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Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees

(acquired immunodeficiency syndrome/envelope glycoprotein 120)

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ABSTRACT **Chimpanzees are susceptible to infection by divergent strains of human immunodeficiency virus type 1 (HIV-1), none of which cause clinical or immunological abnormalities. Chimpanzees were inoculated with one of four strains of HIV-1: human T-lymphotropic virus (HTLV) type IIIB, lymphadenopathy virus (LAV) type 1, HTLV type IIIRF, or an isolate from the brain of a patient with acquired immunodeficiency syndrome. Within 6 months after inoculation with the closely related strains HTLV-IIIB or LAV-1, six chimpanzees developed serum antibodies to the C-terminal half (amino acids 288–467) of the HTLV-IIIB external envelope glycoprotein gp120. Sera from five of those chimpanzees had HTLV-IIIB cell-fusion-inhibiting antibody titers ≥20 at that time, indicating that they neutralized the infecting strain of HIV-1 *in vitro*. No antibodies to the carboxyl terminus of HTLV-IIIB gp120 were observed in sera of chimpanzees inoculated with HTLV-IIIRF or with the brain-tissue strain, and those sera did not neutralize HTLV-IIIB. A rabbit immunized with the C-terminal portion of gp120 acquired neutralizing antibodies that bound to four domains of the HTLV-IIIB external envelope as analyzed by reactivity to 536 overlapping nonapeptides of gp120. One of these domains in the variable region V3, with the amino acid sequence IRIQRGPGRAFVTIG (amino acids 307–321), bound to all chimpanzee sera that neutralized HTLV-IIIB but not to the serum of the HTLV-IIIRF-inoculated chimpanzee that did not neutralize HTLV-IIIB. The HTLV-IIIRF sequence at the same location, ITKGPGRVIYA, was recognized by the serum of the HTLV-IIIRF-inoculated chimpanzee but not by any sera of the HTLV-IIIB-inoculated or LAV-1-inoculated chimpanzees. The HTLV-IIIB residues RIQR and AFV and the HTLV-IIIRF residues lysine and VIYA, flanking a highly conserved β-turn (GPGR), appear to be critical for antibody binding and subsequent type-specific virus neutralization. This neutralization epitope, putatively consisting of a loop between two cysteine residues (amino acids 296 and 331) connected by a disulfide bond, is immunodominant in HIV-1-infected chimpanzees and induces antibodies restricted to the homologous viral strain.**

The efficacy of candidate human immunodeficiency virus (HIV) type 1 vaccines can be studied only in the three anthropoid species susceptible to infection with the virus: humans, chimpanzees, and gibbons. Regions of HIV-1 that might elicit protective immunity in humans are still undefined, although neutralizing epitopes have been shown to reside on the C-terminal portion of the 120-kDa external envelope glycoprotein (gp120) (1).

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Humans who have detectable amounts of cell-free HIV-1 antigen in their serum are at increased risk to develop acquired immunodeficiency syndrome (AIDS) (2–5). Sera of people in that state of aggressive HIV-1 infection typically have low titers of antibodies to the core proteins p17 (6) and p24 (7) and inadequate HIV-1-neutralizing capacity (8–10). Chimpanzees, however, have been infected with several strains of HIV-1 (11–14) without developing clinical or immunological abnormalities during observation periods of 2–5 years. HIV-1 antigen was not detected in the sera of any of the chimpanzees tested (15), antibodies to HIV-1 core protein persisted (16), and sera retained the capacity to neutralize HIV-1 (17–18). All of these findings indicate that chimpanzees can sustain infection with HIV-1 for long periods of time without developing evidence of overt disease. This apparent prolonged latency of HIV-1 infection allows us to study the immunological responses of the chimpanzee that might contribute to the innocuous interaction with the virus.

HIV-1-neutralizing antibodies appeared in sera of infected chimpanzees as soon as did HIV-1-binding antibodies detected by ELISA or by immunoblotting with antigens of the virus strain used in the neutralization assay (18). A correlation between neutralization and binding of antibody to HIV-1 gp120 was suggested, and this neutralizing-antibody response broadened with time (17–19).

MATERIALS AND METHODS

Sera. Sequential serum samples (12, 15) were taken from three chimpanzees (A3D, A86B, and A251) that had been inoculated with the human T-lymphotropic virus (HTLV) strain HTLV-IIIB of HIV-1 and from one (A22) that had been inoculated with the lymphadenopathy virus (LAV) strain LAV-1. Two chimpanzees (A3A and A243B) were inoculated with blood from chimpanzee A22, and one (A304) was inoculated with blood from chimpanzee A243B. One chimpanzee (A233) was inoculated with the HIV-1 strain HTLV-IIIRF and one (A3), with brain tissue of an AIDS patient with encephalopathy. The nine chimpanzees were observed for 13–24 months, and 67 serum samples studied.

Expression and Purification of the HIV-1 ENV Gene Products. The N-terminal and C-terminal portions of the HIV-1 envelope-encoding sequence were expressed as fusions to the 5′ coding region of the *Escherichia coli* galactokinase gene, using the bacterial expression vector pOTSKF33, a derivative of the pAS vector (20) in which the first 56 codons

Abbreviations: HIV-1, human immunodeficiency virus type 1; AIDS, acquired immunodeficiency syndrome; aa, amino acids; HTLV, human T-lymphotropic virus; LAV, lymphadenopathy virus; PEPSCAN, peptide scanning; CFI, cell-fusion inhibiting.

of galactokinase are inserted downstream from and in-frame with the translation initiation codon.

An N-terminal env-encoding fragment extending from the *Kpn* I site to the *Stu* I site [amino acids (aa) 42–204; designated 120N] and a C-terminal env-encoding fragment extending from the *Pvu* II site to the *Bgl* II site (aa 288–467; designated 120C) were prepared from gp120 of HTLV-IIIB, clone BH10 (21). (In this report the amino acids of gp120 are numbered starting with the first methionine in the env open reading frame designated as 1.) The 120N and 120C fragments were inserted in pOTSKF33 downstream from and in-frame with the N terminus of galactokinase. The proteins produced were purified and used to immunize rabbits as described (22).

ELISAs. Wells of microtiter ELISA plates were coated with whole viral extract as described (7) or with 100 μl of purified recombinant protein (1 μg per well) in phosphate-buffered saline (PBS) for 16 hr at room temperature. Then, they were washed with PBS/Tween 20 (0.1%), incubated for 1 hr at 37°C with 4% normal goat serum in PBS/Tween 20 (0.1%), and again washed with PBS/Tween 20. Next, 100 μl of a 1:100 dilution of serum was added, and the wells were incubated for 1 hr at 37°C, washed, incubated with biotinylated goat anti-human IgG heavy and light chains (Zymed Laboratories, Burlingame CA) for 1 hr at 37°C, and washed again. Then, streptavidin-biotinylated horseradish peroxidase complex (Amersham, Buckinghamshire, U.K.) was added, and the wells were incubated for 1 hr at 37°C. After another washing step, bound antibodies were visualized with *o*-phenylenediamine as substrate. The cut-off OD was taken to be mean OD + 2 SD for 20 seronegative samples.

HIV-1 Neutralization Assay. Sup-T1 cells ($1.5–2 \times 10^5$ cells per well) were attached to 96-well microtiter plates as described (12). The medium was removed and human B cells infected with HTLV-IIIB (B24+ cells, washed twice with PBS) were added ($5–25 \times 10^3$ cells per well) in 100 μl of Iscove's modified medium/10% (vol/vol) fetal bovine serum and containing 1:3 dilutions of heat-inactivated (30 min at 56°C) test serum. Reciprocal dilutions of test serum were 20, 60, 180, and 540. After incubation at 37°C for 20 min the plates were centrifuged at low speed for 10 min at 37°C. All experiments were done in duplicate, and each plate included immune and negative control test sera. Syncytia were counted after 24 hr of incubation at 37°C. The dilution of serum producing a 50% reduction of syncytia after 24 hr was taken as the titer of cell-fusion-inhibiting (CFI) antibody.

Antibody-Reactive Peptide Scanning. Overlapping 9-aa-long peptides of HIV-1 gp120 were synthesized and tested as described (23, 24) to determine the reactivity patterns of antisera. In short, scanning for antibody-reactive peptides (PEPSCAN) required the synthesis of 536 overlapping peptides in the gp120 sequence of 545 aa residues. Peptide 1 consists of residues 1–9, peptide 2 of residues 2–10, and so on. The amino acid sequences were derived from the nucleotide sequences of HIV-1 strains HTLV-IIIB (clone BH10) and HTLV-IIIRF (Los Alamos National Laboratory compilation of amino acid sequences of HIV isolates, 1987). The peptides, still coupled to solid supports, were tested against appropriate sera by ELISA. Absorbance values were plotted against the position of the N-terminal aa of the peptide in the total sequence.

RESULTS

Virus-Neutralizing Capacity and Recognition of the N-Terminal (aa 42–204) and C-Terminal (aa 288–467) Portions of HIV-1 gp120. Analysis of sequential sera from HIV-1-infected chimpanzees showed that 72% (48/67) of all anti-HIV-1-positive sera had antibodies to 120C and 67% (45/67) had antibodies to 120N. However, OD/cut-off ratios (OD of the sample divided by the cut-off OD) >8 were seen in 66% (44/67) of the samples in the 120C ELISA but in only 10% (7/67) of the samples in the 120N ELISA. Although neutralizing antibodies were detected almost as frequently in sera containing ELISA antibodies to the the 120N peptide (69%; 31/45) as in sera with antibodies to the 120C peptide (73%; 35/48), marked differences were found when the presence of neutralizing antibodies was correlated with the amounts of ELISA antibodies detected. As shown in Fig. 1, OD ratios ≥ 8 in the 120C ELISA were found in 100% (35/35) of the neutralizing-antibody positive sera but in only 9 samples (28%) that did not contain neutralizing antibody. In the 120N ELISA only 14% (5/35) of the neutralizing-antibody-positive samples and 6% (2/32) of the neutralizing-antibody-negative samples had OD/cut-off ratios ≥ 8. ELISA antibodies to proteins extracted from whole virions of HIV-1 (HTLV-IIIB) appeared in all nine animals included in the study within 3 months after inoculation (Table 1). ELISA antibodies to 120C were detected at the same time as antibodies to whole virions in all HTLV-IIIB-inoculated and LAV-1-inoculated animals, and those antibodies persisted throughout the study period; HTLV-IIIB-neutralizing antibodies also developed in the same chimpanzees within the first year after inoculation. ELISA antibodies to 120N developed in all HTLV-IIIB-inoculated and LAV-1-inoculated animals by the second year after inoculation. In the HTLV-IIIRF-inoculated and brain-inoculated chimpanzees ELISA antibodies to protein extracts of whole HIV-1 virions (HTLV-IIIB) developed as rapidly as they did in the HTLV-IIIB-inoculated and LAV-1-inoculated animals. ELISA antibodies to 120N developed simultaneously in the brain-inoculated animal, but neither ELISA antibodies to the 120C peptide of HTLV-IIIB nor HTLV-IIIB-neutralizing antibodies were detected in sera of either of these chimpanzees within the first year after inoculation (Table 2; Fig. 1).

Sera of the brain-inoculated chimpanzee (Fig. 2) recognized the N-terminal segment of HTLV-IIIB gp120 early after inoculation and later sera continued to bind to the 120N peptide with relatively high OD ratios, but the same sera did not have significant reactions with the C-terminal part of HTLV-IIIB. This contrasted with the antibody responses of HTLV-IIIB-inoculated or LAV-1-inoculated chimpanzees;

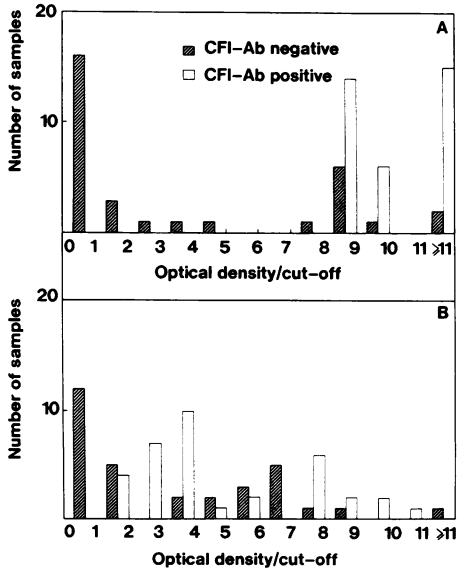

FIG. 1. Reactivity of chimpanzee sera to the 120C peptide (*A*) and the 120N peptide (*B*) of the gp120 of HTLV-IIIB as determined by ELISA. The amounts of ELISA antibodies present in the sera are indicated by sample OD/cut-off OD ratios. Samples with virus-neutralizing antibodies (CFI-Ab positive, see Table 2 and text) are presented separately from samples without detectable neutralizing antibodies (CFI-Ab negative).

Table 1. Development of neutralizing and gp120-binding antibodies to HIV-1/HTLV-IIIB in sera of HIV-1-infected chimpanzees

Time after inoculation, months	HIV-1 inoculum	ELISA			Neut. CFI
		Whole virus	120N	120C	
1 to 3	HTLV-IIIB	3/3	1/2	2/2	1/2
	LAV-1	4/4	1/3	3/3	1/3
	HTLV-IIIRF	1/1	0/1	0/1	0/1
	Brain isolate	1/1	1/1	0/1	0/1
4 to 6	HTLV-IIIB	3/3	2/3	3/3	2/3
	LAV-1	3/3	3/3	3/3	3/3
	HTLV-IIIRF	1/1	0/1	0/1	0/1
	Brain isolate	1/1	1/1	0/1	0/1
7 to 12	HTLV-IIIB	3/3	2/2	3/3	3/3
	LAV-1	4/4	3/4	4/4	4/4
	HTLV-IIIRF	1/1	0/1	0/1	0/1
	Brain isolate	1/1	1/1	0/1	0/1
13 to 24	HTLV-IIIB	3/3	3/3	3/3	3/3
	LAV-1	3/3	3/3	3/3	3/3
	HTLV-IIIRF	1/1	NT	NT	1/1
	Brain isolate	1/1	1/1	0/1	0/1

Results are presented as number of serum samples positive/total number of serum samples tested. Inocula are described in the text. Whole virus ELISA, antigen prepared from detergent-dissociated HIV-1 particles (7); 120N ELISA and 120C ELISA, antigens prepared from the recombinant N-terminal and C-terminal polypeptides of gp120 of HIV-1/HTLV-IIIB. Neut. CFI, cell fusion inhibition [neutralization of infectivity, (12)]. NT, not tested.

only marginally positive reaction with the 120N polypeptide was found by ELISA in serum collected from a chimpanzee (Fig. 3) 6 months after inoculation with HTLV-IIIB, while relatively high titers of neutralizing antibodies and high OD ratios of antibodies to the 120C polypeptide were noted.

Delineation of the Neutalization Epitope in HIV-1 gp120. Rabbits were immunized with the 120N and 120C polypeptides. The anti-120N rabbit serum did not have HIV-1-neutralizing activity, whereas the anti-120C rabbit serum neutralized HTLV-IIIB at a 1:10 dilution. The nonneutralizing serum of the 120N-inoculated rabbit bound to four domains located in the N-terminal portion of gp120: ASDAKAYDTEVHNV (aa 55–68), HACVPTDPNPQEV (aa 72–84), PQEVVLVNVTEN (aa 81–92), and KNDMVEQMHEDIIS (aa 97–110) (Fig. 4). The neutralizing serum of the 120C-inoculated rabbit bound strongly to four domains of the carboxyl-terminal portion of gp120: NNNTRKSIRIQRG (aa 300–312), IQRGPGRAFVT (aa 309–319), EGSDTITLPCRI (aa 409–420), and NSNNESEIFRPG (aa 460–471).

Table 2. Neutralizing and gp120-binding antibodies to HIV-1/HTLV-IIIB in sera of HIV-1-infected chimpanzees

HIV-1 inoculum	Animal	Neut. CFI titer	ELISA		gp120 PEPSCAN
			120N	120C	
LAV-1	A22	180	+	+ + +	+
	A3A	180	+ +	+ + +	+
	A243B	60	+ +	+ + +	+
	A304	540	−	+ + +	+
HTLV-IIIB	A3D	60	+ +	+ + +	+
	A86B	60	+	+ + +	+
	A251	20	+ +	+ + +	+
HTLV-IIIRF	A233	<20	−	−	−
Brain isolate	A3	<20	+ +	−	−

HIV-1 inocula and 120N and 120C ELISA are as in Table 1. CFI titer, reciprocal of serum dilution producing 50% inhibition of HIV-1/HTLV-III-induced cell fusion. ELISA results are expressed as follows: −, sample OD/cut-off OD ratio < 2; +, sample OD/cut-off OD ratio 2–3; + +, sample OD/cut-off OD ratio 3–8; + + +, sample OD/cut-off OD ratio > 8. PEPSCAN, ELISA reactivity with nonapeptides derived from aa 307–321 of gp120 (23, 24).

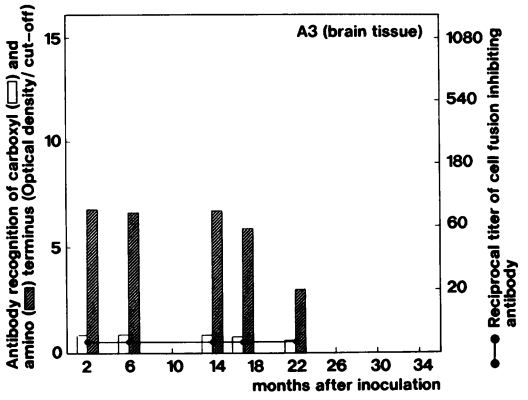

FIG. 2. Comparison between the temporal development of ELISA antibodies to the N-terminal and the C-terminal polypeptides of the gp120 of HTLV-IIIB and the development of virus-neutralizing antibodies (measured by CFI) in sera of a chimpanzee (A3) at intervals after its inoculation with brain tissue from an AIDS patient with progressive encephalopathy.

Sera of chimpanzees infected with the HIV-1 strain originating from brain tissue or with HTLV-IIIRF did not neutralize HTLV-IIIB nor were they bound to the C-terminal portion of HTLV-IIIB gp120. The PEPSCANs of those sera showed no peptide recognition at the serum dilution used (1:250) (Table 2). All HTLV-IIIB-neutralizing sera of both HTLV-IIIB-inoculated and LAV-1-inoculated chimpanzees bound to the recombinant C-terminal portion of HTLV-IIIB gp120 and in particular to the sequence IRIQRGPGRAFVTIG (aa 307–321) (Table 2; Fig. 4).

Type-Specificity of HIV-1-Neutralizing Antibodies Binding to the aa 307–319 Sequence of gp120. Overlapping nonapeptides corresponding to the sequence between the conserved cysteine residues (aa 296 and 331) of HTLV-IIIB and -IIIRF were synthesized and used to study the specificity of chimpanzee antibody binding to divergent regions of gp120 (Fig. 5).

Sera from HTLV-IIIB-inoculated and LAV-1-inoculated chimpanzees bound exclusively to the HTLV-IIIB sequence IRIQRGPGRAFV(TIG) with similar specificities. The corresponding region of HTLV-IIIRF was not recognized, although five amino acids in that region of gp120 are identical in both strains of HIV-1. Serum from the HTLV-IIIRF-inoculated chimpanzee recognized exclusively the HTLV-IIIRF sequence ITKGPGRVI(YAT) and neutralized only HTLV-IIIRF (data not shown), indicating that the HTLV-IIIRF-neutralizing epitope has the same location on the viral

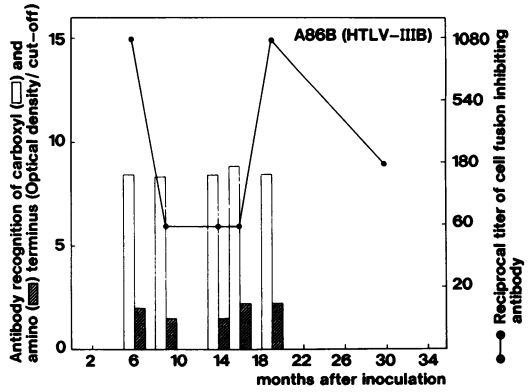

FIG. 3. Comparison between the temporal development of ELISA antibodies to the N-terminal and the C-terminal polypeptides of gp120 and the development of CFI (neutralizing) antibodies in a chimpanzee (A86B) inoculated with HTLV-IIIB.

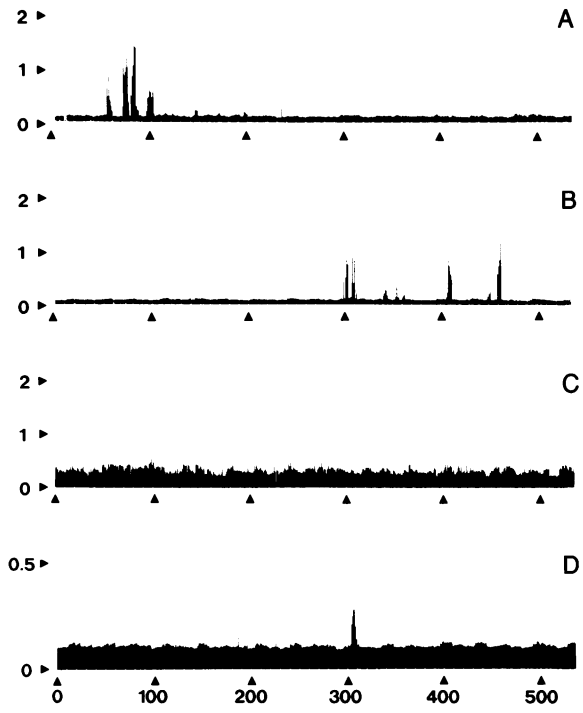

FIG. 4. PEPSCANs for the gp120 protein of HTLV-IIIB. A series of 536 overlapping nine-aa-long peptides were synthesized on polyethylene supports; each was tested for reactivity by ELISA against a panel of sera as described (23, 24). A_{450} values obtained by testing a serum with each peptide are plotted vertically. Prior to retesting, bound antibodies were removed by washing the peptides on their supports three times at 37°C with 8 M urea/0.1% 2-mercaptoethanol/0.1% $NaDodSO_4$ and then several times with phosphate-buffered saline. The numbers on the horizontal axis correspond to the N-terminal amino acid of the nonapeptides. (*A*) Serum (1:1000 dilution) of a rabbit immunized with the 120N peptide of the same strain. (*B*) Serum (1:1000 dilution) of a rabbit immunized with the 120C peptide of the same strain. (*C*) Negative human control serum (1:50 dilution). (*D*) Serum (1:250 dilution) from a chimpanzee infected with LAV-1.

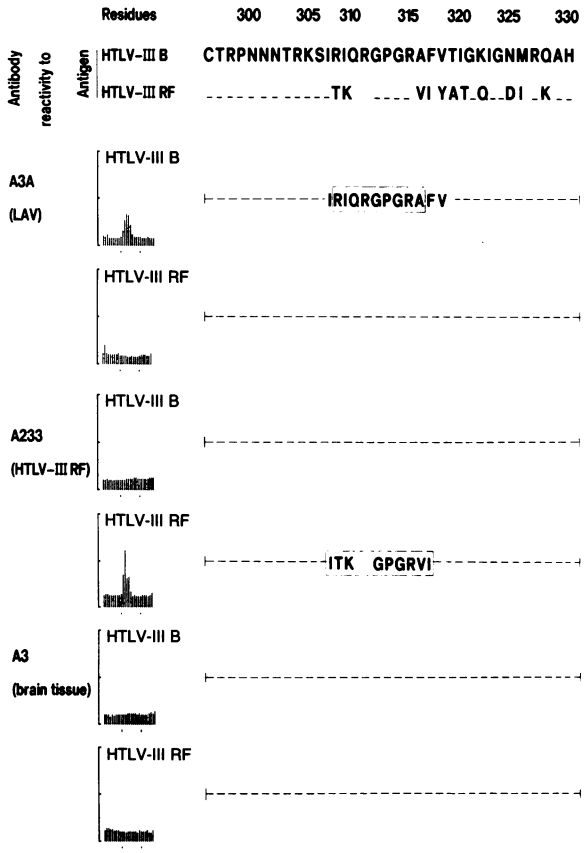

FIG. 5. Reactivity of sera to overlapping nonapeptides covering the region between the cysteine residues at positions 296 and 331. Sera were from one chimpanzee inoculated with LAV-1 (A3A), one inoculated with HTLV-IIIRF (A233), and one inoculated with brain tissue of an AIDS patient (A3). The methodology is described in Fig. 4.

envelope as does the HTLV-IIIB epitope. Serum from a chimpanzee inoculated with brain tissue of an AIDS patient, and also persistently infected with HIV-1, did not bind to either the HTLV-IIIB or the HTLV-IIIRF sequences in the region of aa 296–331 nor did that serum neutralize HTLV-IIIB or HTLV-IIIRF (data not shown).

DISCUSSION

Both the N-terminal (aa 42–204) and the C-terminal (aa 288–467) segments of gp120, the major envelope protein of HIV-1, were antigenic in experimentally infected chimpanzees. Remarkably, the chimpanzee inoculated with HIV-1-infected human brain had an early antibody response to the gp120 N terminus of HTLV-IIIB, indicating that antigenic N-terminal domains of gp120 are constant among divergent strains of HIV-1. Modrow *et al.* (25) have predicted that only one antigenic epitope would be found within the boundaries of this fragment, which is variable and located within the V1 region (aa 127–151). Constant epitopes within the C1 region (aa 30–125) would better account for the data in our study. The fact that immunization of rabbits with the recombinant N-terminal protein induced antibodies that bound exclusively to constant domains within this particular region supports that notion. Antibodies to the gp120 C terminus were generally observed earlier after inoculation than were antibodies to the gp120 N terminus, and antibodies to 120C persisted in larger amounts, indicating that the C-terminal fragment used in the present study contains immunodominant domains. No antibody response to the gp120 C terminus of HTLV-IIIB was detected in chimpanzees inoculated with HTLV-IIIRF or with HIV-1-infected human brain, which indicates that the antigenic domains within the gp120 C-terminal fragment are variable and confirms the predictions of Modrow *et al.* (25). All sera with HTLV-IIIB-neutralizing antibodies had high OD/cut-off ratios of ELISA antibodies to the C terminus of gp120, indicating that variable neutralizing epitopes are immunodominant in chimpanzees. This may explain the previously reported relationship between the intensity of gp120 bands on immunoblots and virus-neutralizing antibodies in chimpanzees (18).

A nonglycosylated segment of gp120 encompassing a region similar to the gp120 C-terminal fragment that we studied, and also expressed in *E. coli*, induced type-specific HIV-neutralizing antibodies in goats (1). We confirmed those findings in rabbits using the gp120 C-terminal fragment as immunogen. The neutralizing rabbit serum recognized two domains in variable region V3, one in variable region V4, and one in variable region V5, three epitopes predicted by Modrow *et al.* based on the secondary structure of gp120 (25). Synthetic peptides encompassing regions 291–307 and 451–477 induced virus-neutralizing antibodies in mice (26). These two domains were also identified by PEPSCAN of the neutralizing rabbit serum raised against the recombinant C-terminal fragment of gp120 (Fig. 4). However, such antibody responses may be species-specific (27). Human antibodies binding to HTLV-IIIB gp120 neutralized HTLV-IIIB but not HTLV-IIIRF, indicating that the external envelope of HIV-1 might contain variable neutralization epitopes inducing only a type-specific response during natural infection (28).

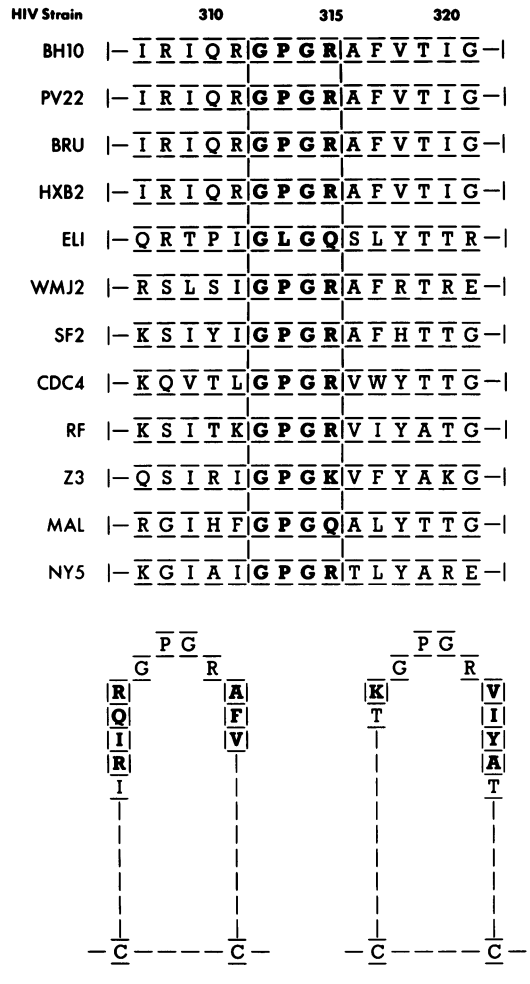

FIG. 6. Variability of sequences in aa positions 307–321 of gp120 deduced for the 12 strains of HIV-1 for which nucleotide sequences have been determined. The conserved β-turn is indicated by boldface type. The putative structural configuration is shown and the amino acids most important for binding are indicated.

HTLV-IIIB-neutralizing sera of chimpanzees recognized predominantly one epitope (IRIQRGPGRAFVTIG) within variable region V3, an epitope also recognized by the rabbit serum raised against the C-terminal polypeptide of gp120. This sequence shows several amino acids with high β-turn potential and elevated values for hydrophilicity, flexibility, and surface probability and has one or two potential glycosylation sites (23) though those are not on the particular peptides discussed above. The HTLV-IIIRF-neutralizing serum recognized exclusively the sequence of HTLV-IIIRF at the same location between the highly conserved cysteine residues 296 and 331.

These results identify a fixed domain between aa 307 and aa 321 as the binding site of naturally occurring and type-specific neutralizing antibodies (Fig. 6). We conclude that residues RIQR and AFV, flanking a conserved β-turn (GPGR), appear to be critical for binding and subsequent type-specific neutralization of the HTLV-IIIB strain of HIV-1. In parallel the lysine and VIYA residues flanking the same GPGR sequence appear to be required for neutralization of the HTLV-IIIRF strain. Because the β-turn is highly conserved within this amino acid sequence (Fig. 6) we hypothesize that the neutralizing epitope is contained within a loop between two cysteines connected by a disulfide bond.

In conclusion, chimpanzees developed HIV-1-neutralizing antibodies directed against a variable domain (V3) within the C-terminal half of gp120 early after infection. The relationship between the presence of these neutralizing antibodies and the prolonged latency and benign course of HIV-1 infection in chimpanzees remains to be studied.

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