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Supporting Online Material

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Materials and Methods
Movies S1 to S5

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Contribution of Human α-Defensin 1, 2, and 3 to the Anti–HIV-1 Activity of CD8 Antiviral Factor

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It has been known since 1986 that CD8 T lymphocytes from certain HIV-1–infected individuals who are immunologically stable secrete a soluble factor, termed CAF, that suppresses HIV-1 replication. However, the identity of CAF remained elusive despite an extensive search. By means of a protein-chip technology, we identified a cluster of proteins that were secreted when CD8 T cells from long-term nonprogressors with HIV-1 infection were stimulated. These proteins were identified as α-defensin 1, 2, and 3 on the basis of specific antibody recognition and amino acid sequencing. CAF activity was eliminated or neutralized by an antibody specific for human α-defensins. Synthetic and purified preparations of α-defensins also inhibited the replication of HIV-1 isolates in vitro. Taken together, our results indicate that α-defensin 1, 2, and 3 collectively account for much of the anti–HIV-1 activity of CAF that is not attributable to β-chemokines.

T lymphocytes that carry the CD8 antigen play a critical role in controlling HIV-1 or simian immunodeficiency virus (SIV) replication in vivo (*1*). The initial control of viremia after primary infection is temporally correlated with the onset of virus-specific CD8 cytotoxic T lymphocytes (CTLs) (*2*, *3*). SIV replication in macaques increases dramatically when a monoclonal antibody (mAb) is used to deplete CD8 T cells (*4*, *5*). Moreover, the strong pressure exerted by cellular immunity in vivo is apparent from the rapid emergence of CTL-escape viruses (*6*, *7*). Although the direct killing of infected cells by CD8 CTLs is important in virus suppression (*1*), soluble factors secreted by CD8 T lymphocytes can also inhibit HIV-1 or SIV replication in vitro. In 1986, Walker *et al.* (*8*) first described the CD8 antiviral factor (CAF), a diffusible molecule secreted by stimulated CD8 T cells from certain HIV-1–infected individuals. Unlike the activity of CTLs, the antiviral activity of CAF is noncytolytic and does not require restriction by major histocompatibility complex class I molecules or cell-to-cell contact. Instead, the activity is believed to be mediated by a heat-stable, acid-stable protein (*9*) with a molecular mass of <20 kD (*10*) or <10 kD (*11*). It is noteworthy that CAF inhibits HIV-1 replication irrespective of viral phenotype or tropism (*9*), but its precise mechanism of action remains unknown, although there are indications that the effect may be at the level of viral transcription (*12*, *13*).

Stimulated CD8 T lymphocytes release CAF in greater than normal abundance from HIV-1–infected persons who are doing well clinically, particularly those characterized as long-term nonprogressors (LTNPs) (*9*, *14*–*16*). In contrast, it is uncommonly detected in CD8 T cells from infected patients with evidence of immunodeficiency (progressors). CAF-like activity has been detected in stimulated CD8 T cells from SIV-infected rhesus macaques (*17*) or African Green monkeys (*18*), HIV-1–infected chimpanzees (*19*), and some healthy uninfected humans (*20*).

Despite tremendous efforts over the past 16 years (*9*), the identity of CAF has remained elusive. In 1995, Cocchi *et al.* (*21*) showed that stimulated CD8 T lymphocytes can secrete β-chemokines (RANTES and the macrophage inflammatory proteins MIP-1α and MIP-1β) that block HIV-1 infection in vitro. However, their antiviral activity was observed against macrophage-tropic viral isolates, but not against T cell line–tropic strains. This dichotomy was later explained by the discovery that the receptor for β-chemokines, CCR5, also serves as the coreceptor for HIV-1 entry into CD4 T cells (*22*–*24*). Thus, it became apparent that β-chemokines can competitively block so-called R5 viruses that use CCR5 as coreceptor, but not so-called X4 viruses that use an alternate coreceptor, CXCR4 (*25*). Such an antiviral profile clearly distinguished β-chemokines from CAF, which can inhibit both types of HIV-1. Moreover, CAF activity could not be eliminated by removing either β-chemokines (*26*, *27*) or SDF 1α (*28*), the ligand for CXCR4, with specific mAbs. Other cytokines have subsequently emerged as possible candidates for CAF, including macrophage-derived chemokine (*29*)

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and interleukin 16 (*30*), but none has stood the test of time (*31*, *32*).

A cluster of small proteins secreted by stimulated CD8 T cells. We have long studied a cohort of LTNPs, many of whom were good producers of CAF (*14*). Supernatant fluids were harvested from stimulated and unstimulated CD8 T lymphocyte cultures derived from three of these LTNPs, as well as from four progressors and 15 normal controls (*33*). Each sample was analyzed on the ProteinChip system (Ciphergen Biosystems, Fremont, CA), which is based on the integration of chemically modified array surfaces with surface-enhanced laser desorption/ionization (SELDI) time-of-flight (TOF) mass-spectrometry (MS) detection (*34*). This technology was chosen because of its power of resolution, high reproducibility, ease of use, and femtomole-level sensitivity (*34*, *35*). As shown in Fig. 1, representative protein mass spectra for two LTNPs and one normal control revealed significant differences in peak pattern between stimulated and unstimulated CD8 supernatants. A cluster of two or three peaks, with molecular masses of 3371.9, 3442.5, and 3486.5 daltons, was found in stimulated cultures. This cluster was detected in cultures of stimulated CD8 T lymphocytes from three of three LNTPs and 11 of 15 normal individuals, but not from four progressors (Fig. 1). A unique peak at 7815.0 daltons, later identified as MIP-1α (see below), was also detected in stimulated samples from two LTNPs. Although plenty of peaks were observed from 8 to 200 kD, no consistent differences were found between stimulated and unstimulated CD8 cultures for the three study groups (*36*). In particular, no peak >8 kD consistently correlated with the presence of CAF activity.

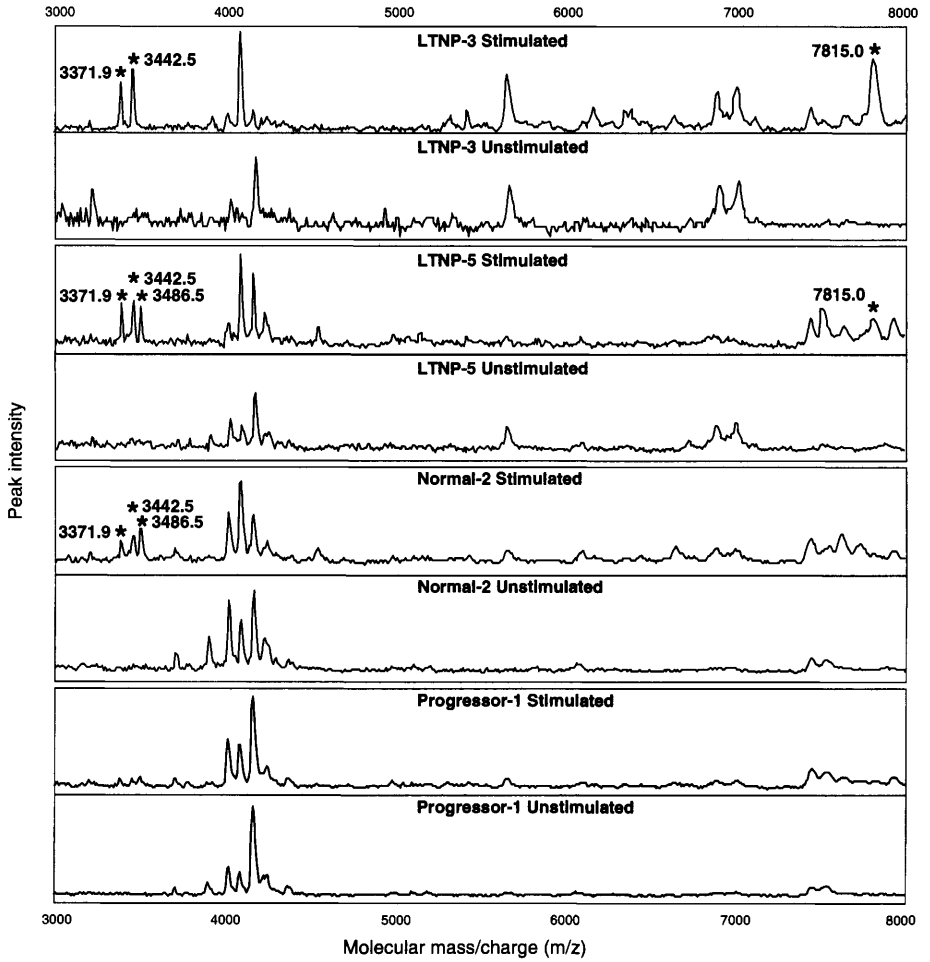

Fig. 1. Representative protein mass spectra of culture supernatants from stimulated and unstimulated CD8 T cells from two LTNPs, one normal individual, and one progressor. Protein peaks that are up-regulated after stimulation are highlighted and their masses are indicated.

Table 1. Changes in molecular mass (m/z) before [–] and after [+] reduction with or without alkylation. n/d, Not detected.

Normal-2	Dithiothreitol			LTNP-3	Acrylamide			Iodoacetamide		
	[–]	[+]	Net change		[–]	[+]	Net change	[–]	[+]	Net change
Peak 1	3371.0	3377.2	6.2	Peak1	3370.0	3804.3	434.3	3371.5	3720.0	348.5
Peak 2	3441.7	3447.8	6.1	Peak2	3441.3	3875.0	433.7	3442.1	3791.3	349.2
Peak 3	3485.8	3491.6	5.8	Peak3	n/d	n/d	n/d	n/d	n/d	n/d
Average			6.0				434.0			348.9
No of disulfide bonds			3				3			3

To further characterize the cluster of peaks between 3.3 and 3.5 kD, culture supernatants from stimulated CD8 T cells from LTNP subject 3 (LTNP-3) and normal control number 2 (Normal-2) were enriched for these proteins as described (*33*). Enriched materials were then treated with dithiothreitol (DTT), acrylamide, or iodoacetamide to probe for the existence of disulfide bonds within each protein in the cluster. The resultant materials were then analyzed by SELDI-TOF-MS. The changes in molecular mass for the three peaks found in Normal-2 upon reduction with DTT are shown in Table 1. Each peak gained ~6 daltons after reduction (fig. S1), which suggests that every protein in the cluster contains three internal disulfide bridges, because DTT reduction would add two hydrogen atoms to form two free sulfhydryl groups for each disulfide bond broken. Furthermore, for peaks detected in the culture supernatant from LTNP-3, reduction and alkylation with acrylamide or iodoacetamide led to increases of ~434 or 349 daltons, respectively (Table 1). Given the molecular masses of acrylamide (71 daltons) and iodoacetamide (57 daltons), the observed mass increases were again consistent with the addition of six acrylamide or iodoacetamide molecules to each protein through six free sulfhydryl groups. This result further confirmed the presence of three intramolecular disulfide bridges in each protein in the cluster. Taken together, these findings raised the possibility that these small proteins are similar and related.

Identification of the protein cluster as human α-defensin 1, 2, and 3. By searching through protein databases (NCBI: www.ncbi.nlm.nih.gov; Swiss-Prot: www.ebi.ac.uk/swissport), we found that the peaks of 3371.9, 3442.5, and 3486.5 daltons correspond precisely to the molecular masses of human α-defensin 2, 1, and 3, respectively, which are peptide antibiotics made principally by human neutrophils (*37*–*39*). Each of these peptides is known to contain three internal disulfide bonds (*40*). Moreover, the absence of the 3486.5-dalton peak in some

cases (e.g., LTNP-3 in Fig. 1) is consistent with the lack of α-defensin 3 in about 10% of the population (*41*). To confirm the identity of these molecules, we first examined whether an mAb specific for human α-defensin 1, 2, and 3 would recognize the protein peaks of interest. We incubated supernatants from stimulated CD8 T lymphocytes from subjects LTNP-3 and Normal-2 with beads only or beads coated with an mAb against α-defensin 1, 2, and 3 or MIP-1α as described (*33*), before testing on protein-chip arrays by using SELDI-TOF-MS. Pretreatment with an mAb against α-defensin 1, 2, and 3 eliminated the cluster of proteins in the range of 3.3 to 3.5 kD (Fig. 2A), without significantly affecting other peaks. Preincubation with an mAb against MIP-1α antibody did not affect the peaks of interest but did result in the removal of a peak at 7815.0 daltons. These findings strongly support the suggestion that this cluster of proteins represents members of the human α-defensin family.

To further confirm this conclusion, the enriched materials previously used for the reduction and alkylation experiments were digested with trypsin and analyzed by tandem mass spectrometry (*33*). Trypsin-digested materials from both LTNP-3 and Normal-2 subjects yielded a unique 1060.50-dalton (Fig. 2B, upper-right inset) fragment, which was further selected and fragmented into smaller ions by collision-induced dissociation in the MS-MS collision cell. The seven unique ions generated by this means (Fig. 2B) were then used in a protein search engine (see legend to Fig. 2B) to look for theoretical fragments of the 1060.50-dalton parent ion. The search yielded a perfect and unequivocal match with a trypsin-digest fragment of human α-defensin 1, 2, and 3. In fact, this peptide is conserved among these three molecules and corresponds exactly to the sequence YGTCIYQGR from amino acid positions 16 to 24 (*42*) (Fig. 2B). We therefore confirmed by protein sequencing that the proteins of interest are indeed members of the human α-defensin family.

Human α-defensin 1, 2, and 3 account for much of the HIV-1–suppressive activity of CAF that is not attributable to β-chemokines. To evaluate the relative contribution of α-defensin 1, 2, and 3 to CAF activity, culture supernatants from stimulated CD8 T cells from subjects LTNP-3 and LTNP-5 were selectively depleted of these molecules by the use of an affinity column or beads coated with a specific antibody (*33*). The antiviral activity, before and after depletion of α-defensin 1, 2, and 3, were compared against a panel of X4 and R5 HIV-1's from various genotypes (*33*) (Fig. 3A). Before depletion, culture supernatants were able to inhibit ~50 to 60% of the replication of all X4 viruses tested. After depletion, however, the inhibition of X4 viruses was largely eliminat-

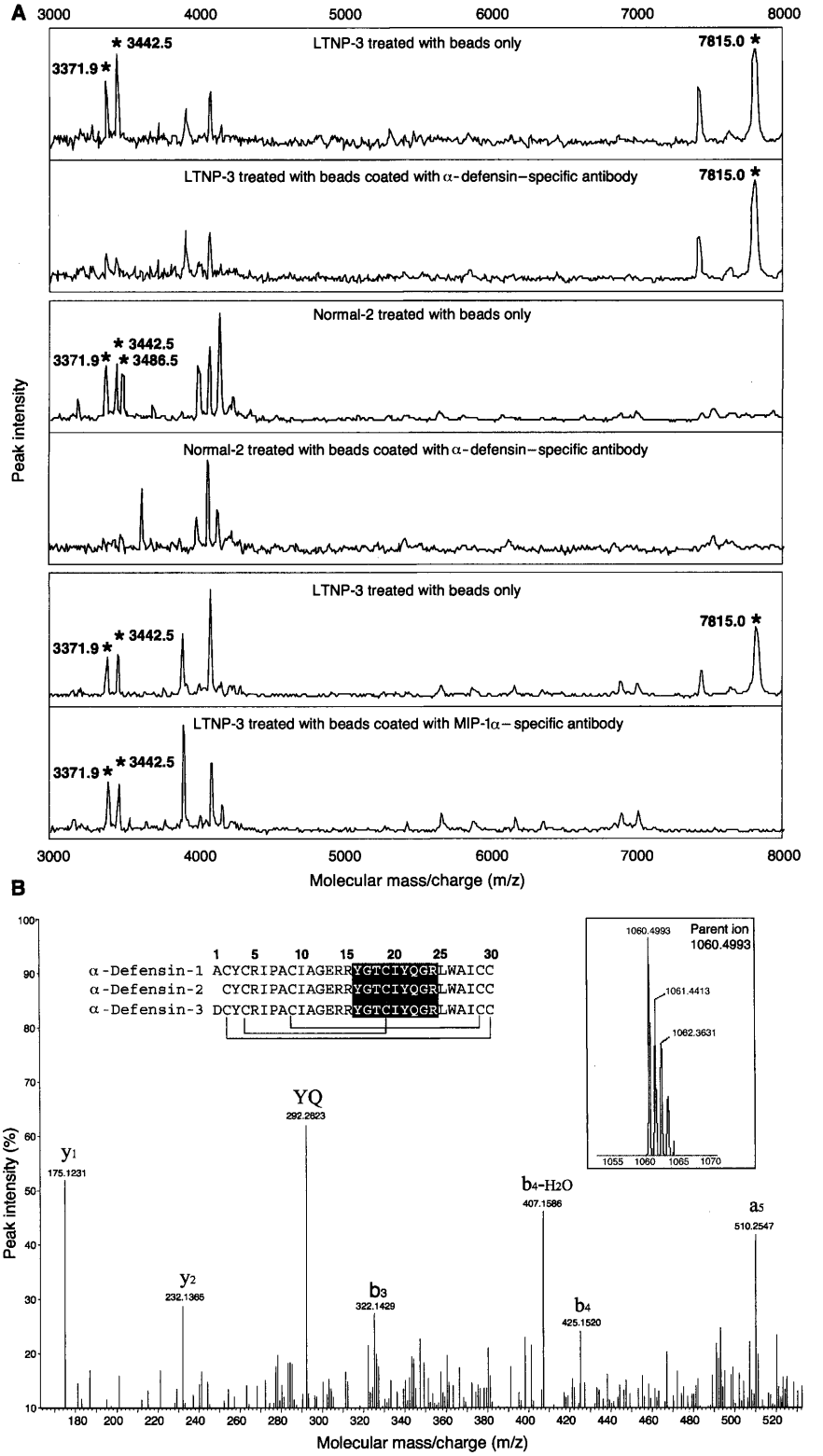

Fig. 2. (**A**) Identification of protein peaks with molecular masses of 3371.9, 3442.5, and 3486.5 daltons as human α-defensin 2, 1, and 3, by using beads coated with an antibody against α-defensin 1, 2, and 3. (**B**) Protein sequencing of a unique 1060.50-dalton peptide fragment after trypsin digestion (upper right), by using tandem mass spectrometry. Unique peaks from collision-induced dissociation of 1060.50-dalton parent ion are indicated. Protein Prospector MS-Tag search of NCBI and SwissProt database showed that the peptide fragment, YGTCIYQGR (highlighted in the upper left), from α-defensin 1, 2, and 3 was the best match with a probability-based Mowse score of 49 (Mascot software from Matrix Science). The next closest match (β-galactosidase precursor, 76.091 kD) had a score of only 17. In such analyses, a Mowse score over 38 is regarded as positive identification or extreme homology.

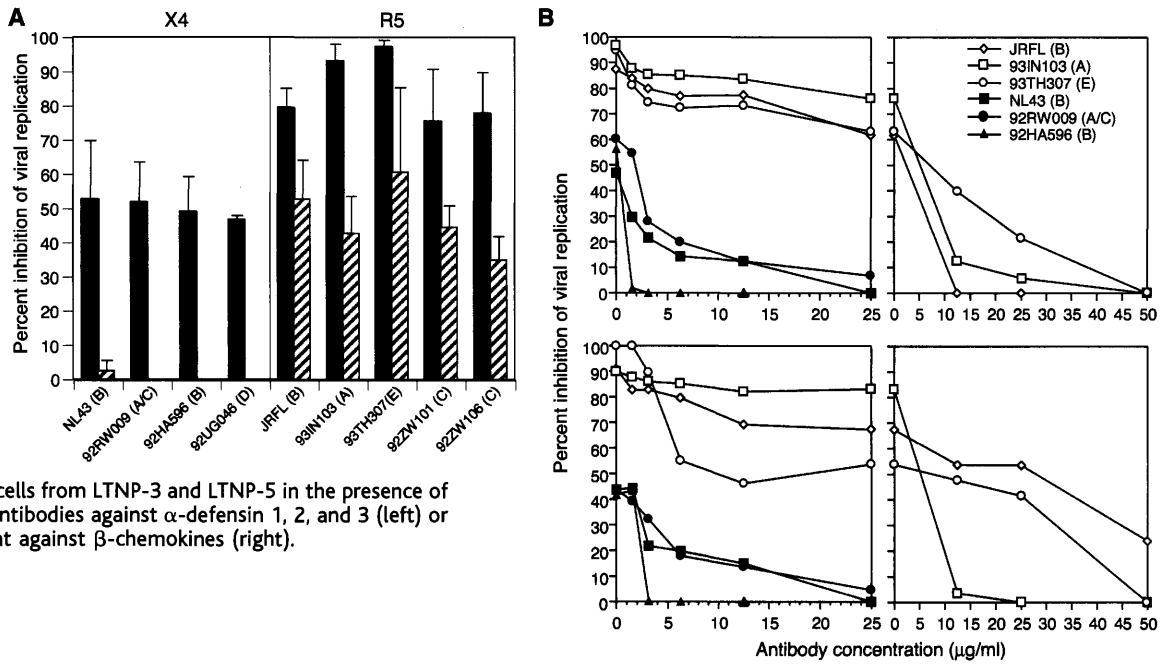

Fig. 3. (**A**) Antiviral activity against a panel of X4 and R5 HIV-1's before (solid) and after (hatched) depletion of α-defensin 1, 2 and 3 from culture supernatants of subjects LTNP-3 and LTNP-5. The names of the viral isolates are as indicated, and the HIV-1 genotypes are shown in parentheses. The error bars indicate the standard deviation from the mean of two independent experiments. (**B**) Antiviral activity of culture supernatants from stimulated CD8 T cells from LTNP-3 and LTNP-5 in the presence of increasing amounts of antibodies against α-defensin 1, 2, and 3 (left) or in combination with that against β-chemokines (right).

ed, which indicated that α-defensin 1, 2, and 3 account for most of the suppressive activity of CAF against X4 viruses. For R5 viruses, there was an average of ~40% reduction in anti–HIV-1 activity after the removal of α-defensins (Fig. 3A). Control experiments with an irrelevant antibody did not result in the loss of antiviral activity (fig. S2).

We next examined whether CAF activity could be neutralized in a dose-dependent manner by the addition to the culture supernatant of an antibody against α-defensin 1, 2, and 3, with and without the addition of antibodies to β-chemokines. Anti–HIV-1 activity of CD8 supernatants from LTNP-3 and LTNP-5 decreased as the concentration of an α-defensin–specific antibody increased (Fig. 3B). For all X4 viruses tested, the suppressive activity of CAF was virtually eliminated when antibody concentration reached 25 μg/ml, although a similar amount of a control antibody had no effect (fig. S3). The inhibitory activity against R5 viruses was also reduced by the addition of an α- defensin–specific antibody, although the effect was not as profound (Fig. 3B and fig. S3). To address the possibility that the residual activity against R5 viruses could be due to β-chemokines (*21*), culture supernatants from subjects LTNP-3 and LTNP-5 were treated with increasing amounts of a mixture (1:1:1) of antibodies against MIP-1α, MIP-1β, and RANTES, together with a fixed concentration (25 μg/ml) of an α-defensin–specific antibody. The residual antiviral activity against three R5 isolates was substantially neutralized at the highest antibody concentration used (Fig. 3B, right panels). Collectively, these results suggest that α-defensin 1, 2, and 3 account for much of the anti–HIV-1 activity in supernatants of stimulated CD8 T lymphocyte cultures that is not attributable to β-chemokines.

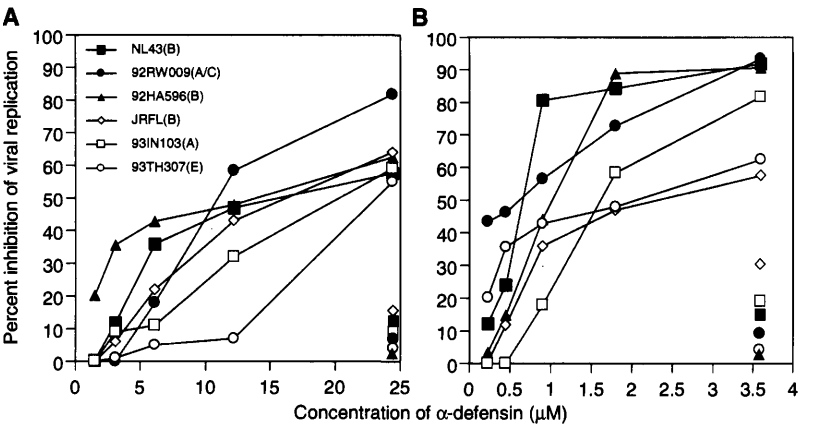

Fig. 4. Anti–HIV-1 activity of commercially available α-defensin 1 and 2 peptides (**A**) and purified α-defensin 1, 2, and 3 (**B**). The unconnected symbols at the lower right corner of each panel denote the antiviral activity of the highest concentration of α-defensins when an α-defensin–specific mAb (25 μg/ml) is also added.

Synthetic and purified human α-defensins can inhibit HIV-1 replication in vitro. We next turned our attention to the testing of synthetic or purified forms of α-defensins. Two products are commercially available: α-defensin 1 and 2 (American Peptide Co., Sunnyvale, CA). With increasing concentrations of a mixture (1:1) of these two synthetic α-defensins, a greater degree of inhibition was observed against six isolates of HIV-1 (Fig. 4), regardless of viral phenotype or genotype. The 50% inhibitory concentrations (IC_{50}) for the mixture ranged from ~11 to 24 μM, and there was no evidence of cytotoxicity associated with these peptides (*36*). Although the antiviral potency of this mixture was not great, it was noted that these commercial products were not pure (fig. S4). Thus, to ensure the specificity of the anti–HIV-1 activity of commercial α-defensin preparations, we repeated virus inhibition assays (*33*) with these peptides but now in the presence of an α-defensin–specific antibody. The antibody indeed neutralized substantially the anti–HIV-1 activity of commercial peptides (Fig. 4, A). This result suggests that the antiviral effect is not mediated by nonspecific contaminants in the commercial preparations; instead, the activity resides in elements that are recognized by the α-defensin–specific antibody.

We also examined the anti–HIV-1 activity of α-defensin 1, 2, and 3 purified from neutrophils of a normal person (*37*, *40*). This preparation contained α-defensin peaks that are virtually indistinguishable by mass spectrometry from those found in supernatants of CD8 T cells from LTNP-5 (fig. S5). It inhib-

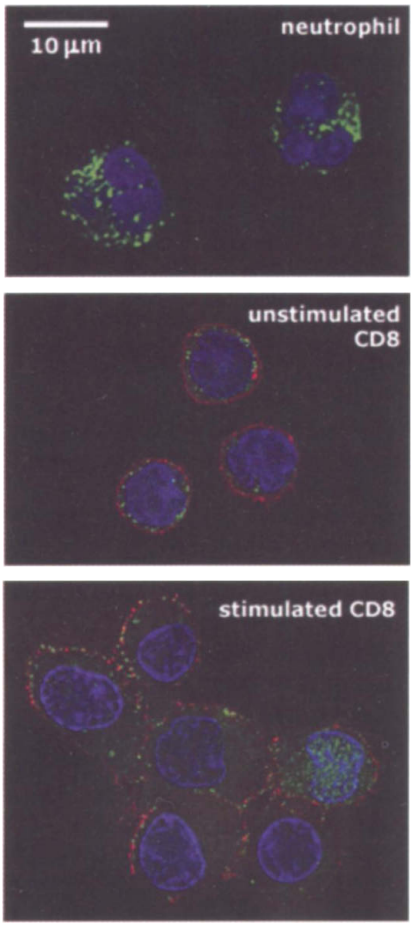

Fig. 5. Immunofluorescence staining of α-defensin 1, 2, and 3 in human neutrophils as well as in unstimulated and stimulated CD8 T lymphocytes. The procedure was carried out as described (*33*) such that α-defensins stain in green, CD8 proteins in red, and nuclei in blue. Cells stained with an irrelevant antibody are shown in fig. S6.

ited HIV-1 replication with IC_{50} of ~0.5 to 2.2 μM (Fig. 4, B) and without cytotoxicity, which suggests that purified α-defensins are about 10 to 20 times as potent against HIV-1 as commercial products. The antiviral effect of purified human neutrophil α-defensins was also substantially reduced or eliminated by the addition of the α-defensin–specific antibody.

A subset of CD8 T lymphocytes express α-defensin 1, 2, and 3. Neutrophils and CD8 T cells purified from several normal blood donors were studied by immunofluorescence (*33*) for intracellular expression of α-defensin 1, 2, and 3. A small fraction of unstimulated CD8 T lymphocytes carried these molecules within small cytoplasmic granules, but in quantities considerably less than that found in neutrophils (Fig. 5). Upon stimulation, some of the CD8 T cells seemed to lose the α-defensin–positive granules, presumably because of secretion into the culture supernatant. However, a small percentage of CD8 T cells were activated to express a higher amount of α-defensins (Fig. 5, cell on the extreme right).

By flow cytometric analysis (*33*), few unstimulated CD8 T lymphocytes expressed appreciable levels of α-defensins (*36*). After 1 day of stimulation, some of the cells containing α-defensin were no longer detectable. However, consistent with the immunofluorescence results, a population of CD8 T cells expressing higher amounts of α-defensins emerged after 2 days of stimulation. These findings further confirm that a small subset of CD8 T cells do indeed harbor and secrete α-defensin 1, 2, and 3, establishing yet another linkage between innate and acquired immune systems.

Discussion. The principal source for α-defensin 1, 2, and 3 is the neutrophil (*37*, *40*). However, additional producers have been described, including NK cells, γδ T cells, B cells, and monocyte/macrophages (*43*), as well as certain epithelial cells (*44*). Thus, it is not too surprising that these molecules are also made by CD8 T lymphocytes, as shown here. Having identified certain α-defensins as a major contributor to CAF, we think it will now be easier to determine the precise subpopulation of CD8 T cells that is producing this family of antiviral factors.

α-Defensin 1, 2, and 3 are cationic molecules, which contain three intramolecular disulfide bonds (*40*) (see Fig. 2B). These peptides can damage the membrane of bacteria, probably only when they are in a proper conformation constrained by disulfide linkages (*40*). We do not know whether this would be true of their anti–HIV-1 activity. Nor do we know the elements within α-defensins that mediate the antiretroviral effect. Likewise, the mechanism of action of defensins on HIV-1 is also unclear, although previous studies on CAF have suggested an effect on viral transcription (*12*, *13*).

Our studies to date do not adequately quantify the potency of α-defensins against HIV-1. Although the commercial products are active in blocking viral infection (Fig. 4), they also contain components with molecular masses that are incorrect for α-defensins (fig. S4). In addition, there is no assurance that proper forms are synthesized even for those peaks that have the right approximate mass. In this regard, it is worth noting two additional points. First, when α-defensin 1 or 2 was individually tested against HIV-1, the inhibitory activity was rather low compared with results for the two defensins combined. It is possible that the anti–HIV-1 activity is mediated by different defensins interacting together, perhaps through the formation of heterodimers. Second, the commercial products together inhibited HIV-1 replication with IC_{50} of 11-24 μM. However, we found that α-defensins purified from human neutrophils blocked HIV-1 infection 10 to 20 times better. Additional studies are necessary to define the true antiviral potency of α-defensins, which in turn will determine their clinical utility in treating HIV-1.

In light of our new findings, it is interesting to note that several defensins have been reported to have antiviral activity in general and anti–HIV-1 activity in particular. Human α-defensin 1 can inactivate herpes simplex virus types 1 and 2, cytomegalovirus, vesicular stomatitis virus, and influenza virus (*45*). Modest anti–HIV-1 activity has been found for defensin-like peptides from guinea pigs, rabbits, and rats (*46*), as well as from insect species (*47*). Last, a human homolog of the monkey θ-defensin, termed retrocyclin, has recently been demonstrated to block HIV-1 infection in vitro (*48*). More studies are required to relate these observations to ours. Nonetheless, we hope that the identification of α-defensin 1, 2, and 3 as a major component of the long-sought-after CAF will open up fruitful avenues of research.

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Figs. S1 to S6

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REPORTS

Guest Transport in a Nonporous Organic Solid via Dynamic van der Waals Cooperativity

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A well-known organic host compound undergoes single-crystal–to–single-crystal phase transitions upon guest uptake and release. Despite a lack of porosity of the material, guest transport through the solid occurs readily until a thermodynamically stable structure is achieved. In order to actively facilitate this dynamic process, the host molecules undergo significant positional and/or orientational rearrangement. This transformation of the host lattice is triggered by weak van der Waals interactions between the molecular components. In order for the material to maintain its macroscopic integrity, extensive cooperativity must exist between the molecules throughout the crystal, such that rearrangement can occur in a well-orchestrated fashion. We demonstrate here that even weak dispersive forces can exert a profound influence over solid-state dynamics.

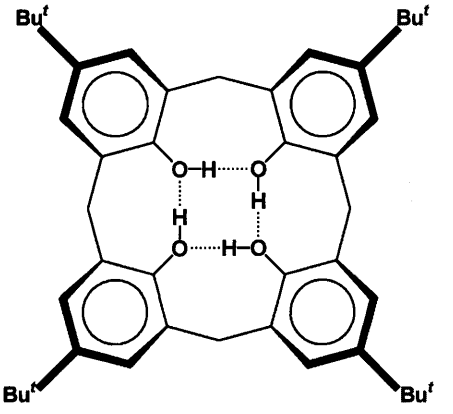
The assembly of organic molecular crystals is primarily controlled by a variety of intermolecular interactions which, in unison, immobilize the building blocks to form stable arrays (*1–6*). When these materials are heated beyond their melting or sublimation points, the cohesive forces are overcome, resulting in increased mobility and disorganization of the molecules. The molecules of a solid can also be mobilized by processes such as dissolution and solid-solid phase changes. The latter can occur as a result of physical stimuli (e.g., temperature, pressure, or radiation) or gain or loss of ancillary molecular components. Whereas inclusion of either a liquid or gaseous guest by a solid matrix is a well-known phenomenon, the mechanisms of such processes are poorly understood. This is particularly true for the organic solid state, where transport of the guest through the solid, and subsequent complexation, usually involves concomitant reorganization of the host lattice (*7*). In all but a few documented cases, guest-induced lattice rearrangement results in severe fracturing of single crystals into polycrystalline material (*8*). When fracturing does not occur, structural analysis of the successive solid phases can provide valuable insight into the dynamic processes that prevail. For instance, unambiguous single-crystal–to–single-crystal transformations preclude a mechanism that entails either complete or localized dissolution of one phase with subsequent growth of another (*9–11*). There have been several reports of monocrystalline host-guest (H-G) inclusion or decomposition reactions of the general type

$$\mathrm{H}\,(s) + n\mathrm{G}\,(l, \text{or } v) \rightleftharpoons \mathrm{H}\cdot\mathrm{G}_n(s) \qquad (1)$$

where it can be shown that the host lattice is porous (*9*, *12–16*). In these instances, it is presumed that the structural integrity of the channels is maintained during the transition, and a plausible mechanism thus simply involves diffusion of a highly mobile guest through these channels until a thermodynamically stable host-guest structure is achieved. This implies, therefore, that the design of an organic system for guest uptake should focus primarily on porosity of the host lattice.

We report the structure of a purely organic solid that does not contain channels, but nevertheless facilitates the diffusion of guest species through its lattice. Furthermore, guest uptake involves a single-crystal–to–single-crystal phase transformation with considerable displacement of the host molecules in two dimensions to give a nonporous inclusion complex. Our results imply that the organic solid state is often much more dynamic than generally believed and that the usually immobile molecules can be reorganized in an orderly fashion by weak dispersive forces.

Crystals of *p*-Bu^t-calix[4]arene (Scheme 1) were prepared by sublimation of **1** at 280°C under reduced pressure. Single-crystal x-ray analysis of the resulting unsolvated form, **1a**, reveals that the calixarene molecules arrange themselves into the well-known bilayer packing motif (*17*). Pairs of offset, facing calixarene molecules can be loosely described as dimers that form skewed capsules, each with an estimated free volume of 235 Å^3. As a result of these relatively large lattice voids, **1a** has a rather low packing efficiency (PE) of 0.59. It should be noted that a polymorphic form of **1**, grown from a tetradecane solution, has recently been published (*18*). The latter structure, **1b**, consists of a well-packed (PE = 0.67) arrangement of calixarene dimers, where each of the two facing molecules inserts one of its Bu^t groups deep into its neighbor's cavity. Purely organic solid-state frameworks rarely contain substantial lattice voids such as those observed in **1a**. Indeed, the



Scheme 1.

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