Novel Anti-CD4 Monoclonal Antibodies Separate Human Immunodeficiency Virus Infection and Fusion of CD4+ Cells from Virus Binding

By D. Healey,* L. Dianda,* J. P. Moore,‡ J. S. McDougal,§
M. J. Moore,∥ P. Estess,¶ D. Buck,¶ P. D. Kwong,**
P. C. L. Beverley,∥ and Q. J. Sattentau*

From the *Academic Department of Genito-Urinary Medicine, University College and Middlesex School of Medicine, London W1; the ‡Institute of Cancer Research, Chester Beatty Laboratories, London SW1, UK; the §Immunology Branch, Center for Disease Control, Atlanta, Georgia 30333; ∥Human Tumor Immunology Group, Imperial Cancer Research Fund, London W1, UK; ¶Becton Dickinson Monoclonal Center Inc., San Jose, California 95131; and the **Department of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Summary

Human immunodeficiency virus (HIV) binds to cells via an interaction between CD4 and the virus envelope glycoprotein, gp120. Previous studies have localized the high affinity binding site for gp120 to the first domain of CD4, and monoclonal antibodies (mAbs) reactive with this region compete with gp120 binding and thereby block virus infectivity and syncytium formation. Despite a detailed understanding of the binding of gp120 to CD4, little is known of subsequent events leading to membrane fusion and virus entry. We describe two new mAbs reactive with the third domain of CD4 that inhibit steps subsequent to virus binding critical for HIV infectivity and cell fusion. Binding of recombinant gp120 or virus to CD4 is not inhibited by these antibodies, whereas infection and syncytium formation by a number of HIV isolates are blocked. These findings demonstrate that in addition to virus binding, CD4 may have an active role in membrane fusion.

CD4 is the high affinity cellular receptor for HIV and is found predominantly on thymus-derived T lymphocytes (1). The structure predicted for CD4 is an extracellular region of four tandem domains having homology with members of the Ig superfamily, followed by a transmembrane domain and a cytoplasmic region (2, 3). HIV binds CD4 via the major envelope glycoprotein, gp120, and recent genetic studies have localized the high affinity gp120 binding site on CD4 to ∼12 amino acids within the NH₂-terminal, Ig-like (V1) domain (4–6). Similarly, residues have been defined in the COOH-terminal half of gp120 that are critical for CD4 binding (7, 8). It is clear that the interaction between CD4+ human cells and cells expressing HIV envelope is sufficient to induce syncytium formation (9–11), and that virus entry is by pH-independent fusion of the virus and cell membranes (12, 13). Little else is known, however, about the events subsequent to binding that lead to membrane fusion.

Recently, evidence from two studies suggests that CD4 may play a role in cell–cell fusion (syncytium formation) ad-

ditional to envelope binding. In the first study, single residue substitutions made in the CDR-3 analogous loop of the V1 domain of CD4 resulted in mutant molecules that were unable to facilitate syncytium formation, even though they mediated both virus binding and infectivity to an extent comparable with wild-type CD4 (14). In the second, chemical mutagenesis of a CD4+ T cell line resulted in CD4 molecules that bound gp120 but mediated syncytium formation poorly (15). Although the mechanism of inhibition of cell fusion was not elucidated in either study, the findings indicate that CD4 participates in events subsequent to gp120 binding that are required for membrane fusion. mAbs reactive with CD4/V1 are highly efficient at inhibiting the binding of gp120 to CD4, and thereby prevent infection and syncytium formation (6, 16–19). In contrast, a mAb reactive with the third or fourth CD4 domains has been shown not to inhibit gp120 binding (18, 19). To investigate whether regions of CD4 other than the gp120 binding site are important in virus infection, we have raised and characterized a panel of CD4

mAbs. Two of these (Q425 and Q428) bind to CD4 without inhibiting HIV/CD4 binding, and yet potently inhibit both HIV infection and syncytium formation in vitro.

Materials and Methods

CD4 mAbs

A panel of CD4 mAbs was prepared by immunization of BALB/c mice with four intraperitoneal inoculations at 3-wk intervals with the CD4-transfected mouse T cell hybridoma 3DT (20), followed by a similar schedule with the CD4$^+$ human T cell line CEM. Mouse splenocytes were fused with the NS-1 myeloma by standard procedures (21), and hybridoma supernatants were screened for: (a) binding to recombinant soluble CD4 (sCD4;[1] from R. Sweet, SmithKline Beecham Laboratories, King of Prussia, PA.; 22) in an ELISA; (b) reactivity with CD4$^+$-transfected mouse cells and human PBL by indirect immunofluorescence and flow cytometry; (c) inhibition of HIV-induced syncytium formation in a cocultivation assay (16); and (d) ability to block purified, recombinant gp120 (prepared in CHO cells by Celltech Ltd., Slough, UK., for the Medical Research Council AIDS-Directed Programme) binding to CD4. Two hybridomas were selected (Q425 and Q428) that produced Ig reactive with CD4 in all of the above assays, and that inhibited syncytium formation, but did not block gp120 binding to CD4. After cloning, purified Ig was used to repeat the above assays and to determine the ability of these mAbs to inhibit HIV infection of CD4$^+$ T cells. Other mAbs used were from the following sources: OKT4, (P. Rao; Ortho Diagnostic Systems Inc., Westwood, MA); MT429 and MT151 (P. Reiber and G. Reithmuller; Munich University, Munich, FRG); and anti-Leu-3a, (from here on referred to as Leu-3a; N. Warner; Becton Dickinson Monoclonal Center Inc., San Jose, CA). L120 and Q4120 were prepared by D. Buck and Q. Sattentau respectively.

CD4 mAb Crosscompetition Studies

CD4 mAbs were labeled with biotin by the following procedure. To mAb at a concentration of 100 μg/ml previously dialyzed against 0.1 M bicarbonate buffer (pH 8.4) was added 50 μl of 1 mg/ml biotin–NHS (Pierce Chemical Co., Rockford, IL) in DMSO. After overnight incubation at 4°C, excess biotin was removed by dialysis against PBS'A'. Cells were incubated with 50 μl of various concentrations of unlabeled CD4 mAb ranging from supersaturating (20 μg/ml) to subsaturating (20 ng/ml), an irrelevant mAb, or medium alone for 30 min. 50 μl of 200 ng/ml of biotinylated CD4 mAb was then added. After 45 min, the cells were washed and 50 μl of a 1/20,000 dilution of streptavidin peroxidase (K377; Dako Corp., Santa Barbara, CA) was added. After a further 45-min incubation, the cells were washed and the reaction developed in o-phenylenediamine in citrate buffer (pH 5). The cells were pelleted and the absorbance of the supernatants was measured at 492 nm after transfer to a separate, flat-bottomed plate. All incubations were carried out at 4°C. Percentage inhibition of labeled mAb binding was calculated by the formula: 100× [(M−T)/(M−C)], where M represents maximum absorbance in the absence of inhibitory mAb, T is absorbance in the presence of test mAb, and C is absorbance in the presence of saturating concentrations of the homologous, unlabeled mAb.

gp120 and mAb Binding Assays

Cell ELISA for Inhibition of gp120 Binding. HPB-ALL cells at a concentration of 5 × 10^4/ml in 100 μl/well were incubated with dilutions of CD4 mAbs or medium alone in 96-well round-bottomed microtiter plates for 30 min, then the appropriate concentration of recombinant gp120 was added. After a further 30-min incubation and washing, 50 μl of 1 μg/ml biotin-labeled gp120 mAb (9284; DuPont Co., Wilmington, DE) (23) was added. Subsequent steps were done as for mAb crosscompetition studies.

Cell ELISA for Inhibition of CD4 mAb Binding by gp120. HPB-ALL cells at 5 × 10^4/ml in 100 μl/well were incubated for 2 h with 10 μg/ml of gp120, a concentration previously shown to supersaturate all CD4 molecules. CD4 mAbs at various concentrations were then added for another 1 h, and the cells were washed and labeled with FITC-conjugated rabbit anti–mouse Ig (Sigma Chemical Co., St. Louis, MO) for 30 min. After further washing, the cells were analyzed by flow cytometry on a FACscan (Becton Dickinson & Co., Mountain View, CA).

Soluble CD4 ELISA. The binding of gp120 to sCD4 captured onto the solid phase by different CD4 mAbs was carried out as follows. 100 μl of 25 μg/ml of purified CD4 mAb in bicarbonate buffer (pH 8.5) was added to each well of a 96-well ELISA plate overnight. After washing, nonspecific binding was blocked by a 30-min incubation with TBS containing 2% Marvel. After further washing, sCD4 at 1 μg/ml was added. The binding of recombinant gp120 was then detected as previously described (24).

HIV Binding Assay

The binding of a concentrated preparation of HIV-1 to CEM cells in the presence or absence of CD4 mAbs was detected by indirect immunofluorescence and cytofluorometry as previously described (25).

Mapping of mAb Binding Regions

Polypeptide Inhibition of mAb Binding. Inhibition studies of CD4 mAb binding were done by previously described protocols (26) with sCD4 and two truncated forms of this molecule: a recombinant, soluble construct consisting of the first two domains of CD4 (amino acids 1–183; from R. Sweet, SmithKline Beecham Laboratories; 6) and a proteolytic cleavage fragment of sCD4 consisting of residues 178–369, which was prepared as follows. Insoluble α-chymotrypsin coupled to agarose (Sigma Chemical Co.) was incubated with 5.6 mM tosyl-L-lysine chloromethyl ketone (TLCK) for 1 h at 26°C in 10 mM CaCl$_2$, 100 mM Tris, pH 8.0. After thorough washing, the TLCK-treated chymotrypsin (5.0 U/ml) was incubated with 1.5 mg/ml sCD4 with constant mixing for 3.5 h at 27°C in 0.15 M sodium chloride, 50 mM DL-methionine, 50 mM Hepes, 30 mM phosphate, pH 7.9. The reaction mixture was centrifuged to remove chymotrypsin and the supernatant applied to a Con A–Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ). The column was washed, and bound protein was eluted with 0.5 M methyl α-D-mannopyranoside (Sigma Chemical Co.). The 0.5-M fraction was subjected to gel filtration on a Sephacryl 100 HR column (Pharmacia Fine Chemicals) in 150 mM NaCl, 50 mM sodium phosphate, 20 mM sodium cacodylate, pH 7.25, 0.01 mM PMSF. The largest peak was pooled and passed down a column of Leu-3a bound to anti–mouse IgG covalently linked to sepharose beads (Sigma Chemical Co.) to remove any residual reactive material. The peak migrated as a homogeneous single band at 30.5 kD on SDS-PAGE. NH$_2$-terminal sequence analysis gave a single sequence (AFQKAS-IVY) for the first 10 cycles, which corresponds to residues 178–187 of the recombinant CD4. Results from amino acid compositional

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[1] *Abbreviations used in this paper:* RT, reverse transcriptase; sCD4, soluble CD4.

analysis were consistent with a CD4 peptide of 178–369 with a calculated molecular mass of 24.5 kD (assuming two biantennary carbohydrate attachments; 27). Limiting concentrations of the CD4 mAbs were incubated for 30 min with various concentrations of the inhibitory polypeptides, then added to SUPT1 cells. Bound mAb was detected with a fluoresceinated anti–mouse Ig antibody. Percentage inhibition of binding was calculated by the formula: $100 \times [(MFc - MFt)/(MFc - MFs)]$, where MF is the mean channel fluorescence without CD4 protein (c), with truncated test peptide (t), and without CD4 mAb (s). All incubations were done at 4°C.

mAb Reactivity with Recombinant Human/Mouse CD4. Molecules of CD4 chimeric between human and mouse have been prepared by homologous recombination in bacteria as previously described (28). Cell surface expression after transfection of the cDNA was detected on mouse EL-4 cells by indirect immunofluorescent staining with the CD4 mAbs, and analyzed by flow cytometry on a FACScan (Becton Dickinson & Co.).

Culture of HIV-infected and -uninfected Cell Lines

H9 cells persistently infected with HIV-1 isolates HTLV-IIIB and HTLV-IIIRF (obtained from R. Gallo, National Institutes of Health, Bethesda, MD) and CBL4 (P. Clapham and R. A. Weiss, Chester-Beatty Laboratories, London, UK) or HIV-2 isolates LAV-2 ROD (L. Montagnier, Pasteur Institute, Paris, France) and CBL20 (P. Clapham), and uninfected lines SUPT1 (J. Hoxie, University of Pennsylvania, Philadelphia, PA), C8166 (R. Gallo), CEM, and HPB-ALL were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS. The CD4-transfected cell lines Hela (Hela T4, from R. Axel, Columbia University, New York, NY) and 3DT (20) were grown in DMEM with 10% FCS and 1 mg/ml G418 (Geneticin; Sigma Chemical Co.) or RPMI 1640 + 10% FCS and 500 μg/ml G418, respectively. HIV-containing supernatants were harvested from 5-d cultures of a 4:1 mixture of uninfected H9 cells with HIV-infected H9, and titrated on C8166 cells by syncytium assay to determine the TCID$_{50}$.

Assays of HIV Syncytium Formation and Infectivity

Syncytium assays were done as previously described (16) by overnight cocultivation of HIV-infected H9 cells with uninfected C8166 cells at a ratio of 1:2, in the presence or absence of inhibitory antibody. Infection of C8166 cells was determined after addition of ~1,000 TCID$_{50}$ of virus in three assays: (a) measurement of syn-

cytium formation; (b) measurement of reverse transcriptase (RT) activity in culture supernatants. This was carried out by the following procedure. 80 μl of a cocktail of 50 mM Tris, pH 8.0, 5 mM DTT, 5 mM KDl, 0.05% Triton X-100, 0.3 mM glutathione, 0.5 mM EGTA, and 50 μg/ml template primer [poly(rA)p.(dT)] was added into each well of a round-bottomed 96-well plate. Test supernatant or medium (20 μl) was added, and the plate was incubated on ice for 10 min. Me[^{3}H]Thymidine 5' triphosphate (20 μl of 0.1 mCi/ml) was added to each well, and the plates were incubated at 37°C for 2 h. The plates were then harvested onto a DE81 filter mat, washed in 2× SSC and 95% ethanol, dried, and counted in a β plate scintillation counter (LKB Instruments, Inc., Gaithersburg, MD). (c) Measurement of soluble p24 HIV core antigen by twin site ELISA. Supernatants were inactivated by heating for 30 min at 56°C in 1% Empigen detergent, diluted 100–1,000-fold into TBS/0.1% Empigen, and 100 μl was added to 96-well microtiter plates containing adsorbed sheep anti-p24 peptide polyclonal antibody, D7320 (Aalto BioReagents, Ireland). Captured p24 was detected with alkaline phosphatase-conjugated mAb EH12E1 (29; obtained through the Medical Research Council AIDS-Directed Programme). The assay was calibrated using purified p24 yeast TyVLP protein (30; prepared by British Bio-Technology Ltd., Oxford, UK; a gift from S. Adams).

Results

Mapping of Q425 and Q428 Epitopes. To localize broadly the epitopes of the CD4 mAbs, binding inhibition studies were done with three forms of CD4: whole sCD4, a recombinant polypeptide consisting of the first 183 amino acids, and a proteolytically cleaved fragment spanning residues 178–369. Table 1 shows that preincubation of all mAbs with 80 nM sCD4 abrogates binding to CD4$^+$ cells. The same molar concentration of the 1–183 peptide inhibited Leu-3a and Q4120 binding, whereas an equivalent concentration of the 178–369 peptide blocked all the other mAbs. Thus, Q425, Q428, L120, MT429, and OKT4 bind in the third or fourth domains. Leu-3a binds in the first two domains of CD4, in accord with previous studies showing V1 reactivity (4, 6, 26), as does Q4120, which we have also shown to bind within V1 (unpublished results). Further characterization was achieved by phenotypic analysis of cells expressing a panel of hu-

Table 1. *Inhibition of CD4 mAb Binding by CD4 Polypeptides*

Polypeptide	Percent inhibition						
	Leu-3a	Q4120	Q425	Q428	OKT4	L120	MT429
Domains 1 + 2 (1–183)*	98‡	96	5	4	6	3	5
Domains 3 + 4 (178–369)	0	2	95	85	67	93	91
sCD4 (1–369)	97	93	100	97	89	100	96

CD4 mAbs at subsaturating concentrations (10–100 ng/ml) were incubated with a molar excess (80 nM) of sCD4-derived polypeptide, then added to 5 × 10^4 HPB-ALL cells. After washing, bound mAb was detected by immunofluorescent staining and flow cytometry.
* Amino acid allocations in polypeptide.
‡ Percent inhibition of mAb binding to CD4$^+$ cells.

Table 2. *Binding of CD4 mAbs to Mouse/Human Chimeras*

Chimeric CD4	Reactivity				
	Leu-3a	Q425	Q428	OKT4	L120
Hu	+ +	+	+ +	+ +	+ +
Mo (22) Hu	+ +	+ +	+ +	+ +	+ +
Mo (79) Hu	−	+ +	+ +	+ +	+ +
Mo (118) Hu	−	+ +	+ +	+ +	+ +
Mo (292) Hu	−	−	−	−	+ +
Mo (305) Hu	−	−	−	−	+ +
Mo	−	−	−	−	+

CD4 mAbs at saturating concentration (10 μg/ml) were incubated with EL-4 cells expressing molecules recombinant between human and mouse CD4. mAb binding was detected by indirect immunofluorescence and flow cytometry. (−) No reactivity (mean fluorescence intensity [MFI] < 5 U above background); (+) weak reactivity (MFI 6–15 U above background; (+ +) strong reactivity (MFI > 16 U above background). Hu, human; Mo, mouse.

man/mouse chimeric CD4 molecules with the CD4 mAbs. Recombinant cDNA molecules were constructed by homologous recombination between human and mouse cDNA, yielding molecules containing various amounts of NH₂-terminal murine sequence joined to human sequence. Transfection of the recombinant cDNA into murine EL-4 cells resulted in cell surface expression of chimeric protein. Table 2 shows that Q425, Q428, and OKT4 require the first 292 residues for binding, and L120 probably binds in the COOH-terminal 67 residues. In contrast, Leu-3a binds within the first 79 amino acids. Taken together with the peptide inhibition data, these results demonstrate that Q425, Q428, and OKT4 react with a stretch of amino acids bounded by

178–292, approximating to the third domain of CD4. Experiments using a panel of cells expressing CD4 molecules containing site-directed mutations showed that Q425 and Q428 require conservation of residues 249–252 for binding (R. Sekaly, personal communication). This finding supports the localization of these mAbs in domain 3 of CD4. In a previous study (26), OKT4 was unreactive with a CD4 molecule lacking the fourth domain, implying that this domain may contain the OKT4 binding site. These data probably reflect gross distortion of CD4 structure in this region, however, since the current study suggests that OKT4 probably binds in domain 3.

Crosscompetition studies between labeled and unlabeled mAbs indicate the spatial proximity between epitopes on an antigen (18, 26). Q425 and Q428 show complete reciprocal inhibition demonstrating closely related, overlapping, or identical epitopes (Table 3). Binding of these mAbs is partially inhibited by OKT4 and L120, implying steric hindrance. The epitope of MT429 is related to those of L120 and OKT4, since binding of labeled MT429 is completely (L120) or partially (OKT4) blocked by these mAbs. As expected, the V1 mAbs Leu-3a and Q4120 did not interfere with Q425 or Q428 binding, nor was there any reciprocal interaction. Neither Q425 nor Q428 binding is inhibited by MT151, a mAb reactive with the first two domains of CD4 (4, 26).

Effect of Q425 and Q428 on Binding of gp120 and Virus. We tested the effect of Q425 and Q428 on gp120 binding to CD4⁺ cells. Neither of these mAbs inhibited gp120 binding at any concentration tested; indeed, a small but reproducible enhancement of gp120 reactivity was detected (Fig. 1) (31). It is unlikely that this effect is related to the inhibition of HIV infection and cell fusion by Q425 and Q428, since other mAbs that also increase gp120/CD4 binding to a similar extent (OKT4 and L120) are unable to inhibit in these assays. Other mAbs showed a pattern of gp120 inhibition similar to that observed in previous studies: both Leu-3a and Q4120 completely blocked gp120 binding at low concentrations,

Table 3. *Crosscompetition between CD4 mAbs*

Biotin-labeled CD4 mAb	Percent inhibition						
	Leu-3a	Q4120	MT151	Q425	Q428	OKT4	L120
Leu-3a	100*	100	0	0	0	0	0
Q4120	100	100	0	0	0	0	0
MT151	0	0	100	0	0	0	0
Q425	0	0	0	100	100	50	50
Q428	0	0	0	100	100	50	100
MT429	0	0	0	0	0	55	100
L120	0	0	0	20	20	65	100

Biotin-labeled CD4 mAbs (200 ng/ml) were incubated with 5 × 10⁴ HPB-ALL cells in the presence or absence of an excess of unlabeled CD4 mAb. Bound labeled mAb was detected with avidin/horseradish peroxidase in an ELISA.
* Percent inhibition of labeled mAb binding to CD4⁺ cells.
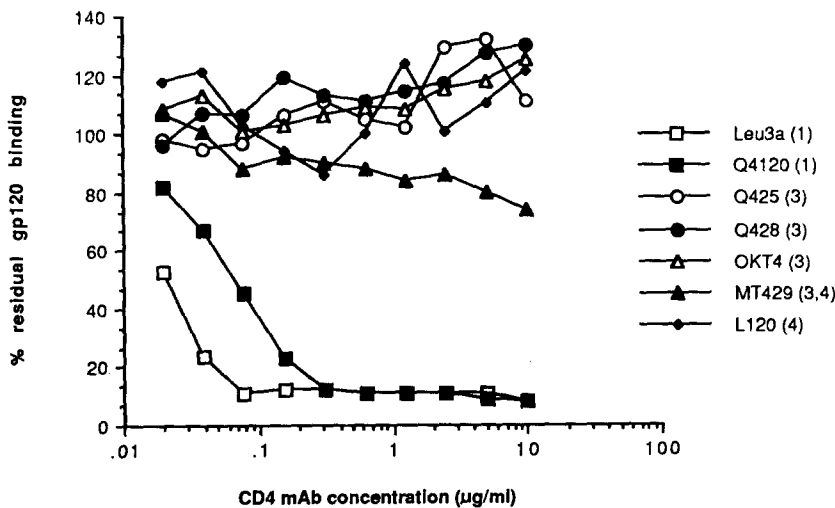
Figure 1. CD4 mAb inhibition of gp120 binding to CD4+ cells. The binding of HIV gp120 (0.5 μg/ml) to CD4+ HPB-ALL cells was measured using a biotinylated anti-gp120 mAb in the presence or absence of prebound CD4 mAbs. Numbers in parentheses indicate the domain of CD4 with which the CD4 mAbs react.

whereas OKT4 and other mAbs binding to the COOH-terminal portion of CD4 did not inhibit binding. We excluded the possibility that Q425 and Q428 might appear not to compete with gp120 binding because the mAbs were displaced by the high affinity interaction of gp120 with CD4, by demonstrating the formation of a trimolecular complex between sCD4, gp120, and Q425 or Q428. The binding of gp120 to sCD4 captured onto the solid phase by various CD4 mAbs was measured in an ELISA (Fig. 2). The capture of sCD4 by L120, Q425, and Q428 resulted in the binding of similar quantities of gp120, demonstrating that none of these mAbs interfere with the gp120/CD4 interaction. Conversely, capture of sCD4 by Leu-3a or Q4120 prevented gp120 binding. To investigate whether Q425 and Q428 were also unable to inhibit binding of whole virions to CD4, we measured the binding of a concentrated preparation of virus in the presence of these mAbs (Fig. 3). Neither of these mAbs nor L120

inhibited even at 100 μg/ml, whereas Q4120 substantially reduced virus binding at 10 μg/ml.

Binding of CD4 mAbs in the Presence of gp120. The binding of sub-saturating concentrations of CD4 mAbs to CD4+ cells was measured in the presence of pre-bound gp120 (Fig. 4). Surprisingly, Q425 and Q428 binding was substantially reduced (by ~50%) by increasing amounts of gp120 in comparison with control reactivity. The reactivity of other mAbs binding to the COOH-terminal region of CD4 (OKT4, MT429, and L120) was unaffected in the presence of gp120, whereas the V1-reactive mAbs demonstrated a dose-dependent reduction in binding with increasing gp120 concentration as expected. Thus, although Q425 and Q428 do not interfere with gp120 binding, in the reciprocal experiment, mAb binding is substantially reduced. This effect is unique to gp120,
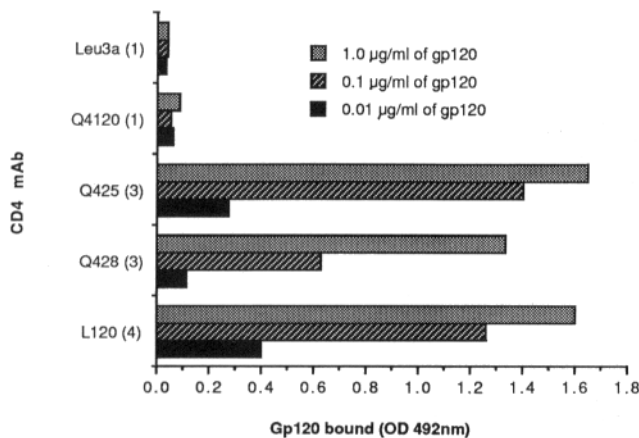
Figure 2. Formation of a trimolecular complex between sCD4, gp120, and Q425 or Q428. sCD4 was captured with CD4 mAbs preadsorbed to an ELISA plate. The binding to the sCD4 of three concentrations of gp120 was then measured by ELISA.
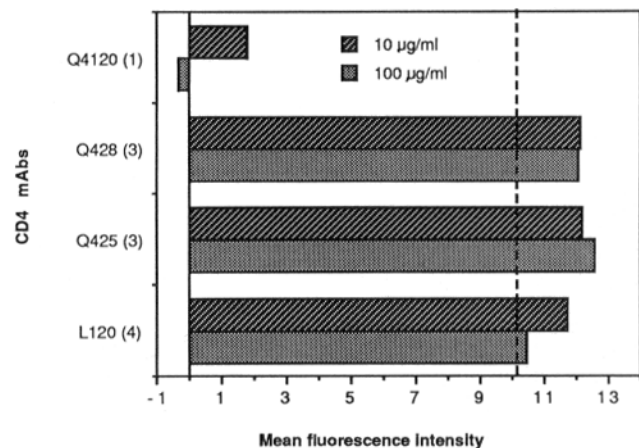
Figure 3. Inhibition of HIV binding to CD4+ cells. The binding of concentrated HIV to CD4+ CEM cells was detected by a FITC-labeled anti-gp120 antiserum in the presence or absence of CD4 mAbs. Results are expressed as the test mean fluorescence indexes minus the background control mean fluorescence. The dotted line indicates the binding of labeled virus in the absence of inhibitory mAb.
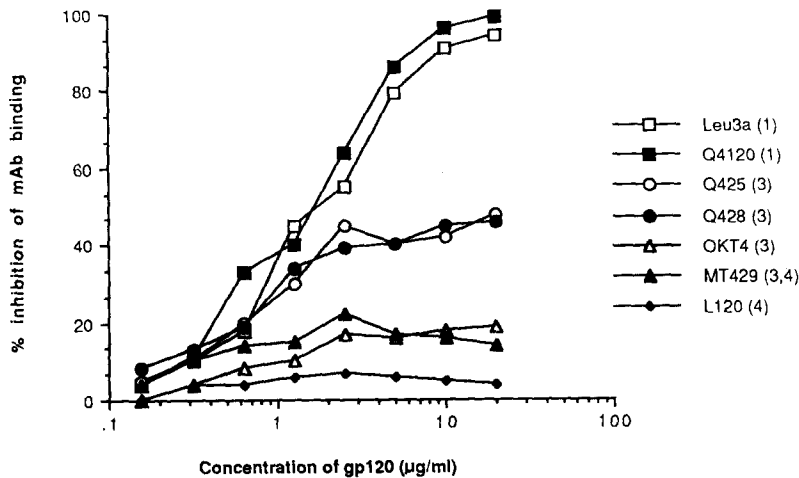
Figure 4. Inhibition of CD4 mAb binding by gp120. The binding of biotin-labeled CD4 mAbs (0.2 µg/ml) to CD4⁺ HPB-ALL cells was measured in the presence of various concentrations of pre-bound gp120. Results are expressed as the percentage inhibition of mAb binding.

since pre-incubation with a panel of eight V1-reactive CD4 mAbs had no effect on Q425 or Q428 binding (data not shown). To investigate whether the reduced binding of Q425 and Q428 reflected a decrease in affinity for CD4 complexed with gp120, the binding of increasing concentrations of these mAbs was tested in the presence or absence of a saturating concentration of prebound gp120 (Fig. 5). There was a clear decrease of about fourfold in the affinity of Q425 and Q428 for CD4 complexed with gp120 as opposed to gp120 alone, which may reflect an allosteric change induced in CD4 by gp120 binding. Interestingly, there was also a small but significant decrease in the saturation binding of these two mAbs, implying a decrease in the number of binding sites available.

Inhibition of HIV-induced Syncytium Formation by Q425 and Q428. We tested the ability of Q425 and Q428 to block syncytia induced by a variety of HIV-1 and HIV-2 isolates (Table 4). Although there was some variation between virus isolates, particularly with respect to strain RF with Q425,

both mAbs inhibited all isolates tested at concentrations within ∼10-fold of those required to saturate 5 × 10⁴ CD4⁺ cells. Both Leu-3a and Q4120 inhibited all isolates at similar concentrations, and with similar potency to Q425 and Q428 relative to their saturation binding. Previous studies have demonstrated that OKT4 is unable to inhibit syncytium formation (18, 19). Other mAbs binding to epitopes in the third or fourth domains of CD4 (MT429 and L120) also have no effect on syncytium formation. To exclude the possibility that the inhibition of syncytium formation by Q425 and Q428 was the result of CD4 internalization, we incubated CD4⁺ cells with Q425 and Q428 overnight at 37°C. No reduction in the level of CD4 was detected (results not shown). Thus, Q425 and Q428 show a previously undescribed inhibition of HIV syncytium formation that is not related to down-regulation of cell surface CD4, or interference with gp120 or whole virus binding.

Inhibition of HIV Infection by Q425 and Q428. Since cell–cell fusion induced in a short-term cocultivation assay of virus-
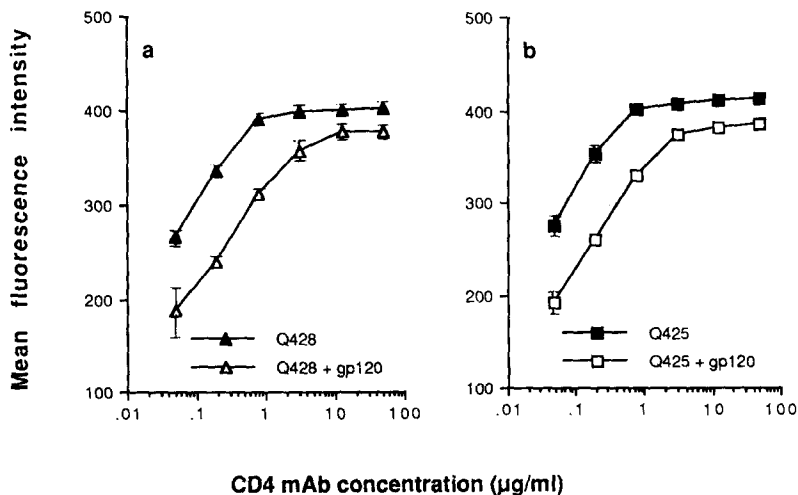
Figure 5. Binding of (a) Q428 and (b) Q425 to sCD4 complexed with gp120. The binding of Q425 or Q428 to CD4⁺ cells previously incubated with a saturating concentration of gp120 was measured by indirect immunofluorescent staining and flow cytometry. Each point represents the mean of three replicates. Error bars indicate ±1 SD.

Table 4. *Inhibition of HIV-induced Syncytium Formation by CD4 mAbs*

| CD4 mAb | Saturation binding[‡] | HIV isolate | | | | |
| | | HIV-1 | | | HIV-2 | |
		IIIRF	IIIB	CBL4	LAV-2	CBL20
	μg/ml			*μg/ml*		
Leu-3a (1)[*]	0.04	0.31[§]	0.31	<0.31	<0.31	<0.31
Q4120 (1)	0.08	<0.3	<0.3	<0.3	<0.3	<0.3
Q425 (3)	0.63	20	1.25	1.25	1.25	0.31
Q428 (3)	0.63	2.5	5.0	2.5	5.0	0.6
MT429 (3,4)	0.63	>20	>20	>20	>20	>20
L120 (4)	0.63	>20	>20	>20	>20	>20

C8166 cells (5 × 10^{4}) were mixed with HIV-infected H9 cells in the presence of various concentrations of CD4 mAbs. After overnight incubation, the wells were scored for inhibition of syncytia.
[*] Domain of CD4 within which mAbs bind.
[‡] Concentration of mAb required to saturate 5 × 10^{4} HPB-ALL cells.
[§] Concentration of mAb required to inhibit syncytia by >80%.

infected and uninfected cells (fusion from without) may be analogous to the mechanism of virus entry into receptor-bearing cells, we investigated the effect of Q425 and Q428 on HIV infection. Table 5 shows the results of incubation of the CD4^{+} T cell line C8166 with cell-free HIV (isolate HTLV-IIIB), in the presence or absence of CD4 mAb. By three criteria (syncytia, RT activity, and soluble p24 antigen release), Q425 and Q428 substantially reduced the titer of HIV measured at various times after infection. Q425 was more effective than Q428 at inhibiting HIV infection, an observation that may imply that these mAbs recognize slightly different epitopes. A decrease in the inhibitory activity of Q428 and, to a lesser extent, Q425 was seen at days 4 and 5, probably reflecting breakthrough of the mAb inhibition by a small number of virus particles. Increasing the dose of these mAbs to 20 μg/ml eliminated this breakthrough (results not shown). Complete inhibition of infection was achieved with the V1 mAb at all time points at 5 μg/ml, implying that mAb competition with HIV for the binding site on CD4 may be a more efficient block to infection than that mediated by Q425 and Q428. The OKT4-like mAb MT429 was unable to inhibit HIV infection at any concentration tested.

Table 5. *Inhibition of HIV Infection of CD4^{+} Cells by CD4 mAbs*

| CD4 mAbs (5 ug/ml) | Syncytium formation | | | RT activity | | | Soluble p24 antigen | | |
	3 d[‡]	4 d	5 d	3 d	4 d	5 d	3 d	4 d	5 d
					cpm			*ng/ml*	
Leu-3a (1)[*]	− [§]	−	−	468	401	430	0	0	10
Q425 (3)	−	−	−	414	558	875	20	20	70
Q428 (3)	−	+	+	517	1,167	3,824	15	40	290
MT429 (3,4)	+ +	+ + +	+ + +	856	3,095	8,219	50	240	3,000
Cells plus virus only	+ +	+ + +	+ + +	1,322	5,509	9,946	30	310	3,300
Cells alone	−	−	−	397	267	300	5	0	0

C8166 cells were incubated with 1,000 syncytium-forming units of HIV in the presence or absence of CD4 mAbs, and incubated for 3, 4, or 5 d before assay.
[*] CD4 domain within which mAb binds.
[‡] Days after infection.
[§] Scoring for HIV-induced syncytia is as follows: (−) no syncytia; (+) <20% syncytia; (+ +) 20–80% syncytia, (+ + +) >80% syncytia.

Discussion

We have demonstrated that two novel CD4 mAbs, Q425 and Q428, which bind within the third extracellular domain of CD4, block HIV infection and cell fusion without inhibiting virus binding. Recent studies have shown that recombinant V1 binds to gp120 with an affinity similar to that of whole sCD4 (6). Hence, mAbs reactive with the first or first and second domains of CD4 directly inhibit gp120 binding and efficiently block HIV infection and cell fusion, whereas a mAb bound more to a membrane proximal region has been shown not to affect HIV binding or syncytium formation (18, 19). Since HIV is thought to enter cells by direct fusion of the virus with the cell plasma membrane without internalization of CD4 (32, 33), mAbs Q425 and Q428 probably interfere with a step after virus binding but before virus entry. The finding that Q425 and Q428 inhibit cell-cell fusion in an overnight cocultivation assay in which virus infection and replication are not required supports the concept that the block to infection is before membrane fusion. The substitution of the third and fourth domains of CD4 for the hinge, transmembrane, and cytoplasmic segments of CD8 results in a chimeric molecule, which when expressed in a CD4⁻ cell line, confers susceptibility to HIV infection (32). Thus, conservation of the region of CD4 to which Q425 and Q428 binds is not required to allow HIV entry into receptor-bearing cells. This implies that the epitopes recognized by these mAbs are not involved in binding a ligand such as a second receptor molecule. The observed inhibition of HIV infection and cell fusion by these mAbs may be explained by the following model. Like some other members of the Ig superfamily (34, 35), CD4 may have a hinge-like region. A study supporting this based on crystallization of sCD4 predicts a hinge between domains 2 and 3 (36). Since CD4 is an extended rod-like molecule of ~125 Å in length (36), and fusion of two membranes probably requires greater proximity than this (37), bending of a hinge may be necessary for fusion to take place. The binding of Q425 or Q428 may restrict such movement. The portion of CD8 replacing domains 3 and 4 of CD4, described above (32), might substitute functionally in allowing flexibility, rendering this molecule permissive for HIV fusion with the cell membrane. An observation potentially relevant to the idea of a hinge is the reduced binding of Q425 and Q428 to CD4 in the presence of gp120. Since binding of the other third- or fourth-domain mAbs is not affected

by gp120, and V1-reactive CD4 mAbs do not mimic gp120 and inhibit Q425 and Q428 binding, the Q425 and Q428 epitopes are uniquely sensitive to bound gp120. The lack of reciprocal inhibition suggests that gp120 may impose a conformational change in CD4, such as bending of the hinge, reducing the affinity of Q425 and Q428 for this region.

An alternative explanation for the inhibition of fusion by Q425 and Q428 is that their binding to CD4 prevents virus-cell or cell-cell membrane fusion by steric interference with the fusogenic apparatus of HIV gp41. At present, we have no way of discriminating between these two hypotheses.

Two recent studies address the possibility that HIV envelope-mediated fusion of CD4-containing membranes may require CD4 activity additional to gp120 binding. Mutations in the CDR-3 analogous region of the V1 domain of CD4 did not disrupt gp120 or HIV binding, but did abrogate syncytium formation (14). Interestingly, cells expressing the mutant forms of CD4 were as susceptible to infection by cell-free virus as those containing wild-type CD4. Thus, a region in the first domain of CD4 adjacent to the gp120 high affinity binding site may have a role in cell-cell fusion. Since the epitopes of mAbs Q425 and Q428 are distant from the CD4/V1 domain, and these mAbs block both HIV infection and cell fusion, it seems likely that these two studies described different mechanisms.

In another study, chemical mutagenesis of CD4⁺ T cells was used to isolate variant CD4 phenotypes (15). Two of these variants had a decreased ability to form syncytia with envelope-expressing cells, but bound gp120 with a similar avidity to wild-type CD4⁺ cells. Although these authors did not attempt infection studies with cell-free virus, and the CD4 variants have not been characterized, it seems possible that mutations between the second and third domains of CD4 may account for the observed results.

We show here that in addition to binding HIV to the cell surface, CD4 may participate in both virus fusion with the cell membrane, an event critical for infection, and HIV envelope-mediated syncytium formation, a potential mechanism for virus spread and cytopathology. This finding may have relevance not only to mechanisms of HIV-mediated membrane fusion, but may also increase our understanding of the receptor-mediated entry of other enveloped viruses.

Address correspondence to Q. J. Sattentau, Howard Hughes Medical Institute, 6th Floor PI. Annex, Columbia University, 722 West 168th Street, New York, NY 10032.

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