkaline phosphatase-conjugated anti-human IgG, IgM, or IgA (Sigma, St. Louis, MO) were added to the plate. After 1 hr incubation at RT and washing with PBS, 100 μL of 0.15 p-nitrophenylphosphate substrate in diethanolamine buffer (pH = 9.8) was added. The optical densities (OD) were determined at 405 nm by StatFax-2600 microplate reader (Awareness Technology, Inc., Palm City, FL). The activity of the aCL antibodies was expressed as international units (GPL, MPL, and APL); for the three isotypes the cutoff was 15 units. The positive samples were confirmed by testing them a second time.

AphL^{TM} ELISA

In cases in which the IgG and/or IgM aCL were positive, the presence of aPL antibodies was confirmed by using a more specific ELISA assay, the APhL^{TM} ELISA kit (Louisville APL Diagnostics, Inc., Doraville, GA). The assay was run as described in the package insert provided by the manufacturer. Values above 15 GPL or 15 MPL were considered positive.

Lupus anticoagulant

For the detection of the lupus anticoagulant activity, two tests were used:

1. Dilute tissue thromboplastin inhibition (dTTI). This was performed as described elsewhere (34). Briefly, 100 μL of thromboplastin (Pacific Hemostasis) diluted 1:500 in saline was added to 100 μL of platelet-poor plasma (PPP) and incubated for 5 min at 37°C. The clotting reaction was started by adding 0.025 M $CaCL_2$, and the clotting time was determined using a Clot-1-coagulometer (RAL S.A., Barcelona, Spain). Determinations were carried out in duplicate. When the ratio patient time/control time was >1.3, the test was repeated using a mixture of 1:1 with normal plasma. If the clotting time was corrected, the platelet neutralization procedure (PNP), using a platelet lysate as a source of phospholipids, was used as a confirmation test.
2. Kaolin clotting time (KCT) (35). Briefly, 100 μL of 0.1% kaolin in PBS was incubated with 200 μL of PPP at 37°C for 3 min. The clotting reaction was initiated by adding 200 μL 0.025 M $CaCL_2$ and clotting time was registered using a Clot-1 coagulometer. Criteria for considering a sample positive and for confirmation were similar to that used for dTTI.

Anti-β_2GPI ELISA

IgG, IgM, and IgA anti-β_2GPI antibodies were determined by ELISA as described (36). In brief, nonirradiated microtiter plates (Maxisorp 439454, NUNC) were coated overnight with 50 μL of a solution containing 20 μg/mL β_2GPI. After washing with PBS-Tween (PBS-Tw) 0.05%, pH 7.4, plates were blocked with 350 μL of 3% bovine serum albumin (BSA) for 2 hr and subsequently washed with the same solution. Patient samples and controls (diluted 1:50 in 3% BSA solution) were added to the plates in duplicate and incubated at RT for 60 min. Plates were subsequently washed three times and incubated with alkaline phosphatase-labelled anti-human IgG, IgM, or IgA for 60 min. Color reaction was obtained as described for the aCL assays. A sample was considered positive when an OD >4 standard deviations (SD) of the average of normal controls (cutoff) was obtained. The activity of these antibodies was expressed as the ratio OD_{405} patient/OD_{405} cutoff. Positive control was kindly provided by Dr. A. Cabral (Department of Immunology and Rheumatology, Instituto Nacional de la Nutricion Salvador Zubiran, Mexico City, Mexico).

Antiprothrombin ELISA

Antiprothrombin antibodies were determined by ELISA (37). Briefly, nonirradiated microtiter wells (Maxisorp 469914-A, NUNC) were coated with 50 μL of a solution containing 5 μg/mL prothrombin overnight at 4°C. Previously 20, 10, and 5 μg/mL were used obtaining similar results. After washing three times with PBS-Tw 0.1%, wells were blocked with PBS-Tw-BSA 1% for 1 hr at RT. After washing three times with PBS, 100 μL of patient samples (positive and normal controls diluted 1:100 in PBS-Tw-BSA) were added to the plates and incubated for 1 hr at RT. After washing three times, 50 μL of conjugates (alkaline phosphatase-labelled anti-human IgG, IgM, and IgA) were added to the plates and the color reaction was developed and measured as described for the aCL assays. The positiveness of samples and the activity of aPT antibodies were determined as in the anti-β_2GPI assay. Positive control was kindly provided by Dr. R. Forastiero, Department of Hematology, Favaloro University, Buenos Aires, Argentina.

Platelet membrane ELISA

This test was used in order to search for aPlt in the serum (38). Briefly, microtiter wells (Maxisorp 469914-A, NUNC) were coated with 50 μL of protein membrane of platelets prepared by sonication at a concentration of 50 μg/mL in phosphate-buffered saline 10 mM, pH 7.4 (PBS)/EDTA 0.33% by overnight incubation at 4°C. The wells were washed with PBS/Tween 0.05% (PBS/Tween) and blocked with 200 μL of PBS-Tw-gelatin (G) 1% during 90 min at RT. After washing with PBS-Tw 50 μL of patient serum, normal and positive control serum diluted 1:20 in PBS-Tw-G was added. After incubating 1 hr at RT, the plates were washed with PBS/Tw. An alkaline phosphatase-labelled anti-human IgG antibody (Sigma) and substrate buffer were added after washing, and the reading was done as in the aCL ELISA test described above. A sample was considered positive when an OD >4 SDs of the average of normal controls (cutoff) was obtained.

Monoclonal antibody-specific immobilization of platelet antigens (MAIPA) (39)

In cases in which the ELISA for platelet membrane was positive, we studied the glycoprotein specificity of autoantibodies using different monoclonal antibodies (mab) for antigen immobilization. In brief, 1×10^8 normal washed platelets were incubated with 100 μL of human serum to be studied and a 25 μL of mab against one of the immunogenic glycoproteins (AP-2, anti-GPIIb-IIIa, a gift from Dr. Thomas J. Kunicki,

Scripps Research Institute, La Jolla, CA; 12-F, anti-GPIa-IIa from Dr. Virgil Woods, University of California, San Diego, CA; anti-GPIb-IX, Pharmingen, San Diego, CA) for 30 min at 37°C. The sensitized platelets were washed and lysed in Tris-buffered saline (TBS) containing 0.5% Nonidet P-40 (N) (Sigma) (TBS-N). The platelet lysates were then centrifuged at 15,000 g for 15 min at 4°C and the supernatants were diluted in TBS-N containing 0.5 mM $CaCl_2$. The diluted supernatants (10 μL, 1:5) were incubated for 90 min at 4°C in microtiter wells (Maxisorp 469914-A, NUNC). After washing, an alkaline phosphatase-labelled anti-human IgG antibody (Sigma) and substrate buffer were added, and the reading was done as in the aCL ELISA test described above. The positiveness of samples and the activity of aPT antibodies were determined as in the Platelet membrane ELISA. An anti-HLA-I monoclonal antibody was used as a control of specificity.

Statistical Analysis

The means of platelet count of patients and controls were compared using Student's *t*-test. The prevalence of each different antibody in patients vs. the control group were compared using the Chi's square test or Fisher test. Five percent level of significance was used.

RESULTS

Prevalence of aPL and aPlt

Seven out of 63 patients (11.1%) presented aCL, four out of 63 (6.3%) anti-β_2GPI, and five out of 63 (7.9%) aPT. The LA test was negative in all of them (Table 1). Fourteen out of the 63 patients (22.2%) tested positive for one or more assays used for aPL detection. Two out of 52 (3.8%) normal controls were aCL positive, one (1.9%) for anti-β_2GPI and two (3.8%) for aPT All were weak positive.

In HIV-infected patients, IgG aCL and IgM aCL were detected more frequently than IgA aCL (Table 2). In the anti-β_2GPI and aPT antibodies, the IgA isotype was more common. The activity of these antibodies, expressed as international units in aCL and as the ratio OD_{405} patient/OD_{405} cutoff in anti-β_2GPI and aPT, was weak in the majority of cases (Table 2). Two patients presented two types of aPL antibodies. One patient presented IgG aCL and IgG anti-β_2GPI and the other presented IgA anti-β_2GPI and IgA aPT antibodies. Six patients presented only aCL, four only aPT, two only anti-β_2GPI, one aCL and anti-β_2GPI, and one aPT and anti-β_2GPI.

Using the "in-house" IgG and IgM aCL ELISA, seven out of 63 sera (11.1%) of HIV-infected patients were positive, but only one of those sera (1.6%) was positive when the APhL^{TM} ELISA Kit was used.

Six out of 63 HIV-infected patients (9.4%) presented aPlt, but only one case was specific for GPIb-IX with weak activity (ratio OD patient/OD cutoff = 1.2). In the other five patients, the specificity for a membrane glycoprotein other than GPIIb-IIIa, GPIb-IX, and GPIa-IIa, was not determined. None of the control sera was positive for antiplatelet antibodies. Two out of six HIV patients who presented aPlt antibodies also had aPL: one had IgG aCL antibodies and the other had IgG and IgM aPT.

TABLE 1. Prevalence of antiphospholipid antibodies in HIV-infected patients and control group

	Patients ($n=63$)		Controls ($n=52$)		
	n	%	n	%	*P*
aCL	7	11.1	2	3.8	NS
Anti-β_2GPI	4	6.3	1	1.9	NS
aPT	5	7.9	2	3.8	NS
Lupus anticoagulant	0	0	0	0	

aCL, anticardiolipin antibodies; aPT, anti-prothrombin antibodies; anti-β_2GPI, anti-β_2 glycoprotein I antibodies.

TABLE 2. Isotype distribution of aCL, anti-β_2GPI, and aPT antibodies in 63 patients with AIDS

	aCL		Anti-β_2GPI		aPT	
	n	%	N	%	n	%
Any isotype[a]	7	11.1	4	6.3	5	7.9
IgG	4 (21.0 ± 1.4)	6.3	1 (2.4)	1.6	2 (1.7 ± 0.6)	3.2
IgM	3 (34.5 ± 3.6)	4.8				
IgA	1 (38)	1.6	2 (3.1 ± 2.0)	4.8	3 (1.6 ± 1.0)	6.3

[a]At least one of the three isotypes positive.
In parenthesis, activity (aCL: GPL, MPL, APL, International units for anticardiolipin antibodies; anti-β_2GPI and aPT antibodies: OD_{405} patient/OD_{405} cutoff). aCL, anticardiolipin antibodies; anti-β_2GPI, anti-β_2 glycoprotein antibodies; aPT, anti-prothrombin antibodies.

Antiphospholipid and Antiplatelet Antibodies Vs. Thrombocytopenia and Thrombosis

Although within the normal range, the platelet count of HIV-infected patients ($202 \pm 63 \times 10^3$ platelets/μL) was significantly lower than that found in controls ($343 \pm 56 \times 10^3$ platelets/μL) ($P < 0.001$). Six of 37 patients (16.2%) presented thrombocytopenia. The platelet count in these patients was $120 \pm 13 \times 10^3$ platelets/μL. Only one patient with aPL and/or aPlt

presented thrombocytopenia, and 11 cases who presented these antibodies did not present thrombocytopenia. No patients presented thrombosis.

DISCUSSION

aPL have been associated with thrombosis, recurrent spontaneous abortions, and/or thrombocytopenia (1,2). aPlt have been associated with thrombocytopenia (3,4). Both types of antibodies have been found in HIV-infected patients (9,21,40). The purpose of our study was to study the prevalence of aPL and aPlt in HIV-infected patients and their association with thrombosis and thrombocytopenia, and thrombocytopenia, respectively.

Prevalence of Antiphospholipid and Antiplatelet Antibodies

IgG and IgM aCL were found in 12.9% of HIV-infected patients. In other studies, the prevalence of aCL has shown a wider range (8–75%) (9,20–22). Other investigations have found that in several stages of the disease, the prevalence of one isotype over the others may vary. For example, the prevalence of IgG aCL was 24–94% and IgM aCL was 0–50% (20,22,23). We found 6.3% of aPT and 6.3% of anti-β_2GPI, as expected. de Larranaga et al. (9) found that HIV-positive patients had a higher frequency of anti-β_2GPI?(13%) and aPT (12%) antibodies than syphilis patients (0 and 4%, respectively), but significantly less than APS patients (61 and 40%, respectively). In SLE, we found 44.4% aCL, 23.3% anti-β_2GPI, and 20.0% aPT (41), and in patients with venous or arterial thrombosis we found 25.5% aCL, 6.2% anti-β_2GPI, and 13.2% aPT (56). In our study, none of our HIV patients had LA. Other studies found LA positive in 1–70% of HIV-infected patients (9,24,42), which was not associated with IgG aCL (23). In this study, aPL antibodies detected by the APhL^{TM} ELISA kit were lower (1.1%) than when detected by the aCL "in-house" assay (11.1%). This is in agreement with previous studies that showed similar findings when samples from patients with leptospirosis, leishmaniasis, and syphilis were tested by aCL, the APhL^{TM} ELISA kit, and the anti-β_2GPI assay (43).

Antiplatelet antibodies were found in 9.4% of HIV-infected patients, but only 1.6% was specific for a platelet glycoprotein. Magnac et al. (26) using Western blot, found antiplatelet antibodies in 6.5% and 20% of non-thrombocytopenic and thrombocytopenic HIV patients, respectively. Riccio et al. (27) found that 50.4% of HIV patients presented increased platelet associated IgG (PAIgG).

Antiphospholipid Antibodies and Thrombosis

In patients with APS, aPL are associated with thrombosis, recurrent spontaneous abortion, and thrombocytopenia (1,2). In all of our HIV-infected patients, no correlation between aPL and thrombosis was found. Interestingly, most studies show that in HIV-infected patients aPL does not correlate with a history of thrombosis (23,24). IgG aCL were found frequently in HIV-infected patients but this does not correlate with biological markers of endothelial injury (20). However, several thrombosis clinic cases have been described (44,45,46).

It is possible that the absence of clinical association can be explained by the fact that the IgG and/or IgM aCL detected by "in house" ELISAs were in the low positive value range and were not specific for APS. Moreover, it is known that LA is more specific than aCL, and no patients presented this type of aPL.

Antiphospholipid and Antiplatelet Antibodies and Thrombocytopenia

We did not find a correlation between aPlt and/or aPL and thrombocytopenia. In our series of patients, around 16% presented discrete thrombocytopenia. Other authors have found that 10–40% of HIV-infected patients develop thrombocytopenia in variable degrees (47–49). However, severe thrombocytopenia is relatively rare, occurring in only 1.5% (30). The mechanisms of thrombocytopenia appear to be multifactorial, involving impaired production and/or an increased destruction: 1) the impaired platelet production may be due to HIV infection of megakaryocytes that express functional CD4 molecules, and 2) a severe thrombocytopenia in HIV-seropositive patients is related to an immune destruction either by aPlt or by immune complexes (30,50–52). In the latter, the thrombocytopenia present in these patients may also be explained by autoimmune destruction: 1) intact virus or viral antigens are adsorbed directly in the surface of platelets, triggering binding of virus-specific antibodies and complement components to platelets, or 2) antibodies formed in response to viral infections may cross-react with glycoproteins present on the platelet (i.e., GPIIb-IIIa, GPIb-IX). In both mechanisms, subsequent clearance of the complex by phagocytic mononuclear system occurs. It has been observed that the anti-HIV-antibodies present in these patients cross-react with platelets due to homology between the gp120 and the GPIIIa (CD61) (53–55).

In summary, we found that approximately 13% of HIV-infected patients presented aCL but only 2% were positive when more specific tests for detection of aPL antibodies were performed. In addition, approximately 10% of these patients presented aPlt antibodies, but

only approximately 2% presented specificity to glycoproteins of platelets. In our group of patients these antibodies were not associated with thrombosis and/or thrombocytopenia.

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