Presence of antibodies to a putatively immunosuppressive part of human immunodeficiency virus (HIV) envelope glycoprotein gp41 is strongly associated with health among HIV-positive subjects

(acquired immunodeficiency syndrome/p15E)

PER JOHAN KLASSE*[†], RÜDIGER PIKORN[‡], AND JONAS BLOMBERG*

*Section of Virology, Department of Medical Microbiology, Lund University, Lund, Sweden; and [‡]Zentrum für Molekulare Biologie, Heidelberg, Federal Republic of Germany

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ABSTRACT The IgG response to gp41 (envelope glycoprotein of M_r 41,000) of the human immunodeficiency virus (HIV) was studied with eight synthetic peptides derived from three different regions of the protein. We tested sera from 17 HIV-seronegative and 68 HIV-seropositive subjects in an enzyme immunoassay. No HIV antibody-negative serum reacted with any of the peptides. The peptide HIV-env 583–599 has a sequence similarity with immunosuppressive peptides derived from the transmembrane proteins of other retroviruses. Antibodies to this 17-mer (HIV-env 583–599; hereafter also referred to as pHIVIS, putative HIV immunosuppressive sequence) were detected in 27 of the 35 sera from healthy HIV-positive persons but only in 1 of the 33 sera from patients with HIV-related disease. Another 17-mer, displaced four amino acids N-terminally from pHIVIS, reacted with fewer of the sera from healthy seropositive subjects than pHIVIS but with no serum from ill seropositive patients. HIV-env 586–603, which shares two-thirds of its sequence with pHIVIS, reacted with the sera from nearly all subjects, regardless of clinical status. The remaining five peptides did not discriminate between healthy and ill seropositive subjects either but gave lower reactivity rates. HIV-positive sera thus exhibited distinct patterns of reactivity with subsequences of gp41. We have mapped two overlapping epitopes within a narrow part of gp41; antibodies to the most N-terminally located of the two—i.e., the pHIVIS-reactive antibodies—might counteract a possible immunosuppressive effect of gp41.

Immunosuppression by human immunodeficiency virus (HIV) may be mediated by a reduction in number of T-helper lymphocytes and by impairment of their function. Much evidence supports the hypothesis that interactions between the heterodimeric HIV envelope glycoprotein (M_r 160,000) and the CD4 molecule cause syncytia formation and the killing of lymphocytes (1–4). HIV gp41, the smaller part of the heterodimer, has been implicated in the cytotoxicity of HIV (4). However, T-helper cells from acquired immunodeficiency syndrome (AIDS) patients are not only abnormally few but also functionally defective (5–7). B-lymphocyte dysfunction in AIDS has been attributed to stimulation as well as to inhibition by HIV (5–8). Soluble factors from cultures of AIDS patients' mononuclear cells inhibit B-cell differentiation and T-cell blastogenic responses to antigen (9, 10).

Several retroviruses contain an evolutionarily conserved sequence in their transmembrane glycoproteins—e.g., p15E of feline leukemia virus (FeLV) and of murine leukemia virus (MuLV) (11–17). A 17-mer, with the MuLV sequence, is immunosuppressive *in vitro* and has six exact amino acid

matches with gp41 of HIV (14). The gp41-derived 17-mer, with this sequence similarity, inhibits CD3 stimulation of lymphocytes *in vitro* (18).

We synthesized and purified eight peptides derived from three regions of HIV gp41. From a first region, the *in vitro*-immunosuppressive 17-mer (HIV-env 583–599; the putative HIV immunosuppressive sequence pHIVIS) was produced. For comparison, we made a peptide with the inverted sequence of pHIVIS (HIV-env:inv 599–583) and two other 17-mers with four amino acid shifts relative to pHIVIS (HIV-env 579–595 and HIV-env 587–603). A 21-mer (HIV-env 586–606) from the same region was made as a further control; antibodies reactive with this peptide are reportedly found in nearly all investigated HIV-positive sera (19). From a second region was synthesized a 13-mer (HIV-env 654–666), recently predicted to correspond to an important epitope (20). From a third region, the C terminus, two peptides (HIV-env 848–863 and HIV-env 854–863) were derived and produced. The C terminus has a sequence similarity with a putative receptor-binding site of interleukin 2; therefore it has been proposed to be immunosuppressive (21).

Antibody reactivity with pHIVIS, but not with the other peptides, was strongly associated with absence of HIV-related disease. Hypothetically, pHIVIS-reactive antibodies curb progression to AIDS.

MATERIALS AND METHODS

Peptide Synthesis. Peptides were derived from the amino acid sequence of the HIV isolate HTLVIII_B, clone BH10 (22). Table 1 shows the sequences of the peptides. The synthesis followed the stepwise solid-phase strategy developed by Merrifield (23) and was done on an automated peptide synthesizer (430A; Applied Biosystems, Foster City, CA). Protected amino acids were obtained from Novabiochem (Basel). For temporary N-terminal protection we used the base-labile N^{α} -9-fluorenylmethoxycarbonyl group. The side chains were blocked in the following ways: *tert*-butyl for aspartic acid, glutamic acid, serine, and tyrosine; 4-methoxy-2,3,6-trimethylbenzenesulfonyl for arginine; *tert*-butoxycarbonyl for lysine; and trityl for histidine and cysteine. A *p*-alkoxybenzyl alcohol resin (24) was used throughout the synthesis. We cleaved the peptides from the resin by treatment with trifluoroacetic acid/thioanisole for 4 hr. Then we

Abbreviations: FeLV, feline leukemia virus; MuLV, murine leukemia virus; HIV, human immunodeficiency virus; pHIVIS, putative immunosuppressive sequence of HIV; EIB, electrophoretic immunoblotting; EIA, enzyme immunoassay; AIDS, acquired immunodeficiency syndrome.

[†]To whom reprint requests should be addressed at: Section of Virology, Department of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

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Table 1. Amino acid sequences of the investigated peptides

HIV-env amino acid position*	Sequence†	Frequency of reactivity	
		+ H	+ D
HIV-env 579–595	G I K Q L Q A R I L A V E R Y L K	12/35	0/33
HIV-env 583–599 (pHIVIS)	L Q A R I L A V E R Y L K D Q Q L	27/35	1/33
HIV-env:inv 599–583	L Q Q D K L Y R E V A L I R A Q L	11/35	3/33
HIV-env 586–606	R I L A V E R Y L K D Q Q L L G I W G C S	31/35	29/33
HIV-env 587–603	I L A V E R Y L K D Q Q L L G I W	31/35	21/33
HIV-env 654–666	E E S Q N Q Q E K N E Q E	4/31	2/31
HIV-env 848–863	R H I P R R I R Q G L E R I L L	24/31	14/31
HIV-env 854–863	I R Q G L E R I L L	19/35	9/33

+ H, healthy HIV-seropositive persons; + D, diseased HIV-seropositive patients.

*The numbering system of Ratner *et al.* (22) was used.

†A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; V, valine; W, tryptophan; Y, tyrosine.

purified the peptides to homogeneity by preparative HPLC (Waters Associates) using a reverse-phase column (Nucleosil C₁₈, 250 × 10 mm) with different gradients (depending on peptide) of 10–90% solution B (acetonitrile/H₂O, 60:40) mixed with solution A (0.1% trifluoroacetic acid in H₂O) at a flow rate of 6 ml/min. We ascertained the amino acid proportions by ion-exchange chromatography. The purity, checked by HPLC, was always at least 98%.

Sera. Sera came from 68 persons confirmed to be HIV antibody-positive by electrophoretic immunoblotting (EIB). Thirty-five of these subjects were healthy, 13 had AIDS, 6 had AIDS-related complex, and 14 had persistent generalized lymphadenopathy according to the Centers for Disease Control criteria (25). The peptides were also tested against 17 HIV antibody-negative sera: 2 from patients with leukemia and the rest from healthy blood donors. Two peptides were tested against 72 additional HIV-negative sera: 6 from leukemia patients and 66 from healthy blood donors.

Enzyme Immunoassay (EIA). The EIA was carried out with modifications of a previously described method (26). One hundred microliters per well of a solution of 10 µg of peptide per ml of phosphate-buffered saline (PBS: 8.0 g of NaCl, 0.2 g of KH₂PO₄, 1.4 g of Na₂HPO₄, and 0.2 g of KCl per liter of H₂O)/1 mM sodium merthiolate (pH 7.4) (PBS/M) or only PBS/M as a control was left overnight at 4°C in 96-well microtitration plates (Nunc Immunoplate II; Nunc). Two hundred microliters per well of a blocking solution containing 4% (wt/vol) bovine serum albumin (fraction V, Boehringer Mannheim) and 0.2% (wt/vol) gelatin (Difco) in PBS/M (pH 7.4) was added and then left for 6 hr at room temperature and subsequently left overnight at 4°C. Before use the plates were washed four times with PBS/0.05% (wt/vol) Tween 20/0.1% (wt/vol) sodium azide (PBS/Tween). Sera were diluted in 3% (wt/vol) bovine serum albumin/0.2% (wt/vol) gelatin/0.05% (vol/vol) Tween 20 in PBS. One hundred microliters of serum diluted 1:20 was added to each well and left to incubate for 1 hr at room temperature on a shaker. The plates were washed five times with PBS/Tween. One hundred microliters of peroxidase-conjugated rabbit immunoglobulins to human IgG γ chains (Dakopatts, Copenhagen) diluted 1:400 in PBS/Tween/0.2% gelatin (wt/vol) was added and incubated on a shaker for 1 hr at room temperature. After washing five times with PBS/Tween, 100 µl of substrate solution per well [40 mg of *o*-phenylenediamine/20 µl of 30% H₂O₂ per 100 ml of buffer (34.7 mM citric acid/66.7 mM Na₂HPO₄ solution, pH 5.0)] was added. Following 1 hr of enzyme reaction at room temperature and in darkness the absorbances were measured in a Titertek Multiskan colorimeter (Flow Laboratories).

Densitometry and EIA Titrations. Densitometry of enzyme immunoblot strips was performed on a random sample of the sera, as described (27), on a Bio-Rad model 620 video

densitometer. Absorbance values were expressed in “color-yield units” (27). A commercial EIA for detection of anti-HIV antibodies (Envacor HIV 1 EIA; Abbott) was used according to the instructions of the manufacturer, except for the serum dilution, which was varied from 1:2 to 1:1000.

Statistical Methods and Computer Programs. We used the Wilcoxon rank sum test and Fisher exact test as included in the Microstat statistical package (Ecosoft, Indianapolis, IN). The PC/Gene program (Genofit, Geneva) was used for computation and plotting of Hopp–Woods indices (28) as averages of six sequential amino acids.

Coating Efficiency of Peptides in EIA. We tested this first by exchange-labeling of the peptides with tritiated water and palladium oxide at pH 3.0 followed by peptide incubation in EIA plates. Second, we measured the binding sites remaining for ¹²⁵I-labeled bovine serum albumin after coating EIA plates with the peptides at various concentrations (data not shown; the methods will be published elsewhere). At a concentration of 10 µg of peptide per ml, which we used in the EIA, all peptides bound to similar extents in both tests; the occupancy of albumin-binding sites was 80–95%. However, at lower concentrations of the peptides, the differences between their coating efficiencies were greater.

RESULTS

Frequency of Reactions. For each serum an absorbance value was calculated from the absorbances of three microtiter wells as the difference between the average absorbance of two peptide-coated wells and a well mock-coated with PBS/M. No HIV-negative serum exceeded the arbitrary cutoff value of 0.100 absorbance unit for any peptide. The HIV antibody-positive sera gave a mosaic pattern of reactivity (Fig. 1). The most sensitive peptide for detection of HIV seropositivity (as defined by EIB positivity) was HIV-env 586–606, the longest peptide in the study: 60 of 68 EIB-seropositive sera were positive for this peptide. The second most frequently reactive peptide was HIV-env 587–603: 52 of 68 reacted. Thirty-eight of 62 HIV-positive sera reacted with HIV-env 848–863 and 28 of 68 reacted with HIV-env 854–863. Twenty-eight of 68 were positive for HIV-env 583–599 (pHIVIS) and 14 of 68 were positive for its inverted counterpart, HIV-env:inv 599–583. The most N-terminally derived peptide, HIV-env 579–595, gave 12 positives of 68 sera. Only 6 of 62 sera reacted with HIV-env 654–666. We titrated the peptide concentration up to 50 µg/ml without seeing any shifts in the relative absorbances obtained with the eight peptides (data not shown).

The specificities of HIV-env 583–599 (pHIVIS) and its inversion were tested further against 66 HIV-negative blood donor sera and 6 HIV-negative sera from patients with

FIG. 1. Frequency of IgG antibody reactivity with eight different peptides derived from the HIV gp41 sequence. The numbers at the top correspond to the eight peptides: 1, HIV-env 579–585; 2, HIV-env 583–599 (pHIVIS); 3, HIV-env:inv 599–583; 4, HIV-env 586–606; 5, HIV-env 587–603; 6, HIV-env 654–666; 7, HIV-env 848–863; 8, HIV-env 854–863. The left margin line between two tick marks represents a serum; the sera are grouped according to the clinical status of the subjects, as indicated. PGL, persistent generalized lymphadenopathy; ARC, AIDS-related complex. Absorbance values above the cutoff are represented as dotted rectangles. The difference between the two groups in proportions above and below the cutoff was tested by means of the Fisher exact test (healthy versus ill): 1, HIV-env 579–595, 12/35 versus 0/33, $P = 1.2 \times 10^{-4}$; 2, HIV-env 583–599 (pHIVIS), 27/35 versus 1/33 (the single ill pHIVIS-positive subject had Kaposi sarcoma and thereby AIDS), $P = 7.9 \times 10^{-11}$; 3, HIV-env:inv 599–583, 11/35 versus 3/33, $P = 0.022$; 4, HIV-env 586–606, 31/35 versus 29/33, $P = 0.61$; 5, HIV-env 587–603, 31/35 versus 21/33, $P = 0.016$; 6, HIV-env 654–666, 4/31 versus 2/31, $P = 0.34$; 7, HIV-env 848–863, 24/31 versus 14/31, $P = 9.0 \times 10^{-3}$; 8, HIV-env 854–863, 19/35 versus 9/33, $P = 0.021$. *, Not done.

leukemia. All yielded absorbances below the stipulated cutoff (data not shown).

Statistical Analysis and Comparison of Groups. Neither the absorbances nor their logarithmic values were consistently normally distributed. Therefore we analyzed differences between healthy and diseased subjects nonparametrically. All original data and statistical test results are given in Figs. 1 and 2. Healthy versus ill gave $P = 1.2 \times 10^{-4}$ (Fisher test), $P < 1.5 \times 10^{-6}$ (Wilcoxon rank sum test) for HIV-env 579–595, and $P = 7.9 \times 10^{-11}$ (Fisher test) and $P < 1.5 \times 10^{-8}$ (Wilcoxon rank sum test) for pHIVIS. The other peptides gave P values > 0.001 with both statistical tests. Since the tests were repeated eight times we considered P values below 0.001/8 highly significant [Bonferroni correction (29)].

Comparison with Other Measures of HIV Seropositivity. We checked if absorbance values for pHIVIS were a mere reflection of abundance of gp41 antibodies. Densitometry was performed on EIB strips of a random sample of eight sera positive and eight negative (of which six were ill) for pHIVIS: the average absolute reflectance values [expressed in color-yield units (27)] for gp41 were 2.0 in both groups.

Sera from 11 pHIVIS-negative ill patients, 6 pHIVIS-positive, and 10 pHIVIS-negative healthy persons were titrated from 1:2 to 1:1000 in a recombinant *gag* and *env* protein HIV EIA (Envacore HIV1 EIA; Abbott). The healthy persons' anti-*gag* titers ranked higher than that of the ill subjects ($P < 1.1 \times 10^{-2}$). The pHIVIS-positive subjects' anti-*gag* titers also ranked higher than that of the pHIVIS-negative patients ($P < 4.9 \times 10^{-3}$). Anti-*env* titers for ill versus healthy subjects gave $P < 0.40$ and for pHIVIS-negative versus pHIVIS-positive subjects gave $P < 0.27$ (Wilcoxon rank sum test).

DISCUSSION

Theoretically, synthetic and recombinant peptides are excellent tools for mapping of epitopes and for exploration of individual immune responses (30, 31). Peptides corresponding to subsequences of gp41 have been used in solid-phase immunoassays for detection of antibodies to HIV and identification of vaccine-relevant epitopes (19, 32–34).

However, epitope mapping by use of short synthetic peptides in EIA is somewhat problematic. Although, in our study, approximately equal amounts of the eight peptides had bound to the EIA wells, their way of binding may have differed; their interaction with hydrophobic surfaces could exclude the conformational simulation of certain epitopes.

The peptide HIV-env 583–599, pHIVIS, which has six amino acid matches with immunosuppressive sequences in other retroviruses (14), inhibits lymphocyte proliferation *in vitro* (15). It was therefore very interesting to find antibodies reactive with this peptide, which were strongly associated with health. We got the highest differentiation between healthy and diseased subjects when all six amino acids matching with the immunosuppressive MuLV sequence (Leu-Gln-...-Arg-...-Leu-Lys-...-Leu) were included (14). Peptides with N- and C-terminally shifted sequences reacted differently: HIV-env 579–595 gave fewer and weaker reactions but only with sera from the symptom-free. Only one serum was positive for HIV-env 579–595 but not for pHIVIS, and the difference was only 0.045 absorbance unit. The C-terminal (587–603) displacement increased the rate of reactivity. It was above the cutoff for 28 sera that were negative for pHIVIS. Vice versa, 3 sera reacted with pHIVIS but not with HIV-env 587–603. Many sera gave highly differing reactivities with these two peptides. There are two hydrophilicity maxima in pHIVIS; HIV-env 587–603 contains only one of them. We suggest that pHIVIS roughly corresponds to an epitope and that the C-terminal shift causes the inclusion of another. Few of the sera reacting with pHIVIS reacted with the inverted peptide. This shows that the pHIVIS-reactive antibodies do not just recognize amino acid residues in a certain order.

The longest peptide used here, HIV-env 586–606, showed a very high rate of reactivity with sera from healthy and from ill persons, in accordance with a previous report (19). HIV-env 654–666, derived from a predicted antigenic site (20), gave the fewest and weakest reactions. Unless this peptide assumed unfavorable conformations in the EIA, it apparently does not correspond to a major epitope.

The two peptides derived from the C terminus of gp41 have a sequence similarity with a putative receptor-binding site of interleukin 2 (21). We note that only antibody reactivity with the longest of the two was somewhat associated with health.

FIG. 2. Quantitative distribution of reactivities to the eight peptides. The absolute absorbance values after background subtraction are represented on the ordinate for each peptide. -C, negative control sera; +H, HIV-positive sera from healthy individuals; +D, HIV-positive sera from patients with HIV-related disease. The absorbances of categories +H and +D for each peptide were compared by the Wilcoxon rank sum test. (Upper) Peptide 1, HIV-env 579-595, $P < 1.5 \times 10^{-6}$; 2, peptide 2, HIV-env 583-599 (pHIVIS), $P < 1.6 \times 10^{-8}$; peptide 3, HIV-env:inv 599-573, $P < 6.1 \times 10^{-3}$; peptide 4, HIV-env 586-606, $P < 0.25$. (Lower) Peptide 5, HIV-env 587-603, $P < 0.018$; peptide 6, HIV-env 654-666, $P < 0.23$; peptide 7, HIV-env 848-863, $P < 8.6 \times 10^{-3}$; peptide 8, HIV-env 854-863, $P < 0.15$.

The widely differing peptide results made it clear that high reactivity with pHIVIS was not a mere reflection of a general tendency to react with gp41-derived peptides. We also compared pHIVIS antibody reactivity with densitometrical gp41 values in EIB and anti-envelope titers in EIA: pHIVIS-positive and -negative sera did not differ in these respects. In contrast, pHIVIS-positive sera had significantly higher anti-gag titers than did pHIVIS-negative sera. All of this agrees with the reports that anti-gp41 antibody levels do not decline as much as anti-p24 antibody levels in progression to AIDS (35).

Why was there such a strong association between the presence of pHIVIS-reactive antibodies and health? Antibodies to the transmembrane protein of MuLV seem to

contribute to the protection against leukemia in AKR mice (36). pHIVIS-reactive antibodies may likewise interfere with a step in the progression to disease. However, a reverse causal relation cannot be entirely ruled out. Nevertheless, such an extremely selective disappearance of antibodies—caused by the immunodeficiency—would be intriguing. Some patients may lack the genetic prerequisites (37) for forming antibodies against an epitope corresponding to pHIVIS. Alternatively, pHIVIS-reactive antibodies become undetectable when they form complexes with gp41-derived factors.

In conclusion, there was a considerable variation between individual sera in their patterns of reactions with peptides derived from the gp41 sequence. Some of this variation may be genetically determined (37) and may affect the progression

to disease. Antibody reactivity with pHIVIS was strongly associated with health. Future studies ought to elucidate the role of pHIVIS in HIV pathogenesis and whether antibodies to pHIVIS can abrogate its immunosuppressive effects; if they can, they may even be therapeutically useful. Prospective studies are needed to decide the usefulness of pHIVIS as a prognostic reagent.

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