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## Mechanism of Killing by Virus-Induced Cytotoxic T Lymphocytes Elicited In Vivo

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The mechanism of lysis by in vivo-induced cytotoxic T lymphocytes (CTL) was examined with virus-specific CTL from mice infected with lymphocytic choriomeningitis virus (LCMV). LCMV-induced T cells were shown to have greater than 10 times the serine esterase activity of T cells from normal mice, and high levels of serine esterase were located in the LCMV-induced CD8<sup>+</sup> cell population. Serine esterase was also induced in purified T-cell preparations isolated from mice infected with other viruses (mouse hepatitis, Pichinde, and vaccinia). In contrast, the interferon inducer poly(I · C) only marginally enhanced serine esterase in T cells. Serine esterase activity was released from the LCMV-induced T cells upon incubation with syngeneic but not allogeneic LCMV-infected target cells. Both cytotoxicity and the release of serine esterase were calcium dependent. Serine esterase released from disrupted LCMV-induced T cells was in the form of the fast-sedimenting particles, suggesting its inclusion in granules. Competitive substrates for serine esterase blocked killing by LCMV-specific CTL, but serine esterase-containing granules isolated from LCMV-induced CTL, in contrast to granules isolated from a rat natural killer cell tumor line, did not display detectable hemolytic activity. Fragmentation of target cell DNA was observed during the lytic process mediated by LCMV-specific CTL, and the release of the DNA label [<sup>125</sup>I]iododeoxyuridine from target cells and the accompanying fragmentation of DNA also were calcium dependent. These data support the hypothesis that the mechanism of killing by in vivo-induced T cells involves a calcium-dependent secretion of serine esterase-containing granules and a target cell death by a process involving nuclear degradation and DNA fragmentation.

The generation of the cytotoxic T-lymphocyte (CTL) response is associated with the clearance of virus-infected cells and the curing of the host during virus infections (32, 37). Most CTL induced in vivo bear the CD8 marker antigen and lyse target cells displaying peptides in the context of class I major histocompatibility complex antigens (28, 37). CTL also recognize alloantigens and play a role in allograft rejection (37), but it seems likely that CTL originally evolved primarily to control infections by intracellular parasites such as viruses.

Until recently, the mechanism of CTL killing was poorly understood, except for the fact that CTL bound to targets via a magnesium-dependent event with antigen and major histocompatibility complex specificity and that calcium ions were required for killing of the target (17). More recent work done mostly with cultured T-cell lines has revealed that CTL have cytoplasmic granules which contain a variety of lysosomal enzymes, an *N*<sup>α</sup>-benzyloxycarbonyl-L-lysine thiobenzyl (BLT) ester serine esterase (24), a cytotoxic perforin protein with some homology to component 9 of complement (12, 18), and tumor necrosis factor-like proteins capable of inducing fragmentation of nuclear DNA (25, 35). A proposed mechanism of killing is that the binding of the target to the CTL transduces a signal across the CTL membrane, resulting in a calcium flux, leading to an increase in cytoplasmic calcium. This catalyzes a variety of events which result in the migration of granules to the point of effector cell-target cell juncture, where the granules and their contents are released onto the target cell membrane. The perforin molecules polymerize into a complementlike ring deposit on the cell membrane, effecting permeability changes and membrane damage (12, 18, 35), and the tumor necrosis factor-like

protein or perforin itself induces prelytic fragmentation of nuclear DNA (8, 27, 37). Cell death usually occurs by apoptosis, a process involving blebbing and fragmentation of the nucleus and cytoplasm into sealed vesicles; this is in contrast to complement-dependent cell killing, which involves an osmotic rupture of the cytoplasmic membrane, leaving the nucleus intact (16).

The above model was based on work done with cultured T-cell lines, but these kinds of studies have the potential for introducing many artifacts. For instance, some cultured CTL lines do not demonstrate the specificity in binding to targets that in vivo-generated CTL do. Some CTL lines lyse a variety of targets in the absence of calcium, arguing for a completely different mechanism of cytotoxicity (23, 29). Further, there are reports that allospecific CTL generated in vivo do not have cytoplasmic granules or significant levels of serine esterase (1, 5); one study indicates that freshly activated allospecific CTL lack granules and significant levels of serine esterase but that granules and serine esterase activity can easily be induced in them in vitro by interleukin-2 (1). Thus, the mechanism of CTL killing has become quite controversial, and there may be different mechanisms (calcium dependent versus independent; granule dependent versus independent).

Paradoxically, the mechanism of CTL killing has not been systematically examined in the system most fundamental to the function and probable evolutionary development of CTL, that of CTL induced in vivo by a virus infection. To address this criticism, we studied the mechanism of lysis by mouse CTL induced in vivo by lymphocytic choriomeningitis virus (LCMV). LCMV induces high levels of CTL, which, dependent on the route of infection, either clear the infection or stimulate a lethal meningoencephalitis (32, 37). The target

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specificities and induction requirements of LCMV-induced CTL are well defined.

In contrast to the studies showing that *in vivo*-induced allospecific CTL lack granules (1, 5), we have found that about 25% of the CD8<sup>+</sup> cells stimulated 7 to 9 days after LCMV infection are large granular lymphocytes (LGL) (3), and over one-half of the CD8<sup>+</sup> cells undergoing blastogenesis are LGL (3, 19). These CTL-LGL are virtually indistinguishable morphologically from natural killer (NK) cell LGL, which can be eliminated *in vivo* by treatment of mice with antiserum to the glycosphingolipid asialo GM1. Thus, the LCMV infection stimulates the conversion of small, resting, nongranular T cells into activated, cytotoxic LGL. LCMV infection of beige mice, which have a point mutation conferring defective granule formation (14), stimulates the production of CD8<sup>+</sup> cells bearing abnormal granule morphology (4). These LCMV-induced beige mouse CTL have about one-half the lytic capacity of LCMV-induced CTL from normal mice. These experiments are consistent with the hypothesis that the mechanism of killing by *in vivo*-induced virus-specific CTL is granule dependent. In this report, we define further the mechanism of killing by *in vivo*-induced virus-specific CTL.

#### MATERIALS AND METHODS

**Infection of mice.** C57BL/6J (*H-2<sup>b</sup>*) and C3H/HeSnJ (*H-2<sup>k</sup>*) male mice (Jackson Laboratory, Bar Harbor, Maine), 4 to 10 weeks old, were used for these experiments. Mice were injected intraperitoneally with 0.1 ml of  $5 \times 10^4$  PFU of LCMV, strain Armstrong,  $1 \times 10^6$  PFU of mouse hepatitis virus, strain A-59,  $1 \times 10^6$  PFU of Pichinde virus, strain AN3739, or  $1 \times 10^7$  PFU of vaccinia virus, strain WR. Unless otherwise stated, spleen leukocytes were harvested 7 to 9 days postinfection. In many of the studies, mice received intraperitoneal injections of antiserum to asialo GM1 (Wako Laboratories, Dallas, Tex.) at 0 and 4 days postinfection. Although high doses of antiserum to asialo GM1 inhibit the CTL response, the lower dose selected for these studies eliminates NK cell activity without inhibiting the CTL response (2, 7, 34).

**Cytotoxicity assay.** A standard microcytotoxicity assay with target cells radiolabeled with sodium [<sup>51</sup>Cr]chromate (Amersham Corp., Arlington Heights, Ill.) was used as described previously (31). Target cells were MC57G (*H-2<sup>b</sup>*) and L-929 (*H-2<sup>k</sup>*), cultivated in Eagle minimal essential medium in monolayers, as described previously (19). Cultivation media were supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum. All assays were run for 5 to 6 h at 37°C.

**BLT serine esterase assay.** In a total volume of 200  $\mu$ l of medium 199 (GIBCO Laboratories, Gaithersburg, Md.) supplemented with 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.),  $1 \times 10^6$  spleen leukocytes were mixed with  $1 \times 10^5$  to  $3 \times 10^5$  target cells and incubated at 37°C for 3 to 4 h in flat-bottomed microdilution plates. The cells were then pelleted in a centrifuge, and 100  $\mu$ l of supernatant was harvested as a source of released serine esterase. This enzyme was measured by combining the supernatant with 100  $\mu$ l of serine esterase buffer, i.e., 0.2 M Tris hydrochloride (J. T. Baker Chemical Co., Phillipsburg, N.J.) (pH 8.1), 0.22  $\mu$ M Ellman reagent (5,5'-dithiobis-2-nitrobenzoic acid; Sigma), and 0.2  $\mu$ M BLT (Calbiochem-Behring, La Jolla, Calif.) (1, 24). After 30 min of incubation at room temperature, the product of the enzymatic reaction was measured by optical density (OD) at a wavelength of 410

nm. The specific release of serine esterase was determined by subtracting the serine esterase released from the effector cells and target cells incubated alone from the serine esterase released from the effector and target cells incubated together. Total serine esterase activity per cell population was determined by incubating the effector cells separately in 100  $\mu$ l of medium 199 plus 100  $\mu$ l of 1% Nonidet P-40 (NP-40) in water. A 100- $\mu$ l sample of this mixture was added to the serine esterase buffer and assayed. Data presented are the means of assays done in quadruplicate. Standard deviations are not tabulated because the variations between replicas in these listed experiments are very low, with standard deviations of <20% of the mean.

**Isolation of serine esterase-containing granules.** Two methods, based on studies by Millard et al. (21) and both successful, were used to isolate serine esterase-containing granules from CTL. Spleen cells were isolated 7 to 9 days after infection of mice which in some cases had been injected with antiserum to asialo GM1 at 0 and 4 days postinfection to eliminate NK cell activity (34). In some cases, the spleen leukocytes were passed through columns of nylon wool to purify the T cells (10). The cells were washed in phosphate-buffered saline, pelleted, and suspended in disruption buffer (0.25 M sucrose, 4 mM EGTA, 100 U of heparin [Sigma] per ml, and 0.01 M HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] buffer [Sigma], pH 7.4) at about  $10^8$  cells per ml. The cells were gently disrupted in a Dounce homogenizer, and the solution was adjusted to 5 mM MgCl<sub>2</sub> and excess DNase (Sigma) to digest the liberated DNA. The suspension was centrifuged at 2,300 rpm for 15 min at 4°C, and the supernatant in 2 ml was loaded onto an 8-ml mixture of 1:1 Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.) and a solution of 0.5 M sucrose and 0.01 M HEPES buffer, (pH 7.4) in a Beckman SW41 ultracentrifuge rotor. The gradients were centrifuged at 25,000 rpm for 30 min in the Beckman ultracentrifuge. Under these conditions of sedimentation, a gradient was spontaneously formed. The gradient was fractionated, and serine esterase activity was monitored in NP-40-treated gradient fractions. Alternatively, the cells suspended in buffer were disrupted by nitrogen cavitation at 450 lb/in<sup>2</sup> for 20 to 30 min while stirring on ice. This solution was adjusted to 5 mM MgCl<sub>2</sub> and incubated with 800 U of DNase I per ml at room temperature for 15 to 30 min. The samples were then filtered through 5- $\mu$ m-pore-size and then 3- $\mu$ m-pore-size filters (Nuclepore Corp., Pleasanton, Calif.). These samples were then centrifuged in Percoll gradients (5-ml sample, 20 ml of 48% [vol/vol] Percoll in a Beckman Ti50.2 rotor).

**Hemolytic assay.** Human or sheep erythrocytes were radiolabeled with chromium-51 and dispensed in microtiter plates at  $10^5$  cells per well. Dilutions of the Percoll gradient fractions containing serine esterase activity were added to the erythrocytes and incubated for 30 to 60 min at 37°C in minimal essential medium. The release of radiolabeled chromium was taken as a measurement of cytotoxicity, similar to standard microcytotoxicity assays. For positive controls in the hemolytic assays, we used cytotoxic granules that we prepared from a Fischer rat LGL tumor line (RNK-16[A]) provided to us by Craig Reynolds, Frederick Cancer Center, Frederick, Md.

**Analysis of DNA fragmentation.** L-929 cells (*H-2<sup>k</sup>*) were infected with LCMV at a multiplicity of infection of 0.2 2 days before use. One day before use, these cells and uninfected control cells were radiolabeled by the addition of 10  $\mu$ Ci of [<sup>125</sup>I]iododeoxyuridine (2,000 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) per ml. After 18 to 24 h of

TABLE 1. Calcium dependence of LCMV-induced CTL-mediated lysis<sup>a</sup>

Expt	Effector	Target	Treatment	% Lysis
1	C57BL/6	MC57G		<1
		MC57G	MgCl <sub>2</sub> + EGTA	<1
		MC57G	MgCl <sub>2</sub> + EGTA + CaCl <sub>2</sub>	<1
		MC57G + LCMV		47
		MC57G + LCMV	MgCl <sub>2</sub> + EGTA	4.6
		MC57G + LCMV	MgCl <sub>2</sub> + EGTA + CaCl <sub>2</sub>	62
2	C3H/HeSnJ	L-929		7.9
		L-929	MgCl <sub>2</sub> + EGTA	1.6
		L-929	MgCl <sub>2</sub> + EGTA + CaCl <sub>2</sub>	2.3
		L-929 + LCMV		63
		L-929 + LCMV	MgCl <sub>2</sub> + EGTA	4.6
		L-929 + LCMV	MgCl <sub>2</sub> + EGTA + CaCl <sub>2</sub>	31

<sup>a</sup> Spleen leukocyte effector cells from mice 8 days postinfection were tested for cytotoxicity against uninfected or LCMV-infected syngeneic targets in the presence or absence of added MgCl<sub>2</sub>, EGTA, and CaCl<sub>2</sub>. Final concentrations were 4 mM MgCl<sub>2</sub>, 2.5 mM EGTA, and 4 mM CaCl<sub>2</sub>. Cytotoxicity assays were 6 h long at effector-to-target cell ratios of 50:1.

exposure to label, the cells were dispersed with trypsin, centrifuge washed twice, and distributed into 6.5-ml polyethylene tubes at 10<sup>5</sup> cells per tube. Effector spleen leukocytes from uninfected control C3H/HeSnJ mice or mice infected 8 days previously with LCMV and treated with antiserum to asialo GM1 on days 0 and 4 postinfection to eliminate NK cell activity were added to the targets in a total volume of 2 ml of RPMI medium and incubated for 6 to 7 h at 37°C. After incubation, the cells were pelleted, and DNA was isolated by a modification of the methods of Duke et al. (6) and Maniatis et al. (15). Briefly, the cells were lysed in 0.2% sodium dodecyl sulfate in TES buffer (10 mM Tris buffer [pH 8.0], 10 mM EDTA, 0.15 M NaCl) containing 100 µg of proteinase K (Sigma) per ml. After digestion overnight at 37°C, the DNA was isolated by sequential extractions with phenol, 1:1 phenol-chloroform, and 48:2 chloroform-isoamyl alcohol. The DNA in the aqueous phase was precipitated overnight at -20°C with 0.3 M sodium acetate (pH 5.2) in ethanol. The DNA was pelleted at 13,000 × g for 20 min and suspended in 10 mM Tris buffer-1 mM EDTA (pH 8.0). For each sample, equal radioactivity ± 10% was loaded onto 1.5% agarose gels. The gels were electrophoresed for 6 h at 120 V in a running buffer containing 89 mM Tris-borate, 89 mM boric acid, and 2 mM EDTA. The gels were then oven dried on glass plates by the method of Jones et al. (9) and exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) with intensifying screens (Du Pont Co., Wilmington, Del.) at -70°C.

## RESULTS

**Calcium dependence of CTL killing.** LCMV-induced spleen leukocytes from C57BL/6 or C3H/HeSnJ mice were tested for their abilities to lyse targets in the presence of EGTA and MgCl<sub>2</sub>, a treatment which selectively removes Ca<sup>2+</sup> from the medium (Table 1). This treatment nearly completely blocked killing against the syngeneic LCMV-infected MC57G and L-929 target cells, respectively. Significant levels of lysis were not detected against uninfected syngeneic targets. Addition of CaCl<sub>2</sub> to the assay mixtures restored the ability of the CTL to lyse the virus-infected syngeneic targets. These experiments therefore indicate that the lysis of these target cells by LCMV-induced CTL is, in 6-h assays, virtually exclusively due to a calcium-dependent mechanism.

**Serine esterase-containing T cells.** BLT ester serine es-

terase activity was examined in T-cell preparations isolated from mice at different times after LCMV infection. T cells were purified by passing spleen cells from NK cell-depleted mice through columns of nylon wool. Moderate levels of esterase activity were found in the T-cell preparations from uninfected mice, and the activities rose significantly with infection, peaking on days 7 through 12 (Fig. 1), correlating with the peak in CTL activity. Thereafter, the serine esterase levels declined. Similar results assaying for a "serine proteinase" have been reported previously by others (27). Induction of serine esterase activity was observed in both C57BL/6 and in C3H/HeSnJ mice. At 9 days postinfection, passage of NK cell-depleted spleen leukocytes through columns of nylon wool resulted in significant (e.g., 55%; Table 2) enrichment of serine esterase activity over unfractionated leukocyte populations, indicating that the T cells were a major source of the enzyme. To show whether

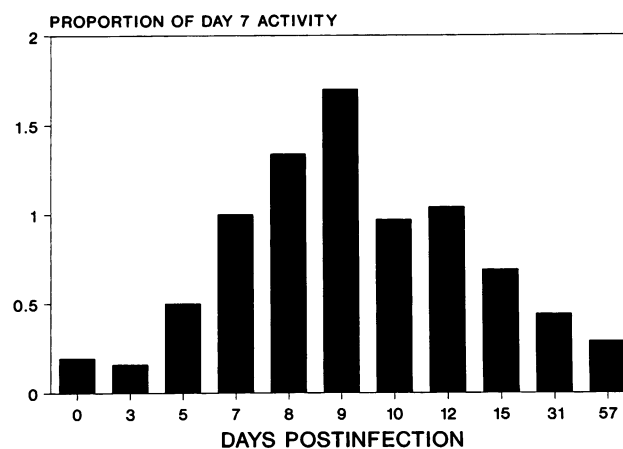


FIG. 1. LCMV-induced serine esterase in T cells. Serine esterase activity was determined in 1:20 dilutions of NP-40 lysates of 10<sup>6</sup> nylon wool-passed spleen leukocytes from NK cell-depleted C57BL/6 mice isolated at different days after LCMV infection. To deplete NK cells, mice received injections of antiserum to asialo GM1 at 0, 4, 7, and 9 days postinfection and the day before harvest of the 31- and 57-day time points. These results are pooled data from two separate experiments. Since the enzyme activities are best comparable within and not between experiments, the day 7 data were normalized as 1, and other data are listed as proportions of the day 7 data.

TABLE 2. Release of serine esterase from C57BL/6 mouse cells upon incubation with LCMV-infected target cells<sup>a</sup>

Effector cells (OD × 1,000)	Serine esterase release (OD × 1,000) induced by:	
	MC57G	MC57G + LCMV
Day 9 (421)		
Control	-9	53
+ MgCl <sub>2</sub> + EGTA	-6	-3
+ MgCl <sub>2</sub> + EGTA + CaCl <sub>2</sub>	-11	36
Day 9; NW passed (654)		
Control	-8	174
+ MgCl <sub>2</sub> + EGTA	-4	14
+ MgCl <sub>2</sub> + EGTA + CaCl <sub>2</sub>	-8	89

<sup>a</sup> Unfractionated or nylon wool (NW)-passed spleen leukocytes from NK cell-depleted mice 9 days after LCMV infection were tested for total serine esterase and for release of serine esterase upon mixture with target cells as described in Materials and Methods. Serine esterase activity is listed as OD × 1,000. The spontaneous releases of serine esterase, which were subtracted from the values presented, were as follows (OD × 1,000): day 9 effector cells, 37; day 9 nylon wool-passed effector cells, 26; MC57G, 4; MC57G plus LCMV, 10. Total serine esterase of effector cells was measured after disrupting effector cells with NP-40 and is listed in parentheses. Final concentrations of additives were 4 mM MgCl<sub>2</sub>, 2.5 mM EGTA, and 4 mM CaCl<sub>2</sub>.

significant levels of serine esterase were located in the CD8<sup>+</sup> T-cell population, which contains the cytotoxic T-cell phenotype, spleen cells from NK cell-depleted mice 8 days postinfection were stained with fluorescein-conjugated anti-Lyt 2 (CD8) antibody (Becton Dickinson and Co., Mountain View, Calif.) and purified by flow cytometry. Compared with controls, elevated levels of BLT serine esterase activity were located in both CD8<sup>+</sup> and CD8<sup>-</sup> populations (Fig. 2), as shown by others for serine proteinase (27). The LCMV-induced CD8<sup>+</sup> cells contained the highest levels of enzyme, which were greater than 20 times the levels of serine esterase activity in CD8<sup>+</sup> cells from unstimulated mice.

To show that the serine esterase activity was in granules, we disrupted splenocytes from C57BL/6 mice 8 days postinfection to release granules, which were sedimented in Percoll gradients as described in Materials and Methods.

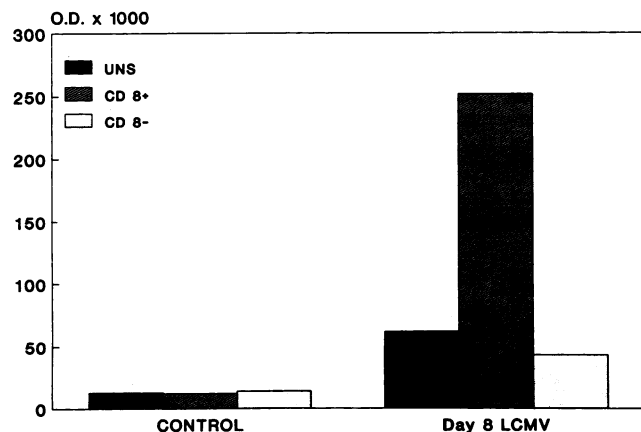


FIG. 2. BLT serine esterase in CD8<sup>+</sup> cells. Spleen cells from NK cell-depleted uninfected or day 8 LCMV-infected mice were stained with antibody to Lyt 2 (CD8) and were either unseparated (UNS) or separated into CD8<sup>+</sup> and CD8<sup>-</sup> cells by a Becton-Dickinson FACS IV flow cytometer. Data listed are serine esterase activities (OD × 1,000) per 3.3 × 10<sup>5</sup> cells.

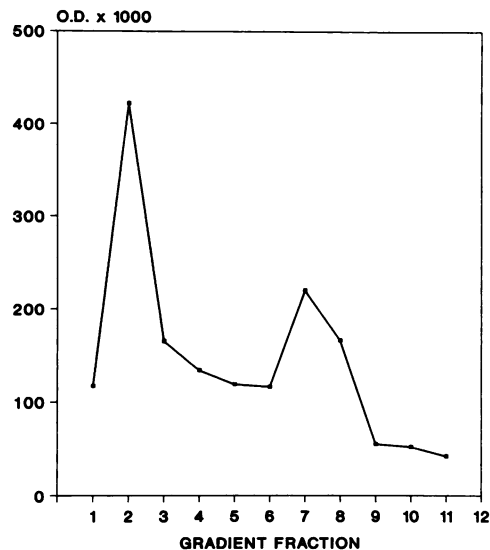


FIG. 3. Serine esterase in subcellular fractions separated in a Percoll density gradient. Spleen leukocytes from C57BL/6 mice 8 days after LCMV infection were disrupted by the Dounce technique and sedimented in a Percoll gradient as described in Materials and Methods. Gradient fractions were tested for serine esterase activity (OD × 1,000) at dilutions of 1:20.

Gradient fractions were collected and examined for serine esterase activity. Most of the activity sedimented close to the bottom of the gradient (Fig. 3), indicating that the serine esterase was packaged in vesicles, as free enzyme would have remained at the top of the gradient. Others have reported similar results with gradients of lysates from cloned cytotoxic cell lines and rat NK cell tumors (5, 21). This experiment is consistent with the hypothesis that in vivo-generated CTL, like CTL clones and NK cell tumors, possess serine esterase-containing granules.

**Serine esterase and granular T cells induced by other viruses.** LCMV is an unusually potent stimulator of the CTL response, and it was possible that our finding of serine esterase-containing granules in LCMV-induced CTL was peculiar only to that virus. We therefore examined serine esterase activity and granule formation in T cells induced by other viruses. Purified T cells from C57BL/6 mice infected with mouse hepatitis virus, Pichinde virus, and vaccinia virus had enhanced serine esterase activity (Fig. 4). Further, granules were seen in >5% of the purified T lymphocytes induced by each virus. This indicates that many viruses are capable of inducing granular T cells bearing serine esterase activity.

**Role of IFN on serine esterase activity.** In vivo-generated allospecific CTL have low levels of serine esterase activity compared with the virus-induced CTL observed here (5, 27). We questioned whether the enhanced levels of serine esterase in virus-induced T cells could be due to the release of interferon (IFN), a cytokine induced to much higher levels in virus infections than in T-cell stimulations with alloantigens. Injection of NK cell-depleted mice with the type 1 IFN inducer poly(I · C) for 2 or 3 days stimulated only a marginal increase in serine esterase activity in the nylon wool-passed T-cell population. In six experiments, the average increase in serine esterase activity per cell was 50 ± 48% (standard deviation). This was not comparable to the major increases seen 7 to 12 days after LCMV infection but was consistent with results showing that LCMV did not enhance the activity

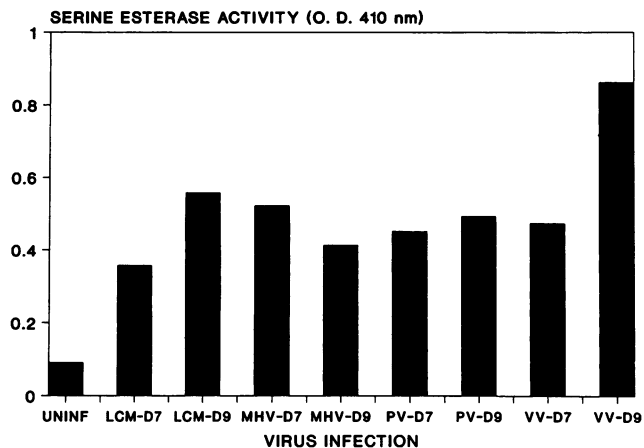


FIG. 4. Serine esterase in T cells induced by various viruses. C57BL/6 NK cell-depleted mice were infected intraperitoneally with LCMV, mouse hepatitis virus, Pichinde virus, or vaccinia virus, and their spleen leukocytes were harvested at 7 or 9 days postinfection. After passage through nylon wool columns,  $10^6$  purified T cells were disrupted with NP-40 and tested for total serine esterase content as described in Materials and Methods. UNINF, Uninfected.

at 3 days postinfection, a time of peak IFN production (Fig. 1). Incubation of purified T cells for 24 to 48 h in vitro with 100 to 10,000 U of IFN- $\beta$  did not stimulate serine esterase activity in that cell population (data not shown).

**Serine esterase release.** Nylon wool-passed splenocytes were mixed with uninfected or LCMV-infected targets for 3 h at 37°C to allow for the release of serine esterase. Exposure of the splenocytes to the appropriate LCMV-infected target caused a release of low but significant levels of the serine esterase from the T cells (Table 2). In 10 experiments with NK cell-depleted C57BL/6 splenocytes, this release of serine esterase averaged 9.3% of unfractionated and 13% of nylon-passed splenocyte (pure T cell) total serine esterase levels. The uninfected syngeneic target stimulated a much lower level of release. These data support the contention that serine esterase-containing granules are released from the in vivo-induced T cells after incubation with the appropriate target. To show whether the release of serine esterase was calcium dependent, we conducted the serine esterase release assays in the presence or absence of calcium. Depletion of calcium by  $MgCl_2$  and EGTA blocked serine esterase release, but reconstitution with calcium restored the ability of the T cells to release serine esterase on incubation with the target (Table 2). To be sure that the release of serine esterase was due to a classic CTL recognition pattern and not due to an undefined contaminant in the virus stock which might directly stimulate T cells, we determined whether the release of serine esterase was only stimulated by virus-infected syngeneic targets. Histocompatible LCMV-infected MC57G cells but not incompatible LCMV-infected L-929 cells or uninfected MC57G cells stimulated serine esterase release from LCMV-induced C57BL/6 T cells (Table 3). Similarly, LCMV-infected L-929 cells but not other targets effectively stimulated serine esterase release from the syngeneic LCMV-induced C3H/HeSnJ effector T cells. This indicates a typical major histocompatibility complex-restricted T-cell recognition pattern.

**Inhibition of cytotoxicity by competitive substrates of serine esterase.** Both T-cell- and tumor necrosis factor-mediated cytotoxicity are reported to be blocked by inhibitors of serine proteases (24, 26). We therefore tested the ability of

TABLE 3. Restriction requirements for serine esterase release<sup>a</sup>

Target cell	H-2	OD $\times$ 1,000 with effector cell:	
		C57BL/6 H-2 <sup>b</sup> (556)	C3H/HeSnJ H-2 <sup>k</sup> (498)
MC57G	<i>b</i>	9	11
MC57G + LCMV	<i>b</i>	99	21
L-929	<i>k</i>	-10	-12
L-929 + LCMV	<i>k</i>	10	67

<sup>a</sup> Nylon wool-passed spleen cells from NK cell-depleted mice 8 days after LCMV infection were used as effector cells. The serine esterase release assay was that described in Materials and Methods. Data are expressed as OD  $\times$  1,000. The spontaneous releases of serine esterase, which were subtracted from the values presented, were as follows (OD  $\times$  1,000): C57BL/6 effector cells, 21; C3H effector cells, 12; MC57G, -3; MC57G plus LCMV, -5; L-929, 28; L-929 plus LCMV, 8. Total serine esterase in the effector population is listed in parentheses.

two serine esterase substrates, BLT methyl ester (the same reagent used in our serine esterase assays) and *p*-toluene-sulfonyl-L-arginine methyl ester (TAME) (Sigma), to inhibit killing by the LCMV-induced CTL. Both substrates inhibited killing in a dose-dependent manner (Fig. 5). These concentrations of substrates did not directly kill the CTL, as preincubation of the CTL for 1.5 h with the inhibitors followed by washing the substrates away resulted in CTL with no significantly impaired cytolytic activity.

**Toxicity mediated by isolated granules.** Granules cytotoxic to erythrocytes can be isolated from some but not all granulated cytotoxic cell lines (12). To determine whether such cytotoxic granules could be isolated from LCMV-induced T cells, we tested Percoll gradient fractions rich in serine esterase activity for cytotoxicity against sheep erythrocytes. Although significant levels of lysis were mediated by gradient fractions from the RNK tumor line, no lysis was detected in the fractions extracted from the LCMV-induced T cells (Fig. 6). In five experiments, no cytotoxicity could be demonstrated with C57BL/6 or C3H/HeSnJ LCMV-induced T cells. On a per cell basis, we estimate that the LCMV-induced T-cell preparations contained less than 1% of the hemolytic activity of the RNK tumor line.

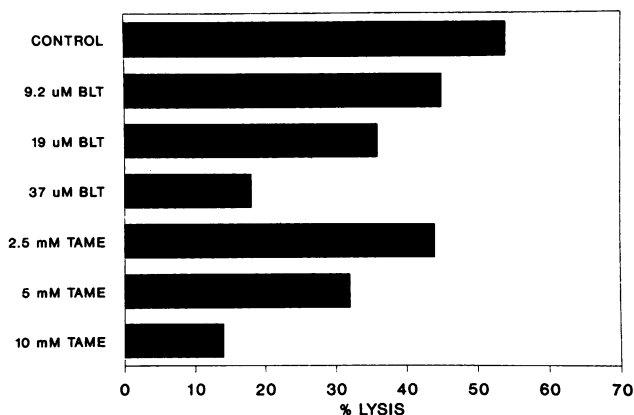


FIG. 5. Inhibition of CTL-mediated lysis by competitive substrates from serine esterase. BLT and TAME at the designated concentrations were included in cytotoxicity assays with LCMV-infected L-929 cell targets and spleen cells from NK cell-depleted C3H/HeSnJ mice 8 days after LCMV infection. Effector-to-target cell ratios were 25:1. In a parallel experiment, pretreatment of T cells for 1.5 h with the inhibitors before adding the washed T cells to the assay did not significantly block killing (control, 32%; 10 mM TAME, 30%; 5 mM TAME, 27%; 35  $\mu$ M BLT, 24%; 18  $\mu$ M BLT, 32%).

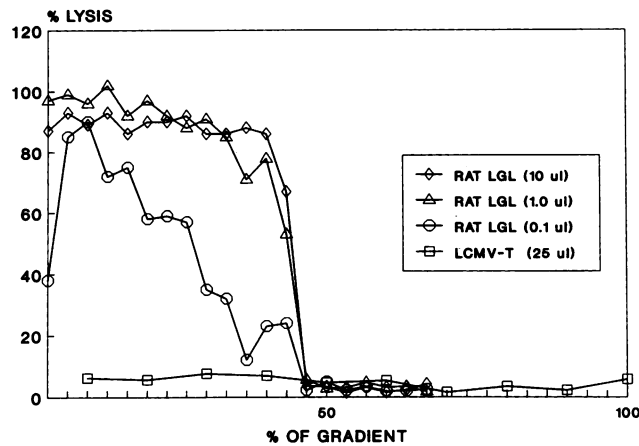


FIG. 6. Hemolytic activity induced by Percoll gradient-fractionated cell lysates. Lysates from  $2.5 \times 10^8$  rat NK tumor cells (LGL) and from  $2 \times 10^8$  purified T cells from NK cell-depleted C57BL/6 mice 8 days after LCMV infection were sedimented in Percoll gradients in a Beckman Ti50.2 rotor as described in Materials and Methods. The indicated volumes of the gradient fractions were tested for hemolytic activity against human erythrocytes. The top of the gradient is oriented to the right, and samples at the top of the gradient from the rat LGL preparation were not tested individually because of previous experiments showing no activity there.

**DNA fragmentation.** Granules from CTL lines are reported to contain proteins that induce DNA fragmentation (22, 25, 35). To determine whether target cell DNA fragmentation is induced by *in vivo*-generated virus-specific CTL, we exposed LCMV-infected and uninfected L-929 cells to C3H mouse splenocytes 7 days after infection of the mice with LCMV. The levels of cytotoxicity against LCMV-infected cells in a 7-h chromium release assay with  $10^5$  target cells per test tube were 57, 34, and 27% at effector-to-target cell ratios of 50, 25, and 12 to 1, respectively, whereas the killing of the uninfected targets was <1% at all ratios. Figure 7 shows the DNA fragmentation induced in parallel cultures of cells prelabeled with [ $^{125}$ I]iododeoxyuridine, a DNA-specific precursor. High levels of effector cell-dose-dependent DNA fragmentation were seen against the infected but not the uninfected targets. This indicates that DNA fragmentation is stimulated by CTL induced by viruses *in vivo*. To determine whether release of DNA from targets required calcium, we analyzed supernatants from [ $^{125}$ I]iododeoxyurine-labeled cells for release of radiolabel after exposure to CTL. The specific release of the DNA radiolabel also required calcium (control, 63%; EGTA plus  $MgCl_2$ , 0.1%; EGTA plus  $MgCl_2$  plus  $CaCl_2$ , 43%; effector-to-target cell ratio = 50). Gel analyses indicated that DNA fragmentation occurred only in the control and calcium-reconstituted samples but not in the calcium-depleted samples (Fig. 7). DNA fragmentation was also blocked by the incorporation of the serine esterase substrates BLT and TAME in the assays (data not shown).

## DISCUSSION

We believe this to be the first demonstration that *in vivo*-induced virus-specific CTL release serine esterase upon exposure to target cells and that these CTL mediate killing of the targets by a process involving fragmentation of nuclear DNA. This work supports and extends several findings very recently published in the LCMV system (3, 4, 11, 13, 19, 20, 27, 36). *In vivo*-generated LCMV-induced T

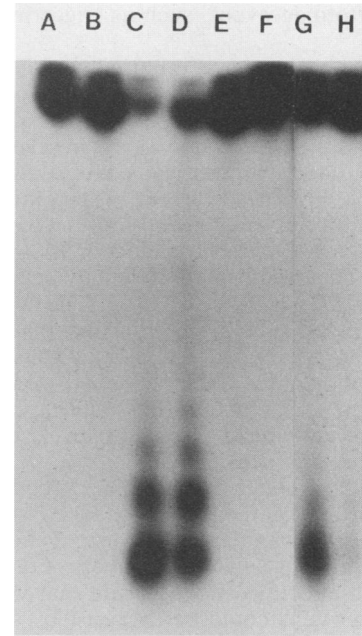


FIG. 7. DNA fragmentation of [ $^{125}$ I]iododeoxyuridine-labeled target cells by LCMV-induced CTL in the presence or absence of calcium. Splenocytes were from NK cell-depleted C3H/HeSnJ mice infected 8 days previously with LCMV. DNA samples of equal counts per minute  $\pm$  10% were loaded onto a 1.5% agarose gel. The target cells, effector cells, effector/target ratios, and treatments, respectively, are listed below. Lanes: A, uninfected L-929 cells, control spleen cells, 50:1; B, LCMV-infected L-929 cells, control spleen cells, 50:1; C, LCMV-infected L-929 cells, day 8 spleen cells, 50:1; D, LCMV-infected L-929 cells, day 8 spleen cells, 25:1; E, LCMV-infected L-929 cells only; F, LCMV-infected L-929 cells, day 8 spleen cells, 50:1, in 4 mM  $MgCl_2$ -2.5 mM EGTA; G, LCMV-infected L-929 cells, day 8 spleen cells, 50:1, in 4 mM  $MgCl_2$ -2.5 mM EGTA-4 mM  $CaCl_2$ ; H, LCMV-infected L-929 cells, day 8 spleen cells, 25:1, in 4 mM  $MgCl_2$ -2.5 mM EGTA-4 mM  $CaCl_2$ .

cells have azurophilic granules (3, 4, 19, 20) and stain histochemically and immunochemically with reagents detecting serine esterase and the perforin protein (11, 13, 36). Further, perforin-specific mRNA has been shown to be present in T cells harvested from organs of LCMV-infected mice at the peak of the CTL response (13). These findings support the contention that the granules contain cytotoxic molecules. However, we were unable to demonstrate hemolytic activity to erythrocytes by granules isolated from LCMV-induced CTL (Fig. 6). This failure to find hemolytic activity in these granules is not surprising, as there are a number of cell lines containing high levels of perforin but poor hemolytic activity, perhaps due to the presence of inhibitors that regulate the response (12). The failure to show that the granules in the LCMV-induced CTL were hemolytic makes it difficult to conclude definitively that they are required for cytotoxicity. Nevertheless, it remains likely that the granules could be involved in cytotoxicity, as LCMV-induced beige mouse CTL have defective granules and defective cytolytic capacity (4). Furthermore, we have shown here that  $Ca^{2+}$  depletion, which prevents granule-associated enzyme release, prevents cytotoxicity (Tables 1 and 2) and that competitive inhibitors of the serine esterase located in the granules block cytotoxicity (Fig. 5). It also is possible that these inhibitors could have affected an earlier

step in the cytotoxic event, such as triggering of the CTL (30).

The fact that others have reported that *in vivo*-induced CTL lack granules and serine esterase leads one to question whether the LCMV-induced CTL are unusual in this regard (15). We therefore examined the T cells in several other virus infections and found that they also contained serine esterase and azurophilic granules (Fig. 4). Thus, the induction of granules and serine esterase seems to be a common property of virus infections. Failures to detect *in vivo*-induced T-cell granules have occurred in studies employing allogeneic cells to stimulate a CTL response (1, 5). The wide variety of cytokines such as IFN or interleukins that are released systemically during virus infections may cause more extensive differentiation of the CTL. However, we were unable to show that poly(I · C), a potent IFN inducer, could stimulate serine esterase activity in T cells at levels comparable to those stimulated by virus infections.

Only a small although reproducibly significant (usually <15%) proportion of the LCMV-induced serine esterase activity in T cells was released by incubation with target cells (Tables 2 and 3). Sufficient numbers of target cells were incubated with effector cells to ensure that virtually all the specific effector cells could be triggered by an appropriate target. This may reflect an insufficient triggering of effector cell function but more likely may be due to the fact that virus infections are polyclonal CTL inducers (33) and that most of the CTL induced during an infection do not react with virus-infected targets with high affinity (33, 34). Further, some serine esterase activity is in the CD8<sup>-</sup> T-cell population, which should not be triggered by these targets, which lack class II major histocompatibility complex expression (Fig. 2). It is likely that a specific release of serine esterase may be even more difficult to find with infections by other viruses which do not stimulate virus-specific CTL as well as LCMV does.

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