Cross-linking of Fas By Antibodies to a Peculiar Domain of gp120 V3 Loop Can Enhance T Cell Apoptosis in HIV-1–infected Patients

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Summary

Previous studies have demonstrated that T cell–reactive antibodies in HIV-1 infection contribute to lymphocyte depletion by cytotoxicity that involves differential membrane targets, such as the 43.5-kD receptor on CEM cells. Here, we show that these antibodies bind Fas as result of a molecular mimicry of the gp120. Both flow cytometry and immunoblotting using the human Fas-transfected mouse WC8 lymphoma revealed positive binding of immunoglobulin G from several patients to a 43.8-kD membrane receptor that also reacts with the CH11 anti-Fas monoclonal antibody. Specificity to Fas was further confirmed to chimeric recombinant human Fas-Fc by ELISA, whereas overlapping peptide mapping of a Fas domain (VEINCTR–N) shared by gp120 V3 loop demonstrated a predominant affinity to the full-length 10-mer peptide. Four anti-Fas affinity preparations greatly increased the subdiploid DNA peak of CEM cells similar to agonist ligands of Fas. In addition, anti-Fas immunoglobulin G strongly inhibited the [^{3}H]thymidine uptake of CEM cells in proliferative assays, inducing a suppression as high as provoked by both CH11 mAb and recombinant human Fas ligand. Since anti-Fas were reactive to gp120, it is conceivable that antibodies binding that domain within the V3 region are effective cross-linkers of Fas and increase apoptosis in peripheral T cells. These results suggest that autologous stimulation of the Fas pathway, rather than of lymphocytotoxic antibodies, may aggravate lymphopenia in a number of HIV-1$^+$ subjects.

Activation–induced apoptosis of antigen-primed CD4$^+$ and CD8$^+$ T lymphocytes has been well documented in peripheral cell cultures from HIV-1$^+$ patients, and has been postulated as a mechanism that is primarily involved in the immunopathogenesis of AIDS (1–4). Chronic immune activation of those cells is indeed thought to significantly enhance their susceptibility to apoptosis (5–7), whereas the subsequent antigenic stimuli may drive the death program to completion (8, 9).

Apoptosis is a signal-dependent suicidal process that is regulated in part by Fas or Apo1/CD95 (10–13), namely a 45-kD membrane receptor that transduces the death signal to its intracellular pathway after ligation with a natural ligand (Fas-L)[1] active in trimeric form (14). Fas-mediated apoptosis makes a physiological contribution within the immune system in suppression of autoreactive T cell clones in the thymus (15), as well as in the regulation of its normal response (16) and cytotoxicity (17). Moreover, the mutated expression of genes encoding either Fas or Fas-L may afford resistance to apoptosis in mature T cells from *lpr* (18) or *gld* (19) mice, respectively. Both phenotype strains suffer from a lymphoproliferative disorder that leads to autoimmune syndromes (20) that are highly similar to SLE. In this disorder, the abnormal expression of soluble Fas (21) is associated with an increased rate of apoptosis in peripheral lymphocytes (22), suggesting the involvement of Fas deregulation in driving the SLE autoreactivity. Further evidence of the role of apoptosis in human autoimmunity has been provided by the demonstration that synovial cells from patients with rheumatoid arthritis are highly subject to death by Fas overexpression (23).

Autoimmunity (24–26) and Fas overexpression (27, 28) have also been described during the HIV-1 infection, even though no linkage between these conditions has been documented so far. We have recently reported that the abnormal overexpression of Fas by T cells in advanced HIV-1 infection correlates with the high responsiveness of the receptor

to its extracellular binding, even when using a monomeric ligand, as provided by mouse IgG1 mAb from the UB-2 clone (29). This finding emphasizes the hypothesis that Fas is somehow involved in the increased in vitro apoptosis of peripheral cells from HIV-1–infected individuals, and that the Fas pathway may play a pathogenic role by aggravating the T cell lymphopenia that is related to the progression of their disease. Aggravation has also been associated to autoimmune phenomena (30–32), and we have illustrated the lymphopenic effect of T cell–reactive autoantibodies in a considerable number of patients, since their serum levels apparently parallel the progression of $CD4^+$ lymphocyte decline (33, 34). As a result of their ability to react with a 43.5-kD marker located on $CD4^+$ clonotypic lymphoblasts of the CEM line, these molecules were found to be powerful inducers of cytolysis in complement-mediated cytotoxicity, when either peripheral T lymphocytes or CEM were used as the cell target (35).

In the present study, we provide evidence that in most patients with variable serum titers of T cell binding antibodies, the molecular target of these reactivities may include Fas. Therefore, activation of the Fas pathway by autoantibodies is at least partly responsible for the increased apoptosis that contributes to T cell depletion because of the receptor's high sensitivity. Since antibodies to Fas are also reactive to a specific epitope shared by the gp120 V3 loop of HIV-1, however, it is conceivable that antibodies primarily elicited to neutralize the virus may cross-react with Fas and activate its function through molecular mimicry. The increment of apoptosis by autologous Fas stimulation rather than lymphocytotoxic antibodies may therefore account for the aggravation of lymphopenia in patients whose HIV-1–neutralizing IgG may include specificity to the viral domain shared by Fas.

Materials and Methods

Study Subjects. Peripheral blood samples were obtained from 74 HIV-1$^+$ individuals at different Center for Disease Control and Prevention (CDC) stages (36) who were hospitalized at the Department of Internal Medicine and Oncology of the University of Bari. The study group included 55 former intravenous drug users, 10 homosexual men who admitted to occasional intravenous drug use, 7 heterosexual women with no history of intravenous drug use, and 2 hemophiliac men. 67 uninfected donors provided control blood samples. All subjects gave their informed consent, and the study was approved by the ethical committee of the University of Bari. In most instances, sera were aliquoted and stored at −80°C until use.

Detection of T cell–reactive Antibodies. Sera were screened for the presence of antibodies to T cell membrane by an optimized ELISA, using the CEM plasma membrane extract as antigen (34). Briefly, the 96 flat-well microtiter plates were coated overnight with 10 μg/ml of lysed membrane and were further incubated for 3 h with sera diluted 1/200. The plates were then supplemented with a goat peroxidase–conjugated anti–human IgG and in parallel with anti-IgM antiserum, and were subsequently read in a micro-ELISA reader (Titertek; Flow Laboratories, Irvine, Scotland) after their OPD (*O*-phenylenediamine) color development. Each

sample was tested in triplicate, and each test included the control group. Patients' sera with absorbance higher than the mean + 3 SD of control values in each isotype test were considered positive for antibody detection.

Anti-Fas Specificity of T Cell–reactive Antibodies. A number of different approaches were adopted to investigate the affinity of CEM-reactive IgG and IgM to Fas. First, we used indirect immunofluorescence analysis with the murine WC8 lymphoma cell line transfected with human Fas as substrate, and its Fas-negative mutant WR19L as a control (37). Preliminary experiments included the preparation of cytocentrifuged cell suspensions from both cell lines, followed by incubation with sera and subsequent treatment with the FITC-conjugated rabbit anti–human IgG and IgM. The slides were then evaluated under a UV microscope. Furthermore, the sera were tested in a flow cytometry assay, using a FACScan® (Becton Dickinson & Co., Mountain View, CA). 3 × 10^5 cells from each line were separately incubated with 50 μl of serum diluted 1/80, and then washed and further treated with the FITC-conjugated antiserum to each class of antibody. Control cell preparations were incubated with anti-T negative sera derived from both patients and controls, whereas an additional check included the treatment of cell suspensions with polyspecific human IgG from commercial preparations, as well as the incubation of cell preparations with only the labeled antisera.

The second approach included immunoblotting analysis, using the membrane lysate from both WC8 and WR19L cell lines as a substrate. Purified plasma membrane extract was obtained by nonionic detergent lysis followed by sequential centrifugations at 400 and 100,000g for clearance of nuclei, as well as the removal of the cytoskeletal component and insolubilized membrane, respectively. The membranes were then run in PAGE and further electroblotted to nitrocellulose. Strips cut from the nitrocellulose sheet were incubated with serum samples diluted 1/50, and were then washed and treated with alkaline phosphatase–labeled antisera to IgG and IgM until the nitro blue tetrazolium chromogenic staining completed the test. The molecular weights of the bands detected by the enzyme-labeled antiserum were evaluated by a standard migration curve obtained in each test by rainbow-colored markers (Amersham International, Bucks, UK).

An evaluation was also made of the specificity detected in both flow cytometry and immunoblotting by an additional ELISA, using the recombinant human Fas (rFas) as antigen. This substrate is a chimeric protein product, since it is conjugated to the Fc portion of human IgG to optimize the biological stability (12). The ELISA was then installed by coating the plates with 10 μg/ml of antigen and subsequent incubation of sera diluted 1/100 for 5 h. Equivalent concentrations of mouse mAb anti–human κ and λ light chains (Immunotech, Marseille, France) were added to the wells, and the test was completed using a goat peroxidase–conjugated anti–mouse IgG. Parallel control ELISA plates coated with Fc fragment alone were prepared with the same mAbs and labeled antiserum to calculate the basic reactivity to Fc potentially present in anti-rFas specificities. The anti-Fc ELISA, including the measurement of IgM rheumatoid factor (RF) levels, was performed concurrently to the anti-rFas assay, whose values were referred to as the relative anti-Fc reactivities. Four RF-positive sera from HIV-1–uninfected patients were also tested as controls.

Evaluation of Specificity to Sequential Peptides of Fas. Sera with positive anti-rFas–reactivity values were also investigated to define their molecular specificity to peculiar epitopes of the Fas molecule by peptides produced by an automated synthesizer (Biochrom Biolynx 4170; LKB, Sweden) by Fmoc chemistry, according to the manufacturer's protocols. Thus, we installed a new

ELISA using as substrate 10-mer overlapping peptides resembling the linear sequence of Fas between 90 and 117 amino acid (aa) positions. Conformity of peptide binding to plates was assessed by parallel tests using KLH as a carrier protein (38) with respect to uncoupled peptides. Since all 10-mer peptides appeared to bind the ELISA solid phase equally efficiently as in their glutaraldehyde–KLH-coupled form, the plates were coated with uncoupled antigen to prevent any cross-reactivity with KLH (39). Peptides included the 8-aa stretch (VEINCTR–N; position: 99–108 of Fas) structurally shared by the third N-glycosylation site of the gp120 V3 loop (40). This sequence is invariably expressed by the HIV-1IIIB strain during both primary infection and advanced disease (41), whereas a single substitution of Glu (E) by Gln (Q) occurs within the domain in the V3 loop from other virus subgroups, including HIV-1MN and HIV-1RF (42). Therefore, we used the 17 overlapping peptides of VEINCTR–N to test its progressive aa antigenicity, whereas the VEINCTRPNN (IIIB strain) and VQINCTRPNY (MN strain) single peptides were also coated in parallel plates. This control was addressed to evaluate a potential prevalence in specific HIV-1 strain infection. After incubation, the plates were supplemented with sera, and the assay was completed as a standard ELISA.

Isolation of Anti-Fas Antibodies. Sequential affinity chromatography was used to purify antibodies to Fas from sera 3, 41, 54, and 59, which displayed the highest reactivity to the antigen in the previous ELISAs. Purified anti-Fas preparations were obtained by recycling absorption of 7–12 ml of serum on Sepharose 4B columns coupled with the WC8 membrane lysate. The absorbed material was then eluted by glycine-HCl acidic buffer (pH 2.8) and promptly adjusted to neutrality by the addition of Tris base (pH 8.3). Next, the eluates were repeatedly passed through WR19L lysate affinity columns to absorb possible unrelated specificities on the Fas–negative parental cell line. In two instances (sera 54 and 59), we optimized these purification experiments by absorption on VEINCTR-coupled Sepharose columns. The protein content of eluted fractions was assessed by SDS-PAGE, whereas aliquots of these affinity-isolated anti-Fas antibodies were dialyzed against PBS and sterilized by filtration for functional studies. In addition, their ability to bind to HIV-1 glycoproteins was also tested by a commercial immunoblotting kit (HIV Blot 2.2; Diagnostic Biotechnology Ltd., Singapore). IgG from HIV-1+ sera unreactive to Fas (Nos. 9 and 34 from group B) were purified by DEAE- cellulose filtration to provide adequate controls to anti-Fas specificities from group A patients.

Functional Tests of Anti-Fas Antibodies. The ability of purified anti-Fas antibodies to induce apoptosis in vitro was investigated by using both CEM cells and peripheral T lymphocytes as the target in separate tests. To this purpose, 5×10^5 CEM or T cells, purified by double-gradient centrifugation from patients 3, 41, and 54, were incubated for 20 h in the presence of 10 ng/ml IL-2 in 24-well plates coated with variable concentrations (0.1–10 μg/ml) of anti-Fas molecules (16). The cells were then washed and stained by the propidium iodide (PI) method to measure the extent of subdiploid DNA content as a degree of apoptosis (43). [³H]thymidine uptake was also measured to determine the inhibition of cell proliferation after anti-Fas incubation (29). The negative controls in both experiments were IgG from anti-CEM negative patients (Nos. 9 and 34), while the positive control included incubation of parallel cell preparations, with the CH11 mAb and the rFas-L at amounts similar to those adopted for anti-Fas antibodies. Control experiments investigated the possible inhibition of anti-Fas antibodies by the VEINCTR peptide and the rgp120 (Intracel, London, UK). To this purpose, anti-Fas molecules were

separately preincubated with variable amounts (up to 7.5 μg/ml of VEINCTR and 1 μg/ml of rgp120) for 3 h and then used in functional tests.

Statistical Analysis. Sera with ELISA OD values higher than mean+3 SD of controls were considered positive. The distribution of subdiploid DNA content in peripheral T cells from patients showing ELISA positivities was also sought by the Wilcoxon test as a nonparametric method. In several instances, Student's *t* test was also used to compare means from different samples within groups.

Results

T Cell–reactive Antibodies in HIV-1+ Patients. Fig. 1 shows the distribution of both IgG and IgM reactivities to the CEM plasma membrane. In line with our previous observations (34), we found that the majority of isolated specificities were of the IgG isotype. This pattern was predominant in seven patients, whereas four showed elevations of IgM only. In addition, a subgroup of five patients was positive for both IgG and IgM, though the OD values of IgG reac-
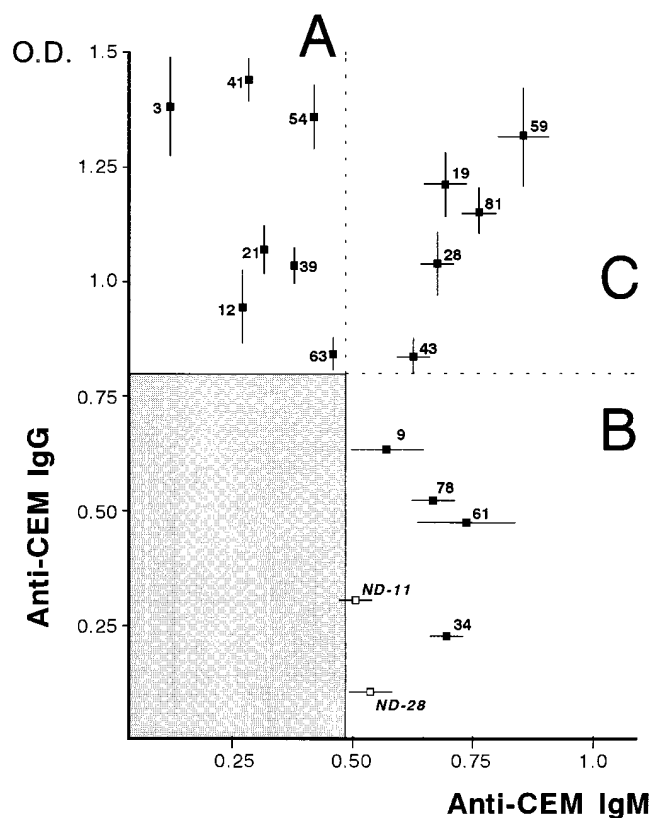

Figure 1. Distribution of serum IgG and IgM reactivities by ELISA to CEM cell membrane in 74 HIV-1–infected individuals at different CDC stages. Positive antibody titers were observed in 16 patients divided into groups A, B, and C in relation to serological elevations of IgG, IgM, or both. Numbers refer to both HIV-1+ patients and two normal donors (*ND*) showing weak yet definite IgM elevations to the membrane. The reactivities of the remaining 58 HIV-1+ sera are included in the shaded area whose positivity limits are the mean + 3 SD of OD values obtained from 67 uninfected controls. SD of single samples are related to outlayer values.
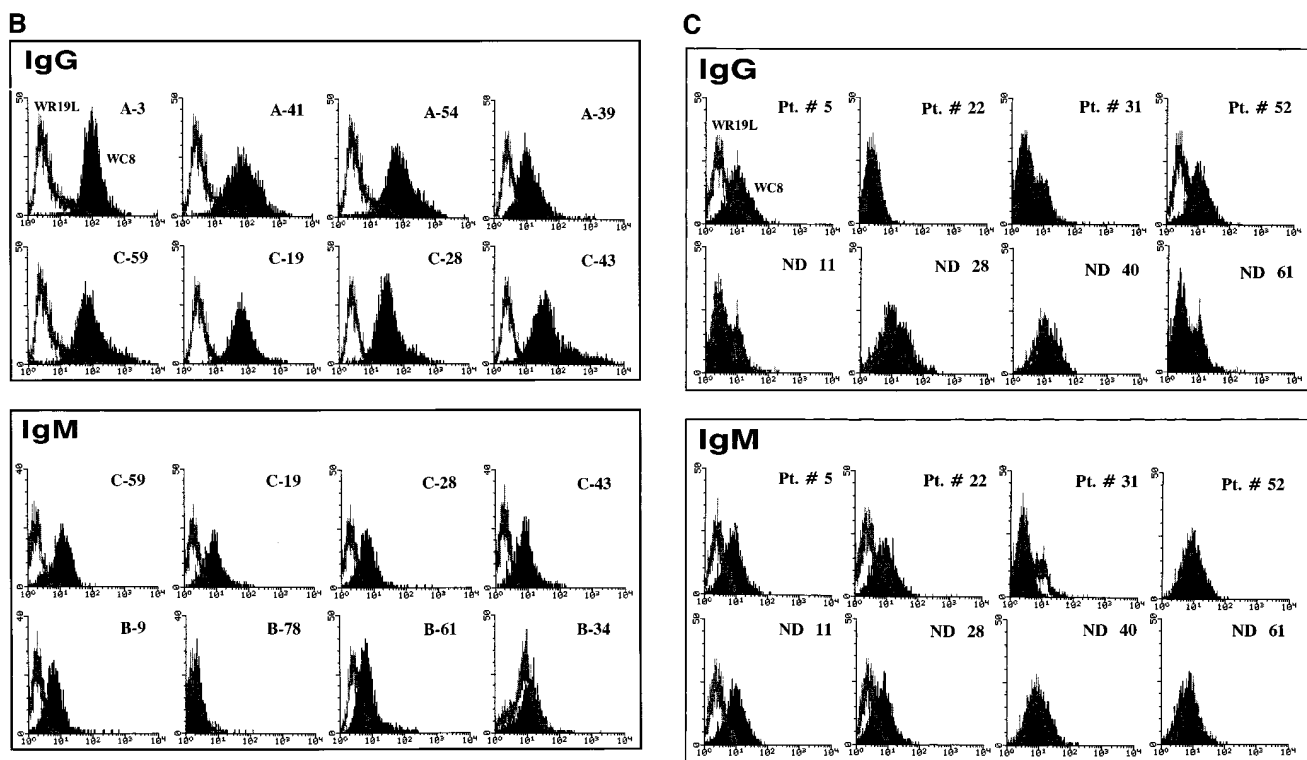
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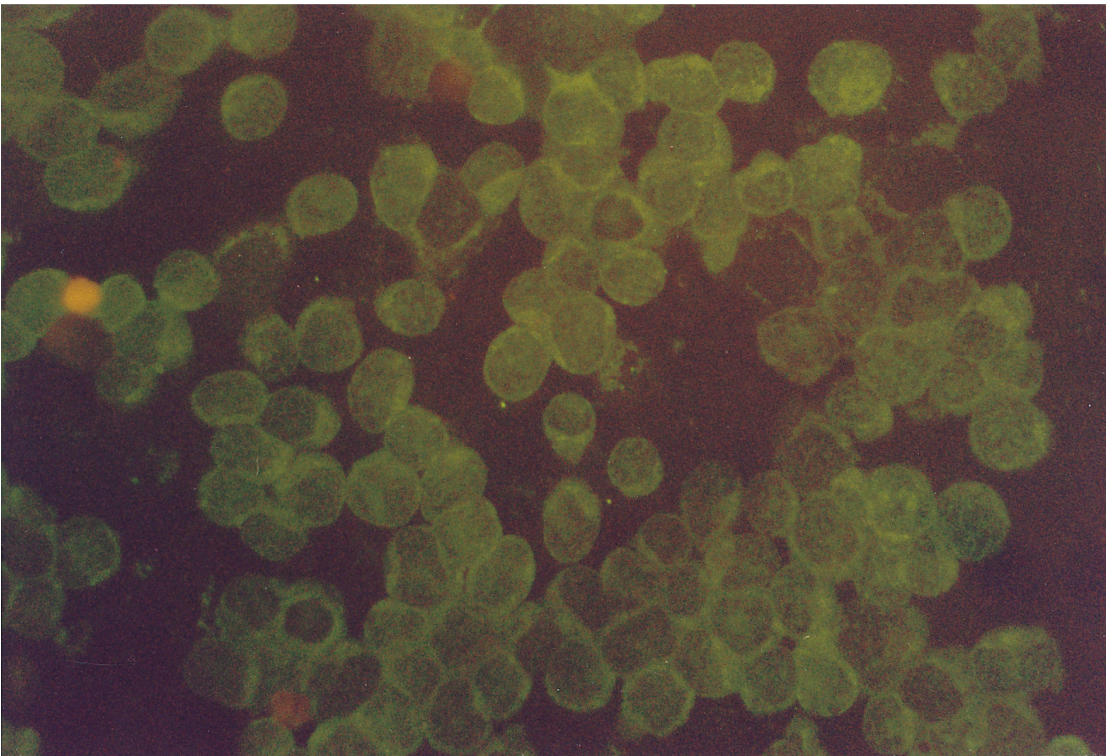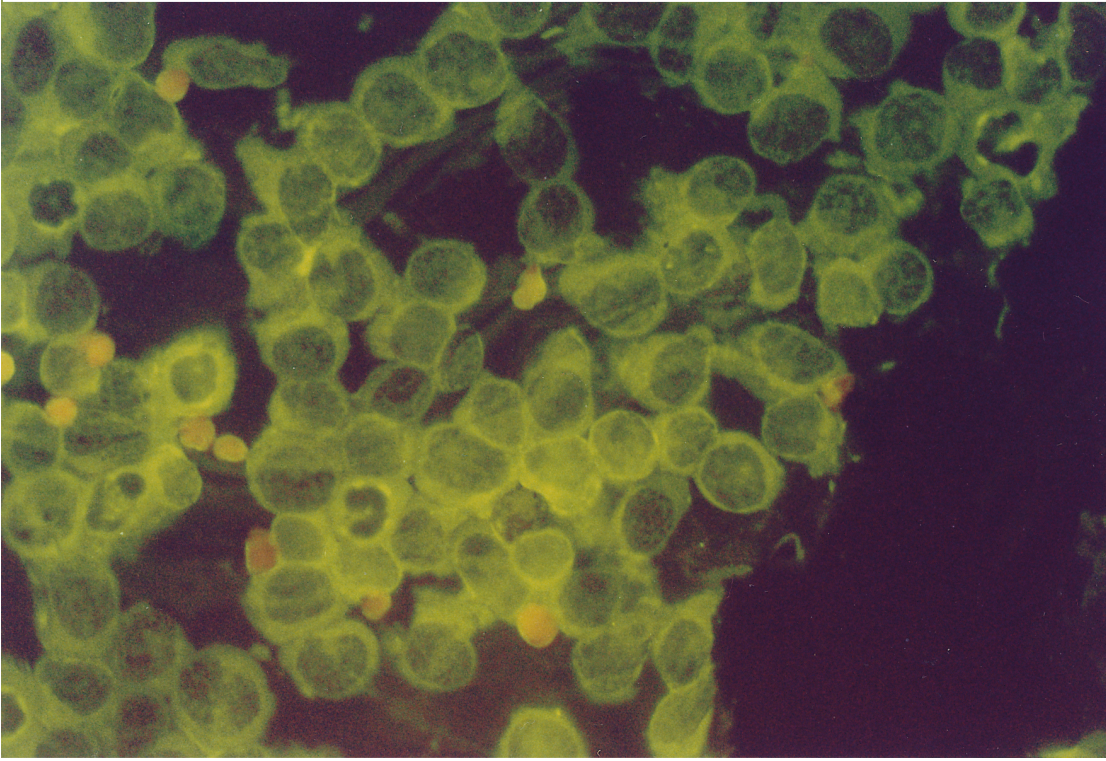
Figure 2. (*A*) Indirect immunofluorescence assay using as substrate the human Fas transfected mouse WC8 lymphoma (*top*) and its Fas–negative mutant WR19L (*bottom*). Representative fluorescence pattern from patient 41, whose serum IgG were reactive to the WC8 membrane with no evidence of specificity to Fas-negative cells. (*B*) Flow cytometry analysis of Fas reactivity of T cell–reactive IgG and IgM detected by ELISA in HIV-1–infected individuals. Positive binding of IgG to the antigen occurred in virtually all patients included in groups A and C, showing high fluorescence intensity to WC8 cell, as compared to its Fas–negative parental line (*WR19L*). By contrast, the reactivity of IgM to WC8 was almost undetectable, since the fluorescence intensity was less than one decade of dynamic range of the Fas–negative control. (*C*) Cytofluorimetric control tests of patients unreactive to CEM membrane and normal donors (*ND*). Representative patterns from both groups consistently showed lack of IgG and IgM reactivity to the human Fas–transfected cell line (*WC8*). *Pt.*, patient.

tivity were constantly higher within each serum. Therefore, we arbitrarily placed these patients in groups A, B, and C, depending on their serum positivities of IgG, IgM, or both. Two control subjects also displayed a moderate, though definite IgM positivity.

CEM Membrane–reactive Antibodies and Specificity to Fas. Preliminary experiments addressed the question whether the T cell–reactive antibodies detected by ELISA were able to bind Fas. Sera from the A, B, and C groups were first investigated by both immunofluorescence and immunoblotting analyses, using the human Fas-transfected WC8 lymphoblasts as the substrate. Results were related to the anti-CEM ELISA, and are expressed in Table 1 along with relative values of peripheral counts of T cells and their subdiploid DNA extent. As shown, although small numbers of comparable patients were available, serum positivities to T cell membranes were in apparent concordance using differential methods, whereas the distribution of the subdiploid DNA extent of group A was apparently different ($P <0.05$) from that of group B. By contrast, CD4$^+$ T cell lymphopenia was always related to the clinical stage, though the lowest values were noted in selected instances from groups A and C. Representative patterns of positive immunofluorescence tests are illustrated in Fig. 2, where the upper section of A

shows the fluorescence obtained by incubating serum 41 with WC8 and further treatment with FITC–anti–human IgG. This pattern was not evident when the same serum was incubated with the Fas-negative parental cell line WR19L (lower section), suggesting a relative specificity of IgG to the transfected antigen. A similar profile of positivity was also observed in the other sera from group A and, to a variable extent, in sera from group C. Conversely, sera from group B constantly showed a pattern of weak fluorescence in tests using FITC–anti-IgM as the related antiserum (data not shown). Similar evidence of feeble reactivity to WC8 was also detected in IgM from the two controls (ND-11 and ND-28) with positive binding to CEM in ELISA.

Cytofluorimetric analysis for anti-Fas is presented in Fig. 2 *B*, which shows a differential pattern of reactivity within the sera tested. While no binding to WR19L was observed in all instances, a variable reactivity to WC8 was recorded in relation to the isotype tested. IgG in sera from groups A and C were highly reactive to the Fas$^+$ cell line, albeit in the presence of slight variations of fluorescence intensity (even more than one decade of dynamic range than WR19L) within different samples. By contrast, IgM in sera from groups B and C uniformly showed a weak to unde-

Table 1. *Clinical and Immunity Parameters of 16 HIV-1$^+$ Patients*

Sera		CDC Stage	Peripheral lymphocytes	CD4$^+$	CD8$^+$	Peripheral T cells with subdiploid DNA (percentage of PI staining)	Anti-Fas IgG FC*	Anti-Fas IgG WB‡	Anti-Fas IgM FC	Anti-Fas IgM WB
			cells/μl	*cells/μl*	*cells/μl*					
Patients										
Group A: No.	3	IV	690	122	467	43.5	+	+	−	−
	12	III	809	212	494	39.6	+	+	−	−
	21	II	1,390	381	724	32.5	+	+	±	−
	39	IV	1,018	172	715	40.8	+	+	−	−
	41	IVC	595	47	380	46.5	+	+	±	−
	54	IV	270	8	165	38.0	+	+	−	−
	63	III	780	190	515	40.8	+	+	−	−
Group B: No.	9	IV	872	73	487	31.5	−	−	±	−
	34	II	2,907	705	1,708	21.9	−	−	±	−
	61	IV	965	153	565	27.5	−	−	−	−
	78	III	706	224	503	33.0	±	−	−	−
Group C: No.	19	III	954	288	527	37.5	+	+	±	−
	28	IV	550	107	308	41.5	+	−	−	−
	43	III	1,615	317	980	29.5	+	−	±	−
	59	IVC	675	28	426	43.2	+	+	±	−
	81	III	1,136	209	690	40.5	+	±	−	−
Uninfected controls										
ND-11		—	2,790	1,786	792	9.7	−	−	±	−
ND-28		—	4,170	2,045	1,215	11.2	−	−	−	−

Clinical and immunity parameters included the amplitude of the apoptotic T cell population, as measured by the content of subdiploid DNA. The peripheral lymphocytes from the 16 HIV-1$^+$ patients showed serological reactivities to CEM membrane antigens and to human Fas–transfected mouse WC8 lymphoma cells. Data from two uninfected controls were presented in relation to their positivity detected in ELISA to CEM membrane.
*FC, flow cytometry. The positivity was related to a fluorescence intensity higher than one decade of dynamic range of the relative control.
‡WB, western blot. Positivity detection of a Fas–related band indicated as +.

tectable affinity to the WC8 membrane, since in most instances, their extent of fluorescence intensity was similar to WR19L cells. This difference supported the contention that IgG rather than IgM are highly reactive molecules to Fas. Control patterns from a number of CEM-unreactive patients and uninfected donors are shown in Fig. 2 *C*. No evidence of positive binding to WC8 cells was demonstrated in both IgG and IgM from these control subjects, although in selected instances, such as in normal donor 28, the low fluorescence of IgM to the Fas$^+$ membrane paralleled the moderate ELISA elevation.

Anti-Fas specificities were also determined by immunoblotting, using both WC8- and WR19L-purified membranes as substrates (Fig. 3). Once again, sera from groups A and C were reactive to a few low molecular weight bands on both lysates (upper section). IgG in most sera, however, showed a variable reactivity to a defined band that was evident only on the WC8 plasma membrane. The target antigen was apparently related to Fas, since it was re-

active to the commercial mAb from clone CH11 and exhibited a similar molecular mass (∼43.8 kD) in our assay. Although more specific than immunofluorescence, however, the binding to blotted Fas by IgG from group C patients was of variable intensity, in contrast with the undetectable IgM reactivity (data not shown). The lower section of Fig. 3 shows the immunoblotting pattern of CEM-unreactive patients and healthy controls. These experiments confirmed the absence of definite specificities to Fas in sera from both control groups.

Binding to Fas was further investigated with an optimized ELISA, using the rFas-Fc as antigen. The amplitude of reactivity to Fas compared with the concurrent activity to Fc is shown in Fig. 4. A broad variation in the ability to bind the antigen was observed. High OD values were detected in group A, particularly in patients 3, 41, and 54, suggesting a remarkable correlation with the anti-CEM ELISA, whereas the total activity to rFas-Fc was apparently modest in group B and still substantial in group C. How-
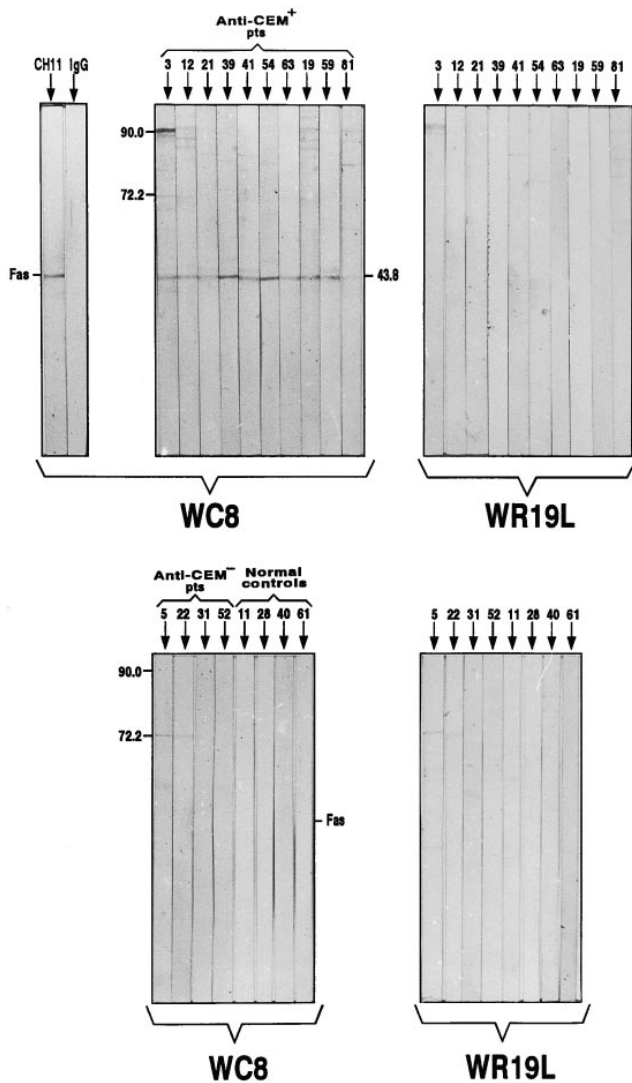
Figure 3. Immunoblotting of anti-Fas specificities in sera from HIV-1⁺ patients and uninfected controls. Although in the presence of undefined bands related to weak reactivity with other antigens, the majority of IgG binding the WC8 cells in the flow cytometry assay were reactive to a 43.8-kD antigen, that was also detected by the anti-Fas mAb from clone CH11. Conversely, no evidence of reactivity was observed when using the Fas–negative membrane (*WR19L*) as control (*upper section*). Sera from patients unreactive to the CEM membrane and uninfected donors showed no evidence of binding to the transfected antigen (*lower section*). Numbers are related to patients and controls. IgG indicate the negative control provided by 0.5 mg/ml of polyspecific IgG incubated with the WC8-blotted membrane.

ever, specificity to rFas was predominant in most sera from group A when compared to the single reactivities to Fc. Both total and IgM specificities to Fc were considerably expressed in sera from group C, suggesting that, in contrast with the potential involvement of RFs in increasing positivities to the T cell membrane, a clear-cut specificity to Fas was definitely present. Conversely, 39 patients unreactive to the CEM membrane showed no similar elevations of anti-Fas specificities in their sera, whereas, as expected, control RF-positive sera were highly reactive to Fc both in

its natural and chimeric forms. In addition, the uninfected controls were constantly unreactive to the antigen (data not shown).

Anti-Fas IgG Show Predominant Reactivity to VEINCTR–N Domain. The next set of experiments investigated whether anti-Fas IgG in sera from groups A and C were reactive to the aa 99–108 stretch of Fas, which is also shared by the V3 loop of gp120. To this purpose, the ELISA was prepared with overlapping peptides of this domain and those resembling the V3-related homology sequence from both HIV-1IIIB and HIV-1MN as the antigen. The results obtained with the most reactive sera (Nos. 3, 41, and 54) showed significant binding starting with peptide [96]GLEVEINCTR[105] (Fig. 5 *A*) and stable until [100]EINCTRTQNT[109], supporting the evidence that the shared stretch alone could constitute the reactive epitope. Interestingly, construction of the peptide with N (Asn) in aa position 108 induced a small but definite increment of reactivity in IgG from serum 54. This was consistent with a potential contribution of Asn to a conformational rather than a linear domain reacting with those antibodies. Moderate and low IgM reactivities (OD <0.31) to the full-length peptide were observed in two sera from group B (Nos. 34 and 78), whereas parallel ELISA tests searching for potential specificities to the peptide in sera from patients who were unreactive to CEM membrane and from uninfected donors revealed a prevalent lack of reactivity in both control groups (data not shown).

Fig. 5 *B* illustrates the pattern of IgG reactivities for the peptides that resemble the homology sequence. Predominant binding to VEINCTRPNN (V3IIIB) was observed in most sera from both groups. The difference between reactivities to VEINCTRPNN(IIIB) and VQINCTRPNY(MN) was evident in several sera (Nos. 3, 21, and 41), whereas the similar values for both peptides by IgG of serum 54 further supported the view that the presence of Asn, rather than the substitution of Glu by Gln, is essential in influencing the antigenicity of both epitopes. As expected, IgG from the controls constantly showed low values of reactivities to both peptides.

Affinity-isolated Anti-Fas Preparations. Serum samples from patients 3, 41, 54, and 59 containing anti-Fas were passed through Sepharose 4B columns coupled with WC8 membrane lysate and were further cleared of nonspecific antibodies by absorption on WR19L affinity columns. Fig. 6 shows the protein analysis of relative eluates detected by SDS-PAGE, using β-mercaptoethanol as the reducing agent. A prevalent IgG content was demonstrable in all samples absorbed on the lysates, even in the presence of traces of IgM, particularly in sera 41 and 59. Similarly, eluates from VEINCTR columns included IgG, although at lower concentrations than in other eluted fractions.

Anti-Fas IgG Are Inducers of Apoptosis in Vitro. The ability of purified anti-Fas preparations to induce cell death was investigated in vitro by different methods, including the measurement of both subdiploid DNA content and proliferative rate inhibition of target cells after their incubation with those antibodies. To this end, we used both CEM cells as a clonotypic model of CD4⁺ cells and, in parallel
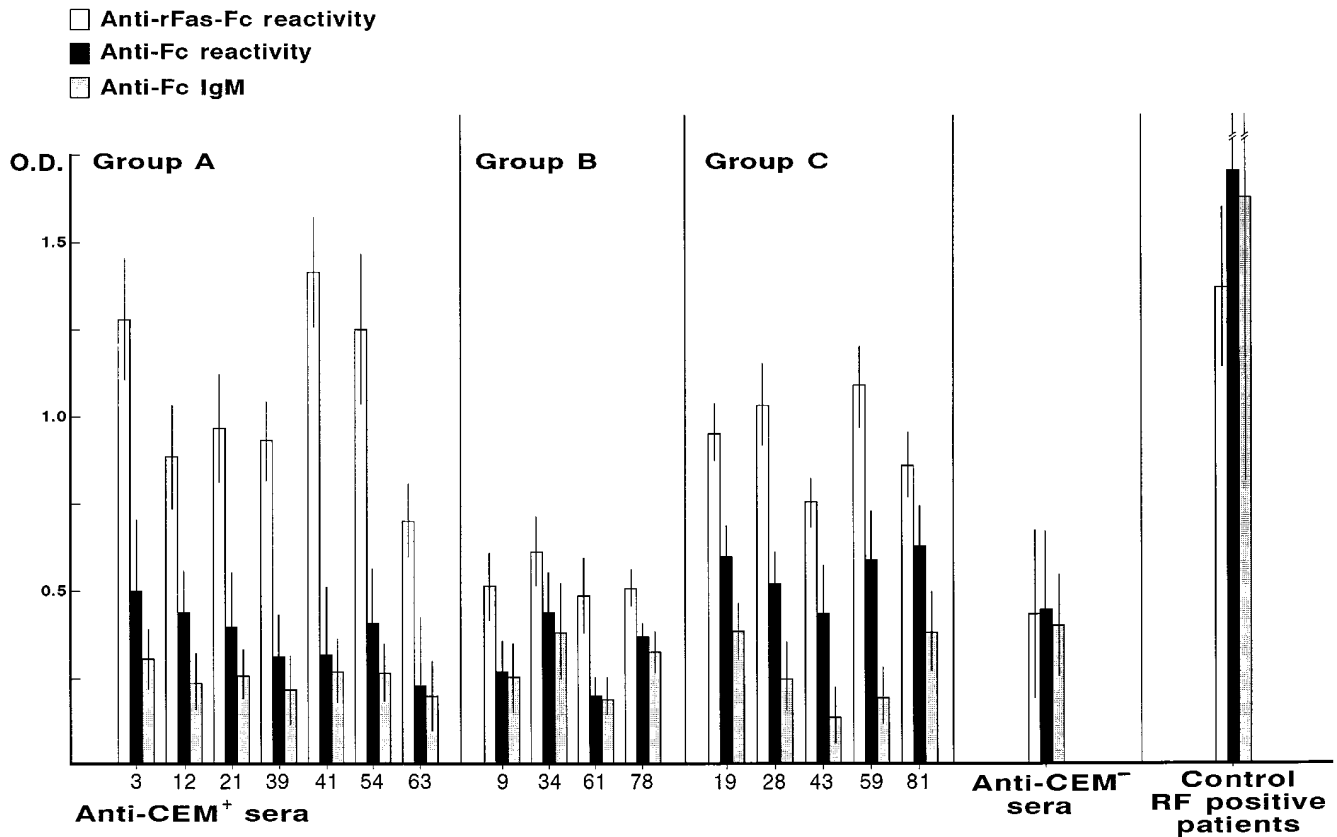
Figure 4. Reactivity to Fas by ELISA using the chimeric rFas-Fc protein in 16 HIV-1+ sera showing positive binding to CEM membrane as compared to 39 anti–CEM–negative sera. Specificity to Fas was predominantly expressed in all instances with respect to either total or IgM-mediated rheumatoid activity. The highest values occurred in patients from groups A (3, 41, and 54) and C (28 and 59), showing serum elevations of T cell–reactive IgG, whereas the sera from patients unreactive to CEM membrane showed mean values of weak affinity to the antigen. By contrast, control sera from four HIV-1–uninfected patients with high RF titers reacted with Fc both in its chimeric and natural forms.

tests, autologous T cells from the peripheral blood of the four patients providing anti-Fas. Positive controls were provided by the anti-Fas from clone CH11 and the rFas-L, whereas purified IgG from two patients of group B (9 and 34) were used as negative controls to anti-Fas. Fig. 7 shows the cytofluorimetric profiles of PI staining of treated CEM cells. As depicted in section B, anti-Fas molecules induced a remarkable increase of the subdiploid DNA peak that was particularly evident at 20 μg/ml or higher concentrations, when its extent ranged from ~63.6 to 92.5%. Both control IgG from patients unreactive to the CEM lysate failed to induce any increase in the apoptotic cell population, whereas as expected, the CH11 and the rFas-L control reagents drove >90% of cells to die (Fig. 7 A). An extension of these experiments included the evaluation of the subdiploid DNA extent in HIV-1 T cells that had been incubated with autologous anti-Fas. A similar pattern to CEM cells with occasional further increment of the apoptotic population was usually observed, whereas no evident differences were noted with heterologous as opposed to autologous anti-Fas preparations (data not shown). Fig. 7 C shows the effect of anti-Fas inhibition by the VEINCTR peptide. Preincubation of anti-Fas antibodies with increasing concentrations

(0.5 to 7.5 μg/ml) of this peptide induced a major suppression of the apoptogen effect, leading to substantial recovery of the euploid DNA peak (M2). This effect paralleled the failure to react with rgp120 in control ELISA, and supported the specificity of the peptide for such anti-Fas agonist molecules.

The inhibitive effect of anti-Fas antibodies on the proliferation of CEM cells is also illustrated in Fig. 8 (*left panel*). Anti-Fas from serum 54 showed the highest inhibition ([³H]thymidine uptake ≤35%) at a concentration >0.1 μg/ml. The similar profile obtained with the anti-Fas from patient 41 at 1 μg/ml was significantly different (*P* <0.02) from the control cell preparations that were treated with purified IgG from group B patients (uptake 100% with ~19,000 cpm). Slightly less inhibition was noted with the anti-Fas from patients 3 and 59. Both preparations suppressed [³H]thymidine uptake of CEM cells by ~50%, showing that antibody concentrations >1 μg/ml were still effective in significantly suppressing their target cells (*P* <0.05). A strong inhibition was induced by both CH11 and rFas-L reagents in control cell suspensions, though their effect at 1 μg/ml was nearly equivalent to anti-Fas preparations from patients 41 and 54. This confirms the
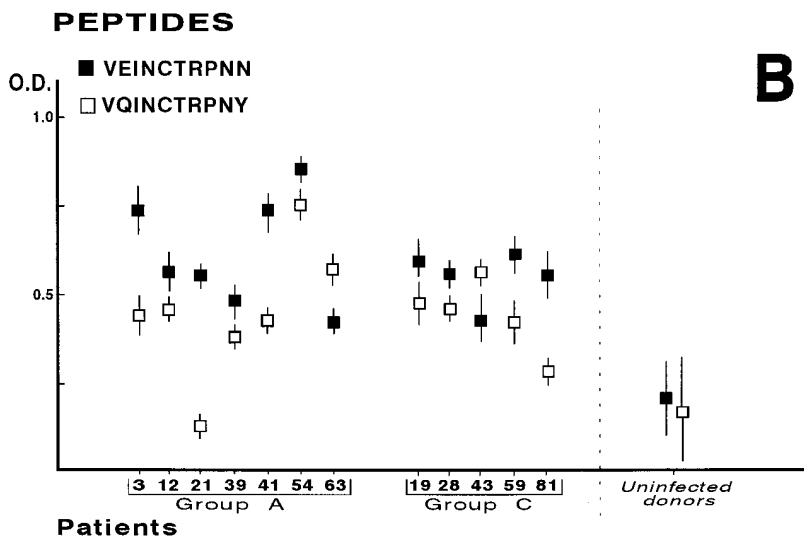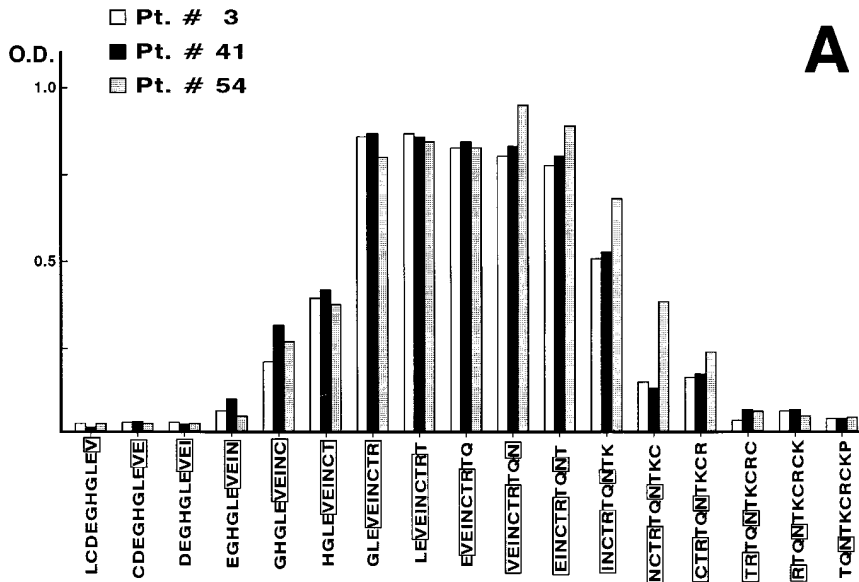
Figure 5. (*A*) Epitope mapping of a domain of Fas (aa position 90–117), including an 8-mer stretch (VEINCTR—N) shared by the V3 loop of gp120. A panel of 17 overlapping 10-mer peptides used as antigens in ELISA revealed a predominant affinity to that domain in sera 3, 41, and 54 although IgG from patient 54 showed a slight increase of reactivity to a subsequent peptide constructed with (N) Asn in the 108 aa position. Specificity to the VEINCTR peptide was almost undetectable in sera from patients unreactive to the CEM membrane. (*B*) ELISA reactivity to synthetic peptides resembling the homology sequence of the aa 99–108 domain of Fas in the V3 loop of HIV-1ᴵᴵᴵB (VEINCTRPNN) and HIV-1ᴹN (VQINCTRPNY) strains. OD values to both peptides were apparently equivalent in most of the anti-Fas IgG in groups A and C. In contrast, sera 3, 21, and 41 showed a slight difference in their affinity to the peptides, suggesting that the replacement of E (Glu) by Q (Gln) was influencing the antigenicity of the epitope.

susceptibility of CEM cells to activate their own Fas pathway after cross-linking with these molecules.

A control of specificity in proliferative assays was also provided using the same anti-Fas antibodies preincubated with increasing amounts of rgp120. Fig. 8 (*right panel*) shows that saturation of anti-Fas from the same patients with >1 μg/ml of virus glycoprotein was able to prevent the observed inhibition of proliferative rate in CEM cells that were treated with 10 μg/ml of anti-Fas. In all instances, the preincubation of these antibodies with a similar concentration of rgp120 restored the normal proliferation of those cells, suggesting the functional inactivation of anti-Fas. Conversely, control IgG unreactive to CEM were not influenced by the virus glycoprotein.

These cross-inhibiting experiments exploring the effect

of anti-Fas saturation with either VEINCTR in PI measurement or rgp120 in proliferation supported the contention that our antibody preparations were specific for both Fas and gp120 to an equivalent extent.

Affinity-isolated Fas-reactive IgG Bind to gp120. The ability of anti-Fas from the four patients to bind to HIV-1 glycoproteins was tested by immunoblotting. Fig. 9 shows the reactivity to gp120 detected in WC8-absorbed anti-Fas IgG from patient 54 compared with the serum pattern of positivity to the virus lysate. Similar binding was observed in IgG from patient 54 and 59, which were purified through VEINCTR columns. A variable positivity of immunoblotting binding was also detected in the other three preparations of anti-Fas derived from WC8-coupled columns (data not shown).
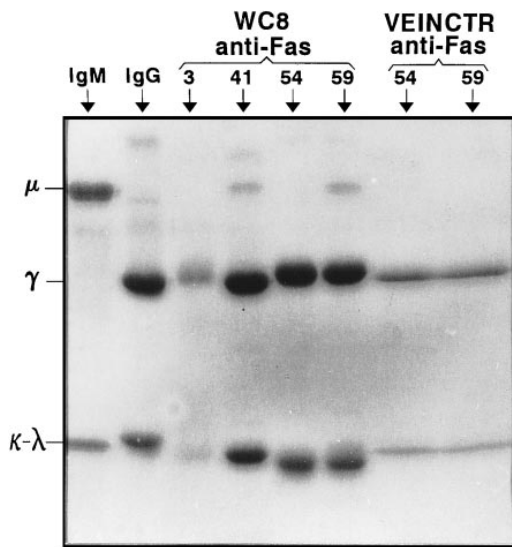
Figure 6. SDS-PAGE pattern of isolated anti-Fas antibody preparations obtained by absorption of sera on affinity columns coupled with the Fas[+] membrane lysate from WC8 cells and further absorption on WR19L lysate columns for clearance of nonspecific reactivities. A prevalent IgG content was observed in those preparations, even though traces of IgM were also present. Parallel absorptions using VEINCTR peptide–coupled affinity columns provided eluates showing a similar content of antibodies in sera 54 and 59. Standard IgM and IgG were myeloma proteins.

Discussion

The present study demonstrates that the described increase of T cell apoptosis in HIV-1 infection could include an antibody-mediated mechanism acting via the Fas pathway. Analysis of serum reactivities in a cohort of HIV-1^{+} patients revealed that IgG to CEM lymphoblast membrane bound Fas in both quantitative cytofluorimetry and immu-

noblotting, using the human Fas-transfected mouse WC8 lymphoma as a substrate, and reacted with the rFas in ELISA. Investigation of Fas epitope specificity by linear sequence peptides documented prevalence of reactivity to a domain including the [99]VEINCTR–N[108] peptide, also shared by the gp120 V3 loop of HIV-1IIIB. Absorption of sera by affinity columns coupling the WC8 membrane lysate provided enriched anti-Fas IgG preparations whose effect in functional studies was nearly equivalent to either CH11 IgM anti-Fas mAb or rFas-L in induction of apoptosis in target cells. These molecules were also reactive to a peculiar epitope of gp120, however, suggesting that the humoral response elicited to that domain of the HIV-1 envelope protein may enhance T cell apoptosis by a molecular mimicry mechanism involving Fas stimulation.

A number of studies have emphasized the role of autoimmune mechanisms in the immunopathogenesis of AIDS. Disturbance of self–nonself recognition resulting from chronic activation of the immune system has been postulated to promote differential autoimmune deregulations, including GVHD condition (44), leukocyte alloimmunization (45), abnormal idiotype interactions (46), and increased cytokine secretion (47). Structural similarities between immunogenic HIV-1 envelope proteins and MHC and other key immunoregulatory molecules have recently been postulated (25, 26, 48) as the source of the chronic immune derangements, leading to the production of autoantibodies and clinical phenomena. Despite the description of peculiar syndromes that resemble idiopathic autoimmune diseases (49), however, the occurrence of autoantibodies that display pathogenic idiotypes which parallel and proceed to the late stages of disease have rarely been detected in HIV-1^{+} patients.

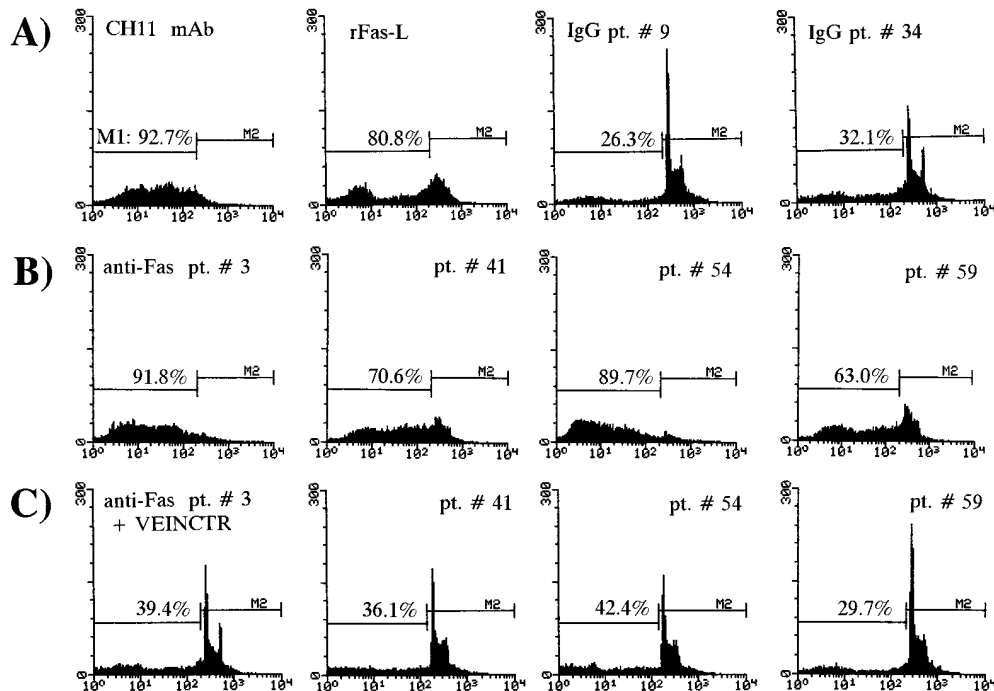Although described in <20% of HIV-1–infected indi-

Figure 7. Functional studies of isolated anti-Fas molecules from patients 3, 41, 54, and 59. (*A*) Percentage of apoptotic CEM cells measured as the extent of PI subdiploid DNA staining (*M1*) induced by 20 μg/ml of agonist ligands of Fas (CH11 mAb and rFas-L) compared to the effect of control IgG from two patients unreactive to CEM membrane. (*B*) The extent of subdiploid DNA in cell preparations that were treated with similar concentrations of the purified anti-Fas from the four patients ranged from ~63–92%, and was apparently equivalent to the effect of functional ligands. (*C*) Preincubation of anti-Fas preparations with 7.5 μg/ml of VEINCTR peptide removed the ability of those antibodies to increase the PI subdiploid DNA peak, suggesting their functional saturation by the related antigen.
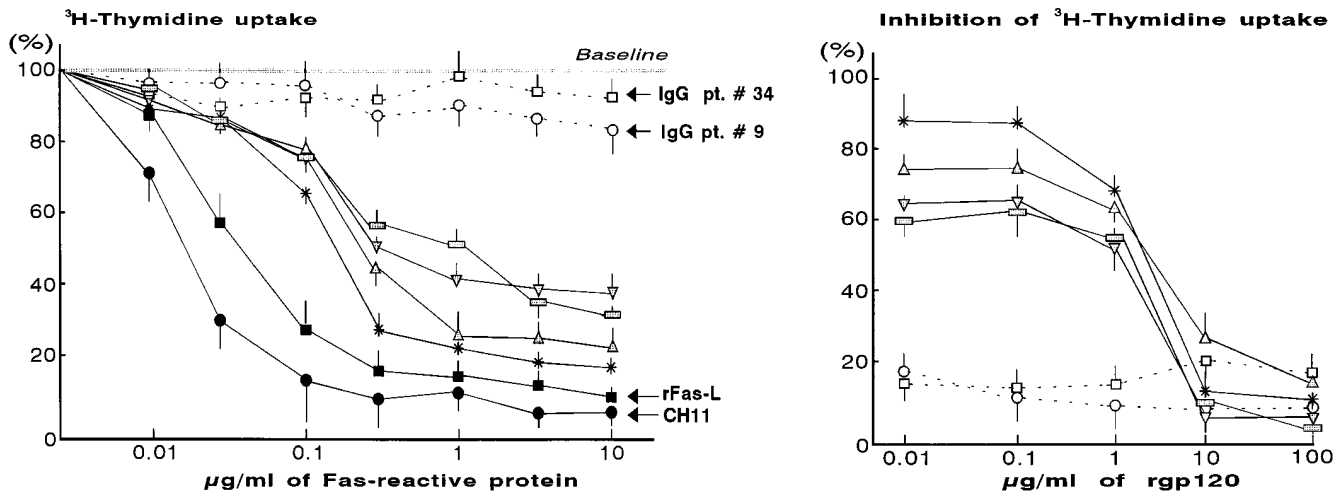
Figure 8. Inhibition of CEM proliferative rate by anti-Fas preparations. Significant inhibition (*P* <0.02) was noted in cell suspensions that were incubated with anti-Fas IgG from patients 41 and 54 by the concentration of 1 μg/ml. A moderate but significant effect (*P* <0.05) was also recorded using anti-Fas preparations 3 and 59 at higher concentrations. The test included both positive and negative controls. The effect of both CH11 mAb and rFas-L was nearly equivalent to anti-Fas antibodies from patients, whereas the control Fas-unreactive IgG from patients 9 and 34 failed to induce significant variations of the baseline [³H]thymidine uptake (*left panel*). Preincubation of anti-Fas with rgp120 induced in all instances a dose-dependent suppression of their inhibitory property on CEM proliferation, which was measured as the percentage of [³H]thymidine incorporation. The effect of control IgG was not influenced by the recombinant glycoprotein (*right panel*).
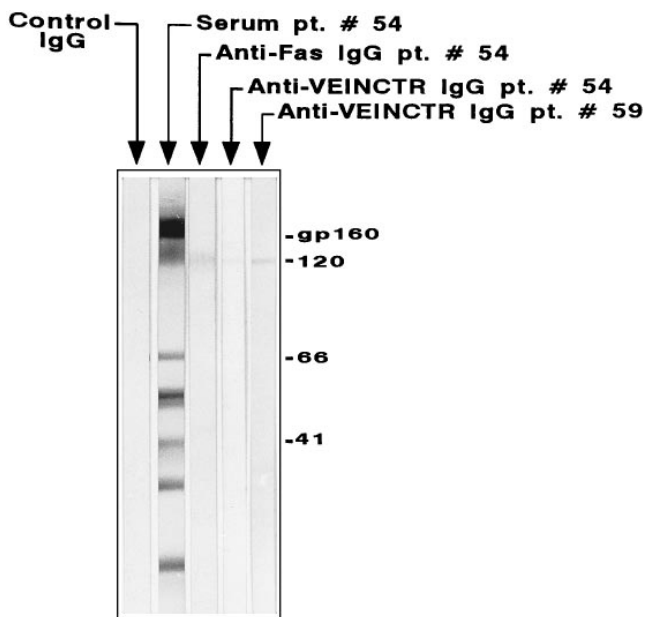

Figure 9. Immunoblotting of isolated anti-Fas IgG from patient 54 on the HIV-1 lysate, as compared with serum pattern of positivities to the viral bands. The antibody was found to react with gp120 in a fashion similar to both antibody preparations (from patients 54 and 59) absorbed on VEINCTR columns. Negative control using commercial human IgG was also provided.

viduals (31, 50, 51), autoantibodies to T cell antigens are suspected to contribute to the immunopathogenesis of AIDS, since their serum levels may parallel the progression of lymphopenia (32, 33, 51). Molecular specificities to the HIV-1 receptor (52, 53) and other membrane antigens (30, 50, 54) have been attributed to such antibodies in affecting the helper function of CD4+ cells, whereas using the CD4+ clonotypic CEM lymphoblasts, we documented that a receptor of ~43.5 kD was able to suppress the cytotoxic properties of those antibodies in competitive inhibition experiments (35). Evidence from this work strongly suggests that the Fas antigen is the primary molecular target of such T cell–reactive antibodies in a number of HIV-1–infected individuals.

Fas is a 45-kD trimeric antigen that is involved in maintaining both consistency of T cell level and self-tolerance within the immune system. Accumulating evidence from recent studies emphasizes the critical role of Fas interaction with its trimeric ligand in the induction of antigen-primed T cell apoptosis (13, 55, 56) and cytotoxic cell killing (17, 57). Further work from independent groups (28, 58) and ourselves (29) reported that, as a result of chronic immune activation related to HIV-1 infection, Fas is significantly overexpressed by peripheral T cells, particularly during advanced disease, and that its responsiveness to differential ligands regularly drives these cells to apoptosis even by

cross-linking with the monomeric IgG1 anti-Fas mAb from clone UB-2. This reagent is a weak inducer of apoptosis in Fas$^+$ cell lines, whereas it may costimulate the proliferation of freshly isolated T cells from uninfected donors (16). Therefore, both overexpression and susceptibility of Fas during HIV-1 infection may be crucial in the increase of peripheral T cell apoptosis.

The main question raised by this study is whether the Fas-reactive IgG detected in several patients are actually implicated in enhancing the extent of T cell apoptosis in vivo. The first answer came from indirect multiparametric analysis. Semiquantitative measurement of the cytofluorimetric PI intensity of DNA staining was enough to show that patients from groups A and C with the highest ELISA reactivities to rFas (Fig 4) showed a parallel increment of their subdiploid DNA T cell population (Table 1). Although the differences between percentages of apoptotic T cells were estimated in a comparatively small number of patients within each group, these data suggest a potential association between the occurrence of anti-Fas antibodies and an increase of apoptosis-mediated lymphopenia. This was manifest particularly in patients with advanced disease from both groups A and C. Meyaard et al. (7) have reported that the degree of T cell apoptosis may not correlate with disease progression. Accordingly, the potential linkage of uniformly accelerated cell death in patients with different CDC stages may depend on their serum elevations of IgG reactive to Fas. Despite the described association, however, our group observed extensive subdiploid DNA content in peripheral T cells from a few patients lacking serum positivities to either Fas$^+$ cell membranes or rFas, thus supporting the assumption that, besides Fas, other mechanisms are involved in driving the increased T cell apoptosis in such patients.

A second interesting point concerned the effect of Fas-reactive IgG in functional studies. These molecules were absorbed by affinity chromatography on Fas$^+$ membrane from WC8 cells and were demonstrated to promptly enhance the subdiploid DNA peak in cultured CEM cells, resulting in an event similar to that provoked by reagents that trigger Fas, such as the CH11 mAb and rFasL (Fig. 7, A and B). Conversely, no effect was observed in control tests using purified IgG that was unreactive to CEM membrane from two patients (Nos. 9 and 34) of group B. These findings were also sustained by the proliferative assays shown in Fig. 8. Again, the presence of anti-Fas preparations at 1 μg/ml or more, particularly from patients 41 and 54, induced a tremendous inhibition of the [³H]thymidine uptake in a fashion similar to that obtained with functional reagents.

The agonist effect of these purified anti-Fas was also cross-inhibited by either VEINCTR or rgp120, resulting in a dose-dependent lack of activity in both functional tests. Since control IgG preparations were ineffective, we concluded that such affinity-isolated anti-Fas were able to activate cytolysis of CEM cells by mimicking the natural Fas-L. Although we obtained equivalent results in experiments using autologous T cells, we reasoned that CEM lymphoblasts were an appropriate CD4$^+$ cell target to avoid the heterogeneity of peripheral lymphocytes at different steps of maturation.

Fine mapping of Fas using a panel of 10-mer overlapping peptides revealed that the entire epitope (VEINCTR–N) shared by the V3IIIB loop of gp120 was highly reactive to the majority of sera from groups A and C (Fig. 5). With the exception of three sera (Nos. 3, 21, and 41), we found that the binding to IIIB (VEINCTRPNN) and MN (VQINCTRPNY) HIV-1 peptides was almost equivalent, and that replacement of Glu by Gln did not influence the relative antibody affinity. By contrast, the presence of Asn on both peptides slightly increased the reactivity of IgG from serum 54, suggesting that a conformational domain of gp120, including Asn, was predominantly recognized by this patient. Conformational V3 loop peptides have been reported to induce specific antibody response to HIV-1 (59). Since the majority of neutralizing antibodies to both IIIB and MN European strains of HIV-1 are prevalently directed to the gp120 V3 loop (42), however, our data suggest that within the wide humoral response to virus, only IgG elicited to that domain of the V3 loop are effective cross-linkers of Fas and are able to induce its trimerization. In this context, it has been demonstrated by Itoh et al. that the cytoplasmic signal–transducing domain of Fas, also termed death domain, is activated by receptor clustering (60). Therefore, the oligomerization of Fas by such anti-V3 IgG may account for the property of these molecules to cross-link the receptor in a self-associated molecular state.

In conclusion, our data indicate that the T cell–reactive antibodies originally described in HIV-1 infection are actually involved in aggravating the lymphopenia, although their mechanism is not complement-mediated cytolysis. Fas-mediated depletion of peripheral T cells may occur as an effect of the humoral response to HIV-1, and is apparently dependent on the fine specificity of IgG to gp120. Since these antibodies react with the described sequence of Fas, it would be of interest to determine the potential reactivity to VEINCTR–N as a screening procedure to select the patients at higher risk of severe lymphopenia, based on autologous Fas activation.

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