JOURNAL OF VIROLOGY, Dec. 1997, p. 9198–9205 Vol. 71, No. 12
0022-538X/97/$04.00+0

The V3-Directed Immune Response in Natural Human Immunodeficiency Virus Type 1 Infection Is Predominantly Directed against a Variable, Discontinuous Epitope Presented by the gp120 V3 Domain

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Received 19 March 1997/Accepted 3 September 1997

The specific binding of antibodies to the V3 loop in sera from human immunodeficiency type 1 (HIV-1)-infected individuals was investigated. Different V3 structures were analyzed as full-length loops or by pepscan. Our data show that on full-length V3 loops, both variable regions on either side of the tip of the loop (GPGRAF) contribute to a common epitope for type-specific antibodies. Type-specific antibodies bound strongly and at high titers to native V3 loops but negligibly once the loop was denatured. In contrast to the type-specific, discontinuous epitope, the linear, conserved epitopes presented by the full-length V3 loop, the tip, the amino-terminal base, and the carboxy-terminal base were not accessible to serum antibody. When the V3 sequences were analyzed with linear peptides, antibodies bound preferentially to peptides containing the conserved GPGRAF sequence. Thus, two different specificities of V3-directed antibodies were detected in patient sera. Unlike group-specific antibodies directed against GPGRAF peptides, lack of type-specific antibodies directed against the discontinuous epitope was correlated with viral escape from autologous neutralization. Our data suggest that the full-length conformation of the V3 loop is accessible predominantly to highly type-specific antibodies present in sera from HIV-1-infected individuals. These antibodies are directed against discontinuous V3 epitopes, not against conserved linear V3 targets. The implications of these findings for viral escape and blockade of infection with V3-based vaccines are discussed.

The V3 loop of human immunodeficiency virus type 1 (HIV-1) gp120 is immunodominant (2, 53) and the major target for neutralizing antibodies (35). In addition, the use of the viral coreceptor CXCR-4 or CCR-5 is determined by V3 sequences of either T-cell line tropic (10) or macrophage tropic viruses (1). The exchange of a macrophage tropic V3 loop for the V3 loop of T-cell tropic strains and vice versa allows the switching of cell tropism (7) and coreceptor usage (5). It is generally accepted that V3-directed antibody can have neutralizing (13, 41) as well as enhancing activity (18). Although the key function of the V3 loop in viral entry has been elegantly shown (5, 43, 50), the significance of V3-directed antibody in blocking receptor binding and controlling HIV-1 replication in vivo is still controversial (6, 19, 23, 25, 32). Whether the levels of neutralizing V3 antibodies correlate directly with the clinical state has not been demonstrated unequivocally (16, 31, 46, 47). In most cases, the antiviral activity directed against the homologous viral strain, the activity type specific to the intrapatient V3 epitopes, was not evaluated. This seems to be important, since the homologous strains would be the most important clinically. However, a probe of the structure of the native gp120 molecule with defined monoclonal antibodies (MAbs) showed that very few conserved, continuous sites were available for antibody binding (29). The V3 loop is especially well exposed on monomeric or oligomeric gp120 and is accessible to various neutralizing MAbs but can simultaneously mask some discontinuous, conserved neutralization epitopes (29, 51). From the work of several groups, it became evident that in natural infection, antibodies are preferentially produced against discontinuous epitopes on the gp120 glycoprotein (28, 30, 44, 45). The V3 loops of clade B strains have a conserved GPGRAF sequence, called tip of the loop, as well as variable sequences on either side (22). Since the V3 loop is hypervariable, both sides create a unique and discontinuous antigenic structure. Therefore, it seems important to study the accessibility of the V3 loop structure to antibodies elicited in natural infection. In some studies, complete V3 structures presented by native gp120 molecules (21) or full-length V3 loops expressed in *Escherichia coli* (36–38) were used as affinity reagents to identify a correlation between neutralizing activity or escape from autologous neutralization and V3 antibody reactivity on a molecular level.

The characterization of HIV-1 variants able to escape from neutralization by V3-directed antibodies has led to the conclusion that the V3 loop is both a linear and a conformational antigenic determinant (8, 26, 48). In previous studies, we reported that the selection and escape of cell-free virus variants (CFV) in AIDS patients is linked to the lack of antibody directed against the V3 loop of the escaped virus (36). By screening serum samples at different stages of the disease, we demonstrated that the lack of type-specific V3 antibody is the result of a continuous and selective antibody loss during disease progression (37). We recently showed that the virus variant, not recognized by V3 antibody, becomes predominant (38). Thus, escape of a unique cell-free variant was shown to be the result of a selectively impaired immune control. In our

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previous studies, the correlation of escape from autologous neutralization and type-specific V3 antibody was identified with full-length V3 structures. However, in studies by different groups, no clear correlation was found between autologous or heterologous virus neutralization and the presence of V3 antibodies (16, 46, 47). In contrast to the methods used in our studies, linear peptides were used as affinity reagents to detect V3 antibodies. Thus, the difference in the observations might be a result of the detection of different V3 antibody subtypes elicited during natural HIV-1 infection.

Therefore, we compared antibody reactivities to conformational and linear V3 loop structures. First, we probed full-length V3 loops expressed as fusion proteins for binding to antibodies present in serum samples from HIV-1-infected individuals. We identified a discontinuous antigenic epitope, represented by both variable regions on either side of the tip of the V3 loop, as the major target for V3-directed type-specific antibodies. Second, we analyzed antibody reactivity to each V3 sequence by pepscan analysis with a 15-mer amino acid window moved by one amino acid at each step. In contrast to the type-specific antibody reactivity against full-length V3 sequences, the minimal condition for antibody binding shown by our pepscan analysis was the presence of the conserved GPGRAF sequence. Importantly, these group-specific antibodies were unable to bind their target sequence when it was presented as part of the full-length V3 loop. Thus, the properly folded full-length V3 loop is accessible to type-specific antibodies but inaccessible to group-specific antibodies directed against the conserved GPGRAF epitope. Our data on the accessibility of the V3 loop compared to that of three-dimensional structures of V3 loops or V3 peptides, based on nuclear magnetic resonance (NMR) or X-ray studies, are discussed.

(This research was conducted by C. Wachsmuth and H. Müller in partial fulfillment of the requirements for a Ph.D. from Hamburg and Bremen Universities, respectively.)

MATERIALS AND METHODS

HIV-1-positive sera. Consecutive serum samples collected from 90 HIV-1-infected German individuals were stored at −70°C. The serum samples from patients were named after coordinates of 96-well plates (A0, A1, . . . B0, B1. . .). All serum samples were anti-HIV-1 antibody positive by enzyme-linked immunosorbent assay (ELISA) and immunoblotting (Pasteur, Paris, France).

Cloning of the V3 loop as a GST-V3 fusion. To study the antibody reactivity of HIV-1-infected individuals directed against full-length V3 loops (Cys to Cys), each V3 loop (C2-03, C2-15, D1-01, D2-15, E1-01, E3-11, F1-01, or F2-06) was cloned and expressed as a fusion with glutathione *S*-transferase (GST-V3) as described previously (37, 38). In brief, the V3 sequences were synthesized as two 109-mer oligonucleotides which form a double-stranded DNA fragment with 5′ GATC and TTAA 5′ overlapping ends. This V3 DNA fragment was cloned directly into the *Bam*HI- and *Eco*RI-digested pGEX3x expression vector. The sequence identity was proven by DNA sequencing.

Purification of GST-V3 fusion proteins. GST-V3 fusion proteins were affinity purified from bacterial lysates by a batch purification method (36, 39). The pGEX3x-V3 transformed *E. coli* DH5α cells were grown to an optical density at 600 nm of 0.7 in 1,000 ml of Luria-Bertani medium. After isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, bacterial growth was continued for 5 h at 37°C. Bacteria were harvested and washed twice with phosphate-buffered saline (PBS). The bacteria pellet was resuspended in GST buffer (1 g/ml; 150 mM NaCl–16 mM Na_2HPO_4–4 mM NaH_2PO_4–1% Triton X-100 [pH 7.5]) and lysed by sonification at 0°C. After centrifugation (20,000 rpm) (SM24; Sorvall), 1 ml of the supernatant was diluted with 4 ml of GST buffer, and 500 μl of a 50% slurry of glutathione agarose beads (Sigma) was added. The beads were washed four times with 5 ml of GST buffer. From the sedimented matrix, the bound GST-V3 protein was eluted in two steps with 500 μl of 5 mM glutathione in 50 mM Tris-HCl (pH 7.5). The purified GST-V3 fusion proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on a 12.5% gel.

Synthesis of V3 peptides. The synthesis of 15-mer V3 peptides immobilized on cellulose membranes was carried out according to the standard spot synthesis protocols (11) with a spot synthesizer (Abimed, Langenfeld, Germany) as described by Kramer et al. (20). The V3 peptide IHIGPGRAFYATGD (amino acid positions 12 to 25 of the V3 loop), designated V3:12–25, was synthesized on an automated 431 peptide synthesizer by 9-fluorenylmethoxycarbonyl chemistry (ABI, Foster City, Calif.). Peptide V3:12–25 was purified by reverse-phase high-pressure liquid chromatography.

Antibody reactivity to GST-V3 fusion proteins. To study the antibody reactivity directed against GST-V3 fusion proteins, 500 ng was applied to each well of a polystyrene 96-well tray (MaxiSorb; Nunc, Wiesbaden, Germany). The trays were stored at 4°C overnight and washed with PBS. Human sera were tested at a dilution of 1:1,000 or in a series of dilutions (100 μl each well for 1 h at 37°C). After four washes with PBS, bound antibodies were detected with secondary goat anti-human immunoglobulin G (IgG)-horseradish-peroxidase (HRP)-conjugated antibodies (1:1,000 dilution, 100 μl in each well for 1 h at 37°C; Bio-Rad), and the trays were washed with PBS containing 0.05% Tween 20. HRP staining was carried out with *o*-phenylenediamine.

Antibody reactivity to V3 peptides. To detect antibodies directed against linear V3 epitopes, synthetic peptides immobilized on cellulose membranes were used (20). The V3 sequence was scanned with a 15-amino-acid window moved by 1 amino acid at each step. Cellulose strips, each containing 21 or 22 spots of 15-mer V3 peptides, were blocked with MNTT buffer (10% low-fat milk, 100 mM NaCl, 10 mM Tris-HCl, 0.05% Tween 20 [pH 7.5]) for 30 min. Serum samples were diluted 1:100 in MNTT buffer and exposed to the cellulose strips for 2 h. Bound antibody was detected with secondary anti-human IgG HRP-conjugated antibodies (1:750 in MNTT; Bio-Rad). Peptide spots were washed three times in NTT buffer (100 mM NaCl, 10 mM Tris-HCl, 0.05% Tween 20 [pH 7.5]) and twice in NT buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.5]). Staining was performed with 4-chloro-1-naphthol (Sigma)–H_2O_2.

Antibody titers directed against the consensus V3 sequence V3:12–25 were tested by ELISA. We used amino-activated 96-well trays that allow covalent binding of the V3:12–25 peptide (CovaLink; Nunc) (40). The peptide was solubilized in water (1 mM), and an equal volume of an aqueous solution of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.1 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added. After 30 min at room temperature, 100 μl of the activated peptide solution was added to each well. After incubation for 18 h at room temperature, the trays were washed three times with PBS. Residual adsorption sites of the wells were blocked with 200 μl of MNTT buffer for 1 h. After a wash with NTT buffer, V3-directed antibody detection was carried out as described before.

Reactivities of V3 antibodies with native and denatured GST-V3 fusion proteins. The reactivities of antibodies with native or denatured proteins were determined according to the method described by Moore et al. (29). In brief, purified GST-V3 protein was denatured by heating for 5 min at 90°C in the presence of 1% SDS and/or 50 mM dithiothreitol (DTT). The native or denatured GST-V3 fusion proteins (500 ng) were bound to nitrocellulose strips with a 96-well dot blot chamber (Bio-Rad). Each strip was blocked with MNTT buffer, and serum was added at a 1:100 dilution. Bound antibodies were determined with anti-human IgG HRP-conjugated antibody as described before.

Three-dimensional model generation. The structures shown in Fig. 6 were retrieved from the Brookhaven database as files 1acy and 1ggi. The visualization of the peptide conformations was done on Silicon Graphics O2 computers with SYBYL software. The structure of E1-01 was obtained by homology modelling from the NMR structure of the Thailand V3 loop published by Gupta et al. (14).

RESULTS

Immune escape of HIV-1 and definition of V3 sequences. To characterize V3-directed antibodies in serum samples of HIV-1-infected individuals, we used V3 sequences of CFV and variants which escaped the autologous antibody response, the so-called infectious CFV (iCFV) (Fig. 1). The V3 sequences of iCFV isolated from the sera of patients D, E, and F (D1-01, E1-01, and F1-01) were not recognized by autologous sera (D0, E0, and F0), in contrast to the neutralized CFV D2-15, E3-11, and F2-06 (36). The V3 sequences of patients D and E differ by only a single amino acid, documenting the high specificity of the V3-directed autologous immune response (D1-01 and D2-15, R10G; E1-01 and E3-11, D26E; Fig. 1). Also, V3 sequences C2-03 and C2-07 are close to each other and reacted positively or negatively with serum antibody. The V3 variant C2-07 showed an A20L amino acid substitution in the GPGRAF motif and was used as an internal control for the detection of antibodies against the tip of the V3 loop, characterized by the GPGRAF or GPGRLF sequences.

V3-directed antibody reactivity against variable, discontinuous V3 epitopes. The V3 sequences shown in Fig. 1 were used as GST-V3 fusion proteins to characterize the V3-directed antibody response against the full-length V3 loop in 90 serum samples from HIV-1-infected individuals. Each of the 90 serum

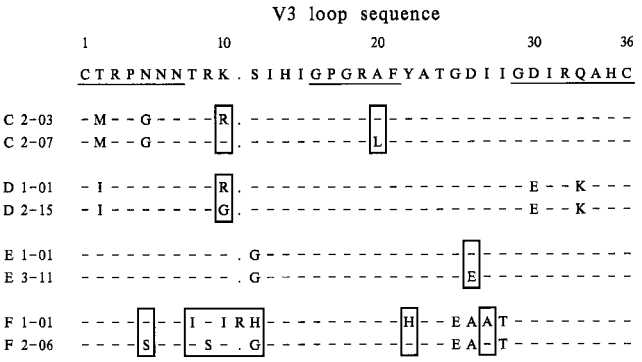

FIG. 1. Sequences of V3 loops used for antibody screening. The V3 sequences were expressed as full-length V3 loops (GST-V3) or synthesized as a series of 15-mer peptides for pepscan analysis. The three conserved secondary structural elements are underlined.

samples was simultaneously positive and negative with different V3 sequences, but all of them contained the conserved IGPGRAFY sequence. Thus, we concluded that the tip of the full-length V3 loop is not accessible to antibodies within the conformation presented by the GST-V3 fusion protein. The result of the screening is exemplified in Fig. 2. We show results of 39 serum samples which reacted either positively or negatively with the paired GST-V3 proteins C2-03 and C2-07, D1-01 and D2-15, E1-01 and E3-11, and F1-01 and F2-06. Sera samples D0, E0, and F0 are autologous serum samples of patients D, E, and F, respectively, which do not react with the V3 sequence of the escaped iCFV (*1-01). In contrast, all three serum samples showed strong reactivity against the V3 sequence of the neutralization-sensitive CFV detected in the patient serum. The screening result demonstrates the high specificity of the immune response, because sera differentiate between closely related complete V3 loop structures. Thus, antibody reacts specifically with V3 sequences that differ by only one amino acid, and little amino acid exchanges, such as D20E between E1-01 and E3-11 or the exchange R10G between D1-01 and D2-15, promote positive or negative reactivity.

From the positive or negative reactivity of closely related GST-V3 proteins, it can be concluded that the center of the V3 loop containing the GPGRAF sequence, which is present in seven of the eight GST-V3 fusion proteins, is not recognized by serum antibody. Thus, V3 antibodies are preferentially di-

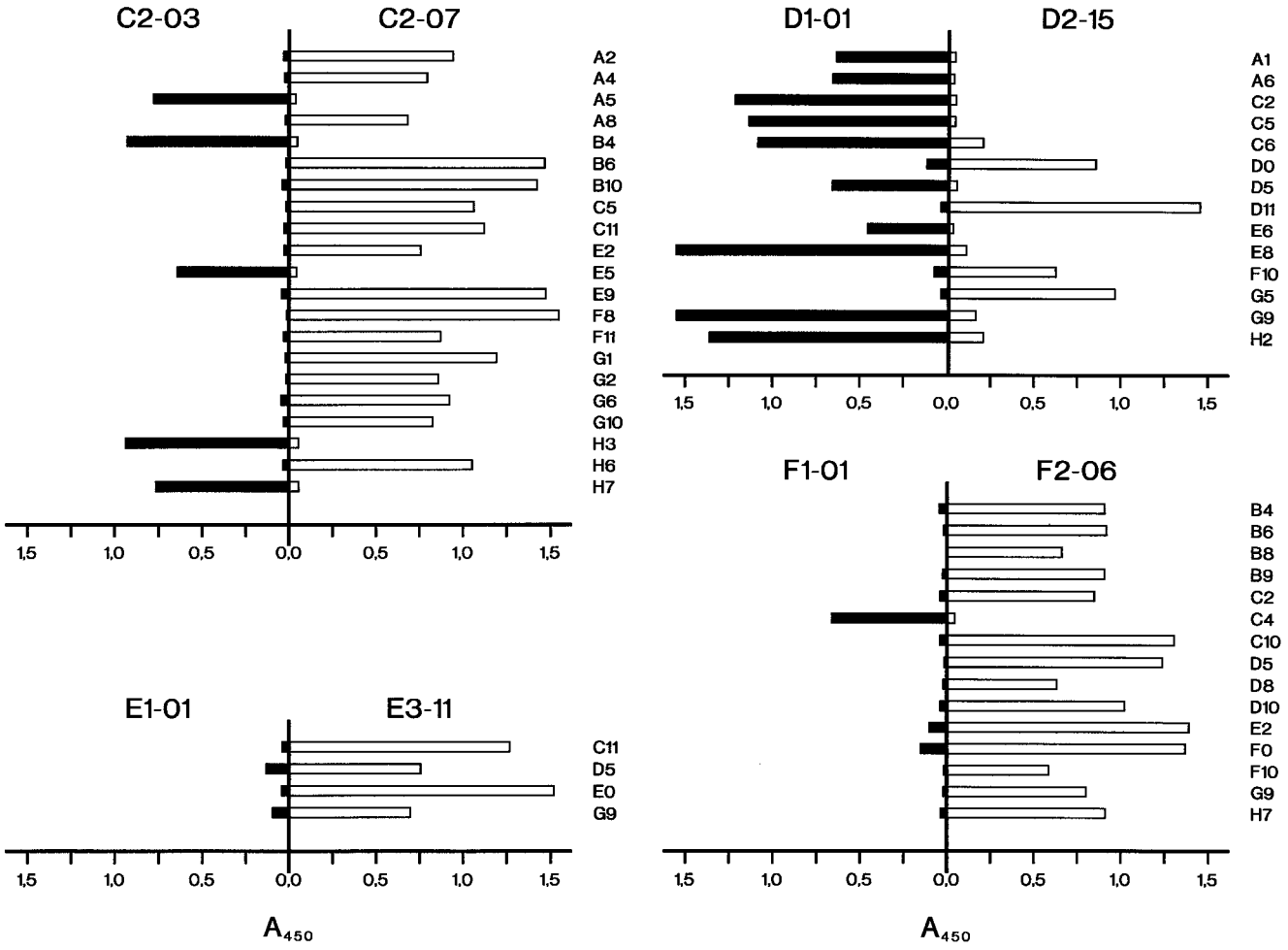

FIG. 2. Reactivity of GST-V3 loop fusion proteins with sera from HIV-1-infected individuals. Five hundred nanograms of each GST-V3 fusion protein was applied to each well of a 96-well plate. Patient serum samples were tested at a 1:1,000 dilution. Black boxes, antibody reactivity to GST-V3 fusion proteins C2-03, D1-01, E1-01, and F1-01; white boxes, V3 reactivity against C2-07, D2-15, E3-11, and F2-06. Results are representative of four experiments.

rected against variable epitopes represented by the two variable regions on either side of the central GPGRAF sequence. It is also evident that neither conserved sequence, the amino-terminal base CTRPNNNTRK nor the carboxy-terminal base GDIRQAHC of the V3 loops is recognized by serum antibody. The precise conformation of the V3 loop that is responsible for human antibody recognition is not certain. However, the V3 loop forms a β-sheet-turn-β-sheet structure, with the GPG sequence as the turn element. We suggest that parts of the V3 loop, like the GPGRAF sequence, are well exposed on native monomeric or oligomeric gp120 but that the conformation of the tip of the loop within the three-dimensional structure of the full-length V3 loop might be immunosilent in vivo.

Detection of V3-directed antibody against conserved, continuous V3 loop epitopes. To determine whether the epitopes mentioned above can be identified on linear V3 loop peptides, we used peptides (15-mer) synthesized on cellulose strips as affinity reagents. Each V3 sequence was scanned with a 15-mer amino acid window moved by one amino acid at each step. The same 39 serum samples used for the screening of the GST-V3 fusion proteins (Fig. 2) were applied to the pepscan analysis for linear V3 epitopes. All sera showed reactivity with peptides containing parts of the central V3 sequence IHIGPGR AFYATGD. The minimal condition for antibody recognition was the presence of the GPGRAF sequence. In contrast to this result, we observed no or weak human antibody reactivity directed against the C2-07 peptides containing the GPGRLF motif. This motif seems to be rare in the viral populations, since it was reactive only with autologous antibodies from patient C0.

In Fig. 3, representative results of the pepscan analysis are shown for sera with positive-negative or negative-positive reactivity to the paired GST-V3 sequences C2-03 and C2-07, D1-01 and D2-15, E1-01 and E3-11, and F1-01 and F2-06. For example, serum sample D5, which showed reactivity with D1-01 but not with D2-15, reacted strongly with the six peptides NNTR(R/G)SIHIGPGRAF, NTR(R/G)SIHIGPGR AFY, TR(R/G)SIHIGPGRAFYA, R(R/G)SIHIGPGRAFY AT, (R/G)SIHIGPGRAFYATG, and SIHIGPGRAFYAT GD. In contrast to the different antibody reactivities obtained with the D1-01 and D2-15 full-length V3s (Fig. 2), differences in reactivity were not observed with the homologous peptides harboring the same R10G amino acid exchange between both GST-V3 sequences (D1-01 and D2-15). Similar results were obtained with sera that recognized only full-length D2-15 but not D1-01, as exemplified by serum sample D11. Identical results were obtained by scanning the E1-01 and E3-11 sequences, which differed by only one single-amino-acid exchange, D26E. Thus, the type-specific immune response documented with the full-length V3s was not seen by pepscan.

In the same way, V3 sequences from patient F (F1-01 and F2-06), with eight amino acid exchanges and variable length, showed no type-specific responses by pepscan analysis (Fig. 3). Again, a type-specific antibody response was detected only with the full-length V3 loops F1-01 and F2-06, as exemplified by the reactivities of serum samples A8 and B6 (both reactive only to C2-07) and of A5 and B4 (both reactive only to C2-03) (Fig. 2). Thus, the conserved GPGRAF motif was shown to be the most important linear target when peptides were used as affinity reagents. It was also observed that C2-07 15-mer peptides, containing the GPGRLF motif, were reactive only with autologous serum C0 but not with heterologous sera (Fig. 3). Again, this result strongly suggests a specificity for the GPGRAF motif in the heterologous sera.

In addition to peptides containing the GPGRAF sequence, 15-mer peptides of V3 loops C2-03 and C2-07 containing the amino acids 2M, 5G, and 10K or 10R were reactive with antibodies. The difference in reactivity obtained might be the result of amino acid exchanges within the conserved CTRPNN NTRK sequence. In all sera tested by pepscan, this conserved sequence and the GDIRQAHC sequence are immunosilent, in contrast to variants of these epitopes that might be present more frequently.

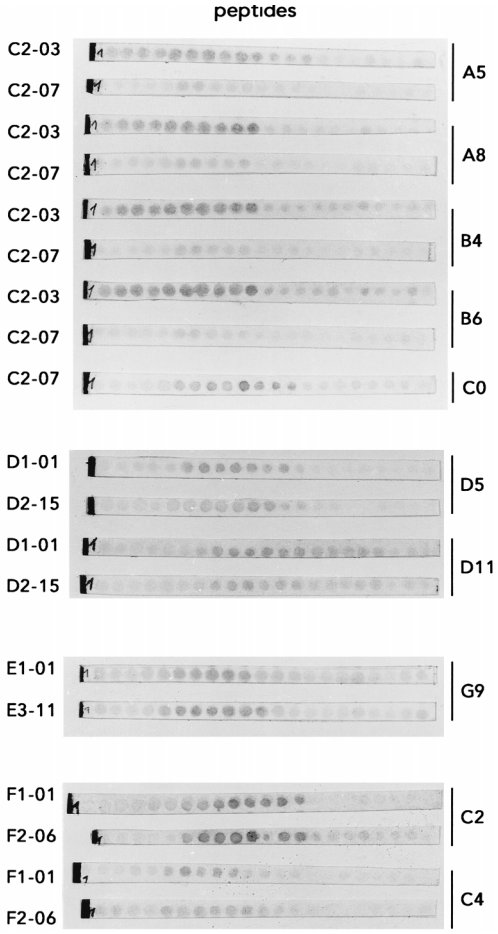

FIG. 3. Spotscan of membrane-bound V3 peptides with sera from HIV-1-infected individuals. Each of the eight V3 sequences (Fig. 1) was scanned with peptides (15 mers) overlapping 14 amino acids. The 35-amino-acid C2-03 sequence was scanned by 21 peptides. The first spot on the left side represents C2-03 peptide CMRPGNNTRRSIHIG, and the last spot represents peptide YATGDIIGDIRQAHC. All peptides were synthesized directly on cellulose strips and incubated with patient serum diluted 1:100 in NTTM buffer. Bound antibody was detected with goat anti-human IgG HRP-conjugated antibody, and 4-chloro-1-naphthol was used as substrate.

Our results for the antibody reactivity directed against the complete conformational V3 loop (GST-V3) demonstrate the presence of highly specific antibodies against closely related V3 sequences. Also, antibodies directed against the conserved GPGRAF sequence were detected in sera from patients. These antibodies bound preferentially to peptides containing the conserved central amino acid sequence GPGRAF.

Comparison of antibody titers against full-length V3 loops and the linear consensus peptide. Endpoint ELISA titers of V3-directed antibodies were measured against all full-length GST-V3 fusion proteins documented in Fig. 1 and a shorter consensus peptide, V3:12–25 (IHIGPGRAFYATGD). In ELISA analyses with 500 ng of the GST-V3 fusion protein, equivalent to 50 ng of full-length V3 loop peptide, we routinely observed

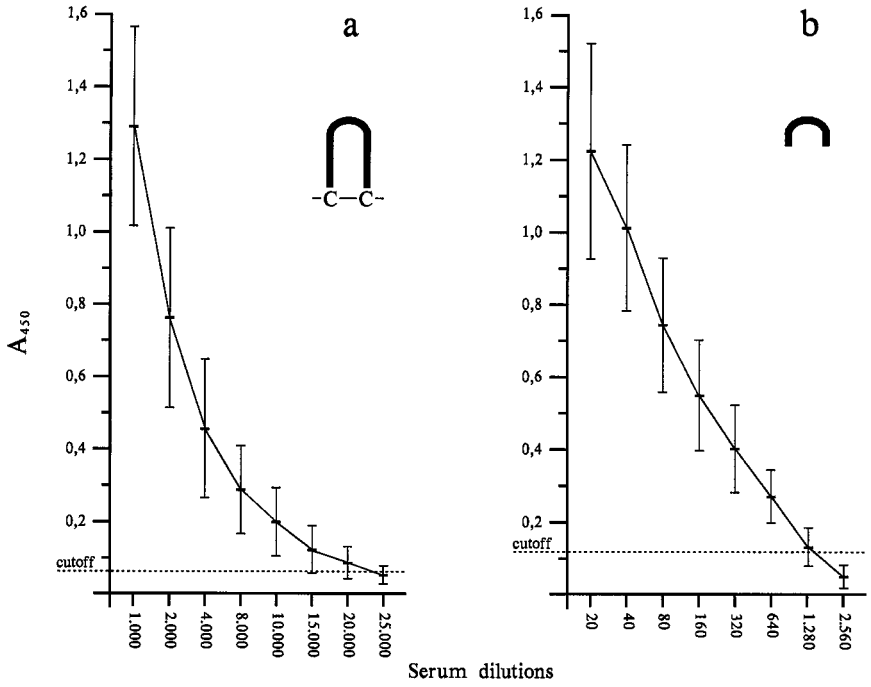

FIG. 4. Endpoint ELISA titers of HIV-1 sera against full-length V3 loops and the 14-residue consensus peptide. (a) Antibody reactivities against the eight GST-V3 fusion proteins. (b) Antibody reactivities against the IHIGPGRAFYATGD peptide.

endpoint titers between 1:10,000 and 1:20,000 (Fig. 4a). To detect antibodies directed against the GPGRAF peptide V3: 12–25, 50 μg was covalently bound to each well of an amino-activated 96-well tray. This procedure was applied to ensure maximum accessibility to antibody. The observed endpoint ELISA titers of the HIV-1 sera against the V3:12–25 peptide used as an affinity reagent ranged between 1:1,000 and 1:2,500. Thus, titers of type-specific V3 antibodies 10-fold higher than those of group-specific antibodies were detected in sera from HIV-1-infected individuals.

Antibody reactivity against native or denatured GST-V3 fusion proteins. To determine whether a V3 epitope, as defined by the amino acid exchange between the paired GST-V3 fusion proteins, was influenced by denaturing reagents, we examined serum reactivity against GST-V3 proteins in the absence of detergent and after denaturation with SDS and/or DTT. In all 90 serum samples investigated, antibodies were detected that bound strongly to native GST-V3 fusion proteins, but binding was significantly reduced once the GST-V3 protein was denatured. These results are exemplified by the analysis of the V3 antibody reactivity in serum samples G9, D5, C11, and E0 (Fig. 5). These sera showed a high specificity for antibody binding to full-length E3-11, while amino acid exchange E26D leading to the E1-01 sequence abrogates all antibody recognition (Fig. 2). Also, in a dot blot assay, V3 antibody bound specifically to the full-length E3-11 V3 loop fusion protein. The binding to the full-length V3 loop was not inhibited by heating at 90°C. In contrast, prior incubation with DTT, SDS, or SDS-DTT led to a complete loss of antibody reactivity. Thus, the loop structure was shown to be heat stable, while the antigenic epitope was destroyed by SDS-DTT denaturation.

These results support the conclusion that the type-specific epitope presented by the V3 loop is discontinuous and can be lost by little amino acid exchanges like D26E, R10K, R10G, and A20L or destroyed by changes of the loop structure by DTT reduction or SDS denaturation. Thus, the epitopes accessible on full-length V3 loops are highly conformational and sensitive to denaturation.

DISCUSSION

Based on data from several groups, it became apparent that, in contrast to sera obtained from HIV-1-infected patients, immune sera generated by gp120/gp160 vaccines are not able to neutralize different primary HIV-1 isolates efficiently (15, 24, 49). It was shown that the inability to neutralize primary isolates was due not only to a quantitative defect in the production of antibody (42). In addition, sera obtained from vaccinees preferentially bound to linear peptides and epitopes accessible within denatured monomeric gp120 (45). In conclusion, there is strong evidence for a basic difference between antibodies elicited by native or recombinant subunit vaccines and those generated by natural infection. Therefore, we were interested in probing the accessibility of the full-length V3 loop to antibodies of HIV-1-immune sera.

In this study, we analyzed the full-length V3 loops expressed and purified from bacterial lysates as a fusion protein. The structure of the V3 loop presented by the GST-V3 fusion is able to bind type-specific antibody for monitoring the selective escape from autologous neutralization (36, 38). The conformation presented by our GST-V3 fusion proteins seems to be identical to one possible conformation of the V3 loop within the native gp120 envelope. The antibodies elicited by natural infection bind the V3 loop with high specificity, since a single point mutation results in the complete loss of reactivity against the particular V3 variant. In a previous study, we monitored the escape of virus variants from autologous neutralization. We observed that variant E1-01 with amino acid D substituted for E (E3-11) has been shown to escape neutralization (36). Also, minor exchanges of G10R between D2-15 and D1-01 led

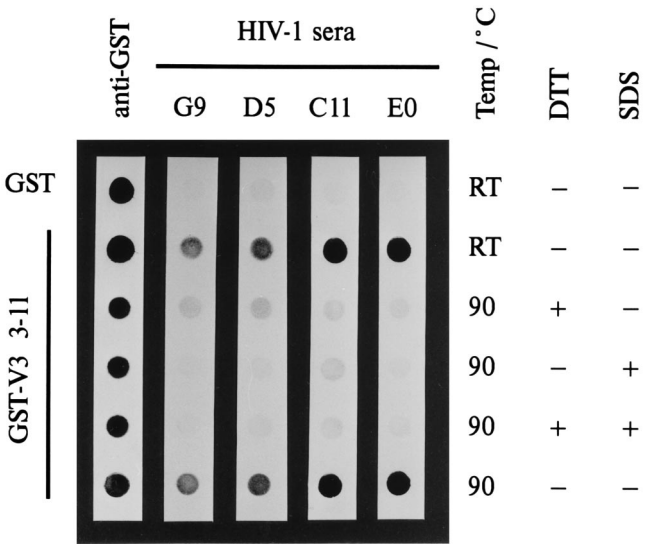

FIG. 5. Reactivities of V3-directed antibodies to native and denatured GST-V3 fusion proteins. Patient sera at a dilution of 1:100 were reacted with native or SDS- and/or DTT-denatured GST-V3 fusion proteins bound to a nitrocellulose membrane. GST-V3 fusion proteins were denatured with 1% SDS and/or 50 mM DTT at 90°C for 5 min. Bound V3-specific antibody was detected with goat anti-human IgG HRP-conjugated antibodies, and 4-chloro-1-naphthol was used as a substrate. Antibodies directed against GST were used as a control.

to a loss of recognition, followed by the escape of D1-01. Simultaneously, all other cell-free variants present in serum were well recognized by V3 antibody. Thus, the presence of neutralized virus in the plasma of AIDS patients was documented. Although iCFVs can be a minority in the cell-free viral population, iCFV escape is correlated with lack of antibody, whereas the majority of cell-free virus is neutralized by autologous serum. Consequently, the iCFV grew out and represented the predominant cell-free virus as well as the predominant virus in T-cells from the spleen and lymph nodes after a period of 1 year (38). These findings are still closely correlated with the autologous type-specific immune response to the full-length V3 conformation presented by the GST-V3 fusion protein. Given the high multiplication rate of HIV-1, it is most probable that each patient contains a specific viral quasispecies inducing a patient-specific immune response of various type-specific antibodies. Therefore, we strongly suggest that type-specific V3 antibodies are an important marker in analyzing autologous neutralization and viral escape in vivo. Besides neutralizing antibodies, the V3 loop is an important gp120 determinant involved in cell tropism and viral entry. Thus, the selective forces of the immune system together with the selection by chemokine receptors are focused in the gp120 V3 region (17, 27, 52). Since the iCFV was by definition the variant that preferentially infected peripheral blood mononuclear cells both in vitro and in vivo, it might also play an important role in vertical transmission.

Several observations suggest that V3-directed antibodies are protective against virus challenge in vivo. One study provided direct evidence that a V3-directed antibody alone is able to protect against virus challenge (9). In the study by Emini et al., chimpanzees were protected by passive immunization with an MAb that was directed to the V3 loop. Since the MAb used by Emini et al. (9) was originally generated in mice, it is not clear if these antibody specificities are induced during natural infection in humans. However, antibodies elicited during natural or experimental immunization are both type and group specific. Early in HIV-1 research, it became apparent that the group-specific neutralizing activity in HIV-1-immune sera was associated with those antibodies that do not bind to native gp120. Also, different variants of the gp120 glycoprotein were inefficient in adsorbing all of the variant- or type-specific antibodies in human serum (15, 33). Thus, a proportion of type-specific antibodies was not adsorbed, probably because of mutated sequences within gp120. Also, group-specific antibody is not adsorbed with native gp120, since these hidden epitopes were exposed only on denatured protein (21, 29).

In our study, we also observed that the group-specific antibodies to the conserved sequences were unable to bind their targets presented by the correctly folded V3 loop. Moreover, we observed that especially the tip of the V3 loop, the GPGRAF sequence, was not recognized by HIV-1-immune sera. Thus, no antibodies recognizing the conserved tip motif were present in the 90 serum samples used in our screening experiments. The conformation of the GPGRAF sequence seems to be responsible for this effect, because linear peptides containing the GPGRAF motif are preferentially recognized.

Our explanation for the exquisite specificity observed with GST-V3 fusion proteins is the S-S-bridged β-hairpin structure (β-strand-type-II-β-turn-β-strand motif). In this conformation, the amino acids flanking the turn motif are placed opposite each other and located like trees on a tree-lined walk (3, 12). Because V3 loops have different flanking residues, each loop will present a unique area of amino acid residues that differs with respect to immunogenicity, even though the V3 sequences are very similar. The discontinuous epitope was sensitive to treatment with DTT or SDS. Since the conformation of the V3 loop was heat stable, only denaturation disrupted the conformation such that no binding of type-specific antibodies was observed.

To further the understanding of the molecular interactions that cause the observed binding specificities, we resorted to published data on three-dimensional structures of V3 loop peptides (Fig. 6). Several groups have investigated three-dimensional structures of different V3 loop sequences by NMR spectroscopy (3, 4, 14) and X-ray diffraction (12, 34). The three-dimensional structures of two V3 peptides in the binding site of neutralizing mouse MAbs were elucidated by X-ray structure analysis. MAb 50.1 (34) recognizes the peptide KRI HIGP, and MAb 59.1 (12) recognizes the peptide IGPGRAF. The turn at the GPG motif is evident in both crystal structures.

A well-defined structure derived by NMR spectroscopy was described for a Thailand HIV isolate V3 loop (14). In contrast, a study of the MN isolate V3 loop (3) resulted in a flexible structure that changes significantly with changes in solvent. Here, two structures are proposed. One conformation in water-trifluoroethanol (70:30) is an S-S-bridged β-hairpin similar to that of the Thailand isolate. The second conformation in water is an open S-S-bridged circle conformation. From this model it is conceivable that the discontinuous epitope is present only in the well-folded V3 loop within the β-hairpin structure, not in an open-circle conformation. All three-dimensional structures discussed show a turn motif at the GPGRAF sequence. However, there are differences between the X-ray data and the NMR structure regarding the orientation of the GPGRAF motif relative to the rest of the V3 loop and the conformation of the GPGRAF motif itself. This becomes evident from Fig. 6 and from comparisons of the published φ angles and ψ angles which deviate from structure to structure by as much as 100° (data not shown). Thus, one could hypothesize that the conserved GPGRAF sequence might be exposed and accessible to antibody in the circular loop and in open-chain peptides but not in a correctly folded V3 loop. Also, the

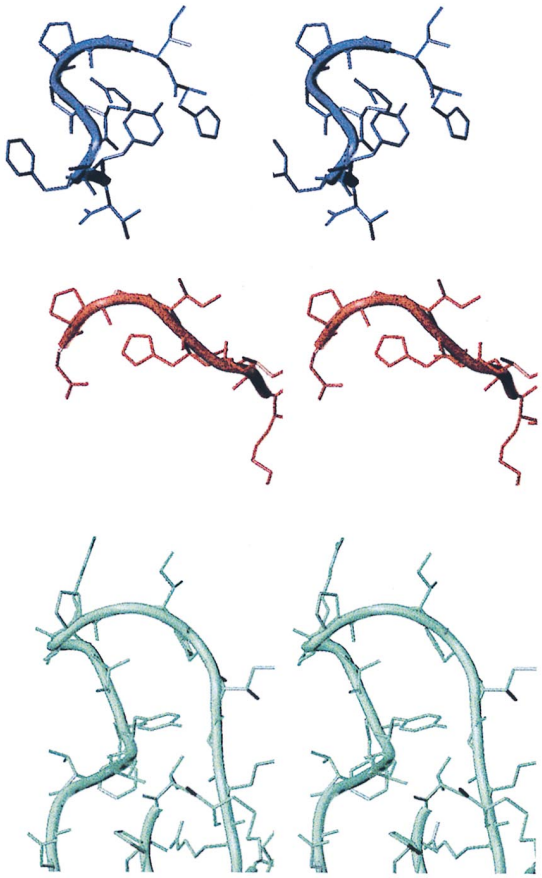

FIG. 6. Three-dimensional models of V3 loop peptides. The conformation of V3 peptides (top and middle) and the structure of the E1-01 V3 loop (bottom) are shown as cross-eyed stereo presentations. The conformation of the two peptides at the top was analyzed by X-ray studies, cocrystallized with MAbs 59.1 and 50.1, respectively (12, 35). The E1-01 conformation was obtained on the basis of NMR data (14). The outline of the backbone and the amino acids are shown as tubes. The three structures are oriented such that the backbone atoms of the peptide sequence HIGP show the best alignment. HIGP is common to all peptides. All three peptides have a loop structure at the proline residue, but there is considerable variation in their relative orientations.

discontinuous epitope which is recognized by the antibodies from human sera is accessible only in the folded β-hairpin structure, not in open-chain peptides.

Indeed, during viral entry, the binding of gp120 to CD4 cells is an important prerequisite for contact with the chemokine coreceptors (43, 50). It was postulated that during this process, a conformational change of gp120 is induced, followed by exposure of hidden gp120 epitopes. One of these epitopes might be the V3 loop that is able to switch from a circular conformation into the known β-hairpin structure. The interaction with CD4 cells and the change of the gp120 structure might be the reason for the different antibody specificities seen in experimental and natural HIV-1 infections.

Our data also demonstrate the presence of antibodies to the conserved sequence, the GPGRAF motif in HIV-1-immune sera. Linear peptides used as affinity reagents showed considerable cross-reactivity. In contrast, we found no binding of group-specific antibody to the full-length V3 loop. Therefore, it can be concluded that these group-specific antibodies are clearly missing in sera from HIV-1-infected individuals. Since the full-length conformation is relevant in binding type-specific antibodies, the various GST-V3 fusion proteins are an important screening mechanism to identify group-specific antibody to conserved epitopes of the correctly folded V3 loop conformation.

In summary, with analysis of the full-length V3 loop of iCFV and non-iCFV, we have been able to increase our understanding of the type- and group-specific immune response to the gp120 V3 loop in natural HIV-1 infection. Finally, it appears reasonable to raise group-specific antibodies to the conserved epitopes of our full-length V3 loops. Even more important is the identification of the structural element that can induce these antibodies in vivo. If these antibodies were able to bind to all of our V3 antigens, they would be important for neutralizing a wide range of HIV-1 strains.

ACKNOWLEDGMENTS

We thank Anette Seifert and Sonja Ziegelmaier for technical assistance.

This work was supported by grant FKZ:01KI 9469/B from the Bundesministerium für Forschung und Technologie and by Sonderforschungsbereich SFB 470 to M.S. C.W. is the recipient of a Wellcome Prize fellowship.

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