Serum reactivities to a panel of phospholipid antigens, including cardiolipin (CL), phosphatidylserine (PS), sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine, were measured by enzyme-linked immunosorbent assay in 196 human immunodeficiency virus-1 (HIV-1) patients with CDC II to IVC clinical disease. Significant levels of IgG to CL, PS, or both were observed in 23 patients lacking evidence of thrombophilic events or any peculiar clinical feature of HIV-1 infection. Fluorescence-activated cell sorting analyses showed that in vitro apoptosis of T cells was increased in patients with high serum anti-PS IgG, whereas the overexpression of Fas/Apo-1 marker was detected in all patients regardless of their antiphospholipid reactivities. Macrophages from patients with significant titers of anti-PS IgG antibodies were not activated by the presence of apoptotic CEM lymphoblasts or by purified anti-PS IgG from the same patients. By contrast, these antibodies greatly improved the effector functions of autologous macrophages in antibody-dependent cellular cytotoxicity (ADCC) assays using $^{51}Cr$-labeled CEM cells, whereas polyclonal IgG were unable to induce an equivalent cytotoxicity in all instances. An increasing effect on ADCC was also observed in tests using macrophages from healthy controls to CEM coated with anti-PS IgG. These results support a potential correlation of anti-PS specificity with T-cell apoptosis in HIV-1 infection. Because PS is exteriorized by apoptotic lymphocytes, its persistence may stimulate antibodies which cooperate with macrophages in the clearance of dead cells by an enhanced ADCC mechanism. This interpretation could explain the absence of thrombophilia in HIV-1 patients with serum elevations of antiphospholipid reactivities.

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anti-PS IgG in HIV-1 infection are indeed related to lymphocyte apoptosis in vivo.

MATERIALS AND METHODS

Patients and controls. Serum samples from 196 HIV-1+ patients were from the Department of Internal Medicine of the University of Bari and the Division of Infectious Diseases of ‘Di Summa’ Hospital (Brindisi, Italy). Patients were classified according to their Centers for Disease Control (CDC; Atlanta, GA) clinical stage. Blood samples were obtained after informed consent and the study was approved by the Ethical Committees of both institutions. Most patients were intravenous drug abusers or ex-abusers; 6 were homosexual males with a concurrent history of occasional drug addiction. Two patients were hemophiliacs. Controls included 46 sera from normals who provided peripheral lymphocytes and macrophages on several occasions and 9 SLE female patients with symptoms of APS.

Antiphospholipid enzyme-linked immunosorbent assay (ELISA) tests and isolation of anti-PS and anti-CL antibodies. A cofactor-independent ELISA was optimized to detect and measure serum levels of antiphospholipid reactivities. Briefly, CL, PS, phosphatidyethanolamine, sphingomyelin, and phosphatidylcholine alcohol preparations (Sigma, St Louis, MO) were diluted at 10 µg/mL in carbonate buffer pH 9.6 containing 1% bovine serum albumin (BSA) and separately incubated overnight at 4°C in flat-bottomed 96-well polystyrene plates. This coating procedure was experienced in our laboratory as the most suitable in reducing the nonspecific binding to plates. After blocking with 0.3% gelatine and 1% milk powder in phosphate-buffered saline (PBS), the plates were supplemented with sera diluted at 1 × 10−1 in PBS containing 5% BSA and incubated at room temperature for 3 hours. Goat peroxidase-conjugated antisera to human IgG and IgM (Jackson Immunoresearch Labs, West Grove, PA) were separately used at appropriate dilutions, and the plates were next developed with o-phenylenediamine solution and read in a microtiter reader at 492 nm (Flow, Irvine, UK). The possible β2-GPI contamination of BSA used in both coating and diluting solutions was not tested in view of the demonstration that only anti-CL antibodies from APS patients are reactive to β2-GPI.13 Each serum was tested in triplicate and the mean absorbance to each phospholipid was compared with the range of reactivity detected in normal sera, although to a much lower extent (<0.21 pg/mL). The PS-reactive antibodies were then eluted by acidic elution (pH 2.8) and adjusted to neutral pH. Protein analyses of these fractions were performed by SDS-PAGE, which showed in all instances a weak, although definite Ig content with sporadic albumin contamination.

Peripheral T-cell analysis. CD3+, CD4+, and CD8+ peripheral cells were counted in a FACSscan (Becton Dickinson, Mountain View, CA). The analysis of cell phenotypes in all patients was simultaneous to the collection of sera for antiphospholipid evaluation and included the study of Fas expression,16,17 namely the 45-kD activation marker acting as apoptosis-inducer by its natural ligand.18 The IgG1 mouse monoclonal antibody (MoAb) to the extracellular Fas from clone UB-2 (MLB, Nagoya, Japan) was used for this purpose in combination with a phycoerythrin-conjugated antimmune antiserum in flow cytometry analysis.

Evaluation of apoptosis. The percentage of apoptosis in fresh T-cell preparations was determined by FACS analysis to measure the extent of propidium iodide (PI) staining.19 A total of 1 × 106 cells from each sample were treated for 3 hours with 70% ethanol solution at 4°C and incubated overnight with 100 µg of PI in the presence of RNase (0.5 mg). Flow cytometry analyses were then performed using the FACSscan with 1,024-channel resolution. The differential light scatter detection parameters were focused on the lymphocyte region,20 and the magnitude of the subdiploid DNA peak in each evaluation was related to the percentage of apoptotic lymphocytes.

The second method included the electrophoresis of cell DNA. A modification of the method of Sellins and Cohen21 was adopted to figure out the DNA fragmentation. Briefly, 2 × 106 cells were lysed with 0.5 mL hypotonic lysis buffer (10 mMol/L Tris, 1 mMol/L EDTA, 0.2% Triton X-100). The lysates were centrifuged at 13,000g for 10 minutes and the supernatant containing DNA was collected. After removing proteins by phenol-chloroform-isomyl-alcohol extraction, the DNA was precipitated overnight with sodium acetate and absolute ethanol at −20°C. Pellets were washed with 70% ethanol, air-dried, and dissolved in 10 mMol/L Tris, 1 mMol/L EDTA, pH 7.4, containing 20 µg/mL of RNase. Electrophoresis of DNA samples supplemented with the loading buffer was performed in 1.5% agarose in TE buffer for 90 minutes at 70 V. DNA was visualized by staining with ethidium bromide under UV light.

Anti-PS IgG and macrophage functions. Because macrophages are involved in phagocytosis of apoptotic cells,22,23 we explored this function in the presence of affinity-purified anti-PS IgG from the HIV-1+ patients. Their activation was tested as an oxidative burst response in the presence of apoptotic target cells, including CEM lymphoblasts as a clonotypic model of CD4+ T cells24 and in several instances peripheral T lymphocytes from HIV-1+ patients. Apoptosis was experimentally induced by 18 hours of incubation with 50 µg/mL of recombinant Fas ligand (rFas-L); kindly provided by Dr S. Nagata, Osaka, Japan). Briefly, 1 × 106 adherent peripheral mononuclear cells from four HIV-1+ patients with isolated serum elevations of PS-reactive IgG were removed from Petri plastic dishes with a cell scraper and incubated with apoptotic CEM cells at 10/1 and 5/1 ratios. This preparation was supplemented with 1 mg/mL of C-ytomic substance in the presence or absence of superoxide dismutase (10 µg/mL; Sigma). The amount of superoxide released (equivalent to the number of moles of reduced Cytotoxicity) and calculated using the formula ∆ΔDNA = 2.1 × 106 mol/L·cm−1·s−1 indicated the degree of macrophage activation. The test was designed to evaluate macrophage susceptibility to activation in the presence of apoptotic cells with and without anti-PS IgG. Next, 50 µg/mL of purified anti-PS IgG from 4 HIV-1+ patients was incubated with the autologous macrophage-CEM preparations. Control experiments included standard activation of each macrophage suspension by phorbol-12-myristate-13-acetate (Sigma). Macrophage anti-PS IgG-mediated cytotoxicity (ADCC) against CEM T lymphoblasts25 was also measured. Briefly, adherent monocyte/macrophages were pretreated overnight at 37°C with 200 U/mL...
Fig 1. ELISA levels of serum IgG to CL and PS detected in 196 HIV-1' patients with no evidence of thrombophilic events, as compared with 9 SLE patients showing antiphospholipid symptoms. Numbers refer to both HIV-1' and SLE sera studied. Positive antibody titers were observed in 23 HIV-1' patients divided into groups A, B, and C in function of their positivity to CL, PS, or both. The reactivities of the remaining 173 HIV-1' sera are included in the shaded area, whose positivity limits are the mean + 3 SD of OD values obtained from 46 healthy donor sera. SD of single samples are related to outlayer values.

Statistical analysis. Sera with ELISA OD values higher than mean (M) + 3 SD of normal controls were considered positive as regards the occurrence of antiphospholipid antibody. The distribution of subdiploid DNA content in T cells from patients grouped in relation to their positive titers of IgG to CL or PS was also sought by the Wilcoxon test as a nonparametric method. In several instances, the Student's t-test was used to compare means of values between groups, whereas the Wilcoxon method was also applied for comparing results of the ADCC assay in the presence of anti-PS and polyspecific IgG.

RESULTS

Antiphospholipid reactivities in HIV-1 infection are directed to CL and PS. Positive titers of IgG with antiphospholipid specificity were observed in 23 HIV-1' patients compared with values of the control sera. Although a low reactivity to either anionic or neutral phospholipids such as sphingomyelin and phosphatidylycholine was detected in a few sera, the prevalent specificities included both CL and PS. Unlike SLE sera, levels of IgM to phospholipids in HIV-1' patients were uniformly low, although in two instances (patients no. 45 and 137) positive levels of IgM to CL were found (data not shown). These sera were unreactive to PS, thus lending further support to the specificity of the ELISA assays. Figure 1 shows the distribution of both anti-CL and anti-PS IgG. As can be seen, positivity to CL, PS, or both was used to form three groups of sera (A, B, and C) in function of their predominant specificity. The majority (11/23) were placed in group C, although in several instances (patients no. 68, 85, 98, 143, 133, and 21), a differential reactivity to the two antigens was recorded. By contrast,
selective specificity to CL or PS was observed in 7 patients from group A and in 5 from group B. The inclusion of patients within each group showed no association with any peculiar feature of HIV-1 infection, because no evidence of thrombophilia was observed in all groups. However, it is of interest that, in several SLE patients with previous vascular symptoms, the titers of pathogenic CL- and/or PS-reactive IgG (SLE patients no. 1, 4, 5, 6, 7, and 9) were lower than in selected HIV-1' sera from both A and B groups (HIV-1 patients no. 81, 83, and 16). In addition, none of the SLE sera showed isolated specificity to PS.

**Staging of HIV-1 infection and antiphospholipid reactivities.** CDC staging as well as the number of peripheral lymphocytes and CD4+ and CD8+ cells are listed in Table 1. As shown, all stages of disease from II to IVC were detected within each group, thus confirming the lack of association of antiphospholipid reactivities with a defined feature of the infection. Both variability and depletion of CD4+ T cells were equally distributed in the three groups. By contrast, we observed an apparent diversity in PI staining of T-cell preparations from individual patients. Peripheral T cells from group B were uniformly increased in their subdiploid DNA content, showing a distribution significantly different from those detected in both group A (P < .01) and group C (P < .1). In addition, the mean value of apoptotic cells (~39.8%) in group B appeared significantly higher than the corresponding value (~13.1%) observed in the control group including 14 healthy donors (P < .01). However, a wide variability of subdiploid T cells was recorded in the 8 evaluable patients from group C, although in selected instances (patients no. 21 and 45) the extent of subdiploid PI staining was as high as the increase detected in single patients from group B.

The percentages of cells expressing Fas are also reported in Table 1. The antigen detected by the IgG1 MoAb from clone UB-2 was broadly expressed in the three groups in a similar fashion to that of most of the remaining HIV-1' individuals with no serum elevations of antiphospholipid reactivities (data not shown). Fas expression was heterogeneous within each group and no direct correlation was shown with the increased levels of PI staining of individual patients from either group B or C.

**Figure 2** shows a differential pattern of spontaneous apoptosis in vitro in T cells from one representative patient

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>CDC Stage</th>
<th>Peripheral Lymphocytes (cells/µL)</th>
<th>CD4+ (cells/µL)</th>
<th>CD8+ (cells/µL)</th>
<th>T Cells With Subdiploid DNA (%) of PI staining</th>
<th>Fas+ T Cells (%)</th>
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<tr>
<td>Group A (anti-CL'-)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>42</td>
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<td>709</td>
<td>124</td>
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<td>420</td>
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<td>78.4</td>
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<td>82.3 ± 8.7</td>
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<td>Control group (14 uninfected donors)</td>
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<td>M ± SD</td>
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<td>42.3 ± 14.6</td>
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</table>

Clinical and immune parameters including the amplitude of apoptotic T-cell population, as measured by content of subdiploid DNA, and the number of Fas+ T cells in peripheral lymphocytes from 23 HIV-1' patients grouped in A, B, and C, in relation to their significant serum levels of IgG to cardiolipin (CL), phosphatidylserine (PS), or both, respectively. The median value of subdiploid DNA containing cells detected in group B was significantly higher as compared with the other groups and a cohort of 14 healthy controls (P < .1 in all instances). Several tests were not done (ND) because of severe lymphopenia.
ANTI-PS IgG AND APOPTOSIS IN HIV-1+ PATIENTS

Fig 2. Patterns of cytofluorimetric PI staining and Fas expression in peripheral T cells from 1 representative CDC II patient from group A (a; anti-CL+ patient no. 83) and 1 from group B (b; anti-PS+ patient no. 78) and electrophoretic evaluation of T-cell chromatin cleavage (c). T cells from patient no. 78 displayed a subdiploid DNA peak three times higher than in patient no. 83. This difference was confirmed by the pattern of DNA fragmentation (c). By contrast, the expression of Fas by T cells was equivalent, as in most patients in the three groups.

from group A and one from group B. Both were classified as having a CDC II infection, although the extent of subdiploid DNA-containing cells of the anti-PS+ patient no. 78 (section b) from group B was at least three times wider than the corresponding value of the anti-CL+ patient no. 83 (section a) from group A. In addition, electrophoresis of T cell DNA from patient no. 83 showed no substantial fragmentation, in contrast with the chromatin cleavage observed in that from patient no. 78 (section c). Conversely, Fas expression on T cells was almost equivalent, and no relationship was established with the increased levels of apoptosis in patients from group B.

Affinity-isolated anti-PS antibodies include predominantly IgG molecules. Serum samples from patients no. 16, 78, 127, and 174 from group B were adsorbed on Sepharose 4B columns coupled with PS from a bovine brain extract. Figure 3 shows the protein analysis of relative eluates detected by SDS-PAGE using β-mercaptoethanol as reducing agent. A prevalent IgG content was demonstrable in all samples, albeit in the presence of scanty albumin contamination and traces of IgM.

Additional experiments were also performed to isolate anti-CL antibodies from group A patients no. 42, 81, and 83, showing low values of subdiploid DNA content in their T cells. After absorption and elution of those molecules by phospholipid-coupled acrylamide columns, their antigenic specificity was retested by the described ELISA assays. No evident reactivity to PS or to other negatively charged phospholipids was observed within the three anti-CL antibody preparations, in contrast with their variable although definite affinity of IgG to CL. This control further supported the specificity of the ELISA methods for both anti-CL and anti-PS antibodies.

Apoptotic T cells induce macrophage activation in vitro.
Both CEM lymphoblasts and T lymphocytes from anti-PS+ patients no. 16, 78, 127, and 174 were used as target cells in the macrophage activation assay to measure C-cytochrome reduction. Apoptosis in lymphoid cells was induced by their incubation for 18 hr with the rFas-L. Figure 4 shows the ultrastructural morphology of CEM cells during three sequential stages of apoptosis (A, B, and C). A progressive breakage of nuclear membrane and cleavage of nuclear masses were evident in the vacuolated cytoplasm, which showed moderate electron density. However, it is interesting to stress the progressive ruffling of the plasma membrane, which was greatly altered and jumbled with evident protrusion and breakage in advanced stages. These cells were incubated with macrophages from the anti-PS+ patients in the presence of cytochrome, as described.

Figure 5 shows that, in macrophage preparations from each of these patients, incubation with apoptotic CEM cells induced weak cell activation, as measured by the increase of anion superoxide production, even in the presence of autologous anti-PS IgG. Indeed, the baseline values of macrophage activation were higher than the corresponding mean rate of the 12 normal controls, suggesting their chronic activation state in vivo. However, a similarly low effect on HIV-1+ macrophage activation was observed in parallel experiments using incubation with autologous T cells pretreated with rFas-L, whereas the amplitude of anion superoxide production remained mostly unchanged in the presence of anti-PS during the reaction. By contrast, we registered a remarkable increase of superoxide release in control macrophage preparations from normals incubated with apoptotic CEM cells. In most instances, the increase was significantly higher than the relative baseline values, whereas parallel tests including 50 μg/mL of pooled anti-PS IgG from the anti-PS+ HIV-1+ infected individuals resulted in a general absence of further macrophage activation.

Anti-PS IgG enhances ADCC function of macrophages. The cytotoxic capability of macrophages from 4 HIV-1+ patients from group B and from 12 controls was explored after the macrophages had been activated overnight with r-IFNγ (Fig 6). The target cells were 51Cr-CEM that had been preincubated with increasing concentrations of anti-PS from the same HIV-1+infected patients or with equivalent amounts of polyspecific IgG. Both HIV-1 macrophages and controls showed an increase in CEM cytotoxicity as the concentration of anti-PS or polyspecific IgG was increased (section A). However, with both HIV-1 and control macrophages, the increase over baseline became significant at ≥25 μg/mL anti-PS (section B), whereas the increase observed with polyspecific IgG did not become significant until the concentration was ≥50 μg/mL (P < .01 in both instances).

A similar, although lower increment of 51Cr release was observed in macrophage preparations from the 12 uninfected controls. In these control experiments, the target cells were preincubated with a pool of anti-PS IgG at similar concentrations than in the autologous tests of HIV-1+ patients. A progressive enhancement of mean macrophage ADCC was detected that paralleled the increasing concentrations of antibody. However, the increase of ADCC induced by the pooled anti-PS IgG in normal macrophages was consistently lower than in the 4 HIV-1+ subjects using autologous anti-PS molecules. Interestingly, the extent of ADCC induced in normal macrophages by 100 μg/mL of anti-PS molecules was significantly higher (P < .05) than the corresponding mean value obtained when polyspecific IgG were used to coat the target cells. Further experiments performed in 3 SLE patients showed that baseline cytolysis of their macrophages was little influenced in the presence of autologous anti-PS IgG. The effect was almost similar to that induced by polyspecific IgG (data not shown). These results supported the contention that anti-PS IgG from HIV-1+ patients enhances the ADCC function in effector cells, including macrophages, and that this effect is particularly evident in experiments using the antibody with autologous cells. By contrast, anti-PS molecules related to the APS in SLE patients are not involved in the ADCC mechanisms investigated in the present study.

Effects of PS and its structural isomers on macrophage functions. Because PS has been postulated as a potential receptor of apoptotic cells that activates the macrophages by its polar head groups, 35 control experiments were aimed at investigating both activation and ADCC function in presence of the PS (phosphatidyl-L-serine) preparation used as antigen in our ELISA, in parallel with glycerophosphoryl-L-serine, phospho-L-serine, and phospho-D-serine. To this purpose, macrophages from patients of groups A and B, in parallel with similar preparations from uninfected individuals, were incubated with these aqueous phospholipids for 30 minutes and then tested in both activation and ADCC assays. Figure 7 shows that preincubation of macrophages with PS and its L-isomers was able to enhance the basic cell activation by increasing the release of anion superoxide in a dose-dependent manner. A comparable enhancement was also observed in the 9 macrophage preparations obtained from the uninfected controls (data not shown), suggesting that the specific recognition of PS and its soluble derivatives by macrophages was an activation-inducing event. On the contrary, macrophage preparations from both patients and controls were not significantly influenced by the phospho-D-serine because their basic superoxide production remained unaffected.

Concerning the influence on ADCC function of macro-
Fig 4. Ultrastructural morphology of apoptosis induced in CEM cells by their incubation with Fas-L. (A) Initial step showing a cell with dark cytoplasm and profiles of rough reticulum (arrow) and partially condensed nucleus with nuclear membrane blebs (arrowhead). (B) Intermediate step showing a cell with a ruffled plasma membrane with a vacuolized cytoplasm and extensive breakage of nuclear membrane. (C) Terminal step showing a cell characterized by the presence of cleaved nuclear masses in a moderately electron dense cytoplasm. The plasma membrane is disrupted with evident protrusions. Original magnifications for (A) through (C) x 15,000.
phages preincubation with phospholipids, no substantial effect was observed on the extent of cytolytic properties to CEM cells. The macrophage killing was apparently unchanged in patients from group B using autologous PS-reactive IgG even in the presence of increasing molar concentrations (mol/L\(^{-10}\) to mol/L\(^{-2}\)) of the phospholipids, with no difference between L- and D-isomers. Similarly, in the 5 patients from group A and in control macrophage suspensions using polyspecific IgG to coat the target cells, PS and its isomers were unable to influence the effector function (data not shown). These results were in line with previous observations suggesting that PS and its L-isomers may interact with some macrophage functions, although they are generally uneffective in Fc-mediated reactions, including phagocytosis.\(^3\)

**DISCUSSION**

Our study focuses on the significance of autoantibodies to phospholipid antigens during HIV-1 infection. By using a panel of antigenic specificities, including both anionic and neutral phospholipids, we identified CL and/or PS as the predominant targets of those molecules in about 12% of HIV-1\(^+\) patients. Serum elevations of IgG to CL, PS, or both were not related to any particular clinical feature or to thrombophilic events. However, in patients with isolated serum anti-PS IgG, fresh T cells showed a uniform pattern
of accelerated apoptosis compared with those from patients presenting anti-CL IgG alone. Flow cytfluorimetry showed that levels of apoptosis in these cells were unrelated to the detection of Fas, the apoptosis-inducing cell activation marker, which was overexpressed in virtually all patients. Macrophages from HIV-1+ patients showing increased T-cell apoptosis were little activated by the PS exposed by both apoptotic CEM cells and autologous T lymphocytes because of their chronic immune activation, whereas target CEM cells coated with increasing amounts of purified anti-PS IgG from 4 HIV-1+ subjects greatly enhanced the ADCC function in these macrophages. This increment of lytic properties was remarkable in tests using macrophages in combination with autologous anti-PS IgG, whereas polyreactive IgG were unable to induce equivalent cytotoxicity. Control macrophage preparations from healthy donors also improved their ADCC in the presence of anti-PS IgG, although to a lesser extent. Therefore, serum elevations of anti-PS IgG in HIV-1+ patients with no evidence of thrombophiliic phenomena could be related to their increased T-cell apoptosis, because PS exhibited by disrupted cell membranes is immunogenic and is also involved in triggering the removal of these cells by macrophages.

Previous studies exploring autoimmunity in HIV-1 infection have reported antibodies to T-cell membrane antigens, including protein and lipid structures, as potentially pathogenic, because their levels correlate with progression of CD4+ T-cell depletion. Indeed, the autoantibodies to phospholipids described in this disease are apparently unrelated to thrombophiliac because of their inability to react with β2GPI cofactor. By contrast, a particular region, namely C284KNKEKKC294 located on the fifth domain of β2GPI, has been recently identified as the molecular target of antiphospholipid antibodies occurring in autoimmunity. In agreement with these observations, increased serum IgG antibodies to CL, PS, or both in our 23 patients were not associated with susceptibility to thrombosis or with a general pattern of severe lymphopenia (CD4+ cells<100 µL). These data suggest that antiphospholipid reactivities in HIV-1 infection do not exert a clear-cut pathogenic role, as reported in APS.

An interesting result of this study was the correlation of anti-PS reactivities with increased levels of T-cell apoptosis. Remarkably, patients from both the B and the C groups showing enhanced subdiploid DNA content produced considerable amounts of anti-PS IgG. Anionic phospholipids, such as PS and phosphatidylethanolamine, are regular constituents of the inner leaflet of the lymphocyte membrane and, because of their asymmetric distribution, are overexpressed in patients with the high level of cell breaking that occurs in apoptosis. In particular, the normal phospholipid packing of plasma membranes is altered during the early stages of apoptosis, and exposure of intact phospholipid head groups could lead to immunization to PS. In keeping with this interpretation, anti-CL patients from group A with no evidence of antibodies to anionic phospholipids showed moderate to low levels of T-cell apoptosis, as shown by both PI staining and minimal DNA fragmentation. To our knowledge, no evidence is available to suggest that anti-CL production is somehow related to breaking of the mitochondria inner leaflet that may result during apoptosis. On the other hand, apoptosis of lymphocytes is accelerated in SLE patients, in which a mechanism of driving antiphospholipid antibodies may also occur, although the difference in cofactor activity in HIV-1+ subjects could explain the different pathogenicity of their molecules.

No substantial correlation was found between T-cell subdiploid DNA content and Fas expression. Although this antigen is able to induce programmed cell death upon ligation with the Fas-L, its overexpression in most of our HIV-1+ patients is perhaps sustained by the chronically active state of T cells, which does not necessarily reflect their tendency to undergo apoptosis. Evidence for the increased levels of Fas in HIV-1 infection has recently been presented. A striking finding of our investigation concerns the role of anti-PS IgG in HIV-1+ patients with increased T-cell apoptosis. Macrophages have been reported to recognize apoptotic cells by a number of receptors, including carboxy-
drates and vitronectin, whereas thrombospondin and PS are suspected of cooperation in phagocytosis. Recent studies also have postulated that phagocytosis and digestion of PS by macrophages may be the signal for these cells to start removing apoptotic lymphocytes via the induction of a specific PS-receptor. In our experiments, we found that apoptotic cells induced a moderate activation of macrophages from the anti-PS subjects, as measured by the small increase in the anion superoxide release. A similar, although slightly higher effect was noted using PS and its L-isomers in parallel tests (Fig 7). No variations were shown with the D-isomer of the phospholipid, suggesting that its polar head groups are weakly sensitive to PS activation as compared with normal macrophages in the presence of both apoptotic CEM cells and PS. Support for this interpretation comes from previous work describing the persistently active state of macrophages during acquired immunodeficiency syndrome.

Anti-PS IgG significantly improved the ADCC function of macrophages from HIV-1* subjects to human T cells, as shown by CEM lymphoblasts. However, the cytolyis was not influenced by PS-preincubation of macrophages because the phospholipid induced a moderate activation, with no effect on the Fc-mediated function. Although tested in a small number of patients, polyspecific IgG were unable to increase the macrophage cytotoxicity to the extent observed with anti-PS IgG. This effect was also reproducible, although to a lesser extent, in uninfected monocyte/macrophages from healthy controls, thus supporting the specificity of anti-PS IgG to the T-cell membrane. This result strongly points to a functional role of such molecules as specific PS receptors in mediating phagocytosis of apoptotic cells by macrophages. A substantial ADCC to HIV-1* coated CEM has also been assigned to macrophages from infected individuals and is suspected of direct participation in the destruction of lymphocytes in vivo that replicate the virus. Recent studies from Fauci and Rosenberg have shown that effector functions of monocyte/macrophages during HIV infection may on binding to phospholipid. J Rheumatol 21:94, 1994


13. McNeil HP, Simpson RJ, Chesterman CN, Krilis SA: Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid binding inhibitor of coagulation: \( \beta_2 \)-glycoprotein I (apolipoprotein H). Proc Natl Acad Sci USA 87:4120, 1990


ANTI-PS IgG AND APOPTOSIS IN HIV-1+ PATIENTS


Antiphosphatidylserine antibodies in human immunodeficiency virus-1 patients with evidence of T-cell apoptosis and mediate antibody-dependent cellular cytotoxicity [see comments]

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