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Immunology

CD4 peptide–protein conjugates, but not recombinant human CD4, bind to recombinant gp120 from the human immunodeficiency virus in the presence of serum from AIDS patients

(human immunodeficiency virus-positive serum/gp120/CD4 peptide)

VICTOR GHETIE*†, CLIVE SLAUGHTER‡§, H. THOMAS WHEELER†, JONATHAN W. UHR*, AND ELLEN S. VITETTA*†¶

Departments of *Microbiology and ‡Biochemistry, §Howard Hughes Medical Institute, and †Cancer Immunobiology Center, University of Texas Southwestern Medical Center, Dallas, TX 75235-9048

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ABSTRACT **Sera from human immunodeficiency virus-positive (HIV$^+$; Walter Reed stage 6) individuals inhibit the interaction between recombinant human CD4 and recombinant gp120 from HIV (rCD4 and rgp120, respectively), thereby interfering with the ability of soluble rCD4 to block infection with HIV or rCD4–toxin conjugates to kill HIV-infected cells. In this report we demonstrate that the inhibitory activity of such sera is caused primarily by anti-gp120 antibodies that do not recognize the CD4 interaction site on gp120. To circumvent the problem of inhibition, we have generated a construct containing a peptide of CD4 (residues 41–84) conjugated to ovalbumin (three to five peptides per molecule). This multivalent conjugate binds to rgp120 and binding is not inhibited by antibodies in HIV$^+$ sera.**

Sera from human immunodeficiency virus-positive (HIV$^+$) individuals inhibit the binding of ^{125}I-labeled recombinant gp120 from HIV (rgp120) to CD4$^+$ cells, whereas sera from HIV$^-$ individuals are only marginally inhibitory (1–4, 20). In one study, those sera that inhibited binding had high titers of anti-gp160/gp120 antibodies, suggesting the involvement of these antibodies in blocking (2). In contrast, other reports suggest that there is no correlation between the ability of sera from HIV$^+$ individuals to inhibit the binding of gp120 to CD4 and their titers of anti-gp120 (3). This discrepancy could be explained by the observation that there are at least two types of anti-gp120 antibodies in the serum of patients with AIDS. One type is directed against the immunodominant epitope in the V3 domain of gp120 (amino acids 308–332). Antibodies against this epitope may not always block the binding of gp120 to CD4 (5–7). Another type is directed against the less immunogenic epitope on C3 (amino acids 397–439) that is involved in CD4 binding (4, 8). The presence of antibody that can block the binding of CD4 to gp120 would seriously undermine CD4-based therapies for patients with AIDS (9, 10, 20). To circumvent this blocking by HIV$^+$ sera, we have used smaller segments of the CD4 molecule. We describe CD4 peptides bound to ovalbumin (OVA) that bind to rgp120 in the presence of antibodies presumably directed against sites on gp120 not involved in its interaction with CD4.

MATERIALS AND METHODS

Preparation of Peptide–OVA Conjugates. CD4-derived peptides were synthesized on an Applied Biosystems model 430A solid-phase peptide synthesizer. These peptides contained (*i*) amino acid residues 40–57 (Gln-Gly-Ser-Phe-Leu-Thr-Lys-Gly-Pro-Ser-Lys-Leu-Asn-Asp-Arg-[^{3}H]Ala-Asp-Ser) to which alanine (penultimate) and cysteine (C-terminal) were added; (*ii*) amino acid residues 81–92 (Thr-Tyr-Ile-Cys-Glu-Val-Glu-Asp-Gln-Lys-Glu-Glu) with [^{3}H]alanine at the N-terminal end; and (*iii*) amino acid residues 41–84 (Gly-Ser-Phe-Leu-Thr-Lys-Gly-Pro-Ser-Lys-Leu-Asn-Asp-Arg-Ala-Asp-Ser-Arg-Arg-Ser-Leu-Trp-Asp-Gln-Gly-Asn-Phe-Pro-Leu-Ile-Ile-Lys-Asn-Leu-Lys-Ile-Glu-Asp-Ser-Asp-Thr-Tyr-Ile-Cys) with [^{3}H]alanine in position 55. The peptides were purified by reverse-phase HPLC and characterized by fast atom bombardment mass spectrometry. A peptide not found in the CD4 molecule and containing 20 amino acid residues (Tyr-Asp-Arg-Pro-Glu-Gly-Ile-Glu-Glu-Glu-Gly-Glu-Arg-Asp-Arg-Asp-Arg-Ser-Gly-Cys) (Immuno-Dynamics, La Jolla, CA) was used as control. OVA (Sigma) (1 ml) dissolved in 0.05 M phosphate buffer with 0.003 M NA_2EDTA (PBE) at 5 mg/ml was mixed with 10 μl of *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Pharmacia) dissolved in dimethylformamide (Pierce) at 80 mg/ml (molar ratio of SPDP:OVA = 24), and the mixture was incubated at 25°C for 1 hr. The thiolated OVA was separated from the small molecules by gel filtration on Sephadex G-25M equilibrated with PBE and, after concentration to 5 mg/ml, immediately mixed with an equal volume of peptide dissolved in PBE at 2–5 mg/ml (molar ratio of peptide:OVA = 5–20). After incubating samples for 2 hr at 25°C, they were passed over a Sephadex G-10 or G-50 column equilibrated with PBE. The first peak, containing peptide–OVA, was pooled and concentrated. The number of peptides coupled to OVA was determined by using an absorption coefficient of 0.73 for OVA and the specific radioactivity of the peptides ($2–4 \times 10^4$ cpm/mg). Values of 8–11 peptide molecules per molecule of OVA were determined for CD4 peptide (40–57) and CD4 peptide (81–92)–OVA conjugates, whereas for CD4 peptide (41–84), there were 3–5 peptide molecules per molecule of OVA. No free -SH groups were detected on peptide–OVA by titration with Ellman's reagent (11).

Preparation of Protein–Horseradish Peroxidase (HRP) Conjugates. The recombinant human CD4 (rCD4)–HRP and IgG–anti-OVA–HRP conjugates were prepared by derivatizing the HRP with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Pierce) (12) and the rCD4 (Genentech) and anti-OVA with *N*-succinimidyl-*S*-acetylthioacetate (Calbiochem) and hydroxylamine.

Absorption of Human Serum on Immobilized Proteins. Protein A, rgp120, or rCD4 was coupled to CNBr-activated Sepharose 4B (Pharmacia). The gels were loaded with 1 ml of human serum and washed with PBE containing 0.5% bovine

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Abbreviations: HIV, human immunodeficiency virus; HRP, horseradish peroxidase; OVA, ovalbumin; rCD4, recombinant human CD4; rgp120, recombinant gp120 from HIV.
¶To whom reprint requests should be addressed.

serum albumin and 0.05% Tween 20 until the absorption at 280 nm of the effluent was zero; total collected volume was 10 ml. Dilutions (1:10) of these sera were used for inhibition experiments. HIV^- sera were obtained from seronegative individuals. HIV^+ sera were obtained from Walter Reed stage 6 (AIDS) patients. All blood donors signed informed consent in accordance with the guidelines of our institutional review board.

Binding of rCD4–HRP to rgp120 and Inhibition by Serum. Wells of a microtiter plate were coated overnight at 4°C with 100 μl of rgp120 (Genentech) (1 μg/ml) in 0.1 M bicarbonate buffer (pH 9.6) and then blocked for 2 hr at 25°C with 200 μl of 0.5% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.05% Tween 20. Dilutions of HIV^+ or HIV^- sera were added (100 μl) and the plate was incubated for 2 hr at 25°C. After washing, 100 μl of rCD4–HRP (5 μg/ml) was added to the wells for 2 hr at room temperature, and the binding of rCD4–HRP was detected by 2,2′-azinodi(3-ethylbenzylthiazoline-6-sulfonic acid) and hydrogen peroxide (Bio-Rad) using an ELISA reader equipped with a 405-nm filter. Linear regression curves were generated by plotting the absorbance vs. the dilutions of serum for each sample. Inhibition of binding of rCD4–HRP to rgp120 was expressed as the dilution of serum giving 50% inhibition of binding. A variant of this binding assay was also performed in which plates coated with rgp120 were blocked with 5% fetal calf serum. Incubation with dilutions of human serum was performed at 37°C for 30 min; this was followed by addition of rCD4–HRP directly to the dilutions of human serum without washing, which was followed by further incubation at 37°C for 30 min.

Binding of Peptide–OVA to rgp120 and Inhibition by Serum. Wells coated with rgp120 and blocked as described above were treated with various concentrations of peptide–OVA or OVA (control) in 100 μl, and the plate was incubated for 2 hr at 25°C. The plates were washed and then incubated with rabbit IgG anti-OVA coupled to HRP (5 μg/ml) for 2 hr at 25°C. Binding of the labeled antibody was detected as described above. Linear regression curves were generated by plotting the absorption vs. the concentration of peptide–OVA.

Inhibition of binding of peptide–OVA to rgp120 was determined in the presence of dilutions of HIV^+ and HIV^- sera. Sera were incubated for 2 hr at room temperature in rgp120-coated wells or 30 min at 37°C before adding the CD4 peptide–OVA with or without washing.

Specificity of rCD4 and Peptide–OVA Binding to rgp120. The specificity of the binding of rCD4–HRP to rgp120 was determined by incubating rgp120-coated wells for 2 hr at 25°C with various concentrations of rCD4, CD4 peptide (40–57)–OVA, CD4 peptide (81–92)–OVA, CD4 peptide (41–84)–OVA, control peptide–OVA, and OVA. The IC_{50} values for rCD4, CD4 peptide (40–57)–OVA, and CD4 peptide (41–84)–OVA were determined graphically. The relative affinity of the CD4 peptides–OVA for rgp120 vs. rCD4 was calculated by dividing the IC_{50} of the peptides by the IC_{50} of the rCD4.

The specificity of the binding of CD4 peptides–OVA to rgp120 was determined by incubating the rgp120-coated plates with different concentrations of rCD4 or OVA for 2 hr at 25°C before adding the CD4 peptides–OVA at concentrations giving an absorption at 414 nm of 0.6–0.8.

RESULTS AND DISCUSSION

Anti-gp120 Antibodies Block the Binding of rCD4 to rgp120. The ability of eight HIV^+ and eight HIV^- human sera to inhibit the binding of rCD4 to rgp120-coated wells of microtiter plates was determined and the results are presented in Fig. 1. The HIV^+ sera inhibited binding; the average dilution of HIV^+ sera giving 50% inhibition of binding was 1:820 ± 1:336 (mean ± SEM). HIV^- sera showed some inhibitory activity [50% inhibition at 1:64 ± 1:56 (mean ± SEM)]. Both values are in agreement with earlier reports (1–4, 20).

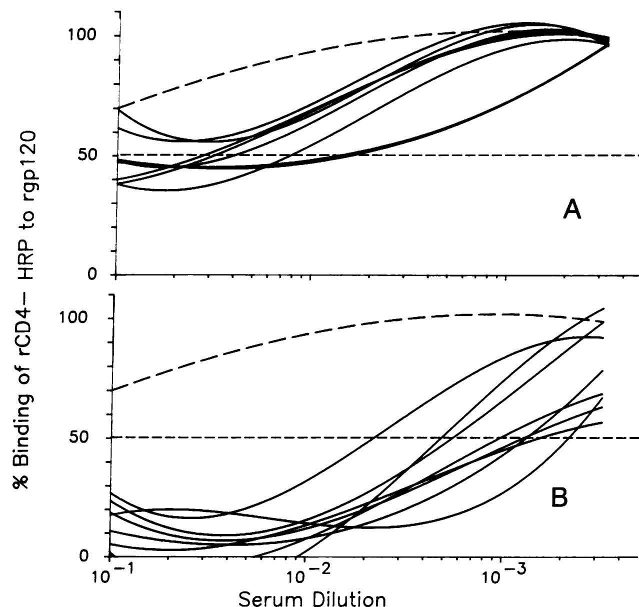

FIG. 1. Inhibition of binding of rCD4–HRP to rgp120 by HIV^- sera (eight individuals) or human serum albumin (– – –) (*A*) or by HIV^+ sera (eight individuals) or human serum albumin (– – –) (*B*). The following dilutions were used: 10^{-1}, 2×10^{-2}, 10^{-2}, 5×10^{-3}, 2.5×10^{-3}, 1.25×10^{-3}, 0.62×10^{-3}, and 0.31×10^{-3} and the curves were computer-generated.

As shown in Fig. 2*A*, the inhibitory activity of a HIV^+ serum was markedly reduced after absorption with rgp120-Sepharose or protein A-Sepharose, but not rCD4-Sepharose, indicating that the inhibitory factor is an immunoglobulin with anti-gp120 activity. Residual inhibitory activity was probably related to incomplete removal of anti-gp120, since

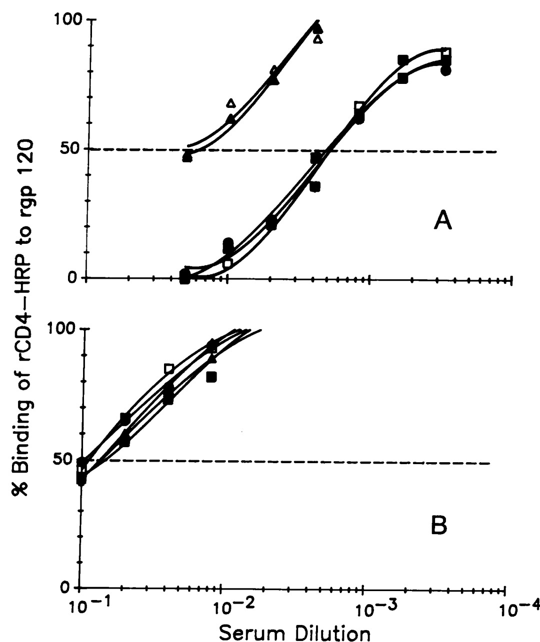

FIG. 2. Absorption of serum on rgp120-Sepharose, protein A-Sepharose, and rCD4-Sepharose. HIV^+ (*A*) and HIV^- (*B*) sera were passed over small chromatographic columns packed with 3 ml of the following gels: rCD4-Sepharose (●); gp120-Sepharose (△); protein A-Sepharose (▲); uncoupled Sepharose 4B (□). ■, Nonchromatographed serum.

only one absorption with rgp120-Sepharose was performed. Similar results were obtained with three other HIV^+ sera tested. The sera from HIV^- individuals were absorbed with the same panel of immobilized proteins, and no reduction of inhibitory activity was observed (one representative serum is shown in Fig. 2*B*). These results indicate that the inhibitory factor in sera from HIV^- individuals does not bind to protein A and is probably not an immunoglobulin. A mannose-binding protein present in normal human serum and reported to inhibit HIV infection of H9 cells (13, 14) is not the blocking factor since it did not bind to mannose-agarose and blocking was not inhibited with mannan (data not shown). Since human serum albumin at a concentration similar to that in the human serum-treated plates gave less inhibition than HIV^- serum (Fig. 1 *A* and *B*), the blocking factor is not albumin.

Anti-gp120 Antibody in HIV^+ Sera Is Not Directed Against the CD4 Binding on gp120. To determine whether the anti-gp120 antibodies in HIV^+ sera are directed against the CD4-binding site on gp120, we used three CD4 peptides that have been reported to bind to gp120. These peptides contain amino acids 40–57 (15), 81–92 (16), and 41–84 (17) of the CD4 molecule. Peptides containing CD4 residues 40–57 and 81–92 did not inhibit the binding of rCD4 to gp120 even at concentrations of 50 μM. However, when these peptides were conjugated to OVA, CD4 peptides 40–57 and 41–84 inhibited binding of CD4 to gp120, whereas CD4 peptide (81–92) or an irrelevant peptide (control) conjugated to OVA was not inhibitory (Fig. 3).

The free CD4 peptide (41–84) (not coupled to OVA) was able to interact with rgp120, as demonstrated by its ability to inhibit the binding of rCD4–HRP to rgp120 (data not shown). The IC_{50} of the CD4 peptide (41–84) was 0.6 μM (vs. 0.008 μM for rCD4), indicating that its binding affinity is 75 times lower than that of rCD4. When this peptide was coupled to OVA, the peptide–OVA conjugate was able to inhibit the binding of rCD4–HRP to rgp120 with an IC_{50} of 0.031 μM, which is 4-fold lower than that observed for rCD4 (0.008 μM). If the inhibition was calculated relative to the concentration of the peptide and not the peptide–OVA, the affinity between the CD4 peptide (41–84) bound to OVA at a molar ratio of 3 was ≈10 times lower than that between rCD4 and rgp120. The ability of the CD4 peptide conjugates to inhibit the binding of rgp120 to rCD4 was not blocked by OVA, further indicating that the interaction between CD4 peptide conjugates and rgp120 is specific.

The binding of CD4 peptide–OVA conjugates to rgp120 was also determined by a direct ELISA. As shown in Fig. 4, CD4 peptides (40–57)–OVA and (41–84)–OVA were able to bind to gp120; CD4 peptide (81–92)–OVA, control peptide–OVA, and OVA alone were not. The amount of CD4 peptide (41–84)–OVA bound at an optical density of 0.2 was approximately three times lower than that of CD4 peptide (40–57)–OVA. This result is in agreement with the inhibition data presented in Fig. 3, which indicate that the CD4 peptide (41–84)–OVA is twice as active as the CD4 peptide (40–57)–OVA (IC_{50} = 0.031 μM vs. IC_{50} = 0.06 μM). If the gp120-coated wells of the microtiter plate were pretreated with intact rCD4, the binding of the CD4 peptides (40–57) and (41–84)–OVA to gp120 was completely inhibited (data not shown), indicating that binding is specific. These results suggest that the CD4 peptide (40–57) is involved in the gp120 interaction site on CD4 and are consistent with other reports that the gp120-binding region of CD4 is located between residues 40 and 57 (15). The lack of binding of CD4 peptide (81–92)–OVA to rgp120 does not prove that this portion of CD4 is unable to react with the rgp120 molecule since the cysteine residue of this peptide has been reported to be involved in binding (16). In these studies, the cysteine residue was used to form the disulfide bond between the peptide and the OVA.

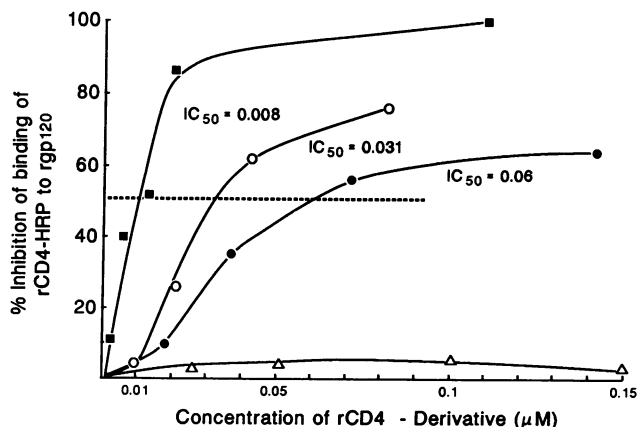

FIG. 3. Inhibition of binding of rCD4–HRP to gp120 by rCD4 and CD4 peptides. ■, rCD4; ○, CD4 peptide (41–84)–OVA; ●, CD4 peptide (40–57)–OVA; △, CD4 peptide (81–92)–OVA; △, OVA.

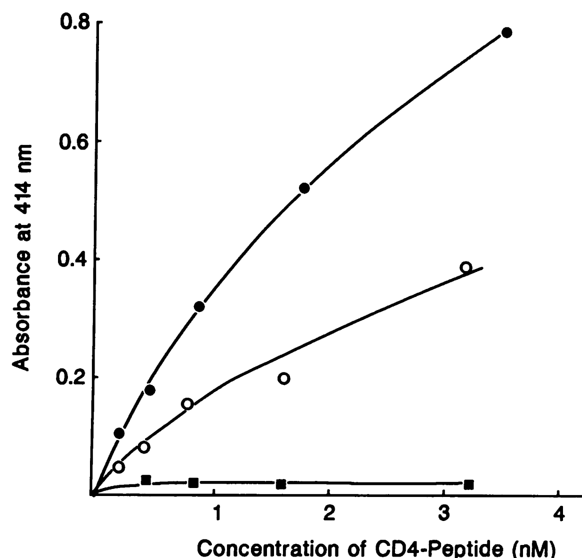

FIG. 4. Binding of CD4-derived peptides to rgp120. ●, CD4 peptide (41–84)–OVA; ○, CD4 peptide (40–57)–OVA; ■, CD4 peptide (81–92)–OVA; ■, control peptide–OVA and OVA.

CD4 Peptide–OVA Conjugates Interact with rgp120 in the Presence of HIV^+ Sera. Since the CD4 peptide (41–84)–OVA conjugate had the highest affinity for rgp120, we used it to determine whether HIV^+ sera could interfere with the binding of this peptide to rgp120. The results indicate that neither the HIV^+ nor the HIV^- sera inhibited the binding of the CD4 peptide–OVA to rgp120 (Fig. 5). The most likely explanation is that the vast majority of anti-gp120 antibodies in HIV^+ sera do not react with the CD4-binding site on gp120 but instead react with adjacent regions of gp120 that are not directly involved in CD4 binding. Crystallographic analysis (18, 19) together with the earlier mentioned binding studies (15–17) suggest that the gp120-binding site on CD4 is located on strand C″ forming a ridge. Hence, the CD4-binding site on gp120 may be a crevice. The inhibitory effect of antibody attached to a region adjacent to this crevice could be steric. When using the CD4 peptide (41–84)–OVA conjugate, one or more of the gp120-binding peptides that protrude from the surface of the carrier protein may gain access to the CD4-binding site on gp120, despite the presence of anti-gp120 antibodies that react with adjacent epitopes.

In summary, our results suggest that this CD4 peptide (41–84) containing portions of strands C″DEF in the V1

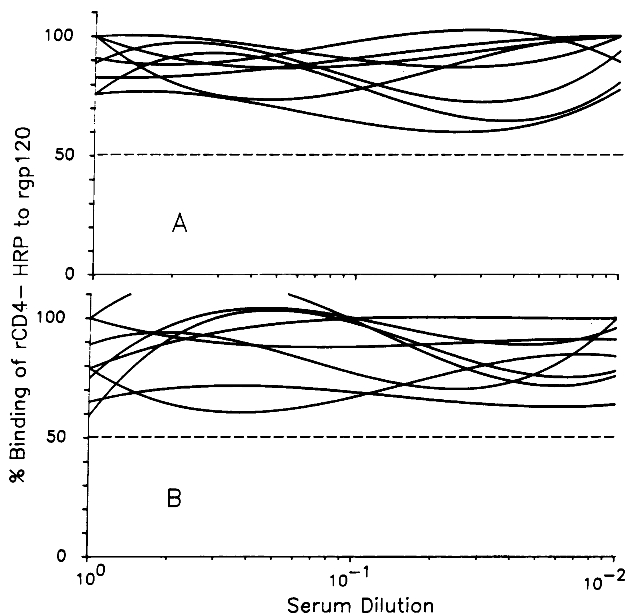

FIG. 5. Inhibition of the binding of CD4 peptide (41–84)–OVA to rgp120 by HIV$^+$ sera (eight individuals) (*A*) and HIV$^-$ sera (eight individuals) (*B*). The following dilutions were used: 10^0, 10^{-1}, 2×10^{-2}, and 10^{-2} and the curves were computer-generated.

domain of CD4 (18,19) coupled to OVA generates a peptide carrier that binds to rgp120 with good avidity, albeit lower than that between rCD4 and rgp120. Furthermore, in contrast to rCD4, CD4 peptide (41–84)–OVA binds to rgp120 in the presence of HIV$^+$ sera. If such CD4 peptide constructs also bind avidly to HIV-infected cells, they may circumvent the inhibitory activity of anti-gp120 antibodies in HIV$^+$ sera. If this is the case, these peptide constructs may inhibit HIV infection even in individuals with high titers of anti-gp120 antibodies.

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