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Immunology

Immune function in mice lacking the perforin gene

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ABSTRACT **Mice lacking the perforin gene were generated by using targeted gene disruption in embryonal stem cells. When infected with lymphocytic choriomeningitis virus (LCMV), perforin-less (−/−) mice showed clear signs of having mounted an immune response based on activation of CD8 T cells but were unable to clear the LCMV infection. This failure to eliminate virus was accompanied by a failure to generate spleen cells capable of lysing LCMV-infected fibroblasts *in vitro*. Spleen cells from LCMV-infected −/− mice were able to lyse hematopoietic target cells after exposure to phorbol 12-myristate 13-acetate and ionomycin, provided the target cells expressed the Fas antigen. Spleen cells from −/− mice also responded to alloantigen in mixed leukocyte culture by blastogenesis and proliferation. The resulting cells were able to lyse hematopoietic target cells, although not as well as spleen cells from +/+ littermates sensitized in the same manner. However, lysis by −/− cells was again seen only if the target cells expressed Fas antigen. We conclude that perforin-less −/− mice retain and express the Fas lytic pathway as expressed *in vitro* but that this pathway is insufficient to clear an LCMV infection *in vivo*.**

Perforin (cytolysin; pore-forming protein), a complement-like protein stored in cytoplasmic granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells, is thought to be a major mediator responsible for the cytolytic properties of these cells (1, 2). When CTL bind to cells recognized as aberrant or foreign ("target" cells), the contents of these granules (which includes a range of other products such as proteolytic enzymes in addition to perforin) are released vectorially onto the target cell. Perforin monomers are thought to assemble into polymeric pore structures that insert into target cell plasma membranes. These structures, in conjunction with other granule components, participate in the induction of rapid, apoptosis-like cell death in target cells (3, 4).

Several observations challenge the notion that perforin is the exclusive lytic mechanism used by cytotoxic effector cells. Peritoneal exudate lymphocytes (PEL) are one of the most potent cytotoxic CD8 CTL known yet are devoid of granules and possess little if any perforin (5). A PEL-derived hybridoma was recently shown by reverse transcriptase-PCR to contain less than one molecule of perforin per cell and yet to retain its cytotoxic function (6). Experiments have shown that CTL degranulation and CTL killing can be uncoupled (7–10) and that killing by CTL can occur in the absence of Ca^{2+} (7, 8), which is required for perforin assembly into pore structures (11). Although these and other results have suggested the existence of cytolytic mechanisms in addition to perforin, analysis of such mechanisms in the presence of perforin has been difficult. To clarify the role of perforin in CTL function and to investigate possible alternative lytic mechanisms, we set out to generate a mouse line completely lacking in perforin by replacing the endogenous perforin genes with nonfunctional gene copies. We describe here the generation of such a mouse and the results of initial analysis of its immune status.

MATERIALS AND METHODS

Production of Mice Lacking the Perforin Gene. A schematic representation of the perforin locus on murine chromosome 10 and the perforin disruption construct p72NeoTK.1 is shown in Fig. 1*a*. The perforin locus extends ≈15 kb along chromosome 10. To generate a disruption construct, a 6.1-kb *Eco*RI–*Sal* I fragment of genomic DNA containing exons 2 and 3 of the perforin gene was isolated from the genomic library clone pfp64 (ref. 12; provided by Joseph Trapani) and maintained in an EMBL3 vector. This fragment was inserted into the cloning vector pSP72 (Promega) in a polylinker *Eco*RI–*Xho* I site sequence. A 1.1-kb neomycin-resistance gene fragment (Neo) from the vector pMC1-NeopA (13) was inserted by blunt-ended ligation into the *Sma* I site of exon 2 of the genomic sequence. To allow selection by fialuridine [FIAU; 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil; a gift of Bristol–Myers Squibb] against cells incorporating the disruption construct by random integration, a herpes simplex virus (HSV) type 1 thymidine kinase (TK) gene fragment was inserted into a *Sal* I site of the pSP72 polylinker, flanking the 3′ end of the perforin genomic sequence of this intermediate construct. The structure of the resulting construct, p72NeoTK.1, was verified by restriction enzyme and Southern blot analyses.

Methods for culturing embryonal stem (ES) cells, DNA electroporation, isolation of ES cell clones, and extraction of cellular DNA were essentially as described by Chen *et al.* (14). Approximately 10^7 AB-1 ES cells (15) were transfected with 20 μg of p72NeoTK.1 linearized with *Bam*HI and grown under selection with G418 and fialuridine. Disrupted clones were identified by Southern blot analysis of ES-cell DNA digested with *Eco*RV. DNA filters were hybridized with the 1.1-kbp 5′ flanking probe, pfpE1 (Fig. 1*a*), prepared by random hexamer labeling with [α-^{32}P]dCTP. The wild-type allele gives a 4.4-kb band in this assay; the disrupted allele is 5.5 kb. AB-1 ES cells containing one disrupted allele were injected into C57BL/6 blastocysts followed by transfer to pseudopregnant foster females [(C57BL/6 × CBA)F_1] *in utero*. Male agouti chimeras were bred to C57BL/6 females, and germ-line transmission was verified by Southern blot analysis of tail genomic DNA as described above (Fig. 1*b*). Homozygous perforin-less (−/−) mice were kept in an ultra-clean isolated mouse room in cages with filter covers.

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Abbreviations: LCMV, lymphocytic choriomeningitis virus; CTL, cytotoxic T lymphocyte(s); MLC, mixed leukocyte culture(s); TCGF, T-cell growth factor(s); PMA, phorbol 12-myristate 13-acetate; pfu, plaque-forming units.

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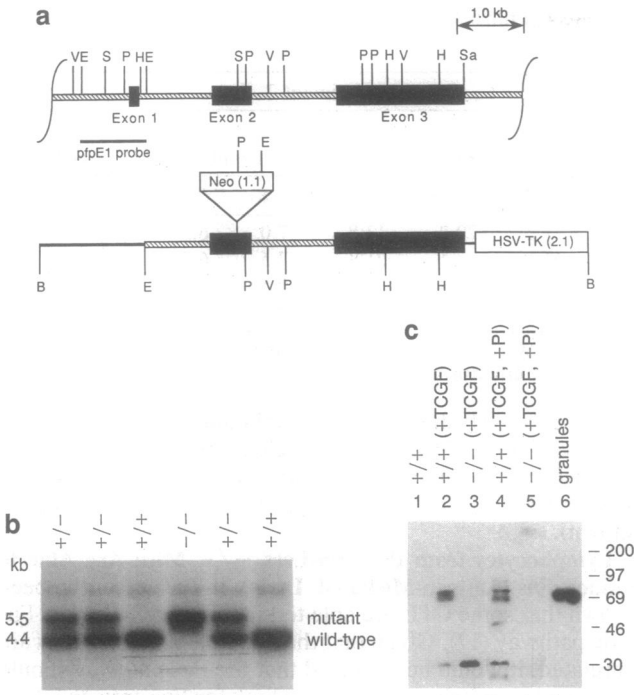

FIG. 1. Generation and characterization of perforin-deficient mice. (*a Upper*) Schematic representation of the perforin genomic locus. Exons are shown as solid boxes, while introns are hatched. The following restriction sites are indicated: E, *Eco*RI; S, *Sma* I; P, *Pvu* II; V, *Eco*RV; H, *Hin*dIII; Sa, *Sal* I; and B, *Bam*HI. The 1.1-kb probe pfpE1 is shown below the genomic map. Note that this sequence contains exon 1 and flanking intronic DNA but does not include sequences contained in the targeting vector. (*a Lower*) Map of the targeting vector p72NeoTK.1 containing a 6.1-kb segment of perforin genomic DNA, with a neomycin-resistance gene (Neo) inserted into a *Sma* I site in exon 2 and a flanking herpes simplex virus thymidine kinase cassette (HSV-TK). (*b*) Southern blot analysis of genomic DNA from heterozygote intermatings. Tail DNA was prepared from 3-week-old mice, cut with *Eco*RV, and subjected to Southern blot analysis as described in text. Wild-type DNA probed with ^{32}P-labeled pfpE1 produces a 4.4-kb band; the 1.1-kb Neo sequence introduced by homologous recombination gives rise to a 5.5-kb band. Genotypes for wild type (+/+), heterozygous (+/−), and perforin-deficient (−/−) mice from a representative litter are shown above each lane. (*c*) Western blot analysis of +/+ and −/− splenocytes. Splenocytes harvested from +/+ (lanes 1, 2, and 4) and −/− (lanes 3 and 5) mice were cultured in the absence (lane 1) or presence (lanes 2–5) of 4% T-cell growth factor (TCGF) for 5 days. Some splenocytes (lanes 4 and 5) were incubated with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) and ionomycin (3 μg/ml) (PI) for 3 hr prior to culture (lanes 4 and 5). As a positive control, a granule extract prepared from CTLL-R8 (12) was also loaded (lane 6). Sizes are shown in kDa.

To verify that disruption of the perforin gene eliminated perforin in the mice, splenocytes were harvested from 8-week-old wild-type (+/+) and perforin-less (−/−) littermates and were cultured in the presence of TCGF (16) for 5 days. Perforin expression in CTL populations is strongly enhanced by TCGF. Both the +/+ and −/− splenocytes were highly granulated after culture. To examine the expression of perforin in these splenocyte populations, the cultures were pelleted, lysed, and subjected to SDS/PAGE and immunoblot (Western blot) transfers on to nitrocellulose. After reaction with perforin-specific antibody coupled with an enhanced chemiluminescence system (Amersham), the blots were washed and exposed to XAR-5 film (Kodak). The results (Fig. 1*c*) show that while wild-type splenocytes have high levels of TCGF-induced perforin, the splenocyte population from the mice lacking the perforin gene has no detectable perforin expression.

Mixed Leukocyte Cultures (MLC) and Cytotoxicity Assays. MLC were initiated by cultivating responder-strain lymph node and spleen cells with irradiated stimulator spleen cells at a 1:1 ratio in culture medium with 5% calf serum. On day 5 or 6, MLC cells were harvested, counted, and assayed for cytotoxicity in a ^{51}Cr-release assay as described (7). Briefly, ^{51}Cr-labeled target cells were washed and incubated with MLC cells at various effector/target ratios in microtiter plates. After 4–5 hr at 37°C, 100-μl samples were removed from each assay well, and the percent ^{51}Cr-release was determined in a γ-scintillation spectrometer. Similar assays were performed with spleen cells from LCMV-infected mice stimulated with 10 ng of PMA and 3 μg of ionomycin per ml. Some target cells were modified by stable tranfection with Fas sense or antisense cDNA. Fas sense cDNA-transfected L1210 (L1210F$^+$; *H-2^d*) cells were provided by Pierre Golstein (17). Fas antisense cDNA-transfected L1210 (L1210F$^-$; *H-2^d*) were prepared with Fas cDNA provided by Pierre Golstein.

Virus Infection. The Armstrong CA1371 strain of lymphocytic choriomeningitis virus (LCMV) was used in these studies (18). Mice were infected with 2×10^5 plaque-forming units (pfu) of LCMV. Infectious LCMV was quantitated by plaque assay on Vero cell monolayers as described (18).

Flow Cytometry. Numbers of CD4 and CD8 T cells and their respective level of expression of the activation marker CD44 (Pgp-1) in the spleens of uninfected as well as LCMV-infected +/+ and −/− mice were determined by flow cytometry on a Becton Dickinson FACScan. Phycoerythrin-conjugated rat anti-mouse CD4 (clone RM4-5), biotinylated rat anti-mouse CD8a (clone 53-6.7), and fluorescein isothiocyanate-conjugated rat anti-mouse CD44 (clone IM7) were purchased from PharMingen. For CD8 staining, biotinylated anti-CD8 was followed by phycoerythrin-conjugated streptavidin (Caltag, South San Francisco, CA).

RESULTS

Immune Responses of Perforin-Less −/− Mice *in Vivo*. When kept in a clean room unconnected to the main mouse colony, −/− mice have thrived and have been healthy for at least 5 months. The lymphoid organs and tissues of −/− mice appear completely normal upon gross examination, and the distribution of CD4 and CD8 T-cell subsets is essentially identical in +/+ and −/− mouse spleens (data not shown).

To test the immune competence of perforin-less −/− mice *in vivo*, they were challenged with LCMV, and their ability to generate an anti-viral CTL response and to resolve the infection was analyzed. Control +/+ and −/− mice were inoculated i.p. with 2×10^5 pfu of LCMV (Armstrong strain), and spleen cells from these mice were tested 8 days later for cytotoxicity against virus-infected fibroblast target cells. As expected, +/+ mice generated a potent LCMV-specific CTL response (Table 1). In striking contrast, spleen cells from −/− mice showed no detectable killing of LCMV-infected targets. This absence of anti-viral cytotoxic activity in −/− mice was not due to lack of activation of CD8 T cells. The spleens of both +/+ and −/− infected mice were enlarged 2–3 times compared with uninfected controls, and there was a >2.5-fold increase in the number of CD8 T cells in both cases. In addition, these CD8 T cells showed increased expression of the T-cell activation marker CD44 (Fig. 2). The proportion of activated (CD44^{hi}) CD8 T cells in −/− mice equaled or exceeded that seen in +/+ mice. Taken together, the results of Table 1 and Fig. 2 show that after LCMV infection there is substantial expansion and activation of CD8 T cells in both +/+ and −/− mice but that the activated CD8 T cells from −/− mice are unable to kill virus-infected fibroblasts. Thus, perforin is a key effector molecule for cytolysis of infected fibroblasts by virus-specific CTL.

Previous studies have shown that CD8 T cells play a critical role in eliminating LCMV infection (19), and cells staining

Table 1. Spleen cells from perforin-less −/− mice are unable to kill virally infected fibroblasts

	^{51}Cr release from target cells, %													
	Experiment 1						Experiment 2							
	LCMV-infected MC57 cells			Uninfected MC57 cells			LCMV-infected MC57 cells				Uninfected MC57 cells			
Effector cells	E:T 50	E:T 17	E:T 5	E:T 50	E:T 17	E:T 5	E:T 40	E:T 20	E:T 10	E:T 5	E:T 40	E:T 20	E:T 10	E:T 5
+/+	67.6	52.6	23.3	−1.4	3.2	−6.2	35.7	27.2	20.2	13.8	7.0	6.5	7.7	5.7
+/+	54.2	36.8	10.5	−2.4	−1.9	−5.1	44.2	33.1	27.1	21.3	7.5	6.3	6.2	3.4
−/−	−1.2	−0.5	−3.1	−5.9	−5.4	−7.7	1.0	1.9	0.8	−0.3	0.5	0.6	0.3	0.9
−/−	−0.9	−2.2	−3.6	−5.7	−5.4	−5.9	0.7	1.5	1.0	1.9	−0.5	0.2	4.6	2.3

Spleen cells harvested from two −/− and two +/+ mice 8 days after infection were tested directly in 4-hr cytotoxicity assays in each experiment with normal or LCMV-infected MC57 (*H-2^b*) fibroblast target cells. E:T, effector/target cell ratio.

positively for perforin have been associated with the LCMV response in mice *in vivo* (20). However, it is not known whether direct killing of virally infected cells by CTL is essential for resolving the infection or whether indirect mechanisms (i.e., antiviral cytokines) are sufficient to eliminate virus. To address this issue, we examined virus levels in various tissues of LCMV-infected −/− and +/+ mice (Table 2). Virus was undetectable in all tissues examined in +/+ mice and in serum, showing the expected acute clearance of LCMV from immunologically normal mice. On the other hand, levels of virus up to 10^4-fold higher than minimal detectable levels were found in all tissues and in the serum of perforin −/− mice. The +/+ mice fully resolved the infection by day 8, whereas the −/− mice continued to harbor high levels of virus in all tissues. Two −/− mice infected with LCMV were followed beyond 8 days. Both mice showed weight loss compared with their +/+ littermates and became increasingly sick over time. Tissue and serum samples taken from these mice when it appeared they were about to expire showed that viral levels continued to increase beyond day 8. These data suggest that virus was not being cleared in some sort of delayed fashion but was continuing to increase in −/− mice with time. This inability to clear the virus was striking because large numbers of cellular infiltrates containing CD8 T cells were present in the tissues of −/− mice (data not shown).

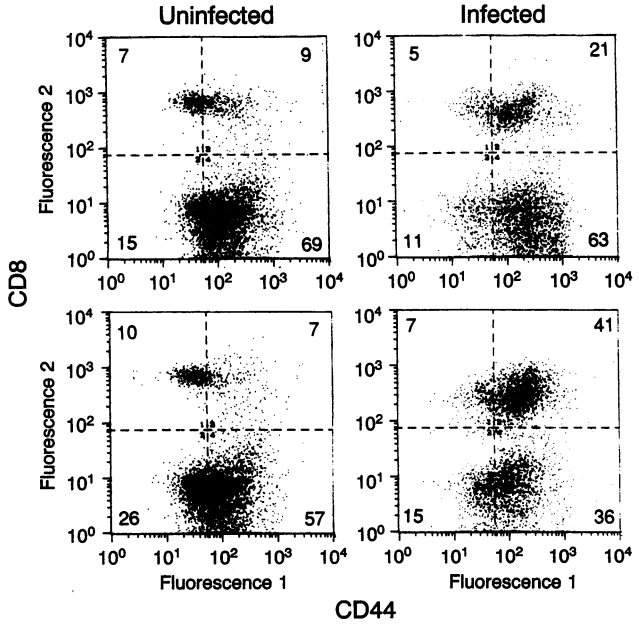

FIG. 2. LCMV-infected perforin-deficient mice have high levels of activated CD8 cells. Splenocytes from uninfected and infected wild-type (*Upper*) and perforin-deficient (*Lower*) mice were isolated 8 days after intraperitoneal infection (2×10^5 pfu of LCMV; Armstrong). These cells were stained by using two-color flow cytometric analysis with fluorescein isothiocyanate-conjugated CD8 and phycoerythrin-conjugated CD44 antibodies. Numbers in each quadrant indicate the percentages of cells tested that stain in that quadrant.

Lymphocytes from Perforin-Less −/− Mice Are Able to Induce Fas Antigen-Mediated Lysis. It has recently been shown that some CTL are able to kill target cells via the Fas lytic pathway (17). Given that the *Fas* and perforin genes are unrelated, it would be expected that the Fas pathway should be intact in perforin-less mice. To look for Fas antigen killing in −/− mice, we activated spleen cells from LCMV-infected mice with PMA and ionomycin. Spleen cells from either −/− or +/+ infected mice were, after activation, able to lyse L1210 (*H-2^d*) target cells transfected with Fas sense cDNA, but not with Fas antisense cDNA (Fig. 3). As has been previously reported (17), this lysis was not sensitive to EGTA. It thus appears that −/− mice still retain the Fas lytic pathway but that this pathway does not enable them to clear an LCMV infection *in vivo*. Since PMA- and ionomycin-activated spleen cells from uninfected mice showed minimal or no Fas-dependent lysis (data not shown) we assume the lysis seen here is from virus-activated CTL rendered non-specific by PMA/ionomycin, and not from natural killer cells.

The ability of lymphocytes from perforin-less −/− mice to mount a cytotoxic response to alloantigen was tested in MLC reactions. Spleen and lymph node cells from −/− and +/+ mice were incubated with irradiated DBA/2 spleen cells for 5 days and tested in a standard 4-hr cytotoxicity assay against ^{51}Cr-labeled target cells. The results are shown in Table 3. Cells from both +/+ and −/− mice responded to DBA/2 spleen cells by becoming blastoid and proliferating. Both cultures showed about 60% large- and medium-size cells by day 5, and the yield of cells was virtually identical in the two cases (data not shown). Experiments 1 and 2 in Table 3 are representative of seven primary MLCs analyzed to date. In some experiments the cytotoxicity of −/− cells after primary MLC was nearly as strong as that of +/+ cells. In most experiments it was substantially lower. The cytotoxicity in all cases was specific for the stimulating-cell major histocompatibility complex (*H-2^d*). The cytotoxicity expressed by +/+ lymphocytes after sensitization in primary MLC was independent of the level of *Fas* expression in the target cells, whereas cytotoxicity expressed by −/− cells after primary MLC was highly dependent on the level of *Fas* expression. These results are also consistent with retention in −/− mice of the Fas lytic pathway.

Experiment 3 in Table 3 is typical of results obtained with lines derived from −/− and +/+ primary MLCs by repeated stimulation with antigen and growth in the presence of TCGF. The level of cytotoxicity expressed by these −/− and +/+ CTL lines is very similar. As with *in vivo*-generated primary CTL stimulated with PMA and ionomycin (Fig. 3), lysis by −/− CTL lines generated and propagated *in vitro* is

Table 2. Perforin-less −/− mice fail to clear an LCMV infection

Mouse	Postinfection day	LCMV titer								
		Liver	Kidney	Lung	Spleen	Pancreas	Thymus	Brain	Salivary gland	Serum
+/+	8	<2.3	ND	<3.1	<2.5	<3.3	ND	<2.8	ND	<1.7
+/+	8	<2.5	ND	<3.2	<2.3	<3.2	ND	<3.1	ND	<1.7
+/+	8	<2.6	<2.8	<3.1	<3.7	<3.8	<3.3	<3.0	<3.4	<1.7
+/+	8	<2.6	<3.1	<3.0	<3.7	<3.8	<3.3	<3.0	<3.1	<1.7
−/−	8	6.8	ND	4.6	5.7	5.4	ND	3.0	ND	3.1
−/−	8	6.9	ND	3.8	6.0	4.9	ND	3.3	ND	3.0
−/−	8	6.7	4.1	4.5	5.9	5.1	5.3	3.2	3.6	4.1
−/−	8	6.7	4.7	4.3	5.9	5.1	6.3	3.0	3.4	3.0
−/−	17	7.5	5.1	ND	7.2	5.2	ND	ND	ND	ND
−/−	21	7.0	5.9	6.5	6.9	6.3	7.2	6.3	6.0	5.6

Values shown are the logarithm of pfu/g or pfu/ml of serum. ND, not done.

highly dependent on expression of *Fas* in the target cells, whereas lysis by +/+ CTL lines is not. However, lysis of Fas^- target cells by +/+ CTL lines is sensitive to EGTA, which would be consistent with exclusive use of a degranulation mechanism against such targets. Lysis of Fas^+ target cells is partially reduced by EGTA, as might be expected if +/+ cells were equipped with both a degranulation and a Fas lytic mechanism.

It has previously been reported that Fas-mediated lysis is Ca^{2+}-independent (17). We find that lysis of Fas^+ targets by −/− CTL generated in primary MLC varied widely in its sensitivity to EGTA from almost completely sensitive to almost completely insensitive. Lysis by −/− CTL lines, on the other hand, was generally insensitive to EGTA or was slightly enhanced by it. Possible reasons for this variability will be presented in the *Discussion*.

DISCUSSION

The results of these studies show clearly that perforin is the key molecule in CTL-mediated killing of LCMV-infected targets and that the ability to overcome an infection with LCMV is absent in mice lacking the perforin gene, despite substantial proliferation and activation of CD8 T cells. Existing evidence points to CD8 T cells as the principal immune mediator in such infections (18), and we presume this to be the locus at which lack of perforin is critical. Failure to clear the LCMV infection is accompanied by a failure to generate cell-mediated cytotoxicity toward virally infected fibroblasts *in vitro*. Thus we would conclude that perforin and the granule exocytosis pathway are also critical in lysing virally infected fibroblast target cells. These results suggest that immune destruction of virus-infected cells *in vivo* is dependent on perforin in the same way as is the lysis of infected fibroblast target cells *in vitro* and that indirect mechanisms

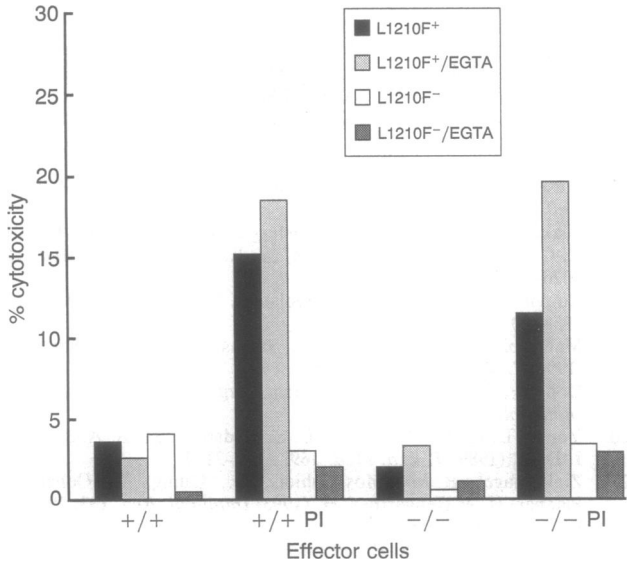

FIG. 3. Fas-dependent cytotoxicity is inducible in wild-type (+/+) (*Upper*) and perforin-deficient (−/−) (*Lower*) splenocyte populations. Splenocytes from +/+ and −/− mice were harvested and incubated for 3 hr in the absence or presence of 10 ng of PMA and 3 μg of ionomycin per ml to induce antigen-nonspecific, Fas-dependent cytotoxic function. These putative effector cells were then added to ^{51}Cr-labeled L1210 cells stably transfected with either a Fas overexpression vector ($L1210\text{-}F^+$) or a Fas antisense expression vector ($L1210\text{-}F^-$). Four-hour ^{51}Cr-release assays were performed as described in text in the absence or presence of 4 mM EGTA/3 mM $MgCl_2$.

Table 3. Generation of cytotoxicity in wild-type (+/+) and perforin-less (−/−) lymphocytes in response to allogeneic stimulation *in vitro*

Exp.	Responder cell	Target cell	*H-2*	EGTA	E:T 20:1	E:T 7:1	E:T 2:1
1	+/+	$L1210\text{-}F^+$	*d*	−	39.0	17.9	9.6
	−/−	$L1210\text{-}F^+$		−	25.2	11.9	3.4
	+/+	EL-4	*b*	−	−0.9	1.9	0.2
	−/−	EL-4		−	2.6	3.1	−0.3
	+/+	$L1210\text{-}F^-$	*d*	−	18.2	11.0	4.4
	−/−	$L1210\text{-}F^-$		−	0.5	1.0	0.9
	+/+	$L1210\text{-}F^+$	*d*	+	2.4	0.6	1.0
	−/−	$L1210\text{-}F^+$		+	6.8	3.1	0.8
2	+/+	$L1210\text{-}F^+$	*d*	−	32.2	23.8	15.8
	−/−	$L1210\text{-}F^+$		−	6.7	4.0	2.6
	+/+	EL4	*b*	−	4.0	3.1	2.2
	−/−	EL4		−	0.7	1.4	0.2
	+/+	$L1210\text{-}F^-$	*d*	−	33.7	20.5	12.3
	−/−	$L1210\text{-}F^-$		−	2.3	1.6	0.5
	+/+	$L1210\text{-}F^+$	*d*	+	23.3	15.1	7.5
	−/−	$L1210\text{-}F^+$		+	11.0	7.3	5.0
3	+/+	$L1210\text{-}F^+$	*d*	−	47.3	32.6	16.1
	−/−	$L1210\text{-}F^+$		−	57.2	41.0	30.5
	+/+	$L1210\text{-}F^+$		+	27.7	21.0	13.3
	−/−	$L1210\text{-}F^+$		+	51.2	46.4	36.0
	+/+	$L1210\text{-}F^-$	*d*	−	76.2	63.8	39.1
	−/−	$L1210\text{-}F^-$		−	8.4	7.1	4.4
	+/+	$L1210\text{-}F^-$		+	6.1	5.8	4.7
	−/−	$L1210\text{-}F^-$		+	11.5	11.6	8.1

Responder lymph node and spleen cells ($H\text{-}2^b$) were cultured with irradiated stimulator spleen cells ($H\text{-}2^d$) for 5 days. Assays at the indicated effector-to-target cell ratio (E:T) were for 5 hr. Spontaneous release was <10% in all cases. Experiment 3 was performed with +/+ and −/− CTL lines obtained after four rounds of restimulation with irradiated DBA/2 spleen cells and growth in the presence of TCGF. $L1210F^+$, L1210 cells transfected with *Fas* sense DNA; $L1210F^-$, L1210 cells transfected with *Fas* antisense DNA.

such as anti-viral cytokines are not sufficient to eliminate virus. It should be noted, however, that direct killing of virus-infected cells may be more critical in eliminating non-cytolytic viruses such as LCMV and hepatitis B, and this mechanism may be of lesser importance in controlling infection by cytolytic viruses. The perforin-less mice should prove useful in addressing this issue and dissecting the mechanisms by which CTL control microbial infections.

Although homozygous perforin-less −/− mice failed to clear LCMV infection, it is our impression that these mice were still experiencing a fair amount of immunopathological damage (data not shown). Infiltration of CD8 cells into liver and other tissues was very prominent in LCMV-infected −/− mice, which failed to thrive and eventually died. The underlying mechanism of the observed weight loss and eventual death is not fully understood, but it is likely that the damage is immune-mediated, since infection of immunodeficient mice with LCMV normally results in a persistent infection with no obvious disease (21).

Although we were unable to detect direct, antigen-specific cytotoxicity in spleen cells from infected −/− mice, we were able to detect antigen-nonspecific cytotoxicity in such spleen cells after treatment with PMA and ionomycin. PMA and ionomycin are well known to cause antigen-specific CTL to kill target cells in an antigen-nonspecific manner (22), and the cytotoxicity seen here may be due to diversion of virus-specific CTL to other targets. It seems likely that this diversion is due to induction of the Fas lytic pathway (17). In all experiments of this type carried out to date, cytotoxicity is blocked virtually completely in target cells expressing *Fas* antisense DNA. The level of cytotoxicity achieved was low, reflecting perhaps a low concentration of effector cells in the spleen and a lack of involvement of a T-cell receptor to focus these virus-plus-$H\text{-}2^b$-specific effector cells onto $H\text{-}2^d$ target cells. However, the cytotoxicity observed was highly dependent on expression of the Fas antigen and was insensitive to EGTA, a characteristic of the Fas pathway (17).

We were able to examine this cytotoxicity more closely in MLC reactions. Both −/− and +/+ spleen cells showed strong signs of reactivity (blastogenesis and proliferation). This is very likely related to the splenomegaly and up-regulation of the CD44 activation antigen on CD8 cells seen *in vivo* after LCMV infection. We saw variable levels of antigen-specific cytotoxicity in −/− MLC cells, varying from 10% to 80% of that seen in control +/+ MLC cells. This cytotoxicity was again strongly dependent on expression of *Fas* in the target cells. Sensitivity to EGTA was variable. Our experience has been that sensitivity of Fas-dependent killing to EGTA depends on the mode of activation of the CTL. CTLs activated by PMA and ionomycin are usually completely insensitive to EGTA. In fact, as shown in Fig. 3, EGTA may actually enhance target cell killing by PMA/ionomycin-activated CTL. We find the EGTA sensitivity in Fas-mediated killing of CTL activated by antigen to be highly variable, depending largely on how recently the CTL were exposed to antigen and the extent to which they are at all able to mediate Fas-dependent lysis (C.M.W., A. Glass, V. Chiu, and W.R.C., unpublished data). Fas-mediated killing by CTL clones and lines subjected to repeated antigen stimulation, particularly in the presence of high levels of TCGF, is rarely sensitive to EGTA.

The relevance of the Fas pathway in CTL function *in vivo* is yet to be established in reactions such as graft rejection, tumor control, and various CD8-mediated immunopathologies. In our experience to date, only hematopoietic cells have proved susceptible to Fas lysis *in vitro*. Cytolysis of such targets by −/− lymphocytes sensitized to alloantigens *in vitro* and by *in vivo*-sensitized spleen cells activated with PMA and ionomycin is essentially completely abolished by the expression of *Fas* antisense DNA in the target cell. This suggests that the Fas lytic pathway may be the only alternate pathway operating in perforin-less mice in such situations. Given the wide range of expression of Fas on cell types *in vivo* including liver (23), it is not obvious at present why the highly activated −/− CD8 cells infiltrating the liver are unable to clear virally infected cells via the Fas lytic pathway. Perforin −/− mice should provide an excellent model in which to assess the *in vivo* relevance of Fas in cell-mediated cytotoxic reactions.

Note Added in Proof. Since the submission of this manuscript, three other reports of perforin-lacking −/− mice have appeared (24–26).

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