

Immunogenicity of an Eight Amino Acid Domain Shared by Fas (CD95/Apo-I) and HIV-1 gp120. I. Structural and Antigenic Analysis

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Abstract

Background: Previous studies have demonstrated that immunoglobulin G (IgG) antibodies to VEINCTR–N, a domain shared by Fas (CD95/Apo-I) and gp120, contribute to T-cell apoptosis during human immunodeficiency virus-type 1 (HIV-1) infection as a result of the agonist cross-linking of Fas. The present work was designed to determine whether these molecules are elicited primarily to HIV-1 or the cell receptor.

Materials and Methods: Sera from 439 HIV-1-infected patients were screened by ELISA for their reactivity to VEINCTR–N. Subjects with significant serum elevations of IgG anti-VEINCTR–N were further investigated. Immunologic parameters, including CD4⁺ and CD8⁺ lymphocyte count, extent of T-cell apoptosis, occurrence of both anti-Fas antibodies and circulating soluble Fas titers, and reactivity to the 8-mer peptides resembling the flank-regions of VEINCTR–N on both gp120 V3 loop and Fas were examined. In addition, the antigenicity of these domains was assessed by biochemical and computerized analyses.

Results: 21 patients with significant levels of IgG to VEINCTR–N showed both an increased extent of peripheral T-cell apoptosis and binding to full-length Fas. A weak, though positive correlation of the anti-

VEINCTR–N activity with its antecedent peptide on Fas was also found. Charge and structural analysis revealed that, although the extended 26-amino acid (a.a.) regions on both proteins were hydrophilic, the Fas peptide adjacent to VEINCTR–N expressed a short β -conformed a.a. sequence in contiguity with a portion of the shared epitope, also in β -sheet conformation. Patterns of antigenicity confirmed an apparent immunodominance of the full VEINCTR–N, based on its homology with the consensus sequence of other members of the tumor necrosis factor (TNF) receptor family. The hypothesis that the high immunogenicity of this region of Fas, rather than gp120, can drive the production of anti-VEINCTR–N antibodies also was supported by the concurrent significant elevations of soluble Fas in almost all of the sera studied.

Conclusions: Our results indicate that a high release of the soluble form of Fas by T cells during the chronic immune activation of HIV-1 infection primes a humoral response against this epitope of Fas as a result of its high antigenicity. This is similar to the antibodies to tumor necrosis factor α (TNF α) receptor (R) (TNF α -R) that occur in response to increased levels of the soluble receptor for TNF during autoimmunity.

Introduction

Sequence homologies in the primary structure of unrelated proteins are considered unusual in biology. New techniques for preparing synthetic peptides have contributed remarkably to the identification of functional polymer(s) within the full-length proteins. However, the occur-

rence of structural similarities, even including short-length linear fragments between endogenous and external proteins, such as bacterial and virus products, may lead to a perturbation of the immune response by molecular mimicry mechanisms currently emphasized in the pathogenesis of autoimmunity (1,2). Accumulating evidence

also suggests that molecular mimicry is involved with human immunodeficiency virus-type 1 (HIV-1)-related autoimmune phenomena, since many structural homologies between the retrovirus and functional molecules of the immune system have been described (3,4). These homologies appear to belong to predominantly *env*-encoded products, particularly gp120 (5), which represents the viral component primarily detected by the immune system when establishing initial molecular contact with CD4⁺ T cells.

We recently identified a functional epitope of Fas (CD95/Apo-I) (6), a membrane receptor that promotes the cellular death by apoptosis (7,8) and acts as the molecular target of serum antibodies in a number of HIV-1-infected individuals (9). This domain, namely VEINCTR-N, is located within the amino acid positions (a.a. pos.) 115–124 of the extracellular portion of the protein (7). It is shared structurally by the third N-glycosylation site of the gp120 V3 loop within a.a. pos. 171–180 (10). Such a linear sequence is invariably expressed by the HIV-1_{IIIIB} strain (11); whereas, it shows a single substitution of Glu (E) by Gln (Q) in other virus subgroups, including HIV-1_{MN} and HIV-1_{RF} (12). Immunoglobulin G (IgG) antibodies to VEINCTR-N induce apoptosis in Fas-sensitive cells and, thus, resemble the biological effect of the natural ligand (Fas-L) of the receptor (13). This also emphasizes the pivotal role of the 8 a.a. stretch in the functional activation of Fas, which is essential for transducing the death signals to the intracellular pathway (14). Serologic elevations of anti-Fas antibodies also have been associated with T-cell depletion and progression to AIDS (9,15), suggesting that the decline of peripheral CD4⁺ cells in the presence of anti-VEINCTR-N specificities includes an apoptotic mechanism mediated by extrinsic activation of Fas. Both the abnormally increased expression and the susceptibility of this receptor to linkage by either agonist or non-agonist ligands (16) are thought to be major events driving T cells to undergo apoptosis in HIV-1 disease (17–19).

We postulated reactivities to VEINCTR-N (6) as the result of the peculiar epitope specificity of HIV-1 neutralizing antibodies to the V3 loop, against which the majority of those molecules

are directed because of its high immunogenicity within the gp120 structure (12). However, agonist anti-Fas antibodies have been detected recently in healthy donors (20), with no evidence of previous immunization to HIV-1. Clinical activity in autoimmunity also has been associated with serum elevations of soluble forms of both Fas and its ligand (21,22) and related antibodies (23). This is an effect of Fas/Fas-L deregulation, resulting in solubilization of such molecules, which are currently regarded as apoptosis-blockers (24).

Since the derangements of Fas/Fas-L described in HIV-1 infection also may result in the release of variable amounts of sFas (25,26) and formation of anti-Fas antibodies that correlate with T cell depletion (15), we investigated whether the apoptogen anti-VEINCTR-N IgG occurring in HIV-1⁺ subjects was interpreted as true anti-Fas molecules, rather than elicited to the V3 loop. The present study focused on the molecular specificity of IgG to VEINCTR-N in a broad group of HIV-1-infected subjects. By using synthetic peptides overlapping the flank regions of VEINCTR-N on both Fas and gp120, we found that the majority of those specificities failed to recognize peptides related to HIV-1; whereas, a predominant reactivity to Fas was confirmed. Thus, we explored both structural and biochemical properties of these parallel domains of Fas and gp120 to evaluate their relative antigenicity. Data from our work suggest that in HIV-1 infection, VEINCTR-N is targeted as a specific component of Fas and shows peculiar immunodominance. In addition, elevations of soluble Fas (sFas) are detectable in the majority of subjects with serum specificities to that domain.

Materials and Methods

Study Subjects

The study included the serological screening of specificities to VEINCTR-N in 439 HIV-1⁺ individuals at different CDC stages (27). Sera were collected prevalently from subjects who were examined and checked periodically during their treatment at the outpatient clinic of the Department of Internal Medicine and Oncology, and from patients included in the "Program of AIDS Prevention," at the Institute of Hygiene and Preventive Medicine, University of Bari. A few patients with advanced disease were also recruited from the Infectious Diseases Division. All subjects gave their informed consent

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and the study was approved by the ethical committee of the University of Bari. Sera were divided into aliquots and stored at -80°C until use. Patients with serological positivities at screening were recalled and asked to provide a further blood sample for immunologic tests. These included peripheral CD4^{+} and CD8^{+} cell counts and evaluation of the extent of the apoptotic T-cell population with subdiploid DNA by propidium iodide (PI) staining (Sigma, St. Louis, MO, U.S.A.). The flow cytometry method, including both immunophenotype analysis and measurement of apoptosis, has been described previously (16).

ELISAs

We installed a screening test for positivity to VEINCTR-N and subsequent assays by measuring the reactivity to several 8-mer peptides resembling the linear sequence of its flank regions on both Fas and gp120. Primary structure of such regions was obtained from the Database Services at (NCBI); (Bethesda, MD) via Internet. The peptides were produced by an automated synthesizer (Biochrom Biolynx 4170; LKB, Vliiby, Bromma, Sweden) by fluorenylmethoxycarbonyl (Fmoc) chemistry and purified by high performance liquid chromatography (HPLC). Microtiter 96-well plates were coated separately with $10\ \mu\text{g}/\text{ml}$ of each peptide and incubated subsequently with sera diluted 1/100 for 5 hr. Conformity of peptide binding to plates was assessed for uncoupled peptides by parallel tests using keyhole limpet hemocyanin (KLH) as a carrier protein (28). Since all 8-mers in their glutaraldehyde-KLH-coupled form appeared to bind the ELISA solid phase with equal efficiency, the plates were coated with uncoupled antigen to prevent any cross-reactivity with KLH (29), as experienced in previous work (6). The plates then were supplemented with a goat peroxidase-conjugated anti-human IgG and, subsequently, read in a micro-ELISA reader (Titertek; Flow Laboratories, Irvine, Scotland) after their OPD (O-phenylenediamine) color development. Negative controls of the assay were provided by the second antibody by itself. Patients' sera with absorbance to VEINCTR-N, calculated as higher than the mean value of the whole HIV-1^{+} population supplemented with standard deviation (3SD), were considered positive and investigated for relative reactivities to 8-mer adjacent peptides on both Fas and gp120.

A further ELISA evaluated the affinity to full-length Fas molecule in anti-VEINCTR-N-posi-

tive sera. This assay used a chimeric recombinant (r) Fas-Fc as antigen (Immunotech, Marseille, France) and was completed as described (6), including the measurement in sera of anti-Fc (RF) levels to calculate the actual anti-Fas reactivities.

Soluble Fas levels were measured in sera from anti-VEINCTR-N-positive patients and in a control group of 67 sera randomly derived from the pool of negative patients. Additional sera from three patients with active systemic lupus erythematosus (SLE) showing elevations of sFas in previous ELISA were included in this assay as positive controls (22), along with a group of 19 healthy donors as negative controls. The test was performed by coating the plates with $2.5\ \mu\text{g}/\text{ml}$ of CH11 anti-Fas (IgM) monoclonal antibody (MoAb) (Immunotech, Marseille, France) in carbonate buffer pH 9.6 and further incubation of sera at 1/10 dilution in phosphate-buffered saline (PBS) containing 10% of fetal calf serum (FCS) and dry milk. After 5 hr incubation, the plates were washed and supplemented with a second anti-Fas MoAb (IgG1 from clone ZB4) and incubated for 2 hr at room temperature. This sandwich ELISA, which makes use of anti-Fas MoAbs of a different isotype, reacts with overlapping domains of Fas (30) and has been optimized recently by our lab. The CH11 IgM as Fas agonist MoAb is unable to induce a full saturation of its antigenic sites, since it is not a precipitating antibody. Therefore, the Fas-antagonist ZB4 IgG1 was found to link its reactive epitopes, even on CH11-coupled Fas molecules. We also verified the use of UB2 IgG1 anti-Fas (Immunotech) as a second antibody of this assay and found a similar reactivity as for ZB4. Thus, the ELISA was standardized with ZB4 for high reproducibility of data and was completed by standard procedure using a labeled goat anti-mouse IgG and color development following the OPD addition. A positive control of known amount was provided by rFas-Fc molecule (Immunotech) at $2.0\ \text{ng}/\text{ml}$. As in previous ELISAs, each serum sample was evaluated in triplicate and positive significance was attributed to values of optical density (O.D.) higher than the mean + 3SD obtained from the entire HIV-1^{+} population.

Charge and Conformational Structure Analysis

The molecular structure of synthetic peptides was investigated by differential methods. Since VEINCTR-N elicited a T-cell-mediated response, resulting in production of specific antibodies, we first used the "TSites" computer software

(MedImmune Inc., Gaithersburg, MD) (31), which predicts both the presence and charge of epitopes reactive to T cells by combining four algorithms for each peptide. This program scans the intrinsic structural properties of the primary a.a. sequence, including alpha-helical periodicity, amphipathicity, and predictive conformation in relation to its a.a. complementarity to HLA class I and II molecules (32). Since it also has been shown to predict immunogenic regions within proteins in most structural studies (33), we explored the full-length sequence of both Fas and gp120, particularly the regions including the 8 a.a. peptides before and after VEINCTR-N.

Evaluation of Immunogenicity and Three-dimensional Models

We assessed the antigenicity of single peptides from both Fas and gp120 to determine whether the linear epitopes were accessible to humoral aspects of the immune system. The structure of the Fas region investigated in the present study is not described in the recent crystallographic assessment of the receptor (34), which starts at a.a. pos. 202. Therefore, we first explored the biochemical properties of Fas-related peptides by evaluating the linear sequence, as well as atomic flexibility and hydrophilicity. The antigenic profile was also assessed by the "Anthprot-Antigenicity Method" program, as suggested by Parker and co-workers (35) and provided by Internet. Briefly, the analysis is based on a predictive method that compares the percentage of each a.a. in single epitopes with the percentage of the residues in the average composition of a protein. Thus, both antigenicity and hydrophilicity values are obtained by calculating the relative occurrence of each a.a. within defined epitopes, with respect to the full-length protein. The three-dimensional (3D) structure of both Fas and gp120 reactive peptides, subsequently, was achieved by computer analyses using "PCImdad" software (Molecular Applications Group, Palo Alto, CA), which provided the visualization of the immunogenic epitopes in three-dimensional models on the computer screen. However, the exact position of the side chain residues, as well as the actual conformational structure, were not known because the complete arrangement must await crystal analysis. Therefore, since Fas belongs to the tumor necrosis factor (TNF) receptor (R) family and, since its domain showed high homology with the corresponding region of TNF α -R

through the presence of a consensus sequence (7), we generated the 3D model of this extracellular portion of Fas, namely the a.a. 101-127 stretch, from the crystallographic coordinates of the sTNF α -R (36). The modeling procedure was also elaborated with the COMPOSER[®] homology modeling program (37) provided by Internet (<http://www.ibcp.fr/>) and all disulphide linkages located within the consensus sequence were joined in analogous conformation to their location on TNF α -R.

Statistical Analysis

The correlation of reactivities to different antigens in ELISA assays was assessed by linear regression analysis.

Results

Anti-VEINCTR-N Reactivity and Immune Parameters

A VEINCTR-N IgG screening of the intact HIV-1⁺ population provided the relative O.D. mean value (0.243 ± 0.039). Therefore, 21 patients with anti-VEINCTR-N reactivity higher than this value, plus 3SD were considered positive. In all instances, their antibody levels were much higher than the control mean value obtained from the group of normal donors (O.D. = 0.117 ± 0.021). Table 1 shows their relative O.D., peripheral lymphocyte counts, and subdiploid T-cell populations. Variable affinity to the peptide was illustrated by the O.D. values range: 0.377 (pt. DI-T07) to 1.163 (pt. DI-F31). Analysis of correlations revealed, in several instances, individual CD4⁺ cell counts inversely related to anti-VEINCTR-N values; whereas, these reactivities were independent of the clinical stage and/or CD8⁺ cell numbers. In addition, although the amplitude of the apoptotic cell population was apparently increased, compared with the reference values from healthy controls (16), the highest reactivities were not associated constantly with the concurrent expansion of the relative cell population with subdiploid DNA (i.e.: pts. DI-F31 and HY-417 with respect to pts. DI-V42, ID-CT06, and ID-CT98). Table 1 also shows the O.D. values of serum reactivity to Fas in these patients. As in a previous work (6), there was a substantial expression of this reactivity in almost all sera and its values were related positively to anti-VEINCTR-N specificity ($R^2 = 0.397$).

Table 1. Immune parameters in 21 HIV-1-infected individuals showing positive values of reactivities to both VEINCTR-N and the full-length Fas molecule in chimeric form.

Patients	CDC Stage	Peripheral Lymphocytes			Peripheral T Cells with Subdiploid DNA (% of PI staining)	Elisa Reactivities (O.D.)	
		Cells/ μ l	CD4 ⁺ Cells/ μ l	CD8 ⁺ Cells/ μ l		Anti-VEINCTR-N	Anti-Fas
1) DI-B03	II	1,390	381	724	32.5 \pm 4.9	0.690 \pm 0.04	0.815 \pm 0.03
2) DI-F12	II	981	301	567	37.5 \pm 6.1	0.795 \pm 0.04	0.509 \pm 0.1
3) DI-F31	IV	690	122	465	43.5 \pm 5.0	1.163 \pm 0.15	1.384 \pm 0.18
4) DI-R10	III	809	212	494	39.6 \pm 11	0.985 \pm 0.11	1.206 \pm 0.09
5) DI-T07	II	2,907	705	1,708	21.9 \pm 6.4	0.377 \pm 0.03	0.705 \pm 0.03
6) DI-V42	IV	550	107	308	41.5 \pm 13	0.890 \pm 0.07	0.511 \pm 0.06
7) HY-011	II	1,415	409	892	27.5 \pm 7.5	0.788 \pm 0.09	1.080 \pm 0.12
8) HY-106	II	1,024	328	590	30.5 \pm 4.5	0.810 \pm 0.09	0.957 \pm 0.09
9) HY-167	II	870	309	427	28.6 \pm 11.2	0.780 \pm 0.05	0.952 \pm 0.11
10) HY-215	III	715	225	430	N.D.	0.668 \pm 0.08	0.515 \pm 0.06
11) HY-227	II	918	301	560	26.5 \pm 9.4	0.720 \pm 0.03	1.230 \pm 0.15
12) HY-304	III	706	224	503	33.0 \pm 5.7	0.538 \pm 0.03	1.035 \pm 0.09
13) HY-417	III	780	190	515	40.8 \pm 12.5	1.090 \pm 0.14	1.561 \pm 0.18
14) HY-432	IV	515	108	390	36.4 \pm 8.5	0.765 \pm 0.05	0.670 \pm 0.08
15) HY-453	II	1,215	412	695	28.6 \pm 5.5	0.480 \pm 0.03	0.812 \pm 0.09
16) HY-512	IV	640	175	406	34.3 \pm 9	0.860 \pm 0.1	1.015 \pm 0.14
17) HY-813	III	814	290	475	N.D.	0.420 \pm 0.03	0.790 \pm 0.09
18) HY-903	II	1,138	220	709	38.5 \pm 6.8	0.415 \pm 0.02	0.563 \pm 0.03
19) ID-CR21	IVc	280	9	168	N.D.	0.630 \pm 0.03	0.702 \pm 0.04
20) ID-CT06	IVc	594	45	386	46.3 \pm 8.2	0.836 \pm 0.09	1.008 \pm 0.07
21) ID-CT98	IVc	389	28	306	41.5 \pm 6.5	0.410 \pm 0.01	0.485 \pm 0.03

Data include relative levels of the peripheral T cell population with subdiploid DNA content. CDC, Centers for Disease Control; PI, propidium iodide; O.D., optical density.

Correlation of Anti-VEINCTR-N Reactivities with Specificities to Fas-related Peptides

ELISA revealed a peculiar pattern of reactivities to these peptides. Figure 1A shows the linear structure with respect to their position on both gp120 and Fas. Therefore, each 8-mer was named as peptide #1 or #2, in relation to its position before or after VEINCTR-N, respectively, within each protein. Figure 1B includes the correlative analyses of anti-VEINCTR-N reactivities in positive sera with relative affinity to each peptide from both proteins. As depicted, no correlation between these reactivities was found in assays using the peptides #1 and #2 of gp120 (up). In both instances, the O.D. values of reactivity to 8-mers revealed a minimal, though unrelated, affinity and the linear regression analysis con-

firmed the lack of correlation between the anti-VEINCTR-N reactivity and each peptide. Conversely, we observed a divergent pattern of reactivities to the peptides derived from the linear sequence of Fas (below). Although the peptide #1 displayed a variable affinity to IgG from several sera with a slight, though definite, correlation of these reactivities with relative anti-VEINCTR-N ($R^2 = 0.131$), no correlation was observed with the O.D. values of specificity to peptide #2 of Fas. These results suggested that sera reactive to VEINCTR-N also showed preferential binding to its antecedent epitope CDEGHGLE, which was located in position 107-114 of Fas, thus, supporting the hypothesis that at least a few a.a. of this stretch could be included within the molecular specificity of anti-VEINCTR-N IgG.

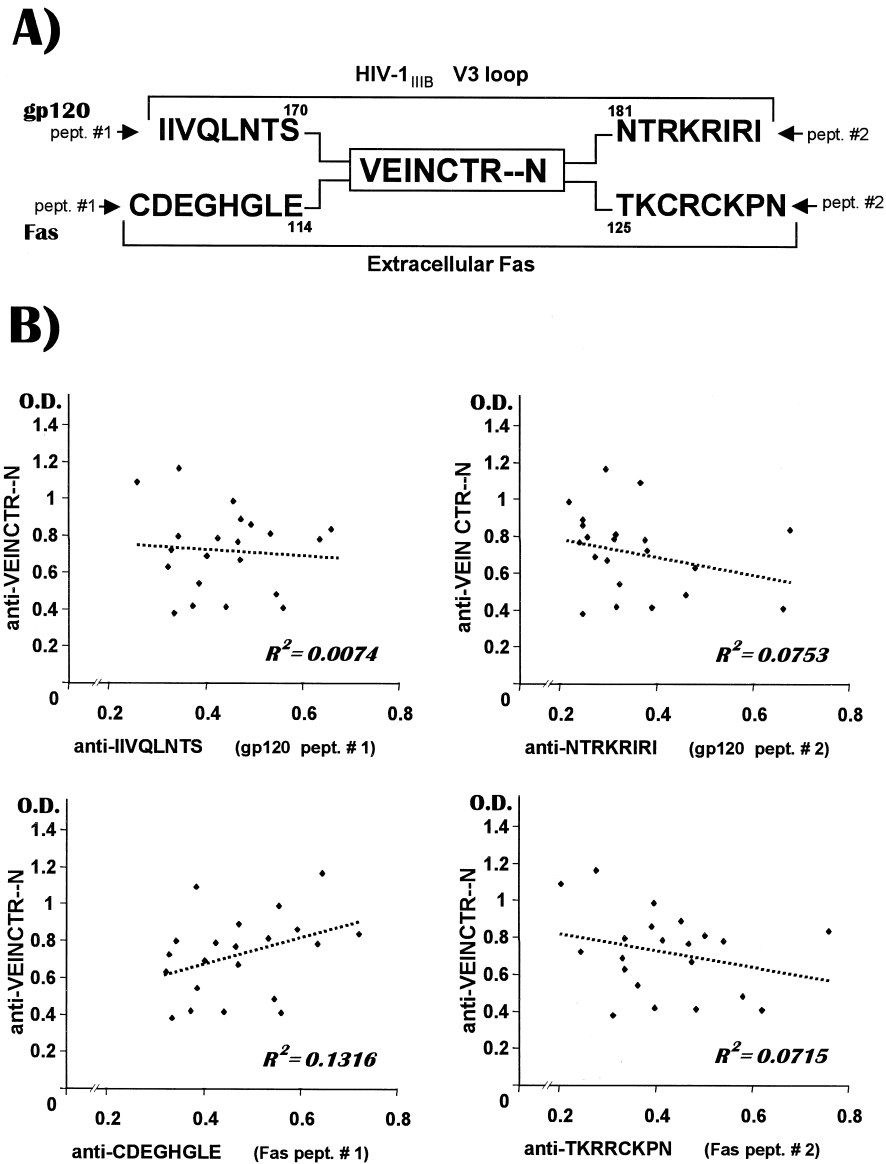


Fig. 1. Primary structure of Fas and HIV-linear peptides. (A) Primary structure of 26 amino acid stretches from both gp120 V3 loop and Fas sharing the homologous sequence VEINCTR–N detected by serum IgG in 21 out of 439 HIV-1-infected individuals. The adjacent 8-mer peptides were synthesized and numbered as peptides #1 and #2 on each protein to evaluate by ELISA their potential involvement in the molecular target of anti-

VEINCTR–N specificities. (B) ELISA correlation of anti-VEINCTR–N activity with that to peptides #1 and #2 from both gp120 and Fas. The only low, though positive, correlation occurred with peptide #1 of Fas; whereas, the reactivities to other peptides were independent of anti-VEINCTR–N positivities. HIV-1_{IIIB}, human immunodeficiency virus-type 1_{IIIB}.

Structural Analyses of Fas and gp120-related Peptides

The next set of experiments investigated the structural variability of VEINCTR–N within both Fas and gp120, in relation to the contiguous peptides on both proteins. Preliminary tests evaluated the biochemical properties of both peptides and Figure 2 illustrates their charge

and relative hydrophathy plots. As shown in Figure 2A, a prevalent positive charge leading to relative hydrophilicity was detected in VEINCTR–N, as well as in the adjacent peptides. The exception was peptide #1 of Fas, which was negatively charged and, therefore, potentially hydrophobic by itself. However, hydrophathy plots in Figure 2B of the 26 a.a.

A) CHARGE

<u>SHARED SEQUENCE:</u>	$\ominus \oplus \oplus \oplus \oplus$ VEINCTR--N	<u>PREVALENCE OF CHARGE</u>
gp120 pept. #1:	$\oplus \oplus$ IIVQLNTS	"
" pept. #2:	$\oplus \oplus \oplus \oplus \oplus$ NTRKRIRI	"
Fas pept. #1:	$\ominus \ominus \oplus$ CDEGHGLE	negative
" pept. #2:	$\oplus \oplus \oplus \oplus$ TKCRCKPN	positive

B) HYDROPATHY PLOTS

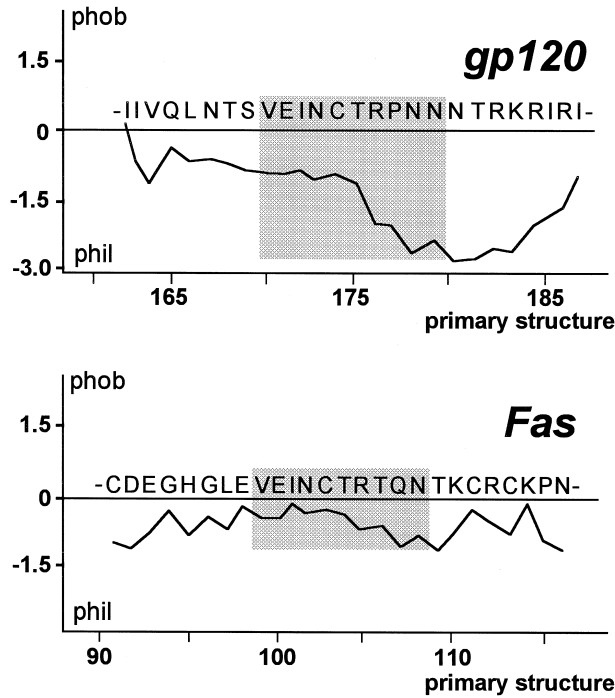


Fig. 2. Analysis of charge and hydrophathy plots of VEINCTR–N and its 8-mer contiguous peptides on both gp120 and Fas. (A) Peptide #1 of Fas shows a prevalence of negative charge, in contrast with other peptides including VEINCTR–N. (B) Both

regions from gp120 and Fas manifest a hydrophilic pattern, though the shared epitope displays higher hydrophilicity in the construct from the virus protein (shaded areas). Pept., peptide.

extended regions of gp120 and Fas (VEINCTR–N plus two 8-mers) revealed the persistence of a negative charge in both proteins, though hydrophilicity values were more pronounced within the epitopes of the viral protein (shaded areas).

The predicted structure of the domains including VEINCTR–N on both gp120 and Fas was also evaluated by combining four algorithms in relation to their primary sequence. Figure 3 shows the results of this analysis, with relative values attributed by the software

to each residue in relation to the possible structure. As represented, the β -sheet conformation was observed on the first four a.a. of VEINCTR–N in both proteins. Interestingly, the peptide #1 of Fas showed a sequence of three a.a. motifs in an adjacent position to VEINCTR–N, which was also assessed as β -sheet in contiguity to the epitope. This result emphasized the hypothesis that the full region of seven residues in β -sheet conformation structure could be immunogenic to the anti-VEINCTR–N IgG.

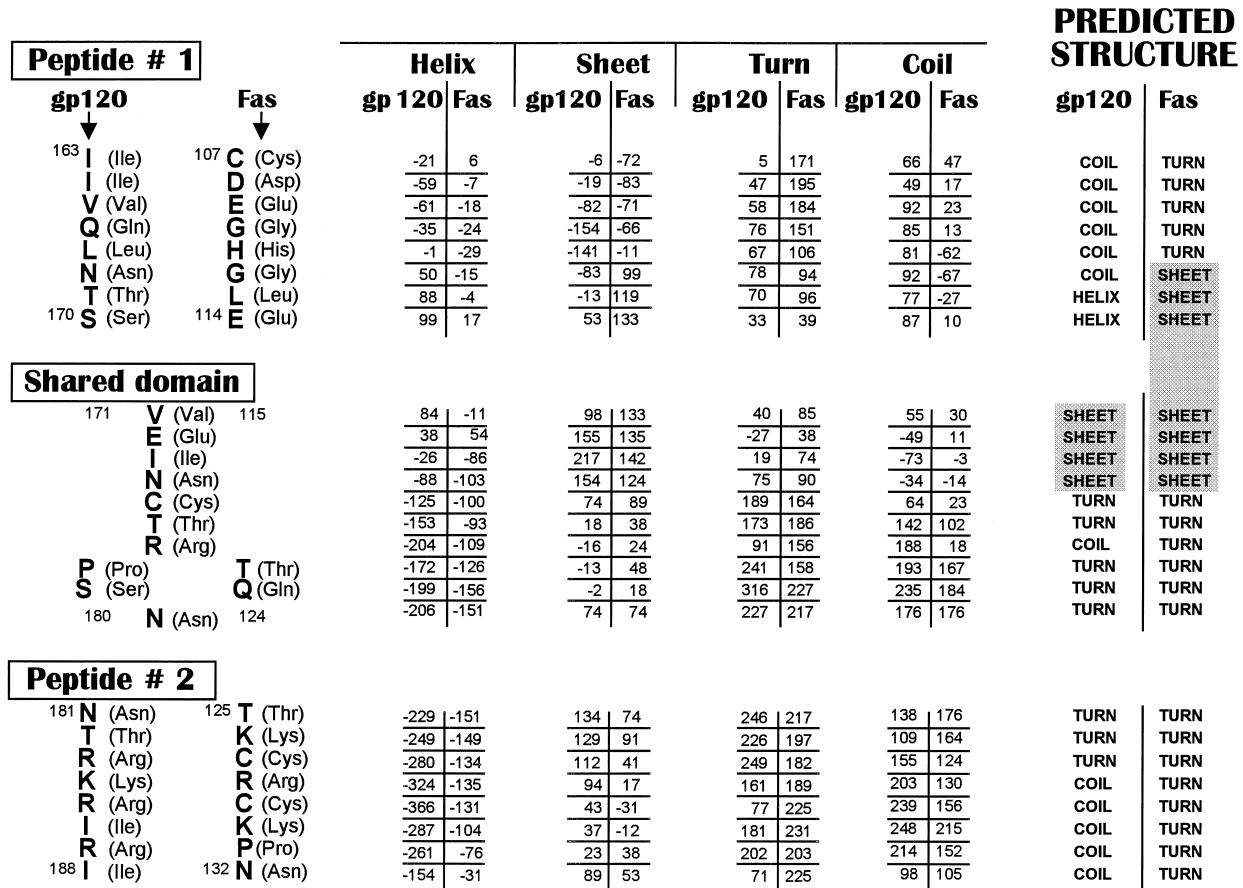


Fig. 3. Structural analysis of multi-peptide domains including VEINCTR-N from both gp120 and Fas, obtained by calculating values of four algorithms of each amino acid residue in relation to the relative primary sequence. The Fas domain was assessed by TSites computer

analysis to show a 7 amino acid (a.a.) sequence in β -sheet conformation composed of three a.a. motifs of peptide #1 in contiguity with a portion of VEINCTR-N. Conversely, the short stretch of four β -conformed a.a. of the shared epitope was isolately expressed within the gp120 domain.

Immunogenicity of the Domain Within the Fas-related Structure

Antigenicity of the polypeptide fragments from both Fas and gp120 was assessed by the Antheprot method (35); whereas, the PCImdad software provided an approach resembling the 3D models. Levels of antigenicity were assigned to each a.a. residue by a scale derived from statistically known epitopes, and Figure 4A shows the relative patterns that included extensive regions of about 50 a.a. residues from both proteins. An apparently similar profile of antigenicity occurred in these regions and directly affected the stretch incorporating VEINCTR-N. However, we observed that the antigenic values of the Fas-related epitope involved the intact domain and two of the β -conformed antecedent residues, namely, Leu 113 and Glu 114. Conversely, antigenicity of

the domain related to gp120 excluded Val 115, Glu 116, and Ile 117 of the shared epitope and was comprehensive of a few subsequent a.a. residues. This analysis suggested that the peculiar position of this epitope could result in potential immunodominance, since it was entirely antigenic within an extended region of Fas. A further structural difference between these domains was related to a discrepancy in Cys residues. The linear sequence of V3 region revealed the presence of a single Cys motif in position 175 within the shared epitope. By contrast, the parallel fragment of Fas evaluated by the Antheprot method (35) (a.a. 100–150) showed as many as 11 Cys residues, predominantly within the highly conserved consensus sequence including VEINCTR-N. Therefore, we verified the homology of this region with the parallel consensus sequence of TNF type I

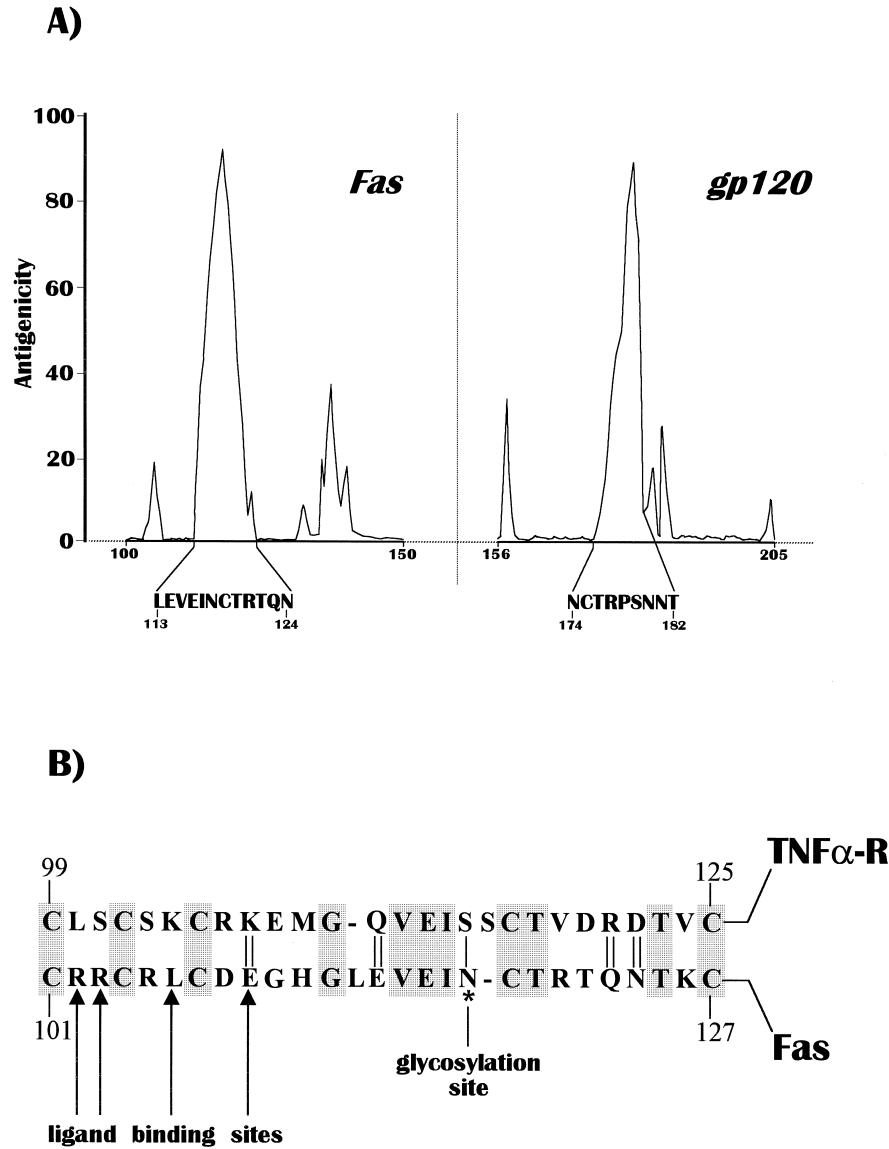


Fig. 4. Antigenicity analysis and linear alignment of the shared peptide on Fas and gp120. (A) Measurement of antigenicity of VEINCTR-N region within 50 amino acid (a.a.) length domains of both Fas and gp120 by the Antheprot method. The computer analysis assigned high antigenicity to a 12 a.a. epitope of Fas, including the intact sequence in contiguity with Leu 113 and Glu 114 of peptide #1. By contrast, similar values of high antigenicity were attributed only in part to the shared epitope on the gp120 domain, since Val 171, Glu 172, and Ile 173 were not antigenic. (B) Linear alignment of a portion of the first consensus sequence of tumor necrosis factor (TNF) type I receptor and Fas incorporating 5 Cys residues in both receptors. Hyphens (-) are introduced to obtain maximal homology. VEINCTR-N was present within the consensus sequence and showed high homology with its parallel domain on tumor necrosis factor- α receptor (TNF α -R). Shaded areas indicate full identity of a.a. residues; whereas, the similarity of charge is depicted by vertical links.

The homology between both stretches was calculated as 42.4% of identity and 60.6% of similarity. Putative glycosylation site is indicated by an asterisk on Asn 118 of VEINCTR-N. The arrows indicate several residues within the consensus sequence of Fas, which are considered relevant for ligand binding to the receptor (39). (C) Three-dimensional molecular model of VEINCTR-N on both Fas and gp120. The conformation related to Fas was obtained by computer evaluation of the high homology with the consensus sequence of TNF α -R. The model also includes the Cys residues (yellow) in identical position, as assembled in disulphide bonds on TNF α -R. This suggests that Cys 119 included within VEINCTR-N is joined to Cys 104 and that the presence of another disulphide link (Cys 107 = Cys 127) in the conformed structure could be critical for antigenicity. Conversely, Cys 175 of gp120 epitope is isolatedly expressed with no involvement in disulphide bonds (carbon atoms are in gray, nitrogen in blue, and oxygen in red, orientation is in green).

Fig. 4. Continued on next page

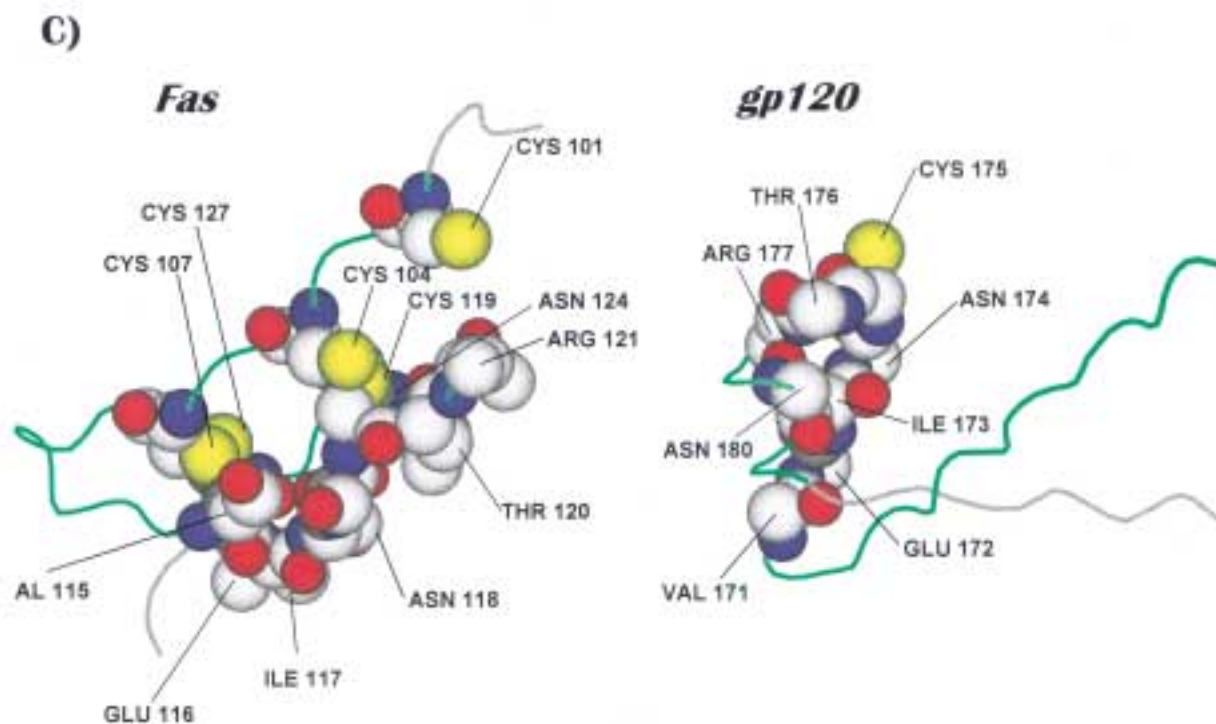


Fig. 4. (Continued)

receptor. Figure 4B shows the pattern of linear alignment. Although the level of sequence identity was calculated as 24.4% between full-length molecules (7), the comparison of 27 a.a. stretches, including 5 homologous Cys residues of the consensus sequence, as well as the VEINCTR-N-related epitope, revealed a high degree of homology; 42.4% of full identity in sequence and 60.6% of similarity based on equivalence of charge in aligned a.a. motifs. This analysis, therefore, confirmed that VEINCTR-N was located in a region of Fas which was conserved highly within its receptor family, and that excess of disulphide links between Cys residues in this domain distinguished such membrane molecules.

The putative immunodominance of the Fas-related domain was also approached by the computer-based structural model depicted in Figure 4C. Construction of models was restricted to the regions investigated in both proteins and included the shared epitope in the median part. The model resembling Fas was optimized by the homology program (37) with TNF α type I receptor, and expressed the conformation related to the consensus sequence with high similarity to the structure of this receptor, especially for the multiple disulphide bonds (left). The model related to the V3 region of gp120 revealed, in fact, a short region, in-

cluding a singularly exposed Cys in position 175 (right). This finding sustained the potential interpretation that unequal exposure of Cys residues could influence antigenicity.

However, although the conformational analysis revealed a potential difference between both domains, the postulated immunodominance of the Fas domain cannot be attributed to simply the presence of multiple disulphide links between Cys motifs, since antigenicity could be further modified by the presence of a glycosylation site on Asn 118. Therefore, its presumptive structural homology to the consensus sequence of TNF α -R as observed here needs crystallographic assessment.

Correlation of sFas Elevations with Anti-VEINCTR-N Levels

Further ELISA investigated the levels of sFas in sera from HIV-1-infected patients with and without anti-VEINCTR-N specificity. To optimize the sensitivity of this assay, we used rFas-Fc as quantitative control and three sera from patients with active SLE as positive controls, in addition to the 19 normal sera included in our ELISAs as negative controls. Figure 5 shows the results. We found an apparent discrepancy of sFas levels in the full HIV-1⁺ population. The majority (18/21) of sera reactive to

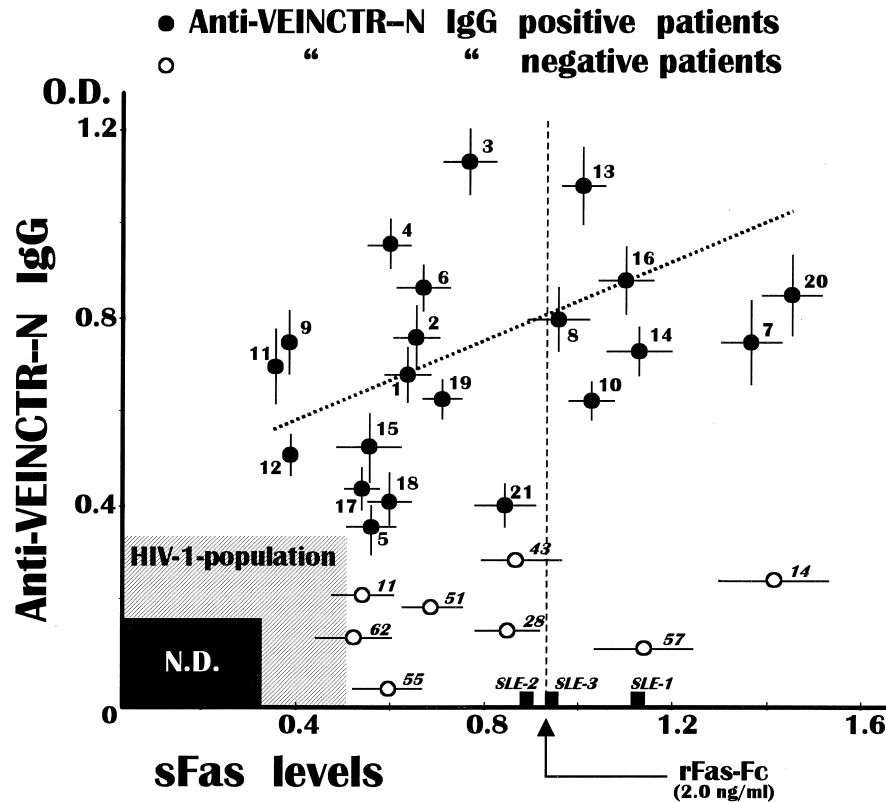


Fig. 5. Distribution of serum levels of soluble Fas in relation to anti-Fas specificities. Serum levels are measured as anti-VEINCTR-N (IgG) activity detected by ELISA in 21 human immunodeficiency virus-type 1 (HIV-1)-infected subjects with definite reactivities to the Fas domain (full symbol) and 67 control patients (empty symbol). Significant titers of the soluble form of the receptor are in linear correlation ($R^2 = 0.168$) with anti-VEINCTR-N levels. In several instances, the amount of sFas was higher than the equivalent value of 2.0 ng/ml of recombinant Fas-Fc (rFas-Fc)

used as quantitative control. By contrast, sFas was significantly present in 8 subjects of the control population and exceeded the reference value only in two instances (pts. #57, and #14). Positive values of sFas were detected in control sera from three systemic lupus erythematosus (SLE) patients. Numbers relate to patients from both groups. The shaded area defines the significance limits ($M+3SD$) of each parameter obtained from the whole HIV-1⁺ population; whereas, the black area refers to those from 19 normal donors.

VEINCTR-N expressed high levels of the soluble form of the receptor, compared with those of both the HIV-1-infected population and normal sera. We also explored the potential relationship between the two values. In several instances, the sFas levels were higher than the 2.0 ng/ml value for the positive control (rFas-Fc) and the linear regression analysis revealed a positive correlation between both parameters within the full group ($R^2 = 0.168$). Conversely, control sera from the anti-VEINCTR-N negative patients showed a prevalent defect of sFas titers, except for 8 of 67 controls (11.9%), whose serum levels of the receptor were higher than the significance limit. These eight sera were re-examined in lower dilutions (1/80, 1/40, and 1/20) in a sensitive ELISA to look for their potential subminimal activity to VEINCTR-N. However, no

significant affinity ($>M + 3SD$) to the peptide was detected in relative tests, confirming that, in this group, sFas levels were independent of the other parameters. As expected, positive controls of sFas levels in SLE sera exceeded the significance limits of both HIV-1-infected population and healthy controls.

These results provided final evidence that significant values of sFas levels occurred predominantly in patients with defined specificity to Fas-related domains including VEINCTR-N in response to its suspected high immunogenicity.

Discussion

This study offers an initial insight into the structural properties of a defined region of Fas

reactive to serum IgG in a number of HIV-1-infected subjects. This domain is also shared by the gp120 V3 loop and was regarded previously as a preferential molecular target of antibodies to HIV-1 (6), as a result of the pronounced immunogenicity of that region within the viral glycoprotein (12). Extensive investigation has confirmed the relative immunodominance of some V3 loop fragments in mouse models by synthetic 8- to 20-mer peptides resembling the primary structure of several domains of the V3 region (38). However, the immunogenicity of VEINCTR-N, namely the epitope shared by Fas, with or without its contiguous 8-mer length peptides, was not evaluated in that study. Since this epitope has been reported to elicit in HIV-1⁺ subjects both a cytotoxic immune response (39) and specific antibodies (6) agonist of T-cell apoptosis through activation of Fas, we investigated the basic immunogenicity of an extensive region including VEINCTR-N on both Fas and gp120 to see whether the immune system detected this domain as HIV-1- or Fas-related antigen.

By exploring serum reactivities to sequential peptides of both proteins, we found that approximately 5% of a large HIV-1⁺ population mainly composed of patients with advanced disease and enhanced T-cell apoptosis showed significant levels of anti-VEINCTR-N IgG. This reactivity is paralleled in most instances by a similar specificity to the full-length rFas and by significant elevations of soluble forms of Fas in sera, supporting the hypothesis that excessive solubilization of the receptor can prime the production of autoantibodies. A striking finding in this context is the predominant reactivity of anti-VEINCTR-N positive sera to peptide #1 of Fas; whereas, no correlation is observed (Fig. 1) for its adjacent peptides on gp120 V3 loop. This difference could indicate that VEINCTR-N is detected as part of a larger domain of the receptor including its contiguous peptide CDEGHGLE (6). On the other hand, a few a.a. residues included in both peptide #1 of Fas and, in a short stretch of seven a.a. in antecedent position, namely, Lys 100, Arg 102, Arg 103, Leu 106, and Glu 109 have been identified recently by Starling et al. (40) as important for ligand binding, thus, supporting our observation of the agonist effect of anti-VEINCTR-N molecules. However, the suspected immunodominance of this region of Fas, with respect to its parallel epitope on the HIV-1 glycoprotein, is not explained suffi-

ciently by the discrepancy in charge within each protein. Although peptide #1 of Fas is the only negatively charged sequence, the constructed 26 a.a. structural epitopes of Fas and gp120 are uniformly hydrophilic and displayed a minimal charge variation.

Structural analysis of these multi-peptide fragments by Parker et al.'s method (35), however, reveal a different pattern of antigenicity for the two epitopes. A prevalent immunogenicity is attributed to the peptide resembling Fas for three reasons. First, a short stretch of three a.a. in β -sheet conformation (GLE) is contiguous to four a.a. residues also conformed in β -sheet, which compose the VEINCTR-N epitope. This may, therefore, originate an extended β -structured region displaying proper antigenicity. Second, the multi-peptide domain shows a relative abundance of Cys residues, which are included in a consensus sequence expressed by members of the TNF receptor family (7). The presence of multiple disulphide bonds within Cys motifs is pivotal in raising the conformational antigenicity of defined domains in proteins (41). And last, the intact VEINCTR-N domain shows high antigenicity, which includes, in part, its antecedent 8-mer on Fas by the Anteprot scale. These findings indicate that, besides the basic difference in linear sequence of this region between Fas and HIV-1, a substantial discrepancy occurs in the conformational structure of both proteins and that the peculiar feature of Fas may account for accretion of immunogenicity in its specific domain. On the other hand, the higher antigenicity of such a Fas-related fragment, compared with the parallel region of gp120, is accurately documented by the ELISAs that explore the fine specificity of VEINCTR-N reactive IgG to the adjacent peptides located on both proteins.

A further interesting aspect of this study is the relation between the putative immunogenicity of the Fas domain and the richness in Cys residues implicated in the consensus sequence. To emphasize the role of Cys in promoting antigenicity, Fadeel and coworkers (41) proved, by elegant studies using multiple synthetic epitopes of Fas reactive to the prototypic anti-Fas MoAb from clone CH11, that intramolecular disulphide bonds between Cys residues at a.a. pos. 84–101, 104–119, and 107–127 within this domain were critical for antibody binding. This region is external to the extracellular death domain and is highly immunogenic, since MoAbs from mouse clones,

including CH11, ZB4 and VB3 bind this epitope with either agonist or inhibitory effect on apoptosis of a few T-cell lines (30). Substitution of single Cys residues in this consensus sequence has been proved to abrogate the molecular binding of some of those MoAbs.

In line with these fine structural analyses, our results support the hypothesis that the peculiar conformation of this consensus sequence could amplify the immunogenicity of this region of Fas, leading to potential immunization. It is conceivable, therefore, that this result could be related to the large amount of soluble receptor released during the course of the infection and/or to its structural degradation by proteolytic enzymes exposing the immunogenic domains. On the other hand, the finding that antibodies to VEINCTR-N are agonist in promoting the cell death in Fas⁺ cells (6) is related to their molecular specificity which, as revealed by the present work, includes specific sites of the Fas-L binding on peptide #1 of Fas (40).

This interpretation is indirectly supported by the comparison of the Fas-related domain with the parallel sequence expressed by the TNF α -R. Our 3D models lack the support of crystallographic definitions of related proteins, which are unavailable. Even so, the computer analysis shows that the VEINCTR-N-associated domain of Fas is highly homologous with the same region of the TNF α -R, because of their identical expression of disulphide bonds among the multiple Cys motifs. Moreover, our observation that antibodies to VEINCTR-N are primarily directed to Fas derives from the observed linear correlation of their levels with circulating titers of soluble Fas. It is possible, however, that the soluble receptor also occurs in serum in complexed form with the anti-VEINCTR-N IgG, since the occurrence of increased levels of circulating immune complexes (42) has been emphasized in HIV-1 disease (43,44). On the other hand, the concomitant presence of serum soluble Fas and related IgG described here is also in line with recent studies describing the occurrence of specificities to TNF α -R in association with variable serum levels of soluble receptor in patients with autoimmune disorders (45) as a result of the chronic T-cell activation, as in HIV-1 disease. By contrast, the anti-TNF α -R antibodies observed in healthy individuals are ineffective, since they are only able to bind the proline-rich epitopes of the receptor in a similar fashion as the natural polyreactive autoantibodies (46).

In conclusion, this work provides evidence that serum anti-Fas specificities in HIV-1⁺ subjects, primarily considered as cross-reacting antibodies, are actually directed to a molecular epitope included in an immunogenic region of the receptor. Their agonist effect on the receptor was demonstrated by ourselves (6) and by others (15). Here we provide evidence of their association with serum elevations of the soluble form of Fas, suggesting that the increased solubilization of the receptor could promote such a humoral immune response, just as increased levels of sTNF type I receptor induce the production of relative antibodies in autoimmunity.

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