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CD4 antigen-based antireceptor peptides inhibit infectivity of human immunodeficiency virus *in vitro* at multiple stages of the viral life cycle

(antiviral agent/syncytium formation)

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ABSTRACT **Benzylated derivatives of peptides corresponding to residues 81 through 92 of the CD4 molecule [CD4-(81–92)] inhibit human immunodeficiency virus 1 (HIV-1)-induced cell fusion and infection *in vitro*. If such peptides are to be considered as candidates in the therapy of HIV infection, it is crucial to know if the anti-HIV efficacy of CD4-based peptides is limited to blockade of infection and virus-induced cell fusion or if other stages of the viral life cycle are affected by these compounds. Accordingly, an *in vitro* quantitative microassay for acute HIV infection was divided into two kinetic phases corresponding to the two general stages of the viral life cycle: (*i*) viral infection and (*ii*) transmission of virus and viral protein products through cell contact or release of free virions. CEM-SS cell cultures were treated with peptide during either the infection or the transmission phase of the assay. When peptides were present during the infection phase, inhibition of syncytium formation correlated with decreased expression of viral core protein p24 and lack of infectious cell centers when cells exposed to virus were washed and replated onto fresh uninfected indicator cells. These data are consistent with complete inhibition of viral infection when peptide is present only during initial exposure to virus. Unexpectedly, parallel inhibition of syncytium formation, decreased p24 levels, and inhibition of infectious cell center formation were also seen even when peptides were added as late as 48 hr after inoculation, during the transmission period of the assay. Since viral binding and penetration are completed well before 48 hr in this assay system, CD4-(81–92) peptide derivatives appear to exert a virostatic effect on cultures already infected with HIV-1, decreasing p24 production, cytopathicity, and cell-mediated infectivity.**

The human immunodeficiency virus (HIV) establishes infection of human T lymphocytes and macrophages by employing the CD4 cell surface antigen as a receptor, as evidenced by blockade of infection by CD4-directed monoclonal antibodies (1, 2) and transfer of susceptibility to HIV-1 infection in otherwise uninfectible human cells by transfection of the CD4 molecule (3). In addition, fusion between HIV-infected cells and CD4-positive uninfected cells may contribute to the cytopathic effects of virus infection seen *in vitro* and *in vivo* (4, 5). Cell fusion processes are also responsible for cell-to-cell spread of the virus *in vivo*. HIV-induced cell fusion, like infection, can also be blocked by monoclonal antibodies against CD4 (6, 7). The crucial role of HIV envelope/CD4 interactions in viral infection has prompted the development of potentially therapeutic CD4-based reagents capable of blocking these interactions. One such reagent has been produced by recombinant DNA-directed synthesis of C-terminally truncated, water-soluble forms of CD4, which have been reported to block HIV infection and cell fusion at concentrations of 10–100 nM (8–13). Another approach is the synthesis of peptide antireceptors, or small peptide fragments of the CD4 molecule designed to competitively inhibit HIV/CD4 interactions and thus block HIV infection and cell fusion. Benzylated derivatives of the peptide TYICEVEDQKEE [CD4-(81–92)][‖] inhibit the formation of syncytia between HIV-1- or simian immunodeficiency virus-infected lymphoid cells and CD4-positive uninfected lymphoid cells or peripheral blood lymphocytes (14). These peptides also inhibit formation of syncytia upon infection of CD4-positive CEM-SS cells with various isolates of HIV-1, as well as HIV-2, in the CEM-SS virally induced syncytium-forming microassay (SFA) (14, 17). Since CD4-(81–92)-based peptides are relatively small and lipophilic, they may be capable not only of blocking extracellular CD4/HIV interactions involved in infection and fusion but also of entering infected cells and blocking other phases of the viral life cycle by binding to sites of CD4/HIV recognition.

We report here the effects of modified CD4-derived synthetic peptides on the infection and transmission phases of the viral life cycle, as well as cell-mediated cytopathicity, in the SFA/infectious cell center (ICC) assay. The antiviral activities of a structurally defined benzylated CD4 peptide, $T_{Bzl}YIC_{Bzl}E_{Bzl}VEDQK_{Ac}EE$ (hereafter referred to as CD4 18), and of a potent, partially purified mixture of modified CD4-derived peptides are described. Finally, we report the unanticipated observation that these small, relatively lipophilic, CD4 derivatives possess a "virostatic" activity—i.e., the ability to inhibit cell-free virus production and, after peptide removal, fusion with uninfected indicator cells, even when added 48 hr after the initial exposure to virus.

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Abbreviations: SFA, syncytium-forming microassay; ICC, infectious cell center; CD4, cluster of differentiation antigen 4; HIV, human immunodeficiency virus.

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‖Numbering of the amino acid residues of the human CD4 molecule in a previous publication by us (14) was after Maddon *et al.* (15), in which the first two cysteine residues are numbered 18 and 86, and CD4-(83–94) corresponds to the amino acid sequence TYICEVEDQKEE. These authors have since reported a sequence and N-terminus assignment error in their original publication. The revised sequence and N terminus of human CD4 are reported by Littman *et al.* in ref. 16. According to the reassigned residue numbers, the sequence TYICEVEDQKEE corresponds to CD4-(81–92). This is the numbering we use in the present report.

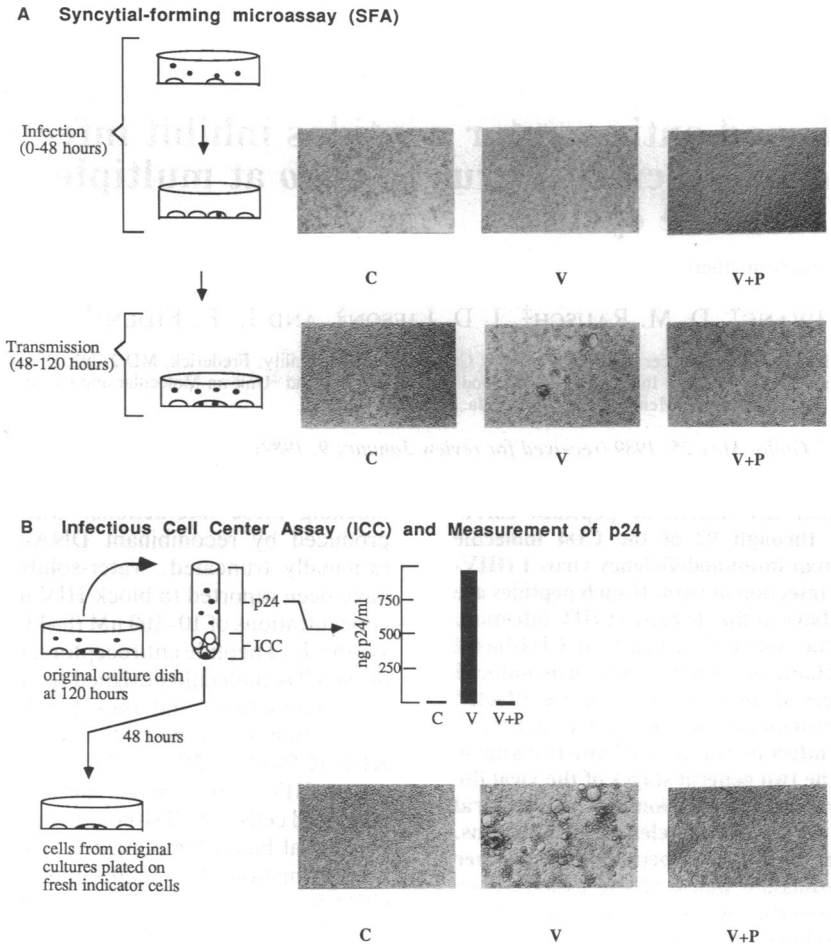

FIG. 1. Infection and transmission phases of SFA. (*A*) Fifty thousand CEM-SS cells per well were plated into 96-well microtiter plates coated with polylysine (17). One hour later medium was removed and fresh medium (C) or thawed, titered virus stocks diluted into RPMI 1640/10% fetal bovine serum and previously incubated at 37°C for 1 hr with (V+P) or without (V) 167 μM CD4-(81–92)-Bzl (14) were added to each well. After 60 min at 37°C, viral inoculum (V, V+P) or medium (C) was removed and replaced with fresh medium (V, C) or medium containing 167 μM CD4-(81–92)-Bzl (V+P). Forty-eight hours later, media were removed and again replaced with fresh medium (V, C) or medium containing 167 μM CD4-(81–92)-Bzl (V+P). (*B*) Seventy-two hours after this medium change, medium was removed, clarified by centrifugation, and prepared for p24 radioimmunoassay (17). Cells were washed with RPMI 1640/10% fetal bovine serum and 50,000 cells were plated onto 50,000 fresh uninfected CEM-SS cells in polylysine-coated microtiter plate wells (19). Syncytia in an entire well were counted for both SFA and ICC, using an inverted phase-contrast microscope. Photographs are 20-fold magnifications of the images of cells filmed with a 35-mm camera under Hoffman modulation optics. Note that in the paradigm shown here, peptide is present during both infection and transmission phases of the assay. Here only the anti-syncytial and anti-infective activities of test compounds are measured. By adding peptide either during the infection or during the transmission phase of the assay, anti-infective activity and inhibition of viral transmission (either by viral release, which is measured by extracellular p24, or by cell–cell contact, which is measured in the ICC assay) can be measured separately and compared (see Tables 2 and 3 and Fig. 2).

MATERIALS AND METHODS

Synthesis of CD4-(81–92)-Derived Peptides. The peptide mixture CD4-(81–92)-Bzl, referred to as "peak 7" in a previous publication (14), was obtained essentially as described previously. Upon purification by C_8 chromatography, all syntheses examined yielded a major peak corresponding to TYIC$_{Bzl}$EVEDQKEE [*S*-benzyl-CD4-(81–92)] and devoid of biological activity and a minor complex peak [corresponding to "peak 7" (14) and here designated CD4-(81–92)-Bzl] containing fusion-inhibiting activity at a nominal concentration of 32 μM. This peak was reproducibly collected from multiple independent syntheses of *S*-benzyl-CD4-(81–92). All batches of CD4-(81–92)-Bzl purified have a nominal potency of 32 μM in the HIV-induced cell fusion assay (5), as initially reported (14). The peptide CD4 18 was synthesized by using conventional fluoren-9-ylmethoxycarbonyl (Fmoc) chemistry (18) in either a batch or automated format, cleaved in trifluoroacetic acid, and rinsed with diethyl ether, and its structure was confirmed by fast atom bombardment mass spectrometry after purification to greater than 90% by reverse-phase chromatography.

Quantitative SFA. Peptides were dissolved in phosphate-buffered saline (PBS; Biofluids, Rockville, MD) or 140 mM $NaHCO_3$ as above and diluted with RPMI 1640 medium to give working stocks. Peptide solutions or medium alone was added to cell-free virus in RPMI 1640/10% fetal bovine serum with penicillin, streptomycin, and antimycotic in a total volume of 50 μl and placed onto 50,000 CEM-SS cells in 96-well microtiter plates coated with poly(L-lysine). Cells had been plated 1 hr previously to allow adherence to the poly(L-lysine) substrate and medium was removed just prior to addition of viral inoculum. After 1 hr of viral inoculation at 37°C, supernatants containing virus were carefully pipetted from the cells and replaced with 100 μl of fresh RPMI 1640/10% fetal bovine serum or RPMI 1640 containing peptide. Forty-eight hours later, medium was again carefully removed by pipette and replaced with either fresh medium or

fresh medium containing peptide. All incubations were carried out at 37°C in a humidified 95% air/5% CO_2 atmosphere. Approximately 72 hr later, cultures were scored for syncytia as previously described (17).

p24 Assay. At the end of the SFA supernatants were removed, centrifuged briefly at 12,000 rpm in a Beckman Microfuge B centrifuge, diluted with lysis buffer (17), and stored at 4°C prior to radioimmunoassay for p24 viral core protein as described (17).

ICC Assay. Cells were collected from duplicate microtiter wells after being scored for syncytia in the quantitative SFA, pooled, washed once in 1 ml of RPMI 1640, suspended in two ml of RPMI 1640, and serially diluted in the same medium. Fifty thousand cells in 100 μl were then added to wells containing 50,000 fresh CEM-SS indicator cells on poly(L-lysine) prepared as described above. Forty-eight hours later, plates were scored for the presence of syncytia, exactly as for scoring of the SFA (see above and ref. 19). Since the incubation period of the ICC is only 48 hr, syncytia formed during this time arise exclusively from fusion of infected input cells and fresh indicator cells, and not from infection and subsequent fusion of indicator cells alone, mediated by free virus particles. Thus, quantitation of syncytia formed in the ICC assay gives an index of the number of infected cells that were present at the end of the preceding SFA.

Preparation of Virus Stocks. HIV-1 isolates HTLV-IIIB and RF-II were propagated from freshly infected H9 cells to maximize the ratio of infectious to defective viral particles, cryopreserved, and titered as working stocks.

RESULTS

The SFA used to assess peptide effects on viral infection and transmission is outlined in Fig. 1. Adherent cells are inoculated from a titred virus stock for 1 hr, after which the inoculum is removed and replaced with fresh medium. The period from viral inoculation to the next medium change 48 hr later is referred to in Fig. 1*A* as the infection phase, since during this period virus binding to the surface of the cell, virus internalization, and intracellular viral replication (in aggregate, infection) has occurred, and production of viral protein products has begun, but cells have not yet produced viral progeny or expressed viral envelope proteins in significant amounts, as evidenced by lack of measurable p24 antigen in culture medium (D.M.R., J. Bess, N. Dunlop, and P.L.N., unpublished observations) and lack of syncytia, formation of which is dependent on the presence of viral envelope glycoprotein on the surface of infected cells (6, 7). Cells inoculated with virus, cells inoculated with virus and treated with peptide, and uninfected cells are indistinguishable at this time (Fig. 1*A*). Incubation is continued to a total of 120 hr after inoculation. This phase of the assay is the transmission phase (Fig. 1*A*). During this period cells infected with virus become competent to infect other CD4-positive cells by release of viral progeny or by cell fusion. Cells inoculated with HTLV-IIIB exhibit multiple syncytia composed of 10–150 fused cells at the end of the transmission phase. Cells treated with virus plus CD4-(81–92)-Bzl are indistinguishable in appearance and number from control cells not treated with virus (Fig. 1*A*).

The SFA alone does not distinguish between blockade of cell fusion and inhibition of viral infection or infectiousness of already virally infected cells. This question was addressed by using the experimental design depicted in Fig. 1*B*. After infection in the presence or absence of peptide, cells were monitored for their potential to transmit virus through release of viral progeny by measurement of viral antigen p24. Cells were monitored for their potential to transmit virus through cell–cell contact, by measurement of ICCs formed after extensive washing of cells to remove peptide and released virus particles, followed by incubation of infected cells with fresh uninfected indicator cells. Incubation of virally infected, otherwise untreated cells in the ICC assay results in formation of multiple ICCs. Cells inoculated with virus and treated with CD4-(81–92)-Bzl showed no infectious cell centers (Fig. 1*B*) and are otherwise indistinguishable from control cells not exposed to virus and transferred onto fresh CEM-SS cells in a mock ICC assay (Fig. 1*B*). Levels of the core viral antigen p24 were likewise greatly decreased in cultures infected with HTLV-IIIB and continuously treated with CD4-(81–92)-Bzl (Fig. 1*B*).

Syncytium formation in the SFA, p24 levels, and syncytium formation in the ICC assay were next measured after continuous treatment with various doses of CD4-(81–92)-Bzl after inoculation of CEM-SS cultures with either HTLV-IIIB or a second HIV-1 isolate, RF-II. Complete inhibition of HIV-1_{RF-II} infection was achieved with 2–4 times more peptide than for inhibition of HTLV-IIIB infection (Table 1). Thus, CD4-(81–92)-Bzl, when present continuously during and after inoculation of CEM-SS cells with two envelope-divergent strains of HIV-1, inhibits not only HIV-induced cell fusion but also viral infection as determined by the inability of inoculated, peptide-treated cells to release p24 antigen and produce ICCs after removal of the CD4-(81–92)-Bzl peptide.

The kinetics of antiviral action of CD4-(81–92) peptide derivatives and their relative potencies in inhibiting HIV infection and HIV-induced cell fusion were further examined by adding peptide only during either the infection (0–48 hr) or transmission (48–120 hr) phases of the SFA (Fig. 2). CD4-(81–92)-Bzl completely inhibited syncytium formation at <25 μM when added during either phase of the assay. The structurally defined compound CD4 #18 also inhibited syncytium formation when added during either the infection or transmission phase of the assay. Unexpectedly, p24 production as well as syncytia generated during the ICC were significantly decreased even when peptides were present only during the final 72 hr of the assay, 48 hr after viral inoculation (Fig. 2). CD4-(81–92)-Bzl was slightly more potent than CD4 #18 in exhibiting this "virostatic" effect. It is unlikely that inhibition of ICC is due to the failure to wash away peptide nonspecifically bound to the CEM-SS cells, since removal of CD4-(81–92)-Bzl by washing immediately after the first hour of the assay resulted in virtually complete loss of antisyncytial activity after inoculation with several viral isolates (N. Dunlop, P.L.N. and L.E.E., unpublished observations).

The virostatic action of CD4-based antireceptor peptides could be due to an obligate decrease in the synthesis of viral

Table 1. Continuous treatment with CD4-(81–92)-Bzl inhibits syncytium formation, p24 elaboration, and subsequent formation of ICCs after HTLV-IIIB or RF-II inoculation of CEM-SS cells

HIV-1	Peptide, μM	SFA, syncytia/well	p24, ng/well	ICC, syncytia/well
HTLV-IIIB	0	120	664	>300
	31	0	2	0
	63	0	3	0
	125	0	3	ND
RF-II	0	168	64	>300
	7.8	111	11	ND
	31	56	5	ND
	125	0	0	0

Experiments were carried out as described in Fig. 1. Values are averages of duplicate (experimental) or octuplicate (control) wells from a single experiment, which was repeated four times with similar results. ND, not determined. Values similar to the no-peptide control were obtained for syncytia in SFA, syncytia in ICC, and p24 by treatment with the control peptide GGa-26 (14), which has an acidic amino acid composition similar to that of the CD4-(81–92)-based peptides, at 100–500 μM.

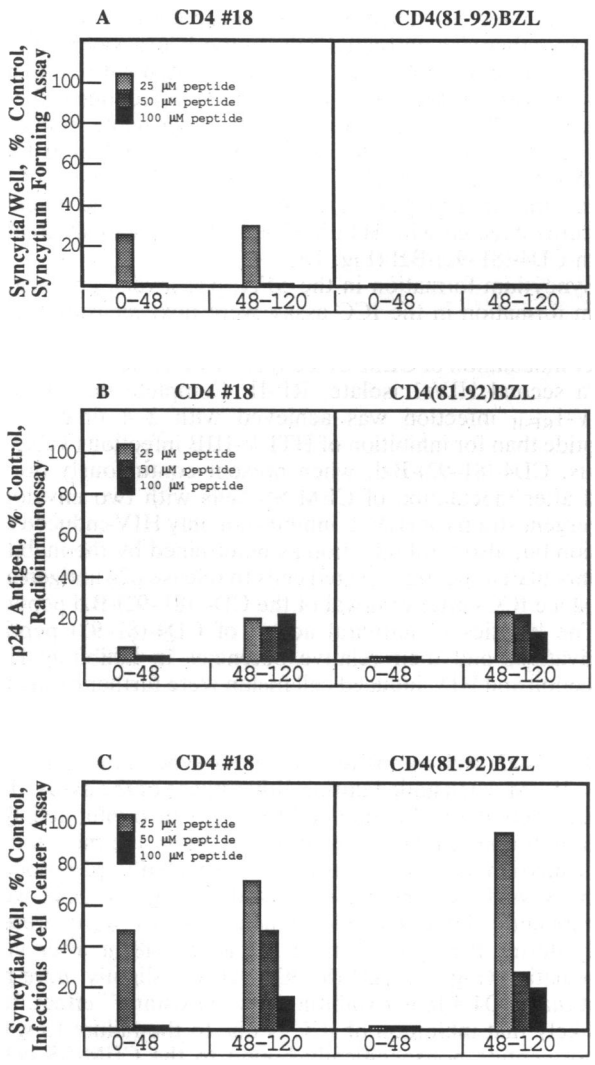

FIG. 2. Treatment with CD4 #18 and CD4-(81–92)-Bzl during infection and transmission phases of the SFA: Syncytium formation, p24 elaboration, and cellular infectivity after HTLV-IIIB infection of CEM-SS cells. CD4 #18 or CD4-(81–92)-Bzl was added during the infection phase (0–48 hr: present from the time of inoculation until 48 hr later, removed and replaced with medium during the last 72 hr of the assay) or the transmission phase (48–120 hr: medium present during the first 48 hr of the assay, replaced with peptide which was present until cultures were scored for syncytia formation 72 hr later) of the assay described in Fig. 1. (*A*) Syncytia per well in SFA as percent of control. The number of syncytia present in each well on day five (120 hr after inoculation) was counted, divided by the number of syncytia present in control wells (i.e., inoculated with virus, no peptide present during assay), and multiplied by 100. The input viral inoculum in the assay shown here was 80 ± 10 (mean ± SEM, n = 8) syncytium-forming units per well. All experimental values are the average of duplicate wells. The experiment shown was repeated twice for CD4 #18 and more than five times for CD4-(81–92)-Bzl with qualitatively and quantitatively similar results. Shading of bars represents the corresponding concentrations of peptides (25, 50, or 100 μM) shown in the upper left-hand key in each of the three portions of the figure. (*B*) p24 antigen measured by radioimmunoassay, expressed as percent of control. Control levels of p24 antigen (i.e., in cultures inoculated with virus, no peptide additions) were 412 ± 39 ng per well (mean ± SEM, n = 8) in this experiment. (*C*) Syncytia per well in the ICC assay, expressed as percent of control. After scoring of syncytia (*A*) and removal of medium for p24 radioimmunoassay (*B*), cells contained in each well were resuspended in culture medium, washed twice, and resuspended in medium, and 50,000 cells from each sample were added to 50,000 fresh CEM-SS indicator cells plated on polylysine in 96-well dishes. Forty-eight hours after cocultivation, each well was scored for syncytia as described (19). Number of syncytia per well in control ICC (indicator cells cultivated with 50,000 cells from culture inoculated with virus only, no peptide present) was 104 ± 6.8 (mean ± SEM, n = 8). As for the initial SFA, no syncytia were observed in ICC from cells carried through the SFA but not exposed to virus. Several control peptides initially reported to be inactive in inhibiting HIV-1-induced syncytium formation (14) also had no activity in inhibiting syncytium formation in SFA and ICC and p24 production, compared with the control without peptides. These peptides, tested at 100 μM, include *S*-benzyl-CD4-(81–92) and GGa-26 (see Fig. 1).

Table 2. CD4-(81–92)-Bzl virostatic activity is dissociable from anti-infective and anti-syncytial activity by peptide dose

Peptide, μM	Syncytia/well	p24, ng/well
0	66 ± 3.6	95 ± 19
6	0, 0	129, 114
25	0, 0	39, 49
50	0, 0	35, 35
100	0, 0	0, 0

Cell cultures inoculated with HTLV-IIIB as described in Fig. 1 were incubated in medium for 48 hr, and medium was replaced with fresh medium or medium containing 6–100 μM CD4-(81–92)-Bzl. Cultures were incubated an additional 72 hr. Syncytia were counted and p24 was measured by radioimmunoassay in culture supernatants as described in the legend to Fig. 2. Values shown are duplicate determinations in a single experiment, typical of three to five additional experiments carried out with these doses of CD4-(81–92)-Bzl. Values shown for controls (cells inoculated with virus in the absence of peptide) are the mean ± SEM for four to eight determinations from a single experiment.

antigens when cell fusion is inhibited after infection, since in the absence of cell fusion, fewer cells, and therefore less protein-synthetic capacity and membrane surface area, are recruited into the process of virus/viral antigen production. Three lines of evidence clearly indicate this is not the case. First, various peptides with similar potencies in inhibiting syncytium formation do not have corresponding potencies as virostatic agents. Thus, $T_{Bzl}YIC_{Bzl}E_{Bzl}VEDQKEE$ (synthesized by K.M.H.) and CD4 #18 both inhibit syncytium formation between HIV-infected and uninfected indicator cells completely at 100 μM, yet only CD4 #18 inhibits p24 production when added 48 hr after infection (data not shown). Second, the dose–response curve for CD4-(81–92)-Bzl inhibition of syncytium formation and p24 production when added 48 hr after infection are quite different: doses of CD4-(81–92)-Bzl between 6 and 50 μM that completely block syncytium formation have no effect on p24 production and ICC formation, although both are completely blocked at 100 μM peptide (Table 2). Finally, anti-CD4 monoclonal antibody anti-Leu-3a, which blocks fusion of HIV-infected cells by binding at or near a gp120-binding epitope of the CD4 molecule and blocking attachment of virus to its cellular receptor (20), inhibited ICC formation when present initially during the infection phase of the assay, but it only partially inhibited ICC formation when present only during the transmission phase of the assay (Table 3). By contrast, CD4-(81–92)-Bzl completely inhibited subsequent ICC formation whether present only during the infection or only during the transmission phase of the assay, as previously observed (Table 3).

DISCUSSION

CD4-(81–92)-Bzl, as well as the structurally defined compound CD4 #18, inhibits syncytium formation upon infection of CEM-SS cells whether present only during the infection (0–48 hr) or only during the postinfection transmission period (48–120 hr) of the SFA for HIV infectivity (Fig. 1). Inhibition of syncytium formation correlates strongly with decreased

Table 3. Anti-Leu-3a completely blocks syncytium formation, but not subsequent ICC formation, when present during the transmission phase of the SFA

Phase	Agent present	Conc., μg/ml	Syncytia/well SFA	Syncytia/well ICC
Infection (0–48 hr)	CD4-(81–92)-Bzl	84	0, 0	0, 0
		166	0, 0	0, 0
	Anti-Leu-3a	5	0, 0	2, 6
		10	0, 0	2, 2
Transmission (48–72 hr)	CD4-(81–92)-Bzl	84	0, 0	3, 4
		166	0, 0	5
	Anti-Leu-3a	5	0, 0	35, 35
		10	0, 0	34, 45

CD4-(81–92)-Bzl or anti-Leu-3a was added during either infection (0–48 hr) or transmission (48–120 hr) phase of the SFA as described in Figs. 1 and 2. Syncytia were scored at 120 hr. Control (cells inoculated with virus only) wells contained 71 ± 4.8 syncytia per well (mean ± SEM). Cells were washed and diluted as described in *Materials and Methods*, and 50,000 cells were plated onto 50,000 fresh CEM-SS indicator cells. Forty-eight hours later, the number of ICCs, as exhibited by the number of syncytia formed, was scored. Control (cells inoculated with virus only) wells contained 160 ± 8.3 syncytia per well (mean ± SEM). CD4-(81–92)-Bzl was prepared as described (14) except that a Vydac C_4 2.5 × 25 cm alkyl silicate HPLC column, rather than a C_8 column, was used to purify the peptide to a specific activity of <32 μM in the HIV-induced cell fusion assay. Anti-Leu-3a was obtained from Becton Dickinson and diluted into RPMI 1640 medium for use in the SFA. Concentrations used were in excess of those required to completely block HIV or gp120 binding to CEM-SS cells (20). p24 levels were not measured due to immunoglobulin interference in the radioimmunoassay (J. Bess, personal communication). Values are shown as duplicate determinations in a single experiment, which was repeated with similar results.

p24 when peptides are added during the initial phase of the assay (0–48 hr) or are present continuously (Table 1, Fig. 2). In addition, antiviral potency is as great when peptides are present during the first 48 hr after infection as when present continuously (compare Table 1 and Fig. 2), demonstrating that the mechanism of action of CD4-(81–92) peptides under these conditions is probably direct inhibition of viral entry after inoculation.

Unexpectedly, when either the CD4-(81–92)-Bzl peptide mixture or the structurally defined CD4 #18 was added at 25–100 μM 48 hr after infection, inhibition of syncytium formation was also accompanied by decreased p24 in culture supernatants and decreased cell-associated infectious activity, after peptide removal, in the ICC assay (Fig. 2). One possible mechanism for this virostatic effect is that the peptide is not removed from the CEM-SS cells upon washing, remaining in the medium and inhibiting syncytium formation during ICC. This is unlikely, since incubation of cells with virus plus peptide during the initial inoculation phase of the assay, followed by removal of peptide from 1 to 120 hr, results in virtually complete loss of antiviral activity, suggesting that the peptide can be completely removed from uninfected CEM-SS cells. A second possibility is that the CD4-(81–92) peptides bind to gp120 both within and on the surface of infected cells, neutralizing infectious and fusogenic viral envelope protein interaction with CD4, and perhaps actually impeding virus maturation or budding, as suggested by the high correlation between inhibition of ICC and decreased levels of p24 detected in culture supernatants. This second hypothesis is supported by the ability of peptides to block binding of both gp120 and gp160 to CD4-positive cells, with potencies similar to those exhibited for antiviral activity (V. S. Kalyanaraman and L.E.E., unpublished data).

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