

## Defensins: Natural Anti-HIV Peptides

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### Abstract

*Mammalian defensins are small cationic antimicrobial peptides predominantly found in leukocytes and epithelial cells engaged in host defense. These peptides act as effector molecules in innate immunity as well as regulators in adaptive immunity. Increasing evidence indicates that defensins are effective inhibitors of HIV-1. While the level of defensins in HIV-1 infected individuals has not been determined, neutropenia and neutrophil dysfunction associated with HIV disease progression may result in altered  $\alpha$ -defensin production. This review provides an overview of the structure and function of defensins, and focuses on the anti-HIV-1 activity of defensins and the mechanism of this activity. Although many questions remain, studying the complex function of defensins in innate immunity against HIV has implications for our further understanding of disease progression and for the development of novel approaches to prevention and therapy. (AIDS Reviews 2004;161-8)*

### Key words

*Defensins. HIV. Innate immunity.*

## Introduction

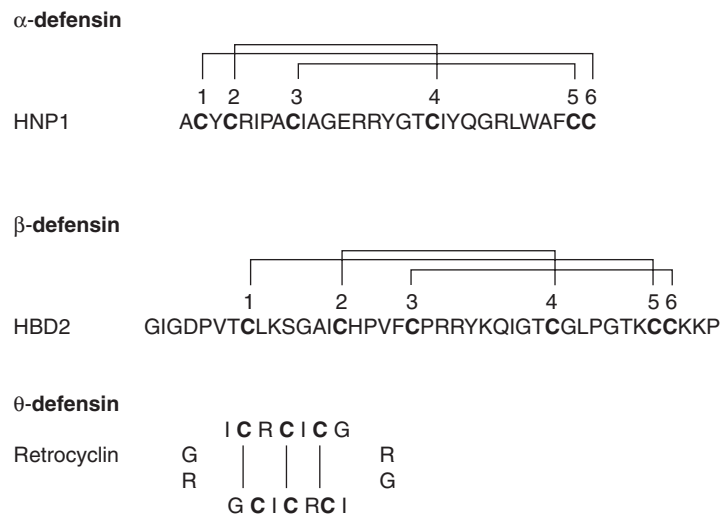
The innate immune system provides the first line of defense for rapidly clearing a wide variety of microbes prior to the development of an adaptive immune response<sup>1,2</sup>. In addition to the ability of the immune system to recognize pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors such as toll-like receptors (TLR)<sup>3</sup>, antimicrobial peptides including defensins and cathelicidins serve as important effectors in innate immunity<sup>4</sup>.

The importance of innate immunity in controlling HIV infection is becoming increasingly appreciated<sup>5-8</sup>. It has been recently shown that GU-rich, single-stranded RNA derived from the HIV-1 genome serves as a natu-

ral ligand for murine TLR7 and human TLR8<sup>9</sup>. There is an inverse correlation between the level of viremia and the ability of natural killer (NK) cells to inhibit HIV replication, which is predominantly mediated through secretion of CC chemokines including macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$  and RANTES that inhibit HIV-1 entry via CCR-5<sup>8</sup>. Similarly, antiviral activity of soluble factor(s) from CD8+ T-cells, known as CD8+ antiviral factor(s) or CAF, is found very early in primary infection, prior to the presence of antibodies against HIV<sup>10</sup>, and correlates with delayed disease progression in HIV-1 infected people<sup>11-13</sup>. Although CC chemokines are known to partially contribute to CAF activity<sup>14</sup>, the full identity of the factor(s) responsible for this inhibition remains elusive. Recently, CAF activity was attributed to  $\alpha$ -defensins 1 to 3<sup>15</sup>. Although it is now clear that  $\alpha$ -defensins are distinct from CAF and are not produced by CD8+ cells<sup>16,17</sup>, they do have anti-HIV activity. Increasing numbers of studies indicate that all subfamilies of mammalian defensins have anti-HIV-1 activity that warrants further exploration of their role in innate immunity against HIV infection and their potential as topical microbicides. This review focuses on the anti-HIV function of defensins and highlights the complex function of defensins in immunity against HIV.

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Table 1. Disulfide pairing of cysteine residues of  $\alpha$ -,  $\beta$ -, and  $\theta$ -defensins

## An overview of mammalian defensins

### Structure

Defensins are small, cysteine-rich, cationic peptides that exhibit anti-microbial activity against a broad spectrum of organisms including gram-positive and gram-negative bacteria and fungi as well as enveloped and non-enveloped viruses<sup>18-22</sup>. Mammalian defensins are classified into three subfamilies:  $\alpha$ -,  $\beta$ -, and  $\theta$ -defensins. Defensins have  $\beta$ -sheet structures stabilized by three disulfide bonds and differ in their distribution and connection of six cysteine (Cys) residues (Table 1<sup>20,23,24</sup>). The linkages of Cys residues in  $\alpha$ -defensins are Cys1-Cys6, Cys2-Cys4, Cys3-Cys5, whereas in  $\beta$ -defensins the linkages are Cys1-Cys5, Cys2-Cys4, Cys3-Cys6<sup>23</sup>. In contrast,  $\theta$ -defensins have a circular structure with the Cys residues linking Cys1-Cys6, Cys2-Cys5, Cys3-Cys4<sup>25</sup>. The  $\alpha$ - and  $\beta$ -defensins are synthesized as large prepropeptides of approximately 95 amino acids in length, which are then processed into small mature peptides of 29 to 42 amino acids in length<sup>23</sup>. The sequences of mature human  $\alpha$ - and  $\beta$ -defensins are shown in Figure 1. The  $\theta$ -defensins are 18 amino acids in length and composed of two  $\alpha$ -defensin-like precursor peptides with nine amino acids, each connected intracellularly via a post-translational head-to-tail ligation<sup>25-27</sup>.

Correct linkages of Cys residues through disulfide bridges are not required for antibacterial functions of

both  $\alpha$ -defensin-1 and human  $\beta$ -defensin (HBD)-3<sup>28,29</sup>. However, correct disulfide bonding is important for the chemotactic activity of HBD-3<sup>28</sup>. Similarly, the direct virion inactivation that has been attributed to  $\alpha$ -defensins 1-3 or  $\theta$ -defensins is completely abolished when disulfide bonds are disrupted by the treatment with dithiothreitol (DTT) and iodoacetamide<sup>30,31</sup>. Thus, the structure of defensins seems to play a different role, depending on function.

### Cell sources and tissue distribution

To date, six human  $\alpha$ -defensins have been identified<sup>24</sup>. Human  $\alpha$ -defensins 1-4 are also called human neutrophil peptides (HNP 1-4) because of their abundance in neutrophils<sup>32</sup>. HNPs 1-3 differ only in the first amino acid (Fig. 1<sup>24</sup>). HNPs 1-4, are all derived from neutrophils; however, HNP-4 comprises less than 2% of total defensins<sup>33</sup>. Although the highest density of HNPs are found in granulocytes, HNPs can be found in NK-cells, B-cells,  $\gamma\delta$  T-cells, and monocytes/macrophages<sup>34</sup>. In addition, HNPs have been detected in placenta, intestinal mucosa, saliva, and cervical mucus plugs that occlude the uterine cervix<sup>35-37</sup>, although the cell source producing these HNPs is unclear. An elevation of  $\alpha$ -defensins has been found in vaginal fluid from patients with lower and upper genital tract infection, suggesting a role for  $\alpha$ -defensins in mucosal immunity against infection *in vivo*<sup>38,39</sup>. Human  $\alpha$ -defensin-5 (HD-5) and 6 (HD-6) are produced predominant-

<b><math>\alpha</math>-defensins</b>	
HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-2	CYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-3	DCYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-4	VCSCRLVFCRRTEL RVGNCLIGGVSFTY <b>CCTR</b> VD
HD-5	A <b>TCY</b> CRTGRCATRESLSGVC <b>EISGR</b> LYRL <b>CCR</b>
HD-6	T <b>CH</b> CRRS- <b>CY</b> STEYSYGTC <b>TV</b> MG <b>I</b> NHR <b>F</b> CCL
<b><math>\beta</math>-defensins</b>	
HBD-1	GNFLTGLGHRSDHYNCVSSGGQCLYSACPIFTKIQT <b>CY</b> RGKAK <b>CK</b>
HBD-2	GIGDPVT-----CLKSGA <b>IC</b> HPVFCPRRYKQIG <b>TC</b> GLPGTK <b>CK</b> CKP
HBD-3	GII <b>N</b> LQKY <b>Y</b> CRV <b>R</b> GGRC <b>AV</b> LS <b>CL</b> PK <b>EE</b> Q <b>IG</b> K <b>CS</b> TRGRK <b>CK</b> RR <b>KK</b>
HBD-4	E <b>F</b> ELDR <b>IC</b> GYGTAR <b>CR</b> KK- <b>CR</b> SQ <b>EY</b> R <b>IG</b> RC- <b>P</b> NT <b>Y</b> AC <b>CL</b> RK <b>W</b> DES <b>LL</b> N <b>RT</b> K <b>P</b>
HBD-5	GLDFSQPFPSGEFAV <b>CS</b> CK <b>L</b> GRGK <b>CR</b> KE- <b>C</b> LENE <b>K</b> PDG <b>NC</b> - <b>R</b> LN <b>FL</b> CC <b>R</b> Q <b>R</b> I
HBD-6	FFDE <b>K</b> CN <b>K</b> L <b>KG</b> T <b>CK</b> NN- <b>C</b> G <b>K</b> NEE- <b>L</b> I <b>A</b> LC- <b>Q</b> SL <b>K</b> CC <b>R</b> T <b>I</b> Q <b>PC</b> GS <b>I</b> ID

**Figure 1.** Primary amino acid sequences of the mature (processed) human  $\alpha$ - and  $\beta$ - defensin families. Defensins contain several positively charged residues (R, K, and H). Cysteine residues contributing to the disulfide bridges are in bold type.

ly by intestinal Paneth cells<sup>23</sup>, although both defensins have been found in the salivary gland, small and large bowel, stomach, and eye<sup>36</sup>, and HD-5 has been reported in the female genital tract<sup>40,41</sup>.

Although 28 human  $\beta$ -defensins have been identified by genomic-based searches<sup>42</sup>, six human  $\beta$ -defensins (HBD 1-6) have been identified and characterized<sup>24</sup>. These peptides are produced mainly by epithelial cells<sup>23,24</sup>. HBD-1 and HBD-2 are produced by epithelial cells, including keratinocytes<sup>43</sup>. HBD-3 is expressed in epithelia of many organs as well as non-epithelial tissues<sup>44,45</sup>. While HBD-1 is constitutively expressed by epithelial cells, HBD-2, and HBD-3 can be induced by viruses, bacteria, microbial products (i.e. endotoxin) or pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-1<sup>23,43,46,47</sup>. Expression of HBD-1 and HBD-2 has been detected in monocytes, macrophages, and monocyte-derived dendritic cells at both RNA and protein levels<sup>48</sup>, indicating that HBD-1 and HBD-2 are not exclusively epithelial cell-associated. Regarding tissue distribution, HBD-1 and HBD-2 have been detected in oral and nasal mucosa, lung, plasma, salivary gland, small and large bowel, stomach, skin, eye, mammary gland, urogenital tracts and kidney<sup>36,49-51</sup>. Constitutive expression of HBD-4 appears to be restrict-

ed to the testis and gastric antrum, although HBD-4 can be induced in human respiratory epithelial cells after exposure to phorbol 12-myristate 13-acetate (PMA) or bacterial infection<sup>52</sup>. HBD-5 and HBD-6 are specifically expressed in human epididymis<sup>53</sup>.

Three  $\theta$ -defensins have been found in leukocytes and bone marrow of rhesus macaque; RTD (for rhesus theta defensin)-1, -2, -3<sup>25-27</sup>. Although RNA transcripts homologous to the rhesus  $\theta$ -defensin gene (DEFT, for defensin theta) are found in human bone marrow, these transcripts contain a premature stop codon in the upstream signal sequence, which abolishes subsequent translation<sup>54</sup>. Interestingly, these pseudogenes containing the same premature stop codon mutation are also found in chimpanzees and gorillas, but not in several Old World monkeys, which have intact DEFT genes<sup>54</sup>. Retrocyclin is an artificially made circular peptide based on the sequence of the mature peptide encoded by the human  $\theta$ -defensin pseudogene<sup>55</sup>.

### **Other functions in the immune system**

Alpha and  $\beta$ -defensins have been shown to play a role in the regulation of both innate and adaptive immunity<sup>24</sup>. They display chemotactic activity for T-lym-

phocytes, monocytes, and immature dendritic cells (DC) and induce cytokine production<sup>24,56</sup>. The chemotactic activity of HBD-1 to HBD-3 for memory T-cells and immature DCs is mediated by CCR6, the receptor for the CC-chemokine CCL20<sup>57,58</sup>. Despite the absence of a significant sequence homology between  $\beta$ -defensins and CCL20, there is a tertiary structural similarity between HBD-2 and the putative receptor-binding region of CCL20<sup>59,60</sup>. Interestingly, many chemokines including CCL20/MIP-3 $\alpha$  display antimicrobial activities<sup>61</sup>.

HBD-2 induces the migration of mast cells through G-protein-phospholipase C coupled receptors, leading to the release of histamine and the generation of prostaglandin D<sub>2</sub><sup>62,63</sup>. Human  $\alpha$ -defensins 1-3 have chemotactic activity for monocytes, T-cells, and immature DCs, but the specific receptor has not been identified<sup>23,24</sup>. This chemotactic activity can be blocked by pertussis toxin, suggesting that a G $\alpha$ -protein-coupled receptor(s) is involved<sup>56,64</sup>. Murine  $\beta$ -defensin-2 can recruit bone marrow-derived immature DCs through CCR6 and induce DC maturation through TLR-4<sup>65</sup>. Taken together, these observations suggest a broad role for defensins in controlling pathogen invasion by acting as effectors, and by enhancing innate immunity as well as regulating adaptive immunity.

### Anti-HIV activities of defensins and their mechanism

When examining the role of defensins as inhibitors of HIV, it is important to carefully define the experimental conditions, as both serum and salt concentrations have been reported to alter their effects. For example, defensins at high concentrations (> 100  $\mu$ g/ml) are known to cause cytotoxicity in the absence of serum by making the target membranes permeable, and the cytotoxicity can be abolished by serum<sup>66,67</sup>. In addition, most defensins display potent antibacterial activities under conditions of low salt<sup>18</sup>. However, serum does not block the chemotactic activities of defensins<sup>57,64</sup>.

### Alpha-defensins

Inhibition of HIV replication by synthetic  $\alpha$ -defensins from guinea-pig, rabbit, and rat was first reported in 1993<sup>68</sup>. These peptides inhibit HIV-1 infection following viral entry, and their antiviral activities are not affected by the presence of serum<sup>68</sup>. Almost 10 years later, native  $\alpha$ -defensins 1-3 purified from human neutrophils are shown to inhibit replication of R5 and X4 strains of HIV-1, including several primary isolates, with 50% in-

hibitory concentration (IC<sub>50</sub>) of 0.5-2.2  $\mu$ M<sup>15</sup>. The IC<sub>50</sub> for a mixture of synthetic  $\alpha$ -defensin-1 and -2 ranges from ~11-24  $\mu$ M (approximately 5 to 10-fold more than the native form) although impurities in synthetic peptides can account for the less potent antiviral activity of the synthetic defensins<sup>15</sup>. Recombinant  $\alpha$ -defensin-1 at a concentration of 1.5  $\mu$ M blocks HIV-1 infection following viral entry, and this antiviral activity is not affected by serum<sup>16</sup>. In addition, there is no cytotoxicity observed at this concentration<sup>16</sup>. Pretreatment of cells with  $\alpha$ -defensin-1, followed by wash-out prior to infection, blocks HIV-1 infection, indicating that direct inactivation of virions is not required for its inhibitory effect<sup>16,17</sup>. In a viral entry assay system, native  $\alpha$ -defensin-1 at ~1.5-2.9  $\mu$ M protects cells from HIV-1 primary isolates<sup>69</sup>. The range of  $\alpha$ -defensins reported to result in significant inhibition of HIV-1 goes from the low micromolar range to an IC<sub>50</sub> of approximately 60  $\mu$ M (~200  $\mu$ g/ml)<sup>15-17,69</sup>. At the latter dose range,  $\alpha$ -defensins may display some cytotoxic affect as well<sup>17</sup>. The inhibitory effect of  $\alpha$ -defensins on HIV-1 infection in these studies were preformed in regular media, indicating that the anti-HIV-1 activity of  $\alpha$ -defensins is not dependent on salt concentrations. The comparison of results of anti-HIV activity of defensin from different subfamilies is summarized in Table 2.

The reported studies are consistent with  $\alpha$ -defensins having at least a dual mechanism to account for their anti-HIV activity. They can inhibit HIV-1 replication by a direct inactivation of the virus as well by affecting the target cells<sup>17,69,70</sup>. In the absence of serum,  $\alpha$ -defensins can act directly on the virus but this effect is abolished by the presence of serum<sup>17,70</sup> or an increase in virus particles<sup>70</sup>. In the presence of serum,  $\alpha$ -defensin-1 can have effects on the target cell resulting in inhibition of HIV-1 replication. Furthermore, these cellular effects appear to block viral replication following entry and reverse transcription in primary CD4 T-cells<sup>16,70</sup>. Recent studies from our laboratory indicate that  $\alpha$ -defensin-1 affects steps prior to completion of nuclear import as well as transcription (T.L. Chang, unpublished results). In a cell-free system,  $\alpha$ -defensin-1 has been shown to inhibit conventional PKC isoforms<sup>71</sup>. Interference with PKC signaling in primary CD4+ T-cells by  $\alpha$ -defensin-1 is associated with its anti-HIV activity<sup>70</sup>, although other signaling pathways may be involved as well. For instance, in macrophages  $\alpha$ -defensins-1 and -2 upregulate CC-chemokines, which could contribute to their inhibition of HIV<sup>72</sup>.

Alpha-defensins are positively charged, and direct binding via charge interactions may account for some of

**Table 2. Summary of anti-HIV-1 activities of defensins**

Defensins	IC <sub>50</sub>	HIV strains	References
Native HNP1-3	0.5-2.2 $\mu$ M	X4, R5 primary isolates	15
Native HNP1-3	200 $\mu$ g/ml (~60 $\mu$ M)	X4, R5	17
Synthetic HNP1-2	11-24 $\mu$ M	X4, R5 primary isolates	15
Recombinant HNP-1	~1 $\mu$ M	X4, R5	16
Synthetic GPNP RbNP-1, RatNP-1	9-12 $\mu$ M	X4	68
Recombinant HBD-2*	9-19 $\mu$ g/ml (~0.2-0.4 $\mu$ M)	X4, R5	76
Recombinant HBD-3*	20-40 $\mu$ g/ml (~0.4-0.8 $\mu$ M)		
Retrocyclin	1-20 $\mu$ g/ml (~0.5-10 $\mu$ M)	X4, R5	55,61
Retrocyclin-1	5-10 $\mu$ g/ml (~2.5-5 $\mu$ M)	X4, R5	69
Retrocyclin-2	2.33 $\mu$ g/ml	primary isolates	
RTD-1	0.9-3.6 $\mu$ g/ml (0.45-1.8 $\mu$ M)	X4, R5	69
RTD-2	1.7-6.7 $\mu$ g/ml	primary isolates	
RTD-3	1.7-3.2 $\mu$ g/ml		

\*IC<sub>50</sub> was determined at a low salt concentration (10 mM phosphate buffer).

their anti-HIV activity as well as their sensitivity to serum in some assays. A recent study indicates that  $\alpha$ -defensins can act as a lectin and bind to gp120 (Kd of 15.8-52.8 nM) and CD4 (Kd of 8 - 34.9 nM) at high affinity. The binding to gp120 is strongly abolished by serum<sup>69</sup>, consistent with the loss of the direct virion effects in the presence of serum<sup>17,70</sup>. They have also been shown to bind to several proteins in blood including  $\alpha$ 1-proteinase inhibitor,  $\alpha$  1-anti-chymotrypsin,  $\alpha$  2-antiplasmin, antithrombin III (ATIII), and C1 complement, suggesting their role in altering inflammatory processes<sup>73,74</sup>. Interestingly, ATIII has been identified as a component of CAF<sup>75</sup>.

### **Beta-defensins**

Induction of HBD-2 and HBD-3 by X4 or R5 viruses has been demonstrated in normal human oral epithelial cells where HIV-1 replication is not detectable<sup>76</sup>. In contrast to the anti-HIV activity of  $\alpha$ -defensin-1, which is independent on salt concentrations, recombinant HBD-2 and HBD-3 can inhibit both X4 and R5 HIV-1 virions with IC<sub>50</sub> of 9-19  $\mu$ g/ml (~0.2-0.4  $\mu$ M) and 20-40  $\mu$ g/ml (~0.4 -0.8  $\mu$ M), respectively, only under a low concentration of salt (i.e. 10 mM phosphate buffer)

and in the absence of serum<sup>76</sup>. HBD-2 and HBD-3 directly inactivate X4 HIV laboratory strain virions, but not R5 virions in regular media without serum as analyzed in GHOST X4/R5 cells. In addition, these peptides can downregulate the HIV-1 coreceptor CXCR4 (but not CCR5) in peripheral blood mononuclear cells (PBMC) in the absence of serum, while HBD-1 neither inhibit HIV-1 replication or downregulate the CXCR4 coreceptor<sup>76</sup>. Using a single cycle infection with VSV pseudotyped HIV-1 luciferase reporter viruses, HBD-1 and HBD-2, but not HBD-3, appeared to have a post-entry inhibitory effect on HIV-1 in primary CD4+ T-cells in the presence of serum (T.L. Chang, unpublished results).

A recent study demonstrates that a single-nucleotide 44 C/G polymorphism in HBD-1 gene is associated with HIV-1 infection in Italian children<sup>77</sup>. The significance of this mutation in HBD-1 in the control of HIV-1 infection remains to be determined.

### **Theta-defensins**

Retrocyclins and RTDs 1-3 act as lectins and inhibit HIV viral entry<sup>31,55,61,69</sup>. Unlike  $\alpha$ - and  $\beta$ -defensins,

retrocyclin does not inhibit HIV-1 replication by direct inactivation of HIV virus particles<sup>55</sup>. Both retrocyclin and RTDs 1-3 block several HIV-1 primary isolates, including X4 and R5 viruses with  $IC_{50}$  of approximate 0.5-5  $\mu$ M (1-10  $\mu$ g/ml)<sup>69</sup>, although the affect of serum in the viral entry assay was not specifically addressed<sup>31,61,69</sup>. Retrocyclin binds to glycosylated proteins including gp120, CD4 but not non-glycosylated gp120 and bovine serum albumin<sup>69</sup>. Interestingly, retrocyclin does not block HIV-1 infection when the reporter viruses are pseudotyped with A-MuLV or VSV-G envelope, although these envelope proteins are also glycosylated<sup>61</sup>. Theta-defensins have high binding affinity to glycosylated gp120 ( $K_d$  of 9.41-340 nM) and CD4 ( $K_d$  of 6- 225 nM) via binding to O-linked and N-linked sugars<sup>31,69</sup>. Serum strongly reduces their binding to gp120<sup>69</sup>. Retrocyclins also have high affinity to galactosylceramide ( $K_d$  of 24.1 nM), a glycolipid, and fetuin ( $K_d$  of 24.1-58.2 nM), a highly abundant glycoprotein in serum<sup>31,69</sup>.

## Clinical aspects

The *in vivo* role of defensins in either HIV-1 transmission or in disease progression is not known. However, it is known that the concentration of  $\alpha$ -defensins at sites of inflammation is associated with the number of neutrophils<sup>39,78</sup>. Although increased neutrophil phagocytosis and oxidative bursts are associated with neutrophils isolated from patients with early stage HIV-1 infection<sup>79</sup>, numerous studies have demonstrated that neutropenia and neutrophil dysfunction are associated with later stages of HIV infection<sup>80-88</sup>. Longitudinal studies of neutrophil function in HIV-1 infected patients and FIV-1 infected cats indicate that a progressive decrease of neutrophil counts occurs during infection<sup>86,89</sup>. Reported abnormal functions associated with these neutrophils include impairment of chemotaxis and phagocytosis, altered expression of surface adhesion molecules, and depressed superoxide production<sup>90</sup>. An increased number of apoptotic neutrophils, which are functionally impaired, are also found in patients with AIDS<sup>80</sup>. In addition, a decrease in neutrophil apoptosis and an improvement in neutrophil function have been shown in patients undergoing highly active antiretroviral therapy (HAART)<sup>91,92</sup>. No study has looked on the association between production of defensins and HIV disease progression. However, the loss of neutrophil number and function with disease progression may result in a loss of production of  $\alpha$ -defensins as well. In light of the *in vitro* activity of  $\alpha$ - and  $\beta$ -defensins

against HIV, a clearer understanding of their potential role *in vivo*, both in transmission and disease progression, is warranted.

## Conclusions

Increasing evidence demonstrates that defensins, long recognized as natural antimicrobial peptides, exhibit anti-HIV activity. However, many questions remain unanswered regarding their role in transmission and disease progression, as well as the potential to exploit this activity for novel microbicide development. While anti-HIV activity of defensins is demonstrable *in vitro*, the relationship between these inhibitory levels and those found at critical mucosal surfaces and at sites of virus replication needs to be determined. Furthermore, the role of defensin modulation of critical cytokine or chemokine production, and subsequent impact on HIV infection and disease progression, needs to be defined. Further studies focused on the contribution of the structure of defensins to their various anti-HIV activities will contribute to their development as novel drugs.

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