Brief Definitive Report

The Target Cell Nucleus Is Not Required for Cell-mediated Granzyme- or Fas-based Cytotoxicity

By Hiroo Nakajima,* Pierre Golstein,‡ and Pierre A. Henkart*

*From the *Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; and the ‡Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, 13288 Marseille Cedex 9, France*

Summary

The requirement for target cell nuclei in the two apoptotic death pathways used by cytotoxic lymphocytes was tested using model effector systems in which the granzyme and Fas pathways of target damage are isolated. Mast cell tumors expressing granzymes A and B in addition to cytolysin/perforin lysed tumor target cells about 10-fold more efficiently than comparable effector cells without granzymes. Enucleated cytoplast targets derived from these cells were also lysed with a similar 10-fold effect of granzymes. In contrast to cytoplasts, effector granzyme expression did not influence lysis of red cell targets. The Fas pathway was assessed using the selected cytotoxic T lymphocyte hybridoma subline d11S, which lysed target cells expressing Fas but not those lacking Fas. Similarly, cytoplasts derived from Fas^+ but not Fas^- cells were also readily lysed by these effector cells. Thus, neither the nucleus itself nor the characteristic apoptotic nuclear damage associated with the two major cell death pathways used by cytotoxic lymphocytes are required for cell death per se.

It has recently become clear that cytotoxic lymphocytes inflict lethal damage on target cells in vitro via two distinct mechanisms that involve granule exocytosis or Fas (1–4). Both mechanisms induce cell death within a few hours, and after the effector cells are fully activated, there is no requirement for protein or RNA synthesis. The dying cells generally display apoptotic features including membrane blebbing, nuclear chromatin condensation, and DNA fragmentation (5–8). Although such apoptotic nuclear damage is a hallmark of this type of cell death, its functional role in the cell death pathway has been unclear. While some schemes for apoptotic death have included it in the sequence of events leading to death (9, 10), recent studies have indicated that enucleated cytoplasts die in response to growth factor withdrawal and antibody-mediated cross-linking of the Fas antigen (11, 12).

We have recently developed model systems that isolate each of the cytotoxic lymphocyte death pathways so that their properties can be studied independently. For the granule exocytosis pathway, the mast cell tumor line RBL has been transfected so that it expresses the CTL lymphocyte granule components cytolysin/perforin, as well as the serine proteases granzyme A and B (8). When all three of these are expressed, RBL transfectants exhibit potent cytotoxic activity, with the granzymes required both for the apoptotic character of target death and for the potent cytolytic activity. For the Fas cytotoxicity pathway, we have developed a selected CTL hybridoma line, d11S, which kills only Fas-bearing target cells after activation by PMA and ionomycin (13). This activation has been shown to result in upregulation of the Fas ligand in these cells, allowing cross-linking of Fas antigen on the target cells and leading to their death (14).

Using these two effector systems, we show here that both lymphocyte cytotoxicity pathways operate efficiently with enucleated target cells. Thus, the prominent nuclear damage accompanying death by both these pathways is not required for cell death, as measured by membrane integrity.

Materials and Methods

Cells. The preparation and properties of RBL mast cell tumor cells lines transfected with either cytolysin alone (RBL-cy) or a combination of cytolysin, granzyme A, and granzyme B (RBL-cy-gza-gzb) have been previously described (8). The d11S hybridoma line using the Fas cytotoxicity pathway exclusively was derived by recloning the d10S line described previously (13, 15). d11S effector cells were preactivated with a 3-h incubation with 5 ng/ml PMA and 3 μg/ml ionomycin before the cytotoxicity assay.

Cytoplast Preparation. Cytoplasts were prepared from target cells using the standard technique (16) with the modified Ficoll concentrations described by Ojcius et al. (17). The purity of cytoplasts was monitored by staining with 5 μg/ml HOECHST 33342 and examination in the fluorescence microscope. The cytoplast preparations used in this study all had purities of >98% and most were >99%. They were loaded with $Na_2{}^{51}CrO_7$ by the standard technique and showed spontaneous release rates very similar to those of the cells from which they were derived.

Cytotoxicity Assays. Target cells were prepulsed with ^{125}I-UdR to label DNA and were then labeled with ^{51}Cr as described (18). All assays were incubated for 4 h at 37°C before harvest. ^{125}I-DNA release was measured after treatment with Triton X-100 (18).

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/05/1905/05 $2.00
Volume 181 May 1995 1905–1909

Results and Discussion

Cytoplast preparations were >98% free of nuclei and approximately one-half the diameter of the tumor cells used (Fig. 1, *A* and *B*). A functional demonstration that these preparations are enucleated was provided by the effect of etoposide, a cytotoxic inhibitor of the nuclear enyzme DNA topoisomerase II (19) (Fig. 1 *C*). In contrast to intact cells, which showed both ^{51}Cr and ^{125}I-DNA release in response to etoposide, cytoplasts were completely resistant to this agent.

Target cell damage via the granule exocytosis cytotoxicity pathway would seem to occur by both membrane damage caused by cytolysin/perforin and by internal damage caused by the entry of granzymes into the target cytoplasm (20). The RBL transfectant expressing cytolysin/perforin (RBL-cy) allows assessment of the former, while the contribution of granzymes can be assessed by comparing RBL-cy with triple transfectants expressing a combination of granzymes A and B as well as cytolysin (RBL-cy-gza-gzb). The mRNA expression levels of these granule components in the RBL transfectants used in this study were within a factor of three of the expression levels in our reference cloned CTL line (Fig. 2 *A*). Previous results indicated that RBL-cy, which showed a potent IgE-dependent ability to lyse red cell targets, were only marginally cytotoxic toward tumor targets (8, 21). Fig. 2, *C* and *D*, confirms this result and further shows that expression of granzymes A and B confers a ~10-fold increase in cytolytic activity, as seen by comparing the RBL-cy ^{51}Cr release titration curve with that of RBL-cy-gza-gzb. In addition, granzyme expression was accompanied by a dramatic increase in the levels of target DNA release accompanying lysis, thus implicating granzymes in both membrane damage and apoptotic nuclear damage associated with this pathway. RBL granzyme expression was also correlated with apoptotic target nuclear morphology (8). Fig. 2, *E* and *F*, shows that for two standard tumor target cell lines, cytoplast lysis by each type of RBL transfectant was similar to that of the parental intact cells. In particular, the ~10-fold difference between the RBL-cy and RBL-cy-gza-gzb transfectants was largely maintained in each case. This result with cytoplasts was particularly striking in comparison with lysis of red cell targets (Fig. 2 *B*), which were potently lysed equally well by RBL-cy and RBL-cy-gza-gzb. Thus, the cytotoxic effect of granzymes, which does not operate in red cells that are easily killed by membrane damage, operates equally well in the presence or absence of nuclei.

Cytolysin/perforin is necessary for target cell damage by the granule exocytosis pathway as shown by the gene knockout results (22) as well as by the lack of cytotoxicity of granzyme-transfected RBL lacking cytolysin/perforin (8, 23). While it is required for measurable cytotoxicity by this pathway, the ability of tumor cells to repair membrane damage (24) means that this alone is an inefficient killing mechanism. This is reflected in the modest cytolytic abilities of RBL-cy clones (reference 8 and Fig. 2, *C* and *D*) as well as the 10-fold greater sensitivity of red cells compared with tumor cells when soluble cytolysin/perforin is added to the medium (25). Recent evidence showing blocking of cytotoxity by loading the target cytoplasm with the granzyme inhibitor aprotinin strongly suggests that the granzymes need to enter the target cell cytoplasm to participate in the cytotoxic process (20). While the nature of the cytoplasmic substrates of these proteases remains to be defined, we have recently described evidence for a synergistic interaction between granzymes A and B (8).

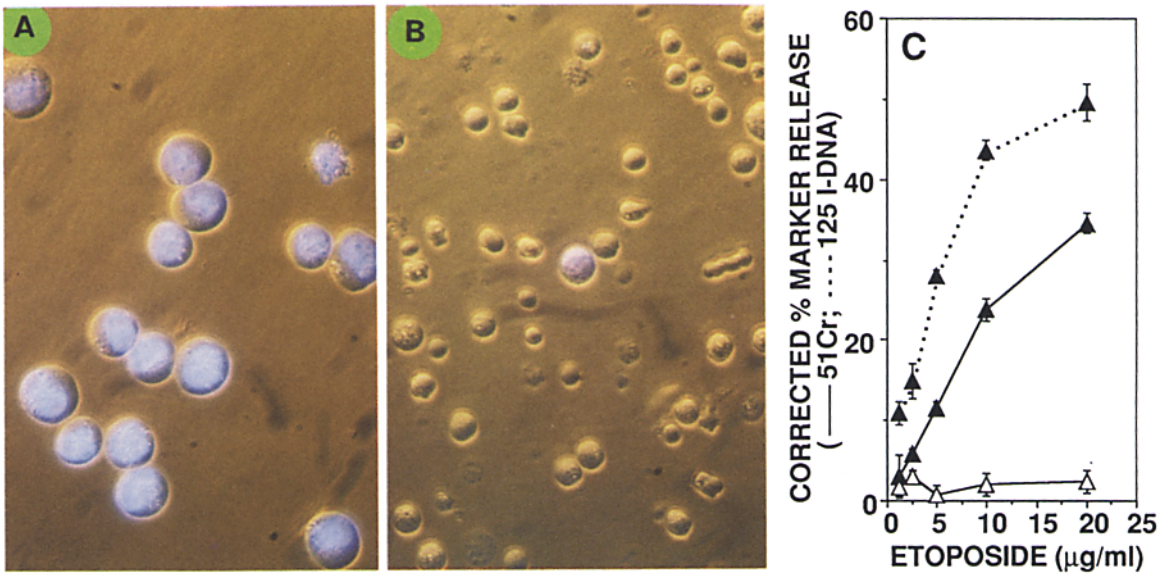

Figure 1. Cytoplast characterization. P815 cells (*A*) and cytoplasts (*B*) stained with the fluorescent DNA-binding dye HOECHST 33342 (*blue*) using combination phase-fluorescence optics. A rare contaminant karyoplast was chosen for illustration in *B*. (*C*) Cytotoxic effect of etoposide on EL-4 cells (*filled triangles*) versus EL-4 cytoplasts (*open triangles*). After labeling, these targets were incubated 18 h with the indicated final concentrations of etoposide, and the release of ^{51}Cr (*solid lines*) or ^{125}I-DNA (*dotted line*) was measured.

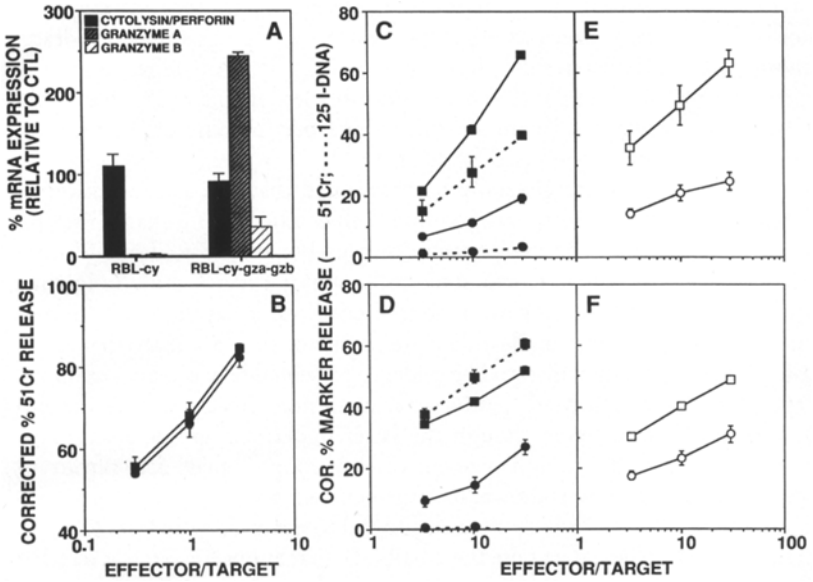

Figure 2. The granzyme death pathway operates in cytoplasts. (*A*) mRNA expression of lymphocyte granule components in RBL transfectants. Northern blots were prepared from separate RNA preparations from four individual RBL-cy clones and two individual RBL-cy-gza-gzb clones and probed with the respective granule component probes as well as β-actin. Phosphorimager analyses of these blots were normalized to β-actin and then expressed as a percentage of the level of reference mouse allo-CTL clone TIM 448. Data are expressed as the mean expression levels of the clones of each type; the error bars represent SEM. *B* shows the equal cytotoxicity of RBL-cy (*circles*) and RBL-cy-gza-gzb (*squares*) on human red cell targets. (*C–F*) Comparisons of the cytotoxicity of RBL-cy (*circles*) and RBL-cy-gza-gzb (*squares*) on EL-4 thymoma target cells (*C*), P815 mastocytoma target cells (*D*), cytoplasts from EL-4 (*E*), or cytoplasts from P815 (*F*). The data show release of ^{51}Cr (*solid lines*) or ^{125}I-DNA (*dashed lines*) after 4 h of incubation, with error bars showing SEM. (*B–F*) Data for each type of RBL transfectant is the mean marker release using the same four RBL-cy and two RBL-cy-gza-gzb clones described in Fig. 1. Additional controls with tumor cell targets treated with cytochalasin B but not spun in the Ficoll gradient showed identical cytotoxicity to nontreated tumor cell targets (data not shown).

One candidate substrate is cdc2 kinase, which was shown to be activated by a granzyme B-like NK cell granzyme (6), thus precipitating a "mitotic catastrophe" giving rise to the prototypical apoptotic nuclear damage. However, these experiments did not address the issue of whether this nuclear damage was part of the chain of events giving rise to cell death or whether it represents a separate branch of destructive events triggered by cytoplasmic mediators such as those recently described (26, 27). Our experiments argue strongly in favor of the latter interpretation, but they do not rule out a role for cdc2 kinase in target cell death, since this enzyme may phosphorylate nonnuclear proteins.

We have used a similar approach to assess whether the nucleus is required for target cell death caused by Fas when it is cross-linked by Fas ligand on the surface of effector cells. As can be seen from Fig. 3 *A*, d11S kills Fas-transfected L1210 cells but not the parental L1210 line, which expresses little or no Fas (13). This death is apoptotic, as seen by prelytic

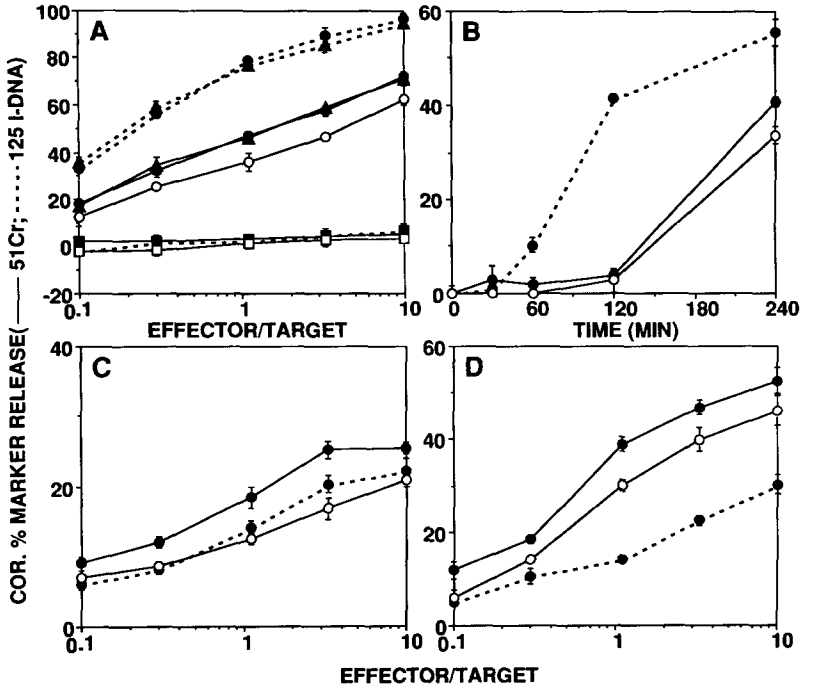

Figure 3. The Fas death pathway operates in cytoplasts. (*A* and *B*) Cytotoxicity using d11S effectors on L1210-Fas$^+$ targets (*circles and triangles*) and L1210-Fas$^-$ targets (*squares*). The triangles represent L1210-Fas$^+$ cells, which were treated with cytochalasin B but not spun in the Ficoll gradient (followed by the same wash as was used for the cytoplasts). *A* shows a 4-h assay, while *B* shows the kinetics using an effector/target ratio of 3.3. (*C* and *D*) 4-h experiments similar to *A*, except that the target cells were 2B4 T hybridoma cells (*C*) and EL-4-Fas$^+$ cells *D*. d11S cells showed no detectable cytotoxicity on EL-4-Fas$^-$ cells (data not shown). In all panels, filled symbols are intact cells and open symbols are cytoplasts; solid lines show ^{51}Cr release and dashed lines show ^{125}I-DNA release.

nuclear damage (^{125}I-DNA release preceding ^{51}Cr release, Fig. 3 *B*). When cytoplasts from L1210-Fas and L1210 tumor cells were tested for their ability to be lysed by d11S, cytoplasts from the former but not the latter were lysed by d11S with titration curves parallel to those with intact tumor cells (Fig. 3 *A*). With cytoplast targets, roughly threefold more d11S cells were required to achieve levels of lysis comparable to that of the intact cells. The kinetics of cytoplast lysis was not significantly different from that of intact cells (Fig. 3 *B*). Cytoplasts from other Fas-bearing tumor cells showed very similar results, as shown in Fig. 3, *C* and *D*. In these cases, d11S also caused lysis of Fas-bearing cytoplasts with similarly 2–3× shifted dose–response curves compared with the parental Fas-bearing tumor cells. This difference between cytoplasts and cells was not attributable to cytochalasin B treatment since L1210-Fas cells treated with cytochalasin B but not subjected to the gravitational field to produce cytoplasts were not different from untreated cells (Fig. 3 *A*). The less efficient lysis of Fas-bearing cytoplasts may suggest that the nucleus, while not required, can influence cell death in this case. However, other differences between cytoplasts and their parental cells (such as the smaller size of cytoplasts) may also be responsible. These results are similar to those of a recent report showing that anti-Fas antibody induces apoptotic morphology changes and inhibition of mitochondrial 3-(4, 5-dimethylthiazol-2-yl)-2, 5-dimethyltetrazolium bromide reduction in cytoplasts (12); in our experiments, Fas cross-linking was provided by effector-bound Fas ligand.

These experiments show that for both defined cytotoxic lymphocyte-mediated death pathways, the nucleus is not required for target cell death. While these death pathways are typically apoptotic in that they result in target death accompanied by characteristic nuclear damage, they are considerably more rapid than most examples of programmed cell death. Earlier results showing that CTL lysis of target cytoplasts was similar (28) or considerably less efficient (29) than intact target cells are difficult to interpret because of the multiple mediators used by CTL.

It has generally been assumed that the various examples of apoptotic programmed cell death share mechanistic steps. Since the most readily measurable apoptotic death features are nuclear, and since cells cannot survive for a long time without nuclear gene transcription, it has been tempting to consider nuclear damage as part of such a widely shared cell death pathway. The evidence presented here provides examples of cell death pathways that do not directly require the nucleus, even though the latter undergoes apoptotic damage; these are similar to the cases of apoptotic cell death from insulin withdrawal or treatment with staurosporine or menadione, which were also found to occur in cytoplasts (11, 12). These data raise the possibility that apoptotic nuclear damage may be an epiphenomenon with respect to cell death generally, although it is quite possible that other examples of slower apoptotic death may be a consequence of nuclear damage. It can also be argued that target cell DNA degradation is relevant to the overall biological function of cytotoxic lymphocytes, which is elimination of intracellular microorganisms, particularly viruses whose nucleic acids may be infectious (30).

We thank Drs. Scott Abrams, John Ashwell, Klaus Ebnet, and Michail Sitkovsky for their helpful comments on the manuscript.

Address correspondence to Dr. Pierre Henkart, Experimental Immunology Branch, National Cancer Institute, Bldg. 10, Room 4B36, NIH, Bethesda, MD 20892.

Received for publication 28 December 1994.

References

1. Kagi, D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science (Wash. DC).* 265:528–530.
2. Kojima, H., N. Shinohara, S. Hanaoka, Y. Someya-Shirota, Y. Takagaki, H. Ohno, T. Saito, T. Katayama, H. Yagita, K. Okumura, et al. 1994. Two distinct pathways of specific killing revealed by perforin mutant cytotoxic T lymphocytes. *Immunity.* 1:357–364.
3. Lowin, B., M. Hahne, C. Mattmann, and J. Tschopp. 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature (Lond.).* 370:650–652.
4. Henkart, P.A. 1994. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. *Immunity.* 1:343–346.
5. Russell, J.H. 1983. Internal disintegration model of cytotoxic lymphocyte-induced target damage. *Immunol. Rev.* 72:97–118.
6. Shi, L., W.K. Nishioka, J. Th'ng, E.M. Bradbury, D.W. Litchfield, and A.H. Greenberg. 1994. Premature p34cdc2 activation required for apoptosis. *Science (Wash. DC).* 263:1143–1145.
7. Yonehara, S., A. Ishii, and M. Yonehara. 1989. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* 169:1747–1756.
8. Nakajima, H., H. Park, and P.A. Henkart. 1995. Synergistic roles of granzymes A and B in mediating target cell death by RBL mast cell tumors also expressing cytolysin/perforin. *J. Exp. Med.* 181:1037–1046.
9. Bissonnette, R.P., F. Echeverri, A. Mahboubi, and D.R. Green. 1992. Apoptotic cell death induced by c-myc is inhibited by bcl-2. *Nature (Lond.).* 359:552–554.
10. Collins, M.K.L., and A.L. Rivas. 1993. The control of apoptosis in mammalian cells. *Trends Biochem. Sci.* 18:307–309.
11. Jacobson, M.D., J.F. Burne, and M.C. Raff. 1994. Programmed cell death and Bcl-2 protection in the absence of a nucleus.

EMBO (Eur. Mol. Biol. Organ.) J. 13:1899–1910.
12. Schulze-Osthoff, K., H. Walczak, W. Droge, and P.H. Krammer. 1994. Cell nucleus and DNA fragmentation are not required for apoptosis. *J. Cell Biol.* 127:15–20.
13. Rouvier, E.M., M.F. Luciani, and P. Golstein. 1993. Fas involvement in Ca^{2+}-independent T cell-mediated cytotoxicity. *J. Exp. Med.* 177:195–200.
14. Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell.* 78:1169–1178.
15. Anel, A., M. Buferne, C. Boyer, A.-M. Schmitt-Verhulst, and P. Golstein. 1994. T cell receptor-induced Fas ligand expression in cytotoxic T lymphocyte clones is blocked by protein tyrosine kinase inhibitors and cyclosporin A. *Eur. J. Immunol.* 24:2469–2476.
16. Wigler, M.H., and I.B. Weinstein. 1975. A preparative method for obtaining enucleated mammalian cells. *Biochem. Biophys. Res. Commun.* 63:669–674.
17. Ojcius, D.M., S. Jiang, P.M. Persechini, P.A. Detmers, and J.D.-E. Young. 1991. Cytoplasts from cytotoxic T lymphocytes are resistant to perforin-mediated lysis. *Mol. Immunol.* 28:1011–1018.
18. Shiver, J.W., L. Su, and P.A. Henkart. 1992. Cytotoxicity with target DNA breakdown by rat basophilic leukemia cells expressing both cytolysin and granzyme A. *Cell.* 71:315–322.
19. Liu, L.F. 1989. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* 58:351–375.
20. Henkart, P.A., M.S. Williams, and H. Nakajima. 1995. Degranulating cytotoxic lymphocytes inflict multiple damage pathways on target cells: a cytolysin/perforin-mediated membrane damage pathway and a granzyme-mediated internal disintegration pathway. *Curr. Top. Microbiol. Immunol.* In press.
21. Shiver, J.W., and P.A. Henkart. 1991. A noncytotoxic mast cell tumor line exhibits potent IgE-dependent cytotoxicity after transfection with the cytolysin/perforin gene. *Cell.* 62:1174–1181.
22. Kagi, D., B. Ledermann, K. Burki, P. Seller, B. Odermatt, K.J. Olsen, E.R. Podack, R.M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature (Lond.).* 369:31–37.
23. Nakajima, H., and P.A. Henkart. 1994. Cytotoxic lymphocyte granzymes trigger a target cell internal disintegration pathway leading to cytolysis and DNA breakdown. *J. Immunol.* 152:1057–1063.
24. Morgan, B.P. 1989. Complement membrane attack on nucleated cells: resistance, recovery, and non-lethal effects. *Biochem. J.* 264:1–14.
25. Henkart, P.A., P.J. Millard, C.W. Reynolds, and M.P. Henkart. 1984. Cytolytic activity of purified cytoplasmic granules from cytotoxic rat LGL tumors. *J. Exp. Med.* 160:75–93.
26. Newmeyer, D.D., D.M. Farshon, and J.C. Reed. 1994. Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell.* 79:353–364.
27. Lazebnik, Y.A., S.H. Kaufmann, S. Desnoyers, G.G. Poirer, and W.C. Earnshaw. 1994. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature (Lond.).* 371:346–347.
28. Siliciano, R.F., and C.S. Henney. 1978. Studies on the mechanism of lymphocyte-mediated cytolysis. Enucleated cells as targets for cytotoxic attack. *J. Immunol.* 121:186–191.
29. Berke, G., and Z. Fishelson. 1977. T-lymphocyte-mediated cytolysis: contribution of intracellular components to target cell susceptibility. *Transplant. Proc.* 9:671–674.
30. Martz, E., and D.M. Howell. 1989. CTL: virus control cells first and cytolytic cells second? DNA fragmentation, apoptosis and the prelytic halt hypothesis. *Immunol. Today.* 10:79–86.