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LYMPHOCYTES AND PROMONOCYTES ATTACH TO THE SYNTHETIC [TYR^{5,12}, LYS^{7}] - POLYPHEMUSIN II PEPTIDE

Benjamin S. Weeks[1*], Motoyoshi Nomizu[2], Akira Otaka[3], Christi A. Weston[1], Akiko Okusu[1], Hirokazu Tamamura[3], Akiyoshi Matsumoto[4], Naoki Yamamoto[5], and Nobutaka Fujii[3]

[1]*Department of Biology, Hamilton College, 198 College Hill Rd., Clinton, New York 13323*
[2]*Laboratory of Developmental Biology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892*
[3]*Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan*
[4]*SEIKAGAKU Corporation, Chuo-ku, Tokyo 103, Japan*
[5]*Department of Microbiology, Tokyo Medical and Dental University, School of Medicine, Bunkyo-ku, Tokyo 113, Japan*

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SUMMARY: The [Tyr^{5,12}, Lys^{7}]-polyphemusin II peptide (T22) has been shown to inhibit HIV-1 replication in lymphocytes. The mechanism of T22 inhibition of HIV-1 replication is not known but may involve T22 competition with HIV-1 for attachment sites on the plasma membrane of targeted cells. Here we find that three human immunocyte cell lines (H9, Jurkat, and U-937) attach to T22. The phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA), has been shown to activate intracellular protein kinase C and to stimulate lymphocyte attachment to various substrates through specific cell surface receptors. Here we find that TPA treatment enhances attachment of the immunocytes to T22 by three- to four-fold. These data demonstrate that T22 binds to immunocyte cell surfaces and support the hypothesis that T22 may inhibit HIV-1 replication by competing with the virus for a common cell surface receptor(s).

Tachyplesin I (T1) is a peptide consisting of 17 amino acids with two disulfide bridges and is derived from Japanese horseshoe crab hemocytes (1). Three additional isopeptides of tachyplesin have been found in both the Japanese horseshoe crab and the American horseshoe crab and are known as tachyplesin II (Japanse) and polyphemusin I and II (American) (2). All four peptides inhibit the growth of Gram-negative and Gram-positive bacteria and are presumed to play a role in the horseshoe crab self-defense system (1-3).

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The most compelling quality of tachyplesin and polyphemusin is their ability to inhibit the replication of the human immunodeficiency virus-1 (HIV-1) in lymphocytes (3-6). Recent research has focused on the development of tachyplesin and polyphemusin analogs which have improved anti-HIV-1 activity. Compared to T1 and fifteen additional analogs the most active peptide is T22, a derivative of ployphemusin II (5). It has been suggested that the T22 peptide interferes with a process after HIV-1 binding to the cell surface but before reverse transcription of the viral genome (6). However it is possible that T22 inhibits HIV-1 binding to the cell surface plasma membrane and prevents internalization of the virus.

Here we investigate the ability of the T22 peptide to interact with cell surface ligands on lymphocytes and promonocytes. This interaction is measured by the ability of these immunocytes to attach to T22. In addition we identify a cell binding site on the T22 peptide. We also investigate the effect of protein kinase C activation on immunocyte binding to T22. Our results indicate that T22 is bound by a cell surface receptor(s), and support the hypothesis that T22 may compete with HIV-1 for cell binding.

Materials and Methods

Synthetic peptides: All synthetic peptides used are listed in Fig. 1. Tachyplesin I (T1) and its analog peptides (T22 and D-T22) were synthesized by 9-fluorenylmethyloxycarbonyl (Fmoc) based solid phase strategy as previously described (7, 8). The branched peptides, Ac-Y16 [(Ac-YIGSRG)$_{16}$K$_8$K$_4$K$_2$KG] and YRK16 [(Ac-YRKG$_{16}$K$_8$K$_4$K$_2$KG], were manually synthesized by Fmoc strategy on low roading Wang-type resin (Fmoc-Gly-resin, 0.1 mmol/g) as previously described (9).

12-0-tetradecanoylphorbol 13-acetate (TPA) and H7: TPA and its control, 4α-phorbol 12, 13-didecanoate12,13-didecanoate (PDD) were obtained from Sigma (St. Louis, MO) and were dissolved in ethanol and stored at -80 °C at a stock concentration of 16 mM. H7 was obtained from SEIKAGAKU Co. (Tokyo, Japan) and was stored at 4 °C at 5mg/ml in ethanol.

Cells and culture: H9, U-937 and Jurkat cells (10-16) were cultured in RPMI 1640 containing 10% fetal bovine serum and pennicillin and streptomycin. For the attachment assays (see below) cells were collected, washed free of serum and placed in medium consisting of soley RPMI 1640.

Attachment assays: The peptides tested were dissolved at 1 mg/ml in distilled water. The peptides were then added to 16 mm diameter wells of a 24 well Costar tissue culture plate to which 0.5 ml of water had been added. Peptides were tested at 1, 10 and 100 μg. The water in the wells thus varied from 500 μl (blank) to 600 μl (100 μg peptide). The water was allowed to completely evaporate overnight, thereby neutralizing volume differences. Next, 2.5 x 10^4 cells in a serum free medium (see above) were plated in the wells and were incubated at 37 °C for three hours. After the attachment period the media and unattached cells were removed by aspiration. The remaining cells were fixed, stained and counted as a measure of cell attachment.

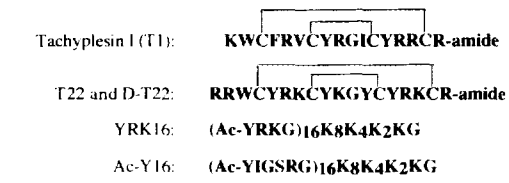

Fig. 1. List of synthetic peptides. *D-T22: all-D-configuration.

The number of cells in three fields were counted at 10x magnification and averaged. At 10x magnification, each field is 1/64 of the 16 mm diameter well. Therefore, the average number of cells per field was multiplied by 64 and then divided by the total number of cells (2.5×10^4). This number was then multiplied by 100 to give the percent of the total number of cells which attached. This number is shown in Fig. 2-4 as the measurement of cell attachment. TPA was added at the time of cell seeding at a concentration of 100 nM and the attachment assay was then allowed to proceed for the usual 3 hours before measuring attachment. H7 was tested by adding 30 mM at the time of TPA addition which, as stated, was also the time of cell seeding.

Results

The two human lymphocyte cell lines, H9 and Jurkat, and one human promonocyte cell line, U-937, attached to the T22 peptide (Fig. 2). Jurkat cells were the most adherent with approximately 30% of the cells attaching to 10 μg of T22 peptide. For H9 cells and U-937 cells 22% and 17 % attached to 10 μg of the peptide. Attachment to 10 μg of the D-configuration T22

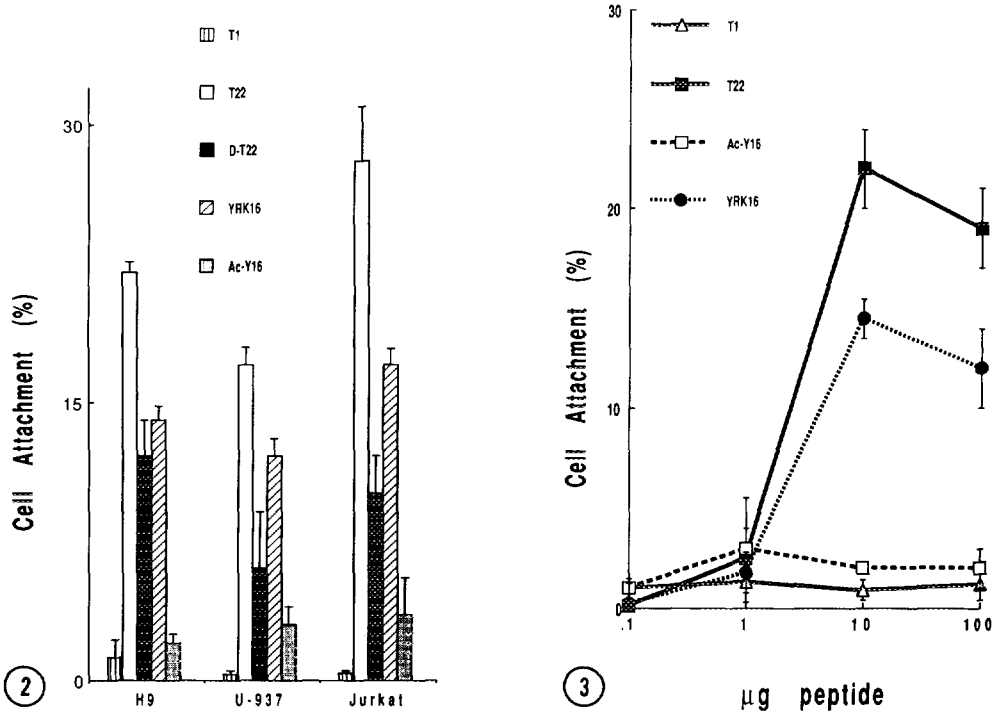

Fig. 2. Attachment of lymphocyte cell lines to the T22 and its related peptides. The percent of cells attached to the synthetic peptides was determined as described in the materials and methods. Here, 10 μg of the peptides was used to coat wells and cells were allowed to attach for three hours in a serum free medium before the unattached cells were removed and the attached cells were counted. Maximum attachment was observed with a well coating of 10 μg and at three hours (Fig. 3).

Fig. 3. H9 cell attachment to the T22, D-T22, YRK16 and Ac-Y16 at different doses. Attachment was determined under the same conditions described for Fig. 2. The laminin YIGSR sequence is an active domain for the attachment of several cell types. H9 cells are known to not attach to this domain (18).

peptide, D-T22, and T1 was tested. Both of these peptides have been shown to have less anti-HIV-1 activity than T22 (17) H9 cell attachment to D-T22 was approximately 50% of that observed for T22, while the U-937 and Jurkat cell attachment to the D-T22 was approximately three-fold less than that observed for T22. The T1 peptide also had less adhesive activity (Fig. 2). This result suggested a correlation between the anti-HIV-1 activity and the cell binding activity of the tachyplesin and polyphemusin analogs.

The T22 peptide has two YRK sequences (5). A multimeric (16mer) YRK peptide, YRK16, was prepared using the multiple antigen peptide system (7) and tested for cell attachment. All cells attached to YRK16 (Fig. 2) which indicated that this sequence is a cell attachment site on the T22 peptide. As a control multimeric peptide, a 16-mer of the laminin YIGSR cell binding domain, Ac-Y16 (9), was also tested for cell attachment. H9 cells do not attach to YIGSR (18), and here we found that neither the lymphocyte or the promonocyte cells attached to Ac-Y16 (Fig. 2).

Attachment of H9 cells to the T22 peptide was maximal at 10 μg with approximately 20% - 25% of the cells attached (Fig. 3). At 100 μg, 19% of the H9 cells to T22 while only 3% attached to Ac-Y16 (Fig. 3). Maximal attachment of H9 cells to YRK16 was also maximal at 10 μg with approximately 15 % of the cells attached (Fig. 3).

The phorbol ester, TPA has been shown to stimulate immunocyte receptor-mediated attachment to various substrates (18). Therefore, the attachment of H9 and U-937 cells to the T22 peptide in the presence of TPA was investigated here. Treatment with 100 nM TPA stimulated a three- to four-fold increase in both H9 and U-937 cell attachment to the T22 peptide (Fig. 4A). Without TPA the maximal attachment of the H9 and U-937 cells to 10 μg of T22 was approximately 22% and 17% respectively. With TPA added, 68 % of the H9 cells and 47 % of the U-937 cells attached to 10 μg of the T22 peptide (Fig. 4A). As a control for the TPA, 4α-phorbol 12, 13-didecanoate (PDD) was tested at 100 nM and this did not stimulate attachment to the T22 peptide (data not shown). Using the H9 cells, the role of protein kinase C in the TPA stimulated attachment was investigated using the protein kinase C inhibitor, H7. Co-treatment of TPA activated H9 cells with 30 μM H7 completely blocked the TPA enhanced attachment to T22 (Fig. 4B). H7 did not cause a reduction of the background attachment to T22. These results demonstrated that activation of cellular protein kinase C increases lymphocyte binding to T22 and suggested that the T22 ligand in immunocyte plasma membranes can be modified by intracellular protein kinase C activation.

Discussion

The tachyplesins and polyphemusins are peptides found in the hemocytes of the horseshoe crab (1, 2). These peptides are potent antimicrobial agents and are presumed to play a role in the horseshoe crab self-defense system. Recently, tachyplesin I and a synthetic analog, T22, was reported to inhibit HIV-1 replication (3-6). The mechanism through which T22 inhibits HIV-1 replication is unknown, but it has been suggested that T22 interferes with a process after virus

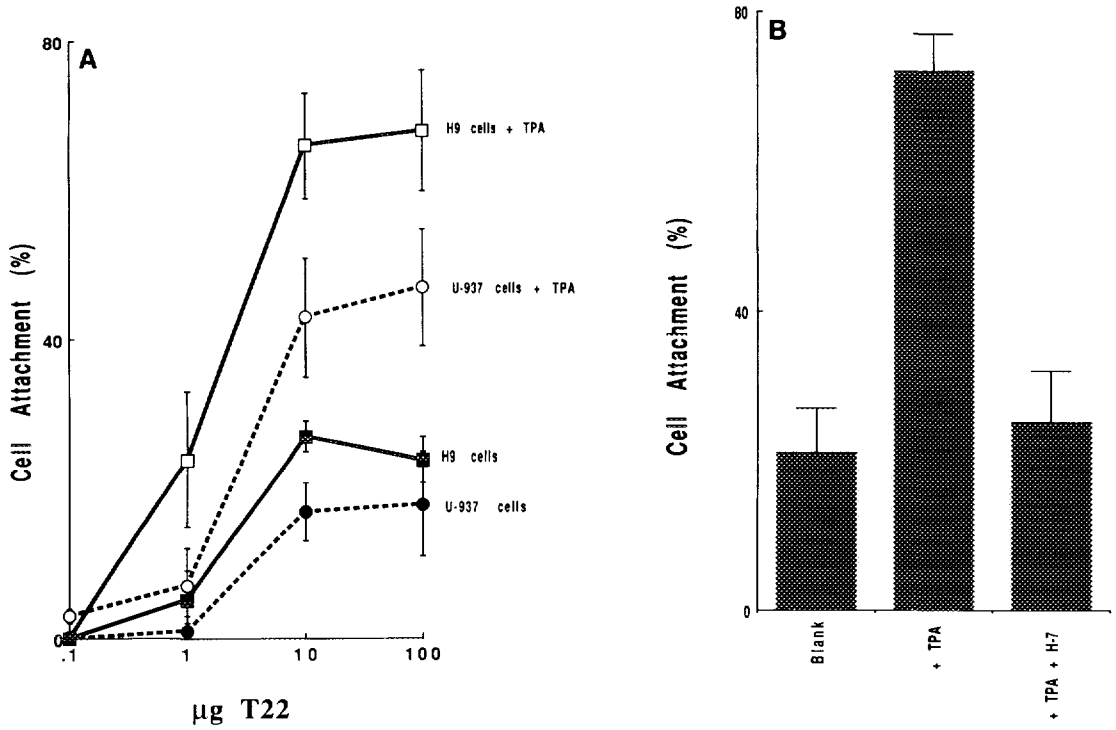

Fig. 4. (A) Treatment of lymphocyte cells with TPA increases their ability to attach to the T22 peptide. H9 and U-937 cells were treated with 100 nM TPA at the time of cell seeding and attachment was allowed to proceed for the standard three hours before the percent of attached cells was determined.
(B) The protein kinase C inhibitor blocks the TPA-induced increase in attachment to the T22 peptide. H9 cells were allowed to attach to 10 µg of the T22 peptide for three hours with 100 nM TPA, or 100 nM TPA and 30 µM H7, or with no treatments (blank). All treatments were added at the time of cell seeding.

binding to lymphocyte plasma membranes (6). However it is possible that the T22 peptide blocks HIV-1 binding to the cell surface plasma membrane thereby inhibiting infection of the cell. Alternatively, T22 may inhibit cell-cell contact and block the direct passage of the virus from infected cells to uninfected cells.

Here we investigate the ability of the T22 peptide to interact with the plasma membranes of human CD4+ lymphocyte and promonocyte cell lines, each of which can be actively infected with HIV-1 (10-16). All of the immunocyte cell lines bind to the T22 peptide. The attachment of the cells to the T22 peptide appears be mediated through the YRK sequence since these cells bind to a synthetic multimeric YRK peptide (YRK16). Activation of protein kinase C in immunocytes has been shown to stimulate cell surface receptor binding activity (18), and here, activation of intracellular protein kinase C increases immunocyte attachment to T22. This further suggests that immunocyte binding to T22 is receptor mediated.

The results of this study focus future research on the identification of the immunocyte receptor(s) for the T22 peptide. The present study also raises the possibility that T22 can compete with HIV-1, or infected cells, for a common cell surface receptor thereby blocking HIV-

1 internalization and replication. Identification of the immunocyte surface receptor for the T22 peptide will contribute to the development of peptides for the prophalaxis HIV-1 infection. Identification of the immunocyte surface receptor for the T22 peptide may also help to further elucidate the fundamental mechanisms of HIV-1 infection.

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