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Expression of mRNAs for Pore-Forming Protein and Two Serine Esterases in Murine Primary and Cloned Effector Lymphocytes

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The cDNAs encoding several proteins present in the granules of cytolytic effector lymphocytes have now been cloned. These include the cytolytic pore-forming protein (PFP) or perforin, and at least six serine esterases (SE), also called granzymes. The cDNA probes for PFP, SE-1, and SE-2 are used here to study the expression of these proteins in murine primary effector lymphocytes. Among the stimuli effective in inducing the expression of PFP, SE-1, and SE-2 were recombinant interleukin-2, the lectin concanavalin A in the presence of phorbol esters, and allogeneic cells in mixed lymphocyte cultures. Some correlation was seen between the levels of PFP and SE mRNAs and cytotoxicity measured in a standard ^{51}Cr release assay. We also examined a panel of 13 cloned cytotoxic T lymphocyte (CTL) lines and found that mRNAs for PFP and SE-2 were expressed in all CTL lines, including some that were previously considered not to produce PFP. Twelve of the 13 CTL lines also proved to possess the mRNA for SE-1. One thymoma cell line, TIMI.4, did not express mRNA for PFP, although it expressed mRNA for SE-1 and SE-2.

Key words: cytotoxic T lymphocytes

The cytoplasmic granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells contain a cytolytic, calcium-dependent, pore-forming protein (PFP, perforin, or cytolysin) and several serine esterases (SE, also called granzymes). According to the pore-formation, or osmotic lysis, model of lymphocyte-mediated killing, the formation of pores on target cell membranes is thought to be responsible for cell death [1–5]. The cDNAs encoding PFP/perforin [6–9] and several SE [10–15] have now been cloned and sequenced. Recent reports have raised questions regarding the role of perforin in CTL-mediated cytotoxicity, as neither cytoplasmic granules nor perforin was detected in

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primary CTL populations that were nevertheless cytotoxic [16–18]. The role of serine esterases in cell-mediated killing is also unclear [19]. To address some of these questions we studied the accumulation of mRNAs for PFP and two SE in murine primary lymphocytes induced to become cytolytic by different stimuli. Since several cloned CTL lines known to be cytotoxic apparently lack hemolytic activity, and by inference lack PFP [20], we also examined the expression of these mediators in an array of murine CTL lines. This is the first study to investigate the simultaneous expression of PFP and serine esterase mRNAs in the same cell populations.

MATERIALS AND METHODS

Cells

Murine CTL cell lines L3, OE4, AB.1, and AB.2 were derived from MLC-generated CTL as described [21,22]. L3 and OE4 are $H2^b$ anti-$H2^d$; AB.1 and 2 are $H2^d$ anti-$H2^b$. CTL cell lines DAB-2, -3, -6, -11, -16, and -17, and BAD-M (BAD-1), are murine alloimmune CTL lines, and along with the thymoma line TIMI.4, were generously provided by Dr. R. Duke (University of Colorado School of Medicine, Denver). The DAB lines are $H2^d$ anti-$H2^b$; BAD-M is $H2^b$ anti $H2^d$ [23]. All CTL cell lines were maintained in modified minimal essential medium (α MEM, Gibco) supplemented with 5% FBS (Hyclone Laboratories) and 10% rat spleen cell-conditioned medium, and stimulated weekly with the appropriate allogeneic cells. Murine CTL cell lines CTLL-2 and CTLL-R8, generated as described [21,22], were maintained in the same medium, but without periodic antigenic stimulation. Murine mastocytoma P815 ($H2^d$) was maintained as a suspension in α MEM/5% FBS. EL-4 ($H2^b$), a mouse lymphoma, was maintained in RPMI 1640/5% FBS.

Mice 8–12 weeks old, C57BL/6 ($H2^b$), CD2F1 ($H2^d$), and DBA/2 ($H2^d$), were purchased from the Trudeau Institute (Saranac Lake, NY). To prepare primary lymphocytes, a single-cell suspension of mouse spleen cells was eluted through a nylon wool column (Fenwal Laboratories, Deerfield, IL). Non-adherent cells were resuspended in T cell growth medium (RPMI 1640/10% FBS/2 mM glutamine/5×10^{-5} M 2-mercaptoethanol). The cells were stimulated with either recombinant human interleukin-2 (rIL-2, generously provided by the Cetus Corporation, Emeryville, CA) or a combination of 20 μg/ml concanavalin A (Con A, Sigma) and 10 ng/ml phorbol myristyl acetate (PMA, Sigma) and cultured at 37°C in a humidified incubator with 5% CO_2. For mixed lymphocytic cultures (MLC), spleen cells from C57BL/6 mice were cultured with irradiated (2,000 rads, using a ^{137}Cs source) spleen cells from DBA/2 mice at a ratio of 1:1 (MLC #1); the stimulator cells were not passed through a nylon wool column. For MLC #2 the stimulator and responder cells were from C57BL/6 and DBA/2 mice respectively.

Plastic adherent lymphokine-activated killer cells (LAK) were prepared by a modification of the protocol of Vujanovic et al. [24]. Briefly, the nylon-wool-purified spleen cells were incubated with 1000 U/ml rIL-2. After 48 h the non-adherent cells were removed and the adherent cells were incubated a further 48 h and then collected.

Peritoneal exudate lymphocytes (PEL) were harvested from Balb/c mice ($H2^d$) that had been injected with EL-4 ($H2^b$) cells intraperitoneally 10 days earlier [25]. PEL were collected by peritoneal lavage and passed through a nylon wool column. The cells were washed and resuspended in α MEM/10% FBS.

Cytotoxicity Assay

The cytotoxicity of MLC-derived cells was determined by 4 h ^{51}Cr release assay as described [21], using P815 or EL-4 cells as targets. To assess cytotoxic activity of stimulated spleen cells we used lectin-dependent cell cytotoxicity (LDCC) assays, which were performed as above, except that 5 μg/ml Con A was included in the incubation medium.

cDNA Clones

Murine PFP cDNA and SE-1/Hanukah Factor (HF)/Granzyme A cDNA clones were obtained as described [9,13]. The SE-2/CTLA-1/Granzyme B cDNA clone [12] was generously provided by Dr. P. Golstein (CNRS, Marseilles-Luminy). Human γ-actin cDNA clone [26] was obtained from Dr. J. Trapani (Sloan-Kettering Institute, New York).

Northern Blots

Total RNA was extracted from tissue culture cells by the guanidinium method as described [27]; 10–15 μg RNA was analysed by formaldehyde-agarose gel electrophoresis by standard techniques [28]. The RNA was transferred onto GeneScreen Plus (NEN) membrane according to the manufacturer's instructions and baked at 80°C for 2 h in a vacuum oven. The blots were prehybridized for 2 h in a solution containing 50% formamide, 10% dextran sulfate, 1.0 M NaCl, 1% sodium dodecyl sulfate, and 100 μg/ml sonicated herring sperm DNA. Radioactive ^{32}P-labeled cDNA probes were prepared by nick translation and added to the prehybridization solution. After washing, the blots were autoradiographed at −70°C on Kodak XAR films. Intensity of signal was quantitated by densitometric scanning on an LKB ultroscan XL laser densitometer (Pharmacia). The signal intensities obtained with PFP, SE-1, and SE-2 probes were compared with the intensity seen with actin cDNA probe and expressed numerically as a quantitative measure of the relative change in the mRNA level. Where indicated, the signal intensities were quantitated on autoradiograms exposed for shorter or longer durations than those shown in the figures. The blots were stripped according to manufacturer's instructions and sequentially rehybridized with other probes.

RESULTS

Cloned CTL Cell Lines

Previous work [20] has shown that PFP, as assessed by hemolytic activity, was not present in some CTL cell lines known to be cytotoxic. Northern blot analysis with PFP cDNA probes showed the expression of perforin mRNA in all 13 CTL cell lines tested, but not in the thymoma line TIMI.4 (Fig. 1, data not shown for TIMI.4 and DAB 16 and 17). The CTL line AB.2, which was previously considered to be PFP negative, also expressed PFP. All 13 CTL lines, and TIMI.4, expressed both SE-1 and SE-2 mRNAs, with the exception of CTLL-R8, which did not express SE-1. For comparison, the signal intensities for these transcripts were normalized against the signal intensity for actin obtained on the same blot (Fig. 1, lower panel).

Primary Cells

Spleen cells stimulated with rIL-2 expressed a 2.9 kb mRNA specific for PFP. The ratio of perforin to actin mRNAs increased markedly, and correlated well with the

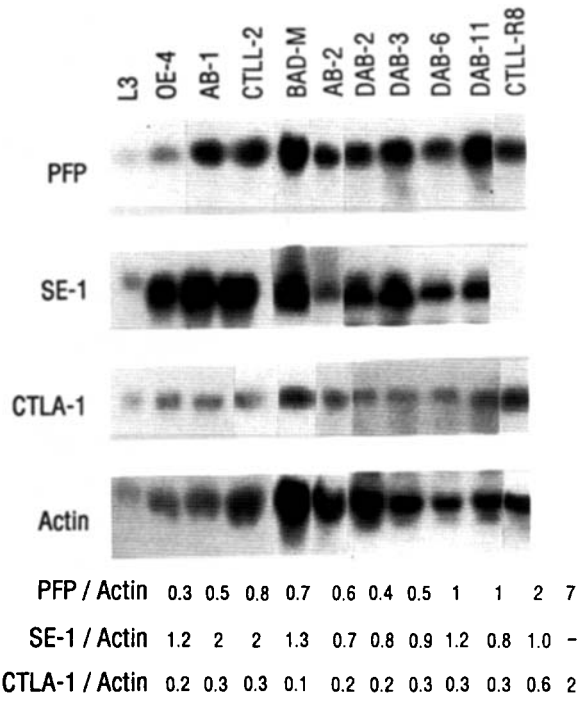

Fig. 1. Expression of PFP and SE in CTL lines. Total cellular RNA (10–15 μg) obtained from CTL lines grown in vitro was electrophoresed, transferred to nylon membranes, and sequentially hybridized with ^{32}P-labeled cDNA probes as described in Materials and Methods. The ratio of signal intensities with PFP and SE probes to the intensity with actin probe was expressed numerically as described in Materials and Methods. CTLA-1 is used to investigate the expression of SE-2. CTLL-R8 hybridized with PFP was exposed for autoradiography for only 6 h, as compared to the others which were exposed for 24 h.

increase in cytotoxicity (Fig. 2, compare upper and lower panels). It should be emphasized that the PFP/actin ratio is a rough estimate of the increase in PFP mRNA, since the ratio would be affected by small changes in the actin mRNA level. Stimulation with rat spleen-cell-conditioned medium, which contains IL-2, gave similar results (data not shown). Plastic adherent LAK cells, which have been cultured for 5 days in the presence of high concentrations of rIL-2, are very actively cytolytic and produce abundant mRNA for PFP. Messenger RNA levels for SE-1 and SE-2 also showed similar changes.

When spleen cells were stimulated with Con A and PMA, both PFP and SE-2 mRNA levels increased gradually over 5 days (Fig. 3), while the SE-1 mRNA level did not change appreciably. These changes could not be correlated with cytotoxicity as cytotoxicity levels were low.

In MLC, the expression of PFP, SE-1, and SE-2 mRNAs appeared to increase during the 6 days of culture, but comparison of signal intensities with those obtained with actin cDNA probe was difficult because of RNA degradation observed in all cell samples obtained from MLC (Fig. 4, and data not shown).

We also attempted to characterize PEL in terms of the expression of PFP and SE, but failed to observe any measurable expression of PFP, SE-1, or SE-2 in the PEL tested

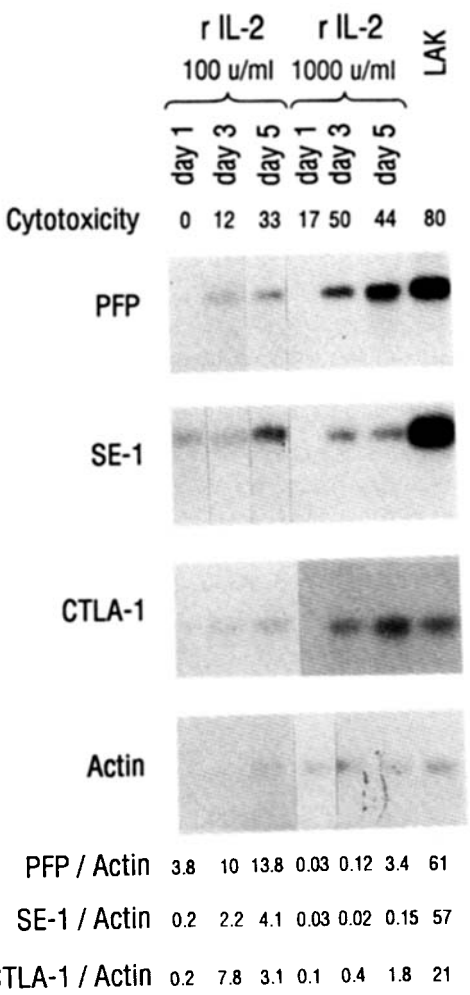

Fig. 2. Expression of PFP and SE in spleen cells stimulated with IL-2. Spleen cells in tissue culture dishes were stimulated with either 100 U/ml or 1,000 U/ml of recombinant human IL-2 for 1, 3, or 5 days. LAK cells were prepared as detailed in Materials and Methods. Cytotoxicity was tested by LDCC in a ^{51}Cr release assay. RNA yields on day 1 were very low with both concentrations of IL-2. Northern blots were prepared and hybridized as in Figure 1.

(data not shown). However, all PEL samples obtained according to published protocols [25] gave largely degraded RNA, precluding us from drawing reliable conclusions.

DISCUSSION

Whether PFP and SE are the mediators of cytotoxicity in CTL lines remains an open question. We approach this problem by investigating the expression of mRNAs encoding these proteins in T lymphocytes. The expression of mRNA does not necessarily imply the production of the corresponding proteins, and our results should be interpreted in this light. The presence of PFP mRNA in all CTL lines studied, and its absence in the

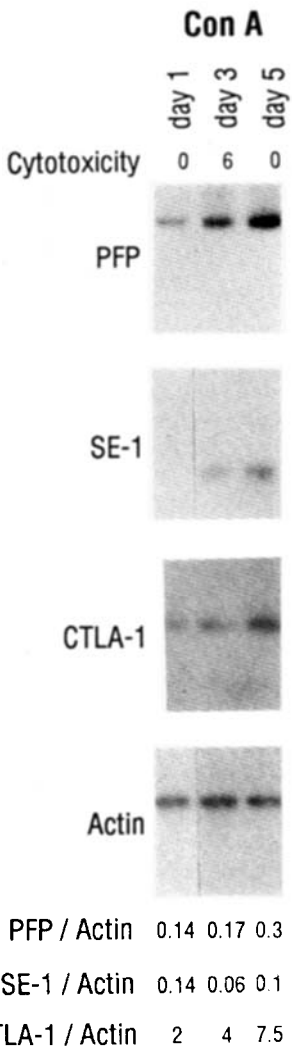

Fig. 3. Expression of PFP and SE in spleen cells stimulated with Con A and PMA. Spleen cells in tissue culture were stimulated with 20 μg/ml Con A and 10 ng/ml PMA for 1, 3, and 5 days. Cytotoxicity was tested by ^{51}Cr release in an LDCC assay. Northern blots were prepared and hybridized as in Figure 1.

thymoma line TIMI.4, lend support to, but cannot prove, the osmotic lysis model. Since PFP was not demonstrable in some primary CTL [16–18], its role as a cytotoxic mediator has been controversial. We show its presence in spleen cells activated by three different stimuli—rIL-2, Con A with PMA, and MLC. Since these studies were conducted with bulk populations of splenic T cells including both $CD4^+$ and $CD8^+$ lymphocytes, it is not possible to draw definitive conclusions as to which of the subpopulations express the PFP mRNA. We are investigating this question, and our preliminary data (not shown) suggest PFP is produced mainly, but not exclusively, by the $CD8^+$ lymphocyte subset.

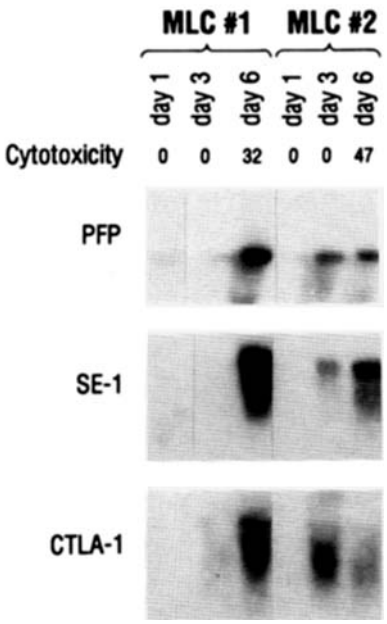

Fig. 4. Expression of PFP and SE in spleen cells stimulated in MLC. Mixed lymphocyte cultures were set up as described in Materials and Methods. Northern blots were prepared and hybridized as in Figure 1. Cytotoxicity was tested in a ^{51}Cr release assay by using as targets P815 ($H2^d$) in MLC #1 and EL-4 ($H2^b$) in MLC #2. Very low yields of RNA were obtained on day 1 of both MLC.

Our finding that PFP and SE are expressed by stimulated primary lymphocytes and by cloned CTL lines suggests that both types of cells could use essentially similar mechanisms of cytotoxicity. It remains possible that primary CTL generated as PEL do not express PFP, as suggested by Berke and Rosen [17,18]. We were unable to resolve this question due to technical limitations.

Serine esterases in CTL remain enzymes in search of a function. PFP and SE appear to be induced by the same stimuli and over a similar period of time. Their ubiquitious presence, in both primary CTL and cloned CTL lines, suggests they play an important, as yet undetermined, role in CTL biology.

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