

## SERINE ESTERASE AND HEMOLYTIC ACTIVITY IN HUMAN CLONED CYTOTOXIC T LYMPHOCYTES

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Cytotoxic T lymphocytes (CTLs) are prominent components of immune defenses against virus infections because of their ability to destroy virus-infected cells. Extensive studies indicate that when the cells of cloned murine CTL cell lines adhere to and recognize epitopes on another cell (a target cell), dense cytoplasmic granules within the CTL migrate towards and discharge their contents at the CTL target cell junction (reviewed in reference 1). One component of the granules is a complement (C9)-like hemolytic protein (termed perforin [2-6] or cytolyisin [1]) that can form nonspecific ion channels in cell membranes; the subsequent massive changes in intracellular concentrations of  $Ca^{2+}$  and other ions are probably responsible for target cell death (7-9). The granules also contain large amounts of a neutral serine esterase that cleaves the synthetic lysine derivative benzyloxycarbonyl-L-lysine-thiobenzyl ester (BLT)<sup>1</sup> (10-13). The enzyme (referred to here as BLT-serine esterase) is one of the most abundant proteins (~1%) in murine CTL cell lines; although its function, and that of several other granule-associated serine esterases, is unknown, some evidence suggests that it may also be involved in target cell destruction (10, 11, 13, 14). If these molecules are essential for granule-dependent cytolytic activity, homologous proteins would be expected to be present in the CTLs from diverse species. Do cloned human CTL cell lines express the BLT-serine esterase and hemolytic activities that are so prominent in murine CTL cell lines? To answer this question, we have tested several cloned human CTL cell lines for the presence of serine esterase and hemolytic granules. Our results show that human CTLs have a serine esterase homologous to the murine enzyme, albeit at about one-tenth the level found in the corresponding murine cloned cell lines, but we were unable to detect any evidence of hemolytic activity in the human cells. The findings suggest that an alternative mechanism of target cell lysis may be employed by the human CTLs examined.

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<sup>1</sup> *Abbreviations used in this paper:* BLT, benzyloxycarbonyl-L-lysine-thiobenzyl ester; DFP, diisopropylfluorophosphoridate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); TLCK, *N*- $\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

## Materials and Methods

**Mixed Lymphocyte Culture.** Venous blood was sterilely collected from human volunteers and anticoagulated with sodium heparin (25 U/ml; Ellins-Sims, Inc., Cherry Hill, NJ); the blood was mixed with an equal volume of RPMI 1640, then layered over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged at 2,500 rpm for 15 min. Cells at the Ficoll-serum interface were collected, washed twice with RPMI, and resuspended at a concentration of  $10^6$  cells/ml in the presence of  $5 \times 10^5$ /ml irradiated (45 Gy) allogeneic EBV-transformed B cells, in KH medium (RPMI 1640, 6 mM Hepes, 0.06 mM 2-ME [Sigma Chemical Co., St. Louis, MO], 1.6 mM L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin [Gibco Laboratories, Grand Island, NY], 10% heat-inactivated pooled human serum [Flow Laboratories, Inc., McLean, VA]). After 5–7 days, lymphocytes were cloned by limiting dilution.

**Rheumatoid Synovial Membrane.** Synovial membranes were obtained from patients undergoing joint replacement for rheumatoid arthritis in accordance with the human studies guidelines of the Massachusetts Institute of Technology and the Massachusetts General Hospital. Membranes were placed in K medium (the same as KH medium, except that 10% heat-inactivated FCS is substituted for human serum) with 30 U/ml rIL-2, and after 1 wk cells in the supernatant were cloned by limiting dilution.

**CTL Cloning by Limiting Dilution.** Aliquots from the bulk T cell cultures were diluted to 10 viable cells/ml in KH medium to which was added 5% PHA supernatant (15), 50 U/ml human rIL-2 (the gift of Biogen Research Corp., Cambridge, MA), and B cells plus cells from the bulk MLC (each at  $5 \times 10^5$  cells/ml and irradiated with 45 Gy). The mixture was distributed into flat-bottomed 96-well culture plates (Costar, Cambridge, MA) at 0.1 ml/well, equivalent to 1 viable cell/well. After 7 and 14 d, an additional 0.1 ml of KH medium supplemented with PHA supernatant (5%), IL-2 (50 U/ml), and irradiated (45 Gy) B cells ( $7.5 \times 10^5$ /ml) was added to each well. Clones were generally visible after 10–20 d, and were then expanded into 24-well culture plates. Clones were gradually weaned to KH medium supplemented with 5% PHA supernatant and 20–25 U/ml rIL-2, and restimulated at five 10-d intervals. The added B cells were allogeneic EBV-transformed cells.

**Cytotoxicity Assays.**  $^{51}\text{Cr}$ -release killing assays were performed as described (16); when measuring lectin-dependent cytolytic activity, Con A (Vector Laboratories, Inc., Burlingame, CA) was added to a final concentration of 10  $\mu$ g/ml.

**PMSF Inhibition of Cytolysis.** Various numbers of T cells were incubated for 15 min at 37°C in K medium containing PMSF at 0, 2, 4, and 6 mM (PMSF was added in DMSO; the final concentration of DMSO in all instances was 1%). Cytotoxicity was assayed as above except that a 2-h assay was used to minimize the effect of new protein synthesis.

**T Cell Surface Markers.** mAbs to CD3, CD4, and CD8 (OK-T3, OK-T4 and OK-T8, Ortho Diagnostic Systems Inc., Westwood, MA) and FITC-labeled rabbit anti-mouse (Fab')<sub>2</sub> (CooperBiomedical, Inc., Malvern, PA) were used to stain T cells (17), which were then analyzed by fluorescence microscopy or in an Ortho Diagnostic Systems Inc. 50 H flow cytometer.

**Serine Esterase Activity.** Enzyme levels were determined as previously described (10).

**CTL Membranes.**  $10^9$  cells of a cloned human CTL cell line, HE-14, were harvested and the membranes purified and solubilized with 0.5% NP-40 in PBS as previously described (11); about 97% of the total BLT-esterase activity present in the cell lysate resided in the pelleted membranes.

**Inhibition of Serine Esterase Activity.** Aliquots of NP-40 solubilized HE-14 membranes containing about 1 U of BLT-esterase activity were diluted to 0.1 ml with 0.5% NP-40 in PBS, and 2  $\mu$ l of the following inhibitor solutions were added: PMSF, 100 mM in DMSO; *N*- $\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 25 mM in DMSO; *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 25 mM in DMSO; and diisopropylfluorophosphoridate (DFP), 100 mM in propylene glycol. After 30 min at 37°C, serine esterase activity was measured as above.

**SDS-PAGE of DFP-labeled Cell Extracts.** 0.4 ml of NP-40 extracts of the murine CTL cell line G4 or of HE-14 cell membranes were labeled with 20  $\mu$ Ci of 1,3-[ $^3\text{H}$ ]DFP in

## 530 SERINE ESTERASE AND HEMOLYTIC ACTIVITY IN HUMAN CTL

propylene glycol (5.2 Ci/mmol; New England Nuclear, Boston, MA) as described (10), and subjected to discontinuous SDS-PAGE on a 10% gel as per Laemmli (18). Gels were briefly stained with 0.1% Coomassie Brilliant Blue (Bio-Rad Laboratories, Richmond, CA) to locate molecular weight markers, then after destaining were impregnated with Autofluor (National Diagnostics, Inc., Somerville, NJ), dried, and exposed at  $-70^{\circ}\text{C}$  on Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY).

*pH Optimum of Serine Esterase Activity.* Aliquots containing  $\sim 1$  U of BLT-serine esterase were diluted to 0.1 ml with buffered saline (130 mM of NaCl, 20 mM Tris phosphate, pH ranging from 5.0–9.5). 10  $\mu\text{l}$  of 20 mM BLT was added, followed after 30 min at room temperature by 0.9 ml of 2.2 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 300 mM TrisCl, pH 8.1. The absorbance at 412 nm was determined 30 s later, to allow ample time for the cleaved BLT to react with the DTNB without having significant amounts of additional enzymatic cleavage of the BLT. At each pH value, the spectrophotometer was blanked to an identically treated sample free of added esterase, to control for any pH effect on the spontaneous hydrolysis of BLT.

*Dot Blot Analysis of cDNA Inserts.* A  $\lambda\text{gt}10$  phage cDNA library was constructed from the human cloned CTL line HE-14 as described (19), and screened with a mixture of [ $^{32}\text{P}$ ]kinase-labeled oligonucleotide probes (20) corresponding to the regions around the active site serine and histidine residues of the murine HF (21) and C11 (22) genes. The cDNA insert from one of the identified clones was compared by dot blot analysis (23) with the insert of a murine cDNA clone known to have a C11 sequence (Verret, C. R., E. B. Reilly, M. S. Pasternack, R. Mattaliano, and H. N. Eisen, manuscript in preparation) using the individual oligonucleotide probes.

*Serine Esterase Secretion by Human CTLs.* CTLs and  $^{51}\text{Cr}$ -labeled JY targets were suspended in RPMI 1640 plus 1 mg/ml BSA (Boehringer Mannheim Diagnostics, Inc., Houston, TX);  $8 \times 10^5$  CTLs in 0.1 ml were added to a variable number of target cells (also in 0.1 ml) to give final CTL/target ratios ranging from 4:1–0.25:1. After 3 h at  $37^{\circ}\text{C}$ , the supernatants from duplicate wells were pooled; 50  $\mu\text{l}$  were taken to determine serine esterase activity, and 75  $\mu\text{l}$  taken to measure release of  $^{51}\text{Cr}$ . Total esterase activity in the CTLs was determined from NP-40 lysates; the baseline esterase activity from JY alone (which is mostly composed of reducing activity rather than enzymatic activity) was determined by incubating targets without CTLs and treating as above.

*Percoll Density Gradient Centrifugation.* CTLs were harvested, washed with PBS, resuspended in relaxation buffer (0.1 M KCl, 3 mM NaCl, 3.5 mM  $\text{MgCl}_2$ , 1.25 mM EGTA (Sigma Chemical Co.), 10 mM Pipes, pH 6.8, with ATP (both Sigma Chemical Co.) added to 1 mM just before use and subjected to Percoll density gradient centrifugation as described (11). After centrifugation, 0.8-ml fractions were collected from the bottom of the tube using capillary tubing attached to a peristaltic pump. To detect serine esterase activity, 20–25- $\mu\text{l}$  aliquots of each fraction were placed in 96-well flat-bottomed assay plates (Immulon-1; Dynatech Laboratories, Inc., Alexandria, VA) and 0.125 ml of a standard reaction mixture (0.1 mM BLT, 1 mM DTNB in 300 mM TrisCl, pH 8.1) was added to each well. After 5 min at room temperature, the absorbance at 414 nm was measured using a Titertek Multiskan scanner (Flow Laboratories, Inc.) blanked to relaxation buffer plus reaction mixture alone. Hemolytic activity in 20–50- $\mu\text{l}$  aliquots was measured as described (11) using as targets either sheep erythrocytes (Colorado Serum Co., Boulder, CO; stored at  $4^{\circ}\text{C}$  in Alsevier's solution, then washed and resuspended in Hepes-buffered saline just before use) or fresh human erythrocytes (collected sterily into sodium-heparin, 25 U/ml, then washed and resuspended in Hepes-buffered saline).  $\sim 3 \times 10^7$  erythrocytes were used for each sample. The absorbance of released hemoglobin was measured at 414 nm on a Titertek Multiskan scanner blanked to PBS.

*Freeze-Thaw Assay for Hemolytic Activity.*  $5 \times 10^6$  CTLs (HE-14, ML-4) or murine CTLs (2C) were washed twice in PBS plus 1 mM EDTA, then resuspended in 0.05 ml of the same buffer. Samples were frozen in a dry ice-ethanol bath for 10 min, then thawed in cold water, centrifuged for 5 min at 8,000 rpm and  $4^{\circ}\text{C}$ , and the supernatants were stored on ice until assayed. Aliquots of supernatants obtained after one, two, and three freeze-thaw cycles were tested for hemolytic activity as above.

*Other Cell Lines.* JY (HLA-A2,-B7) was obtained from the Tissue Culture Center at MIT, and CCRF-SB (HLA-A1,2-B12,17-Cw2) and RPMI 7666 (HLA-A3,w29,-B7,12) were obtained from the American Type Culture Collection (Rockville, MD). JY and CCRF-SB were derived by EBV transformation of peripheral blood lymphocytes; RPMI 7666 is a B cell lymphoma line. 6B-7 is a CTL clone derived from a transplanted kidney during an episode of rejection (24, 25) and was the gift of Dr. James Kurnick, Massachusetts General Hospital.

## Results

*BLT-Serine Esterase Activity.* Table I summarizes the levels of BLT-serine esterase found in NP-40 lysates of 20 human T cell lines. All but three (1E-12, ML-47.2, and 5D-4) were cytotoxic, exhibiting 30–80% specific lysis of  $^{51}\text{Cr}$ -labeled target cells during a 4-h assay at a CTL/target ratio of 20:1. For comparison, three human B cell lines and one murine CTL were also assayed. The murine CTL 2C had an enzyme level ( $94 \text{ U}/10^6$  cell equivalents) similar to that previously described for other cloned murine CTLs (10). The human B cell lines had only trace activity in the BLT assay ( $<0.5 \text{ U}/10^6$  cells). In contrast, the level of serine esterase in human cytolytic T cells ranged from 3.9–21  $\text{U}/10^6$  cells, with an average of 9.1  $\text{U}/10^6$  cells; no consistent difference was seen between  $\text{CD4}^+$  and  $\text{CD8}^+$  cell lines. While the levels in three noncytotoxic  $\text{CD4}^+$  human T cell lines were perhaps slightly lower than in cytotoxic T cells, they were distinctly higher than in non-T cells; this is in contrast to murine T cell lines in long-term culture, where the enzyme levels in nearly all noncytotoxic ( $\text{CD4}^+$ ) T cells is  $<1\%$  of the level in cytotoxic T cells (10). However, the level of esterase activity seen in all the human CTL clones was consistently lower than in murine CTLs.

The levels of BLT-serine esterase activity are very low in murine thymocytes, but increase more than 50-fold after activation by Con A, in parallel with the appearance of cytotoxic activity (10). Similarly, the enzyme levels in fresh human peripheral blood lymphocytes were also very low ( $\sim 0.5 \text{ U}/10^6$  cells), but increased along with the appearance of specific cytolytic activity during the course of a mixed lymphocyte culture, to 1.5  $\text{U}/10^6$  cells after 5 d and 3.8  $\text{U}/10^6$  cells after 12 d.

*Inhibitors of BLT-Serine Esterase.* One of the distinguishing features of the murine T cell-specific serine esterase is its pattern of susceptibility to protease inhibitors: it is inhibited by PMSF and DFP, but not by TLCK or TPCK (10). Similarly, after a 20-min incubation at room temperature with 2 mM PMSF, the human BLT-serine esterase was almost completely inhibited (97% reduction in esterase activity), whereas a similar incubation with 0.5 mM TLCK or 0.5 mM TPCK did not significantly inhibit the enzyme ( $-1\%$  and  $4\%$  inhibition, respectively) (Table II). A single addition of 2 mM DFP resulted in only 34% inhibition, probably because of instability of DFP in an aqueous environment at pH 8; increased inhibition (to 68%) was seen with a second addition of DFP. As with murine CTLs (10), preincubating intact human CTLs with PMSF markedly decreased their cytolytic activity in a 2-h  $^{51}\text{Cr}$ -release assay (Fig. 1); 15 min in 6 mM PMSF reduced cytotoxicity by  $\sim 75\%$ , while not affecting the viability of either the CTLs (as assessed by trypan blue exclusion) or the targets (as assessed by release of  $^{51}\text{Cr}$ ).

## 532 SERINE ESTERASE AND HEMOLYTIC ACTIVITY IN HUMAN CTL

TABLE I  
BLT-DTNB Assay for Serine Esterase Activity

Cell line	Source	Type	Specificity*	Esterase level
				<i>U/10<sup>6</sup> cells</i>
JY	EBV-pbl	B cell	—	0.2
CCRF-SB	EBV-pbl	B cell	—	0.3
RPMI 7666	Lymphoma	B cell	—	0.1
2C	Murine CTL	CD8 <sup>+</sup>	H2-L <sup>d</sup>	94
HE-14	MLC	CD4 <sup>+</sup>	JY	11.7
HE-17	MLC	CD4 <sup>+</sup>	LDK	4.2
HEJ-20	MLC	CD4 <sup>+</sup>	JY	21.0
ML-21	MLC	CD4 <sup>+</sup>	JY, CCRF-SB	19.4
HEB-13	MLC	CD8 <sup>+</sup>	HLA-B7	9.9
HEB-34	MLC	CD8 <sup>+</sup>	HLA-A2	5.8
ML-4	MLC	CD8 <sup>+</sup>	HLA-A2	4.1
ML-32	MLC	CD8 <sup>+</sup>	HLA-B7	4.6
ML-37.4	MLC	CD8 <sup>+</sup>	HLA-A2	15.4
1B-1	MLC	CD8 <sup>+</sup>	LDK	9.6
1B-6	MLC	CD8 <sup>+</sup>	LDK	4.4
1D-12	MLC	CD8 <sup>+</sup>	CCRF-SB	3.1
5C-5	MLC	CD8 <sup>+</sup>	LDK	7.5
1B-5	MLC	CD8 <sup>+</sup>	LDK	4.2
1A-3	MLC	CD8 <sup>+</sup>	LDK	3.9
1D-1	Synovium	CD8 <sup>+</sup>	LDK	13.0
6B-7	Renal graft	CD8 <sup>+</sup>	?	11.4
1E-12	Synovium	CD4 <sup>+</sup>	Non-killer	3.4
ML-47.2	MLC	CD4 <sup>+</sup>	Non-killer	5.6
5D-4	MLC	CD4 <sup>+</sup>	Non-killer	2.2

\* Where appropriate, specificity was determined in a 4-h <sup>51</sup>Cr-release assay using various B-cell lines as targets (7). LDK, lectin-dependent killer, denotes cells cytolytic only in the presence of Con A.

TABLE II  
Inhibition of HE-14 Serine Esterase Activity

Inhibitor	Concentration	Esterase activity	Inhibition
	<i>mM</i>	<i>OD<sub>412</sub></i>	%
None	—	0.608	—
PMSF	2.0	0.018	97
TLCK	0.5	0.615	1
TPCK	0.5	0.585	4
DFP	1 × 2.0	0.403	34
DFP	2 × 2.0	0.192	68

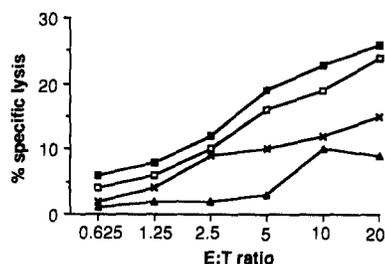


FIGURE 1. Inhibition of cytolytic activity of a human cloned CTL cell line (HE-14) by PMSF. HE-14 cells were preincubated with PMSF for 15 min, then assayed for killing activity against  $^{51}\text{Cr}$ -labeled JY. (■) no PMSF; (□) 2 mM PMSF; (×) 4 mM PMSF; (▲) 6 mM PMSF.

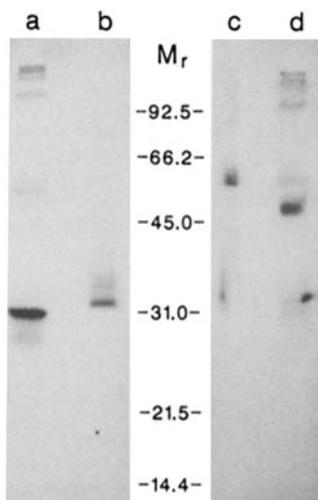


FIGURE 2. Relative molecular mass ( $M_r$ ) of  $[^3\text{H}]\text{DFP}$ -labeled proteins in lysates of human and murine cloned CTL cell lines. NP-40 extracts of murine G4 cells (lanes *b* and *c*) or of human HE-14 cell membranes (lanes *a* and *d*) were labeled with 1,3- $[^3\text{H}]\text{DFP}$  and subjected to discontinuous SDS-PAGE on a 10% gel. Lanes *a* and *b* were run under reducing conditions, *c* and *d* under nonreducing conditions.

**SDS-PAGE of  $[^3\text{H}]\text{DFP}$  Labeled BLT-Serine Esterase.** Because human CTLs exhibit less BLT-serine esterase activity than murine CTLs, only limited amounts of  $[^3\text{H}]\text{DFP}$  affinity-labeled enzyme from unfractionated cell lysates could be loaded onto a gel. To overcome this limitation, NP-40-solubilized HE-14 cell membranes (which contained ~97% of the BLT-serine esterase activity present in these cells) were labeled and subjected to SDS-PAGE under reducing and nonreducing conditions; similarly labeled extracts of the murine CTL line G4 were included for comparison (Fig. 2). As shown in Fig. 2, under reducing conditions the tritium label from HE-14 appeared predominately in a band of  $M_r$   $31 \times 10^3$ ; the murine cell extract shows a more diffuse band at  $M_r$   $30\text{--}34 \times 10^3$ . Under nonreducing conditions, the  $^3\text{H}$ -labeled proteins had a higher apparent molecular mass ( $M_r$   $\sim 51 \times 10^3$  for human and  $\sim 59 \times 10^3$  for mouse), indicating that the human enzyme, like the mouse enzyme (11, 13) is probably a disulfide-linked dimer.

**pH Dependence of BLT-Serine Esterase.** The pH optimum for the BLT-serine esterase activity in murine CTLs is about 8 (13; our unpublished results), much higher than the optimal pH for macrophage esterases (pH 3–4). The activity of the serine esterases from HE-14 cell membranes and 2C whole-cell lysates was measured at various pH values ranging from 5 to 9.5 (Fig. 3). For both enzymes, activity was negligible at pH 5, and increased in parallel to a maximum at pH 8–9.

**Homology of Human Serine Esterase cDNA With the Murine HF Gene.** To further

## 534 SERINE ESTERASE AND HEMOLYTIC ACTIVITY IN HUMAN CTL

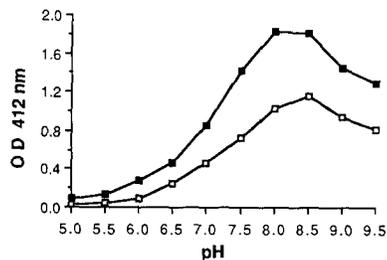


FIGURE 3. pH dependence of serine esterase activity in human and murine CTLs. Equal aliquots of NP-40 solubilized whole cell lysate (mouse) or cell membranes (human) were assayed for amount of BLT-serine esterase activity at various pH values. (■) human CTL clone HE-14; (□) mouse CTL clone 2C.

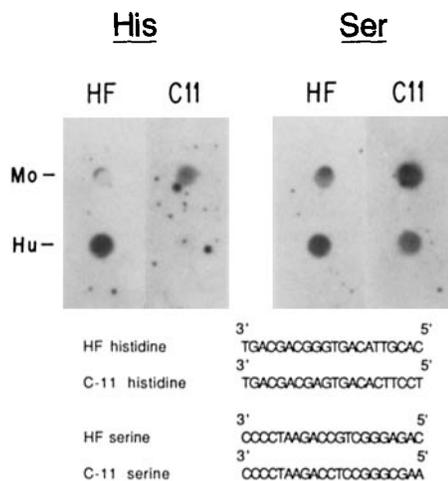


FIGURE 4. Hybridization of inserts from human and mouse cDNA clones with oligonucleotide probes derived from genes for two murine T cell-specific serine esterases (HF and C11). A mixture of oligonucleotide probes with sequences corresponding to regions around the active site serine and histidine residues of the mouse HF and C11 genes was used to screen a  $\lambda$ gt10 phage cDNA library from the human cloned CTL cell line HE-14. The cDNA insert from one of the identified clones (Hu) was compared by dot blot analysis with the insert of a mouse cDNA clone (Mo) known to have the C11 sequence (Verret, C. R., et al., manuscript in preparation). In the blots shown, the four [ $^{32}$ P]kinase-labeled oligonucleotide probes were used individually: HF at the serine-183 (Ser) and histidine-41 (His) sites (21) and C11 at the serine-184 (Ser) and histidine-44 (His) sites (22). In RNA dot blot assays, it reacted consistently with RNA from human T cell lines; it also reacted with two EBV-transformed B cell lines, but not with a human B cell lymphoma cell line (data not shown). The four probes are shown below.

characterize the human BLT-serine esterase, a cDNA library was constructed from the human CTL line HE-14 and screened with a mixture of synthetic oligodeoxynucleotide probes corresponding in sequence to the regions around the active site serine (Ser) and histidine (His) residues of the murine serine esterase genes termed HF (21) and C11 (22). Dot blots of the insert from one of the strongly hybridizing cDNA clones, together with a previously isolated murine cDNA clone having a sequence identical to the published sequence for murine C11, were separately hybridized with each of the oligonucleotide probes (Fig. 4). The human cDNA clone hybridized strongly with the His probe from the murine HF gene but not with the His probe from the murine C11 gene, indicating homology between the human cDNA clone and the murine HF gene. The intensity of hybridization to the Ser probes also support this conclusion, although less definitively because the probes crosshybridize with both the murine HF and C11 genes.

*Serine Esterase Secretion.* Many murine CTLs release some (10–60%) of their total serine esterase activity into the medium when they interact with an excess of their target cells (11, 26). Two human CTL lines (HE-14, HEJ-20) were similarly tested for serine esterase secretion when incubated with up to a fourfold excess of the appropriate  $^{51}$ Cr-labeled target cells. No significant release of BLT-

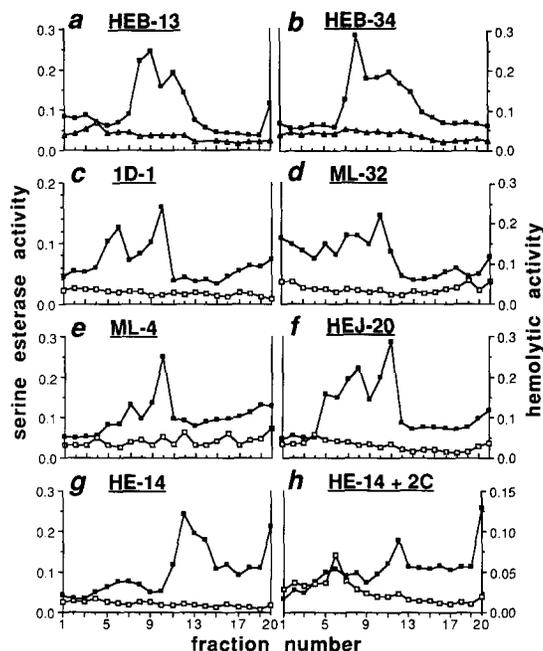


FIGURE 5. Percoll density gradient centrifugation of nitrogen-cavitated human and murine CTLs. Fractions were monitored for serine esterase activity (■) and for hemolytic activity (□, when measured with sheep erythrocytes; △, when measured with fresh human erythrocytes). Number of cells analyzed: (a)  $5 \times 10^7$  HEB-13; (b)  $1.4 \times 10^8$  HEB-34; (c)  $5 \times 10^7$  1D-1; (d)  $5 \times 10^7$  ML-32; (e)  $8 \times 10^7$  ML-4; (f)  $9 \times 10^7$  HEJ-20; (g)  $1 \times 10^8$  HE-14; (h)  $1 \times 10^8$  HE-14 +  $2 \times 10^7$  2C. Only the 2C cells were of murine origin.

serine esterase activity into the medium was detected over 3 h, although considerable target cell lysis (as high as 0.8 target cell lysed per CTL) was observed.

**Percoll Density Gradient Centrifugation.** When disrupted by nitrogen cavitation and subjected to discontinuous Percoll gradient centrifugation, murine CTLs exhibit both a large high-density peak of BLT-serine esterase activity that coincides with the peak of hemolytic activity, and a small low-density peak of BLT-serine esterase activity (11, 12). Seven human CTL lines (two CD4<sup>+</sup>, five CD8<sup>+</sup>) were similarly analyzed by Percoll density centrifugation (Fig. 5, a-g). Each gradient exhibited two peaks of activity, with the high-density peak being only about the same size as the low-density peak, instead of much larger as with murine CTLs.

**Hemolytic Activity.** As shown in Fig. 5, none of the Percoll gradients contained any hint of hemolytic activity, even when the aliquots tested were 20 times larger than those sufficient to demonstrate considerable hemolysis from murine CTL lines. To test the possibility that this lack of hemolytic activity was due to the presence of an inhibitor, rather than the absence of the hemolytic protein per se,  $10^8$  HE-14 cells were mixed with  $2 \times 10^7$  murine CTL (2C) cells, and the mixture subjected to nitrogen cavitation and Percoll density centrifugation (Fig. 5h). The amount and position of the peak with hemolytic activity from the murine CTL 2C matched the results from gradients prepared from 2C alone, and coincided with the high-density serine esterase peak. Thus, the absence of hemolytic activity in the human CTL cell lysate was not due to the presence of an inhibiting substance, at least not one that was also capable of inhibiting the murine hemolytic activity. The location of the murine and human high-density BLT-serine esterase peaks coincided in this experiment and also when separate gradients of murine cells and HE-14 were run in parallel (data not shown).

## 536 SERINE ESTERASE AND HEMOLYTIC ACTIVITY IN HUMAN CTL

TABLE III  
Hemolytic Activity After Freeze-Thaw Lysis of Murine and Human CTLs

Number of freeze-thaw cycles	CTL cell equivalents tested	Hemoglobin release (specific hemolysis)					
		2C (murine)		HE-14 (human)		ML-4 (human)	
		OD <sub>414</sub>	%	OD <sub>414</sub>	%	OD <sub>414</sub>	%
1	2 × 10 <sup>5</sup>	2.073	85	0.056	-1	0.062	-1
	1 × 10 <sup>6</sup>	2.379	98	0.059	-1	0.058	-1
	2.5 × 10 <sup>6</sup>	2.369	98	0.053	-1	0.057	-1
2	2 × 10 <sup>5</sup>	0.415	15	0.068	0	0.051	-1
	1 × 10 <sup>6</sup>	2.312	96	0.046	-1	0.057	-1
	2.5 × 10 <sup>6</sup>	2.397	99	0.050	-1	0.051	-1
3	2 × 10 <sup>5</sup>	0.148	3	0.055	-1	0.069	0
	1 × 10 <sup>6</sup>	1.710	70	0.050	-1	0.058	-1
	2.5 × 10 <sup>6</sup>	2.152	89	0.054	-1	0.059	-1

Detergent lysis of erythrocytes, which resulted in an absorbance of 2.416, was taken as 100% hemolysis; untreated erythrocytes (spontaneous hemolysis) resulted in an absorbance of 0.074.

To look for hemolytic activity by an alternative method, we assayed suspensions of freeze thawed cells (27) from two human CTL lines (HE-14, ML-4) in parallel with the murine CTL line 2C. As can be seen in Table III, the hemolytic activity from murine CTLs is easily detected in this manner; in contrast, detectable hemolytic activity was not released from the human CTLs.

### Discussion

Taken together, all of these results show that cloned human T cell lines contain a homologue of the murine T cell-specific BLT-serine esterase. The human and murine enzymes are similar in several respects: (a) both are expressed in T cells but not in B cells; (b) they have similar patterns of inhibition by small organic molecules (i.e., they are inhibited by PMSF and DFP, but not by TLCK or TPCK); (c) both enzymes can be affinity labeled with [<sup>3</sup>H]DFP, and SDS-PAGE analysis under reducing conditions revealed the labeled proteins to have similar apparent molecular weights ( $M_r$  ~30 × 10<sup>3</sup> for the human enzyme and ~30-34 × 10<sup>3</sup> for the murine enzyme); (d) a cDNA clone from a library prepared from a human cloned T cell line (HE 14) hybridized with two synthetic oligonucleotide probes that correspond to nucleotide sequences surrounding the active site serine and histidine of the HF gene, which encodes the principal murine T cell-specific BLT-serine esterase (28); (e) just as the BLT-serine esterase activity in murine thymocytes increases markedly after stimulation with Con A, the activity in human peripheral blood lymphocytes also increased after allogeneic stimulation.

However, there are also some important differences between the murine and human BLT-serine esterases: (a) the enzyme level in the cloned human T cell lines is only about one-tenth that present in cloned murine CTLs; (b) whereas with murine T cells it was found initially that the enzyme is expressed at high levels (30-200 U/10<sup>6</sup> cells) in cytolytic clone T cell lines but not in noncytolytic (helper) cloned T cell lines (which typically had ≤1 U/10<sup>6</sup> cells), we have seen

here that in the cloned human T cell lines the enzyme is present at about the same level in both cytolytic (either CD4<sup>+</sup>,CD8<sup>-</sup> or CD4<sup>-</sup>,CD8<sup>+</sup>) and noncytolytic (CD4<sup>+</sup>,CD8<sup>-</sup>) lines. Interestingly, a recent study has shown that, in mice also, the CD8<sup>+</sup> (Lyt-2<sup>+</sup>) and CD4<sup>+</sup> (L3T4<sup>+</sup>) bulk T cell populations derived from 6-d mixed lymphocyte cultures have similar levels of BLT-serine esterase, despite substantial difference in cytolytic activity (27). It is possible that the previously observed differences between cytolytic and noncytolytic murine CTLs is a consequence of these cells having been maintained in culture for long periods (i.e., 1-5 yr); the cloned human T cell lines tested here were in culture for no longer than 6 mo; (c) most murine CTLs are triggered to secrete their BLT-serine esterase by interaction with target cells; two similarly stimulated human CTL clones did not release a detectable level of BLT-serine esterase during interaction with their target cell; (d) the apparent molecular weights of the reduced and nonreduced [<sup>3</sup>H]DFP-labeled human enzyme are  $\sim 31 \times 10^3$  and  $51 \times 10^3$ , respectively, whereas the corresponding values for the murine enzyme are  $30-34 \times 10^3$  and  $59 \times 10^3$ , respectively. While the murine enzyme is probably a homodimer (11, 13), the present data would suggest that the human enzyme exists as a heterodimer consisting of a DFP-reactive subunit of  $\sim 31 \times 10^3 M_r$  and a nonreactive subunit of  $\sim 20 \times 10^3 M_r$ ; however, the possibility that the human enzyme is a homodimer with an unusually high electrophoretic mobility cannot be ruled out. It may be noted parenthetically that other human-mouse homologues differ structurally to a surprising extent: the CD8 glycoprotein is a homodimer on human T cells but a heterodimer (Lyt-2,3) on murine T cells (29).

Perforin plays a central role in the exocytosis model of target cell destruction by CTLs (2-6) and NK cells (30-33). It was therefore surprising that in none of the 17 human CTL clones analyzed could we find evidence of hemolytic activity using procedures that readily reveal such activity in cloned murine CTLs. Since the human CTLs do not contain a demonstrable inhibitor of perforin activity, it would appear that if perforin activity is present at all, it is either at exceedingly low levels (<2% of the level in mouse cells) (33) or in an unusually labile form. Whether perforin content of cells strictly parallels perforin hemolytic activity is not known. Recent observations by Berke (34) and others (e.g., 35-38) suggest that release of cytolytic granules with their included perforin may not be the only mechanism by which murine CTLs can rapidly destroy target cells. The absence of the characteristic hemolytic activity of perforin, the lack of detectable serine esterase secretion during interaction with specific target cells, and, perhaps, the low overall level of BLT-serine esterase, all suggest that the human CTLs examined may likewise kill their target cells by a perforin-independent or granule-independent mechanism.

### Summary

Target cell lysis by most murine cytotoxic T lymphocytes appears to be mediated by a complement (C9)-like protein called perforin, contained in high-density cytoplasmic granules. These granules also contain high levels of serine esterase activity, which may also play a role in cytolysis. Analysis of 17 cloned human cytotoxic T lymphocytes revealed the presence of serine esterase that is

very similar to its murine counterpart in substrate and inhibitor specificities, pH optimum, and molecular mass; dot blot hybridization with synthetic oligonucleotides corresponding to the active sites of two known murine CTL esterases suggests homology to the murine enzyme HF. However, serine esterase was present at only ~10% of the level found in murine CTLs, and was not secreted during CTL-target cell interaction; moreover, hemolytic activity could not be detected in any of the seven cell lines tested. The results suggest that the human CTLs examined here kill their target cells by a mechanism different from that used by most cloned murine CTLs.

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540 SERINE ESTERASE AND HEMOLYTIC ACTIVITY IN HUMAN CTL

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