program MLPHARE [Z. Otwinowski, in *Isomorphous Replacement and Anomalous Scattering*, W. Wolf, P. R. Evans, A. G. W. Leslie, Eds. (SERC Daresbury Laboratory, Warrington, Cheshire, U.K., 1991), pp. 80–85] in the CCP4 software package. Phases were improved by density modification [K. Y. J. Zhang and P. Main, *Acta Crystallogr.* **A46**, 377 (1990)] and twofold averaging [T. A. Jones, in *Molecular Replacement*, E. J. Dodson, Ed. (SERC Daresbury Laboratory, Warrington, Cheshire, U.K., 1992), pp. 91–105] at 3.5 Å resolution and then extended to 3 Å. The extension was made in steps of 0.1 Å and consisted of averaging, phase combination with the MIR phases, and density modification. The main chain was traced with O with skeletonized electron density, and the initial model built from a database of refined structures [T. A. Jones and S. Thirup, *EMBO J.* **5**, 819 (1986); T. A. Jones, J.-Y. Zou, S. W. Cowan, M. Kjeldgaard, *Acta Crystallogr.* **A47**, 110 (1991)]. The initial complete model had an *R* factor of 49.9% for reflections in the resolution range of 7.5 to 2.0 Å. Two rounds of simulated annealing [A. T. Brünger, J. Kuriyan, M. Karplus, *Science* **235**, 458 (1987)] and rebuilding reduced the *R* factor to 25.0%. Six cycles of standard least squares refinement and rebuilding gave a final *R* factor of 18.1% for all measured unique reflections in the resolution range 7.5 to 1.8 Å. This model contained 7038 nonhydrogen atoms corresponding to the complete amino acid sequence of both molecules, two *N*-acetyl glucoseamine molecules (one *N*-glycosylation site per molecule at Asn^{270}), two *o*-iodobenzyl-1-thio-β-D-glucose molecules, 529 water molecules, and one calcium atom (in a special position on the twofold axis). The model was tightly restrained with rms deviations in bond lengths, bond angles, fixed dihedral angles of 0.008 Å, 1.7°, and 1.4°, respectively. One nonglycine residue (Ser^{99}) has deviant main chain torsion angles, and 12 residues have "pep-flip" values greater than 2.5 Å. The two molecules in the asymmetric unit have an rms fit of 0.2 Å for all atoms. The surface in Fig. 3 was generated with Voidoo [G. J. Kleywegt and T. A. Jones, *Acta Crystallogr.* **D50**, 178 (1994)].

30. C. Divne *et al.*, data not shown.
31. We thank U. Heinemann for Cα coordinates of the β-glucanase before their general release and P. Laamanen and T. Kuurila for skillful technical assistance. This work has been supported by the Swedish Natural Science Research Council, Nordisk Industrifond, and Alko Limited. The coordinates have been deposited at the Protein Data Bank at Brookhaven.

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Fas and Perforin Pathways as Major Mechanisms of T Cell–Mediated Cytotoxicity

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Two molecular mechanisms of T cell–mediated cytotoxicity, one perforin-based, the other Fas-based, have been demonstrated. To determine the extent of their contribution to T cell–mediated cytotoxicity, a range of effector cells from normal control or perforin-deficient mice were tested against a panel of target cells with various levels of Fas expression. All cytotoxicity observed was due to either of these mechanisms, and no third mechanism was detected. Thus, the perforin- and Fas-based mechanisms may account for all T cell–mediated cytotoxicity in short-term in vitro assays.

T cell–mediated cytotoxicity has been studied over many years (*1*). Two mechanisms have been defined at the molecular level. A perforin-based mechanism (*2*) was confirmed by the low cytotoxic activity of activated lymphoid cell populations from perforin-deficient (P°) mice obtained by gene targeting (*3*). This mechanism seems to require molecules other than perforin, including certain serine esterases (*4*). Independently, a Fas-based mechanism was molecularly defined through involvement at the target cell level (*5*) of the cell death–transducing molecule Fas (APO-1) (*6*) and at the effector cell level of a Fas ligand (*7*). We investigated whether these two mechanisms could account for all T cell–mediated cytotoxicity.

We first examined the specific antialloge-

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neic cytotoxicity generated in mixed leukocyte cultures (MLCs) in vitro (*8*). Wild-type C57BL/6–anti-C3H (H-2b–anti–H-2k; b–anti-k) MLC cells lysed thymocytes from wild-type C3H (k) mice in a 4-hour ^{51}Cr release assay (*9*) (Fig. 1A). They also lysed thymocytes from *lpr* mutant k mice (*10*), which express little or no Fas (*11*) and are thus unable to be lysed by the Fas-based mechanism (Fig. 1B). When P° b–anti-k MLC cells, which are unable to lyse through the perforin-based pathway, were used as effector cells, some cytotoxicity still occurred on wild-type thymocytes (Fig. 1C), but not on *lpr* thymocytes (Fig. 1D). The simplest interpretation for this absence of cytotoxicity is that when effectors were unable to exert perforin-based lysis and targets were unable to be lysed through the Fas pathway, no other mechanism operated. In confirmation of other results (*3*, *5*), both perforin- and Fas-based mechanisms were antigen-specific because in all cases k, but not C57BL/6 (b), thymocytes were lysed (Fig. 1, A through C).

Similar evidence was obtained with nonantigen-specific stimuli. MLC cells from wild-type mice could be induced by phorbol 12-myristate 13-acetate (PMA) plus ionomycin to lyse syngeneic target cells through the Fas-based mechanism and by concanavalin A (Con A) to lyse the same syngeneic target cells by a mechanism that was not Fas-based (*12*). C57BL/6–anti-BALB/c (b–anti-d) MLC cells, either wild-type or P°, were tested against syngeneic b thymocytes, either wild-type or *lpr*. In medium alone, no cytotoxicity was detected in this syngeneic combination (Fig. 1, E through H). In the presence of PMA plus ionomycin, wild-type MLC cells lysed wild-type thymocytes (Fig. 1E), but not *lpr* thymocytes (Fig. 1F), confirming that in this system PMA plus ionomycin reveals the Fas-based pathway exclusively (*12*). The same results were obtained with P° MLC cells (Fig. 1, G and H), leading to the conclusion that P° cells could be induced by PMA plus ionomycin to exert Fas-based cytotoxicity against syngeneic cells.

Lysis of both wild-type and *lpr* thymocytes by wild-type MLC cells stimulated by Con A (Fig. 1, E and F) confirmed that Con A triggered a non–Fas-based mechanism of cytotoxicity. Con A also triggered lysis by P° MLC cells of wild-type thymocytes (Fig. 1G) but not of *lpr* thymocytes (Fig. 1H). The simplest interpretation of these findings is that Con A induces both perforin- and Fas-based mechanisms. Neither can operate when P° effectors, which are unable to exert perforin-based cytotoxicity, are used with *lpr* target cells (Fig. 1H). Thus, in this experimental system (Fig. 1, E through H), although Con A could trigger both perforin- and Fas-based cytotoxicity, no other cytotoxicity mechanisms were revealed.

These conclusions also held for experiments that used target cells other than thymocytes. Wild-type or P° b–anti-d MLC cells were tested on L1210 or L1210-Fas tumor target cells, which express (*5*) little or more significant amounts of Fas, respectively. Whereas both target cells were lysed to about the same extent by wild-type mouse MLC cells (Fig. 2A), L1210 cells were lysed less efficiently than L1210-Fas cells by P° MLC cells (Fig. 2B) and by d11S cells, which can be considered prototypic Fas-based killer cells (Fig. 2C) (*5*, *13*). Cytotox-

icity by wild-type or P° MLC cells on these tumor target cells was antigen-specific because syngeneic tumor target cells were not lysed (*14*). Inclusion of antibody to Fas (JO2) in solution, which in overnight tests lysed Fas-bearing target cells (*15*), did not lyse L1210 or L1210-Fas cells during a 4-hour ^{51}Cr release assay. It therefore could be used to specifically inhibit Fas-based T cell–mediated cytotoxicity, indicating that it recognized an epitope on Fas near the site recognized by the Fas ligand. This antibody almost completely inhibited d11S-mediated, but not wild-type MLC, cytotoxicity (Fig. 2, A and C) and almost entirely blocked the residual lysis of L1210 cells by P° cells (Fig. 2B). Taken together, these results indicated that, when tested on these tumor target cells, the strong specific antiallogeneic cytotoxic activity by P° MLC cells was also Fas-based.

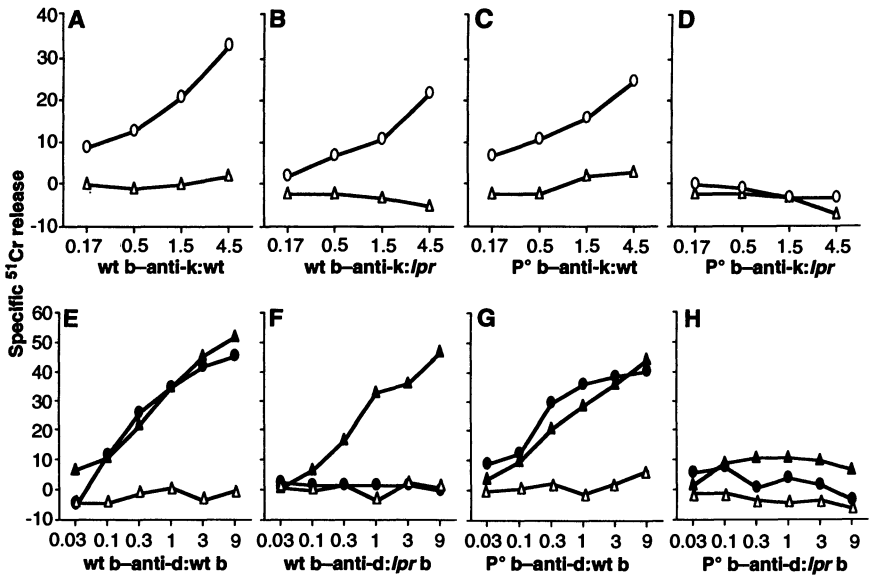

Fig. 1. Mechanisms of T cell–mediated cytotoxicity in MLC cells. (**A** through **D**) Specific antiallogeneic C57BL/6 anti C3H (b–anti-k) MLC cells were tested against either C57BL/6 (b, triangles) or C3H (k, circles) thymocytes. (**E** through **H**) In a separate experiment, C57BL/6 anti BALB/c (b–anti-d) MLC cells were tested against syngeneic C57BL/6 thymocytes, either without added stimuli (open triangles), in the presence of a mixture of PMA and ionomycin (filled circles), or in the presence of Con A (filled triangles). (A and E) Both MLC effectors and thymocyte targets were wild-type (wt). (B and F) Wild-type effectors tested on *lpr* thymocytes. (C and G) P° effectors tested on wild-type thymocytes. (D and H) P° effectors tested on *lpr* thymocytes. Cytotoxicity was expressed as specific ^{51}Cr release after subtraction of spontaneous ^{51}Cr release, which was 17 to 28% in (A) through (D) and 26 to 34% in (E) through (H).

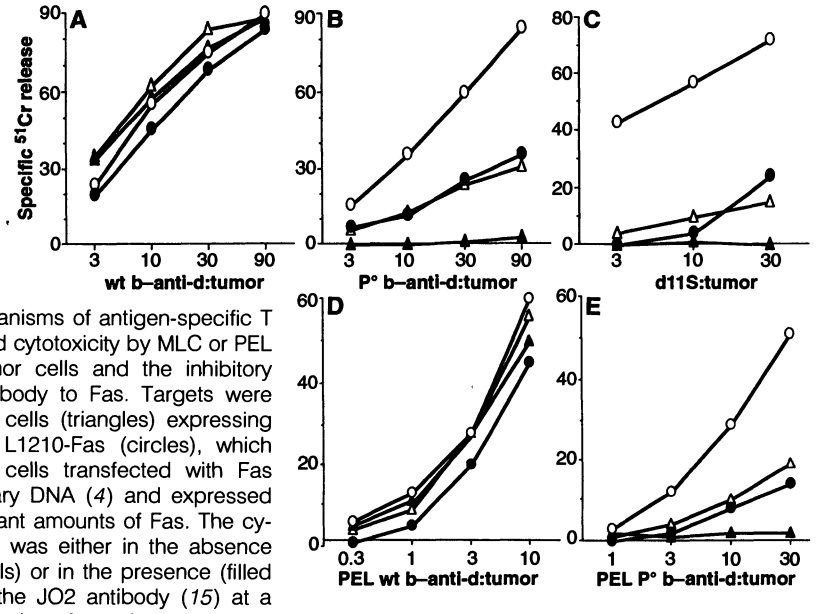

Fig. 2. Mechanisms of antigen-specific T cell–mediated cytotoxicity by MLC or PEL cells for tumor cells and the inhibitory effect of antibody to Fas. Targets were either L1210 cells (triangles) expressing little Fas or L1210-Fas (circles), which were L1210 cells transfected with Fas complementary DNA (*4*) and expressed more significant amounts of Fas. The cytotoxicity test was either in the absence (open symbols) or in the presence (filled symbols) of the JO2 antibody (*15*) at a final concentration of 5 μg/ml. Effector cells were (**A**) wt b–anti-d MLC cells, (**B**) P° b–anti-d MLC cells, (**C**) d11S cells activated with a 3-hour incubation in the presence of PMA and ionomycin (*4*), (**D**) wt b–anti-d PEL cells, and (**E**) P° b–anti-d PEL cells. Blocking by antibody to Fas was specific because it did not significantly affect the perforin-based T cell–mediated cytotoxicity of wild-type effector cells, whereas it inhibited most of the Fas-based T cell–mediated cytotoxicity of d11S cells. Antibody to Fas (1 μg/ml) gave about the same amount of inhibition (*14*), indicating that concentrations higher than 5 μg/ml may not inhibit to a greater extent. Cytotoxicity was expressed as specific ^{51}Cr release after subtraction of spontaneous ^{51}Cr release, which was 9 to 14%.

Similar results were obtained with other types of cytotoxic T cells. Antiallogeneic peritoneal exudate lymphocytes (PELs) (*16, 17*) generated in vivo in wild-type or P° mice gave results similar to those obtained with MLC cells (Figs. 1 and 2, A and B) when tested on thymocytes (*14*) and on tumor cells (Fig. 2, D and E). Major histocompatibility complex class I–restricted cytotoxic cells from wild-type or P° C57BL/6 mice, specific for lymphocytic choriomeningitis virus (LCMV), were tested 8 days after intravenous infection with 200 plaque-forming units of strain LCMV-WE on Fas-expressing RMA and Fas-negative MC57G target cells (Fig. 3, E and F) (*18, 19*). If first incubated with a main epitope peptide of LCMV glycoprotein (*9, 20*), both MC57G and RMA target cells were lysed by wild-type effector cells (Fig. 3, A through D), but only the Fas-expressing RMA cells were lysed by P° effector cells (Fig. 3D). This lysis by P° effector cells was completely blocked by antibody to Fas (Fig. 3D), reinforcing the conclusion that Fas-based cytotoxicity accounts for all of the detectable lysis induced by P° virus peptide–specific cytotoxic T lymphocytes. Taken together, these results showed that mouse lymphocyte populations, either those that are lectin driven or those that are antigen-specific, raised either in vivo or in vitro, exerted T cell–mediated cytotoxicity in short-term assays through both perforin- and Fas-based mechanisms and apparently only through these mechanisms. However, this conclusion of only two mechanisms may not apply to in vitro cytotoxicity tests of longer duration or involving other target cells or to some in vivo situations.

A close examination of Figs. 1 and 2 also showed that, when tested on target cells such as thymocytes or L1210-Fas, the extent of Fas-based cytotoxicity and that of perforin-based cytotoxicity may differ by a factor of only about 3 in terms of effector-to-target cell ratios required to obtain the same ^{51}Cr release values. This observation is at variance with other estimates in which the Fas pathway in the presence of Mg^{2+}-EGTA was about 10% of total cytotoxicity (*5*). Fas-based cytotoxicity with P° effector cells was inhibited by Mg^{2+}-EGTA. This observation apparently reflects a requirement for

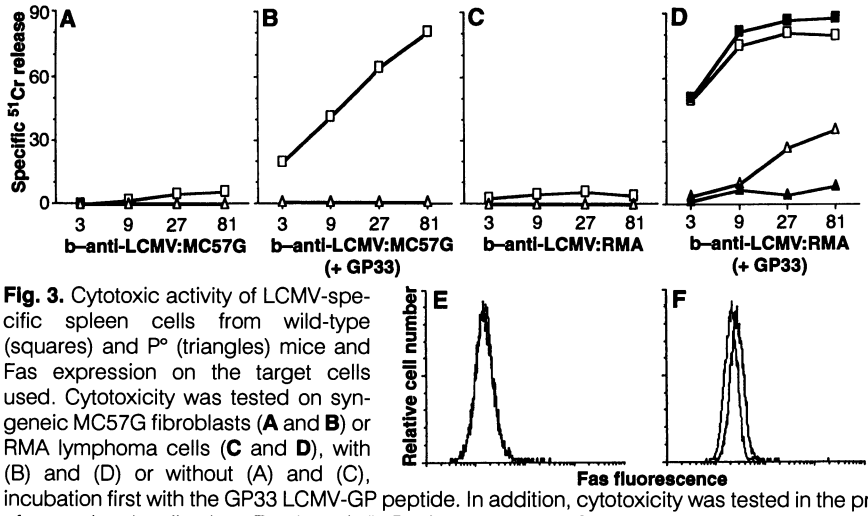

Fig. 3. Cytotoxic activity of LCMV-specific spleen cells from wild-type (squares) and P° (triangles) mice and Fas expression on the target cells used. Cytotoxicity was tested on syngeneic MC57G fibroblasts (**A** and **B**) or RMA lymphoma cells (**C** and **D**), with (B) and (D) or without (A) and (C), incubation first with the GP33 LCMV-GP peptide. In addition, cytotoxicity was tested in the presence of monoclonal antibody to Fas (5 μg/ml) (D) (filled symbols). Cytotoxicity was expressed as specific ^{51}Cr release after subtraction of spontaneous ^{51}Cr release, which was 16 to 21%. The Fas expression patterns of MC57G cells (**E**) and of RMA cells (**F**), stained with either second-stage reagent alone (left curves) or monoclonal antibody to Fas and second-stage reagent (right curves), were analyzed by cytofluorometry (*18*).

Ca^{2+} for the induction, as opposed to the execution, of Fas-based cytotoxicity (*21*).

Absence of perforin resulted in the abolition of a major component of T cell–mediated cytotoxicity, thus formally demonstrating the existence of a perforin-based mechanism (*3*). The experiments reported here show that effector cells from perforin-deficient mice can still exert Fas-based cytotoxicity on Fas-expressing target cells, whether this cytotoxicity was triggered by antigens or was nonspecific. Thus, perforin was required neither for the expression nor for the induction of the Fas pathway. This conclusion is consistent with the observation that simple transfection of Fas ligand complementary DNA could confer cytotoxic activity to fibroblast-like COS cells (*7*). Reciprocally, the perforin-based mechanism did not require Fas. Thus, there may be little or no overlap between the perforin- and Fas-based mechanisms.

The perforin-based pathway seems to involve granule exocytosis, whereas the Fas pathway involves a cell-bound ligand-receptor interaction. The coexistence of two such different mechanisms that ensure antigen-specific, T cell–mediated cytotoxicity raises questions of their origin, perhaps related to mast cell degranulation and developmental pathways, respectively, of their connections with the T cell receptor complex, and of their role. Two mechanisms of cytotoxicity might increase cytotoxic efficiency, at least on certain target cells. Also, the Fas- and perforin-based mechanisms may not have completely overlapping roles in vivo. Perforin is required for clearance of LCMV, elimination of some tumor cells, and some pathophysiological manifestations (*3*). The perforin pathway may ensure most of the classical defense roles, directed against, for example, infected syngeneic cells ("modified self"). On the other hand, Fas-based cytotoxicity may have a regulatory role, directed against activated syngeneic lymphocytes ("activated self") (*12*). These findings about two different mechanisms of T cell–mediated cytotoxicity may also be relevant in some studies of tumor and graft rejection and other pathological circumstances.

REFERENCES AND NOTES

1. P. Perlmann and G. Holm, *Adv. Immunol.* **11**, 117 (1969); J.-C. Cerottini and K. T. Brunner, *ibid.* **18**, 67 (1974); P. Golstein and E. T. Smith, *Contemp. Top. Immunobiol.* **7**, 273 (1977); C. S. Henney, *ibid.*, p. 245; E. Martz, *ibid.*, p. 301; G. Berke, *Prog. Allergy* **27**, 69 (1980); in *Fundamental Immunology*, W. E. Paul, Ed. (Raven, New York, 1989), pp. 735–764; J. D.-E. Young, *Physiol. Rev.* **69**, 250 (1989); J. Tschopp and M. Nabholz, *Annu. Rev. Immunol.* **8**, 279 (1990); R. C. Duke, in *Apoptosis: The Molecular Basis of Cell Death*, L. D. Tomei and F. O. Cope, Eds. (Cold Spring Harbor Laboratory Press, New York, 1991), pp. 209–226; M. Sitkovsky and P. Henkart, *Cytotoxic Cells* (Birkhauser, Boston, 1993).
2. P. A. Henkart, *Annu. Rev. Immunol.* **3**, 31 (1985); E. R. Podack, *Immunol. Today* **6**, 21 (1985).
3. D. Kägi *et al.*, *Nature* **369**, 31 (1994).
4. J. W. Shiver *et al.*, *Cell* **71**, 315 (1992); L. Shi *et al.*, *J. Exp. Med.* **176**, 1521 (1992); J. W. Heusel *et al.*, *Cell* **76**, 977 (1994).
5. E. Rouvier, M.-F. Luciani, P. Golstein, *J. Exp. Med.* **177**, 195 (1993).
6. S. Yonehara *et al.*, *ibid.* **169**, 1747 (1989); B. C. Trauth *et al.*, *Science* **245**, 301 (1989).
7. T. Suda *et al.*, *Cell* **75**, 1169 (1993).
8. For MLCs, stimulator spleen cells were treated with mitomycin C (final concentration of 25 μg/ml for 1 to 6×10^7 cells per milliliter, M-0503; Sigma) for 30 min at 37°C in the dark, followed by three washes. Mixtures of 2×10^7 responder spleen cells and 8×10^6 stimulator cells were incubated in 15 ml of medium [RPMI 1640, supplemented with final concentrations of 1 mM sodium pyruvate, 2 mM glutamine, 2.5×10^{-5} M β-mercaptoethanol, and 10% fetal bovine serum (FBS)] in a tissue culture flask (Falcon 3013) kept upright at 37°C in a water-saturated, 6% CO_2 atmosphere for 5 days.
9. Tumor cells (2×10^6) in 7.5 ml of RPMI 1640, supplemented with 5% FBS, were labeled overnight at 37°C in 6% CO_2 with 300 μCi of sodium [^{51}Cr]-chromate. Thymocytes (2 to 3×10^7) in 0.2 ml of RPMI 1640, supplemented with 10% FBS, were labeled with 100 μCi of sodium [^{51}Cr]chromate for 1 hour at 37°C in 6% CO_2. After labeling, all cells were washed three times. Each V-shaped well of 96-well microtiter plates received, in a total volume of 200 μl, target cells (10^5 thymocytes or 10^4 tumor cells), effector cells in the indicated ratios, and either medium or a mixture of PMA (final concentration of 5 ng/ml, P-8139; Sigma) and ionomycin (final concentration of 0.5 μg/ml, 407952; Calbiochem) or Con A (final concentration of 10 μg/ml). Microplates were centrifuged for 1 min at 1500 rpm and incubated for 4 hours at 37°C. After another centrifugation, 100-μl aliquots of the supernatants were assayed for radioactivity. The fraction of the total radioactivity released was then calculated, and the results, averaged from triplicates, were expressed as percent specific ^{51}Cr release (percent experimental ^{51}Cr release minus percent ^{51}Cr release from target cells alone). For experiments with peptide-labeled target cells, the GP33 LCMV-GP peptide (amino acids 33 through 41: KAVYNFATM) (*20*), a main LCMV epitope peptide in H-2b mice, was included at a concentration of 10^{-6} M during the incubation with sodium [^{51}Cr]chromate. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
10. C57BL/6J-*lpr* and C3H-*lpr* mice were obtained from Jackson Laboratory (Bar Harbor, ME) and bred at the CIML. C57BL/6 (b), C57BL/6 P° (P° b), C3H (k), and BALB/c (d) mice were bred at Sandoz or at the CIML.
11. R. Watanabe-Fukunaga *et al.*, *Nature* **356**, 314 (1992).
12. F. Vignaux and P. Golstein, *Eur. J. Immunol.* **24**, 923 (1994).
13. PC60-d11S (d11S) was a subclone of the d10S clone, shown to exert Fas-based T cell–mediated cytotoxicity (*5*) and derived by serial subcloning (*5, 22*) from the PC60 cytotoxic hybridoma cell (*23*).
14. F. Vignaux *et al.*, unpublished data.
15. J. Ogasawara *et al.*, *Nature* **364**, 806 (1993).
16. PELs were developed by Berke and associates (*17*) as a model of specific cytotoxic T cells raised in vivo. Wild-type or P° C57BL/6 mice were injected intraperitoneally (IP) with 2×10^7 L1210-Fas and boosted similarly at least 3 weeks later. Peritoneal cells were sampled 5 days after the boost injection, enriched in PELs by one passage through a nylon wool column, and tested for cytotoxic activity. The P° mice rejected IP-injected L1210-Fas cells and also P815 and RDM4 cells (*14*).
17. G. Berke, K. A. Sullivan, B. Amos, *J. Exp. Med.* **135**, 1334 (1972).
18. To analyze Fas cell surface expression, we incubated tumor cells with normal hamster serum, followed by biotin-conjugated hamster monoclonal antibody JO2 to mouse Fas (*15*) and fluorescein isothiocyanate–conjugated avidin. Viable cells were separated by a combination of forward and side-light scatter on a FACScan cytofluorometer (Becton Dickinson) and analyzed with logarithmic scales.
19. H.-G. Ljunggren *et al.*, *J. Immunol.* **142**, 2911 (1989); P. C. Doherty, W. E. Biddison, J. R. Bennink, B. B. Knowles, *J. Exp. Med.* **148**, 534 (1978).
20. H. P. Pircher *et al.*, *Nature* **346**, 629 (1990).
21. F. Vignaux *et al.*, in preparation.
22. P. Golstein, M.-G. Mattéi, C. Foa, M.-F. Luciani, in *Apoptosis and the Immune Response*, C. D. Gregory, Ed. (Wiley, New York, in press).
23. A. Conzelmann *et al.*, *Nature* **298**, 170 (1982).

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