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Peptide T inhibits HIV-1 infection mediated by the chemokine receptor-5 (CCR5)

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Abstract

Peptide T, which is derived from the V2 region of HIV-1, inhibits replication of R5 and dual-tropic (R5/X4) HIV-1 strains in monocyte-derived macrophages (MDMs), microglia, and primary $CD4^+$T cells. Little to no inhibition by peptide T was observed with lab adapted X4 viruses such as IIIB, MN, or NL4-3 propagated in $CD4^+$ T cells or in the MAGI entry assay. The more clinically relevant R5/X4 early passage patient isolates were inhibited via either the X4 or R5 chemokine receptors, although inhibition was greater with R5 compared to X4 receptors. Virus inhibition ranged from 60 to 99%, depending on the assay, receptor target, viral isolate and amount of added virus. Peak inhibitory effects were detected at concentrations from 10^{-12} to 10^{-9} M. Peptide T acted to block viral entry as it inhibited in the MAGI cell assay and blocked infection in the luciferase reporter assay using HIV virions pseudotyped with ADA envelope. These results using early passage virus grown in primary cells, together with two different entry reporter assays, show that peptide T selectively inhibits HIV replication using chemokine receptor CCR5 compared to CXC4, explaining past inconsistencies of in vitro antiviral effects.

Keywords: Peptide T; Macrophage; HIV-1; Entry inhibitor; Therapeutic

1. Introduction

The envelope protein of HIV interacts with CD4 and one of the two major chemokine receptors, CXCR4 (Feng et al., 1996) and CCR5 (Choe et al., 1996; Deng et al., 1996) to enter cells and select chemokines inhibit infectivity (Cocchi et al., 1995; Bleul et al., 1996). HIV-1 establishes infection primarily via interactions with the β-chemokine receptor R5 (CCR5) in macrophages (Choe et al., 1996; Hill and Littman, 1996; Michael and Moore, 1999) and viral strains,

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called 'R5', which use this receptor predominate during initial spread and the early phases of infection. An octapeptide derived from the gp120 V2 region (Levy et al., 1984; Luciw et al., 1984), which we called peptide T (D-Ala_1-Peptide-T-amide, 'DAPTA'), inhibited gp120 binding and infectivity of uncharacterized patient virus grown in activated PBMC cultures (Pert et al., 1986), prevented gp120 neuronal degeneration (Brenneman et al., 1988a,b), inhibited gp120 signaling (Zorn et al., 1990; Liapi et al., 1998) and prevented the neuroendocrine effects of gp120 to suppress growth hormone secretion (Mulroney et al., 1998). Homologous pentapeptides are present in the V2 region of all HIV-1 env proteins and function as potent chemoattractants (Ruff et al., 1987b) and gp120 antagonsists (Brenneman et al., 1988a; Ruff et al., 1987b; Redwine et al., 1999).

Peptide T was proposed to function as a viral entry inhibitor by blocking CD4 dependent gp120 binding (Pert et al., 1986), at that time considered to occur via CD4. Recent reports show that peptide T preferentially inhibits β-chemokine chemotaxis and binding in a CCR5 dependent manner (Redwine et al., 1999; Raychaudhuri et al., 1998). The identification of the two major entry co-receptors (Feng et al., 1996; Bleul et al., 1996) suggested additional targets for peptide T effects. In this report we show that peptide T acts as an HIV-1 entry inhibitor and preferentially targets chemokine receptor CCR5, one of the two major HIV-1 co-receptors with CD4.

2. Materials and methods

2.1. Peptides

D-Ala_1-Peptide-T-amide ([d-A_1STTTNYT-NH2, 'DAPTA') was synthesized under GMP conditions by Peninsula Laboratories, Belmont, CA. D-Ala_1, D-Tyr_7-peptide T-amide ('D-Y-DAPTA') was synthesized by Phoenix Pharmaceuticals, Menlo Park, CA. All peptides were purified to $>95\%$ homogeneity and verified by HPLC isolation, amino acid analysis, and sequencing using an ABI 470A gas-phase sequencer with on-line HPLC. Peptides were dissolved in sterile water and stored as aliquots at 0.1 mM stock (peptide T, -20 °C) and vortexed vigorously prior to use.

2.2. Cells

Purified human monocytes were prepared from healthy adult human donors by centrifugal elutriation ($>95\%$ pure) and cultured for 7–14 days in RPMI (Gibco) containing 5% human AB plasma, 2 mmol/l glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), to generate monocyte-derived macrophages (MDMs) prior to use in infection assays. In some experiments monocytes were obtained by centrifugation of mononuclear cells over lymphocyte separation media (LSM, Organon Technica Corp., Durham, NC) at $1500 \times g$ for 45 min. The mononuclear cell layer was collected and incubated in gelatin coated flasks for 45 min at 37 °C, followed by washing off of the non-adherent cells with Dulbecco's modified Eagles Medium (DMEM). After detachment with ethlenediamine tetra-acetic acid (EDTA), monocytes were resuspended in DMEM supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), and non-essential amino acids and plated in 24 well culture plates at a density of 10^6 cells/ml. The monocytes were greater than 96% pure as determined by fluorescence activated cell sorting using monoclonal antibody (mAb) against CD 14 (LeuM3).

Human fetal brain tissues were obtained from 16- to 22-week-old aborted fetuses under a protocol approved by the Human Subjects Research Committee at Children's Hospital, Philadelphia, PA. These cultures were prepared using a previously described technique (Chao et al., 1992). Briefly, brain tissues were dissociated after a 45 min trypsinization (0.125%) and were seeded into 75 cm^2 Falcon culture flasks (Fisher Scientific, Pittsburgh, PA) in DMEM (Sigma) containing 10% heat-inactivated serum (Hyclone Laboratories, Logan, UT) and penicillin/streptomycin (Gibco). On day 10–12 of culture, harvested cells (microglia) were seeded into 96-well plates.

2.3. HIV-1 stocks

HIV-1_{BaL} (BaL), 2.3 ng/ml p24; HIV-1 92US727, 20.3 ng/ml p24; HIV-1 92US077, 23 ng/ml p24;

HIV-1 92HT596, 22 ng/ml p24; and HIV-1 92HT593, 57.1 ng/ml p24), NL4-3, MN, RF, IIIB were obtained from the NIH AIDS Research and Reagent Program.

2.4. HIV infections

The anti-viral effects of peptide T were studied in several systems. MDM at a concentration of 1×10^6 cells per ml in 24-well plates were cultured for 7–14 days in growth medium (RPMI, 5% human AB serum). At the beginning of the experiment the cells were washed with serum free RPMI and were treated with peptide T (DAPTA) at the indicated concentrations, or vehicle (medium), for 1–2 h at 37 °C, 5% CO_2. Cultures were washed two times to remove unadsorbed virus and cultured in growth medium containing peptide T at indicated concentrations. Supernatants from day 7 or 14 cultured MDM's were sampled. Cultures were re-fed with peptide T and 50% fresh medium after the day 7 sample. The p24 antigen determination was made using commercial kits according to the manufacturer's instructions (CoulterELISA).

2.5. Flow cytometric analysis of intracellular p24 antigen

Intracellular p24 antigen was stained in MDMs cultured for 7 days on low adherence 24 well culture trays (Falcon). Cells were treated in culture wells with 10 μg/ml Brefeldin A (Sigma, St. Louis, MO) for 4 h at 37 °C to inhibit protein transport and removed by vigorous pipetting. Cells were surface stained with FITC-CD14. After washing three times in PBS the cells were fixed with 4% paraformaldehyde for 20 min at 4 °C, then permeabilized in 1 ml with 0.1% saponin. PE labeled *anti*-HIV core antibody (KC-57, Beckman-Coulter, Brea, CA) was added and the cells incubated for 45 min in the dark. After washing the cells were left undisturbed for 10 min, resuspended and analyzed by flow cytometry using a FACSCAN (Beckton-Dickinson, MountainView, CA). A PE-IgG1 antibody was used as an isotype control. Uninfected monocytes were used as a negative control, and an *anti*-HIV serum was used to block the binding of PE-labeled anti core KC-57. Monocytes were gated using CD14 and 25,000 events were collected. Data was analyzed using FlowJo software (Tri Star, Inc., San Carlos, CA).

2.6. MAGI/MAGI-CCR5 infection assay

A HeLa cell line (MAGI) that both expresses high levels of CD4 and contains a single integrated copy of a β-galactosidase gene that is under the control of a truncated human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) (Kimpton and Emerman, 1992), and this cell line with the CCR5 gene (MAGI-CCR5) (Pirounaki et al., 2000), were obtained from the NIAID AIDS Repository. To determine chemokine receptor subtype sensitivity of peptide T, MAGI and MAGI-CCR5, are used as described below, and were adapted from earlier protocols (Kimpton and Emerman, 1992; Harrington and Geballe, 1993; Vodicka et al., 1997; Pirounaki et al., 2000). Briefly, 10,000 MAGI or MAGI-CCR5 cells/well were seeded into a 96-well plate. One day later the medium was removed and dilutions of peptide T or MIP-1β were added in Opti-MEM medium (Gibco BRL, Life Technologies) with 20 μg/ml DEAE-Dextrin. The plates were cultured for 60 min, 37 °C, 5% CO_2, and then dilutions of virus added with culture for a further 1.5 h. The viral inoculum was removed and wells washed twice with 0.2 ml Opti-MEM medium and fresh media (150 μl/well DMEM) added. After culture for a further 46 h. The cells were fixed for 5 min at room temperature with 1% formaldehyde, 0.2% glutaraldehyde in PBS, washed twice with PBS, stained with 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM MgCl, and 0.4 mg/ml 5-bromo-4-chloro-3-indoloyl-β-D-galactopryanoside (X-gal stain, Inalco Pharmaceuticals, St. Luis Obispo, CA) in PBS at 37 °C, 5% CO_2, for 50 min, and washed twice with PBS. Blue foci were counted microscopically and infection levels were recorded as blue focus units (BFU/well). Background levels of BFU/well were typically < 3 in all assays.

2.7. Luciferase reporter virus entry assay

Recombinant luciferase-encoding HIV virions pseudotyped with M-tropic ADA Env were used to study HIV infection of peptide T treated microglia. The plasmid encoding HIV ADA was provided by John Moore (Aaron Diamond AIDS Research Center, New York, NY), and the NL4-3 luciferase virus backbone was provided by Ned Landau (Aaron Diamond AIDS Research Center). In brief, NL4-3 Luc-R-E-virus stocks pseudotyped with ADA virus were generated by transfecting 293T cells with NL4-3 Luc-R-E- and pSV7d-base expression vectors encoding HIV ADA. Virus containing supernatants were collected at 48 h post-transfection and frozen at −70 °C. Microglia at a concentration of 10^6 cells per ml in 48-well plates were infected for 4 h with 100 μl of the supernatants (50 ng of p24). At 72 h post-infection, the cells were lysed in 150 μl of 0.5% Triton-X-100 in PBS. Luciferase activity was determined in 50 μl of each lysate in a Wallac Trilux Microbeta luminometer (Wallac, Turku, Finland).

2.8. Statistical analysis

The data were analyzed using the Student's *t*-test for paired samples.

3. Results

3.1. Peptide T inhibits HIV-1_{BaL} production in MDMs

We studied the effect of peptide T (DAPTA) on the spread of BaL, a CCR5 specific lab adapted isolate of HIV-1. Monocyte-derived macrophages were prepared from peripheral blood by adherence and cultured for 5 days prior to use in 24-well trays (10^6 cells/well). The cells were treated with peptide T for 60 min at the indicated doses and infected with HIV-1_{BaL} virus (1 ng p24) for 1 h. Virus was then washed away and peptide T re-added and cultures maintained with weekly feeding, 50% media change, for 14 days. Supernatants were sampled from duplicate cultures for p24 antigen, a measure of viral replication, at day 7 and 14.

In the presence of peptide T virus production 7 and 14 days post-infection was suppressed with maximum inhibition from -1.5 to $-2.0\ \log_{10}$ observed at 10^{-12}–10^{-9} M doses (Table 1). At higher peptide T doses (e.g. 10^{-7} M) inhibition was less. Inhibition with peptide T was most apparent at earlier time periods as virus was breaking through by day 14, although still suppressed compared to untreated cultures. Inhibitory effects were also greater with lower virus levels. Subsequent experiments were therefore conducted at day 7 or earlier in order to focus on initial events in virus production, such as ligand binding and viral entry.

To further characterize conditions for detecting anti-viral activity, the effect of different virus

Table 1
Inhibition of HIV-1_{BaL} production from monocyte derived macrophages (MDMs) by peptide T

	DAPTA (M)	p24 (pg/ml)/s.e.m.	Inhibition ($\log_{10}$)
Experiment #1			
Day 7	0	8792/766	
	10^{-12}	146/98	−1.8
	10^{-9}	255/66	−1.5
	10^{-7}	287/54	−1.5
Day 14	0	53,579/8354	
	10^{-12}	16,477/2368	−0.5
	10^{-9}	11,278/1875	−0.7
	10^{-7}	13,646/1422	−0.6
Experiment #2			
Day 7	0	2476/645	
	10^{-12}	24/13	−2.0
	10^{-9}	76/45	−1.5
	10^{-7}	413/66	−0.8

Monocytes were cultured for 5 days to generate monocyte-derived macrophages (MDMs) which where then infected with HIV-1_{BaL} virus (1 ng/culture), with or without (vehicle only control) or peptide T at the indicated concentrations. Supernatants were sampled and tested on days 7 and 14 for p24 antigen production by ELISA kit. Experiments 1 and 2 differ in the source of donor monocytes. Each data point is the mean of duplicate determinations. Inhibition of p24 production was observed in the presence of peptide T at concentrations from 10^{-12} to 10^{-7} M at days 7 and 14 in comparison to vehicle treated cultures.

concentrations on peptide T activity was determined in cultured monocytes (day 7) after differentiation into MDMs and upregulation of CCR5 (Wang et al., 1998). Receptor effects should be sensitive to virus and receptor concentrations, with the most potent effects detectable at lower concentrations of each. Cells were infected with HIV-1_{BaL} concentrations of 0.2 and 0.02 ng p24/well, corresponding to a multiplicity of infection of approximately 1 and 0.1 infectious particle per cell.

We measured both extracellular (supernatant) and intracellular p24 of day 7 p24 antigen production in the presence of different concentrations of peptide T. The results are presented in Fig. 1. Peptide T caused a dose dependent inhibition of BaL production as judged by decreased p24 antigen in the supernatant, which was associated with decreased number of p24 positive MDMs. The inhibitory effect was most pronounced at the lower (0.02 ng p24) (80%), compared to the higher (0.2 ng p24 (69%) viral inoculum and significant inhibition was detectable at peptide T concentrations of 10^{-12} M. The decrease of supernatant virus is therefore likely a result of decreased initial infection.

3.2. Peptide T inhibits HIV-1 primary isolate production in MDMs

Of interest was the effect of peptide T on primary viral isolates as these have been observed to differ in drug sensitivity compared to lab adapted strains (Turner et al., 1992). Day 7 MDMs were infected with the indicated amount of three early passage viruses: HIV-1 92US727 (1 ng virus/1.5×10^6 MDMs/1.0 ml) is a CCR5 tropic isolate, while HIV-1 92US077 (1.15 ng virus/1.5×10^6 MDMs/1.0 ml) and HIV-1 92HT593 (1.42 ng virus/1.5×10^6 MDMs/1.0 ml) are dual-tropic (CCR5/CXCR4). P24 antigen production was determined from day 7 post-infection MDM supernatants. Peptide T maximally inhibited all three primary isolates by greater than $2 \log_{10}$ at concentrations between 10^{-12} and 10^{-10} M. (Fig. 2). Infection of MDMs by the two dual-tropic isolates is presumably occurring via the CCR5 receptor pathway.

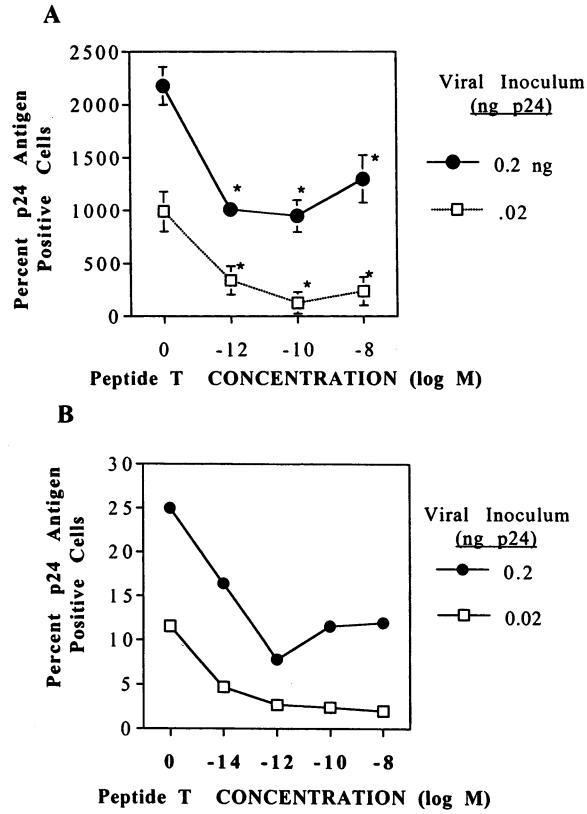

Fig. 1. Inhibitory effect of peptide T on intracellular and extracellular p24 antigen production of HIV-1_{BaL}. MDM were cultured for 7 days and infected with HIV-1_{BaL} virus at 2 or 0.2 pgms p24/10^6 cells, with or without added peptide T, as indicated. Supernatants were sampled and tested on day 7 for p24 antigen. (A) Extracellular (supernatant) production by ELISA kit. Each data point is the mean of two determinations $\pm$ s.e.m. Statistical analysis was by *t*-test (*, $P \leq 0.05$) for peptide T vs. vehicle treated cultures; (B) FACS analysis of intracellular p24 using directly labeled *anti*-p24 mAb (Coulter) as detailed in the Section 2.

3.3. Specific effect of peptide T on entry of R5 compared to X4 HIV-1 isolates

To determine chemokine receptor subtype sensitivity for peptide T inhibition we tested a panel of laboratory HIV-1 isolates with known receptor specificity using MAGI and MAGI-CCR5 cells containing the HIV-LTR-β-galactosidase reporter gene as infection targets. This assay employs a single cycle of virus replication and is sensitive to entry inhibitors (Pirounaki et al., 2000). Blue Fo-

cus Forming Units (BFU/well) were counted microscopically and results expressed as blue-focus units (BFU)/ well ± s.e.m. for peptide T treated compared to vehicle only controls from triplicate determinations.

Peptide T inhibited HIV-1_{BaL} infection in MAGI-CCR5 cells (Fig. 3). The greatest inhibitory effect was detected at 0.1 nM (80%) and the EC_{50} was approximately 10^{-11} M. Peptide T (10^{-10} M) was as efficacious as MIP-1β (100 ng/ml, 1.4 nM) in suppressing HIV-1_{BaL} entry. HIV-1_{BaL} has been previously shown to infect macrophages via CCR5 (Choe et al., 1996; Deng et al., 1996) and in controls for the experiments here fails to infect MAGI cells, which lack the CCR5 receptor (Table 2, Fig. 3). A selective effect of peptide T on CCR5-tropic HIV-1 isolates was apparent as peptide T (0.1–10 nM) inhibited only slightly the laboratory adapted CXCR4 isolates IIIB (17%) and MN (32%) in MAGI cells (Table 2).

We examined the apparent selectivity of peptide T (10^{-9} M) inhibition further by testing several early passage patient isolates in MAGI-CCR5 (Fig. 4A) and MAGI (Fig. 4B) cells using different doses of viral inocula. Peptide T inhibited

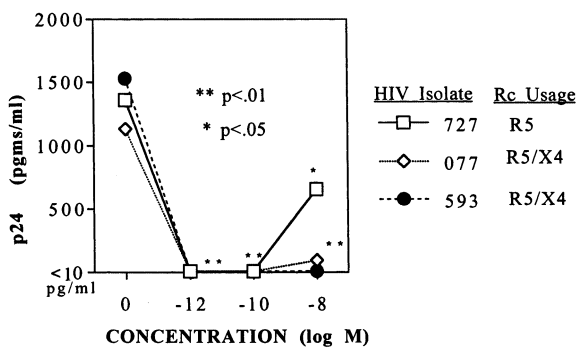

Fig. 2. Peptide T inhibits infection of primary early passage HIV-1 isolates in MDMs. MDMs cultured for 7 days were treated with peptide T as indicated for 1 h prior to infection with the following primary, early passage HIV-1 isolates for 90 min: HIV-1 92US727, 20.3 ng/ml p24; HIV-1 92US077, 23 ng/ml p24; and HIV-1 92HT593, 57.1 ng/ml p24. Virus production was quantified by p24 ELISA of supernatant samples at day 7 post-infection. Data are the means of duplicate determinations. Statistical analysis was by *t*-test (**, $P \leq 0.01$; *, $P \leq 0.05$).

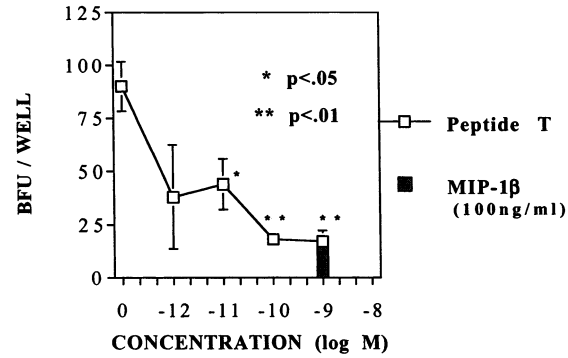

Fig. 3. Entry inhibition by peptide T on MAGI-R5 expressing cells. To determine chemokine receptor subtype sensitivity of peptide T, MAGI-CCR5 cells containing the HIV-LTR-β gal, were treated with vehicle only (control), peptide T or MIP-1β (1.4 nM), as indicated, and infected with HIV-1_{BaL}, a CCR5-tropic isolate. Infection in this system is CCR5 dependent and is considered a measure of receptor-sensitive viral entry. Data are expressed as blue focus-forming units (BFU)/culture well and are the mean and s.e.m. of triplicate determinations. Statistical analysis was by *t*-test (**, $P < 0.01$; *, $P < 0.05$), for peptide T vs. vehicle treated cultures.

($P < 0.01$) at all viral doses and showed greater magnitude of inhibition for R5 isolates compared to dual-tropic R5/X4 isolates. The specificity of the assay is shown in that the R5 isolates BaL and 92US727 did not infect MAGI cells which express only the CXCR4 receptor.

The differential sensitivity of CCR5 vs. CXCR4 chemokine receptor utilization was confirmed by infection of primary $CD4^+$ T cells with laboratory adapted HIV isolates of defined receptor utilization. Cells were treated with doses of peptide T as indicated and infected with the CXCR4 viruses, MN or NL4-3, the CCR5 tropic BaL and the dual tropic 92HT596 and 92HT593 isolates. Results in Table 3 show that peptide T had little to no inhibitory activity on the CXCR4 adapted laboratory isolates MN or NL4-3. Two early passage dual-tropic viruses showed varied sensitivity to peptide T inhibition, from slight (24%) to substantial (94%). Interference with CCR5 has previously been shown to efficiently inhibit the replication of some dual tropic HIV-1 strains, both in lymphoid tissue (Glushakova et al., 1999) and PBMC's (Ghezzi et al., 2001) which therefore may be more similar to a CCR5 tropic virus in terms of entry.

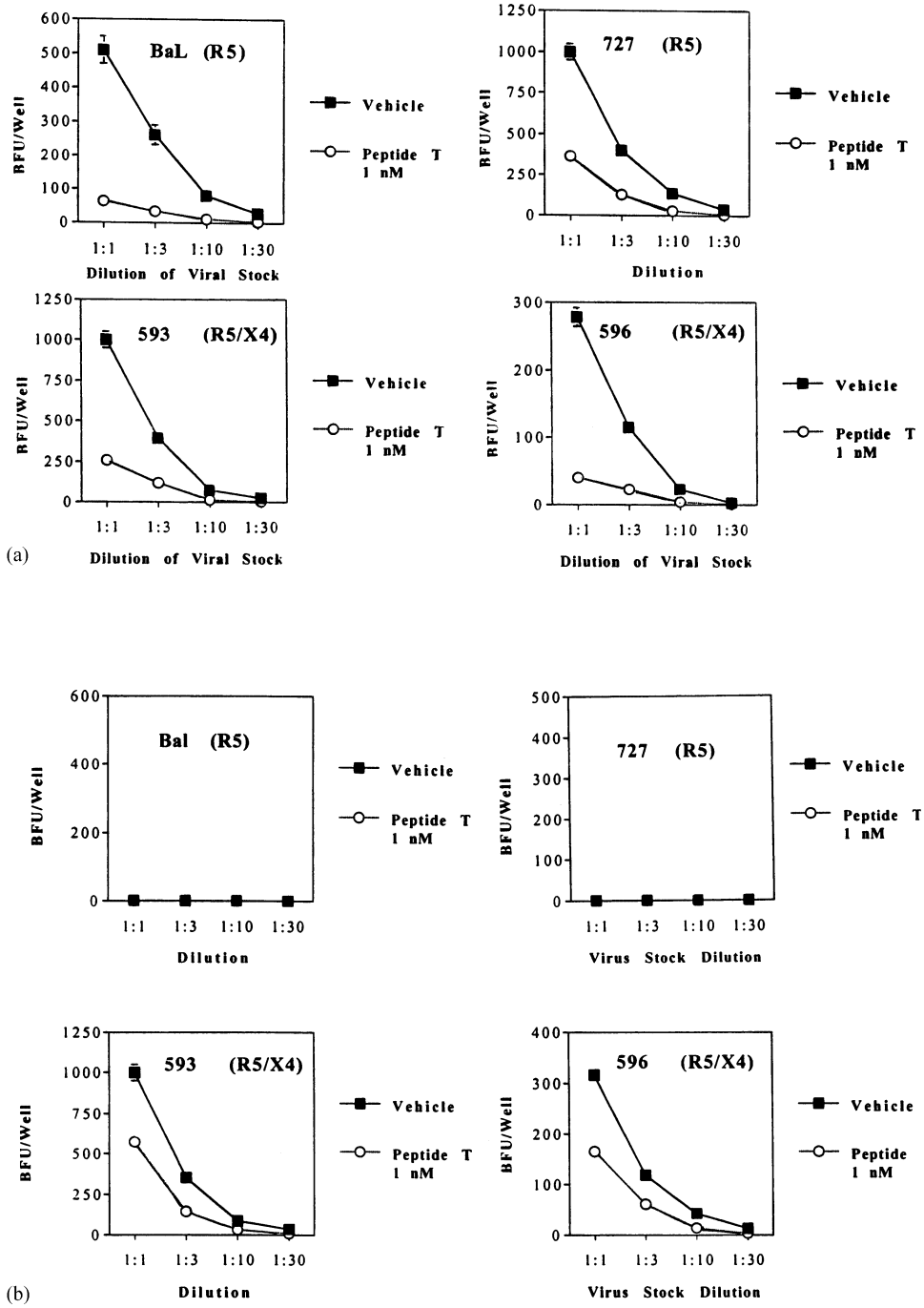

Fig. 4. Peptide T preferentially inhibits entry of R5 early passage isolates. MAGI-CCR5 (A) and MAGI (B) cells were infected with the indicated dilutions of viral stocks (see Section 2 for p24 concentrations) in the presence of 10^{-9} M peptide T or vehicle as control. The HIV-1 isolates are indicated in each panel, with additional details in Section 2. The corresponding chemokine receptor utilization is indicated above the legend. Other details are as described in Fig. 3 and Section 2. Data presented are the mean and s.e.m. of triplicate determinations. Statistical analysis was by t-test and peptide T (1 nM) resulted in significant inhibition ($P < 0.01$) in the six panels where an infection was established.

3.4. Effect of peptide T on infection of microglial cells

It was of interest to determine if peptide T suppression of HIV could be demonstrated for other cells of monocytic origin, such as microlgia, which are significant sources of virus production in the brain. We therefore tested the suppressive effect of peptide T on microglia infected with recombinant luciferase-encoding HIV particles pseudotyped with M-tropic (ADA) env, a strain that uses CCR5 as the major fusion co-receptor for entry. Microglial cultures prepared from human fetal brain tissues were treated with peptide T at 10^{-12} and 10^{-10} M prior to infection with pseudotyped virions. After 3 days cells were lysed and luciferase activity, a measure of viral entry, determined. The results, Fig. 5, show that peptide T was highly suppressive, causing a 96% reduction in virus production at low concentration (10^{-12}) in yet a different CCR5 dependent model, sensitive to entry inhibitors.

4. Discussion

Peptide T had potent anti-viral effects in several different systems. In cultured MDMs the magnitude of suppression by peptide T varied depending on viral isolate, but nearly 2 $\log_{10}$ (Table 1), or greater (Fig. 2), decrements in viral antigen production were detected at a concentration of 10^{-12} M. Peptide T was active in suppressing early passage patient isolates, which use either of the two major co-receptors R5/X4, in their natural target cells, macrophages (Fig. 2) and $CD4^+$ lymphocytes (Table 3). Peptide T was also active in two different reporter systems which detect entry inhibitors, MAGI/MAGI-CCR5 cell lines with expressed chemokine receptors, or a test which used virions pseudotyped with the R5 envelope from HIV-1_{ADA} to infect microglia.

A dose response was evident, with peak anti-viral activity occurring over a range from 10^{-12} to 10^{-9} M (Figs. 1–5, Table 1). The inhibitory effect was often bi-phasic (Figs. 1, 2 and 5). An explanation for this effect is a ligand–ligand interaction has been recently reported for heterodimers of MIP-1α and MIP-1β (Ennan Guan et al., in press). We have observed 'laddering' of peptide T on SDS gels and circular dichroism studies confirm multimerization. Receptor desensitization at higher doses may also contribute to this effect.

The inhibition of macrophage tropic isolates, both the laboratory adapted viruses BaL, and ADA, as well as early passage clinical isolates in

Table 2
Minimal effect of peptide T on 4 isolates in the MAGI cell reporter entry assay

Cells	Virion	Treatment	BFU per well/s.e.m.	% Inhibition
MAGI	IIIB	None	209/16	
	IIIB	DAPTA (10^{-10} M)	185/5	11 ($P \leq 0.05$)
	IIIB	DAPTA (10^{-9} M)	172/10	18 ns
	IIIB	DAPTA (10^{-8} M)	173/8	17 ($P \leq 0.05$)
	MN	None	50/5	
	MN	DAPTA (10^{-10} M)	73/8	0 ns
	MN	DAPTA (10^{-9} M)	34/6	32 ($P \leq 0.05$)
	MN	DAPTA (10^{-8} M)	43/3	14 ns
	BaL	None	2/0.8	–
	None	None	2/0.3	–

To determine chemokine receptor subtype sensitivity of peptide T, MAGI cells, which express CD4 and CXCR4 with the HIV-LTR-β galactosidase gene, were treated with peptide T for 1 h and infected with the lab adapted X4-tropic viruses IIIB or MN (0.03 ml of undiluted viral stock) for 2 h. Cells were cultured for a further 46 h and blue focus-forming units (BFU) counted. Data are the means and s.e.m. of triplicate cultures. Statistical analysis was by *t*-test, ns was not significantly different from vehicle treated.

Table 3
Peptide T did not inhibit lab adapted X4 HIV-1, but did inhibit early passage R5/X4 (dual-tropic) viruses in primary CD4$^+$ T cells

Virion (Rc usage)	Treatment	p24 (pg/ml)/s.e.m.	% Inhibition
MN (X4)	None	2270/385	–
MN (X4)	DAPTA (10^{-10} M)	2243/216	0
MN (X4)	DAPTA (10^{-8} M)	2074/426	9
NL4-3 (X4)	None	3760/129	–
NL4-3 (X4)	DAPTA (10^{-10} M)	3740/287	0
NL4-3 (X4)	DAPTA (10^{-8} M)	4010/363	0
593 (R5/X4)	None	2216/212	–
593 (R5/X4)	DAPTA (10^{-10} M)	1675/519	24
593 (R5/X4)	DAPTA (10^{-8} M)	1722/186	22
596 (R5/X4)	None	2412/174	–
596 (R5/X4)	DAPTA (10^{-10} M)	140/66	94
596 (R5/X4)	DAPTA (10^{-8} M)	574/122	76

Purified CD4$^+$ T cells were activated with PHA and cultured in IL-2 containing media for 3 days prior to treatment with peptide T and infection with HIV-1 isolates with varied receptor tropism, as indicated. P24 levels were determined at day 7 post-infection and are the means of replicate cultures. Peptide T was partially suppressive for 92HT593, highly suppressive for the 92HT596, but did not inhibit the R4-tropic laboratory isolates MN and NL4-3.

MDMs (Table 1, Fig. 1) or microglia (Fig. 5), suggested CCR5 as a receptor target. The ability to show receptor-mediated anti-viral effects would be expected to be highly sensitive to the amount of virus and number of viral receptors in each of the methods. Not surprisingly, peptide T inhibition was most easily detected at lower input virus multiplicities (Figs. 2 and 3) and was more potent as an anti-viral in primary cultured monocytes, microglia, or CD4$^+$ cells (Tables 1 and 3, Figs. 2 and 5) transfected MAGI-CCR5 cells (Figs. 3 and 4) which express higher receptor levels.

Peptide T acted at an early step. Inhibition of BaL, as well as several early passage R5 or R5/X4 isolates in the MAGI/MAGI-CCR5 cell assay (Table 2, Figs. 3 and 4) was shown and this test is considered to be sensitive to entry inhibitors (Pirounaki et al., 2000).

The specificity of peptide T action for either of the two major HIV-1 co-receptors, CCR5 (Choe et al., 1996) and CXCR4 (Feng et al., 1996; Bleul et al., 1996) was determined. Our studies using viruses with different chemokine receptor specificities and different cellular targets (MDMs, MAGI-CCR5, microglia), suggest that the chemokine receptor CCR5 is a preferential target of peptide T antiviral effect. This conclusion could be confirmed since peptide T blocked BaL infection of MAGI-CCR5 cells (Figs. 3 and 4), with peak inhibitory effect at 0.1 nM (80%). The anti-viral effects of peptide T were not limited to R5 isolates as early passage R5/X4 isolates which infected MAGI cells via the CXCR4 receptor were sensitive to peptide T inhibition (Fig. 4B),

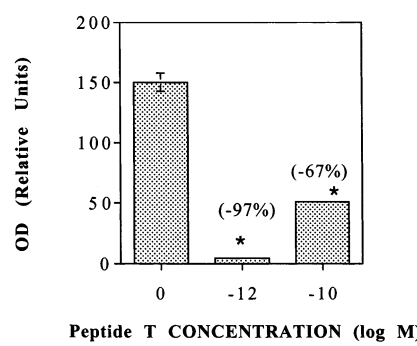

Fig. 5. Luciferase reporter assay for entry/early infection inhibition by peptide T in microglia. NL4-3 Luc-R-E virus pseudotyped with HIV-1_{ADA} was used to infect brain-derived microglia treated with peptide T at 10^{-10} or 10^{-12} M or vehicle only (control). At 72 h postinfection, the cells were lysed and luciferase activity was determined spectrophotometrically. The percent inhibition by peptide T is indicated. The mean and s.e.m. of duplicate determinations is presented (*, $P < 0.05$).

but less so than via the CCR5 pathway (Fig. 4A), again confirming a selective action of peptide T for R5 compared to X4 mediated infection.

Infection of primary $CD4^+$ T cells by dual-tropic clinical isolates was also inhibited by peptide T (Table 3), however little or no inhibition was observed with laboratory adapted X4 isolates such as IIIB, MN, or NL4-3 as tested on MAGI cells (Table 2), or in direct infections of purified primary $CD4^+$ T cells (Table 3). The failure of peptide T to act on HIV isolates adapted to grow in cultured cell lines compared to patient isolates grown in primary cells further explains (Ruff et al., 1987a) the failure to find peptide T inhibition using cell free supernatants from infected H9IIIB cells (Sodroski et al., 1987) and reveals subtle differences in chemokine receptor utilization by HIV isolates which are not well understood.

A direct effect of peptide T on CCR5 seems the most plausible explanation for the observed specificity of anti-viral effects. Although the neuroprotective effects of peptide T in brain cell cultures may be due to release of β chemokines (Brenneman et al., 1999), peptide T neither induced the release of the anti-viral β-chemokines RANTES, MIP-1α, or MIP-1β (Cocchi et al., 1995) from day 7 cultured MDMs after 2 or 24 h treatment (data not shown) or $CD4^+$ T cells, nor substantially down-modulated MDM or CD4 chemokine receptors CCR5 or CXCR4.

The conservation of a family of related, bioactive, short peptides in V2 (discussed in Ruff et al., 1987a(Ruff et al., 1987b), which, in addition to blocking infectivity also antagonize gp120 neurotoxic (Brenneman et al., 1988a,b) and growth hormone suppressing effects (Mulroney et al., 1998), as well as blocking gp120 signaling (Zorn et al., 1990; Liapi et al., 1998), and β-chemokine chemotaxis (Redwine et al., 1999; Raychaudhuri et al., 1998) is suggestive of an important receptor mediated function contributed by this site in *env*. Our earlier studies of CD4 dependent, direct ^{125}I-gp120 (Pert et al., 1986) or ^{3}H-peptide T (Ruff et al., 1987b) binding support a role for the peptide T site in *env* receptor interactions. The active, synthetic, peptide T analogs have been shown to have a stable β-turn (Picone et al., 1988; Cotelle et al., 1990) which is required for receptor potency, although it is unknown whether these peptides fully recapitulate a functional *env* structure.

While most discussion of the determinants of CCR5 tropism implicate the V3 region (Hwang et al., 1991; Simmons et al., 2000), or other sites (Rizzuto and Sodroski, 2000), and exclude a binding role for V2 based upon continued infectivity, albeit of lower efficiency, of T cell line adapted X4 virus with deletions in the V1/V2 region (Cao et al., 1997), other evidence supports a role for V2 in co-receptor binding (Groenink et al., 1993; Koito et al., 1994; Labrosse et al., 2001). Substitutions in the peptide T region of V2 allow envelope proteins to assume a conformation competent for CD4-independent CCR5 binding (Kolchinsky et al., 1999; Shieh et al., 2000) suggesting an *env* interaction with CCR5 mapping, in part, to the V2/peptide T site. Variability may enhance viral immune escape providing a survival advantage, while preserving receptor interaction.

In addition to possible direct effects of peptide T via CCR5 binding (Redwine et al., 1999), indirect effects involving functionally, or even physically interacting G-protein coupled receptors (GPCRs) may occur (Gines et al., 2000; Li et al., 2001). GP120 shares sequence homology to the GPCR ligands vasoactive intestinal peptide (VIP) and growth hormone releasing hormone (GHRH) via the peptide T/V2 sequence (Brenneman et al., 1988b; Mulroney et al., 1998) and low doses of *env* proteins antagonize the action of both these peptides at their cognate receptors, results which have relevance to the pathogenesis of dementia and wasting (Brenneman et al., 1988a; Zorn et al., 1990; Mulroney et al., 1998). Receptor cross-regulation (Richardson et al., 2000), by which interacting receptors control physiology, may underlie the various effects of peptide T on chemokine receptors, including effects on gp120 and chemokine binding and signaling. Additional studies will be required to clarify the details of how these receptors may interact to modulate one another.

Macrophages act as major reservoirs of HIV in the body (Levy, 1993) and microglia are the main source of virus in the brain (Koenig et al., 1986; Johnson et al., 1996; He et al., 1997), whose pathogenic secretory products cause neuro-AIDS (Pert et al., 1988; Price et al., 1988; Hill et al.,

1993; Xiong et al., 2000). The majority of blood isolates from either asymptomatic patients or those with AIDS (Li et al., 2001), as well as brain isolates (He et al., 1997), use only CCR5 for entry. Macrophages also form a long-lived reservoir of non-latent virus which is difficult to treat (Sonza et al., 2001). The anti-viral activity of peptide T on macrophage infection, shown here for the first time, may have treatment relevance.

Although current HIV combination therapies have proven to be highly effective, there is a need to identify new targets against HIV as a result of drug-resistant viruses that have emerged and the side effects associated with the long-term use of current therapies (Kilby et al., 1998; Simmons et al., 1997; Trkola et al., 2001; Baba et al., 1999). Drugs which suppress CCR5-using isolates of HIV-1 which work by different mechanisms may prove useful in combination therapy as epidemiological studies show greater survival in persons with non-functional chemokine CCR5 receptors (Huang et al., 1996; Dean et al., 1996). The development of non-toxic treatment modalities, like peptide T (Kosten et al., 1997; Heseltine et al., 1998), may be useful to provide therapeutic options during structured treatment interruptions (Kolchinsky et al., 1999) should this regimen prove useful. Two placebo-controlled studies of peptide T for neuro-AIDS have shown cognitive improvements with no significant toxicities (Kosten et al., 1997; Heseltine et al., 1998). Peptide T's effects on patient virus levels have not been similarly well studied but are the subject of an ongoing clinical evaluation.

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