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Minimal Sequence Requirements for Synthetic Peptides Derived from the V3 Loop of the Human Immunodeficiency Virus Type 1 (HIV-1) to Enhance HIV-1 Binding to Cells and Infection

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We previously demonstrated that a 23-mer peptide (DB3) derived from the V3 loop of the surface glycoprotein of HIV-1 MN strain was able to bind to soluble CD4 and enhance HIV-1 infection. The mechanism and structural features required for these biological activities were studied by using shortened DB3 derivatives and DB3 analogs carrying single amino acid substitutions. We found that peptides in which the aromatic amino acid in position 15 or 16 had been replaced by an uncharged hydrophobic residue (DB3-I15 and DB3-I16), analogs in which positively charged amino acids were replaced by corresponding D-enantiomers, and shortened DB3-derivatives lost both enhancing activity and ability to bind to soluble CD4. Other peptide variants in which a positively charged amino acid was replaced by asparagine at positions 3 (DB3-N3), 6 (DB3-N6), and 19 (DB3-N19), respectively, retained both enhancing and binding activities, although with different efficiencies. The CD4 binder peptides DB3 and DB3-N19, but none of the CD4 nonbinder peptides, enhanced CD4 expression on peptide-treated cells as well as gp120 binding to both CD4+ cells and soluble CD4. These findings strongly suggest that the peptide/CD4 interaction induced an increase in both CD4 expression and CD4/gp120 binding affinity, which in turn mediated the enhancement of viral infection. A model of the structural conformation of DB3 peptide required for its biological activities is discussed.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) isolates display a high degree of genetic variability and several differences in their biological properties *in vitro*. Studies of the relationship between the biological phenotype and the primary and conformational structures of the surface viral glycoprotein gp120 (SU) evidenced the relevance of the SU third variable region (V3) to viral infectivity (Ivanoff *et al.*, 1992; Schulz *et al.*, 1993), cell tropism (Chesebro *et al.*, 1992; Westervelt *et al.*, 1992), and syncytium-inducing capability (Fouchier *et al.*, 1992; De Jong *et al.*, 1992). It was also shown that amino acid substitutions and conformational changes in the V3 domain modulate these biological properties (Page *et al.*, 1992; Shioda *et al.*, 1992; Stamatatos and Cheng-Mayer *et al.*, 1992). The V3 domain most likely plays a role during the infection process at a virus–cell postbinding level; indeed, antibodies to the principal neutralizing domain (PND), located within the V3 loop, prevent viral infection and fusion between HIV-1-infected cells and uninfected CD4-bearing cells (Palker *et al.*, 1988; Javaherian *et al.*, 1989), but not gp120 binding to the CD4 cellular receptor (Skinner *et al.*, 1988; Celada *et al.*, 1990). It also emerged that gp120 binding to CD4 triggers conformational changes in the former, and an increased exposure of gp120/V3 and gp41 epitopes (Sattentau and Moore, 1991), which play a crucial role in the virus–cell membrane fusion process. Single amino acid substitutions within the V3 loop dramatically affect HIV-1 ability to induce cell fusion (Freed and Risser, 1991; Chiou *et al.*, 1992).

We previously demonstrated that a synthetic peptide patterned on the PND of the HIV-1 MN strain bound to the CD4 molecule at the V1/V2 domain site identified by monoclonal antibodies MT151 and B66 (Autiero *et al.*, 1991), and enhanced HIV-1-induced syncytium formation and infection in CD4+ target cells (De Rossi *et al.*, 1991). Peptides that were designed from the PND sequences of other viral strains exhibited less or no infection enhancement, and these differences paralleled a different affinity for CD4 binding (Autiero *et al.*, 1991; De Rossi *et al.*, 1991; Dettin *et al.*, 1993). Voneche *et al.* (1993) recently demonstrated that short di- and tripeptides containing a hydrophobic amino acid and/or a positive net charge strongly enhanced syncytium formation induced by bovine leukemia virus, most likely by interacting with the target cell membrane.

The present study addressed the minimal amino acid sequence and structural features that were required by

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TABLE 1

Amino Acid Sequence of Synthetic Peptides

	1																					23
DB3	Y	N	K	R	K	R	I	H	I	G	P	G	R	A	F	Y	T	T	K	N	I	I	G
DB3-NH2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	G-NH_2
DB3-N3	—	—	N	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
DB3-N6	—	—	—	—	—	N	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
DB3-N19	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	N	—	—	—	—
DB3-I15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	—	—	—	—	—	—	—	—
DB3-I16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	—	—	—	—	—	—	—
DB3-DH8	—	—	—	—	—	—	—	H	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
DB3-D	—	—	K	R	K	R	—	H	—	—	—	—	R	—	—	—	—	—	K	—	—	—	—
DB3-R	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—					
DB3-M							—	—	—	—	—	—	—	—	—	—	—	—					
DB3-S										—	—	—	—	—	—								

Note. DB3 is patterned on the PND of the HIV-1 MN strain. DB3-NH2 derivative has an amide function instead of a carboxy function at position G^{23}. In the analogs DB3-N3, DB3-N6, and DB3-N19, positively charged lysine or arginine was substituted with the uncharged asparagine. In derivatives DB3-I15 and DB3-I16 aromatic amino acid phenylalanine or tyrosine was substituted with the hydrophobic isoleucine. In DB3-DH8 and DB3-D, histidine and all seven positively charged amino acids, respectively, were replaced with their corresponding D-enantiomers. DB3-R, DB3-M, and DB3-S were obtained by deleting the carboxy-terminus (DB3-R) or both the carboxy- and amino-terminus (DB3-M and DB3-S) regions of DB3. —, indicates identity with the DB3 sequence; ⌐¬, indicates the positions of the D-enantiomers.

synthetic peptides derived from the V3 loop of HIV-1 in order to enhance syncytium formation and viral infection; we also studied the mechanism(s) underlying these biological effects.

MATERIALS AND METHODS

Peptides

The peptides employed in this study are shown in Table 1. Peptides were prepared by solid-phase synthesis using an automated peptide synthesizer (431 A apparatus, Applied Biosystems). The Boc method (Stewart and Young, 1984) was employed to synthesize the shortened derivatives DB3-R, DB3-M, DB3-S, and DB3-D, while the Fmoc technique (Atherton and Sheppard, 1989) was used to synthesize all the other peptides studied. The peptides were purified by ion-exchange chromatography, and reverse-phase high-performance liquid chromatography. Their purity exceeded 95%, and examination of their amino acid composition by the Pico-tag method (Bidlingmeyer *et al.*, 1984) gave the expected results.

Cells and virus

HIV-1 infection and CD4 modulation experiments were performed using CD4+ cells from the MOLT-3 T lymphoid cell line. Cells were maintained in RPMI medium (Flow Laboratories, Irvine, UK) supplemented with 10% fetal calf serum (Flow), 2% L-glutamine (Flow), and 50 μg/ml gentamycin, and cultured under standard conditions. H9/HTLV-IIIB cells, productively infected by HIV-1 strain IIIB (Ratner *et al.*, 1985), constituted the virus source. Supernatants from H9/HTLV-IIIB cells were collected, centrifuged at 2000 *g* (Minifuge GL, Heraeus, Hanau, Germany) for 15 min to remove cells, and then filtered through 0.22-μm filters (Millipore, Bedford, MA). Virus titer was determined by the reverse transcriptase (RT) assay, as previously described (De Rossi *et al.*, 1990).

Viral infection

Cells (1×10^5) were resuspended in 100 μl of medium containing serial twofold dilutions of peptides, plated in 96-microwell plates (Falcon Microtest III, Becton Dickinson Labware, Lincoln Park, NJ), and after 1 hr infected with 10 μl of an HIV-1 preparation containing 1×10^5 cpm/ml of RT activity (0.01 cpm RT/cell); 24 hr later, the plates were centrifuged and washed to remove residual p24 antigen and peptide, and fresh medium was added. Four days after infection, supernatants were collected and quantitatively assayed for p24 HIV-1 capsid antigen using a commercially available kit (HIV-1 p24 core profile ELISA; DuPont de Nemours, Wilmington, DE).

Cytofluorimetric analysis

MOLT-3 cells (5×10^5) were incubated in 1 ml final volume with serial twofold dilutions of peptides for 30 min at 37°, and then washed in ice-cold phosphate-buffered saline (PBS). To evaluate CD4 expression, cells were stained with fluorescein-conjugated (FITC) monoclonal antibodies (MAb) Leu3a (Becton Dickinson, Mountain View, CA), OKT4, and OKT4a (Ortho Diagnostic, Raritan, NJ), and then analyzed on an EPICS-ELITE flow cytometer (Coulter Electronics, Hialeah, FL). Viable cells were gated on the basis of forward and 90° light scatter parameters, and at least 5000 cells were collected in each fluorescence histogram.

To quantify CD4 molecules per cell, commercially available fluorescence quantitation kits were employed, as previously reported (Amadori *et al.*, 1992). Briefly, the mean fluorescence intensity (MFI) of the samples was quantified in terms of molecules of equivalent soluble fluorochrome (MESF). A standard MESF curve was obtained by plotting MESF values of FITC-labeled microspheres of known fluorescein content (Quantum-TM, Becton Dickinson) against MFI values; sample MESF was derived by interpolating MFI values of MAb-stained samples with the reference curve. The fluorescein/protein ratio of Leu3a antibody was calculated using cell-sized microbeads (Simply Cellular, Becton Dickinson); the antibody binding sites per cell were then calculated as the ratio of MESF per cell/(fluorescein/protein ratio).

To evaluate viral gp120 in cells exposed to HIV-1, an anti-gp120-FITC MAb (American Biotechnologies Inc, ABT, Cambridge, MA) was used. After staining, cells were fixed in cold 1% formaldehyde/PBS to prevent virus contamination (De Rossi *et al.*, 1994), and then analyzed cytofluorimetrically.

To evaluate viral gp120 binding to peptide-treated and untreated cells, 5×10^5 cells were incubated for 30 min with 2 μg gp120-FITC (ABT), and then analyzed cytofluorimetrically. The MFI of peptide-treated and untreated samples was then quantified in terms of MESF, as described above.

Binding of gp120- and V3-derived peptides to immobilized sCD4

Recombinant soluble CD4 (sCD4, Genentech Inc., San Francisco, CA) was covalently coupled to a Sepharose 4B matrix, as previously described (Autiero *et al.*, 1991; Cammarota *et al.*, 1992). This immobilized sCD4 retained a number of testable epitopes that could be detected by several anti-CD4 MAbs, as well as the ability to bind gp120-derived peptides (Autiero *et al.*, 1991, 1992). Aliquots of 3×10^5 cpm of I^{125}-labeled gp120 (sp act 5×10^8 cpm/μg) were added to 0.1 ml of CD4–Sepharose resin resuspended in 15 m*M* potassium phosphate containing 150 m*M* NaCl, pH 7.1, and the mixture was incubated for 1 hr at room temperature. After extensive washing, the amount of bound I^{125}-labeled gp120 was determined. The specificity of gp120 binding to sCD4 was determined by competition with anti-Leu3a MAb, which prevents gp120 binding to CD4 (Sattentau *et al.*, 1986); 20 μg/ml of Leu3a MAb determined an 80% reduction in binding, while a similar amount of irrelevant MAb (Anti-HLA-DR D1.12 Mab) caused no reduction in binding (Autiero *et al.*, 1991). The amount of residual ^{125}I-labeled gp120 after competition with excess anti-Leu3a MAb was considered the experimental background, and was subtracted from sample data. The irrelevant leu-15-gastrin peptide (EGPWLEEEEEAYGWLQF) was used as a control to evaluate the specificity of the DB3 effect.

The binding of unlabeled peptides to CD4–Sepharose matrix was measured as previously reported (Autiero *et al.*, 1991). Briefly, 0.5 μmol of each peptide was loaded onto 1 ml of the CD4–Sepharose matrix. Nonadsorbed material was eliminated by extensive washing; adsorbed material was eluted at low pH, neutralized, and quantified spectrophotometrically. The specificity of this binding assay was previously demonstrated (Autiero *et al.*, 1991).

ELISA for gp120-sCD4 binding analysis

The ELISA procedure was performed according to Moore (1990) with slight modifications. Microtiter plates (Falcon) were coated with anti-CD4 MAb (OKT4, Ortho Diagnostic) by overnight incubation at 4°, using 100 μl of antibody at a concentration of 2 μg/ml in carbonate buffer (pH 9.6). After washing with PBS containing 0.05% Tween-20, 100 μl of blocking buffer (PBS–10% bovine serum albumine, BSA) was added to each well for 2 hr at room temperature; after three washings, 100 μl of soluble CD4 (sCD4, ABT) at a concentration of 1 μg/ml in PBS/2% BSA was added to each well. After 1 hr at 37°, unbound material was removed by washing; 100 μl of gp120 (ABT) at a concentration of 4 μg/well, which was shown in preliminary experiments to bind 50% of the sCD4, was added to each well with or without scalar dilutions of the peptides. After 1 hr at 37°, unbound gp120 was washed away. To determine the gp120 bound to sCD4, 100 μl of peroxidase conjugated anti-gp120 MAb (ABT), diluted 1:100 in diluting buffer (PBS, 10% BSA, 10% normal goat serum, and 0.05% Tween 20), was added to each well, and the plates were then incubated for 1 hr at 37°; 50 μl per well of a specific peroxidase substrate (ABTS, Vector Laboratories, Burlingame, CA) was added to develop color, and absorbance values were determined by reading the plates at 405 nm with an automated spectrophotometer (Titertek multiscan, Flow Laboratories).

RESULTS

Effect of peptides on HIV-1 infection

MOLT-3 cells were exposed for 1 hr to serial twofold dilutions of the peptides listed in Table 1, and then infected with HIV-1. After 24 hr, the cells were centrifuged, the supernatants were removed to eliminate residual p24 HIV-1 antigen, and fresh medium without peptide was added. Four days after infection, the supernatants were collected and assayed for p24 protein. The cytotoxicity of the peptides was evaluated in parallel cultures which underwent the same treatment, except for viral infection. DB3 peptide (14 μ*M*) increased p24 production more than 10-fold, compared with peptide-untreated, infected control cells (Fig. 1A), thus confirming its previously described enhancing effect on HIV-1 infection (De Rossi *et al.*, 1991). At higher DB3 concentrations, p24 values fell, most likely due to peptide cytotoxicity and consequently

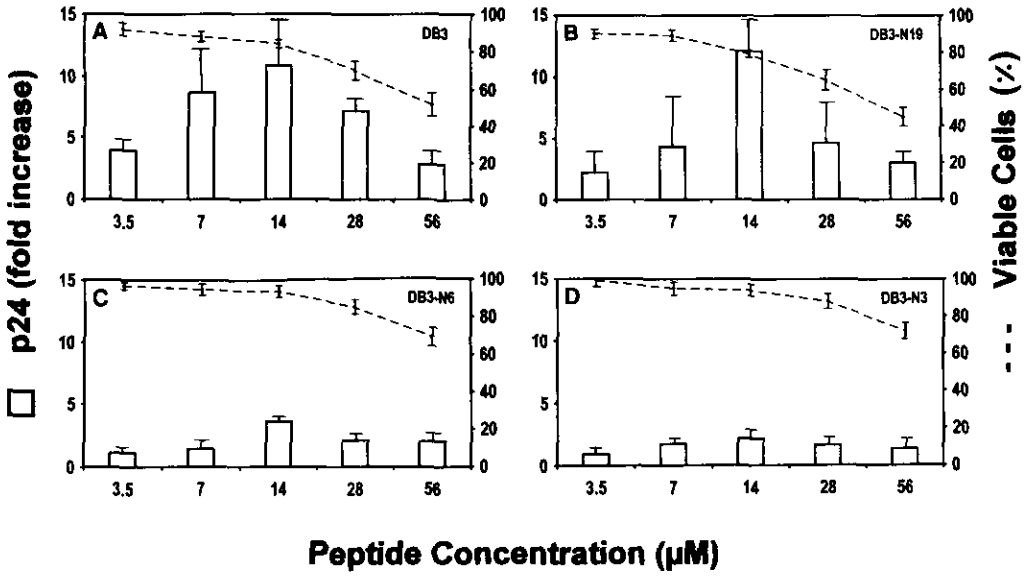

FIG. 1. Activity of peptides on HIV-1 infection. 10^5 MOLT-3 cells were treated for 1 hr with serial twofold dilutions of peptides and then infected with HIV-1; after 24 hr, culture supernatants were replaced with fresh medium without peptide. p24 antigen levels were determined in culture supernatants 4 days postinfection. Data are expressed as the p24 fold-increase compared with the HIV-1-infected peptide-untreated control, and represent the mean values ± standard deviation of three separate experiments. Peptide cytotoxicity was determined by incubating 10^5 cells in triplicate wells at the same peptide concentrations; after 24 hr culture supernatants were replaced with fresh medium without peptide. On Day 4, the percentage of viable cells, compared with the peptide-untreated control, was determined by trypan blue dye exclusion.

lower numbers of viable cells in culture, but were nonetheless consistently higher than those in untreated control cells. Among the DB3-derived peptides, DB3-N19 showed the strongest enhancing effect on HIV-1 infection; at a concentration of 14 μM, p24 production was 12-fold higher than the control value. As observed with DB3, p24 levels fell at higher concentrations in parallel with increased peptide cytotoxicity (Fig. 1B). Peptides DB3-N6 (Fig. 1C) and DB3-N3 (Fig. 1D) had a lower enhancing effect on HIV-1 infection, and at 14 μM, p24 values were respectively 3.6- and 2.1-fold higher than control levels; both peptides were also less cytotoxic than DB3 and DB3-N19. The other peptides tested showed no enhancing effect on HIV-1 infection, and a slight cytotoxicity (10%) only at the highest (56 μM) concentration employed. Interestingly, repeated experiments with peptide DB3-R showed a persistently lower p24 production (50% compared with untreated infected control); the inhibitory activity of this peptide appeared to be dose-independent (not shown).

Binding of DB3-derived analogs to immobilized sCD4

Binding of DB3 and DB3-derived analogs to sCD4 coupled to Sepharose matrix was measured as reported under Materials and Methods. Peptides DB3-N3, DB3-N6, and DB3-N19 exhibited at least 50% of the binding activity shown by DB3 (Fig. 2). As reported above, these three analogs could also enhance HIV-1 infection, although with different efficiencies. In particular, the substitution of lysine with asparagine (DB3-N19) caused an increase in enhancing activity, but not a proportional increase in CD4 binding. All the peptides that lacked enhancing activity also showed a considerably reduced ability to bind to the sCD4–Sepharose matrix (Fig. 2).

Peptides that enhance viral infection also modulate CD4 expression

We previously demonstrated that CD4 expression was increased in cells exposed to DB3 peptide (De

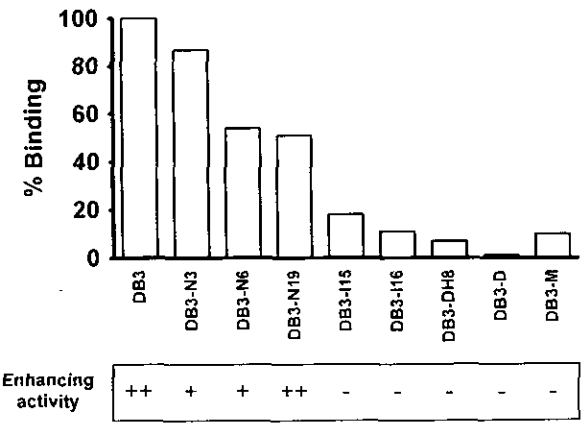

FIG. 2. Binding of peptides to immobilized sCD4. The amount of peptide retained by sCD4 coupled to the Sepharose resin was measured as described under Materials and Methods. Equal amounts of each peptide (0.5 μmol) and of CD4-Sepharose (1 ml) were used. The results are expressed as the percentage of the binding value of DB3 peptide which is normalized to 100%. The bottom line refers to virus infection enhancing activity: more than 10-fold (++), more than 2-fold (+), and less than 2-fold (−) compared with peptide-untreated HIV-1-infected control.

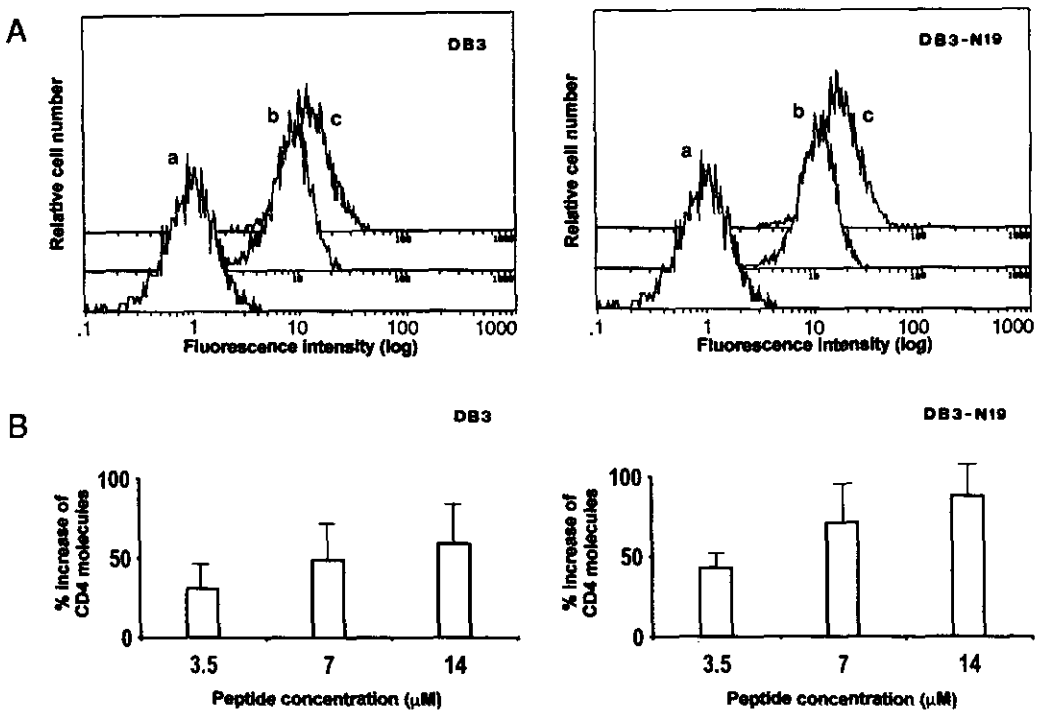

FIG. 3. Effect of peptides on CD4 molecule expression. MOLT-3 cells (5×10^5) were incubated in 1 ml final volume with serial twofold dilutions of peptides for 30 min, and then analyzed by flow cytometry using Leu3a MAb. (A) Flow cytometry histograms of (a) background staining of anti-mouse (RAM-IgG)-FITC-labeled cells, (b) specific staining of Leu3a-FITC-labeled peptide-untreated cells, and (c) specific staining of Leu3a-FITC-labeled cells exposed to 14 μM of DB3 or DB3-N19 peptide. (B) Increase in CD4 molecule number in cells treated with different peptide concentrations. Values were obtained, as detailed under Materials and Methods, and represent the mean ± standard deviation of three separate experiments.

Rossi *et al.*, 1991). To further investigate this phenomenon and its relationship to the peptide enhancing effect on HIV-1 infection, we quantified the number of CD4 molecules following cell exposure to different peptides. These experiments were performed on 3-day plated MOLT-3 cells, when CD4 molecule expression ranged from 1.3×10^4 to 1.6×10^4 molecules/cell. At a peptide concentration of 14 μM, DB3 and DB3-N19-treated cells showed an increase of 59 and 88%, respectively, in the number of CD4 molecules on their cell surface (Fig. 3). In line with their lower enhancing effect on HIV-1 infection, peptides DB3-N3 and DB3-N6 also showed a lower effect on CD4 expression; at a concentration of 14 μM, the increase was 18 and 23%, respectively. All the other peptides tested had no effect on CD4 expression (not shown).

The increase in CD4 expression was accompanied by a rapid increase in fluorescinated gp120 binding to the surface of DB3 and DB3-N19-treated cells over the same short (30 min) period of time (Fig. 4). At a peptide concentration of 14 μM, DB3 and DB3-N19-treated cells showed an increase in gp120 binding of 128 and 185%, respectively, compared with controls.

A time–course analysis of the events accompanying the exposure of the DB3-treated cells to viral inoculum evidenced a higher amount of CD4 epitopes reacting with OKT4 and OKT4a MAbs before infection, and a consistently higher number of gp120 positive cells in the first 2 hr following HIV-1 exposure, compared with untreated infected control cells (Table 2). The increase in HIV-1 binding to DB3-treated cells was demonstrated in both the number of cells bound, estimated by the percentage of gp120 positive cells, and the amount of viruses bound to the cell, evaluated by the MIF parameter. Concomitant with the viral infection, the OKT4a epitope, which maps to the gp120 high-affinity receptor (Sattentau *et al.*, 1986; Arthos *et al.*, 1989), decreased in both peptide-treated and untreated cells. Forty-eight hours after infection, 56 and 16% of peptide-treated and untreated cells, respectively, expressed viral gp120 (Table 2); the dramatic CD4 loss observed in the peptide-treated cells at this time point may reflect masking by gp120 protein and CD4 down-regulation during virion release (Hoxie *et al.*, 1986; Sattentau *et al.*, 1986; Stevenson *et al.*, 1987).

To detail the effect of DB3 on the first cycle of viral infection, peptide-treated and untreated cells were exposed in parallel to HIV-1 (1 cpm RT/cell). Twelve hours later, cells were incubated with Leu3a MAb (2 μg/1 $\times$ 10^6 cells) to prevent the spread of virus progeny; 48 hr postinfection, the cells were stained with a MAb anti-p24 viral capsid protein (KC57 RD-1, Coulter) and analyzed cytofluorimetrically. In Leu3a-exposed cultures, 38 and 13% of DB3-treated and untreated cells, respectively,

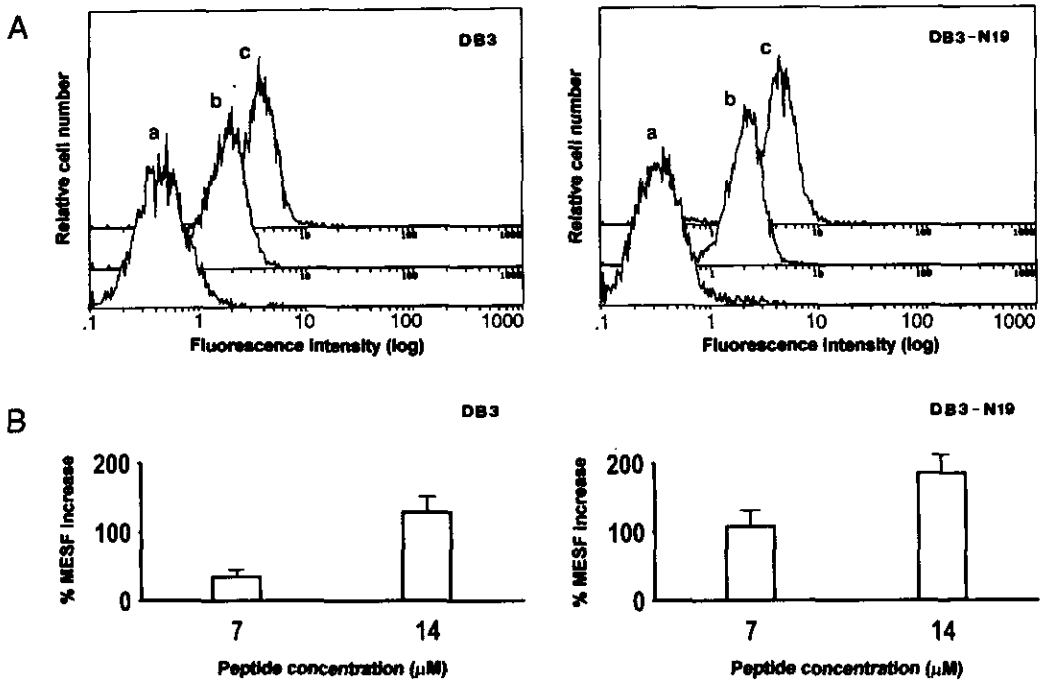

FIG. 4. Effect of peptides on gp120 binding to MOLT-3 cells. Cells (5×10^5) were exposed to serial twofold dilutions of the peptides for 30 min, and then incubated with 2 μg of gp120-FITC for 30 min at 37° and analyzed by flow cytometry. (A) Flow cytometry histograms of (a) background staining of RAM-IgG-FITC-labeled MOLT-3 cells, (b) specific staining of gp120-FITC-labeled peptide-untreated cells, (c) and specific staining of *gp120-FITC-labeled cells treated with 14 μM DB3 or DB3-N19 peptide. (B) Increase in gp120 binding in cells treated with different peptide* concentrations. The mean intensity fluorescence of the samples was quantified in terms of MESF (molecules of equivalent soluble fluorochrome) as detailed under Materials and Methods; MESF values are the mean ± standard deviation of three separate experiments.

were p24 positive; in the non-Leu3a-exposed controls, these figures were 57 and 18%, respectively. These data clearly demonstrated that the enhancing effect of DB3 on viral infection was primarly due to an increase in HIV-1 binding to the cells, which led to a larger number of HIV-1 infected cells right from the first cycle of HIV-1 replication.

Peptides enhancing HIV-1 infection increase CD4 affinity for gp120

The above experiments indicated that the binding of viral gp120 or the virus itself to the DB3 or DB3-N19-treated cells was at least 3 times more efficient, compared with untreated cells. This increase may be ex-

TABLE 2

Kinetics of HIV-1 Infection in Peptide-Treated Cells

Time after HIV-1 infection[a]	MOLT-3 cells						DB3-treated MOLT-3 cells[b]					
	OKT4		OKT4a		gp120		OKT4		OKT4a		gp120	
	%	MFI[c]	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
0	99	477	99	483	0		99	525	99	525	0	
30 min	99	463	99	407	3	223	99	468	99	428	10	381
1 hr	99	443	99	343	4	221	99	432	99	365	11	410
2 hr	99	429	99	277	6	212	99	417	99	273	13	367
48 hr	84	388	71	362	16	320	29	260	15	300	56	320

[a] Untreated and DB3-treated cells were infected with HIV-1 (IIIB) at high multiplicity of infection (1 cpm RT/cell). Prior to infection (Time 0) and at different postinfection times, cells were analyzed by flow cytometry for CD4 expression using OKT4 and OKT4a MAbs, and for viral gp120 using an anti-gp120-FITC MAb.

[b] DB3-treated cultures were exposed to peptide (14 μM) starting 30 min before infection, until the cells were harvested (i.e., up to 48 hr after infection).

[c] MFI, mean fluorescence intensity.

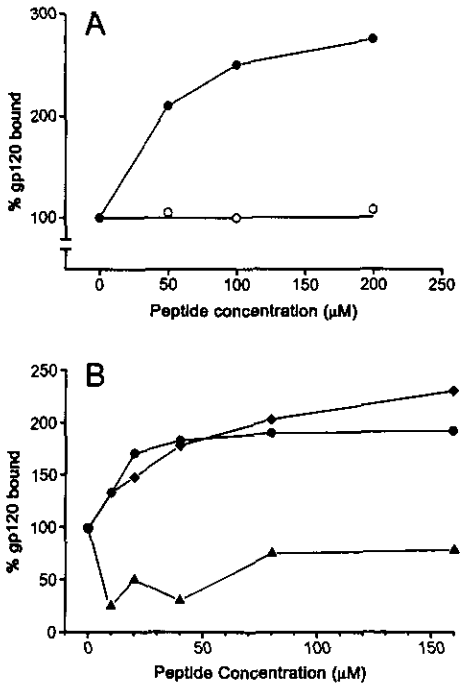

FIG. 5. gp120 binding to sCD4. (A) sCD4 was covalently coupled to a Sepharose matrix. Binding of ^{125}I-labeled gp120 to CD4–Sepharose matrix in the presence of increasing concentrations of DB3 (●) and an irrelevant control peptide (leu-15 gastrin) (○) was measured as described under Materials and Methods, and plotted as the percentage of gp120 binding in the absence of peptide (100% binding). (B) sCD4 was tethered to the solid phase via adsorbed OKT4 MAb. gp120 (0.4 μg/well) was added to the microwells in the presence of increasing concentrations of peptides DB3 (●), DB3-N19 (◆), and DB3-R (▲). gp120 bound to sCD4 was detected using anti-gp120 MAb. The data are plotted as percentages of the gp120 bound to sCD4 in the absence of peptides (100% binding).

plained only in part by the rise in the absolute number of CD4 receptors on the peptide-treated cells, because it was less than twofold higher than that in controls. It is thus conceivable that these enhancing peptides also increased CD4 receptor affinity for viral gp120. To investigate this aspect, we analyzed the binding of ^{125}I-labeled gp120 to sCD4 coupled to a Sepharose 4B matrix. Measurements of gp120 binding in the presence of scalar dilutions of DB3 indicated that increasing peptide concentrations induced a parallel increase in gp120 binding; equimolar amounts of an irrelevant peptide had no effect (Fig. 5A).

We then analyzed gp120 binding to sCD4 in the presence of serial twofold dilutions of peptides carrying amino acid substitutions. For this purpose, binding was measured by using sCD4 adsorbed onto 96-well plates. As detailed under Materials and Methods, in this assay sCD4 is tethered to the solid phase via adsorbed OKT4 MAb; as the latter binds to the membrane proximal domain of CD4, gp120 binding to this molecule is not affected (Moore, 1990). At DB3 and DB3-N19 concentrations of 10 μ*M*, the increase in CD4-bound gp120 was 30 and 33%, respectively. This increase was dose-dependent, and at 40 μ*M* an increase of 100% was reached with both peptides (Fig. 5B). Unlike the other tested peptides, which had no effect, DB3-R decreased gp120 binding to sCD4 at lower concentrations (5 to 40 μ*M*). This observation was consistent with the partial inhibition of infection mediated by this peptide.

DISCUSSION

We previously demonstrated that DB3, a peptide designed from the PND of the V3 loop of the HIV-1 MN strain, is able to bind to a site of the V1/V2 domain of the CD4 molecule (Autiero *et al.*, 1991) and enhance CD4 expression and HIV-1 infection (De Rossi *et al.*, 1991). The present study shows that this peptide is also able to increase gp120 binding both to sCD4 and to CD4 expressed in peptide-treated cells. As both CD4 expression and gp120 binding were enhanced as soon as 30 min after cell exposure to peptide, it is unlikely that these phenomena were due to a *de novo* synthesis of CD4 molecules. Instead, these findings strongly suggest that the interaction of DB3 with its binding site in the V1/V2 region induces a conformational transition leading to a CD4 configuration with a slower rate of endocytosis and a greater gp120 affinity. CD4 undergoes endocytosis at different rates in different cell types (Pelchen-Matthews *et al.*, 1991). Conformational changes might modulate this event, thus leading to an increase in the number of CD4 molecules exposed on the cell membrane. This notion is strengthened by the finding that DB3 peptide partially blocked TPA-induced endocytosis of CD4 (De Rossi *et al.*, 1991). Moreover, the observation that DB3 increased gp120 binding to immobilized sCD4 further supports the greater affinity of the CD4 molecule for viral gp120 after peptide exposure. Notably, as the CD4 high-affinity binding site for gp120 appears to be distinct from the binding site for DB3 (Autiero *et al.*, 1991), enhancement of gp120 binding to CD4 is likely to depend on a molecular cross-talk between distinct CD4 determinants.

The increase in both CD4 molecule number and affinity for gp120 permitted the infection of a larger fraction of cells, possibly at a higher multiplicity. This was demonstrated by infection kinetics studies in which DB3-treated cultures showed an increase in both the number of gp120 positive cells and the amount of viruses bound to the cells, during virus entry, and a large number of infected cells right from the first cycle of viral replication.

Among the peptides we analyzed, only those retaining a significant CD4 binding activity maintained their enhancing effect, although with different efficiencies; peptides with a severely reduced binding capacity were totally deprived of their enhancing effect on viral infection. In addition, the substitution of an amide group at the carboxy-terminus of DB3 (DB3-NH2) abrogated enhancing activity; we previously observed that

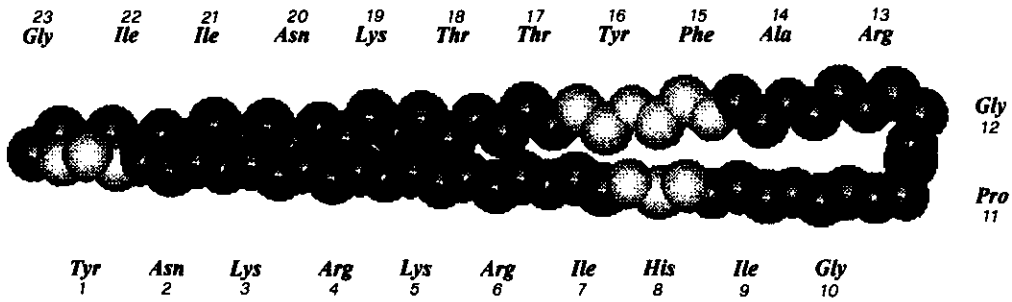

FIG. 6. Proposed model for the active conformation of the DB3 peptide backbone. Light grey spheres represent aromatic amino acids. H^8, F^{15}, and Y^{16} might generate an aromatic area; Y^1 (positively charged) and G^{23} (negatively charged) amino acids might stabilize the peptide's conformation through a salt bridge.

this modification led also to a significant impairment in CD4 binding ability (Autiero *et al.*, 1991). Therefore, although the correlation may be imperfect, we conclude that the ability of DB3 and DB3-N19 peptides to enhance infection is directly mediated by their interaction with the CD4 molecule. Besides supporting the specificity of the peptide/CD4 interaction, these findings might also suggest that the peptide assumes its active conformation at the receptor site.

In reference to the conformational aspects, the observed biological activities of the DB3-derived peptides led us to postulate a model characterized by the presence of: (a) a β-turn, probably of type II, involving the GPGR sequence in the center of DB3; (b) two regions, Y^1-I^9 and $A^{14}-G^{23}$, in an extended chain or β-sheet; (c) a salt bridge within the protonated amino group of Y^1 and the carboxy group of G^{23}; and (d) hydrophobic interactions within the side-chains of H^8, F^{15}, and/or Y^{16}, which are closely located and consequently could generate an aromatic area that might be relevant to peptide/receptor recognition (Fig. 6). The GPGR propensity for a β-turn arrangement is supported by several investigations (La Rosa *et al.*, 1991, Chandrasekhar *et al.*, 1991), as well as by our structural studies (Dettin *et al.*, 1993). Moreover, in line with the observation that biological activity is lost in the DB3-NH2 peptide, an extended chain disposition of the Y^1-I^9 and $A^{14}-G^{23}$ sequences would stabilize the active conformation through the formation of a salt bridge between the protonated NH_2 group of Y^1 and the carboxylate anion of G^{23}. Such a conformation would favor an interaction between H^8 and the aromatic rings of F^{15}, and/or Y^{16}. The finding that substitution of the aromatic residues with isoleucine (peptides DB3-I15, DB3-I16) abolished peptide activity supports the relevance of this aromatic interaction to the peptide's functional activity. We also found that substitution of H^8 with its D-enantiomer (peptide DB3-H8), which changed the histidine side-chain orientation due to the inverted configuration, or substitution of all the positively charged residues with their corresponding enantiomers (peptide DB3-D), completely abrogated the peptide's activity, thus confirming a specific and not a merely electrostatic interaction between the peptide and the CD4. In agreement with the conformational model proposed above, substitution of K^{19} with asparagine (peptide DB3-N19) slightly increased the peptide's biological activity, probably by further stabilizing its active conformation by removing the repulsive interaction between R^4 and K^{19}.

These observations may help achieve a better understanding of the role of V3 in HIV-1 infection. It is worth mentioning that in HIV-1 strain SF13, H^8 and Y^{16} contribute to the virus' ability to infect CD4+ T cell lines (Stamatatos and Cheng-Mayer, 1993); furthermore, in a large panel of patient isolates, the finding that the highest variability involved the amino acid at residue sites 8 and 15 led to the proposal that these two variable residues might be equivalently placed to influence sequence heterogeneity in the V3 loop (Milich *et al.*, 1993). Our observation that amino acid substitutions at residues 8, 15, and 16 compromised the peptide's biological properties may further indicate that these residue sites in the peptide's active conformation, as well as in the V3 loop, are adjacent to each other across the sheet, and that their interactions contribute to their functional activity.

It was shown that after gp120 binding to CD4, conformational changes occurred in both the receptor molecule (Celada *et al.*, 1990) and the virion envelope, including an increased exposure of the V3 loop (Sattentau *et al.*, 1991, 1993). It was also suggested that while a single gp120–CD4 interaction might initiate virus binding, virus adsorption and penetration into cells would require subsequent multiple gp120 interactions with the receptor (Layne *et al.*, 1990). The V3-derived peptides might mimic, at least in part, V3 loop function; their interaction with the accessory site in the V1/V2 region of CD4 could lead to conformational changes in the receptor molecule, and cooperatively affect virus binding to its high-affinity receptor located on the V1 domain of the CD4 molecule (Arthos *et al.*, 1989). The finding that different V3-derived peptides have different effects on HIV-1 infection may partially explain the ob-

servation that different viral variants infect CD4+ cells with different efficiencies.

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REFERENCES

Amadori, A., De Silvestro, G., Zamarchi, R., Veronese, M. L., Mazza, M. R., Schiavo, G., Panozzo, M., De Rossi, A., Ometto, L., Mous, J., Barelli, A., Borri, A., Salmaso, L., and Chieco-Bianchi, L. (1992). CD4 epitope masking by gp120/anti gp120 antibody complexes. *J. Immunol.* **148,** 2709–2716.

Arthos, J., Deen, K. C., Chaikin, M. A., Forwald, J. A., Sathe, G., Sattentau, Q. J., Clapham, P. R., Weiss, R. A., McDougal, J. S., Pietropaolo, C., McDougal, J. S., Pietropaolo, C., Axel, R., Truneh, A., Maddon, P. J., and Sweet, R. W. (1989). Identification of the residues in human CD4 critical for the binding of HIV. *Cell* **57,** 469–481.

Atherton, E., and Sheppard, R. C. (1989). "Solid-Phase Peptide Synthesis, A Practical Approach." IRL Press/Oxford University Press, Oxford.

Autiero, M., Abrescia, P., Dettin, M., Di Bello, C., and Guardiola, J. (1991). Binding to CD4 of synthetic peptides patterned on the principal neutralizing domain of the HIV-1 envelope protein. *Virology* **185,** 820–828.

Autiero, M., Abrescia, P., Dettin, M., Di Bello, C., and Guardiola, J. (1992). Affinity chromatography of CD4 ligands. *J. Immunol. Res.* **4,** 6–11.

Bidlingmeyer, B. A., Cohen, S. A., and Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Biochemistry* **336,** 93–104.

Cammarota, G., Scheirle, A., Takacs, B., Knorr, R., Doran, D. M., Guardiola, J., and Sinigallia, F. (1992). Identification of a CD4 binding site on the Beta2-domain of HLA-DR molecules. *Nature* **356,** 799–801.

Celada, F., Cambiaggi, C., Maccari, J., Burastero, S., Gregory, T., Patzer, E., Porter, J., McDanal, C., and Matthews, T. (1990). Antibody raised against soluble CD4-rgp120 complex recognizes the CD4 moiety and blocks membrane fusion without inhibiting CD4-gp120 binding. *J. Exp. Med.* **172,** 1143–1150.

Chandrasekhar, K., Profy, A. T., and Dyson, H. J. (1991). Solution conformational preferences of immunogenic peptides derived from the principal neutralizing determinant of the HIV-1 envelope glycoprotein gp120. *Biochemistry* **30,** 9187–9194.

Chesebro, B., Wehrly, K., Nishio, J., and Perryman, S. (1992). Macrophage-tropic human immunodeficiency virus isolates from different patients exhibit unusual V3 loop envelope sequence homogeneity in comparison with T-cell-tropic isolates: Definition of critical amino acid involved in cell tropism. *J. Virol.* **66,** 6547–6554.

Chiou, S. H., Freed, E. O., Panganiban, A. T., and Kenealy, W. R. (1992). Studies on the role of the V3 loop in human immunodeficiency virus type 1 envelope glycoprotein function. *AIDS Res. Hum. Retroviruses* **8,** 1611–1618.

De Jong, J. J., De Ronde, A., Keulen, W., Tersmette, M., and Goudsmit, J. (1992). Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino acid substitution. *J. Virol.* **66,** 6777–6780.

De Rossi, A., Calabro', M. L., Panozzo, M., Bernardi, D., Caruso, B., Tridente, G., and Chieco-Bianchi, L. (1990). In vitro studies of HIV-1 infection in thymic lymphocytes: A putative role of the thymus in AIDS pathogenesis. *AIDS Res. Hum. Retroviruses* **6,** 287–298.

De Rossi, A., Pasti, M., Mammano, F., Panozzo, M., Dettin, M., Di Bello, C., and Chieco-Bianchi, L. (1991). Synthetic peptides from the principal neutralizing domain of human immunodeficiency virus type 1 (HIV-1) enhance HIV-1 infection through a CD4-dependent mechanism. *Virology* **184,** 187–196.

De Rossi, A., Ometto, L., Roncella, S., D'Andrea, E., Menin, C., Calderazzo, F., Rowe, M., Ferrarini, M., and Chieco-Bianchi, L. (1994). HIV-1 induces down regulation of bcl-2 expression and death by apoptosis of EBV immortalized B cells: A model for a persistent "self-limiting" infection. *Virology* **198,** 234–244.

Dettin, M., De Rossi, A., Autiero, M., Guardiola, J., Chieco-Bianchi, L., and Di Bello, C. (1993). Structural studies on synthetic peptides from the principal neutralizing domain of HIV-1 gp120 that bind to CD4 and enhance HIV-1 infection. *Biochem. Biophys. Res. Commun.* **191,** 364–370.

Fouchier, R. A. M., Groenink, M., Kootstra, N. A., Tersmette, M., Huisma, H. G., Miedema, F., and Schuitemaker, H. (1992). Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *J. Virol.* **66,** 3183–3187.

Freed, E. O., and Risser, R. (1991). Identification of conserved residues in the human immunodeficiency Virus type 1 principal neutralizing determinant that are involved in fusion. *AIDS Res. Hum. Retroviruses* **7,** 807–811.

Hoxie, J. A., Brass, L. F., Pletcher, C. H., Haggarty, B. S., and Hahn, R. H. (1986). Alterations in T4 (CD4) protein and mRNA synthesis in cells infected with HIV. *Science* **234,** 1123–1127.

Ivanoff, L. A., Dubay, J. V., Morris, J. F., Roberts, S. J., Gutshall, L., Sternberg, E. J., Hunter, E., Matthews, T. J., and Petteway, S. R. (1992). V3 loop region of the HIV-1 gp120 envelope protein is essential for virus entry. *Virology* **187,** 423–432.

Javaherian, 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., and Matthews, T. J. (1989). Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. *Proc. Natl. Acad. Sci. USA* **86,** 6768–6772.

La Rosa, G. J., Davide, J. P., Weinhold, K., Waterbury, J. A., Profy, A. T., Lewis, J. A., Langlois, A. J., Dreesman, J. R., Boswell, R. N., Shadduck, P., Holley, L. H., Karpus, M., Bolognesi, D. P., Matthews, T. J., Emini, E. A., and Putney, S. D. (1990). Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. *Science* **249,** 932–935.

Layne, S. P., Merges, M. J., Dembo, M., Spouge, J. L., and Nara, P. (1990). HIV requires multiple gp120 molecules for CD4-mediated infection. *Nature* **346,** 277–279.

Milich, L., Margolin, B. and Swanstrom, R. (1993). V3 loop of the human immunodeficiency virus type 1 Env protein: interpreting sequence variability. *J. Virol.* **67,** 5623–5634.

Moore, J. P. (1990). Simple methods for monitoring HIV-1 and HIV-2 gp120 binding to soluble CD4 by enzyme-linked immunosorbent assay: HIV-2 has a 25-fold lower affinity than HIV-1 for soluble CD4. *AIDS* **4,** 297–305.

Page, K. A., Stearns, S. M., and Littman, D. R. (1992). Analysis of mutations in the V3 domain of gp160 that affect fusion and infectivity. *J. Virol.* **66,** 524–533.

Palker, T. J., Clark, M. E., Langlois, A. J., Matthews, T. J., Weinhold, K. J., Randall, R. R., Bolognesi, D. P., and Haynes, B. F. (1988). Type-specific neutralization of the human immunodeficiency virus with antibodies to env-coded synthetic peptides. *Proc. Natl. Acad. Sci. USA* **85,** 1932–1936.

Pelchen-Matthews, A., Armes, J. E., Griffiths, G., and Marsh, M. (1991). Differential endocytosis of CD4 in lymphocytic and non lymphocytic cells. *J. Exp. Med.* **173,** 575–587.

Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Joseph, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayed, J., Chang, N. T., Gallo, R. C., and Wong-Staal, F. (1985). Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* **313,** 277–284.

Sattentau, Q. J., Dalgleish, A. G., Weiss, R. A., and Beverley, P. C. L. (1986). Epitopes of the CD4 antigen and HIV infection. *Science* **234,** 1120–1123.

Sattentau, Q. J., and Moore, J. P. (1991). Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. *J. Exp. Med.* **174,** 407–415.

Sattentau, Q. J., Moore, J. P., Vignaux, F., Traincard, F., and Poignard, P. (1993). Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. *J. Virol.* **67,** 7383–7393.

Schulz, T. F., Reeves, J. D., Hoad, J. G., Tailor, C., Stephens, P., Clements, G., Ortlepp, S., Page, K. A., Moore, J. P., and Weiss, R. A. (1993). Effects of mutations in the V3 loop of HIV-1 gp120 on infectivity and susceptibility to proteolytic cleavage. *AIDS Res. Hum. Retroviruses* **9,** 159–166.

Shioda, T., Levy, J. A., and Cheng-Mayer, C. (1992). Small amino acid changes in the V3 hypervariable region of gp120 can affect the T-cell-line and macrophage tropism of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **89,** 9434–9438.

Skinner, M. A., Langlois, A. J., McDanal, C. B., McDougal, J. S., Bolognesi, D. P., and Matthews, T. J. (1988). Neutralizing antibodies to an immunodominant envelope sequence do not prevent gp120 binding to CD4. *J. Virol.* **62,** 4195–4200.

Stamatatos, L., and Cheng-Mayer, C. (1993). Evidence that the structural conformation of the envelope gp120 affects human immunodeficiency virus type 1 infectivity, host range, and syncytium ability. *J. Virol.* **67,** 5635–5639.

Stevenson, M., Zhang, X. H., and Volsky, D. J. (1987). Down-regulation of cell surface molecules during noncytopathic infection of T cells with human immunodeficiency virus. *J. Virol.* **61,** 3741–3748.

Stewart, J. M., and Young, J. D. (1984). "Solid-Phase Peptide Synthesis." Freeman, San Francisco.

Voneche, V., Callebaut, I., Lambrecht, B., Brasseur, R., Burny, A., and Portetelle, D. (1993). Enhancement of bovine leukemia virus-induced syncytia formation by di- and tripeptides. *Virology* **192,** 307–311.

Westervelt, P., Trowbridge, D. B., Epstein, L. G., Blumberg, B. M., Li, Y., Hahn, B. H., Shaw, G. M., Price, R. W., and Ratner, L. (1992). Macrophage tropism determinants of human immunodeficiency virus type 1 in vivo. *J. Virol.* **66,** 2577–2582.