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Immunizations of monkeys with synthetic peptides disclose conserved areas on gp120 of human immunodeficiency virus type 1 associated with cross-neutralizing antibodies and T-cell recognition

(B-cell epitopes/T-cell epitopes/vaccine development/*Macaca fascicularis*)

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ABSTRACT **Site-directed immunization was employed to identify sites on the envelope glycoprotein gp120 for antibody-mediated neutralization of human immunodeficiency virus type 1 (HIV-1). Antisera were raised in monkeys (*Macaca fascicularis*) against a series of 40 overlapping synthetic peptides covering the entire amino acid sequence of gp120 from the HTLV-IIIB strain of HIV-1. Immune sera against 12 of these peptides were reactive with gp120 by immunoblotting analysis, and antisera raised against 5 peptides, corresponding to amino acids (aa) 152–176, 193–218, 206–230, 248–269, and 307–330, were highly efficient in neutralizing HIV-1 (HTLV-IIIB) infectivity *in vitro*. Admixture of individual neutralizing anti-peptide monkey sera resulted in increment in neutralizing antibody titer. Antisera with reactivity to the relatively conserved regions defined by aa 152–176, 193–230, and 248–269 also neutralized to different extents the infectivity of the five Swedish clinical isolates of HIV-1 tested. Only a few HIV-1-infected people were found to make antibodies to these three conserved domains of gp120 as judged by ELISA using synthetic peptides as antigens. Three of the peptides (aa 152–176, 248–269, and 307–330) that induced neutralization antibodies also induced interleukin 2 production and lymphocyte proliferation when added to cultures of peripheral blood mononuclear cells from monkeys immunized with the corresponding peptides, indicating that these domains accommodate T-cell recognition sites. The results have obvious implications for the rational design of subunit vaccines against HIV-1 infection.**

A major role has been ascribed to the envelope glycoproteins gp120 and gp41 of human immunodeficiency virus type 1 (HIV-1) as targets for antibody-mediated neutralization of virus infectivity (1–4). HIV-1 neutralizing antibodies have been raised in experimental animals using native (5) and recombinant gp41 and gp120 (1, 6). Synthetic peptides derived from these proteins have been used as immunogens and have been shown to elicit a neutralizing antibody response (7–10). These studies have disclosed a major neutralizing antibody determinant located in the hypervariable region 3 (V3) of HIV-1 gp120. An additional B-cell epitope associated with neutralizing antibody activity has been identified in the second conserved region (C2) defined by amino acids (aa) 248–269 of gp120 (8).

We have attempted to identify neutralizing B-cell epitopes on HIV-1 gp120 by immunizing macaques with a series of 40 synthetic peptides covering in an overlapping fashion the entire primary sequence of gp120. Because antibody responses are critically dependent on cognate B cell–T helper cell interactions, we also examined synthetic peptides shown to contain neutralizing B-cell epitopes for their ability to stimulate a T-cell response in monkeys. We have identified at least four topographically distinct regions of HIV-1 gp120 entailing prominent neutralizing B-cell epitopes. Three of these are relatively conserved regions, two of which appear also to harbor T-cell recognition sites.

MATERIAL AND METHODS

Peptide Synthesis and Coupling of Peptides to Carrier Protein. Solid-phase peptide synthesis was performed using an Applied Biosystems 430A peptide synthesizer as described (11, 12). Sequence data for HTLV-IIIB were from ref. 13. An N-terminal cysteine was added to each peptide to facilitate coupling to carrier protein. Forty peptides covering the entire sequence of HIV-1 gp120 were synthesized, with lengths of 17–29 aa and overlapping each other by ≈50% (Table 1). Peptides were covalently coupled to ovalbumin (grade V, Sigma) at an ≈10:1 (peptide/ovalbumin) molar ratio by using *N*-succinimidyl 3-(2-pyridyldithio)propionate (Pharmacia) under the conditions given by the manufacturer.

Immunizations and Collection of Blood Samples. Three- to five-year-old male and female monkeys (*Macaca fascicularis*) were immunized by three consecutive intramuscular injections of 100 μg of ovalbumin-conjugated peptides emulsified in Freund's complete (first injection) or incomplete (second and third injections) adjuvant given 3 weeks apart. Blood was collected from the femoral vein before immunization and 1 or 2 weeks after the final immunizations. Preimmune and immune sera were prepared and stored at −20°C. One monkey was used for immunization with each individual peptide. Peptides found to contain B- or T-cell epitopes and peptides representing regions found by other investigators to be of interest (e.g., peptide gp120-24) were used for immunization of additional monkeys. Peripheral blood mononuclear cells (PBMCs) were obtained by sequential gelatin sedimentation of heparinized blood samples followed by Ficoll/Hypaque (Pharmacia) density centrifugation; interphase PBMCs were washed twice with isotonic phosphate-buffered saline (PBS: 10 mM sodium phosphate/150 mM NaCl, pH 7.4) and resuspended in RPMI 1640 supplemented with 5% fetal bovine serum (FBS) and antibiotics.

ELISA. Synthetic peptides at 1 mg/ml in 10% acetic acid were diluted to 10 μg/ml with 0.05 M sodium carbonate buffer (pH 9.6) and 100-μl aliquots were added to individual wells of M24 polyvinyl Microtiter plates (Dynatech). After

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Abbreviations: HIV-1, human immunodeficiency virus type 1; PBMC, peripheral blood mononuclear cell; FBS, fetal bovine serum; IL-2, interleukin 2; PHA, phytohemagglutinin; $TCID_{50}$, median tissue culture infective dose(s); aa, amino acid(s).

Table 1. The 40 overlapping gp120-derived synthetic peptides and their ability to induce antibodies that react with gp120 on Western blots

No.	Sequence (one-letter code)	aa*	gp120 reactivity
1	MRVKEKYQHLWRWGWRWGTMLLGMLMIC	1–28	–
2	GMLMICSATEKLWVTVYYGVPVWK	23–46	–
3	GVPVWKEATTTLFCASDAKAYDTE	41–64	+
4	CASDAKAYDTEVHNVWATHAC	54–74	+
5	VHNVWATHACVPTDPNPQEVVLVNV	65–89	–
6	VPTDPNPQEVVLVNVTENFNMWKNDM	75–100	+
7	TENFNMWKNDMVEQMHEDIISLWDQSL	90–116	+
8	VEQMHEDIISLWDQSLKPCVKLTPLC	102–126	+
9	KPCVKLTPLCVSLKCTDLKNDTNTN	117–141	–
10	VSLKCTDLKNDTNTNSSSGRMIMEK	127–151	–
11	SSSGRMIMEKGEIKNCSFNISTS	142–164	–
12	GEIKNCSFNISTSIRGKVQKEYAFF	152–176	+
13	IRGKVQKEYAFFYKLDIIPIDNDTTSYT	165–192	–
14	YKLDIIPIDNDTTSYTLTSCNTSVITQAC	177–205	–
15	LTSCNTSVITQACPKVSFEPIPIHYC	193–218	+
16	PKVSFEPIPIHYCAPAGFAILKCNN	206–230	+
17	APAGFAILKCNNKTFNGTGPCTNVSTVQC	219–247	–
18	KTFNGTGPCTNVSTVQCTHGIRPVVST	231–257	–
19	THGIRPVVSTQLLLNGSLAEEE	248–269	–
20	QLLLNGSLAEEEVVIRSANFTDNAK	258–282	–
21	VVIRSANFTDNAKTIIVQLNQSVEIN	270–295	–
22	TIIVQLNQSVEINCTRPNNNTRKS	283–306	–
23	CTRPNNNTRKSIRIQRGPGRAFVTI	296–320	–
24	IRIQRGPGRAFVTIGKIGNMRQAH	307–330	+
25	GKIGNMRQAHCNISRAKWNNTLK	321–343	–
26	CNISRAKWNNTLKQIDSKLREQF	331–353	–
27	QIDSKLREQFGNNKTIIFKQSSG	344–366	–
28	GNNKTIIFKQSSGGDPEIVTHSFN	354–377	–
29	GDPEIVTHSFNCGGEFFYCNSTQ	367–389	–
30	CGGEFFYCNSTQLFNSTWFNSTW	378–400	–
31	LFNSTWFNSTWSTEGSNNTE	390–409	–
32	STEGSNNTEGSDTITLP	401–417	–
33	GSDTITLPCRIKQFINMWQE	410–429	–
34	CRIKQFINMWQEVGKAMYAPPISGQIR	418–444	–
35	VGKAMYAPPISGQIRCSSNITGLL	430–453	+
36	CSSNITGLLLTRDGGNNNNESE	445–466	–
37	LTRDGGNNNNESEIFRPGGGDMR	454–476	+
38	IFRPGGGDMRDNWRSELYKYKV	467–488	–
39	DNWRSELYKYKVVKIEPLGVA	477–497	–
40	VKIEPLGVAPTKAKRRVVQREKR	489–511	+

*Amino acid numbering according to Muesing *et al.* (13).

incubation at 4°C overnight, the wells were washed with PBS and the remaining protein binding sites were blocked by incubation with 5% FBS in PBS for 30 min at 37°C. Monkey serum samples in serial 2-fold dilutions (starting dilution 1:100) or human serum samples diluted 1:50 were tested in an indirect ELISA (11). Horseradish peroxidase-conjugated goat anti-monkey antibodies to monkey whole IgG (Organon Teknika–Cappal) or alkaline phosphatase-conjugated goat anti-human IgG antibodies (The Jackson Laboratory) were used as second antibody, respectively. Enzyme chromogen substrates were *o*-phenylenediamine or *p*-nitrophenyl phosphate (Sigma). Color development was monitored at 490 nm (monkey serum ELISA) or 405 nm (human serum ELISA). Cutoff levels for monkey sera were 3 times the absorbance value obtained with corresponding preimmune serum at the dilution 1:100 and for human sera were twice the sum of the mean absorbance value obtained with six uninfected controls plus 3.3 times the SD.

Immunoblot Analysis of Monkey Hyperimmune Sera. Pre- and postimmunization sera were assayed for IgG antibody activity against PAGE-separated denatured HIV-1 whole virus lysate proteins with a commercial Western blot kit (Diagnostic Biotechnology, Singapore). The enzyme-conjugated anti-human IgG provided with the kit (previously shown to crossreact with monkey IgG) was employed as developing reagent.

Lymphocyte Proliferation Assay. PBMCs were dispersed in round-bottomed 96-well plates (Nunc) at 2×10^5, 1×10^5, and 5×10^4 cells per well in Hepes- and bicarbonate-buffered Iscove's medium (GIBCO) supplemented with 10% FBS (Biological Industries, Beth Haemek, Israel) and antibiotics. Synthetic peptides were added to the microcultures at 10, 1, and 0.1 μg/ml. Concanavalin A (Sigma, 100 μg/ml) and ovalbumin (Sigma, 10 μg/ml) were added to separate cultures. Culture plates were incubated at 37°C in a humid atmosphere containing 7.5% CO_2. After 4 days, 25 μl of culture supernatant was collected from each well and kept frozen at −70°C until assayed for interleukin 2 (IL-2) activity (see below). Next, 20 μl of culture medium containing 1 μCi (37 kBq) of [6-^{3}H]thymidine (Amersham) was added to each well. After 16 hr, the cells were collected on filters with an automated cell harvester (Inotech, Wohlen, Switzerland), and the incorporation of [^{3}H]thymidine into DNA was determined with an automated argon-activated β scintillation counter (Inotech). Results are expressed as stimulation indexes (SI), defined as the ratio of the arithmetic mean of the amount of radioactivity incorporated in triplicate cultures exposed to test peptide to that incorporated in corresponding replicate control cultures exposed to irrelevant peptides. SI values equal to at least 2.4 [i.e., twice the sum of the mean SI plus 3.3 times the SD of replicate cultures exposed to irrelevant peptides (confidence interval, $P < 0.001$, Student's *t* test)] were considered as significantly increased. The latter SI was determined as the ratio of radioactivity incorporated in cultures of monkey PBMCs ($n = 6$ animals) exposed to 18 irrelevant peptides to that incorporated in replicate cultures exposed to medium only. The IL-2 content of individual PBMC microcultures was determined (14) using CTLL-2 cells as responding cells. IL-2 in supernatants from test and replicate control cultures was determined by extrapolation from a standard curve generated by culturing CTLL-2 cells with known amounts of recombinant human IL-2 (Genzyme).

Cells and Virus Stocks. HIV-1 infectious stocks of HTLV-IIIB-infected H9 cells (15) and SF-2-infected HUT-78 cells (provided by J. A. Levy, San Francisco) were used. The cells were maintained in RPMI 1640 (GIBCO) supplemented with 20% heat-inactivated FBS, penicillin (GIBCO, 100 units/ml), and streptomycin (GIBCO, 100 μg/ml). Continuous tests of cell lines for contamination with mycoplasma (16) were negative. Virus stocks were prepared by standard procedures (15) and frozen at −90°C. One virus stock of HTLV-IIIB with endpoint titer of 40,000 median tissue culture infective doses ($TCID_{50}$) and one stock of SF-2 with endpoint titer of 10,000 $TCID_{50}$ were used throughout the study.

Five isolates of HIV-1 ("street" strains) were recovered by cocultivation of 10^7 PBMCs from five HIV-1-infected individuals who had developed AIDS with 10^7 blood-donor PBMCs, which had been stimulated for 3 days with phytohemagglutinin (PHA; Difco) at 2.5 μg/ml. Cell cultures were maintained in RPMI 1640 plus 10% FBS, IL-2 (10% T-cell growth factor, Cellular Products), Polybrene (2 μg/ml), antibiotics, and hydrocortisone acetate (5 μg/ml). Half of the medium was replaced every third to fourth day and fresh PHA-stimulated cells were added at intervals of 7 days. The culture supernatants were continuously assayed for HIV-1 p24 antigen, and when positive the virus isolates were frozen as stocks at −90°C. Endpoint titers of the patient isolates ranged from 100 to 400 $TCID_{50}$.

Neutralization Assays. Monkey sera were assayed for their capacity to neutralize HTLV-IIIB and SF-2 infectivity by (*i*) inhibition of HIV-1-induced syncytium formation, (*ii*) inhibition of p24 antigen production, and (*iii*) inhibition of HIV-1

antigen positivity of cells as determined by indirect immunofluorescence. HIV-1 street-strain neutralization was monitored by p24 antigen production only. Stock virus was diluted to 100 $TCID_{50}$ and mixed with serial 2- or 4-fold dilutions of heat-inactivated serum starting at 1:8 (HTLV-IIIB and SF-2 neutralization) or 1:10 (HIV-1 street-strain neutralization). A guinea pig hyperimmune serum with high HTLV-IIIB neutralizing activity (kindly provided by L. Åkerblom, Uppsala, Sweden) served as positive control. After incubation for 16 hr at 4°C (and also as specified in *Results* for 30 min at 37°C), the serum/virus mixture was added to 10^6 H9 cells and incubated for 60 min at 37°C. The cells were then washed once and placed in 24-well plates with 2 ml of growth medium [RPMI with 20% FBS, antibiotics, and Polybrene (2 μg/ml)] per well.

Cells were examined for the presence of syncytia 5–8 days after infection. Dilutions (1:10 to 1:1000) of supernatants from infected cell cultures were collected 10 days after initial virus inoculation and assayed for the presence of p24 with a commercial ELISA kit (Abbott antigen test HIVAG-1, Abbott). The number of infected cells was determined 10–15 days postinfection by indirect immunofluorescence on acetone-fixed suspensions (16), using a pool of sera from HIV-infected individuals and fluorescein-conjugated goat anti-human IgG antibody (BioMerieux, Charbonnier les Bains, France) as primary and secondary reagents, respectively. Results are expressed as mean neutralization titer, defined as the reciprocal of the highest serum dilution that reduced HIV-induced syncytium formation, production of p24 antigen, or number of HIV-specific immunofluorescent cells by at least 90%. When such a reduction was obtained only at the last serum dilution step, a titer value equal to the reciprocal value of this last dilution was given if the reduction was 90–95%; the titer was given a value greater than the reciprocal of the last dilution if the reduction obtained at the last dilution step was >95%.

Neutralization of street strains was performed on PHA-stimulated PBMCs. Two dilutions (1:10 and 1:40) of heat-inactivated preimmune and hyperimmune sera of monkeys were mixed with street strains of virus diluted to 100 $TCID_{50}$. After incubation at 4°C for 16 hr, the mixtures were used to infect 2×10^6 PBMCs in complete medium [RPMI 1640 supplemented with 10% FBS, 10% T-cell growth factor, Polybrene (2 μg/ml), and hydrocortisone (5 μg/ml)]. After 1 hr of incubation at 37°C the cells were washed and placed in 24-well plates with 2 ml of complete medium. Virus replication was monitored at day 10 postinfection by measuring p24 in the culture supernatants as above. No nonspecific inhibitory effects on infectivity of any of the five street strains were obtained with monkey preimmune sera. Duplicate testing of the guinea pig hyperimmune serum and selected anti-peptide monkey sera consistently gave the same neutralizing titer, indicating a minimal intra-test variation.

RESULTS

Antibody Responses in Monkeys Immunized with Synthetic Peptides. Immunization of monkeys with ovalbumin-conjugated peptides induced an antibody response to the homologous peptide as determined in ELISA in all animals but one immunized with gp120-39. High titers (>6400) of anti-peptide antibodies were elicited with most of the peptides. Of the 40 sera tested, 12 recognized the full-length gp120 on immunoblots (Table 1). The corresponding preimmune sera were completely negative.

Neutralization of HTLV-IIIB. After immunization of the first group of monkeys with the series of 40 different gp120 peptides, virus-neutralizing activity was detected in antisera raised against five of the synthetic peptides (gp120-12, -15, -16, -19, and -24) with all three assays used (i.e., inhibition of p24 antigen synthesis, reduction of HIV-induced syncytia, and reduction of relative number of antigen-positive cells; Table 2). The presence of neutralizing antibody epitopes in the four domains defined by peptides gp120-12, gp120-15 and -16, gp120-19, and gp120-24 was confirmed in a second group of monkeys. The neutralizing titer obtained with antiserum to gp120-24 was low (≤8) in the first immunized monkey but high (>128) in the second monkey (Table 2). A guinea pig hyperimmune serum with high HTLV-IIIB neutralizing titer served as positive control. Incubation of serum with virus for 16 hr at 4°C was chosen to minimize the chance of missing HIV-1 neutralizing activity in any serum, since in our hands this neutralization protocol is approximately twice as sensitive as when serum is incubated with virus for 30 min at 37°C. For positive sera, neutralizing activity was confirmed, albeit at a lower titer, by preincubating the serum with virus for 30 min at 37°C. No nonspecific inhibition of HIV infectivity was seen with any of the preimmune sera of the monkeys. As the peptides gp120-12, -15/-16, -19, and -24 represented different regions of gp120, we also tested the neutralizing activity of combinations of the corresponding antisera. All combinations of antibody mixtures neutralized HTLV-IIIB infectivity at a titer exceeding the neutralizing titer obtained with each individual serum; the serum combinations consistently gave >95% reduction in p24 antigen production, syncytium formation, and frequency of antigen cells at the last serum dilution tested (1:128).

Cross-Neutralization of Street Strains of HIV-1. The sera neutralizing HTLV-IIIB were also tested for their ability to neutralize five different clinical isolates of HIV-1. Three to five of these Swedish street isolates of HIV-1 were neutralized by four of the HTLV-IIIB neutralizing immune sera corresponding to peptides gp120-12, gp120-15, gp120-16, and gp120-19 (Table 3). The antiserum raised against peptide gp120-24 that was used in this experiment, although highly efficient in neutralizing the infectivity of the HTLV-IIIB strain, failed to reduce the infectivity of any street isolate (Table 3). In another experiment, cross-neutralization was also tested using the HIV-1 strain SF-2. Only sera from monkeys immunized with peptides gp120-12 and gp120-19 were able to neutralize SF-2 virus infectivity, although the neutralizing titers were significantly lower against SF-2 (titer = 8) than against the homologous HTLV-IIIB strain (titers = 64 and 32, respectively) (data not shown).

Antibody Response of HIV-Infected Humans to gp120 Peptides. To see whether antibodies of HIV-infected individuals recognize the neutralizing epitopes of gp120 identified above, ELISAs using each of the 40 gp120 peptides as antigen were performed. Seventy-five HIV-1-positive serum samples were analyzed for IgG reactivity against the individual peptides (Fig. 1). Most sera (82%) contained IgG antibodies against the C-terminal peptide, gp120-40. Very few of the sera from HIV-1-infected humans showed significant ELISA activity against the peptides that elicited neutralizing antibody responses in monkeys (9%, 11%, 23%, 4%, and 14% to peptides gp120-12, -15, -16, -19, and -24, respectively) (Fig. 1).

***In Vitro* Proliferative Responses to Immunizing Peptides.** PBMCs from monkeys immunized with each of the five synthetic peptides found to induce production of neutralizing antibodies were tested for their ability to proliferate and to produce IL-2 when exposed *in vitro* to "recall" (immunizing) peptide. Two monkeys immunized with each peptide were tested.

Three of these peptides induced proliferation of the lymphocytes when added to cultures of PBMCs from animals previously immunized with the corresponding peptide conjugated to ovalbumin. Thus, PBMCs of two monkeys immunized with peptide gp120-12 responded to recall peptide, giving SI values (mean of triplicate cultures) of 9.8 and 4.7. PBMCs of monkeys immunized with gp120-19 gave SI values

Table 2. Neutralization of HIV-1 (HTLV-IIIB) infectivity by monkey hyperimmune sera to synthetic peptides from gp120 according to the sequence of HTLV-IIIB

Anti-peptide hyperimmune serum	Virus neutralization endpoint titer					
	p24 detection		Fusion inhibition		Relative number of antigen-positive cells	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
gp120-12	64	32	64	32	64	8
gp120-15	128	ND	>128	ND	64	ND
gp120-16	64	32	128	32	64	32
gp120-19	32	32	32	32	32	8
gp120-24*	8	<8 (>128)	8	<8 (>128)	8	<8 (>128)
Positive control	640	160	640	160	640	160

ND, not done; positive control, guinea pig hyperimmune serum.
*Antiserum against gp120-24 from the first immunized monkey was used in these experiments; results with antiserum from a second immunized monkey are shown in parentheses.

of 3.1 and 4.6 and PBMCs of one monkey immunized with gp120-24 gave a SI of 6.0. Likewise, the same peptides also induced production of IL-2 (data not shown).

Peptides gp120-15 and -16 failed to induce proliferation or IL-2 production when added to cultures of PBMCs from monkeys (four animals) immunized with either of these peptides. In support of the specificity of the results, peptides gp120-12, -19, and -24, when added to cultures of PBMCs from at least three other monkeys immunized with a nonoverlapping peptide, failed to induce significant proliferative responses or IL-2 production (data not shown).

DISCUSSION

We have identified five synthetic peptides with sequences derived from that of HIV-1 gp120 (HTLV-IIIB strain) that are capable of inducing the production of neutralizing antibodies in subhuman primates when used as immunogens after coupling to a carrier protein. Three of these peptides also appear to accommodate T-cell recognition sites, as seen by their ability to induce proliferation and IL-2 secretion in cultures of PBMCs from immunized macaques.

We chose to synthesize peptides of at least 17 aa having 50% overlaps to reduce the possibility of missing any B-cell neutralizing epitope. Antibodies to the peptide used for immunization were demonstrated in ELISA in all monkeys but one receiving peptide gp120-39. We cannot, however, exclude that gp120 may contain additional neutralizing epitopes recognized by primates, as most ovalbumin-conjugated peptides were injected into only one monkey each. We were not able to detect neutralizing epitopes in the C-terminal half of gp120, although such epitopes have been reported by others (7, 9) in peptide-immunized rodents. It remains to be defined whether these epitopes are species-restricted or would have been recognized by immunization of a larger number of macaques.

Neutralizing anti-peptide antibodies, except for anti-gp120-19, also recognized the full-length gp120 on immunoblots. The inability of the HIV neutralizing antibodies induced by peptide gp120-19 to recognize the intact gp120 may indicate that the configuration of the epitope recognized by these antibodies was destroyed by SDS/PAGE. Eight peptides, five of them located in the N-terminal half, induced antibodies that bound to gp120 in immunoblotting analysis but did not inhibit virus infectivity. This might suggest that the regions containing these epitopes are not on the surface of the natively folded protein. Three of the peptides (gp120-35, -37, and -40) that induced immunoblot-positive antibodies that did not neutralize HIV-1 were from the region aa 430–511, i.e., the C-terminal part of gp120. However, Neurath *et al.* (9) reported that peptides from this region induced neutralizing antibodies in rabbits. Perhaps the *in vitro* neutralization assay employed by those authors was more sensitive than the one we used, or perhaps rabbits recognize other epitopes than those recognized by primates.

One peptide, gp120-24, corresponding to the middle/right portion (aa 307–330) of the major neutralization domain of the V3 region of the protein, induced type-specific neutralizing antibodies as has also been reported by others (17–19). However, rabbit antibodies to the hexapeptide GPGRAF, also contained in gp120-24, may neutralize HIV-1 strains other than HTLV-IIIB, including the MN strain (20).

The gp120-24 peptide was also found to harbor a major T-cell recognition site and it largely overlaps with aa 303–337, a region shown by other investigators (21) to accommodate a T-cell epitope. This is of particular importance, since a vaccine against AIDS, to be efficient, is likely to comprise components that activate T cells capable of providing help to

Table 3. Cross-neutralization of HIV-1 street-strain infectivity by monkey hyperimmune sera to synthetic peptides from gp120 according to the sequence of HTLV-IIIB

Anti-peptide hyperimmune serum	Virus neutralization endpoint titer by p24				
	73	5004	5049	5092	5220
gp120-12	10	<10	<10	40	>40
gp120-15	>40	>40	40	>40	>40
gp120-16	40	40	40	>40	>40
gp120-19	<10	10	10	10	10
gp120-24	<10	<10	<10	<10	<10

HIV-1 strain designations (73, 5004, etc.) head each column.

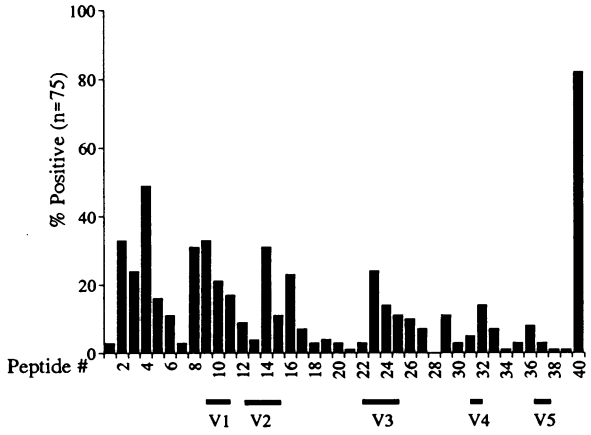

FIG. 1. Serum reactivity of 75 HIV-1-positive human sera against 40 synthetic peptides covering the entire gp120. Cutoff value was the mean of six negative control sera plus 6 SD. Horizontal bars below the abscissa indicate the approximate location of the five variable regions (V1–V5) of gp120.

cognate B cells committed to the production of virus neutralizing antibodies (22, 23). The techniques used in the present study cannot discriminate among functionally distinct T-cell subpopulations.

Two neutralizing domains (Peptides gp120-15/-16 and gp120-19) were found in the C2 conserved region of the protein. All three peptides were found to be capable of inducing broadly cross-neutralizing antibodies. The gp120-19 peptide is almost identical to a peptide found by Ho *et al.* (8), but not by others (24), to induce neutralizing antibodies in rabbits. The gp120-19 peptide also displayed T-cell activating properties in two out of two immunized monkeys. To our knowledge, gp120-15 and -16 represent a previously undescribed neutralizing region in HIV-1. However, neither gp120-15 nor gp120-16 appeared to harbor a T-cell recognition site identifiable by the techniques employed. Whether the domain defined by these two peptides entails more than one neutralizing antibody epitope has yet to be determined. Preliminary experiments using immune sera from monkeys immunized with truncated variants of gp120-15 or -16 suggest that this region might entail two separate neutralizing epitopes.

Another domain, corresponding to peptide gp120-12, was found to accommodate at least one site that induced neutralizing antibody. This region was particularly interesting since (*i*) it represents a discrete but partly conserved region located between the variable regions V1 and V2 of gp120, (*ii*) antibodies elicited in monkeys immunized with the corresponding peptide cross-neutralized the infectivity of SF-2 as well as that of three out of five HIV-1 street isolates, and (*iii*) this region appears to accommodate at least one T-cell recognition site. Recent experiments indicate that one such bona fide T-cell epitope is recognized not only by T cells from monkeys immunized with gp120-12 but also by T cells from all monkeys immunized with a peptide half-overlapping with peptide gp120-12, suggesting that recognition of this region by immune T cells may be subject to limited genetic restriction (K.E., P.H., A.V., S.J., B.S., J.H., and C.C., unpublished data). These observations raise intriguing questions since <10% of HIV-1-infected humans examined in this study had detectable antibodies reactive with gp120-12. The C-terminal half of gp120-12 overlaps a peptide (aa 164–187), corresponding to the N-terminal part of hypervariable region V2, that was shown (9) to induce HTLV-IIIB neutralizing antibodies in rabbits.

As serum antibody reactivity to the peptides gp120-12, -15, -16, and -19, all corresponding to relatively conserved regions of the glycoprotein, appeared infrequently in HIV-1-infected human individuals, it would be interesting to correlate antibody reactivity to these peptides with clinical outcome of HIV-1 infection.

Although interpretations of the results of our study with peptides from conserved regions of gp120 are limited because macaques cannot be challenged with HIV-1 and thus neutralization efficacy of antibodies induced could not be tested *in vivo*, it is indicated that combinations of appropriate synthetic peptides might prove useful in the construction of a vaccine for protective immunity to HIV-1.

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1. Lasky, L. A., Groopman, J. E., Fennie, C. W., Benz, P. M., Capon, D. J., Dowbenko, D. J., Nakamura, G. R., Nunes, W. M., Renz, M. E. & Berman, P. W. (1986) *Science* **233,** 209–212.
2. Nara, P. L., Robey, W. G., Pyle, S. W., Hatch, W. C., Dunlop, N. M., Bess, J. W., Kelliher, J. C., Jr., Arthur, L. O. & Fischinger, P. J. (1988) *J. Virol.* **62,** 2622–2628.
3. Thomas, E. K., Weber, J. N., McClure, J., Clapham, P. R., Singhal, M. C., Shriver, M. K. & Weiss, R. A. (1988) *AIDS* **2,** 25–29.
4. Zagury, D., Bernard, J., Cheynier, R., DesPortes, I., Leonard, R., Fouchard, M., Reveil, B., Ittele, D., Lurhuma, Z., Mbayo, K., Wane, J., Salaun, J.-J., Goussard, B., Dechazal, L., Burny, A., Nara, P. & Gallo, R. C. (1988) *Nature (London)* **332,** 728–731.
5. Matsushita, S., Robert-Guroff, M., Rusche, J., Koito, A., Hattori, T., Hoshino, H., Javaherian, K., Takatsuki, K. & Putney, S. (1988) *J. Virol.* **62,** 2107–2114.
6. Krohn, K., Robey, W. G., Putney, S. D., Arthur, L., Nara, P., Fischinger, P., Gallo, R. C., Wong-Staal, F. & Ranki, A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4994–4998.
7. Ho, D. D., Sarngadharan, M. G., Hirsch, M. S., Schooley, R. T., Rota, T. R., Kennedy, R. C., Chanh, T. C. & Sato, V. L. (1987) *J. Virol.* **61,** 2024–2028.
8. Ho, D. D., Kaplan, J. C., Rackauskas, I. E. & Gurney, M. E. (1988) *Science* **239,** 1021–1023.
9. Neurath, A. R., Strick, N. & Lee, E. S. Y. (1990) *J. Gen. Virol.* **71,** 85–95.
10. Palker, T. J., Clark, M. E., Langlois, A. J., Matthews, T. J., Weinhold, K. J., Randall, R. R., Bolognesi, D. P. & Haynes, B. F. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1932–1936.
11. Horal, P., Svennerholm, B., Jeansson, S., Rymo, L., Hall, W. W. & Vahlne, A. (1991) *J. Virol.* **65,** 2718–2723.
12. Horal, P., Hall, W. W., Svennerholm, B., Lycke, J., Jeansson, S., Rymo, L., Kaplan, M. H. & Vahlne, A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5754–5758.
13. Muesing, M. A., Smith, D. H., Cabredilla, C. D., Benton, C. V., Lasky, L. A. & Capon, D. J. (1985) *Nature (London)* **313,** 450–455.
14. Gillis, S., Ferm, M. M., Ou, W. & Smith, K. A. (1978) *J. Immunol.* **120,** 2027–2033.
15. Popovic, M., Sarngadharan, M. G., Read, E. & Gallo, R. C. (1984) *Science* **224,** 497–500.
16. Jeansson, S. & Brorson, J. E. (1985) *Exp. Cell Res.* **1,** 181–188.
17. Goudsmit, J., Debouck, C., Meloen, R. H., Smit, L., Bakker, M., Asher, D. M., Wolff, A. V., Gibbs, C. J., Jr. & Gajdusek, D. C. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4478–4482.
18. Looney, D. J., Fisher, A. G., Putney, S. D., Rusche, J. R., Redfield, R. R., Burke, D. S., Gallo, R. C. & Wong-Staal, F. (1988) *Science* **241,** 357–359.
19. Putney, S. D., Matthews, T. J., Robey, W. G., Lynn, D. L., Robert-Guroff, M., Mueller, W. T., Langlois, A. J., Ghrayeb, J., Petteway, S. R., Weinhold, K. J., Jr., Fischinger, P. J., Wong-Staal, F., Gallo, R. C. & Bolognesi, D. P. (1986) *Science* **234,** 1932–1935.
20. LaRosa, G. J., Profy, A. T., Bolognesi, D. P., Herlihy, W. C., Putney, S. D. & Matthews, T. J. (1990) *Science* **250,** 1590–1593.
21. Javherian, K., Langlois, A. J., McDanal, C., Ross, K. L., Eckler, L. I., Jellis, C. L., Profy, A. T., Rusche, J. R., Bolognesi, D. P., Putney, S. D. & Matthews, T. J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6768–6772.
22. Cease, K. B., Margalit, H., Cornette, J. L., Putney, S. D., Robey, W. G., Ouyang, C., Streicher, H. Z., Fischinger, P. J., Gallo, R. C., DeLisi, C. & Berzowsky, J. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4294–4253.
23. Cornette, J. L., Margalit, H., DeLisi, C. & Berzofsky, J. A. (1989) in *Methods in Enzymology*, ed. Dinarello, C. (Academic, New York), Vol. 178, pp. 611–634.
24. Ronco, J., Charbit, A., Dedieu, J.-F., Mancini, M., Michel, M.-L., Henin, Y., O'Callaghan, D., Kaczorek, M., Girard, M. & Hofnung, M. (1991) *AIDS Res. Hum. Retroviruses* **7,** 1–2.