

# HUMAN $\alpha$ -DEFENSIN-1 AND RHESUS $\theta$ -DEFENSIN-1 INHIBIT HIV-1 REPLICATION BY DIFFERENT MECHANISMS

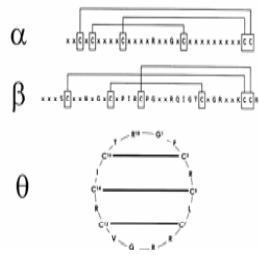
Aprille L. Matthews, Lesley R. White, Patti Tran, Michael E. Selsted, and David Camerini

University of California, Irvine, California USA

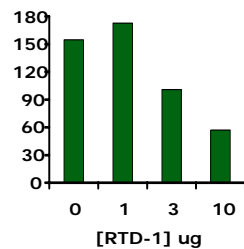
**Background:** Defensins are cationic peptides employed by the innate immune system to protect against viral, fungal, and bacterial infection.  $\alpha$ ,  $\beta$  and  $\theta$  defensins all have anti-HIV-1 activity, but the mechanisms by which they inhibit HIV-1 replication have not been fully characterized.

**Methods:** We measured loss of cell surface CXCR4 and CCR5 following incubation with HNP-1 and RTD-1 in a GHOST cell line uniformly expressing CCR5, CXCR4 and CD4. We assayed defensin effects on HIV-1 replication in GHOST and in PBMC cultures. HIV-1 infection was measured by the presence of the viral capsid protein p24 in tissue culture supernatant, by internal cell staining for p24 or by GFP expression. Defensins were incubated with cells and with virus at various times pre and post infection and at 4°C or 37°C. We used both R5 and X4 strains of HIV-1 for infections.

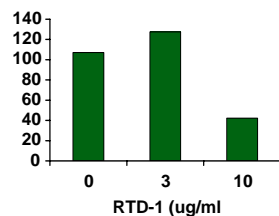
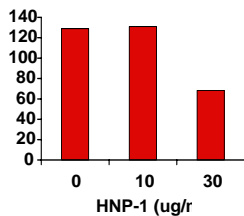
**Results:** We observed down-modulation of CXCR4 by the human  $\alpha$ -defensin, HNP-1, and the rhesus macaque  $\theta$ -defensin, RTD-1. Incubation of cells with defensins at 37°C induced a dose-dependent loss of CXCR4, but not CCR5. We also found that RTD-1 inhibited both X4 and R5 HIV-1 infection in GHOST cells in a dose-dependent manner when added at the time of infection but had no activity when added four hours later. In contrast, HNP-1 inhibited X4 and R5 HIV-1 replication in PBMC when added at the time of infection and up to sixteen hours later, but had no activity in GHOST cells. HBD-2 also inhibited R5 and X4 HIV-1 replication in PBMC. RTD-1 inhibited anti-CXCR4 MAb binding to CXCR4 indicating that RTD-1 may bind CXCR4. RTD-1 also directly inactivated X4 HIV-1 when RTD-1 was pre-incubated with HIV-1 prior to infection. HNP-1 directly inactivated both X4 and R5 HIV-1.



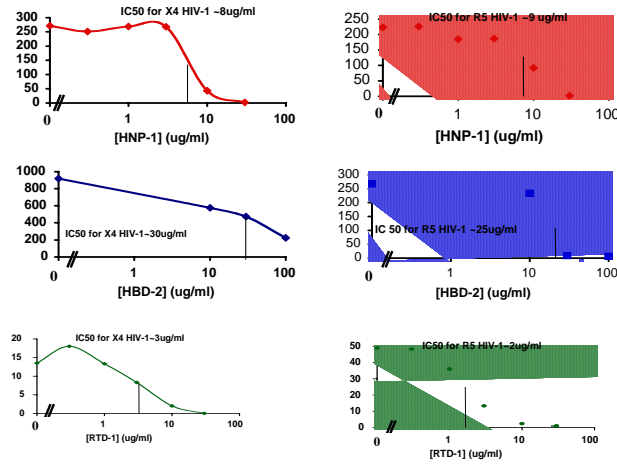
**Fig. 1.** Schematic diagrams of the three defensin subfamilies. Invariant amino acids in the  $\alpha$  and  $\beta$ -defensins are specified, and the distinguishing disulfide motifs are defined by connecting solid lines.



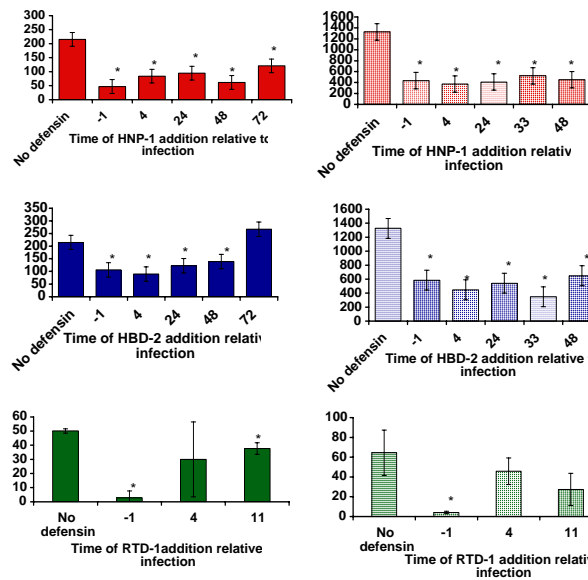
**Fig. 2.** RTD-1 blocks MAb binding to the second extracellular loop of CXCR4. RTD-1 was incubated with GHOST-R5X4 cells, at the indicated concentrations for 1 hour at 4°C in PBS + 0.02% Sodium Azide. The cells were then stained with R&D Systems MAb 44717 and analyzed by flow cytometry. RTD-1 was unable to block MAb binding to either the ECL2 or N-terminus of CCR5 (data not shown).



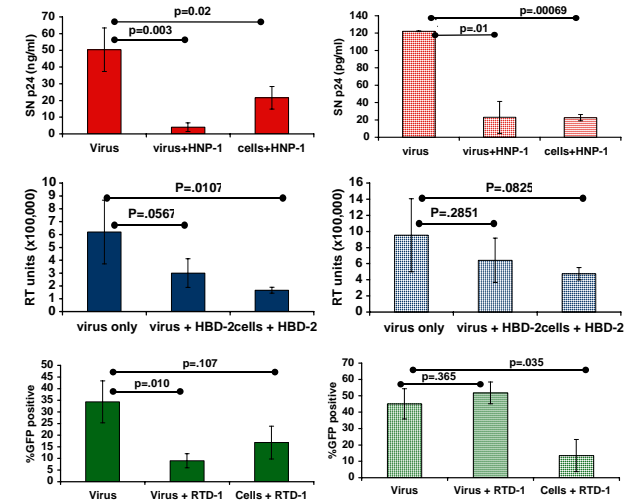
**Fig. 3.** Down-modulation of CXCR4 by HNP-1 and RTD-1. HNP-1 or RTD-1 were incubated with GHOST-R5X4 cells, at the indicated concentrations for 3 hours at 37°C, in Iscove's media without serum or other additions. The cells were then incubated with an anti-CXCR4 MAb whose binding was not affected by RTD-1 and analyzed by flow cytometry. The left panel shows HNP-1 mediated down-modulation of cell surface CXCR4, while the right panel shows the effect of RTD-1.



**Fig. 4.** IC<sub>50</sub> for HNP-1, HBD-2 and RTD-1 on X4 and R5 viruses. PHA-stimulated PBMC or GHOST R5/X4 cells were incubated for one hour at 37°C with varying concentrations of defensins. Cells were washed in PBS and then spininfected with NL4-3 (X4) or JR-CSF (R5) HIV-1 at an MOI of 0.1. GHOST cells were harvested 36 hours post infection and analyzed for GFP. Supernatant from PBMC was harvested at 72 hours and analyzed for viral capsid protein p24 by ELISA. IC<sub>50</sub> values were calculated from the resulting graphs.



**Fig. 5.** Time of addition studies with HNP-1, HBD-2 and RTD-1. HNP-1 (red bars) or HBD-2 (blue bars) were added to PHA-stimulated PBMC one hour prior to infection with NL4-3 (solid bars) or JR-CSF (checked bars) or four, twenty-four, thirty-three, forty-eight or seventy-two hours later at 10ug/ml. The results shown are from p24 ELISA of the media at seventy-two hours post infection. RTD-1 (green bars) was added to GHOST-R5X4 cells one hour prior to infection with NL4-3 (solid bar) or JR-CSF (checked bar) or four or 11 hours post infection. GHOST-R5X4 cells were harvested 36 hours after infection and analyzed by flow cytometry for GFP. Bars represent the mean of triplicate samples in one experiment representative of 3 experiments. Error bars show the standard deviation of the triplicate samples. Asterisks denote significant difference from the no defensin control (p<0.01).



**Fig. 6.** Virus inactivation by HNP-1, HBD-2 and RTD-1. JR-CSF (checked bars) or NL4-3 (solid bars) were incubated with 10  $\mu$ g/ml HNP-1 (red bars), HBD-2 (blue bars) or RTD-1 (green bars) for one hour on ice. The virus and defensin mix was then centrifuged on a Sephadex G-25 column to remove defensin. The defensin free virus was then added to PBMC or GHOST-R5X4 cells. Alternatively, defensin was incubated with PHA-stimulated PBMC or GHOST-R5X4 cells for one hour at 37°C and washed away prior to the time of infection with JR-CSF or NL4-3. PBMC supernatant was collected 3 days after infection and analyzed by ELISA for the presence of viral capsid p24 (HNP-1) or by assaying for the presence of reverse transcriptase (HBD-2). GHOST-R5X4 cells were harvested 36 hours after infection and analyzed by flow cytometry for GFP (RTD-1). Data shown is from a representative experiment. Error bars represent the standard deviation from the mean of triplicate samples. P values shown were calculated using the students t test.

## CONCLUSIONS:

- RTD-1 blocked binding of an anti-CXCR4 MAb that binds the second extracellular loop of CXCR4
- Both RTD-1, and HNP-1 downmodulated CXCR4, but not CCR5 on GHOST-R5X4 cells. However, downmodulation required higher concentrations of defensins than did inhibition of HIV-1 replication.
- RTD-1 inhibited R5 and X4 HIV-1 replication when added at the time of infection but not when added four hours later to GHOST-R5X4 cells. RTD-1 also inhibited HIV-1 replication in PBMC (Data not shown).
- HNP-1 inhibited R5 and X4 HIV-1 replication in PBMC when added at the time of infection or up to 48 hours post infection. HNP-1 did not inhibit R5 or X4 HIV-1 replication in GHOST-R5X4 cells (data not shown).
- HBD-2 inhibited R5 and X4 HIV-1 replication in PBMC when added at the time of infection or up to 48 hours post infection.
- HNP-1 inactivated both R5 and X4 HIV-1 when incubated prior to infection. RTD-1 inactivated X4, but not R5 HIV-1 when incubated prior to infection. HBD-2 was unable to inactivate either R5 or X4 HIV-1.
- RTD-1 inhibited HIV-1 replication by blocking access to CXCR4 and inactivating X4 HIV-1, while HNP-1 inactivated R5 and X4 HIV-1 and blocked viral replication at a post-entry step.