AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 16, Number 4, 2000, pp. 367–372
Mary Ann Liebert, Inc.

Short Communication

HIV Virions and HIV Infection *in Vitro* Are Unaffected by Human Granzymes A and B

CARL E. MACKEWICZ,[1] JUDY LIEBERMAN,[2] CHRISTOPHER FROELICH,[3] and JAY A. LEVY[1]

ABSTRACT

Granzymes are a family of serine proteinases commonly found in the granules of $CD8^+$ T cells. In HIV infection, $CD8^+$ cells show cytotoxic and noncytotoxic antiviral activities. The latter is mediated, at least in part, by a secreted $CD8^+$ cell antiviral factor, CAF. Because of the antiviral nature of $CD8^+$ cells, we examined the potential anti-HIV activity of free granzymes that can be found in $CD8^+$ cell culture fluids. Pretreatment of $CD4^+$ T cells with granzyme A or granzyme B had no effect on their susceptibility to infection with HIV, nor did incubation of the granzymes with HIV virions alter their infectivity. Continuous culture of acutely infected $CD4^+$ T cells with granzyme A or B showed no effect on cell viability or the replication of HIV. The findings of this study suggest that free granzymes do not control HIV infection and spread in $CD4^+$ T cells.

GRANZYMES (GRANULE ENZYMES) are serine proteinases found in the granules of $CD8^+$ T cells and natural killer (NK) cells (reviewed in Ref. 1). Granzyme A (a tryptase) and granzyme B (an aspase) are by far the most abundant of the five known human granzymes, and together with perforin make up the majority of the nonproteoglycan granule protein content. Granzymes are thought to function primarily in concert with perforin to mediate the antigen-targeted killing of virus-infected or allogeneic cells by cytotoxic lymphocytes. Targeted delivery of granule constituents leads to the perforin-mediated entry of granzymes into the target cell cytoplasm and nucleus, where the granzymes activate cell death pathways. Granzyme A induces a newly described caspase-independent pathway[2] and granzyme B induces the proteolytic cleavage of one or more members of the caspase cascade as well as other cellular substrates.[1] The end result is in either case the apoptotic death of the cell.

In HIV infection, $CD8^+$ cells are thought to play a major role in the immunologic control of virus replication. The immune response to infection results in an increased number of activated $CD8^+$ T cells[3–5] and a corresponding increase in $CD8^+$ T cells that express granzymes, in both the peripheral blood and lymph node compartments.[6–9] Increased levels of granzymes have also been found in the plasma of HIV-infected individuals relative to seronegative individuals,[10] possibly reflecting excess antigen-targeted granule release or the constitutive synthesis and secretion of granzymes by $CD8^+$ T cells.[11]

Both cytotoxic[12] and noncytotoxic[13] anti-HIV functions of $CD8^+$ cells have been well documented. The latter activity does not involve cell killing, does not require HLA compatibility, and is mediated, at least in part, by a soluble $CD8^+$ cell antiviral factor (CAF).[13] CAF has not been identified, but it appears to be unlike other known cytokines and chemokines.[14–16] Granzymes are secreted by $CD8^+$ cells, are found at elevated levels in HIV infection, have cytokine-inducing activity[17,18] and have antiviral activity against vesicular stomatitis virus (VSV) and adenovirus.[19,20] Therefore, we evaluated whether free granzymes have antiviral activity against HIV and if granzymes might be linked to CAF activity.

We first tested whether pretreatment of $CD4^+$ cells with granzymes affect the susceptibility of these cells to HIV infection, possibly by directly cleaving a cell surface protein involved in HIV entry or by inducing the production of regulatory cytokines. Phytohemagglutinin-P (PHA, 3 μg/ml; Sigma, St. Louis, MO)-stimulated $CD4^+$ cells (3×10^6 cells/ml) from HIV-seronegative control subjects were incubated in serum-free

[1]Department of Medicine and Cancer Research Institute, University of California, School of Medicine, San Francisco, California 94143.
[2]Center for Blood Research and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115.
[3]Rheumatology Research Laboratory, Women's Hospital B643, Evanston Northwestern Healthcare, Evanston, Illinois 60201.

AIM-V medium (GIBCO-BRL, Gaithersburg, MD) for 2 hr with a 5-μg/ml concentration of purified or recombinant granzyme A, granzyme B, or their inactive S → A mutant controls prior to infection. Recombinant human granzyme A and its inactive analog S → A granzyme A (*Escherichia coli* expressed), and recombinant murine granzyme B and its inactive recombinant analogue S → A granzyme B (baculovirus expressed), were prepared as described.[21–23] The proteolytic activity of recombinant granzyme A was 2.86×10^6 M^{-1}•sec^{-1}, using Z-Arg-SBzl as the substrate, and the activity of recombinant granzyme B was 4.5×10^4 M^{-1}•sec^{-1} using BAAD-SBzl as the substrate. The activity of recombinant murine granzyme B on human target cells is similar to that of human granzyme B.[23] In the inactive mutants, the active-site serine is changed to an alanine, rendering the enzymes proteolytically inactive. The recombinant proteins were virtually free of contaminating endotoxin. The purification of human granzyme A (167 units/μg; 1 unit hydrolyzes 1 nmol of BLT per minute) from interleukin 2 (IL-2)-stimulated T cell granules and granzyme B (6.1 units/ug; with BAADT substrate) from the YT NK cell line has been described.[24] After incubation with granzyme, the cells were washed twice and inoculated with 600 median tissue culture infective doses ($TCID_{50}$) of HIV-1_{SF2} molecular clone.[25] The cells were incubated for 1 hr at 37°C, then washed three times, and 3×10^5 infected cells were cultured in 600 μl of complete medium, which consisted of RPMI 1640 medium (Mediatech, Herndon, VA) containing 10% heat-inactivated (56°C for 30 min) fetal calf serum (FCS), 1% antibiotics (penicillin [100 ug/ml] and streptomycin [100 μg/ml]), 2 mM glutamine, and recombinant IL-2 (100 U/ml; Glaxo-Wellcome, Research Triangle Park, NC) in a 48-well culture plate in quadruplicate. The cultures were passed and monitored for reverse transcriptase (RT) activity[26] every 3 days.

Exposure of $CD4^+$ T cells to purified or recombinant forms of granzyme A did not affect their sensitivity to subsequent infection and replication of HIV. For example, treatment of the $CD4^+$ cells with purified human granzyme A or recombinant granzyme A resulted in peak RT levels (±SD) of 341,000 ± 76,000 and 446,000 ± 108,000 cpm/0.5 ml, respectively, which was not appreciably different from the RT levels observed in the untreated (392,000 ± 105,000 cpm/0.5 ml) and the inactive S → A granzyme A-treated (445,000 ± 49,000 cpm/0.5 ml) $CD4^+$ cell cultures. The virus replication kinetics were also not affected. A similar lack of effect was observed with purified granzyme B and recombinant murine granzyme B (data not shown).

To test the possibility that the granzymes can affect HIV replication by directly inactivating HIV virions via cleavage of an external protein such as gp120, the granzymes or their inactive S → A mutant controls were added to 750 $TCID_{50}$ of HIV-1_{SF2} (diluted in AIM-V serum-free medium) to yield a 5-μg/ml concentration of granzyme or the inactive S → A control. The mixtures were incubated for 2 hr at 37°C before being used to infect 3×10^6 PHA-stimulated $CD4^+$ cells for 1 hr. The cells were washed and cultured in triplicate as described above. Treatment of HIV virions with either purified or recombinant granzyme A did not significantly alter their infectivity, as indicated by similar peak levels of virus replication (Fig. 1) and replication kinetics (data not shown), relative to the untreated and inactive S → A control. Treatment with purified granzyme B yielded similar results (Fig. 1). Treatment of HIV virions with recombinant granzyme B, however, usually

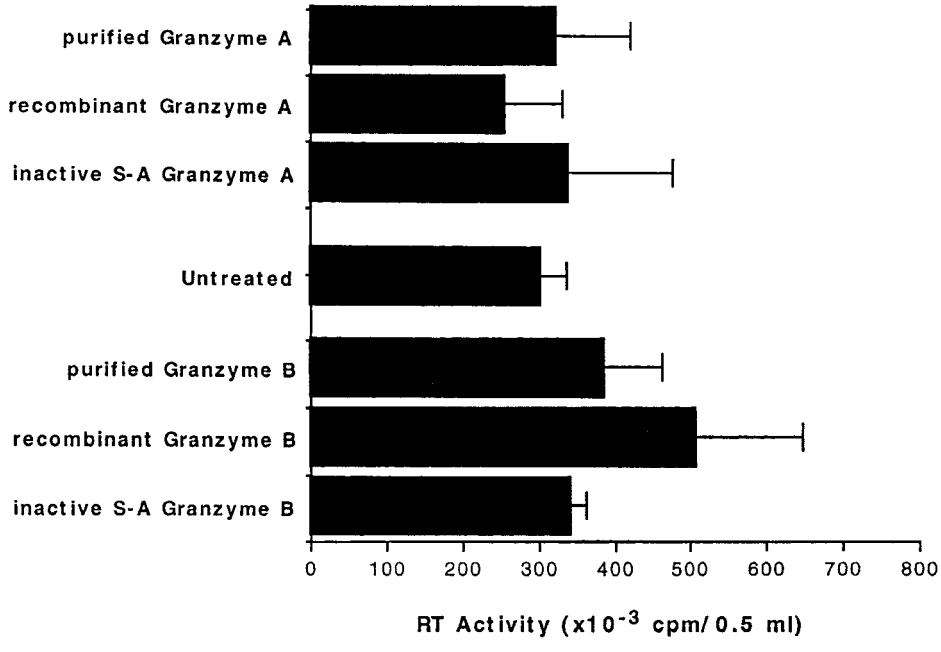

FIG. 1. The effect of granzyme A and granzyme B on virion particle infectivity. HIV virions were incubated in the presence of diluent (untreated) or a 5-μg/ml concentration of the indicated forms of granzyme A or granzyme B for 2 hr at 37°C. $CD4^+$ lymphoblasts were then infected with the treated or untreated virus and cultured. HIV replication (indicated by RT activity) at the peak of virus production (usually day 9–12) (±SE) is shown.

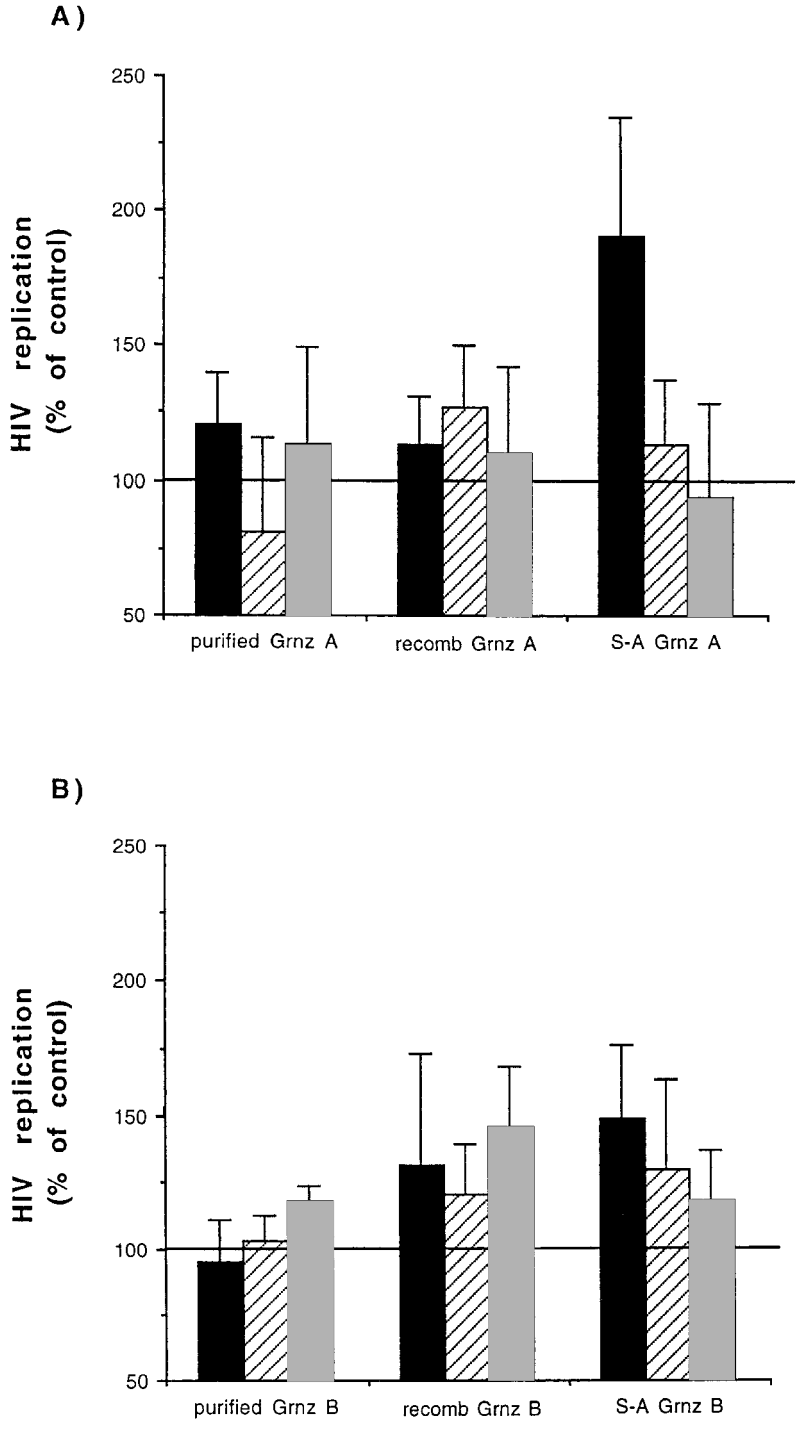

FIG. 2. The effect of granzyme A and granzyme B on HIV replication in $CD4^+$ T cells. PHA-stimulated $CD4^+$ T cells, acutely infected with HIV-1_{SF2}, were cultured (in triplicate) alone or in the continued presence of (**A**) purified or recombinant granzyme A (black columns, 0.05 μg/ml; hatched columns, 0.5 μg/ml; gray columns, 5 μg/ml) or its inactive S $\rightarrow$ A control (S-A Grnz A) or (**B**) purified or recombinant granzyme B (Grnz B) or its inactive S $\rightarrow$ A control (S-A Grnz B). The amount of HIV replication, relative to the untreated control ($\pm$SE), in the presence of each granzyme concentration reflects the peak RT activity of the treated $CD4^+$ cell cultures divided by that of the untreated $CD4^+$ cells, multiplied by 100. The peak RT activity of the untreated infected $CD4^+$ cells ranged from 90,000 to 220,000 cpm/0.1 ml.

resulted in slightly increased HIV replication, but this result did not reach significance ($p = 0.15$, Student t test) relative to either the untreated or the S → A granzyme B control (Fig. 1). In addition, recombinant granzyme B treatment of HIV virions did not alter their infectivity of the Jurkat derivative T cell line 1G5 (data not shown). These results indicate that neither granzyme A nor granzyme B proteolytically inactivated HIV virions.

We next evaluated whether culturing acutely infected $CD4^+$ T cells in the continued presence of granzymes would affect HIV replication. PHA-stimulated $CD4^+$ T cells were inoculated with approximately 3000 $TCID_{50}$ of HIV-1_{SF2}. After a 1-hr incubation, the cells were washed and plated (in triplicate) in 96-well culture plates (10^5 cells/well) in a final volume of 200 μl of complete medium. The infected $CD4^+$ cells were cultured alone or in the presence of various concentrations of granzymes (diluted in AIM-V medium), which were added at the initiation of culture and every 2 days thereafter on cell passage. The amount of HIV replication in the cultures was determined every 2 days by measuring RT activity in culture fluid samples.

In repeated experiments, both purified and recombinant forms of granzyme A and granzyme B usually induced slightly elevated levels of HIV production relative to the untreated control (Fig. 2), without any detectable effect on cellular viability. The enhancing effect often showed inconsistent dependency on dose and never reached significance. Both of the inactive mutants of recombinant granzyme A and granzyme B also enhanced HIV replication at various doses comparable to the active forms. Because granzymes have been shown to induce the production of certain cytokines from peripheral blood mononuclear cells (PBMCs), including IL-6 and tumor necrosis factor α (TNF-α),[17] the slight increase in HIV production might be related to increased release of these cytokines. Thus, continuous exposure of acutely infected $CD4^+$ T cells to granzyme A or granzyme B did not affect their viability or reduce their ability to replicate HIV.

In other studies, the level of granzyme B in various $CD8^+$ cell culture fluids containing or not containing CAF activity was measured by enzyme-linked immunosorbent assay (ELISA).[10] These culture fluids were prepared as described.[14] $CD8^+$ cells, purified from the PBMCs of HIV-seropositive individuals, were cultured in complete medium with anti-CD3-coupled immunomagnetic (IM) beads at a bead-to-cell ratio of approximately 4:1. After 3 days, the IM beads were removed and serum-free AIM-V medium was added. Every 2 days thereafter, culture fluids were collected and the cells passaged with fresh AIM-V medium. The cell density was adjusted to 2×10^6/ml at each passage. Fluids were assayed for anti-HIV activity as described.[14] All the $CD8^+$ cell culture fluids tested showed substantial levels of granzyme B protein ranging from 3 to 9 ng/ml (Table 1). The amounts of granzyme B protein released into these fluids did not correlate with the extent of their anti-HIV suppressing activity.

This study examined the potential antiviral role in HIV infection of free granzyme A and granzyme B, the most abundant granzymes. The effect of granzymes on three parameters relating to the infection and replication of HIV in $CD4^+$ T cells was studied. Neither human granzyme A nor granzyme B substantially affected (1) $CD4^+$ T cell sensitivity to infection by HIV, (2) HIV virion infectivity (Fig. 1), or (3) ongoing virus replication in acutely infected $CD4^+$ T cells (Fig. 2). These findings suggested that the elevated levels of free granzymes in the blood of HIV-infected individuals[10] do not play a direct role in the control of HIV.

Both recombinant and purified natural granzymes were examined in these studies and the concentrations of the proteolytically active granzymes used were sufficient to induce the various other biological activities reported for granzymes. These include cytokine production,[17,18] inactivation of VSV,[19] perforin-mediated apoptosis,[1,2,23] the proteolytic activation of the thrombin receptor,[27,28] and cleavage of extracellular matrix proteoglycans.[29,30] Thus, biologically relevant levels of granzymes were examined, suggesting that the lack of effect on the parameters studied cannot be explained by inappropriate granzyme concentrations.

In contrast to our results with HIV, free granzymes have been reported to exhibit antiviral activities in other systems. The addition of purified granzyme B, but not granzyme A or perforin, to VSV-infected U937 cells resulted in prompt apoptotic cell death and was associated with the rapid degradation of viral RNA and inhibition of infectious virus production.[19] Similarily, incubation of Jurkat T cells with adenovirus in the presence of granzyme B leads to apoptosis of infected cells.[20] From these studies and others that document the endocytic uptake of granzyme A and B,[31–33] it has been proposed that granzymes efficiently enter the cell through a receptor-dependent endocytic pathway.[1] Perforin or virus (e.g., VSV and adenovirus) then facilitates the release of the granzyme to the cytosol. In our study, therefore, the granzymes are likely endocytosed by the $CD4^+$ T cells, but the failure to observe cell death suggests HIV is unable to deliver the internalized proteinases from endocytic vesicles to the cytosol and nucleus.

The identity of the $CD8^+$ cell antiviral factor (CAF) is not known but it appears to differ from other cytokines and chemokines.[14–16] The present studies indicate that neither free granzyme A nor granzyme B has intrinsic anti-HIV activity. In addition, taken together with the lack of correlation between

TABLE 1. GRANZYME B LEVELS IN $CD8^+$ CELL CULTURE FLUIDS

Sample	*Anti-HIV activity*[a]	*Granzyme B level (ng/ml)*[b]
A	65	3
B	50	7
C	55	4
D	60	3
E	54	9
F	43	8
G	11	7
H	0	5
I	0	7
J	0	8

[a]The extent of anti-HIV activity in culture fluids from activated $CD8^+$ cells from HIV-infected individuals is indicated as the percent suppression of HIV replication in $CD4^+$ cells measured as described.[14]

[b]Granzyme B levels were determined by ELISA.[10] The sensitivity of detection was about 50 pg/ml.

the level of granzyme B and suppressing antiviral activity in $CD8^+$ T cell culture fluids (Table 1), our findings suggest that granzymes are not directly responsible for CAF-like antiviral activity.

ACKNOWLEDGMENTS

We thank Michael Luther (Glaxo-Wellcome Corporation) for providing the recombinant IL-2 used in these studies. Susan Ridha is thanked for her technical assistance. We thank Paul Beresford and Zhan Xia for preparing granzymes. This research was supported by grants from the National Institutes of Health (RO1AI30350 and RO1 AI42519) (J.A.L.) and by National and Chicago Area Chapter Arthritis grants (C.J.F.). We thank Ann Murai and Kaylynn Peter for help in preparation of the manuscript.

REFERENCES

1. Froelich CJ, Dixit VM, and Yang X: Granule mediated apoptosis: A matter of viral mimicry. Immunol Today 1998;19:30–36.
2. Beresford PJ, Xia Z, Greenberg AH, and Lieberman J: Granzyme A loading induces rapid cytolysis and a novel form of DNA damage independently of caspase activation. Immunity 1999;10:585–594.
3. Giorgi JV, Ho H-N, Hirji K, Chou C-C, Hultin LE, O'Rourke S, Park L, Margolock JB, Ferbas J, and Phair JP: $CD8^+$ lymphocyte activation at human immunodeficiency virus type 1 seroconversion: Development of HLA-DR+ CD38− $CD8^+$ cells is associated with subsequent stable $CD4^+$ cell levels. J Infect Dis 1994; 170:775–781.
4. Landay AL, Mackewicz C, and Levy JA: An activated $CD8^+$ T cell phenotype correlates with anti-HIV activity and asymptomatic clinical status. Clin Immunol Immunopathol 1993;69:106–116.
5. Prince HE and Jensen ER: Three-color cytofluorometric analysis of CD8 cell subsets in HIV-1 infection. J Acquir Immune Defic Syndr 1991;4:1227–1232.
6. Tenner-Racz K, Racz P, Thome C, Meyer CG, Anderson PJ, Schlossman SF, and Letvin NL: Cytotoxic effector cell granules recognized by the monoclonal antibody TIA-1 are present in $CD8^+$ lymphocytes in lymph nodes of human immunodeficiency virus-1-infected patients. Am J Pathol 1993;142:1750–1758.
7. Devergne O, Peuchmaur M, Crevon MC, Trapani JA, Maillot MC, Galanaud P, and Emilie D: Activation of cytotoxic cells in hyperplastic lymph nodes from HIV-infected patients. AIDS 1991;5: 1071–1079.
8. Koopman G, Wever PC, Ramkema MD, Bellot F, Reiss P, Keehnen RMJ, Ten Berge IJM, and Pals ST: Expression of granzyme B by cytotoxic T lymphocytes in the lymph nodes of HIV-infected patients. AIDS Res Hum Retroviruses 1997;13:227–233.
9. Trimble LA and Lieberman J: Circulating CD8 T lymphocytes in human immunodeficiency virus-infected individuals have impaired function and downmodulate CD3 zeta, the signaling chain of the T-cell receptor complex. Blood 1998;91:585–594.
10. Spaeny-Dekking EH, Hanna WL, Wolbink AM, Wever PC, Kummer AJ, Swaak AJ, Middeldorp JM, Huisman HG, Froelich CJ, and Hack CE: Extracellular granzymes A and B in humans: Detection of native species during CTL responses in vitro and in vivo. J Immunol 1998;160:3610–3616.
11. Isaaz S, Baetz K, Olsen K, Podack E, and Griffiths GM: Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway. Eur J Immunol 1995;25: 1071–1079.
12. Walker BD and Plata F: Cytotoxic T lymphocytes against HIV. AIDS 1990;4:177–184.
13. Levy JA, Mackewicz CE, and Barker E: Controlling HIV pathogenesis: The role of noncytotoxic anti-HIV activity of $CD8^+$ cells. Immunol Today 1996;17:217–224.
14. Mackewicz CE, Ortega H, and Levy JA: Effect of cytokines on HIV replication in $CD4^+$ lymphocytes: Lack of identity with the $CD8^+$ cell antiviral factor. Cell Immunol 1994;153:329–343.
15. Mackewicz CE, Barker E, and Levy JA: Role of β-chemokines in suppressing HIV replication. Science 1996;274:1393–1395.
16. Mackewicz CE, Levy JA, Cruikshank WW, Kornfeld H, and Center DM: Role of IL-16 in HIV replication. Nature (London) 1996; 383:488–489.
17. Sower LE, Froelich CJ, Allegretto N, Rose PM, Hanna WD, and Klimpel GR: Extracellular activities of human granzyme A. Monocyte activation by granzyme A versus alpha-thrombin. J Immunol 1996;156:2585–2590.
18. Sower LE, Klimpel GR, Hanna W, and Froelich CJ: Extracellular activities of human granzymes. Cell Immunol 1996;171:159–163.
19. Hommel-Berrey GA, Bochan MR, Montel AH, Goebel WS, Froelich CJ, and Brahmi Z: Granzyme B independently of perforin mediates noncytolytic intracellular inactivation of vesicular stomatitis virus. Cell Immunol 1997;180:1–9.
20. Froelich CJ, Orth K, Turbov J, Seth P, Gottlieb R, Babior B, Shah GM, Bleackley RC, Dixit VM, and Hanna W: New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. J Biol Chem 1996;271:29073–29079.
21. Beresford PJ, Kam CM, Powers JC, and Lieberman J: Recombinant human granzyme A binds to two putative HLA-associated proteins and cleaves one of them. Proc Natl Acad Sci USA 1997; 94:9285–9290.
22. Xia Z, Kam CM, Huang C, Powers JC, Mandle RJ, Stevens RL, and Lieberman J: Expression and purification of enzymatically active recombinant granzyme B in a baculovirus system. Biochem Biophys Res Commun 1998;243:384–389.
23. MacDonald G, Shi L, Velde CV, Lieberman J, and Greenberg AH: Mitochondria-dependent and -independent regulation of granzyme B-induced apoptosis. J Exp Med 1999;189:131–143.
24. Hanna WL, Zhang X, Turbov J, Winkler U, Hudig D, and Froelich CJ: Rapid purification of cationic granule proteases: Application to human granzymes. Protein Express Purif 1993;4:398–404.
25. Sanchez-Pescador R, Power MD, Barr PJ, Steimer KS, Stempien MM, Brown-Shimer SL, Gee WW, Renard A, Randolph A, Levy JA, Dina D, and Luciw PA: Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). Science 1985;227: 484–492.
26. Hoffman AD, Banapour B, and Levy JA: Characterization of the AIDS-associated retrovirus reverse transcriptase and optimal conditions for its detection in virions. Virology 1985;147:326–335.
27. Suidan HS, Clemetson KJ, Brown-Luedi M, Niclou SP, Clemetson JM, Tschopp J, and Monard D: The serine protease granzyme A does not induce platelet aggregation but inhibits responses triggered by thrombin. Biochem J 1996;315:939–945.
28. Suidan HS, Bouvier J, Schaerer E, Stone SR, Monard D, and Tschopp J: Granzyme A released upon stimulation of cytotoxic T lymphocytes activates the thrombin receptor on neuronal cells and astrocytes. Proc Natl Acad Sci USA 1994;91:8112–8116.
29. Simon MM, Simon HG, Fruth U, Epplen J, Muller-Hermelink HK, and Kramer MD: Cloned cytolytic T-effector cells and their malignant variants produce an extracellular matrix degrading trypsin-like serine proteinase. Immunology 1987;60:219–230.
30. Froelich CJ, Zhang X, Turbov J, Hudig D, Winkler U, and Hanna

WL: Human granzyme B degrades aggrecan proteoglycan in matrix synthesized by chondrocytes. J Immunol 1993;151:7161–7171.
31. Pinkowski MJ, Hobman M, Heibein JA, Tomaselli K, Li F, Seth P, Froelich CJ, and Bleackly RC: Cytosolic granzyme B is sufficient to induce apoptotic cell death. Blood 1998;92:1044–1053.
32. Trapani JA, Jans P, Smyth MJ, Froelich CJ, Williams EA, Sutton VR, and Jans DA: Perforin-dependent nuclear entry of granzyme B precedes apoptosis, and is not a consequence of nuclear membrane dysfunction. Cell Death Differ 1998;5:488–496.
33. Jans DA, Sutton VR, Jans P, Froelich CJ, and Trapani JA: BCL-2 blocks perforin-induced nuclear translocation of granzymes concomitant with protection against the nuclear events of apoptosis. J Biol Chem 1999;274:3953–3961.

Address reprint requests to:
Jay A. Levy
Department of Medicine
University of California, San Francisco
Box 1270
513 Parnassus Ave., #S-1280
San Francisco, California 94143-1270