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Continuous Epitopes of the Human Immunodeficiency Virus Type 1 (HIV-1) Transmembrane Glycoprotein and Reactivity of Human Sera to Synthetic Peptides Representing Various HIV-1 Isolates

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Immunoreactive regions of human immunodeficiency virus type 1 (HIV-1) gp41 were mapped by reacting HIV-1 antibody-positive human sera with overlapping synthetic peptides which covered the transmembrane protein. Three immunoreactive domains were identified, and five different and partially overlapping epitopes recognized by HIV-1-positive human sera were found within one immunodominant region. The effect on antibody recognition after single amino acid substitutions within one defined epitope was also studied. The reactivity of various HIV-1-positive sera to synthetic peptides with amino acid substitutions representing known isolates suggests an important substitution in the major epitope of African HIV-1 strains.

After infection with human immunodeficiency virus type 1 (HIV-1), structural and nonstructural proteins are presented to the immune system and induce an antibody response (1, 9). Purified antigens from infected cells (3, 19, 20), recombinant proteins (16, 22), and synthetic peptides (2, 8, 11, 15, 17, 21, 23) have all been employed in assays to demonstrate anti-HIV antibodies. Studies on antibody reactivity to synthetic peptides corresponding to amino acid sequences of the transmembrane HIV-1 gp41 have defined a relatively conserved immunodominant region between amino acids (aa) 587 (Ala) and 618 (Ser) in the external amino-terminal part of this protein (2, 6–8, 11, 15, 17, 21, 23).

In the present report, we describe experiments in which synthetic peptides covering the entire HIV-1 gp41 sequence, except for a hydrophobic region (aa 685 to 709), were employed to define all the immunoreactive domains that contain continuous B-cell epitopes and to map the precise location of such epitopes within the most immunoreactive peptide of the protein. We have also investigated the tolerance of antibody recognition for aa substitutions in one defined epitope and the reactivity of sera to peptides corresponding to sequences of the immunodominant region of gp41 from different HIV-1 isolates.

Serum samples from a large collection of HIV-1-positive sera (originating from Sweden, the United States, and East Africa) were used. HIV-1 seropositivity was confirmed by Western blot (immunoblot) analysis on all sera. Sera were not classified according to clinical features or stage of disease. Sera were assayed against synthetic peptide antigens that were covalently coupled to bovine serum albumin (BSA) fraction V (Boehringer GmbH, Mannheim, Germany) by using *N*-succinimidyl 3-(2-pyridyldithio) propionate, (Pharmacia, Uppsala, Sweden) as described by the manufacturer. The peptide-BSA conjugates (100 μg/ml) were passively coated onto microtiter plates (Maxisorp; Nunc AS, Roskilde, Denmark), and the remaining free binding sites in the wells were blocked with 3% BSA in phosphate-buffered saline (PBS). Serum samples were tested against each peptide at a dilution of 1:50 (in PBS containing 1% BSA and 0.05% Tween 20).

Mapping of antibody-binding domains of gp41. The serum immunoglobulin G (IgG) reactivity of 96 randomly selected Swedish HIV-1-positive serum samples against 21 overlapping synthetic peptides (Table 1) spanning the entire HTLV-IIIB-encoded gp41, except for the hydrophobic region at aa 685 to 709, was tested by enzyme-linked immunosorbent assay (ELISA). Solid-phase peptide synthesis was performed on an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Foster City, Calif.). A carboxyl-terminal cysteine was added to all peptides to facilitate coupling to the carrier protein. The t-Boc synthesis protocol was used, the peptides were removed from the solid-phase resin, and the protective side chains were removed (by use of hydrogen fluoride) as described by the manufacturer. Three different nonoverlapping immunoreactive domains at aa 594 to 634, 640 to 672, and 781 to 818, as defined by the peptides A5 and B1, B3 and B4, and CT5 and CT4, respectively, were identified (Fig. 1). One peptide (A5) reacted with all 96 HIV-positive serum samples, confirming the presence of a highly immunodominant region of gp41 in the amino-terminal portion of the protein (2, 6, 8, 11, 17, 21, 23). The A5 peptide has subsequently been tested against several thousand HIV-1 antibody-positive serum samples from different parts of the world and shown a sensitivity better than 99.9% for antibody detection when tested against sera of European and U.S. origin. In sera of East African (mainly Tanzanian) origin, the sensitivity is considerably lower, approximately 95% (data not shown). The B4 peptide reacted with 19 (20%) of the 96 serum samples assayed, and the CT4 peptide reacted with 29 (30%) of the serum samples.

Topography of the antibody-binding domains of gp41. Experiments were performed to determine whether the three antibody-binding domains of gp41 represented by the peptides A5, B4, and CT4 were exposed on the surface of the folded protein. Two human HIV-1-positive serum samples (116 and 190) that expressed IgG activity against peptides A5, B4, and CT4 were immunoadsorbed with whole-virus antigen and were then assayed for residual IgG activity against the three peptides by ELISA. Sera from HIV-1

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TABLE 1. Amino acid sequences of the 21 overlapping gp41 peptides synthesized

Peptide designation	Sequence (one-letter code)[a]	Amino acid nos.
A0	AVGIGALFLGFLGAAG	517–532
A2	GAAGSTMGAASMTLTVQARQ	529–548
A3	VQARQLLSGIVQQQNNLLRAIEA	544–566
A1	RAIEAQQHLLQLTVWGILQLQAR	562–584
A4	QLQARILAVERYLKDQQLL	580–598
A5	DQQLLGIWGCSGKLICTTAVPWN	594–616
B1	TAVPWNASWSNKSLEQIWNNMTWM	611–634
B2	IWNNMTWMEWDREINNYTSLI	627–647
B3	INNYTSLIHSLIEESQNQQEKNE	640–662
B4	EESQNQQEKNEQELLELDKWA	652–672
B5	ELDKWASLWNWFNITNWL	667–684
D3	VNRVRQGYSPLSFQTHLPTP	710–729
D2	LPTPRGPDRPEGIEE	726–740
D1	DRPEGIEEEGGERDRDRSIRLV	733–754
D0	RSIRLVNGSLALIWDDLRSLC	749–769
CT6	LRSLCLFSYHRLRDLLLIVTR	765–785
CT5	LIVTRIVELLGRRGWEALKY	781–800
CT4	ALKYWWNLLQYWSQEKNSAVS	797–818
CT3	KNSAVSLLNATAIAVAEGTD	813–832
CT2	AEGTDRVIEVVQGSACRAIRHIPR	828–850
CT1	RHIPRRIRQGLERILL	846–861

[a] Sequence data are derived from the work of Muesing et al. (13).

seropositive asymptomatic persons were immunoadsorbed with HIV-1 whole-virus antigen (Triton X-100-solubilized HIV-SF2-infected HUT-78 cells) and tested by ELISA for antibody reactivity to synthetic peptides representing different immunogenic domains of gp41. Whole-virus antigen (10.5 mg of protein) was coupled to 1.05 g of cyanogen bromide-activated Sepharose 4B (Pharmacia), and 0.5-ml gel columns were prepared in a glycine-NaOH buffer (pH 8.8), as described by the manufacturer. Sera were immunoadsorbed by incubating 400 μl of diluted (1:200) serum with the HIV antigen gel for 1 h at room temperature. Nonbound antibodies were eluted with the glycine-NaOH buffer, and 100-μl fractions were collected and tested for IgG reactivity

TABLE 2. Remaining reactivities of two human HIV-1 antibody-positive serum samples to synthetic peptides after immunoadsorption to HIV-1 whole-virus antigen

Peptide	A_{405} of serum sample[a]:	
	116	190
A5	0.22	0.32
B4	0.06	0.02
CT4	0.01	0.11

[a] A_{405} value by ELISA of the fraction collected from an immunoadsorption column containing the highest antibody reactivity to the respective peptide is presented. For reactivity of these sera to the peptides prior to immunoadsorption, see Fig. 2. More than one fraction was reactive against the A5 peptide.

to the peptides by ELISA. Only anti-A5 peptide IgG activity could be demonstrated after adsorption (Table 2).

To extend these findings, we assayed antipeptide IgG bound to the immunoadsorption columns containing whole-virus antigen by peptide ELISA after desorption at pH 2.5. After immunoadsorption of the human sera (at a dilution of 1:2) to gel-coupled whole HIV-1 virus antigen, the gels were washed with 1.5 ml of the pH 8.8 glycine-NaOH buffer and then with 1.5 ml of glycine buffer, pH 4.5 and pH 3.5, respectively. Thereafter, bound antibody was desorbed with 1 column volume of glycine buffer (pH 2.5), and 100-μl fractions were collected and diluted (see Results) in PBS buffer (containing 1% BSA and 0.05% Tween 20) and tested for reactivity to the peptides by ELISA. The absorption values obtained by ELISA of serial dilutions of the HIV-1-positive human serum samples 116 and 190 prior to adsorption and the desorption fraction containing the highest antibody reactivity to the peptides tested at the dilutions of 1:50 and 1:100 are presented in Fig. 2. It can be seen that with both sera the immunoadsorption column preferentially bound antibodies to the B4 and CT4 peptides. However, some residual A5 activity could also be seen after desorption.

Rabbit immune sera raised against the peptides corresponding to the three immunoreactive domains of gp41 were

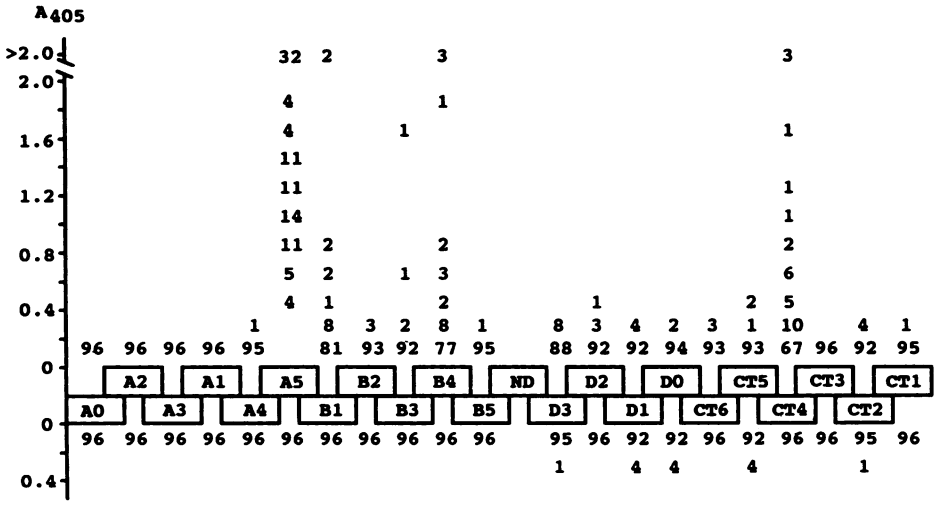

FIG. 1. Seroreactivity in a standard indirect ELISA. Reactivities of 96 HIV-1-positive serum samples (above the abscissa) and 96 blood donor serum samples (below the abscissa) to 21 overlapping gp41 peptides are shown. Boxes containing designations of the peptides show the relative positions of the peptides in the protein. The numbers of HIV-1-seropositive and -seronegative serum samples that gave an A_{405} value by ELISA within each 0.2 absorbance unit interval are depicted above and below each peptide. ND, Not done.

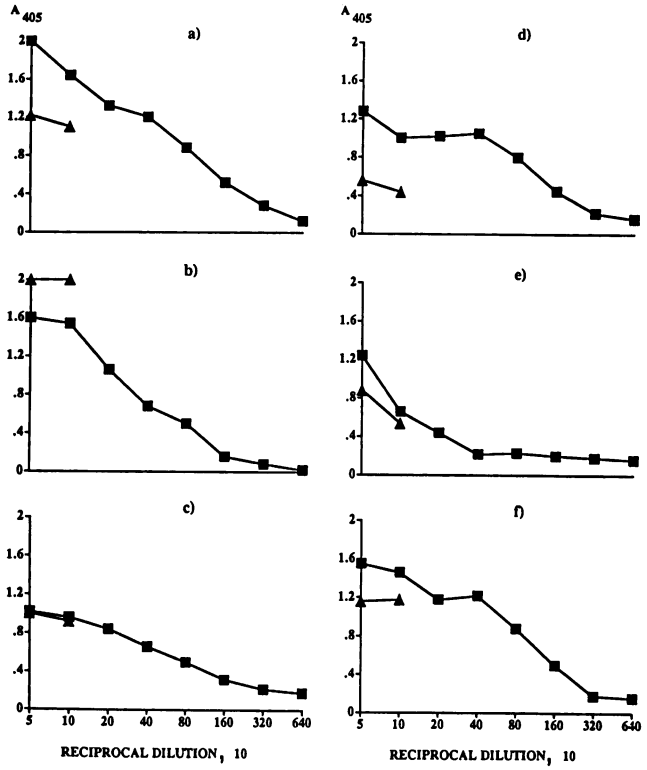

FIG. 2. Reactivities of two HIV-1-positive serum samples to peptides A5 (a and d), B4 (b and e), and CT4 (c and f) before immunoaffinity chromatography against HIV-1 whole-virus antigen (■) and after elution of adsorbed antibody (▲). Serum sample 116 (a, b, and c) and serum sample 190 (d, e, and f) were used. Depicted are the absorbance values obtained with serum samples before immunoadsorbtion at twofold dilutions ranging from 1:50 to 1:6,400 and with the desorption fraction containing the highest antibody reactivity to the peptides at dilutions of 1:50 and 1:100.

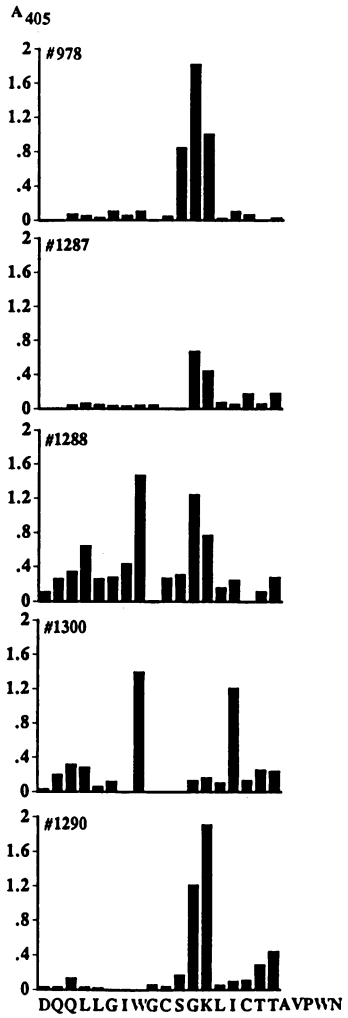

FIG. 3. Epitope mapping of the immunodominant region of gp41 as represented by the A5 peptide. Five HIV-1-seropositive serum samples were assayed by ELISA against 18 hexapeptides overlapping one another by 5 aa. Each bar represents the absorbance value obtained with the hexapeptide having the amino-terminus amino acid depicted below the bar. Absorbance values obtained with an HIV-1 seronegative serum sample with the respective peptide (never exceeding 0.2 absorbance unit) have been subtracted in the figure. In each experiment, control peptides (Pro-Leu-Ala-Gln and Gly-Leu-Ala-Gln) were assayed with a monoclonal antibody (provided with the kit) specific for the Pro-Leu-Ala-Gln peptide.

obtained by immunization with the peptides coupled to rabbit serum albumin by using *N*-succinimidyl 3-(2-pyridyldithio) propionate as described above and administering 50 μg intramuscularly four times, 2 weeks apart, with 0.5 ml of Freund's complete or incomplete adjuvant. These sera were tested against HIV-1 proteins by Western blot analysis. Anti-A5 immune sera did not recognize gp41 or the uncleaved gp160. Anti-B3 antibodies, also reactive with the B4 peptide, gave positive bands for both gp41 and gp160 by Western blot analysis. Anti-B4 antibodies that did not cross-react with the B3 peptide and anti-CT4 immune sera were negative by Western blot analysis (data not shown).

Mapping of epitopes in the A5 region of gp41. Truncated versions of the original A5 peptide, A5/1 (DQQLLGIWGC SGKLIC), A5/2 (CSGKLICTTAVPWN), A5/3 (KLICTT AVPWN), and A5/4 (CTTAVPWNASW), were synthesized and tested by ELISA for reactivity with the panel of 96 confirmed HIV-1-positive serum samples. Of these samples, 100 and 96% reacted with the peptides A5/1 and A5/2, respectively. These peptides had the overlapping sequence CSGKLIC. Although the A5/3 peptide had only a 4-aa overlap (KLIC) with the A5/1 peptide, it was still recognized by 83% of the sera. In contrast, the A5/4 peptide, which shares only 1 aa with the A5/1 peptide, was recognized by only three serum samples. These results suggested that the A5 peptide contained more than one epitope and that one major epitope seemed to be located at the A5/1-A5/2 overlap. To precisely delineate the epitopes within the 23-aa region of gp41 represented by the A5 peptide, 18 hexapeptides (overlapping one another by 5 aa) were tested by ELISA (at a dilution of 1:200) with five HIV-positive human serum samples (Fig. 3). The epitope-scanning kit from Cambridge Research Biochemicals (Cambridge, United Kingdom) was used to manually synthesize the small peptides on polyethylene pins, as originally described by Geysen et al. (4, 5), utilizing f-moc chemistry as described by the manufacturer. Five partially overlapping epitopes could be distinguished: LLGIWG, WGCSGK, GKLICTT, ICTTAV, and TAVP WN. No single serum sample showed reactivity to all individual epitopes. Two serum samples (978 and 1287) reacted to only one epitope, centered around the hexamer

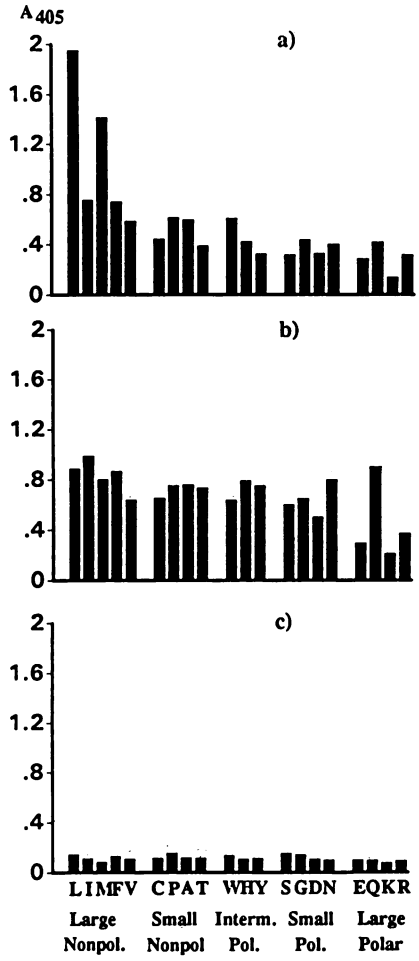

FIG. 4. Antibody recognition after amino acid substitutions in the defined epitope GKLICT. Leucine was replaced by all 19 of the other naturally occurring amino acids. (a and b) ELISA reactivity patterns of two HIV-1-positive serum samples; (c) pattern of an HIV-1-negative serum sample. Each bar represents the absorbance value obtained with the peptide having the amino acid substitution indicated by the one-letter code below the bar at the bottom of the figure. Peptides are grouped according to similarity in chemical properties of the substituted amino acid. The replacement ELISA was performed by using the epitope-scanning kit as described in the text. Interm., Intermediate; Pol., polar.

GKLICT. When four negative control blood donor serum samples were assayed, less than 0.2 U of absorbance was observed with all of the 18 hexamer peptides.

Effects of amino acid substitutions on antibody recognition. The effect of amino acid substitutions on the ability of human serum antibody to recognize HIV gp41 epitopes was tested by using the epitope defined by the sequence GKLICT. The third amino acid (leucine) was replaced by all 19 of the other aa. Two serum samples (978 and 1288) recognizing this epitope and one negative-control serum sample were assayed for antibody recognition of the original and 19 variant synthesized hexapeptides. With serum sample 978, which gave high values of adsorption to the original GKLICT peptide (Fig. 4a), antibody recognition decreased when the leucine was replaced by any other amino acid except for methionine. With the somewhat less reactive serum sample 1288 (Fig. 4b), antibody recognition did not vary significantly after any of the amino acid substitutions, except for the negatively charged amino acid glutamate and the positively charged amino acids lysine and arginine. The absorbance values obtained with the HIV-negative serum samples are presented in Fig. 4c.

Antibody reactivity to peptides of the immunodominant region of gp41 representing different sequenced isolates. In contrast to the results obtained when panels of HIV-1-positive serum samples of Western origin (Europe and the United States) were tested, some serum samples in panels from East Africa showed low or no reactivity against the gp41 A5 peptide with sequences derived from the HTLV-IIIB isolate (data not shown). To determine whether such sera were reactive to the immunodominant region of gp41 of other HIV-1 isolates, nine variant peptides representing the A5 region and corresponding to HIV-1 strains other than HTLV-IIIB (14), or variants thereof, were synthesized. The ELISA reactivity patterns of eight HIV-1-positive serum samples of East African origin (selected from a panel of more than 500 assayed serum samples as representative of the different reactivity patterns seen in the larger panel) against these variant A5 peptides are shown in Table 3. The most prominent effect on antibody reactivity was seen with the replacement of leucine by histidine in the GKLICTT epitope, as exists in the MAL and ELI strains of HIV-1 (serum samples 1 to 3 versus serum samples 4 to 7). The position of an epitope at this location was also supported by the absence of reactivity of these serum samples to the peptide when isoleucine was replaced by tyrosine, as seen in the sequence of the peptide representing the NY5 strain.

TABLE 3. ELISA reactivities of eight selected HIV-1-positive serum samples of East African origin against 10 different synthetic peptides representing eight different sequenced isolates (14) and two variants (A54 and A51)

Peptide sequence	Reactivity[a] against serum sample no.:								HIV isolate
	1	2	3	4	5	6	7	8	
DQQLLGIWGCSGKLICTTAVPWN	++	++	++	++	−	−	−	−	HTLV-IIIB
------F----------	+++	+++	+++	++	+++	−	−	−	CDC4
----------L---------	++	++	++	−	−	−	−	−	HXB3
----------T-----	++	++	++	−	−	−	−	−	WMJ2
------M--------Y---T-----	−	−	−	−	−	−	−	−	NY5
S------L----------T-----	++++	+++	++++	−	−	−	−	−	Z3
------L------H----------	−	−	−	+++	++++	++	+	−	A54
------M------H----------	−	−	−	++++	++++	+	+	−	A51
--R---M------H-----F-----	−	−	−	++++	++++	++	+	−	MAL
----------H-----N-----	−	−	−	++++	++++	+++	+++	++	ELI

[a] −, none reactive; +, < 3× cutoff; ++, > 3× < 6× cutoff; +++, > 6× < 9× cutoff; ++++, > 9× cutoff. Cutoff, mean of A_{405} values of six negative control serum samples + 6 standard deviations.

Serum sample 8 seemed to react to an epitope to the right of GKLICTT only when the alanine was replaced by an aspargine. In addition to reacting to the GKHICT epitope, serum sample 5 seemed to react to an epitope to the left of this when an isoleucine was replaced by a phenylalanine (HTLV-IIIB versus CDC4). The fact that serum samples 1 and 3 reacted more strongly to the peptide corresponding to Z3 than to the peptides corresponding to HTLV-IIIB and WMJ2 on one hand and the A54 peptide on the other indicated the presence of an epitope at the amino terminus of the peptide recognized by the two serum samples when the aspartic acid was replaced by a serine.

In the present study, we have demonstrated three different antigenic domains in gp41 accommodating continuous epitopes. The defined domains demonstrated here were situated within rather conserved regions of gp41 (14). However, we cannot exclude the possibility of additional epitopes residing within variable regions possibly not well represented by the HTLV-IIIB sequence.

The results of the present study indicate that some epitopes of the gp41 region defined by the A5 peptide are not exposed on the surface of the folded protein and support earlier studies which suggested that the immunodominant domain between aa 594 to 618, which corresponds to a hydrophobic peak in hydrophilicity plots (13), is probably hidden in the folded protein (11). This would suggest that the A5 region is first presented to the immune system after processing of the protein by antigen-presenting cells and further that anti-A5 antibodies are not consumed in situations of high antigen load, as seen in early and late infection (10, 12). This might provide an explanation for the high sensitivity of detection of anti-HIV antibodies with peptide ELISAs using peptides from this region of gp41 as antigen. Furthermore, peptides from this region of the transmembrane glycoprotein of both HIV-1 and HIV-2 react type specifically (6, 17). By the criteria named above, the B3 and possibly the CT4 regions are exposed when the protein is folded.

Fine mapping by using 6-aa-long peptides encompassing the A5 domain revealed five different epitopes, partially overlapping each other. However, none of the five serum samples studied recognized all five epitopes. The differences in epitope recognition observed with the five serum samples suggest a difference in processing and/or antigen presentation. In a previous study, Norrby et al. (18) identified only one or possibly two epitopes in the immunodominant region of gp41 recognized by HIV-1 antibody-positive human sera; this was done by using variants of a 23-aa peptide in which 1 aa had been either deleted or replaced by a Gly and by recording the numbers of human serum samples with decreased ELISA reactivity after amino acid deletion or substitution. The higher number of epitopes found in the present study is probably due to the differences in the techniques employed.

Our results are also similar to those of Gnann et al. (7) and Norrby et al. (18) in that we found a major antibody recognition site in the region of aa 604 to 612. This region seemed to contain two different but overlapping epitopes. After we replaced Leu by all other amino acids in one of these epitopes, a prominent reduction in antibody binding was observed for all amino acid substitutions except for Met when a serum with a strong reactivity to the original sequence was tested. However, antibodies of apparently lower avidity tolerated the substitutions of Leu by all other amino acids except for the charged amino acids Glu, Lys, and Arg.

The consequences of relatively conservative amino acid changes within an epitope as well as localization and numbers of epitopes within the A5 region of gp41 could also be derived to some extent from the results of seroreactivity of African sera to peptides corresponding to HIV-1 strains other than HTLV-IIIB. These results also indicated a prominent epitope located at aa 605 to 610 (GKLICT). Gnann et al. (6) reported that some HIV-1-positive sera from Zaire reacted to a 12-aa peptide spanning the GKLICT epitope only when the Leu at position 607 was replaced by a His. We present here similar results obtained with sera from East Africa. Thus, at least in relation to the GKLICT epitope, there would seem to be at least two prevailing strains of HIV-1 in Africa. This should be considered when immunoassays based on peptides for the detection of HIV-1-infected blood are designed, particularly if such assays are to be used for screening donor blood in Africa. Indeed, synthetic peptides representing immunodominant regions of different HIV-1 isolates might provide a more exact tool for epidemiological studies.

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