Lack of the CD8+ Cell Anti-HIV Factor in CD8+ cell granules

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Abstract

In HIV infection, CD8+ cells show cytotoxic and noncytotoxic anti-HIV activities. The latter function is mediated, at least in part, by a secreted antiviral protein, the CD8+ cell antiviral factor (CAF). Because antiviral effector molecules, such as perforin and granzymes, reside in the exocytic granules of CD8+ T cells, we examined the possibility that granules contain CAF-like activity. CD8+ cells from HIV-infected individuals showing strong CAF-mediated antiviral activity were induced to release their granule constituents into culture media. Within one hour post-stimulation, high levels of granzyme B (a primary granule constituent) were found in the culture fluids of previously activated CD8+ cells. The same culture fluids contained none, or very low, amounts of CAF activity, as measured with HIV-infected CD4+ cells. Maximal levels of CAF activity were not observed until five or seven days post-stimulation, consistent with typical CAF production kinetics. In addition, extracts of granules purified from antiviral CD8+ cells did not show any CAF activity, whereas the cytoplasmic fraction of these cells showed substantial levels of antiviral activity. These findings suggest that CAF does not reside at appreciable levels in the exocytic granules of antiviral CD8+ T cells.
Introduction

In HIV infection, two types of cellular antiviral responses have been described associated with CD8+ T cells. One response, mediated by cytotoxic T lymphocytes (CTL), involves the cytolysis of infected cells in an HLA-restricted manner (1). Cytolysis results from the targeted release of the contents of exocytic granules from the CD8+ cell. These granules contain perforin and granzymes which act together to induce apoptosis in the targeted cell (2, 3). In addition to these effector molecules, other immune factors reside in the exocytic granules of CD8+ T cells, such as granulysin and β-chemokines (4, 5). Naive T cells do not have detectable granules, but once activated, the granule content of the CD8+ T cell increases, reaching a peak level 5 to 10 days later (6).

The second type of CD8+ cell-mediated anti-HIV response involves the suppression of HIV replication in cultured CD4+ cells in the absence of cell killing (7). This CD8+ cell noncytotoxic antiviral response (CNAR) is not HLA-restricted, blocks HIV transcription, and is associated with the production of a novel CD8+ cell antiviral factor (CAF) (7-10). CAF lacks identity to other known cytokines, including granzymes and chemokines (7, 11, 12). Its peak production occurs 5-9 days after activation of the CD8+ cells (7, 11). Because of the antimicrobial content of CD8+ T cell granules, and the similarity in kinetics of synthesis of granules (6) and CAF production, we sought to determine whether CAF resides in the exocytic granules of CD8+ T cells of HIV-infected individuals.

Materials and Methods

Subjects

Heparinized peripheral blood samples were obtained by venipuncture from HIV-1 seropositive donors previously characterized for their CD8+ cell anti-HIV activity. The HIV-
infected individuals were clinically healthy males with CD4+ T-cell counts of >500 cells/µl and viral loads <6000 copies/ml. Two subjects were on antiretroviral therapy. Blood samples from HIV-seronegative donors were provided by Blood Centers of the Pacific (San Francisco, CA). This study received the approval of the Committee on Human Research, University of California, San Francisco (UCSF).

**Induction of granule release from antiviral CD8+ cells**

CD8+ T cells from HIV-infected individuals, previously shown to be good producers of CAF, were isolated from the heparinized blood by immunomagnetic bead (IM) (Dynal, Lake Success NY) separation as described (13). The purity of the isolated CD8+ cells was always >95% as determined by flow cytometry. The cells were washed x 3 and cultured in complete RPMI 1640 medium (Mediatech, Herndon, VA) containing 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS), 2 mM glutamine, 1% antibiotics (100 µg/ml penicillin; 100 µg/ml streptomycin) (Tissue Culture Facility, University of California, San Francisco) and 100 U/ml recombinant IL-2 (rIL-2, provided by Glaxo Wellcome, Research Triangle Park, NC). The cells were then plated in 24-well plates at a density of 4x10^6 cells/ml in the presence of either 25 ng/ml of phorbyl ester (PMA) plus 100 ng/ml ionomycin (Sigma Chemicals, St. Louis, MO), or anti-CD3 IM beads (at a 4:1 bead:cell ratio) (11), or left untreated. After 1 and 3 hours of culture, all of the culture fluid was collected (stored at –80°C), and the cultures were replenished with fresh medium. The untreated and anti-CD3 IM bead-treated CD8+ cell cultures were maintained with passing and collecting fluids on day 3 and every two days thereafter (11). In all cases, the anti-CD3 IM beads were removed from the cultures after the initial 3-day activation period.

Previously activated CD8+ cells were obtained from the cultures stimulated with anti-CD3 IM beads (see above) 9 to 11 days after the initial activation. They were washed three times and
then given a second round of stimulation analogous to the first round. In each case, the viability (measured by trypan blue dye exclusion) of these previously stimulated CD8+ cells was greater than 85%.

**Subcellular fractionation and isolation of CD8+ cell granules**

Antiviral CD8+ cells were stimulated for 3 days with anti-CD3 IM beads, then separated from the IM beads and passaged at a density of 2x10^6 cells/ml into serum-free AIM-V medium (Gibco-BRL, Gaithersburg, MD) supplemented with 200 U/ml of rIL-2. The cultures were passed every two days thereafter, collecting samples at each passage for monitoring CAF activity. On days 7 or 9, the CD8+ cells (80-93% viable) were washed twice with phosphate buffered saline (PBS) and then disrupted in a cavitation bomb as described (14). In brief, the cells were adjusted to about 5x10^8 cells/ml in cold relaxation buffer prior to placing in a chilled cavitation bomb (Parr Instrument Co., Moline, IL) on ice. The cavitated material was then processed through several centrifugation steps to remove cell fragments, debris, and nuclei. The resulting supernatant was centrifuged to pellet the granules, leaving the cytoplasmic supernatant. The pelleted granules were resuspended in 1-1.5 ml of PBS, then put through four freeze/thaw cycles to burst the granules. Ultracentrifugation of this material resulted in a fraction containing soluble granule contents and a pellet of granule membranes. Because various proteins have been found associated with the granule membrane (including granzymes), we treated this fraction with 1 M NaCl to dissociate bound proteins. These solubilized proteins were recovered following ultracentrifugation to remove the granule membranes. The three fractions of interest, the cytoplasm, the soluble granule contents, and the granule membrane-associated proteins, were all immediately frozen at -80°C prior to analysis.
Quantification of granzyme B

The level of granzyme B in CD8+ cell culture fluids was measured by ELISA (15). In the subcellular fractions of CD8+ cells, granzyme B levels were determined with a functional assay based on their enzymatic activity (14).

Assay for CAF activity

The extent of anti-HIV activity in the CD8+ cell culture fluids was measured using a standardized acute virus infection assay (11). In brief, phytohemagglutinin-P (3 µg/ml; Sigma Chemicals)-stimulated CD4+ cells cultured from HIV seronegative subjects were acutely infected for one hour with 4,000 TCID50 of HIV-1SF2, a β-chemokine-insensitive isolate (16). The infected CD4+ cells (>90% pure by flow cytometry) were washed to remove free virus, and 10^5 cells were plated per well in a 96-well culture plate. Unless noted otherwise, the target cells were cultured in triplicate in a 50% dilution of the test fluid (i.e. CD8+ cell culture fluid, subcellular CD8+ cell fraction, or control medium or buffer). The cultures were passed every two days monitoring for reverse transcriptase (RT) activity (17). Fresh CD8+ cell supernatant, test material, or control medium was added at each passage. Using this assay for CAF activity, the maximum amount of reduction in RT activity typically observed is between 50-80% (7, 11).

Results

Induction of exocytic granule release from CD8+ cells

Freshly isolated CD8+ T cells from HIV-infected individuals were induced to release exocytic granules by short-term stimulation with PMA plus ionomycin, or anti-CD3 IM beads. Both stimuli have been shown to induce high levels of granule release from murine and human CTLs within one hour of treatment (6). After one and three hours of exposure to either of these
activating stimuli, the levels of granzyme B (a major constituent of T cell exocytic granules) in the culture fluids were relatively low (<15 ng/ml) and not substantially different from the levels found in untreated CD8+ cells (Fig. 1A). In the same 1 h and 3 h fluids, little anti-HIV activity was detected (≤ 25% reduction in HIV replication). However, maximal levels of CAF production were observed at days 5 and 9 in the anti-CD3 IM bead-treated cultures, as expected for the typically cyclical kinetics of CAF production (7, 11). Previous studies have shown that the granzyme B levels in CD8+ cell culture supernatants from days 5 to 13 after stimulation with α-CD3 IM beads are relatively low or absent (12). Stimulation with PMA plus ionomycin led to massive death in the CD8+ cells after 1-2 days of culture (as expected), so CAF production could not be followed similarly in these cultures.
Figure 1. Granule release (granzyme B levels) and CAF production from antiviral CD8+ cells.

Freshly isolated CD8+ cells (A) from an HIV-infected subject showing strong CAF activity were left untreated (■) or activated with anti-CD3 IM beads (●) or with PMA plus ionomycin and cultured as described in the methods. (B) On day 9 or 11, CD8+ cells from anti-CD3 IM bead-activated cultures were washed, then treated as in (A). At the indicated time points, samples of culture fluid were taken and frozen at -80°C. Granzyme B levels in the culture fluids were measured by ELISA (detection limit of 50 pg/ml). CAF activity in the culture fluids, indicated as the percent suppression of HIV replication (+/− SD error bars), was measured in a standardized β-chemokine-insensitive virus acute infection assay (see text). The data are representative of two experiments (each with a different subject).

Because of the apparent lack of granule release from the freshly isolated CD8+ T cells from HIV-infected individuals, we evaluated granule release from the CD8+ cells previously activated
with \( \alpha \)-CD3 IM bead treatment. CD8+ cells actively producing CAF, taken 9 to 11 days after initial activation, were re-stimulated to induce granule release in an analogous fashion to the treatment of the freshly isolated cells (Fig. 1B). Both PMA plus ionomycin and \( \alpha \)-CD3 IM beads induced release of high levels of granzyme from the CD8+ cells within one hour of treatment (generally from 85 to \( >200 \) ng/ml), suggesting effective granule exocytosis. CD8+ cells in the untreated cultures, however, produced low levels of granzyme B, between 17 and 25 ng/ml (Fig. 1B). This result was likely due to residual synthesis following the initial stimulation, which was between 20 and 31 ng/ml at days 9 and 11 (data not shown). CAF levels in the fluids from the untreated and the re-stimulated cultures were minimal after 1 h and 3 h, never reaching more than 25% reduction of HIV replication (Fig. 1B). In contrast, strong CAF production was seen in the cultures 5 days after CD8+ cell stimulation (Fig. 1B). This finding is again consistent with the kinetics of CAF production from freshly isolated stimulated CD8+ cells (Fig. 1A) (7, 11). These results suggest that CAF is likely not at appreciable levels in exocytic granules, but instead is secreted late after activation.

**CAF activity in subcellular fractions of CD8+ cells**

To further evaluate the possibility that CD8+ cell granules contain CAF antiviral activity, we analyzed extracts of granules purified from antiviral CD8+ cells. Purified CD8+ cells from HIV-infected individuals were ruptured by cavitation and the constituents processed to yield subcellular fractions representing the cytoplasm, the soluble granule contents, and the granule membrane-associated components (see Methods). The latter fraction was prepared (using high salt extraction) because some of the proteins residing in granules associate with the granule membrane. Granzyme B levels were used as an indicator of granule purification efficiency and were measured by an assay dependent on the enzymatic activity of this protein. The results indicate that the fractionation
procedure was mild enough to leave proteins (e.g. granzyme B) functionally intact. Representative results from three of six subjects studied are presented (Table): two that showed typical CAF production at 5-7 days (subjects A and B) and one that lacked production of CAF (subject C). In the CD8+ cell extracts, low or no granzyme B was detected in any of the cytoplasmic fractions, whereas the highest levels were found in the soluble granule fractions indicating ideal granule isolation (Table). The amount of granzyme B in the soluble granule fractions ranged from about 650-5000 ng. This amount is about 10-60% of that released by a CD8+ CTL cell line (on a per cell basis) following induction of granule exocytosis (15) (Table, Fig. 1).

Appreciable CAF activity was only found in dilutions of cytoplasmic fractions, and only in those fractions from CD8+ cells that produced CAF in culture (Fig. 2; Table, subjects A and B). In some cases, the granule fractions showed enhancement of HIV replication but these findings were not dose-dependent nor found consistently in the subjects. The antiviral activity in the one granule membrane-associated fraction was associated with toxicity of the CD4+ cells, and thus does not reflect CAF activity (Fig. 2A). Except for this one case, the dilutions of the various subcellular fractions did not affect the target CD4+ cell viability or growth (data not shown).
<table>
<thead>
<tr>
<th>Subject</th>
<th>CAF activity (% Suppression)</th>
<th>Fraction</th>
<th>Concentration (nM)</th>
<th>Total volume (ml)</th>
<th>Total yield (ng)</th>
<th>CAF activity (see Fig. 2)</th>
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<tr>
<td>A</td>
<td>46</td>
<td>Cytoplasm (organelle-free)</td>
<td>0</td>
<td>2000</td>
<td>0</td>
<td>+</td>
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<td></td>
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<td>1500</td>
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<td></td>
<td></td>
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<td>200</td>
<td>2870</td>
<td>-</td>
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<tr>
<td>B</td>
<td>61</td>
<td>Cytoplasm (organelle-free)</td>
<td>0.9</td>
<td>1000</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soluble granule contents</td>
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<td>1000</td>
<td>1973</td>
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<tr>
<td></td>
<td></td>
<td>Granule membrane-assoc.</td>
<td>193</td>
<td>100</td>
<td>617</td>
<td>-</td>
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<tr>
<td>C</td>
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<td>1000</td>
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<td>100</td>
<td>4845</td>
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</table>

These results are representative of 6 subjects studied. The activity of the CD8+ cell antiviral factor (CAF) was measured as described in the text. Fluids were collected at 9 days (subject A) or 7 days (subjects B and C) after CD8+ cell stimulation\(^13\). The total number of CD8+ cells processed was \(13.7 \times 10^8\) (subject A) and \(5.0 \times 10^8\) (subjects B and C).

The granzyme B concentration is measured with an enzymatic assay (see text).

*The total granzyme B yield was calculated from the respective concentration and total volume.

The total volume of each fraction was ~170-300-fold (1700-3000-fold in the case of the membrane-associated fraction) less per number of cells than that used to obtain the CAF-active culture supernatants.
Figure 2. Antiviral activity of subcellular fractions isolated from CD8+ cells of HIV-infected subjects. Subcellular fractions, obtained during the purification of intracellular granules, were diluted in the respective fractionation buffer and added to acutely infected CD4+ cells in a standardized β-chemokine-insensitive virus assay for CAF activity. ■ cytoplasmic fraction; □ soluble components of granzymes; □ granule membrane. The percent suppression of HIV replication (+/- SD error bars) was determined by dividing the amount of peak RT activity (day 7) in the culture fluids treated with the test sample by that treated with the respective buffer control, x 100. The amount of peak RT activity in the control infected CD4+ cell cultures was always greater than 200,000 cpm/0.1 ml. Some toxicity was noted in the least diluted granule membrane extract of subject A (*), which explains the reduction in RT activity observed. Cultured CD8+ cells from subjects A and B
showed production of CAF in cell culture; those from subject C did not (Table). Negative values indicate enhancement of HIV replication.

**Discussion**

CD8+ T lymphocytes can suppress HIV replication in a non-cytotoxic manner. At least part of this activity is mediated by production of a novel CD8+ cell antiviral factor, CAF (7). The present studies were conducted to determine whether CAF resides in granules where other effector components such as perforin, granzymes and granulysin can be found. The results indicate that granules of CD8+ T cells from HIV-infected individuals do not contain the antiviral activity associated with CAF (Figures 1, 2). Instead, an anti-HIV activity was found in the cytoplasmic fractions of these antiviral CD8+ cells (Fig. 2, Table). This antiviral activity observed in the cytoplasmic fraction was only associated with CD8+ cells that produced CAF, suggesting that the anti-HIV activity observed in these fractions resulted from CAF. This conclusion is supported by the lack of demonstrable CAF-like activity associated with granule exocytosis immediately following mitogenic stimulation of CD8+ cells (Fig. 1). CAF production, however, did become detectable 3 to 5 days later as has been commonly demonstrated with activated CD8+ cells from healthy HIV-infected individuals (Fig. 1) (7, 11). These findings suggest CAF is not stored at substantial levels in the cell but requires new synthesis for its production.

The present results are consistent with previous work in our laboratory showing that the granule constituents, granzymes A and B and granulysin, do not show anti-HIV activity and that CAF activity in CD8+ cell culture fluids does not correlate with the granzyme concentrations in these fluids (12, 18). Moreover, these studies also
indicate that CAF does not appear to be produced with typical kinetics of cytokine production by CD8+ T cells, which is highest early after activation, then decreases after one to three days.

The absence of CAF activity in the extracts of exocytic granules does not likely result from inactivation of the antiviral factor during protein purification. The recovered granzyme B was still enzymatically active and we have observed stability of CAF activity following several freeze-thaw procedures with CD8+ cell supernatants (unpub. obs). The results of this study, therefore, support the conclusion that CAF does not reside in appreciable amounts in exocytic granules but is directly secreted by CD8+ cells.

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References


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