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DIFFERENTIAL EFFECTS OF A HYDROPHOBIC TRIPEPTIDE ON HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)-INDUCED SYNCYTIUM FORMATION AND VIRAL INFECTIVITY

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The synthetic hydrophobic peptide, Z-D-Phe-L-Phe-Gly, was shown previously to inhibit the infectivity of paramyxoviruses and the fusion of Sendai virus with liposomes. We examined the ability of this peptide to inhibit HIV-1 infectivity in A3.01, Sup-T1, and H9 cells and syncytium formation between these cells and chronically infected H9 cells. Although the peptide inhibited syncytium formation in a dose-dependent manner, its effect on virus infectivity was very limited. Our results suggest that the mechanisms of interaction of the HIV-1 envelope glycoprotein gp120/gp41 with the target cell membrane leading to membrane fusion may be different in cell-cell and virus-cell fusion. © 1995 Academic Press, Inc.

Oligopeptides with amino acid sequences similar to those of the hydrophobic N-termini of paramyxovirus F_1 or the orthomyxovirus HA_2 fusion proteins were shown previously to be specific inhibitors of cellular infection by measles, Sendai, SV5 and influenza A viruses [1,2]. The peptide Z-D-Phe-L-Phe-Gly inhibited plaque formation by measles virus in CV-1 cells at a 50% effective concentration of 0.2 μM. This peptide also inhibited the fusion of phospholipid vesicles induced by acidic pH and the fusion of Sendai virus with liposomes, although at much higher concentrations [3], as would be expected from the relative inefficiency of the peptide in inhibiting the infectivity of Sendai virus [2]. Nevertheless, the relative potency of inhibition of these fusion reactions by a series of peptides was the same as that of cellular infectivity.

Human immunodeficiency virus type 1 (HIV-1) predominantly utilizes the T-cell surface antigen CD4 as a receptor for infection of susceptible cells (reviewed in [4]). Binding of the viral

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envelope glycoprotein gp120 to CD4, is thought to induce conformational changes in both molecules, resulting in the interaction of the hydrophobic N-terminus of gp41 with the target cell membrane, followed by pH-independent membrane fusion [5-8]. HIV-infected cells can also undergo fusion with uninfected $CD4^+$ cells [9,10]. Here we have investigated whether the peptide Z-D-Phe-L-Phe-Gly could inhibit the infection of $CD4^+$ T-cell lines by HIV-1, and HIV-1-induced syncytium formation.

MATERIALS AND METHODS

Compounds: Carbobenzoxy-D-Phe-L-Phe-Gly (Z-D-Phe-L-Phe-Gly) was purchased from Sigma (St Louis, MO). Recombinant soluble CD4 (sCD4) was provided by Genentech (South San Francisco, CA).

Cells and viruses: A3.01 cells were obtained from T. Folks (Centers for Disease Control, Atlanta, GA), Sup-T1 cells from J. Hoxie, and H9 cells from R. C. Gallo (both through the AIDS Research and Reference Reagent Program, NIAID, NIH, Bethesda, MD. H9/HTLV-IIIB cells were kindly provided by J. Mills (San Francisco General Hospital). Viral p24 antigen was determined in cell-free supernatants by the antigen capture assay described previously [11]. All cell lines were maintained at 37°C, under 5% CO_2 in Medium A consisting of RPMI 1640 medium (Irvine Scientific, Santa Clara, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), penicillin (50 units/ml), streptomycin (50 μg/ml) and L-glutamine (2 mM). The cells were split 1 : 6 and fresh medium was added every 3 to 4 days. The virus and infected cells were handled in a BSL-3 facility. HIV-1_{LAI} was provided by F. Barré-Sinoussi (Pasteur Institute, Paris), propagated in human lymphoblastoid leukemia (CEM) cells, purified as described [12] and stored at -70°C. This preparation (provided by M. Jennings, Department of Medical Pathology, University of California, Davis) was propagated in A3.01 cells. The supernatant of chronically infected H9/HTLV-IIIB cells was used as a source of the HIV-1_{IIIB} isolate. The culture supernatants were harvested at times of peak p24 production, filtered through Millipore filters of 0.22 μm pore diameter, and stored in 1 ml aliquots at -80°C. The reverse transcriptase activity of the virus stock solutions was 2.5 x 10^6 and 8.4 x 10^4 cpm/ml for HIV-1_{LAI} and HIV-1_{IIIB}, respectively, as detected by the method of Hoffman *et al.* [13]. The p24 concentrations of the two isolates were 2.6 μg/ml for HIV-1_{LAI} and 0.11 μg/ml for HIV-1_{IIIB}. The tissue culture infectious dose, 50% endpoint ($TCID_{50}$), was determined as described by Johnson and Byington [14]. The infectious titer per 100 ng p24 antigen was: 4.6 x 10^5 $TCID_{50}$ (both on A3.01 and H9 cells) for HIV-1_{LAI} and 1.2 x 10^4 $TCID_{50}$ (on H9 cells) for HIV-1_{IIIB}.

Assay for CD4-dependent HIV-1-induced cell fusion: Syncytium formation was evaluated by inverted phase contrast microscopy at 25 x magnification [15]. After 16-20 h of co-cultivation in Medium A, syncytium formation had proceeded to its full extent and cell fusion was typically extensive enough to warrant a 3+ score. A score of 4+ was assigned to numerous large syncytia with a low number of viable cells, and 5+ to numerous syncytia and no viable cells. Diluents alone served as a negative control; and sCD4, known to block HIV-1-induced syncytium formation, served as a positive control for inhibition of cell fusion. The hydrophobic peptide was first dissolved in methanol, and further dilutions were prepared in Medium A. This residual amount of methanol did not affect syncytium formation or HIV-1 infectivity (data not shown).

HIV infection of cells: Cells (2 x 10^7/ml) were exposed to HIV-1 for 2 h at 37°C, washed three times to remove unbound virus, resuspended at 0.5-1 x 10^6 cells/ml in Medium A and cultured in 24-well culture plates (2 ml per well). Viral inocula were standardized by their p24 content and cultures were infected at a concentration of 2 or 4 ng p24 per 1 x 10^6 cells, as described in the figure legends. Where indicated, cells were infected in the presence of the peptide, then washed three times and cultivated further in the absence of antibody. The peptide was present *only* during the initial exposure of cells to the virus, therefore the level of p24 antigen determined in the culture medium several days later is a measure of the effect of the peptide on the initial infection by cell-free virus, i.e. virus-cell fusion. Since the cells were infected at very low MOI (see figure legends) and subsequently washed, the measured p24 values reflect *de novo* viral replication, and not any input virus. Control cells were treated similarly but not exposed to the

virus. Every 2, 3 or 4 days, 1 ml of the supernatant was removed and fresh medium was added. The p24 content of the harvested medium was measured by ELISA [11].

Detection of CD4: The presence of cell surface CD4 was examined by direct immunofluorescence assay, using fluorescein isothiocyanate (FITC)-conjugated Leu3 antibody (Becton-Dickinson), with FITC-conjugated mouse IgG as a control. The fluorescence distribution was analyzed with a flow cytometer (FACScan; Becton-Dickinson, Mountain View, CA).

RESULTS

Inhibition of HIV-1-induced syncytium formation by Z-D-Phe-L-Phe-Gly: We investigated the effect of the peptide on syncytium formation between chronically infected H9/HTLV-IIIB cells and three uninfected CD4+ T-cell lines, A3.01, Sup-T1 and H9. To characterize the inhibitory effect of the peptide on cell-cell fusion under the conditions used in the infectivity studies, we performed the assay in complete medium. The peptide inhibited syncytium formation with all three cell lines in a concentration-dependent manner (Figs. 1A, 2A, and 3A). Although syncytium formation was more efficient with Sup-T1 cells (score 5+), than with A3.01 and H9 cells (score 4+) in the absence of the peptide, the inhibitory activity of the peptide was comparable.

The effect of Z-D-Phe-L-Phe-Gly on infection by cell-free virus: We examined whether the inhibitory effect of the peptide Z-D-Phe-L-Phe-Gly on HIV-mediated cell-cell fusion

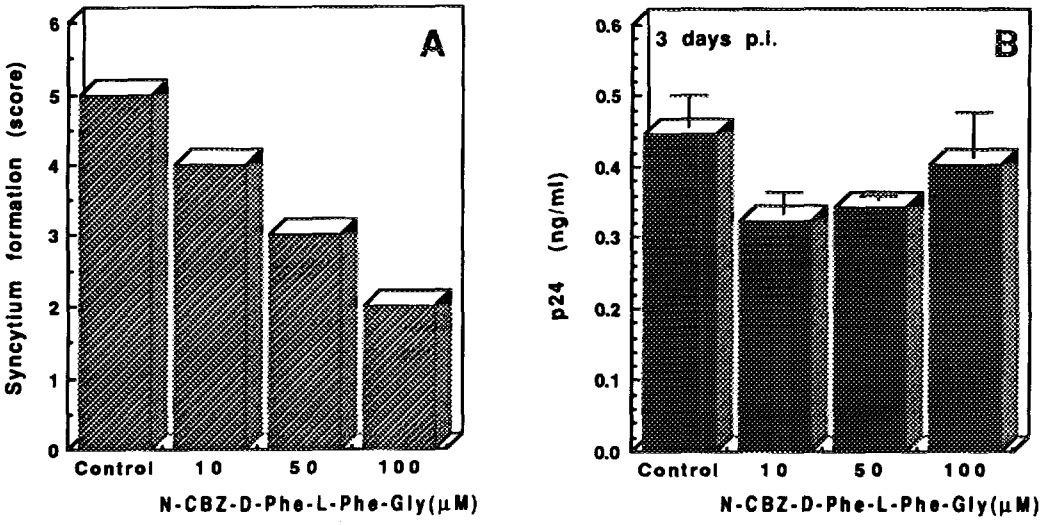

Figure 1. Effect of Z-D-Phe-L-Phe-Gly on syncytium formation between Sup-T1 and H9/HTLV-IIIB cells (A) and HIV-1_{LAI} replication in Sup-T1 cells (B). (A) Appropriate dilutions of peptide in 200 μl of complete medium were added to wells containing uninfected Sup-T1 cells, and chronically infected H9/HTLV-IIIB cells were then added. After 16 h of cocultivation in the continuous presence or absence of peptide, syncytium formation was scored (score 1+ = 1). (B) Sup-T1 cells were exposed to HIV-1_{LAI} at 2 ng p24 per 10^6 cells (at an MOI of 0.009), in the presence of the peptide as described in Materials and Methods. p24 antigen was determined in cell culture supernatant on day 3 post-infection. Data represent the mean ± standard deviation of p24 determinations in duplicate, in supernatants of two replicate wells (n=4).

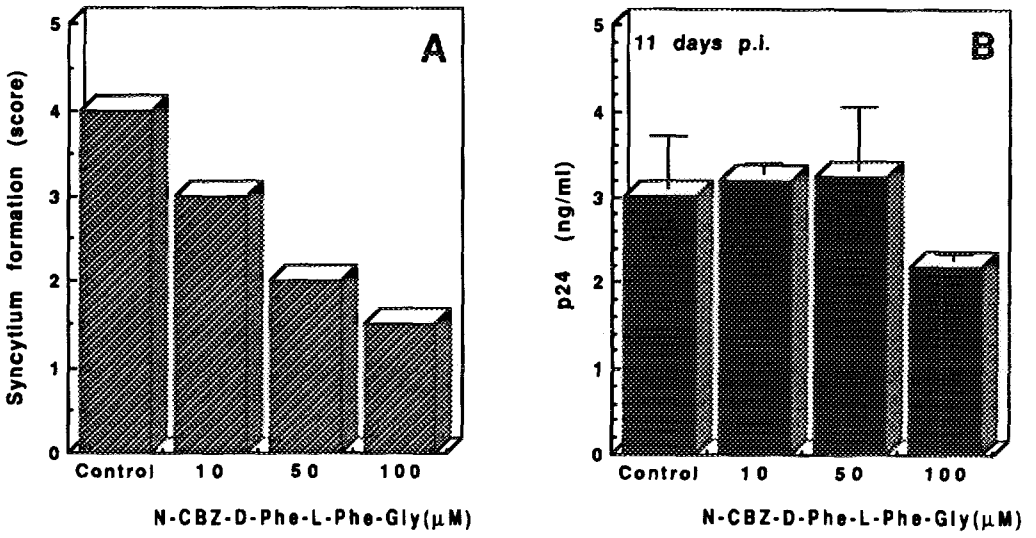

Figure 2. Effect of Z-D-Phe-L-Phe-Gly on syncytium formation between A3.01 and H9/HTLV-IIIB cells (A) and HIV-1_{IIIB} replication in A3.01 cells (B). (A) Appropriate dilutions of peptide in 200 μl of complete medium were added to wells containing uninfected A3.01 cells, and H9/HTLV-IIIB cells were then added. (B) A3.01 cells were exposed to HIV-1_{IIIB} at 4 ng p24 per 10^6 cells (at an MOI of 0.0004), in the presence of peptide as described in Materials and Methods. p24 antigen was determined in cell culture supernatant on day 11 post-infection. Other details were as in Figure 1.

was also exerted on infection by cell-free virus (virus-cell fusion). A3.01, Sup-T1, or H9 cells were infected with HIV-1_{LAI} or HIV-1_{IIIB} in the absence or presence of the peptide. FACS analysis indicated that 93%, 98%, and 59% of A3.01, Sup-T1, and H9 cells, respectively, were $CD4^+$. The background-subtracted mean fluorescence was 99, 140, and 43 (arbitrary units), for A3.01, Sup-T1, and H9 cells, respectively. The effect of the peptide on HIV-1 infectivity in these cells was very limited, and did not appear to have a uniform concentration dependence. When Sup-T1 cells were infected in the presence of 10, 50, and 100 μM peptide, the production of p24 antigen in the culture supernatant was inhibited by 29% (the difference from the control was statistically significant; $p \leq 0.025$), 25.4% (significant; $p \leq 0.005$), and 11.1% (not significantly different from the untreated control; $p \leq 0.375$), on day 3 post-infection, respectively; after 5 days the inhibition was 32.2%, 45.2%, and 28.6%, respectively (Fig. 1B and data not shown). The peptide at 10 and 50 μM did not affect viral p24 production in A3.01 cells determined on day 11 post-infection (Fig. 2B). At 100 μM, Z-D-Phe-L-Phe-Gly reduced the infectivity of the virus by 27% (statistically significant; $p \leq 0.025$). The peptide inhibited the infectivity of the virus in H9 cells to a slightly greater extent. The amount of p24 produced by day 7 post-infection was reduced by 30% ($p \leq 0.025$, compared to controls), 40% ($p \leq 0.005$), and 20% ($p \leq 0.05$), at 10, 50, and 100 μM, respectively (Fig. 3B).

DISCUSSION

Although the molecular mechanisms of HIV-1-cell fusion and HIV-1-induced cell-cell fusion are generally presumed to be similar, some differences between the molecular requirements

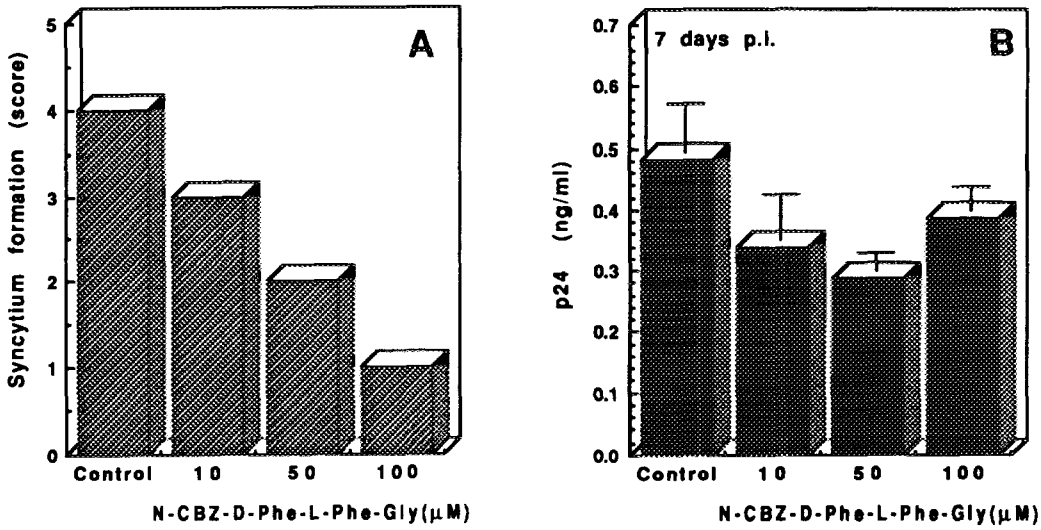

Figure 3. Effect of Z-D-Phe-L-Phe-Gly on syncytium formation between H9 and H9/HTLV-IIIB cells(A) and HIV-1_{IIIB} replication in H9 cells (B). (A) Appropriate dilutions of peptide in 200 μl of complete medium were added to the wells containing uninfected H9 cells, and H9/HTLV-IIIB cells were then added. (B) H9 cells were exposed to HIV-1_{IIIB} at 4 ng p24 per 10^6 cells (at an MOI of 0.0004), in the presence of peptide as described in Materials and Methods. p24 antigen was determined in cell culture supernatant on day 7 post-infection. Other details were as in Figure 1.

of viral entry and syncytium formation have been reported [16-21]. We previously reported that while a monoclonal antibody raised against the complex of soluble CD4 and gp120 (mAb F-91-55) inhibits syncytium formation between CD^+ T-cell lines and chronically HIV-infected cells, it has only a limited effect on virus infectivity [22]. The experiments reported here indicate that the peptide Z-D-Phe-L-Phe-Gly inhibits syncytium formation in a dose-dependent fashion, but its effect on viral infectivity is minimal and not apparently dose-dependent. In the infectivity experiments the peptide was present only during the initial infection period; this is the crucial period for viral entry, since trypsin treatment of cells after this period does not affect the level of infection [23]. A peptide ("DP-178") derived from gp41 residues 643-678 (HIV-1_{LAI} isolate) was also found to be a more potent inhibitor of cell-cell fusion than of infection by cell-free virus, about 10 to 100 times higher concentrations being required to inhibit infection [24]. Studies on the role of different regions of the HIV envelope protein in membrane fusion have shown that virus entry is disrupted less than syncytium formation by changes in gp41 residues 566 to 596 [25]. Mutations in the N-terminal fusion domain of HIV-2 gp41 severely reduce the capacity of the virus to induce syncytia, but only slightly affect virus infectivity [26]. It is therefore likely that the membrane fusion reactions accompanying virus entry and cell-cell fusion have different molecular requirements. One possibility is that viral gp120/gp41 can interact more effectively than cellular gp120/gp41 with accessory molecules in the cell membrane that may be involved in the membrane fusion reaction [27,28]. Another consideration is that the intermembrane complexes formed by the interaction of gp120/gp41 and target cell CD4 during cell-cell fusion are much weaker and more prone to inhibition by Z-D-Phe-L-Phe-Gly, than those formed during virus-cell membrane fusion,

particularly since the surface density of the viral envelope proteins on the virus membrane is expected to be higher than on the plasma membrane.

The mechanism by which Z-D-Phe-L-Phe-Gly inhibits cell-cell fusion is not known. Yeagle et al. [29] have proposed that the peptide inhibits the formation of certain intermediate lipid structures in the fusion pathway. Longer hydrophobic peptides derived from the N-terminus of gp41 also inhibit cell-cell fusion, as well as infectivity [30]. These peptides may interact with the same sites on the target membrane that the N-terminal region of viral gp41 is thought to interact to initiate membrane fusion, thereby competitively inhibiting the fusion reaction. Determining the binding site of these peptides may help in the design of more potent inhibitors of viral infection.

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