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Anti-HIV-1 activity of CD4 synthetic oligopeptides representative of the putative gp120 binding site

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Summary

Two CD4 oligopeptides, corresponding to residues (37–53) and (37–55) of the V1 domain of CD4, which recent structural studies propose as the most likely binding site of HIV-1 gp120, have been chemically synthesized by solid-phase techniques, modified by the addition of two side-chain protected cysteines at both termini and purified by HPLC. Their ability to inhibit the infectivity of human immunodeficiency virus type 1 (HIV-1) (HTLV-IIIB, RF and GB8 strains) in different cell lines was monitored by the production of progeny virus, p24 and reverse transcriptase activity in the culture supernatants and by electron microscopy. The results indicated that the peptides inhibited HIV-1 infectivity in a dose-dependent fashion without any detectable cytotoxicity.

Introduction

CD4 is a glycoprotein which is associated with class II molecules in the recognition process between T cells and antigen-presenting cells (Doyle and Strominger, 1978; Gay *et al.*, 1987; Sleckman *et al.*, 1987; Robey and Axel, 1990). CD4 is also the receptor for human immunodeficiency virus (HIV) and plays a key role in determining the cell tropism of the virus and in promoting some of its cytopathic effects both *in vitro* and *in vivo* (Dalgleish *et al.*, 1984; Klatzman *et al.*, 1984; McDougal *et al.*, 1986; Maddon *et al.*, 1986). HIV binds to CD4 through the N-terminus of gp120, the virus envelope glycoprotein (Peterson and Seed, 1988; Clayton *et al.*, 1988; Mizukami *et al.*, 1988).

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CD4 is essential for virus infection as shown by the susceptibility to HIV infection of $CD4^+$ cells and by transfection experiments whereby expression of this molecule in non-permissive human cells allowed replication of HIV. For these reasons CD4 has been viewed as a potential therapeutic tool to block *de novo* infection.

Previous reports have already shown that the soluble/anchor-minus form of CD4 molecule (SCD4) is effective in inhibiting HIV infectivity *in vitro*, and clinical trials are ongoing (Traunaker *et al.*, 1989). Attempts are also being made to improve the pharmacokinetic profile of CD4 by genetic or chemical fusion to Ig fragments and to increase its therapeutic potential by conjugation with toxin (Chaudhary *et al.*, 1988; Capon *et al.*, 1989). Recently, a few studies have been carried out to evaluate the anti-HIV-1 effectiveness of synthetic oligopeptides representative of different portions of the receptor molecule (Jameson *et al.*, 1988). Conflicting results have emerged in relation to the particular amino acid (aa) sequence responsible for the antiviral activity of the peptides and the role of the chemical modifications thereupon. In this regard, Arthos *et al.* (1989) have shown that mutations within the region of, approximately, residues 41–52, homologous to the complementarity-determining region 2 (CDR2) of immunoglobulin light chains, affect gp120–CD4 interactions. On the other hand, a number of different authors (Lifson *et al.*, 1988; Nara *et al.*, 1989; Hayashi *et al.*, 1989) have shown that peptides belonging to the CDR3 domain of CD4, namely fragments spanning residues 81–92, are capable of blocking HIV-1 syncytium formation and infectivity. The retention of benzyl-protecting groups on Cys-84 and Glu-85 was found to be important for biological activity. In the present paper we report on the anti-HIV-1 activity of two selectively modified synthetic CD4 peptides in comparison to the activity exhibited by the soluble CD4 molecule.

Results

Peptide synthesis

As shown in Table 1, peptides A and B encompass the amino acid sequences of the CD4 CDR2 region. Products were >98% pure as determined by high-performance liquid chromatography (HPLC) in different solvent systems and showed the expected amino acid composition. The primary structure was confirmed by Edman degradation.

Both A and B contain two protected cysteines deliberately inserted at the C and N-termini to allow disulphide bond formation and the formation of a loop structure, for conformational restriction experiments. Since, at the present stage of the study, both cysteines are blocked by acetoamido methylation (Acm groups), these peptides are free to move and to accommodate themselves into the putative natural interaction site. Peptide C is a synthetic control peptide representative of the C-terminal region of horse heart cytochrome C, sequence (81–104), unrelated to A and B, which was synthesized, purified and characterized as reported for the CD4-derived peptides.

Inhibition of HIB-1 growth in cultured cells

The data are presented in Fig. 1 as an example of a dose–response curve and are summarized in Table 2 as the peptide ED_{50} values for different pairs of viral strains and cell lines. It appears that the two CD4 peptides, like sCD4 (Fig. 1a,b), inhibit virus yield in a dose-dependent manner, when added to cultures directly or after a short pre-incubation with the virus suspension. ED_{50} values were of the order of 10 μg/ml (5 μM) irrespective of the virus strain and the cell type used. These values may represent a slight over-estimation, since the peptide content in the sample was 80% by weight as determined by amino acid analysis. sCD4 was more effective (Table 2), by at least one order of magnitude in terms of weight (0.3 μg/ml) and two in molar terms (50 nM), with an ED_{50} close to that already reported (Arthos *et al.*, 1989). Production of both p24 (Fig. 2) and RT (not shown) was dramatically decreased in HIV-1 III B-infected H9 cells treated with the peptides compared with untreated cells. Inhibition was dependent on oligopeptide concentration and reached maximum at 10 μg/ml. Electron microscopy did confirm the measurements of the biological parameters of viral replication (not shown). No antiviral activity was exhibited by the unrelated horse heart cytochrome C peptide, which was used as an internal negative control.

Assay of cell cytotoxicity

Peptides A and B at 200 μg/ml were virtually devoid of cytotoxicity as shown by the growth curve of treated and untreated H9 cells over a time period (3 days) allowing at least three cycles of replication (Fig. 3). Signs of cytotoxicity only appeared at doses exceeding 500 μg/ml (50-fold higher than those at which antiviral activity was observed). The limited amounts of pure reagents did not permit an ED_{50} value to be obtained.

Discussion

Recent reports have appeared in the literature about the effectiveness of CD4 oligopeptides as inhibitors of HIV-1 replication. However, the definition of the biologically active amino acid sequence is still controversial, since the functional and structural properties of both the CD4 molecule and the HIV glycoprotein have not been dissected. In particular, it is not totally clear as yet which of the different external regions of CD4 is the most crucial for gp120 binding. Homologue-scanning mutagenesis studies (Landau *et al.*, 1988; Clayton *et al.*, 1988; Arthos *et al.*, 1989; Lamarre *et al.*, 1989; Sattentau *et al.*, 1989) have indicated that a relevant role might be played by the CDR2-like domain, in the stretch from amino acids 40–50. An approach using alanine-scanning mutagenesis, on the other hand, has produced evidence that mutations within the CD3-like region cause disruptions of gp120 CD4 interactions at least as pronounced as those mediated by mutations in the CDR2-like domain (Ashkenazi *et al.*, 1990). The importance of this domain (amino acids 37–52), as the most exposed N-terminal region of CD4 which is probably involved in the gp 120 recognition process, has

Table 1. Synthetic peptides.

(A) Sequence (37–53) of CD_4

H-Cys (Acm)-Leu -Gly -Asn-Gln -Gly -Ser-Phe -Leu-Thr-Lys -Gly -Pro -Ser -Lys-Leu -Asn-Asp
37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53
-Cys (Acm)-NH_2

(B) Sequence (37–55) of CD_4

H-Cys (Acm)-Leu -Gly -Asn-Gln -Gly -Ser-Phe -Leu-Thr-Lys -Gly -Pro -Ser -Lys-Leu -Asn-Asp
37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53
-Arg-Ala-Cys (Acm)-NH_2
54 55

(C) Sequence (81–104) of horse heart cytochrome C

H-Ile -Phe -Ala -Gly -Ile -Lys -Lys -Lys-Thr -Glu -Arg -Glu -Asp -Leu-Ile-Ala -Tyr -Leu-Lys -Lys
81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100
-Ala -Thr-Asn -Glu -OH
101 102 103 104

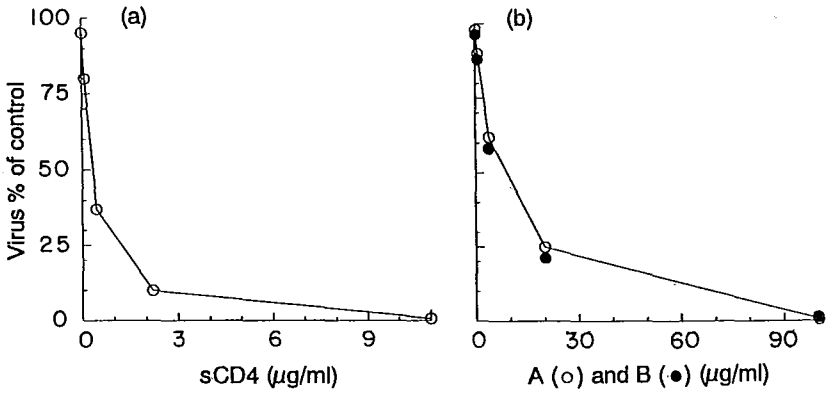

Fig. 1. Effects of sCD4 and oligopeptides A (open circles) and B (closed circles) on the replication of HIV-1 RF strain in C8166 cells. Progeny production was assayed as reported in the experimental section and in the legend to Table 2.

Table 2. Effects of sCD_4 and synthetic fragments thereof on HIV-1-infectivity.[a]

Cells and virus strains	Peptide A	Peptide B	ED_{50} (μg/ml) CD_4	CC[b]
H9 (IIIB)	13	11	0.4	>500
C8166 (RF)	10	10	0.3	ND
JM (GB8)	10	10	0.3	ND

a. Progeny production was assayed by syncytia formation in newly infected cells. $CCID_{50}$ values were determined according to Reed and Muench (1938).
b. CC is the amino acid (81–104) sequence of heart horse cytochrome C. ND, not done.

been further highlighted by very recent crystallographic data (Wang *et al.*, 1990; Ryu *et al.*, 1990). It therefore appears that this sequence ought to be part of a newly designed peptide to confer it with an anti-HIV-1 activity. For the above considerations we have synthesized two oligopeptides spanning the sequences (37–53) and (37–55), which are called A and B. In view of conformational studies which are underway and of possible mobility restriction, two side-chain-blocked cysteine residues were linked to the two termini of both peptides. Experiments performed with three different pairs of viral strains and cell lines, using a back titration assay and measuring p24 and RT, show that both A and B are effective in inhibiting HIV-1 replication. The two oligos have a comparable activity which is, however, much lower (virtually two orders of magnitude in molar term) than the one exhibited by the whole CD4 molecule. The addition of residue (54–55) in B is apparently not crucial for antiviral activity (Jameson *et al.*, 1988). A totally unrelated synthetic peptide of similar M_r representative of the C-terminal region of horse heart cytochrome C was devoid of any effect.

The inhibition of HIV-1 infectivity is, thus, in line with previous results using either longer peptides (25–58) (Jameson *et al.*, 1988) or the entire V1 domain (Arthos *et al.*, 1989). Differences in activity observed could reflect the specific requirement for a certain degree of structural complexity by our peptides, and support the view that the gp 120-binding site of CD4 consists of several discontinuous segments (Ashkenazi *et al.*, 1990). On this basis, it could also be explained why CD4 oligopeptides representative of a different portion of the CD4 molecule (i.e. amino acids 81–92) possess antiviral activity (Nara *et al.*, 1989).

Dose–response effects and lack of cytotoxicity by A and B would suggest a kind of selectivity. The molecular basis for it could reside on the specific sequence we employed and its relation with gp120, but an interference with other processes, such as exocytosis (Nara *et al.*, 1989), or with molecular targets different from the viral glycoprotein, cannot be excluded. Since A and B are endowed with a certain degree of mobility, they could freely adapt themselves to a complementary region of interaction. The alternative possibility that they could give rise to tertiary structures, although still under study, seems quite remote. Therefore, if the block of HIV infectivity is operating at the

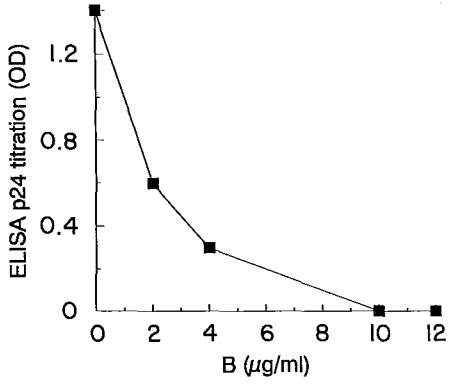

Fig. 2. Effect of oligopeptide B on HIV-1 (IIIB) replication in H9 cells. Assessment of virus growth by p24 ELISA titration was performed as described in the Materials and Experimental procedures. Uninfected H9 cells gave an OD value of <0.1.

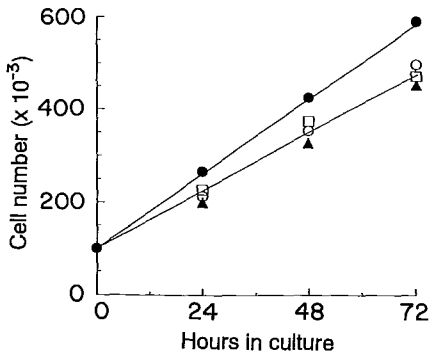

Fig. 3. Growth of H9 cells in the presence of different oligopeptides at a concentration of 200 μg/ml. Cells were originally seeded at a concentration of 10^5 cells/ml. Untreated control cells are represented by open squares; C-treated cells by closed circles; A- and B-treated cells by open circles and closed triangles, respectively.

level of the gp120–CD4 interaction, the role the peptides play has to be akin to that of a simple ligand, for they can not mimic, in classical terms, the function of a receptor structure.

From our investigation we cannot rule out the possibility that other CD4 regions may interfere with HIV infectivity, possibly through gp120 binding.

As for the design of more powerful anti-HIV-1 peptides, our study would indicate that a certain degree of structural complexity which accounts for long-range effects and protein folding is clearly needed to grant the active CD4 region the potential for a high-affinity binding to gp120.

Dissection of the three-dimensional structure of both the HIV-1 glycoprotein and CD4, which is currently ongoing, will certainly contribute to a more rational peptide design.

Materials and Experimental procedures

Chemicals

Starting resins, Boc-protected amino acids in preloaded cartridges and all solid-phase synthesis reagents were supplied by Applied Biosystems (Foster City, CA).

Synthesis, hydrofluoric acid (HF) cleavage and purification of peptides

Assembly of the polypeptide chains was carried out by step-wise techniques on solid support by using an Applied Biosystem 431A automated peptide synthesizer starting with 0.5 mmol of Boc-Cys(Acm)-MBHA-resin. The following side-chain protection was used: acetamidomethyl (Acm) for cysteine, benzyl for serine and threonine, p-chlorocarbobenzoxy for lysine, cyclohexyl for aspartic acid. Boc removal was accomplished by using one 2-min wash and one 20-min treatment with 65% trifluoroacetic acid in methylene chloride. Boc protected amino acids were activated by dicyclohexylcarbodiimide and introduced as hydroxybenzotriazole esters. Each residue incorporation was followed by a 'capping' cycle using acetic anhydride. At the end of the synthesis, the peptide-resin was suspended in 500 μl of anisole and treated with 10 ml of HF. The reaction mixture was stirred at 0°C for 1 h, the HF was evaporated under vacuum and the solids were triturated with 50 ml of 10% AcOH. After lyophilization the peptides were purified by ion-exchange chromatography using a Protein Pack SP 5PW Waters column (0.8 × 7.5 cm) with different gradients of solution B (10 mM $Na_2HPO_42H_2O$, pH 6) at a flow rate of 1 ml/min. Each peptide was then further purified by reverse-phase C_{18} HPLC on a Delta Pack, 15 μ, 300 A Waters column (7.8 × 30 cm) using 0.1% trifluoroacetic acid with a gradient of acetonitrile containing 0.1% trifluoroacetic acid. A purity >98% for each peptide was determined by analytical chromatography on a Resolve 5 μ, 100 A Waters column (3.9 × 15 cm). The amino acid composition of the synthesized peptides, after hydrolysis with 6 M HCl for 22 h at 110°C, was determined with a Carlo Erba automated amino acid analyser AAA3A28 and gave the expected compostion. Soluble CD4 was obtained from Microgenesis (West Haven, CT).

Viruses and cells

H9, C8166 and JM cells were routinely grown in RPMI-1640 medium supplemented with 10–20% foetal calf serum (FCS), antibiotics and L-glutamine. The HIV-1 HTLV-IIIB, RF and GB8 strains were titrated by syncytia formation, essentially as described previously (Arthos *et al.*, 1989).

Assay of antiviral activity

H9, C8166 and JM cells were originally seeded in 25-cm flasks at a concentration of 3 × 10^5 cells/ml in 12 ml final volume. Cultures were infected with the HIV-12 HTLV-IIIB, RF and GB8 strains, respectively, at a virus multiplicity of 10 $CCID_{50}$. Before being added to cell cultures, virus suspensions were incubated with different concentrations of CD4 and oligopeptides for 5 min at room temperature. After the absorption period, cells were incubated at 37°C for 5–7 days. Supernatants were then tested for progeny virus (Reed and Muench, 1938). ED_{50} values were obtained, essentially as described elsewhere (Palù *et al.*, 1986a), by relating the progeny virus production in the newly infected cells to the peptide concentration. In another set of experiments, H9 cells were infected with HIV-1 IIIB in the presence of the synthetic oligopeptides and viral growth was directly assessed by syncytia formation, electron microscopy, p24 (ELISA; DuPont, Willmington, DE) and reverse transcriptase (RT) titration, as reported previously (Filice *et al.*, 1988).

Assay of cell cytotoxicity

H9 cells were seeded at a concentration of 100000 cells/ml in RPMI-1640 supplemented with 20% FCS in the presence of increasing concentrations of oligopeptides and incubated for a period of 72 h. At this time-point viable cells were counted by trypan blue staining in a conventional haemocythometer (Palù *et al.*, 1986b).

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References

Arthos, J., Deen, K.C., Chaikin, M.A., Fornwald, J.A., Sathe, G., Sattentau, Q.J., Clapham, P.R., Weiss, R.A., 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.

Ashkenazi, A., Presta, L.G., Marsters, S.A., Camerato, T.R., Rosenthal, K.A., Fendly, B.M., and Capon, D.J. (1990) Mapping the CD4 binding site for human immunodeficiency virus by alanine-scanning mutagenesis. *Proc Natl Acad Sci USA* **87:** 7150–7154.

Capon, D.J., Chamow, S.M., Mordenti, J., Marsters, S.A., Gregory, T., Mitsuya, H., Byrn, R.A., Lucas, C., Wurm, F.M., Groopman, J.E., Broder, S., and Smith, D.H. (1989) Designing CD4 immunoadhesins for AIDS therapy. *Nature* **337:** 525–531.

Chaudhary, V.K., Mizukami, T., Fuerst, T., FitzGerald, D.J., Moss, B., Pastan, I., and Berger, G. (1988) Selective killing of HIV-infected cells by recombinant human CD4-*Pseudomonas* axotoxin hybrid protein. *Nature* **335:** 369–372.

Clayton, L.K., Hussey, R.E., Steinbrich, R., Ramachandram, H., Hussain, Y., and Reinherz, E.L. (1988) Substitution of murine for human CD4 residues identifies amino acids critical for HIV-gp 120 binding. *Nature* **335:** 363–366.

Dalgleish, A.G., Beverley, P.C.L., Clapham, P.R., Crawford, D.H., Greaves, M.F., and Weiss, R.A. (1984) The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312:** 763–767.

Doyle, C., and Strominger, J.L. (1987) Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* **330:** 256–259.

Filice, G., Cereda, P.M., and Varnier, O.E. (1988) Infection of rabbits with human immunodeficiency virus. *Nature* **335:** 366–369.

Gay, D., Maddon, P., Sekaly, R., Talle, M.A., Godfrey, M., Long, E., Goldstein, G., Chess, L., Axel, R., Kappler, J., and Marrack, J. (1987) Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. *Nature* **328:** 626–629.

Hayashi, Y., Ikuta, K., FuJii, N., and Kato (1989) Inhibition of HIV-1 replication and syncytium formation by synthetic CD4 peptides. *Arch Virol* **105:** 129–135.

Jameson, B.A.A.J., Rao, P.E., Kong, L.E., Hahn, B.H., Shaw, G.M., Hood, L.E., and Kent, S.B.H. (1988) Location and chemical synthesis of a binding site for HIV-1 on CD4 protein. *Science* **240:** 1335–1339.

Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guetlard, D., Hercend, T., Gluckman, J.C., and Montagnier, L. (1984) T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* **312:** 767–768.

Lamarre, D., Ashkenazi, A., Fleury, S., Smith, D.H., Sekaly, R.P., and Capon, D.J. (1989) The MHC-binding and gp120-binding functions of CD4 are separable. *Science* **245:** 743–746.

Landau, N.R., Warton, M., and Littman, D.R. (1988) The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4. *Nature* **334:** 159–162.

Lifson, J.D., Hwang, K.M., Nara, P.L., Fraser, B., Padgett, M., Dunlop, N.M., and Eiden, L.E. (1988) Synthetic CD4 peptide derivatives that inhibit HIV infection and cytopathicity. *Science* **241:** 712–716.

McDougal, J.S., Kennedy, M.S., Sligh, J.M., Cort, S.P., Mawle, A., and Nicholson, J.K.A. (1986) Binding of HTLV-III/LAV to T4+T cells by a complex of the 110K viral protein and the T4 molecule. *Science* **231:** 382–385.

Maddon, P.J., Dalgleish, A.G., McDougal, J.S., Clapham, P.E., Weiss, R.A., and Axel, R. (1986) The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell* **54:** 65–72.

Mizukami, T., Fuerst, T.R., Berger, E.A., and Moss, B. (1988) Binding region for human immunodeficiency virus (HIV) and epitopes for HIV-blocking monoclonal antibodies of CD4 molecule defined by site-directed mutagenesis. *Proc Natl Acad Sci USA* **85:** 9273–9277.

Nara, P.L., Hwang, K.M., Rausch, D.M., Lifson, J.D., and Eiden, L.E. (1989) CD4 antigen-based antireceptor peptides inhibit infectivity of human immunodeficiency virus *in vitro* at multiple stage of the viral life cycle. *Proc Natl Acad Sci USA* **86:** 7139–7143.

Palù, G., Meloni, G.A., Von Berger, J., and Masotti, L. (1986a) On the complex nature of the antiviral activity of coumermycin A_1: its interference with the replication of herpes simplex virus type 1. *Antiviral Res* **6:** 19–32.

Palù, G., Palumbo, M., Antonello, C., Meloni, G.A., and Marciani-Magno, S. (1986b) A search for potential antitumor agents: Biological effects and DNA binding of a series of anthraquinone derivatives. *Mol Pharmacol* **29:** 211–217.

Peterson, A., and Seed, B. (1988) Genetic analysis of monoclonal antibody and HIV binding sites on the human lymphocyte antigen CD4. *Cell* **54:** 65–72.

Reed, L.J., and Muench, H. (1938) A simple method of estimating fifty per cent end points. *Am J Hyg* **27:** 493–497.

Ryu, S.E., Kwong, P.D., Truneh, A., Porter, T.J., Arthos, J., Rosenberg, M., Dai, X., Xuong, N.H., Axel, R., Sweet, R.W., and Hendrickson, W.A. (1990) Crystal structure of an HIV-binding recombinant fragment of human CD4. *Nature* **348:** 419–426.

Robey, E., and Axel, R. (1990) CD4: collaborator in immune recognition and HIV infection. *Cell* **60:** 697–700.

Sattentau, Q.J., Arthos, J., Deen, K., Hanna, N., Healy, D., Beverley, P.C.L., Sweet, R., and Truneh, A. (1989) Structural analysis of the human immunodeficiency virus-binding domain of CD4. *J Exp Med* **170:** 1319–1334.

Sleckman, B.P., Peterson, A., Jones, W.K., Foran, J.A., Greenstein, J.L., Seed, B., and Burakoff, S.J. (1987) Expression and function of CD4 in a murine T-cell hybridoma. *Nature* **328:** 351–353.

Traunecker, A., Schneider, J., Kiefer, H., and Karjalainen, K. (1989) Highly efficient neutralization of HIV with recombinant CD4-immunoglobulin molecules. *Nature* **339:** 68–70.

Wang, J., Yan, Y., Garrett, T.P.J., Liu, J., Rodgers, D.W., Garlick, R.L., Tarr, G.E., Husain, Y., Reinherz, E.L., and Harrison, S.C. (1990) Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. *Nature* **348:** 411–418.