Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11571–11575, November 1994
Immunology

A null mutation in the perforin gene impairs cytolytic T lymphocyte- and natural killer cell-mediated cytotoxicity

(gene targeting/cell-mediated cytotoxicity)

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Communicated by Hans J. Müller-Eberhard, July 15, 1994 (received for review April 12, 1994)

ABSTRACT **Lymphocyte-mediated cytotoxicity has been proposed to consist of the polarized secretion of granule-stored perforin leading to target-cell lysis. Nevertheless, perforin-independent pathways were postulated to explain the cytolytic activity of apparently perforin-free lymphocytes and the DNA degradation found in dying target cells. To evaluate the role of perforin, we used gene targeting in embryonic stem cells to produce mice lacking perforin. Mice homozygous for the disrupted gene have no perforin mRNA. The mice are healthy. Activation and granzyme A secretion of perforin-free cytolytic T cells are unaltered. The killing activity of cytolytic T cells as well as natural killer (NK) cells, however, is impaired but not abolished. Approximately one-third of the killing activity remains when lysis of 3T3 fibroblast targets and the apoptotic cell death of YAC-1 NK targets are analyzed. We conclude that perforin is a crucial effector molecule in T cell- and NK cell-mediated cytolysis. However, alternative perforin-independent lytic mechanisms also exist.**

The mechanisms of cytolytic T lymphocyte (CTL)- and natural killer (NK) cell-mediated lysis have been studied extensively in recent years. One proposed mechanism, known as the granule-exocytosis model, consists of the polarized secretion of granule-stored molecules by the lymphocytes, after the formation of CTL–target cell conjugates. Isolated granules are cytotoxic on their own (1, 2), indicating that they harbor effector proteins responsible for cell killing. Contained within the granules are perforin, a pore-forming, membranolytic protein (3, 4); granzymes, a family of serine esterases (5); calreticulin, a Ca^{2+}-binding protein (6); TIA-1, an RNA-binding protein (7); and proteoglycans (8).

Perforin is the only protein which is cytolytic in its isolated form. After granule exocytosis, perforin monomers are released into the conjugate juncture and, in the presence of Ca^{2+}, bind to the target cell membrane and polymerize into pore-like transmembrane channels, leading to lysis (9). Nevertheless, perforin does not induce DNA degradation that is observed during lymphocyte attack (10, 11). Recent data suggest that apoptosis may be induced by a combination of perforin and granzymes and/or TIA-1 by as yet unknown mechanisms (7, 12, 13).

Some investigators have questioned whether perforin is the chief effector molecule of CTL-induced killing, since most of the evidence in favor of the granule-exocytosis model was obtained with CTL lines cultured *in vitro* in the presence of high levels of interleukin 2 (for a debate, see ref. 14). Some CTL clones analyzed killed target cells in the absence of Ca^{2+} (15, 16), yet the lytic activity of perforin and successful exocytosis of granular proteins are strictly Ca^{2+}-dependent. Therefore, alternative, Ca^{2+}-independent pathways were proposed, such as those involving the Fas receptor (17).

Numerous experiments have been performed to assess the role of granules and in particular the role of perforin in cytotoxicity. For example, perforin antisense oligonucleotides partially suppressed CTL-mediated cytolysis (18), and the expression of perforin into granules of mast cells rendered these cells hemolytic (19). Although these data have suggested a crucial role of perforin in cell killing, direct evidence is still lacking.

MATERIAL AND METHODS

Cloning of the Mouse Perforin Gene and Construction of the Targeting Vector. A 35-kb fragment containing the perforin gene was isolated by screening a cosmid library derived from DNA of NIH 3T3 mouse fibroblasts (Stratagene). A *Pst* I-fragment containing exon 2 and part of exon 3 was subcloned into pBluescript SK(+) (Stratagene). The targeting construct was made by insertion of a neomycin-resistance (*neo*) gene (pMC1neopolyA; Stratagene) into a single *Sph* I site of the third exon, for positive selection. The herpes simplex virus thymidine kinase gene was placed into pBluescript SK(+) at the 5′ end of the perforin sequence, allowing negative selection.

Electroporation and Selection of Embryonic Stem (ES) Cells. D3 ES cells (a gift of Frank Hilberg, Bender, Vienna) were routinely grown on γ-irradiated (4′500rad) neo^r primary embryonic fibroblasts isolated from the MTKneo3 transgenic mouse line (received from C. Ovitt, European Molecular Biology Laboratory, Heidelberg) in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 15% fetal bovine serum, nonessential amino acids (GIBCO), 0.1 mM 2-mercaptoethanol (Sigma), and leukemia inhibitory factor (ESGRO, 10^3 units/ml; GIBCO). Electroporation with 20 μg of linearized (*Kpn* I-cut) targeting vector was performed with a single pulse at 500 μF/280 V by a Gene Pulser (Bio-Rad) electroporator. After 24 hr, cells were transferred into selection medium containing G418 (300 μg/ml, dry powder; GIBCO) and 2 μM ganciclovir and were incubated for 10–12 days. Doubly resistant colonies were picked into phosphate-buffered saline, trypsinized, and analyzed by PCR. The following oligonucleotide primers were used to identify the rearranged perforin locus: primer 1 (5′-TGG-GCA-GCA-GTC-CTG-GTT-GGT-GAC-CTT-3′), complementary to genomic DNA that lies downstream of the perforin sequence contained within the targeting vector, and primer 2 (5′-ATT-CGC-AGC-GCA-TCG-CCT-TCT-ATC-GCC-3′), complementary to sequences at the 3′ terminus of the *neo* gene.

Southern Blot Analysis. Genomic DNA (15 μg) was digested with *Eco*RV (Boehringer) for Southern blot analysis. The 620-bp *Sph* I–*Pst* I (PCR I) fragment from the perforin

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Abbreviations: CTL, cytolytic T lymphocyte; NK, natural killer; ES cell, embryonic stem cell.

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gene, residing within the targeting vector, and the 512-bp *Pst* I–EcoRV (SB I) fragment, downstream of the targeting construct, were used as probes.

PCR Analysis. RNA was isolated from a 5-day mixed lymphocyte culture (10^7 cells) (21). cDNA was prepared with the cDNA Cycle kit (Invitrogen), and perforin-specific message was amplified by PCR (94°C for 1 min, 1 min at 60°C, and 3 min at 72°C for 30 cycles) using primers for exon 2 (5′-GTG-GCC-GCA-GCC-AAG-GTC-CAC-TC-3′) and exon 3 (5′-TGG-GCA-GCA-GTC-CTG-GTT-GGT-GAC-CTT-3′); the expected amplification product is 900 bp. Control TIA-1 message was amplified in the same reaction mixture with primer 1 (5′-CTC-CAG-CTC-CAA-AGA-GTA-C-3′) and primer 2 (5′-CGG-GAT-CCT-CAC-TGG-GTT-TCA-TAC-3′); the size of the amplification product is 500 bp.

Northern Blot Analysis. This was performed on Genescreen membranes (NEN) according to the manufacturer's protocol, using as probe the random primer-labeled *Bst*XI fragment containing the entire perforin open reading frame.

Generation of Chimeric Mice. Approximately 10-15 ES cells were injected into C57BL/6J blastocysts. Injected blastocysts (10–20) were transferred to the uteri of pseudopregnant NMRI females at 2.5 days postcoitum. Germline chimeras were identified by the presence of agouti coat color in the F_1 progeny.

Cytotoxicity Assay of CTL. Alloreactive CTLs were generated in a 5-day mixed lymphocyte culture (22). Responder spleen cells (2.5×10^6 per ml) were from adult (5- to 6-week-old) mice with the normal perforin gene (+/+), and mice heterozygous (+/−) and homozygous (−/−) for the disrupted perforin gene. The mice were of *H-2*^{b} haplotype (129/C57BL/6J). Stimulators were irradiated spleen cells (2.5×10^6 per ml, 3000 rads; 1 rad = 0.01 Gy) from DBA/2 (*H-2*^{d}) mice. Target cells were haplotype-matched P815 mastocytoma cells (*H-2*^{d}) and 3T3.A31 fibroblasts (H-2^d). EL-4 cells (*H-2*^{b}) were used to measure major histocompatibility complex (MHC)-unrestricted cytotoxicity. Cellular cytotoxicity and DNA degradation were assayed as described (23): P815 cells and EL-4 cells (10^6 cells in DMEM with 5% fetal bovine serum) were first labeled for 1 hr with 4 μCi (148 kBq) of ^{125}I-Urd (Amersham) in a final volume of 200 μl. The cells were then incubated in the presence of 100 μCi of sodium [^{51}Cr]chromate (Amersham) for an additional hour. 3T3.A31 fibroblasts were labeled with ^{51}Cr (2 μCi per well) in 96-well plates for 12–16 hr (24).

Isolation of NK Cells and Cytotoxicity Test. To activate NK cells *in vivo*, mice were inoculated intraperitoneally with poly(I·C) (100 μg in phosphate-buffered saline; Sigma) 16 hr prior to the experiment (20). For the cytotoxicity assay, spleens were excised and homogenized, and cells were purified over a Ficoll (Pharmacia) gradient in order to eliminate red blood cells. The cell suspension was then washed three times in DMEM with 5% fetal bovine serum and tested, as described for CTLs, in a standard 4-hr ^{51}Cr/^{125}I-release assay using YAC-1 tumor cells (American Type Culture Collection) as specific targets.

T-Cell Activation. The activation of T cells during the mixed lymphocyte reaction was followed by measuring the concentration of interferon γ in the supernatant of 2.5×10^7 responder cells, after 5 days of culture (25).

Degranulation of CTLs. Ninety-six-well plates (Dynatech) were coated with rat anti-mouse CD3 monoclonal antibody 17A2 (26). Subsequently, activated lymphocytes from a mixed lymphocyte reaction were added (10^5 cells per well) in serum-free medium and incubated for 4 hr at 37°C. The amount of secreted granzyme A was measured by the BLT-esterase assay (27).

RESULTS

Targeting Strategy. The perforin gene contains three exons spanning a region of ≈1.6 kb (28). Only the last two exons code for the protein. To disrupt the perforin gene by homologous recombination, a targeting construct was made by subcloning a 3.4-kb *Pst* I fragment containing part of exon 2 and the complete exon 3 (Fig. 1*A*). Exon 3 was interrupted by insertion of a *neo* cassette, thereby interrupting the putative transmembrane domain of perforin that is thought to consist of two amphipathic α-helices spanning amino acid residues 167–221 (29). Mutations in the analogous region of complement component C9 were shown to lead to a nonfunctional protein (30). A herpes simplex virus thymidine kinase gene was placed 3′ of the perforin sequence in order to allow the use of a positive/negative selection strategy (31).

Detection of Targeted Clones and Generation of Perforin −/− Mice. Successfully targeted ES cell clones were first identified by PCR and then confirmed by Southern blotting using *Eco*RI- and *Eco*RV-digested ES cell DNA and an internal (PCR I) and external (SB I) probe (data not shown). Of 349 doubly resistant clones screened, two were positive in the PCR screen and showed an additional band in the Southern blot analysis, indicative of targeted disruption of the perforin gene. None of these clones had additional random integrations of the targeting vector.

The two ES cell clones with the disrupted perforin gene were injected into C57BL/6J blastocysts and the embryos were reimplanted into NMRI foster mothers. One clone gave rise to chimeric animals. One male chimera transmitted the disrupted allele to all offspring. Males and females heterozygous for the perforin mutation were then crossed to pro-

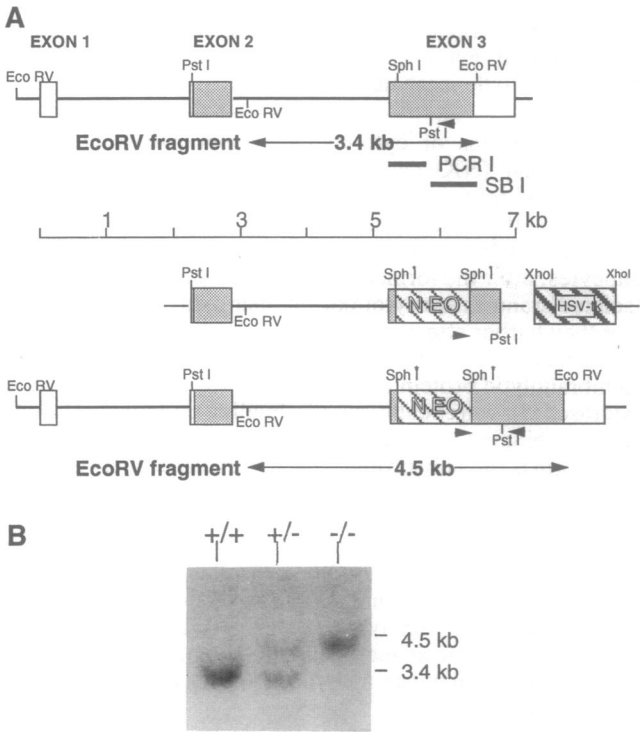

FIG. 1. Gene targeting of the perforin locus. (*A*) Structure of the murine perforin locus, perforin targeting vector, and homologous-recombination allele. Translated exon sequences are depicted as stippled boxes, and untranslated sequences as open boxes. Introns are represented as bold lines. Probes used for Southern blots are PCR I, the internal probe, and SB I, the external probe. Genomic restriction fragments detected by Southern blotting are indicated by a double-arrowed line. Primer sites for PCR are represented as arrowheads. Hatched boxes represent the neomycin-resistance gene and herpes simplex virus thymidine kinase gene. (*B*) Representative Southern blot analysis of progeny of +/− × +/− mating. Tail-biopsy genomic DNA was digested with *Eco*RV and probed with the SB I fragment. The wild-type allele is 3.4 kb, and the homologous-recombination allele shows the predicted 4.5-kb fragment.

duce homozygotes. A representative Southern blot of tail genomic DNA from a heterozygote × heterozygote mating is shown in Fig. 1*B*. Heterozygous mutant mice as well as homozygotes appeared to be healthy.

Lack of Perforin Transcripts in Homozygous Mutants. To evaluate perforin expression, spleen cells of homozygotes (perforin −/−) and control mice (perforin +/+ and +/−) were isolated and stimulated with γ-irradiated DBA/2-derived splenocytes and the perforin RNA was analyzed by PCR after 5 days. A perforin-specific amplification product of ≈900 bp was detected in perforin +/+ and +/− mice, whereas no transcript was discernible in spleen cells of −/− animals (Fig. 2*A*). The TIA-1 RNA was amplified at similar levels in all samples, indicating that the lack of perforin message in −/− cells was not due to defective cDNA synthesis.

When the splenic RNA was subjected to Northern blot analysis, no 2.9-kb perforin message was detectable in cells from −/− animals (Fig. 2*B*). Moreover, a reduced amount of perforin message was observed in cells from heterozygous animals.

Cytotoxicity of T Lymphocytes. To investigate the role of perforin in CTL-mediated cytotoxicity, spleen cells derived from −/−, +/−, and +/+ mice (*H-2*^{b}) were activated in a bulk mixed lymphocyte culture with irradiated allogeneic DBA/2 (*H-2*^{d}) spleen cells for 5 days. The allo response and activation of perforin-lacking T cells were normal, since the production of interferon γ was identical in all cultures (Fig. 3) and the T-cell subset distribution was not altered (data not shown). Moreover, no difference in the distribution of lymphocyte populations upon allogeneic stimulation was observed (data not shown).

The cytotoxic activity of the responder cells 5 days post-stimulation was assessed in a standard ^{51}Cr release assay against *H-2*^{d} alloantigen-carrying P815 mastocytoma cells (Fig. 4*A*). Although the cytolytic activity of T cells from +/− mice was comparable to the effector activity of cells from

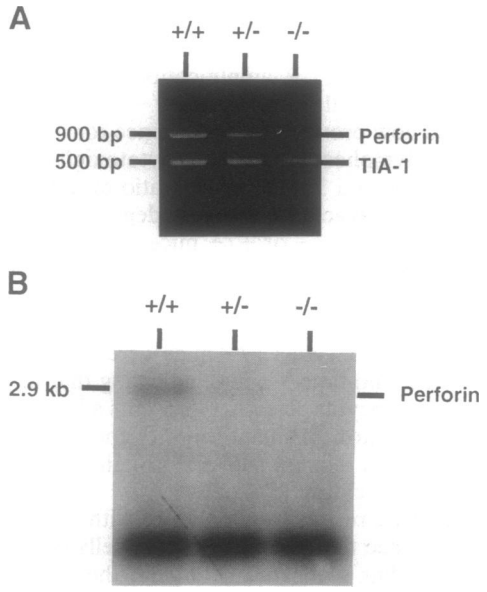

FIG. 2. Absence of perforin message in lymphocytes derived from perforin −/− mice. (*A*) PCR analysis of spleen cells isolated from +/+, +/−, and −/− mice and allogeneically activated for 5 days. The cDNA was amplified with perforin-specific primers, resulting in a 900-bp product. The 500-bp band corresponds to the amplification product from the T-cell specific TIA-1 message, which was included for control purposes. (*B*) Northern blot analysis of the activated spleen cells. Ten micrograms of total RNA was loaded in each case. The band migrating at ≈1.4 kb is nonspecific but demonstrates equal loading of RNA.

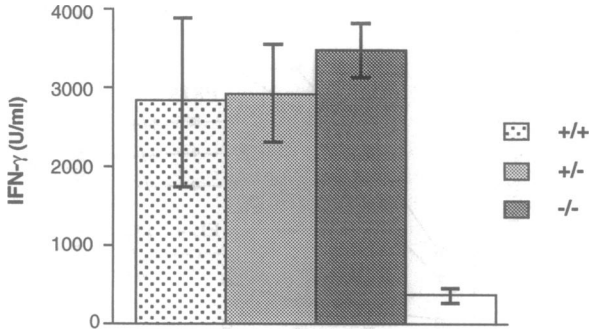

FIG. 3. Unaltered interferon γ production in perforin-free lymphocytes in mixed lymphocyte culture. Interferon γ (IFN-γ) concentration [units (U)/ml] was determined in supernatants of spleen cells (*H-2b*) derived from perforin +/+, +/−, and −/− mice 5 days after activation by irradiated spleen cells of DBA/2 (*H-2*^{d}) mice. The interferon γ production in the absence of a stimulatory signal is shown with the open bar.

wild-type mice, a slightly lower activity was consistently noticed with the +/− lymphocytes (for example, 63% versus 43% lysis at a 10:1 killer-to-target ratio). In contrast, cells derived from homozygous −/− mice did not develop a significant CTL response against these target cells. Only at a large excess of cytolytic cells over target cells did some of the cells lose the ^{51}Cr label. This killing activity was above the spontaneous lysis observed with the irrelevant EL-4 target (*H-2*^{b}).

During target cell lysis by lymphocytes, plasma membrane impairment is preceded by the characteristic signs of apoptosis—i.e., DNA condensation and fragmentation. These latter events are undetectable in a 4-hr ^{51}Cr release assay but require the determination of radioactively labeled DNA fragments released into the cytoplasm of the target cell. After 4 hr, complete DNA fragmentation was detected in targets attacked by T cells derived from +/+ and +/− animals, even at low killer-to-target ratios (Fig. 4*A*). Again, +/− cells were weaker effector cells than the +/+ lymphocytes. In spite of the low ^{51}Cr release observed with perforin −/− cells, fragmentation of the genomic DNA was clearly detected and reached levels up to 50% at a 100:1 effector-to-target cell ratio (10% in the ^{51}Cr assay).

Fibroblasts are known to be more resistant to CTL-mediated cytolysis than the highly susceptible P815 cells (32). Moreover, DNA ladder formation, one of the hallmarks of apoptosis, is not detectable in these target cells. When lymphocytes of +/+ and +/− mice were added, 60% of 3T3 cells succumbed to this attack after a 7-hr incubation at a 100:1 excess of killer cells (Fig. 4*B*). Perforin-free lymphocytes were only 3 times less efficient in lysing the fibroblasts.

Cytotoxicity of NK Cells. NK cells, which constitutively produce perforin, are believed to utilize a killing mechanism similar to CTLs. We therefore analyzed the effect of the defect of perforin on NK cell cytotoxic capacity. The ^{51}Cr release from YAC-1 cells, the typical NK target, was measured by using poly(I·C)-stimulated NK effector cells. NK cells isolated from normal (+/+) mice exhibited 2-fold higher lytic activity than NK cells derived from heterozygous +/− mice (Fig. 4*C*). In NK cells lacking perforin, the effector activity was reduced almost to background levels. As in CTLs, the apoptosis-inducing activity of NK cells was diminished only 3-fold in perforin −/− cells as reflected by the considerable amount of DNA fragments recovered in the YAC-1 cytoplasm (Fig. 4*C*).

Exocytosis of Cytotoxic Granules. Perforin is stored in cytoplasmic granules together with other proteins—in particular, granzyme proteases. We could not exclude that the diminished cytotoxicity was due merely to an inefficient

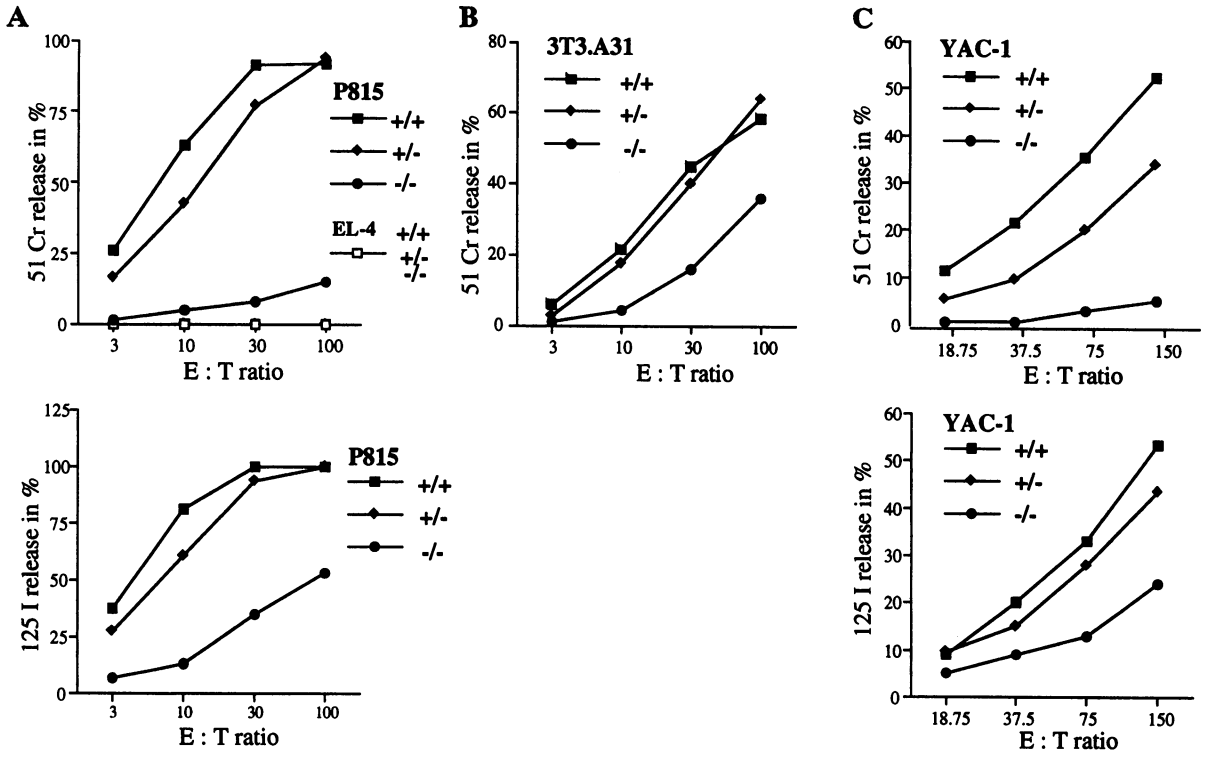

FIG. 4. Lack of CTL and NK cytotoxic activity. (*A*) The CTL activity of the responder cells after 5 days of stimulation in a mixed lymphocyte culture was assessed in a standard 4-hr ^{51}Cr-release assay against P815 cells (*Upper*) and in a 4-hr ^{125}I-Urd DNA fragmentation/release assay (*Lower*). (*B*) As in *A*, but 3T3 fibroblasts (*H-2^d*) were used as target cells. ^{51}Cr release was assessed after 7 hr. (*C*) Cytotoxicity of NK cells. Splenic NK cells were added to YAC-1 cells, and ^{51}Cr and ^{125}I-Urd release was determined after 4 hr. E, effector; T, target.

granule fusion and/or exocytosis event. Perforin comprises high-affinity lipid binding sites and may act as a fusogen of membranes, as has been demonstrated for the structural homologue complement component C9 (33). Upon incubation with immobilized anti-CD3 antibody, cytotoxic granules fuse with the plasma membrane and release their contents into the supernatant, where the secreted proteins can be detected by immunological or enzymatic means (34). Fig. 5 shows that comparable amounts of granzyme A are released from +/+, +/−, and −/− T cells.

DISCUSSION

Although perforin has been shown to participate, at least in part, in CTL- and NK cell-mediated cell killing, the existence of alternative, perforin-independent, mechanisms has been suggested. We decided to use the gene knockout technology in ES cells to generate perforin-deficient mice to unambiguously evaluate the contribution of perforin. A replacement vector was devised which disrupts the putative transmembrane domain of perforin, making it unlikely that a truncated protein will display lytic activity. Indeed, no perforin 2.9-kb message and 900-bp PCR amplification product were detected in activated cells of perforin −/− mice.

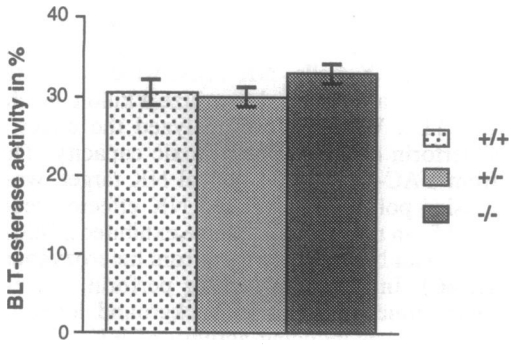

FIG. 5. Exocytosis of cytotoxic granules. Granzyme A esterolytic activity in the supernatant of spleen cells allogeneically activated for 5 days was determined after a 4-hr incubation with immobilized anti-CD3 antibodies. Granzyme A activity (percent of total granzyme A activity) was detected using the lysine *S*-benzyl ester (BLT) substrate. The total amount of granzyme A activity of the various mixed lymphocyte cultures was similar (±15%).

Our results now provide direct evidence for a crucial role of perforin in lymphocyte-mediated cytotoxicity. For P815 target cells, at least a 30-fold higher ratio of killer to target cells was required to achieve the same degree of lysis as with T cells derived from +/− or +/+ mice. The difference was less pronounced when fibroblasts were used as targets, where only a 3-fold reduction of the cytotoxic activity was observed.

T cells from heterozygous +/− mice were consistently less active than the wild-type T cells, indicating that in primary cultures of lymphocytes perforin represents a limiting factor. This is in agreement with an antisense oligonucleotide study (18), where a 2-fold reduction of perforin directly correlated with a decrease in cytolytic activity.

Almost identical results were obtained with NK cells, thus providing evidence that the two effector cells use similar if not identical killing mechanisms. Again, the ^{51}Cr-releasing activity was almost entirely abolished, whereas DNA fragmentation in target cells was only partly impaired.

The involvement of perforin in the cytotoxic process may be explained in several ways. The simplest interpretation is that the absence of perforin results in the lack of transmembrane pores in the target cell membrane. Incorporation of isolated perforin into membranes induces massive ion conductance (35). Since, in perforin-lacking CTLs, no toxic lethal massive Ca^{2+} influx will ensue, target cells resist the attack of the killer cells. However, other explanations cannot

be excluded *a priori*. Not only may the absence alter the biogenesis of the secretory granules so that they become incompetent for the storage of other proteins, but perforin might play the role of a fusogen akin to viral proteins, thus allowing the fusion of granule and cell membrane. Moreover, the absence of perforin may lead to a defective activation of T cells, including altered cytokine release. The absence of all these cellular activities would result in an impaired release of granule harbored proteins or potentially lytic cytokines and thus to apparent nonactive killer cells. This supposition seems unlikely, however. Our results show (*i*) a normal activation of T lymphocytes as reflected by unchanged interferon γ secretion and (*ii*) an unaffected T-cell receptor-induced exocytosis of granzyme A.

CTLs are known to efficiently induce apoptosis in target cells. DNA degradation, an indicator of ongoing apoptosis, was impaired as well in target cells attacked by perforin-free cells, although not to the same extent as the plasma membrane lysis. In fact, with P815 cells, only a 3-fold reduction in DNA degradation was seen with perforin-negative T cells, in spite of the 30-fold higher resistance in plasma membrane lysis. Almost identical results were obtained with NK effector cells. This indicates that other, perforin-independent mechanisms are operational which cause the slow apoptotic death. Since granzymes are not cytotoxic in the absence of perforin, the Fas-based cytotoxic pathway which is known to lead to apoptotic cell death (36, 37) is the most likely candidate.

Perforin message has been shown to be highly increased in T cells at the site of lesions in various disease models, including murine choriomenengitis, herpesvirus infection, nonobese diabetic mice, myocarditis, graft rejection, and rheumatoid arthritis. It will therefore be interesting to analyze these *in vivo* models in perforin knockout mice to evaluate the contribution of the pore-forming protein.

Note. The analysis of another perforin-deficient mouse line has been reported by Kägi *et al.* (38), providing convincing evidence that perforin $-/-$ mice failed to clear lymphocytic choriomenengitis virus. However, Kägi *et al.* did not observe residual lysis of NK target cells and fibroblasts by perforin-free CTLs and concluded that perforin accounted for 100% of the lytic activity against most target cells. The reasons for this discrepancy are unclear.

We gratefully acknowledge the technical assistance of C. Mattman, P. Allegrini, and E. Burnier. The advice and help of Drs. Michel Aguet (University of Zürich), Frank Hilberg (Bender, Vienna), and Edith Hummler (University of Lausanne) were essential for the establishment of the knockout technique. The helpful comments and suggestions of Drs. J. Mariansky, R. MacDonald, and H. Acha-Orbea at our research center are gratefully acknowledged. This work was supported by grants from the Swiss National Science Foundation.

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