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Immunology

Identification of human neutralization-inducing regions of the human immunodeficiency virus type 1 envelope glycoproteins

(ELISA/gp120/gp41/epitope/peptide)

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ABSTRACT **Four major neutralizing regions of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein were identified and characterized with a panel of 80 HIV-1 antibody-positive human sera. Levels of neutralizing antibodies against the HIV-1 strains IIIB, SF2, and RF were compared with reactivity in ELISAs against peptides that correspond to certain regions of the HIV-1 envelope. A correlation between high neutralizing activity and strong seroreactivity against specific peptides suggested that the corresponding regions might be involved in neutralization. This was further substantiated by using peptides to inhibit neutralization by a panel of 10 HIV-1 antibody-positive sera. The positions of three neutralizing sites, defined earlier mostly by antisera from animals, were confirmed in the present study. Human sera thus recognize the strain-specific third variable region of gp120 (amino acids 304–318), the C-terminal end of gp120 (amino acids 489–508), and the conserved region in the intracellular part of gp41 (amino acids 732–746). It is likely that these different regions mediate help rather than self-sufficient neutralization. Furthermore, a human neutralizing region was detected in a conserved part of gp41 (amino acids 647–671). Accordingly, neutralizing antibodies directed to this region were found to be cross-reactive between HIV-1 strains. Peptides corresponding to these four regions were able to inhibit neutralization mediated by serum from HIV-1 antibody-positive individuals. These results indicate that this conserved B-cell epitope of the HIV-1 envelope elicits a virus-neutralizing antibody response during natural infection in humans and may therefore be considered for inclusion in a vaccine against HIV-1.**

Identification and characterization of epitopes that play a role in the induction of protective immunity are crucial for development of vaccines that may prevent human immunodeficiency virus (HIV) infection or modify clinical progression of an established infection. HIV type 1 (HIV-1)-specific antibodies from infected individuals have been found to inhibit viral infectivity *in vitro* (1, 2). However, the *in vivo* role of neutralizing antibodies in prevention of virus infection and disease progression is not fully understood. In general, neutralizing antibodies are found in relatively low titers. They may be either strain specific or, as seen later in the infection, more broadly reactive. Several reports have indicated that neutralizing antibodies are directed to various regions of HIV-1, such as gp120 (3–6), gp41 (4, 7), and p17 (8). Of particular interest is the third variable region (V3 region) of gp120 between amino acids 296 and 331, which has been suggested as the major neutralizing site of HIV-1 (5, 6, 9). However, results in humans and chimpanzees indicate that emergence of neutralization-resistant variants is not accompanied by a change in the primary amino acid sequence of the V3 domain. Other parts of the envelope may change and influence sensitivity to neutralization, either directly or indirectly, by imposing a conformational change (10, 11). Recent observations indicate that antibodies against the V3 region are either strain specific or broadly cross-neutralizing (12), depending on the paratope reactivity.

Most of the studies on potential neutralizing epitopes have been performed by immunizing animals with peptides or recombinant proteins, and the corresponding antisera have been analyzed for neutralizing activity. The aim of this study was to define epitopes of the HIV-1 envelope glycoprotein that are involved in virus neutralization by *human* sera.

MATERIAL AND METHODS

Study Population. Sera from 80 HIV-1-seropositive individuals from Sweden and the United Kingdom were used. They represented all clinical stages of the HIV-1 infection. All sera were not included in all experiments. Where a smaller number were used, they were always randomly selected. The study of frequency of reactivity presented in Table 1 was performed with another group of 50 Swedish patients representing all clinical stages of HIV-1 infection.

HIV Neutralization Assays. The HIV-1 prototype virus strains IIIB and RF used for neutralization were kindly obtained from R. Gallo (National Institutes of Health, Bethesda, MD) and SF2 was from J. Levy (University of California, San Francisco).

Neutralization by inhibition of viral infectivity. This assay has been extensively described elsewhere (13). Briefly, cell-free virus supernatant (IIIB, 50 times the tissue culture infective dose) was preincubated with serial dilutions (seven 2-fold steps starting with 1:10) of sera, and then the serum/virus mixture was added to 1×10^5 H9 cells. At day 7 supernatants were collected and analyzed by HIV-antigen capture ELISA (14). Neutralization was defined as ≥80% reduction of p24 viral antigen in the supernatant as compared to p24 content when virus was incubated in the presence of HIV antibody-negative sera. HIV-1 antibody-positive sera with known neutralization titers were included in each test.

Vesicular stomatitis virus (VSV) pseudotype neutralization assay. Three different HIV-1 strains, IIIB, SF2, and RF, were used in the VSV pseudotype neutralization assay. VSV pseudotypes bearing the different HIV-1 envelope glycoproteins were prepared and assayed as described (2, 15). Neutralization assays were carried out by mixing serial serum dilutions, five 5-fold steps starting with 1:10, with 200 plaque-forming units of pseudotype virus. After incubation for 1 hr at 37°C, the mixtures were assayed for residual pseudotype

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Abbreviations: HIV-1, human immunodeficiency virus type 1; VSV, vesicular stomatitis virus; V3 region, third variable region.

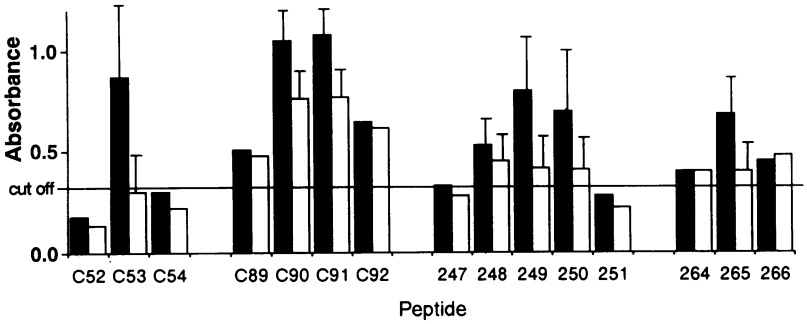

FIG. 1. Mean absorbance values (serum dilution, 1:100) of seroreactivity to sequentially overlapping peptides (15-mer peptides overlapping with 10 amino acids) representing the IIIB sequence (see Table 1 for specificity). The sera were divided into a neutralization-positive group (titer > 10; $n = 49$; solid bars) and a neutralization-negative group (titer < 10; $n = 17$; open bars). Neutralization was tested by inhibition of IIIB infectivity assay. Values above the mean absorbance value + 3 SD of negative controls were considered positive.

infectivity. The neutralizing titers were expressed as the highest serum dilution giving ≥80% reduction of plaque-forming units.

Peptides and Peptide Enzyme-Linked Immunosorbent Assay (ELISA). Solid-phase synthesized (16) 15-amino acid peptides based on the IIIB sequence (17) were used as antigens in ELISAs. The amino acid sequences were numbered according to the Los Alamos data base (18). Peptides that represented the V3 region of the MN strain (18) were also used. Five of these peptides (LP1–LP5) contained extra cysteine residues and were actively cyclized by disulfide bridges. This has been done to increase the exposure of immunogenic regions by conformational changes. These peptides were kindly provided by J. Trojnar (Ferring AB, Malmö, Sweden). The peptide ELISA has been extensively described elsewhere (13).

Inhibition of Neutralization by Peptides. Human sera diluted in three 5-fold steps starting with 1:20 were preincubated with 4 μg of peptide in 100 μl for 2 hr at 37°C, after which a neutralization assay was performed (as above). The monoclonal antibody F58/H3 (19) was used in the same way in a final concentration of 2 μg/ml.

Statistics. Student's *t* test was used for the statistical analysis.

RESULTS

Correlation of Virus-Neutralizing Activity and Reactivity to Peptides Corresponding to Different Regions of the HIV-1 Envelope. To identify linear immunogenic epitopes in humans, a group of 50 HIV-1 antibody-positive sera was tested against all overlapping 15-mer peptides representing the whole IIIB HIV-1 envelope by using an ELISA. Peptides reacting with >40% of the serum panel were selected for further study. Other regions that are less immunodominant could possibly function as neutralization epitopes, but in this study we have concentrated on the most immunogenic regions. Another group of 80 HIV-1 antibody-positive sera was tested for reactivity to these peptides (and to the adjacent peptides of these regions) and for neutralizing activity against three different HIV-1 strains (Fig. 1, Table 1).

Amino acids 304–318 of gp120. Seroreactivity to peptide C53 correlated with a strong neutralizing response against IIIB ($P < 0.0005$). Peptide C53 represents the IIIB sequence of the central part of the V3 region of gp120 (amino acids 304–318). The adjacent peptides C51–C52 (amino acids 294–313) and C54–C58 (amino acids 309–343) spanning the rest of the V3 region lacked correlation with neutralization. In line with previous findings, the neutralizing site in the V3 region appeared to be strain specific since the antibody response to this region (represented by peptide C53) correlates with neutralization of IIIB but not with the SF2 or RF strains.

We have further characterized the reactivities to the V3 region by using peptides (LP1–LP5, A12, and A13) representing the amino acid sequence derived from the MN strain of HIV-1. Seroreactivity to peptide LP2 representing the central part of the MN-specific V3 region showed a correlation with neutralization against RF but not against IIIB or SF2. Seroreactivity to peptide LP4, representing the C-terminal site of the V3 region, correlated with RF- and SF2-specific neutralization but not with IIIB. Reactivity to the other MN peptides, LP1 (amino acids 298–315), LP3 (amino acids 318–334), and LP5 (amino acids 332–348), did not correlate with neutralization of any of the virus strains. Two additional peptides representing the MN sequence were also tested. Seroreactivity to peptide A12, like that to peptide

Table 1. Sequences and amino acid numbers of the peptides used and their correlation with seroreactivity of human sera and neutralization of three HIV-1 strains

Peptide	Strain	Amino acid number	Sequence	Neutralization*				Seroreactivity, %
				IIIB	VSV–IIIB	VSV–SF2	VSV–RF	
gp120								
C53	IIIB	304–318	RKSIRIQRGPGRAFV	++	++	−	−	83
A12	MN	309–321	RKRIHIGPGRAFY	ND	−	−	+	98
LP2	MN	310–325	KRCIHIGPGRAFYCTKN	ND	−	−	+	95
LP4	MN	321–335	YTTCNIIGTIRQAHC	−	−	+	+	100
C90	IIIB	489–503	VKIEPLGVAPTKAKR	++	++	−	−	50
C91	IIIB	494–508	LGVAPTKAKRRVVQR	++	++	−	−	55
gp41								
248	IIIB	647–661	EESQNQQEKNEQELL	+	+	−	−	16
249	IIIB	652–666	QQEKNEQELLELDKW	++	++	+	−	56
250	IIIB	657–671	EQELLELDKWASLWN	+	+	−	−	47
265	IIIB	732–746	GIEEEGGERDRDRSI	++	++	++	−	12

The HTLVIIIB strain was used in two different neutralization assays. Positive correlations are indicated with ++ ($P < 0.005$) or + ($P < 0.01$); − means no significant correlation. The VSV–SF2 correlation was done by comparing the group of sera with low neutralizing titers (≤50) with the group of sera with high neutralizing titers since all sera could neutralize SF2. Only results from peptides with a positive correlation are shown. Additional peptides that were tested, which lacked correlation, are presented in *Results*. Underlined C (cysteines) are substitutes in the natural sequence.

*Neutralizing activity against the three HIV-1 strains indicated.

LP2, correlated with neutralization to RF but not to IIIB or SF2. Seroreactivity to peptide A13 (amino acids 328–342) showed no correlation to neutralization of any of the strains.

Amino acids 489–508 of gp120. Also, seroreactivity to peptides C90 and C91 correlated with a high neutralizing activity against IIIB ($P < 0.005$) but not against RF and SF2, indicating strain-specific neutralization is also seen in this region. Reactivity to the adjacent peptides C89 and C92 did not correlate with neutralization of any of the strains tested.

Amino acids 647–671 of gp41. Interestingly, the antibody response to peptide 249 (amino acids 652–666 of the IIIB-derived sequence) was high in sera with neutralizing activity against IIIB and SF2 ($P < 0.005$ and $P < 0.01$, respectively) but not against RF. Correlation of peptide reactivity and neutralization was less pronounced when peptides 248 ($P < 0.01$) and 250 ($P < 0.01$) were tested and disappeared when compared to the flanking peptides 246–247 (amino acids 637–656) and 251 (amino acids 662–676) for IIIB. Moreover, high reactivity to peptide 249 was paralleled by high neutralizing titers in individual sera (data not shown). The results show that neutralizing antibodies directed to this region are more broadly reactive than those directed against the previously described regions in gp120.

Amino acids 732–746 of gp41. Another region of interest is represented by peptide 265 (amino acids 732–746 of the IIIB-derived sequence). Similar to region 647–671, seroreactivity against this region correlated with high neutralizing titers against IIIB ($P < 0.005$) and SF2 ($P < 0.005$) but not with RF. Reactivity to the flanking regions (amino acids 722–741 and 737–771, respectively) did not correlate with neutralization of any HIV-1 strain tested.

Additional regions that were tested without finding a correlation between seroreactivity and neutralization were represented by overlapping 15-mer peptides from the following regions: amino acids 119–133, 204–218, 274–293, 512–526, 547–561, 582–616, 622–636. The correlation of neutralizing activity with reactivity against a certain peptide was not simply an effect of high general HIV IgG titers, tested against viral lysate, since none of the specific peptide seroreactivities correlated with high HIV lysate IgG titers (data not shown). Nor did the neutralizing titers in the sera correlate with HIV lysate IgG titers. In summary, by relating peptide reactivity and neutralizing activity of a panel of human sera against three strains of HIV-1, four regions of interest were detected in the HIV-1 envelope. The results suggest that the two regions in gp41 display a certain degree of cross-reactivity.

Peptide Blocking of Virus Neutralization. The next set of experiments was designed to demonstrate that the correlation between the neutralizing capacity of a serum and seroreactivity in ELISA to a certain peptide was due to the epitope(s) represented by that peptide. For this purpose, human sera were preincubated with different peptides before being assayed in the neutralization tests. A reduction in neutralizing capacity of a serum would indicate that the peptide blocked the corresponding neutralizing antibody. A peptide was said to block neutralization when there was a 4-fold increase of p24 antigen in the culture medium compared with serum not preincubated or preincubated with irrelevant peptides, as defined in *Material and Methods*.

Mouse monoclonal antibody F58/H3 (12, 19) with a high neutralizing titer (1:1000) against IIIB was used to evaluate the blocking system. This antibody is directed to the conserved part of the V3 region of gp120 including the GPGR sequence. Neutralization by the F58/H3 antibody was completely inhibited by preincubation with peptide C53, which represents this region (data not shown). None of the other peptides tested was able to affect neutralization by F58/H3. No specific monoclonal antibodies to peptides C91, 249, or 265 are available.

When human sera were preincubated with peptides in order to reduce the neutralizing capacity, a more complex picture was seen (Fig. 2). This is expected since neutralizing antibodies to several epitopes might be present in one serum. Ten sera were analyzed in these experiments. Neutralization was reduced by at least one of the four peptides in all of the sera. There was a proportional absorption of neutralizing activity with the respective peptides in the various serum dilutions. The highest possible serum dilution is shown in Fig. 2. All sera had high neutralization titers and it is assumed that they contained several potent populations of antibody. No correlation was found between the magnitude of neutralization titer and blocking by a specific peptide or a certain pattern of peptide blocking. Neutralization was not blocked by any of 14 other 15-mer peptides tested. Eight of them represented adjacent regions to the above mentioned peptides (C52, C54; C89, C92; 247, 251; 264, 266) and six of them represented conserved and variable regions of the gag and env proteins (p17, amino acids 48–62; p24, amino acids 168–182; gp120, amino acids 284–298, 379–393, and 419–433; gp41, amino acids 542–556, 562–576, and 822–836). Taken together these results indicate that the peptides representing the four regions described above each are able to completely or partially inhibit neutralization. Individual human sera greatly differ in their neutralization blocking pattern, suggesting that antibody production to immunogenic regions of the envelope is highly variable.

DISCUSSION

We have defined four regions of the HIV-1 envelope that appear to be important for neutralization by *human* sera. Epitopes involved in neutralization and the degree of cross-reactivity at each epitope were detected by two means. In the first assay, neutralizing capacity against different HIV-1 strains in sera from HIV-1-infected individuals was compared with antibody response to peptides representing all antigenic regions of the HIV-1 envelope gene. In the second assay, epitopes crucial in virus neutralization were substantiated by blocking the neutralizing activity in a serum by preincubation with peptides. In this way we defined four important regions for neutralizing antibodies in human sera. Two epitopes were located on gp120, one in the V3 region (amino acids 304–318) and one in the C-terminal end (amino acids 489–508), as reported previously (5, 6, 20). The two other epitopes were located on gp41. One of these epitopes has been described previously and is apparently located in a region on the C-terminal half of gp41 (amino acids 732–746) (7). The other epitope, to our knowledge, not reported previously, is in a conserved region of the N-terminal half of gp41 (amino acids 647–671).

Several determinants of the HIV-1 envelope have previously been suggested to be involved in virus neutralization. The majority of the studies have focused on the variable region (V3) of gp120 (5, 6, 9). Our results show that indeed the central part of the V3 region is involved in neutralization. The V3 region is, however, not the only neutralizing epitope. Reactivity with peptides representing the IIIB sequence of the V3 region correlated with neutralization specific for IIIB but not for RF or SF2. Similar results were obtained with the corresponding MN peptide LP2, in that virus neutralization appeared to be strain specific. However, reactivity to a peptide corresponding to the C-terminal part of the MN-specific V3 domain showed correlation to neutralization of RF and SF2. The broader reactivity seen with this MN-derived peptide is encouraging since, by using peptides representing the MN sequence, instead of the IIIB sequence, of the V3 region as a putative immunogen, a broader antibody response may be detectable. If all peptides used in the present study would have been derived from the MN sequence, the

chance may have been higher of finding cross-reactive antibodies. In view of the variability of the V3 domain it is interesting to observe that some of the other regions we describe here are conserved and may therefore be better targets for functional antibodies in a vaccine.

The other site we detected on gp120 is located in the C-terminal end (amino acids 489–508) of the protein. The C terminus of gp120 has been suggested to be an antibody-dependent cellular cytotoxicity epitope (21). Palker *et al.* (20) have demonstrated its high immunogenicity. The overall seroreactivity against this site, represented by peptide C91 in our experiments, is 55%. Seroreactivity to peptides representing the IIIB-derived sequence correlated with neutralizing activity for IIIB virus infectivity inhibition and VSV pseudotype neutralization but not for SF2 or RF. In addition, in 6 of 10 sera, peptides representing this site could help to inhibit sera in virus neutralization assays.

The same results were also seen with peptides representing a part of a conserved region on gp41 (amino acids 647–671). The amino acid sequence is exactly the same in the four HIV-1 strains (IIIB, MN, RF, and SF2) and is also conserved between HIV-2 and simian immunodeficiency virus isolates. The site has not previously been described as a neutralizing epitope in either immunized animals or HIV-infected humans, although a potential B-cell epitope has been predicted within this region. The frequency of seroreactivity to this region (peptide 249) in HIV-1-infected subjects was 56%. This potential neutralizing epitope was also more broadly reactive than the V3 region, since IIIB- and SF2-specific neutralization correlated with reactivity to the 249 peptide. Interestingly, in consecutive serum samples from HIV-1-infected individuals, reactivity to this region correlated very well with the presence of neutralizing antibodies, whereas the amount of general anti-HIV IgG and anti-HIV V3 antibodies did not (unpublished data). Teeuwsen *et al.* (22) have reported a human monoclonal antibody that mapped in this region (amino acids 643–692) and reacted with three HIV-1 isolates (IIIB, RF, and SF2), suggesting that antibodies directed to this region are cross-reactive. Also, all sera tested inhibited the binding of the monoclonal antibody to HIV-1. Since the human monoclonal antibody lacks neutralizing activity it probably does not react with exactly the same epitope as we detect in human sera by neutralization. Furthermore, other human monoclonal antibodies specific for conformational determinants within this region (amino acids 644–663) could mediate antibody-dependent enhancement and antibody-dependent cellular cytotoxicity but not neutralization (23, 24). Thus, this region seems to induce antibodies with different immunologic functions. This phenomenon has also been seen with two murine monoclonal antibodies reacting with apparently the same epitope in the V3 region, where both of them could neutralize HIV-1 but only one could mediate antibody-dependent cellular cytotoxicity (19).

The fourth epitope was located in a conserved site (amino acids 732–746) of gp41. Immunizing animals to this site (amino acids 735–752) has been previously shown to induce

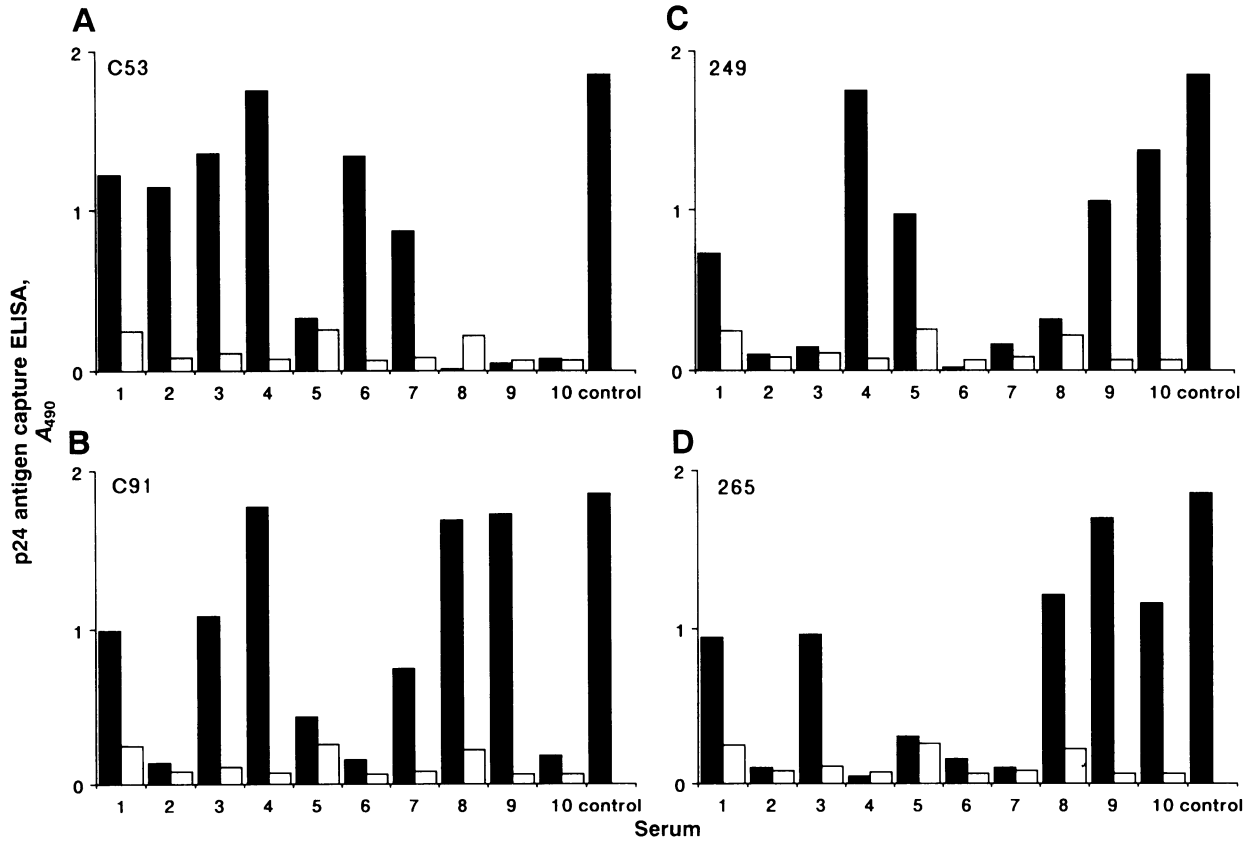

FIG. 2. Peptides (see Table 1 for specificity) were preincubated with 10 different HIV-1-seropositive and neutralizing sera (dilution, 1:20, 1:100, or 1:500; optimal value given) and one HIV-1 antibody-negative control serum prior to the neutralization assay (inhibition of HIV-1 HTLVIIIB infectivity assay). Solid columns show the serum neutralization after preincubation with peptides. Open columns represent the sera (at the same dilution) without preincubation with peptides. A high absorbance value indicates virus replication (lack of neutralization). None of the peptides could inhibit virus replication by itself without any serum present. Each experiment was performed three times with reproducible results. All experiments were compared with a specific control for calibrating the antigen ELISA and for comparing different experiments. The data are from one experiment performed at the same time for all sera. Individual neutralization titers of the sera are as follows: 1, 160; 2, >640; 3, >640; 4, 80; 5, 40; 6, 320; 7, 160; 8, 40; 9, 80; 10, 80. The peptide inhibition is shown at dilution 1:20 for sera 4, 5, and 8–10, at dilution 1:100 for sera 1, 6, and 7, and at dilution 1:500 for sera 2 and 3.

antibodies that neutralize HIV-1 and block fusion (7, 25). This region is also a potent immunogen when inserted in the VP1 domain of poliovirus (26). The epitope is probably not normally available for antibodies on the virion but might be recognized by antibodies after CD4 binding and thus block the putative fusion event. By using human serum in the present study reactivity to this region correlated in a broad fashion to neutralization of IIIB and SF2 (but not RF). The mechanism by which this site could contribute to neutralization is not clear.

The pattern of absorption of neutralizing antibodies varied between sera. The neutralization capacity could be reduced in all sera but not by control peptides from gag and other env regions. Eight of 10 sera could be reduced in neutralization capacity by more than one peptide. This implies that neutralization of HIV most often is due to antibody binding to more than one site. It has not been demonstrated that the sites, one by one, are able to induce neutralization. For the V3 region this has been shown previously (12, 27) and perhaps the additional regions alone are not enough for self-sufficient neutralization *in vivo*. Discontinuous sites may become recognized that also mediate such help.

Future studies may include purification of antibodies from HIV antibody-positive human sera with specific peptides from neutralizing regions, although several investigators have failed in such experiments. The neutralizing regions could also be further specified by using peptides with single amino acid substitutions. In conclusion, the four B-cell epitopes described in this study as well as other protective T-cell epitopes may be included in a vaccine against HIV. In this putative vaccine it will also be important to identify and exclude regions for undesired immune responses like immunosuppression, autoimmunity, and enhancement of viral infectivity.

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