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The Regulation of CD95 Ligand Expression and Function in CTL

Jie-hui Li,* Dalia Rosen,* Denise Ronen,* Christian K. Behrens,† Peter H. Krammer,† William R. Clark,‡ and Gideon Berke*‡

Previous studies with CTL lines and CTL hybridomas have suggested that functional CD95 (APO-1/Fas)-ligand (CD95L) expression on effector CTLs is a consequence of specific CTL-target recognition and TCR triggering of newly transcribed CD95L. Such a control on the expression of CD95L could provide a double safeguard for killing only cognate target cells. Here the regulation of CD95L expression and function was tested in in vivo primed, allogeneic peritoneal exudate CTL (PEL) from perforin-deficient (P0) mice. CD95L-based, PEL-mediated cytotoxicity was blocked by brefeldin A, an inhibitor of intracellular protein transport, but not by the protein synthesis inhibitor emetine, the immunosuppressive drug cyclosporin A, or the DNA transcription inhibitor actinomycin D. CD95L mRNA transcripts in freshly isolated PEL were shown by RT-PCR; CD95L surface expression was evident by staining with Fas-Fc as well as CD95 Abs. Undiminished CD95L expression and cytoidal activity were found in PEL incubated for 48 h in culture, without adding Ag, mitogen, or cytokines. PEL expressed functional CD95L, yet exhibited target cell-specific killing, except when encountering high CD95-expressing cells. The results indicate that PEL use CD95L probably expressed in the Golgi and/or on the cell surface and do not require newly transcribed CD95L upon target cell conjugation. Hence the TCR-triggered recruitment of preformed CD95L, rather than its biosynthesis, controls CD95L-based specific lysis induced by CTL.


Cytotoxic T lymphocytes constitute a primary immune surveillance system that can recognize and destroy foreign cells, or autologous cells expressing foreign or mutated self proteins. Clearly, at least two distinct cytidical mechanisms are used by CTL in destroying such cells (1, 2). In the degranulation pathway, the secreted lytic protein perforin and a family of CTL-specific enzymes are thought to be responsible for target cell destruction (3). A perforin-independent killing pathway (4) is now recognized because perforin-deficient CTL-hybridomas (5), CTL clones (6), and mice (7–9) still possess a high degree of CTL-mediated lytic activity, most probably through the binding of CD95 (APO-1/Fas) on the target cell surface membrane by CD95 ligand (CD95L)3 expressed on the surface of CTL (2, 10, 11). It is widely believed that cross-linking of CD95 triggers a cascade of intracellular protein-protein interactions and proteolytic activities, culminating in apoptosis of the target cell (12–14).

CD95L is expressed after primary T cell activation, a process inhibited by the immunosuppressive agent cyclosporin A (CsA) (15–17). Little is known, however, about the regulation of CD95L expression and function in effector CTL (18). In CTL lines, transient CD95L expression is induced upon TCR engagement (15, 19), by CD3 Abs (20), by the polyclonal stimulator Con A (21), or by PMA and ionomycin (PI) (15, 17, 21). Induction of CD95L expression in CTL is calcium dependent and sensitive to macromolecule synthesis inhibitors; however, CD95L function in triggering CD95-based apoptosis is not (22). Based on studies conducted mainly with CTL lines and T cell hybridomas, transcriptional regulation of CD95L expression and function in CTL action against cognate target cells has been proposed (2). With that model, TCR-based recognition of the Ag presented by the MHC at the target cell surface transduces within the effector cell a transcriptional signal(s) that triggers CD95L gene expression. Enhanced CD95L mRNA expression is assumed to be due to increased transcription, although an increase in message stability has not been ruled out. Swift but transient expression of the CD95L protein then allows CD95L engagement at the target cell surface, signaling its demise. Obviously, failure to recognize the target would not signal CD95L expression nor its implementation. It has also been proposed that functional CD95L expression involves translocating previously made CD95L from storage compartments to the cell surface, or transforming CD95L from an inactive to a functional form (18). Here, we have taken a closer look at the regulation of CD95L expression and function on effector CTL, using PEL, a mouse model system of in vivo primed CTL (23).

Materials and Methods

Cell lines and mice

C57BL/6 (H-2b) T cell leukemia EL4 and DBA/2 (H-2b) mastocytoma P815 were carried as ascites in syngeneic mice or maintained for short periods in culture. Leukemia L1210 of DBA/2 (H-2b) and BW of AKR (H-2b) were cultured in vitro. LF* is an L1210 variant transfected with mouse CD95 overexpression construct (kindly provided by Dr. Pierre Golstein, Centre d’Immunologie, Marseille-Luminy, France) (10). LF- is another L1210 subline that expresses little CD95 Ag because of transfection.

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3 Abbreviations used in this paper: CD95L, CD95 ligand; CsA, cyclosporin A; PL, PMA and ionomycin; PEL, peritoneal exudate CTL; P0, perforin knockout; NCS, newborn calf serum; PER, PEL blasts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BFA, brefeldin A; ER, endoplasmic reticulum; ActD, actinomycin D.

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with a CD95 antisense construct (24). All cells were cultured in RPMI 1640 containing heat-inactivated FCS (5%), sodium pyruvate (1 mM), HEPES (10 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and β-mercaptoethanol (5 × 10⁻⁴ M). The CTL line AB.1 (H-2b anti-H-2d) has been previously described (25) and was maintained in vitro by periodic stimulation with irradiated C57BL/6 spleen cells and a minimal level of Con A cell growth factors (supernatant of Con A-stimulated rat splenocytes) required to support growth. To activate the AB.1 cytolytic function, cells were incubated for 2 h with PMA (Sigma; final concentration of 25 ng/mL) and ionomycin (Sigma, St. Louis, MO; final concentration of 0.5 μg/mL). The CTL hybridoma d11s (kindly provided by Dr. Pierre Golstein) exerts CD95-CD95L-based cytotoxicity when activated with P1 (10).

Perforin-knockout (P0, H-2b) mice have been previously described (9). Two- to four-month-old C57BL/6, BALB/c, and P0 mice were supplied by the Animal Breeding Center of the Weizmann Institute.

Preparing and culturing PEL

PEL were generated, prepared, and purified as previously described (26). Briefly, P0, C57BL/6, and BALB/c mice were injected i.p. with allogeneic tumor cells LF⁺ (H-2d) or EL4 (H-2b) (25 × 10⁶/mouse). Eight to eleven days after a primary allogeneic immunization, or 4 to 5 days after a secondary stimulation (given 6–12 wk after priming), the mice were sacrificed, and their peritoneal cavities were rinsed with PBS supplemented with 5% heat-inactivated newborn calf serum (PBS-NCS). The resulting crude peritoneal exudate cells were centrifuged, resuspended in medium, and incubated on nylon wool columns at 37°C to deplete adherent cells such as B cells and macrophages. After 60 min, the nonadherent cells were eluted by rinsing the columns with cold PBS-NCS. The eluted cells (PEL) contained >95% T cells, 80 to 90% of which were CD8⁺, about half of which formed specific conjugates. PEL blasts (PEB) were derived from PEL upon incubation in recombinant human IL-2 (500 U/mL), as previously described (5).

Cytotoxicity assay

A standard ⁵¹Cr release assay was used. Target cells were incubated with Na⁵¹CrO₄ (1 h at 37°C) and washed twice with PBS-NCS before use. Lytic assays were conducted in U-shaped, 96-well microtiter plates with 3 × 10⁵ labeled target cells per well, and effector cells at the indicated ratios. The plates were centrifuged to promote conjugate formation and incubated at 37°C for 4 to 5 h and then recentrifuged. One hundred microliters of supernatant from each well was harvested, and its radioactivity was determined in a gamma counter. The percentage of cytotoxicity was calculated as follows: % cytotoxicity = [(experimental release – spontaneous release)/ (total release – spontaneous release)] × 100.

RT-PCR of CD95L mRNA transcripts

Total RNA was isolated from various CTL and control cells by TRI REAGENT (Molecular Research Center, Cincinnati, OH). Titan One Tube RT-PCR System (Boehringer Mannheim, Mannheim, Germany) was used to analyze these RNAs for CD95L expression compared with GAPDH expression. In this system, reverse transcription and PCR are performed in a single step. Each 50-μL reaction mixture contained 2 μg RNA, 15 pmol downstream primer (5′-CTT CTC TTA GCA CCA GAT CC-3′), 15 pmol upstream primer (5′-TCT CCA TTA GCA CCA GAT CC-3′), nucleoside 5′-triphosphate NTP (0.2 mM), DTT (5 mM), MgCl₂ (1.5 mM), RNase inhibitor (10 U), 5 μL RT-PCR buffer (10 μL), and 1 μL enzyme mixture containing avian myeloblastosis virus RT and Expand High Fidelity (Boehringer Mannheim). Each sample was mixed, briefly centrifuged, overlaid with 30 μL mineral oil, and placed in the thermocycler (Pro便携式实时PCR控, MJ Biotech). The staining procedure by these CD95L Abs followed that described for Fas-Fc except for the necessary variations. a) Staining by the Alexis Ab was in 30 μL of 8 μg/mL. b) In staining by the Pharmingen Ab, cells were first incubated in 30 μL (40 μg/mL) of anti-mouse CD95L, washed, and secondarily incubated in 30 μL (5 μg/mL) of FITC-goat anti-mAb (Zymed, San Francisco, CA). c) In staining by the Oncogene Ab, cells were first incubated in 30 μL (8 μg/mL) polyclonal Rb anti-mAb CD95L, washed, and secondarily incubated in 30 μL (20 μg/mL) FITC-goat anti-Rb Ab (Jackson ImmunoResearch).

Results

CD95L expression and function in CTL

Unlike perforin-mediated lysis, CD95-based cytotoxicity induced by CTL can occur in Ca²⁺-free medium (10, 16, 21, 29) and is inhibited by CD95 Abs (30) and by Fas-Fc (31). Using perforin-deficient (P0) mice (H-2b) and L1210 cells (H-2b) transfected with CD95 or CD95-antisense (LF⁻ and LF⁺, respectively), we have found that P0 anti-LF⁺ (or anti-LF⁻) (b anti-d) PEL-mediated lysis of LF⁺ cells was calcium independent and inhibitable (to about 15% of the control) by either a CD95 Ab or Fas-Fc. The P0 PEL showed poor cytotoxic activity toward either LF⁺ (H-2b) or third party (H-2b) EL4 cells (Fig. 1). These results confirmed that the cytotoxic activity of P0 anti-LF⁺ PEL is indeed CD95 based and Ag specific.

Because CD95L-based, Ag-independent cytotoxicity has been observed with reactivated CTL lines and hybridoma cells, but not with nonreactivated effectors (10, 11), constitutive, sustained expression of CD95L on in vivo primed CTL has been overlooked. In vivo primed PEL are highly specific killers (23). If PEL expressed CD95L and did not require additional TCR-based stimulation, they might kill nonspecifically high, but not low, CD95-expressing cells. We have tested this hypothesis with BALB/c anti-EL4 PEL (d anti-b), shown to exhibit potent, specific cytotoxic activity (Table I), using LF⁻ and LF⁺ target cells (both are d). In the absence of Ca²⁺ (4 mM Mg²⁺/2 mM EGTA), a condition in
Table I. Cytocidal activity of BALB/c anti-EL4 PEL

<table>
<thead>
<tr>
<th>Specificity</th>
<th>H-2 Type</th>
<th>CD95 Expression (% cells)</th>
<th>% ^51Cr Released at E:T of</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>EL4</td>
<td>b</td>
<td>34.7</td>
<td>74.3</td>
<td>49.4</td>
</tr>
<tr>
<td>EL4 + EGTA</td>
<td>b</td>
<td>34.7</td>
<td>49.2</td>
<td>42.9</td>
</tr>
<tr>
<td>P815</td>
<td>d</td>
<td>30.2</td>
<td>2.0</td>
<td>0.0</td>
</tr>
<tr>
<td>L1210</td>
<td>d</td>
<td>22.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CD95 dependence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LF^+ + EGTA</td>
<td>d</td>
<td>70.0</td>
<td>21.1</td>
<td>14.5</td>
</tr>
<tr>
<td>LF^+ + EGTA</td>
<td>d</td>
<td>9.4</td>
<td>3.6</td>
<td>3.4</td>
</tr>
</tbody>
</table>

^a BALB/c anti-EL4 (d anti-b) PEL, 4 days after a secondary alloimmunization were mixed with the ^51Cr-labeled targets (3 x 10^5 cells/well); lysis was measured after 5 h at 37°C.

Expression of CD95L mRNA transcripts and cell surface CD95L in PEL

To determine whether the CD95-based cytocidal activity exhibited by PEL correlated with the active expression of CD95L, CD95L mRNA transcripts in PEL were tested by PCR. Figure 2a shows that the nonactivated hybridoma d11s and CTL line AB.1, P0 PEL blasts (PEB), BALB/c anti-EL4 PEL, P0 anti-LF^+ PEL, but not control LF^+ or EL4, expressed CD95L mRNA. The CD95L PCR was performed with the same RNA preparations used for GAPDH control PCR.

Given the PCR data, we next investigated whether PEL express cell surface CD95L by testing Fas-Fc binding to PEL. Fas-Fc is a soluble fusion protein composed of mouse CD95 and the Fc portion of human IgG and is capable of binding CD95L (28). Although the specificity of Fas-Fc binding to membrane-bound CD95L is not absolute, its blocking of CD95L-based PEL action was robust and comparable to that of the CD95 Ab (Jo2) (Fig. 1). Using FACS analysis, we found that freshly isolated PEL of various origins, at peak lytic ability after primary or secondary immunization, expressed CD95L (Fas-Fc binding) without requiring stimulation by cognate target cells (Fig. 2b, A and B). The small differences in ligand staining intensity among the various PEL populations tested (Fig. 2b, A, C, and E) probably reflected experimental variations. The FACS experiments were conducted with different batches of cells, various settings of the cytometer, and newly prepared reagents. CD95L expression on PEL detected by Fas-Fc staining was validated by staining with three independently derived CD95L Abs (Fig. 2c).

To exclude the possibility that stable CD95L expression on PEL was due to residual exposure of the PEL to antigenic stimulation in vivo, the PEL were harvested, depleted of adherent cells on nylon wool columns, and then incubated in vitro for 48 h, without deliberate antigenic or IL-2 stimulation, and CD95L expression and cytocidal activity were monitored. Figure 2b, C, D, E, and F, shows that CD95L continued to be expressed on in vitro cultured PEL, which also maintained undiminished lytic activity (Table II). We found that the CTL line AB.1 (d anti-b) showed Fas-Fc staining even without activation (Fig. 2b, I and J), although its (non-specific, CD95L-based) lytic activity was enhanced after PI stimulation, as previously reported (27). Interestingly, PI stimulation of P0 anti-LF^+ PEL affected neither their cytocidal activity against cognate, high CD95L-expressing LF^+ nor against the low Fas-expressing target LF^+.

FIGURE 2. CD95L mRNA transcripts and cell surface expression of CD95L. a, RT-PCR. Lanes: 1, d11s (not PI activated); 2, P0 PEB (derived from P0 anti-LF^+ PEL); 3, PEL (BALB/c anti-EL4, 9 days after primary alloimmunization); 4, P0 PEL (P0 anti-LF^+; 4 days after secondary alloimmunization); 5, AB.1 (not PI activated); 6, LF^+; 7, EL4. b, FACS analysis of CD95L expression on PEL and CTL lines stained by Fas-Fc, c, CD95 staining of PEL detected by CD95L Abs from Alexis (M), PharMingen (N), and Oncogene (O) (see Materials and Methods).
EL4 lysis by PI was noted in a few experiments, whereas the lysis of noncognate LF<sup>+</sup> was markedly enhanced and was not Ca<sup>2+</sup>-dependent (data not shown).

**Transcriptional and translational control of CD95-based lymphocytotoxicity**

The immunosuppressive agent CsA selectively inhibits those T cell activation pathways associated with an increase in intracellular Ca<sup>2+</sup>. A cytoplasmic, T cell-specific component of the transcription factor NF-AT, necessary for IL-2 gene transcription, must be dephosphorylated by the Ca<sup>2+</sup>/calmodulin-dependent protein phosphatase calcineurin for NF-AT to translocate into the nucleus and activate transcription. Calcineurin is inhibited by binding to a complex of CsA and cyclophilins (34, 35). Table III shows that, even with 100 nM of CsA, the lysis of LF<sup>+</sup> mediated by P0 anti-LF<sup>+</sup> PEL was only slightly inhibited. To ascertain the efficacy of the CsA used in blocking lymphocyte activation, we determined its effects on the lymphoproliferative response during a two-way mixed lymphocyte reaction (P0 vs BALB/c). As little as 5 nM CsA blocked the incorporation of [<sup>3</sup>H]thymidine by 95% (Table III), thus proving the efficacy of the inhibitor used. These results indicate that transcriptionally regulated (and CsA-sensitive) activation is not required in the course of CD95-based, PEL-induced lysis.

The requirements for de novo RNA synthesis were tested using actinomycin D (ActD), a powerful inhibitor of DNA transcription. ActD at 0.5 µg/ml completely inhibited the proliferation of the tumor cell lines P815, L1210, EL4, and LF<sup>+</sup> (not shown). With up to 3 µg/ml of ActD, only a slight inhibition of P0 anti-LF<sup>+</sup> PEL activity was evident (Table III). Next the requirements for protein synthesis were tested by Emetine, a potent, nonreversible inhibitor of protein synthesis of mammalian cells. With 0.5 to 2.5 µM of Emetine, [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine incorporation into protein of the CD95-LF<sup>+</sup> pathway was reduced to 10% of the control with only 1 to 15% inhibition of the lytic CTL activity at 50% lysis (Table III). Apparently, new DNA transcription or protein synthesis is not needed by the CD95-LF<sup>+</sup> pathway of PEL action, which supports our theory that PEL express CD95L before cognate binding of the respective target.

**Inhibition of the CD95-LF<sup>+</sup> pathway of PEL-mediated killing by BFA**

BFA blocks constitutive protein transport by disrupting the Golgi apparatus (36, 37). Since PEL-mediated cytotoxicity and most intracellular constitutive protein transport are not calcium dependent,
we tested whether BFA would also inhibit the P0 PEL-mediated killing. Table IV shows that, with BFA at 2.5 and 10 μg/ml, cytotoxicity of LF⁺ decreased by 85%. BFA is known to have other intracellular targets, and its effects are concentration dependent and influenced by the cell type and their biologic state (36, 37). However, no toxic effect against either the effector or target cell was detected by eosin dye exclusion at the working concentrations and up to 15 μg/ml (data not shown); at the highest concentrations used (10 μg/ml), BFA had only a minimal effect on 51Cr-release from LF⁻ cells (4.2% at 10 μg/ml, Table IV). Furthermore, BFA only marginally (16%) affected the lysis of LF⁻ mediated by C57BL/6 anti-LF⁻ PEL (Table IV), excluding the possibility that BFA inhibition of P0 PEL-induced lysis was due to its effects on PEL viability or interference with the recognition and signaling of target apoptosis. Hence, it was concluded that BFA inhibited lymphocytotoxicity by blocking the constitutive transport pathway of CD95L membrane expression. PEL express functional CD95L via the constitutive pathway of intracellular protein transport rather than by regulated secretion. This was consistent with the reduced Fas-Fc staining of cell-surface CD95L, as a result of incubating P0 PEL and the AB.1 CTL line with BFA (Fig. 2b, G, H, K, and L).

Discussion
After activation by Ag, by mitogen, and particularly by PI, certain CTL lines and hybridomas were found to express CD95L and kill CD95-expressing targets specifically and even nonspecifically (10, 38). Similarly, secreted CD95L, CD95L-expressing tumor cell lines, as well as transfected nonlymphoid COS cells can kill CD95-expressing target cells (11). On the other hand, the hallmark of CTL-mediated killing is that it is highly specific and MHC restricted, making constitutive, sustained expression of functional CD95L on effector CTL unlikely. Target cell specificity for lysis induced by CD95L-expressing CTL has therefore been explained by the activation of the CD95L gene and the transient surface membrane expression of CD95L upon (but not before) specific effector CTL-target conjugation and TCR triggering (2). It has been argued that constitutive expression of CD95 on tissues would make constitutive expression of CD95L on effector CTL quite dangerous (39), further supporting the above control mechanism.

We have found that effector CTL such as PEL show sustained expression of functional CD95L without apparent Ag stimulation (Table II; Figure 2b, C–F) and in the presence of inhibitors of transcription and translation (Table III). Similar CD95L-expressing cytotoxic CTL (CD8⁺CD45RA⁺CD27⁺) not requiring in vitro stimulation have been recently isolated from human blood (40). Thus, the proposed transcriptional regulation of CD95L expression and cytolytic function (2) must apply to the initial activation steps of naive (precursor) and the reactivation of memory CTL, CTL lines and hybridomas, but not to the action of effector CTL in vivo such as PEL, where constitutively expressed CD95L can signal target cell apoptosis upon TCR-mediated cognate interaction. Hence, the control of specific CD95L-based CTL action is at the cognitive TCR-mediated level, not the gene expression of ligands.

CD95-based lymphocytotoxicity induced by PEL has been shown to be blocked by BFA (Table IV). Importantly, killing by perforin-expressing PEL was not inhibited (Table IV). Neither CD95 expression nor cell apoptosis induced by CD95 Abs was affected by BFA (41). This indicated that BFA blocking was due to interference with constitutive CD95L expression, as was confirmed by FACS staining (Fig. 2). On the other hand, PEL-induced lysis was only marginally inhibited by the protein synthesis inhibitor Emetine (Table III), but not at all by the immunosuppressive drug CsA, or by the DNA transcription inhibitor ActD (Table III).

From RT-PCR for CD95L mRNA transcripts (Fig. 2a) and Fas-Fc staining of cell surface CD95L (Fig. 2b), effector CTL, such as PEL, apparently express CD95L on the surface, not requiring fresh TCR triggering of CD95L gene expression brought about by the cognate target cells. Cytotoxic PEL expressed functional CD95L upon removal from the peritoneal cavity and continue to express it for at least 2 days under in vitro conditions (Table II; Figure 2b, C–F).

Upon CTL-target conjugate formation, we postulate that the constitutively expressed CD95L, either present at or drawn from the ER/Golgi complex to the CTL/target contact site under the influence of TCR, triggers signaling through CD95 receptors on the targets, ultimately leading to their apoptosis. Interactions of molecules other than TCR-MHC and CD95-CD95L (e.g., LFA-1) may further modulate the outcome of the CTL/target cell encounter.

The proposed model does not preclude bystander killing. That TCR-mediated cognate recognition enhances low level of Fas-based killing of bystander targets has already been demonstrated (32, 33) and confirmed by us with PEL (not shown). Importantly, bystander killing is a two-step event (32). In the first step of cognate recognition/killing, bystanders are spared. It is only in the second stage that the activated CTL, now expressing more adhesion molecules (notably LFA-1), bind to and exert low level killing against Fas-expressing bystanders. That the killing of bystanders does not occur simultaneously with the killing of the cognate target supports a focal distribution on the cell surface of newly recruited CD95L (Fig. 3).

Why then is in vivo CD95L expression on CTL not dangerous to CD95-expressing neighboring cells? In humans, regulating surface membrane CD95L expression by metalloproteinase has been thought to prevent the accumulation of the membrane-bound form of the ligand, thus avoiding bystander killing (45, 46). On the other hand, in studies on CD95-based CTL action, selected or artificially transfected high CD95-expressing target cells are used (e.g., LF⁺

in mouse, SKW in humans, etc.); and third party and bystander killing is frequently seen (32). Although BALB/c PEL do lyse high CD95-expressing LF cells nonspecifically, and even more so as bystanders, they still preferentially kill the cognate target cells (Table I), even though EL4 express less CD95 than LF. This reflects the selective advantage of cognitive binding affected by the T cell receptor, which then facilitates effective interaction of CD95L with target cell CD95. In our experience, most tissues in vivo express the selective advantage of cognitive binding affected by the T cell CD95-dependent cytotoxicity in Ca2⁺ death shortly after the onset of target cell contact with the CTL, CD95-expressing LF killing is frequently seen (32). Although BALB/c PEL do lyse high express CD95L EXPRESSION AND FUNCTION IN CTL

Whereas the Ca2⁺ granulation and CD95-CD95L are quite different (27, 47).

standers, except perhaps in isolated diseases such as hepatitis and myocarditis, where Fas expression on the target organ is high.

The kinetics of the two pathways of lymphocytotoxicity (de-granulation and CD95-CD95L) are quite different (27, 47). Whereas the Ca2⁺-dependent degranulation pathway leads to cell death shortly after the onset of target cell contact with the CTL, CD95-dependent cytotoxicity in Ca2⁺-free media has an extended lag period (27, 47, 48). The lag phase may reflect the time required to attract sufficient CD95L for surface membrane expression and its implementation to achieve effective killing, as well as for downstream signaling of apoptosis. Note also that a lag period of several hours is required for CD95-based apoptosis triggered by a powerful CD95 Ab such as Jo2.

The regulation of CD95L expression seems to vary widely (15, 20, 22, 24, 27, 47, 49). The characteristics of the different CTL lines and hybridomas probably determine how they express CD95L. Actually, IL2, Con A, PI, and TCR triggering can all lead to CD95L expression in T cells (10, 22, 49). Hence, multiple signaling pathways may exist. Naive and memory CTL do not express CD95L, whereas cytotoxic effectors do, as clearly demonstrated by Hamann et al. (40). PEL, like naive and memory cells, are small- to medium-sized nondividing lymphocytes (7–9 microns in diameter); however, they express potent, specific cytoidal activity (26) and may be regarded as activated, cytoidal effectors such as those described by Hamann et al. In vivo studies, we have proved that these cells originate from Ag-stimulated dividing lymphoblasts (50). The small cytoidal PEL, upon IL-2 treatment, undergo blast transformation into large, dividing cytoidal blasts (51), expressing CD95L. Late-stage PEL (e.g., 17–20 days and longer after priming) exhibit no cytoidal activity and do not express CD95L; an extended PI treatment is required for their reactivation. The data presented here suggest that, like the triggering of T cell cyto-

kines, CD95L expression and function in CTL is intrinsically related to the differentiation and activation state of the cells and is not directly dependent on continuous Ag-mediated TCR signaling.

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