Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 247–251, January 1989
Immunology

Cloning, analysis, and expression of murine perforin 1 cDNA, a component of cytolytic T-cell granules with homology to complement component C9

(cytotoxic lymphocyte/pore-forming proteins/cytotoxic mechanisms/primary structure)

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Communicated by Herman N. Eisen, September 14, 1988

ABSTRACT **The nucleotide sequence coding for the cytotoxic T-lymphocyte (CTL) protein perforin 1 (P1) has been determined and the corresponding protein sequence has been derived. Murine CTL cDNA libraries contained in the vector λgt11 were screened by using a monospecific antiserum to purified P1. Three recombinant phages were isolated and their cDNA inserts were sequenced. The derived protein sequence contains 554 amino acids and displays, as expected, considerable homology with certain functional domains in the complement components C9, C8α, C8β, and C7. The identity of P1 cDNA clones was verified by prokaryotic expression and the reactivities of antisera produced to the expressed proteins. In addition, antisera were produced to two synthetic peptides located in the center and C-terminal portions of P1. All antisera reacted with purified P1. In Northern blot analyses, P1 cDNA probes recognized a 2.9-kilobase mRNA only in CTL. Perforin mRNA was found in all cloned CTL and in all mixed lymphocyte reactions that gave rise to cytotoxic cells. Perforin mRNA was also detected in virus-specific CTL that had been generated *in vivo* and isolated from liver tissue of mice infected with lymphocytic choriomeningitis virus. The cell-specific expression of perforin is consistent with its postulated role in cytolysis.**

Cytotoxic T lymphocytes (CTL) are of primary importance for the immunological control of virus infection (1, 2). Together with natural killer (NK) cells, CTL may play a role in tumor surveillance (3), in transplantation rejection (4), and in graft-versus-host reactions (5).

The mechanism of lymphocyte-mediated cytolysis appears to involve more than one pathway ultimately leading to the destruction of the target cell membrane and nucleus (6–8). An early event in target cell lysis by CTL and NK cells is the vectorial secretion onto target cell membranes of cytolytic granules contained in killer cells (9–12). Cytolytic granules contain several related serine proteases (13–17), proteoglycans (18), and cytotoxic proteins (19–21). One of the major cytotoxic proteins found in granules is perforin 1 (P1). Incubation of granules or of purified P1 (12) with erythrocytes or nucleated cells in the presence of calcium results in lysis and the formation of membrane lesions of 160-Å diameter. These transmembrane lesions are indistinguishable from membrane lesions formed on target cells by intact NK and CTL (19, 20). By morphological criteria, P1 membrane lesions resemble those formed by complement components C5b–9 during hemolysis. This observation, and immunological crossreactivity of P1 with C6, C7, C8, and C9, has led to the suggestion that P1 and terminal complement components share primary sequence homology (sequence for C6 is not available) (22, 23).

Here we report definitive information on the sequence homology between C9 and P1[§] and provide evidence for a role of P1 in the cytolytic process of lymphocytes.

MATERIALS AND METHODS

Materials. Restriction enzymes, the large (Klenow) fragment of DNA polymerase, and T4 DNA polymerase were obtained from Boehringer Mannheim. Deoxyribonucleotides and dideoxyribonucleotides were purchased from Pharmacia. The 17-nucleotide universal primer for M13 sequencing was obtained from Collaborative Research and Bio-Rad. 5-[α-[^{35}S]thio]dATP (400 Ci/mmol, 1 Ci = 37 GBq) as well as [α-^{32}P]dCTP (3000 Ci/mmol) were obtained from Amersham. Oligonucleotides used as primers for DNA sequencing were synthesized on a DuPont Coder 300 DNA synthesizer using the phosphoramidite methodology. pEX plasmids 1–3 and host *Escherichia coli* strain pop 2136 were kindly provided by K. K. Stanley (EMBL, Heidelberg). The λgt11 cDNA libraries 3F9 and AD9 were made from CTL clones as described (24). A CTL cDNA library from a hapten-specific clone (25) was kindly provided by Pamela Ohashi (University of Toronto).

Antibody Screening. Monospecific rabbit antiserum to murine P1 (12) was absorbed with detergent lysates of *E. coli* Y1090 before being used for screening (26). From 100,000 recombinant plaques, a single positive phage was plaque purified in four subsequent platings. Longer cDNA clones of 1.8 and 2.9 kilobases (kb) were obtained by using the 600-base-pair (bp) insert of the original clone as a hybridization probe.

DNA Sequencing. Phage cDNA inserts were excised with *Eco*RI and subcloned in the pTZ18U plasmid (Bio-Rad). Sequencing was performed by using the "shotgun" protocol and dideoxynucleotide chain termination method exactly as described (27). The 500 bp at the 5′ end and 1100 bp at the 3′ end were sequenced by using synthetic 17-mer primers spaced every 200 bp on either strand to prime synthesis instead of the universal 17-mer M13 primer. DNA sequences were analyzed with the Beckman Microgenie computer software (28).

Peptide Synthesis. On the basis of the deduced amino acid sequence of P1, peptides 15 and 18 amino acids long were synthesized by using the fluoren-9-ylmethoxycarbonyl (Fmoc) methodology on a DuPont Coder 250. The peptides were coupled with 0.025% glutaraldehyde to ovalbumin at a 1:1 ratio (wt/wt) and used as immunogens to prepare specific rabbit antisera. Hyperimmune sera were tested against pu-

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Abbreviations: P1, perforin 1; NK, natural killer cells; CTL, cytotoxic T lymphocytes.

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§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (accession no. J04148).

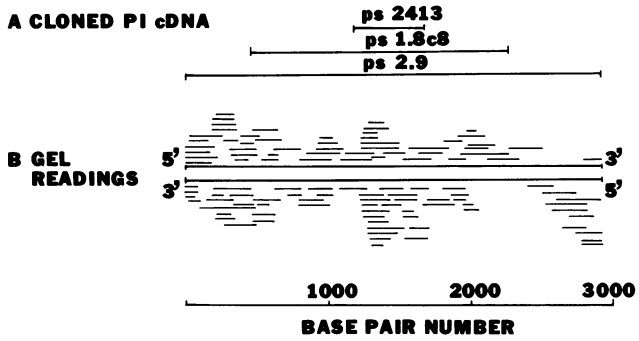

FIG. 1. Strategy used for sequencing P1 cDNA. (*A*) Relative positions of the three isolated and sequenced cDNA clones. (*B*) Individual sequences used to assemble complete P1 cDNA sequence.

rified P1 by Western blot analysis. In the case of the 15-residue peptide, the corresponding hyperimmune serum was also tested against ghosts of P1-lysed erythrocytes. P1-lysed membrane ghosts were prepared by lysing sheep erythrocytes with P1 in 5-fold excess of the amount required for 100% lysis. The membranes were then pelleted in an Eppendorf table-top centrifuge and washed twice in 1.0 mM EDTA. The final pellet was then analyzed by Western blotting subsequent to sodium dodecyl sulfate/PAGE.

pEX Subcloning and Prokaryotic Expression of P1 cDNA. *Alu* I fragments of P1 cDNA were subcloned in pEX1–3 as described (29) and used to transform *E. coli* pop 2136. pEX1, -2, and -3 are frame-shifted by one nucleotide each and thus allow expression of inserted DNA in all three reading frames. Three clones reactive with anti-P1 antisera were further studied by DNA sequence analysis and immunogenicity. *E. coli* inclusion bodies were prepared by sonication followed by centrifugation at 14,000 × *g* for 10 min. The pellet was washed once with 0.15 M NaCl/10 mM Tris·HCl, pH 7.5, and then used to immunize rabbits. Inclusion bodies analyzed by sodium dodecyl sulfate/PAGE were composed of approximately 20–50% fusion protein.

Northern Blot Analysis. Total RNA prepared by CsCl step gradients (30) or by the protocol of Chomczynski and Sacci (31) was analyzed by using standard Northern blot procedures for P1 mRNA.

RESULTS

Isolation of P1 cDNA Clones by Prokaryotic Expression. Approximately 100,000 recombinant clones from a CTL library (3F9) contained in the λgt11 expression system were screened with the P1 antiserum described previously (23). One clone (ps2413) was isolated that directed the synthesis of a perforin–galactosidase fusion protein reactive with anti-P1 in Western blots. In addition, the 600-bp insert cDNA of this clone showed limited homology to C9 and was therefore used to obtain a 1.8-kb clone (ps1.8c8) and full-length clones of P1 cDNA (ps2.9) by screening of additional CTL libraries. The longest DNA insert obtained was 2.9 kb, which agreed with

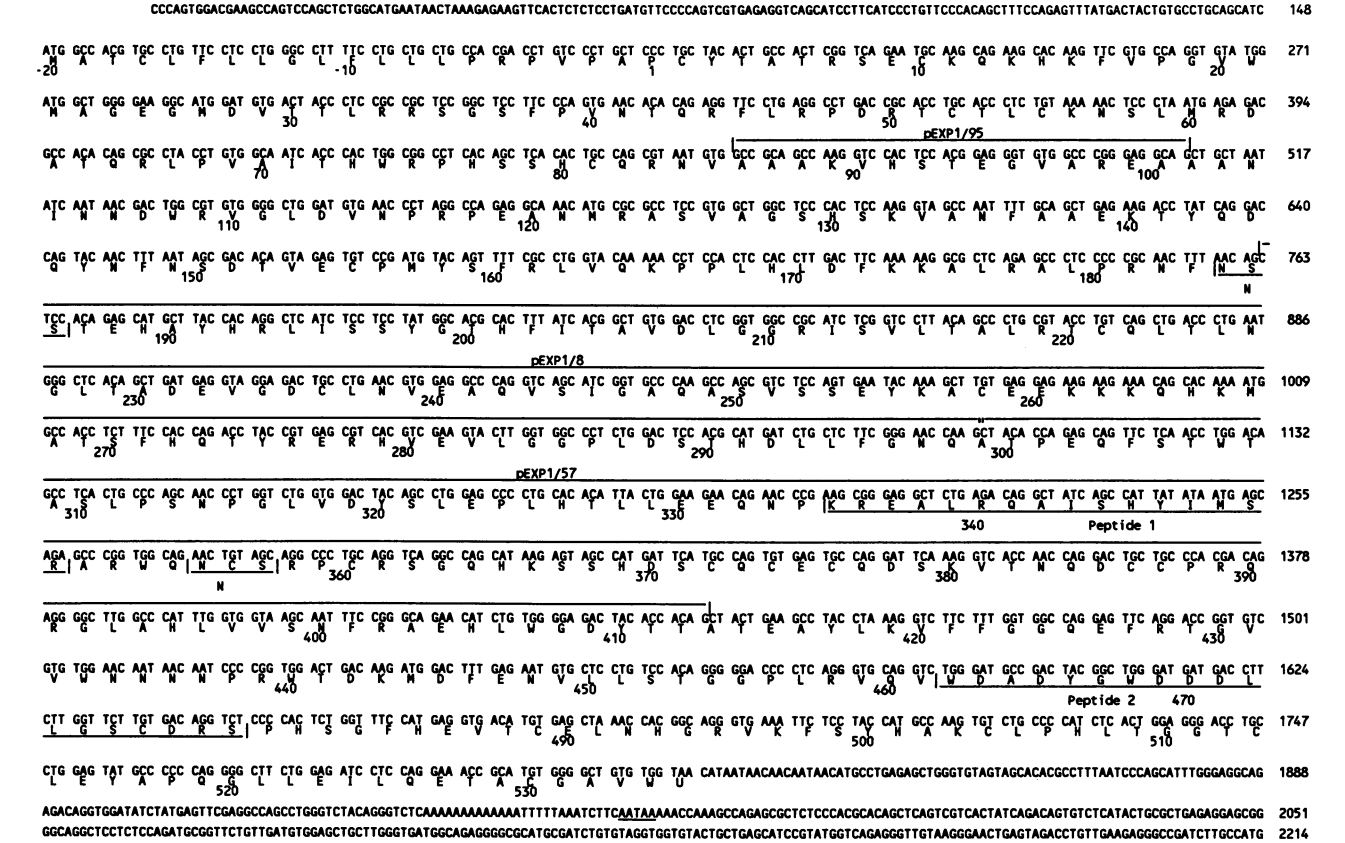

FIG. 2. Nucleotide and derived amino acid sequence of murine P1. Amino acids (standard one-letter symbols) are enumerated below each line and nucleotides are numbered above each line. The two N-linked glycosylation sites are underlined and marked "N." Also shown are the sequences of both pEXP1 clones and synthetic peptides. pEXP1 cDNA clones have been shown, including the *Alu* I sites. pEXP1/95 constituted the 5′ end of ps1.8c8.

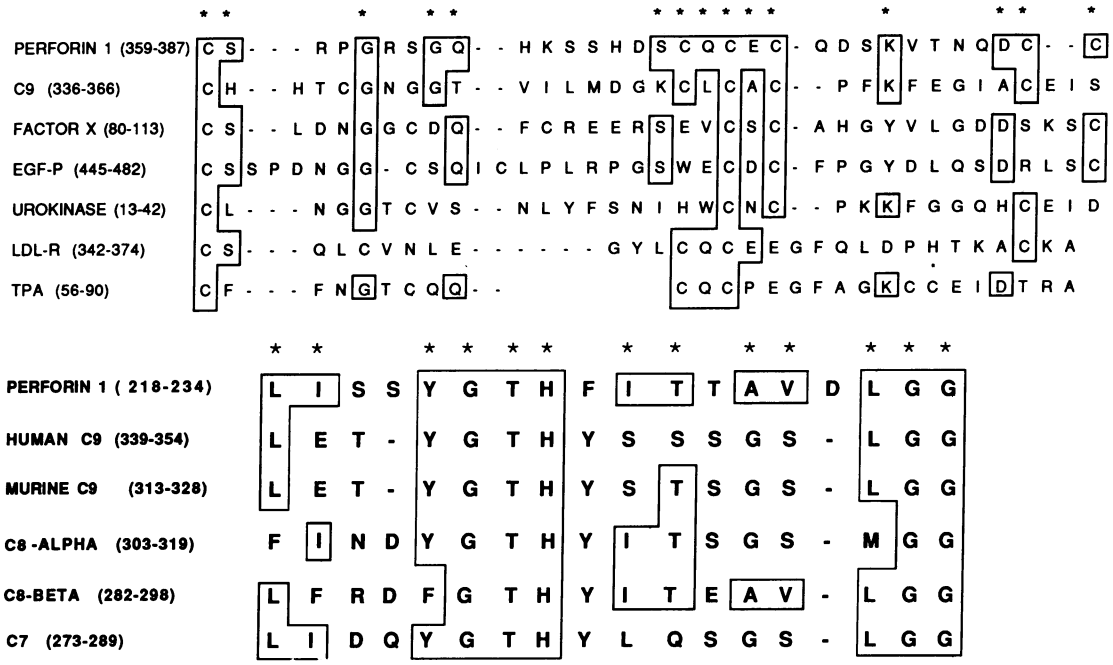

FIG. 3. (*Upper*) Homology of P1 with other proteins containing the cysteine-rich domain of epidermal growth factor receptor precursor (EGF-P). Asterisks were placed in the P1 sequence wherever the same amino acid was found to occur in at least two other proteins. LDL-R, low density lipoprotein receptor; TPA, tissue plasminogen activator. (*Lower*) Homology of P1 to the putative membrane-binding domain of C9, C8α, C8β, and C7 (32–35).

the size of mRNA expressed by CTL and hybridizing with the original clone.

Sequence of Perforin cDNA and Derived Protein Sequence. The DNA sequence was obtained by using the M13–shotgun strategy, combining the universal M13 primer with synthetic primers complementary to sequences already obtained (Fig. 1). Ambiguous G+C-rich sequences in addition were sequenced with reverse transcriptase as DNA polymerase.

The 2.9-kb perforin cDNA contained one open reading frame beginning with an ATG start codon and continuing for 554 codons to a TAA termination site (Fig. 2). The derived amino acid sequence predicts a leader peptide with a probable cleavage site at position 1 (Fig. 2). Thus the mature protein contains 534 amino acids. The sequence contains two potential glycosylation sites and, as in the case of the terminal complement proteins, no typical transmembrane domain.

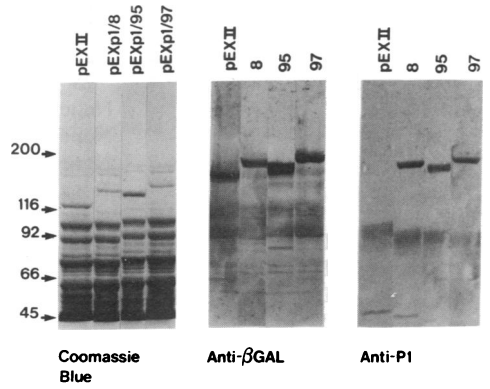

FIG. 4. Reactivity of pEXP1 plasmid fusion proteins with anti-P1 and anti-β-galactosidase antisera. Each clone was induced to express its fusion proteins as described (29) and the proteins were separated by electrophoresis on a sodium dodecyl sulfate/5–12.5% polyacrylamide gradient gel. Coomassie blue staining is shown on the left, where positions of molecular mass standards (kDa) are indicated. After transfer to nitrocellulose, the proteins were stained with 1:200 dilutions of antisera.

Homology to C9. Computer alignment shows homology of the C-terminal part of C9 with the N-terminal part of P1. These regions include a putative membrane-spanning amphipathic helix and the cysteine-rich epidermal growth factor receptor precursor domain (Fig. 3). The spacing of the two domains also is retained. P1 does not contain the cysteine-rich domain homologous to the low density lipoprotein receptor which is found in all terminal complement proteins (32–35).

Verification of Perforin cDNA as Coding for P1. An immunological approach was taken to prove the identity of the P1 clones. Two synthetic peptides (see Fig. 2 for sequence) and three in-frame P1–β-galactosidase fusion proteins (Fig. 4) produced in *E. coli* were used to immunize rabbits. The antisera were tested in Western blots for reactivity with purified P1. Fig. 5 shows the results of this analysis, indicating that the antisera produced from *E. coli* extracts (anti-fusion protein) detect a protein isolated from cytolytic granules with all the characteristics of P1. Similarly, the anti-peptide antisera reacted with P1 and stained erythrocyte membranes previously lysed with P1 (Fig. 6). As these

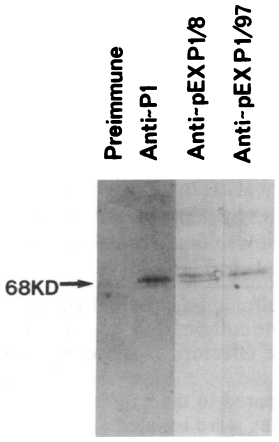

FIG. 5. Western blots with P1-specific antisera generated by immunization with P1 fusion proteins. pEXP1/8 and pEXP1/97 were used to immunize rabbits after preparation of inclusion bodies from *E. coli*. pEXP1/57 antiserum was weakly reactive and its immunoblot is not reproduced here.

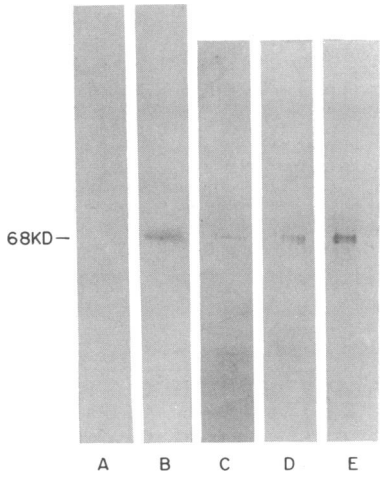

FIG. 6. Western blots of purified P1 and P1-lysed erythrocyte ghosts with antisera prepared against peptide 1. P1-lysed ghosts were prepared as described in the text. Purified P1 was used as the antigen in lanes A, B, and D, while P1-lysed ghosts were run in lanes C and E. Lane A was immunostained with preimmune sera, lanes D and E were immunostained with anti-P1, and lanes B and C were immunostained with anti-peptide 1 antisera. The same reactivity against purified P1 was obtained with antisera made against peptide 2.

membranes were washed to remove unbound protein, the positive reaction indicated that P1 inserted and lysed the erythrocytes and that this inserted P1 reacts with anti-peptide antisera. Of all known granule components, only P1 inserts into erythrocyte membranes.

Cell-Specific Expression of Perforin. Northern blot analysis of RNA extracted from various tissues and cells (Table 1) indicates that perforin is expressed specifically in cytolytic lymphocytes and not in other cells or tissues. A heavily loaded Northern blot of HY3-AG3 shows a 2.9-kb band and some degradation products (Fig. 7). All CTL clones analyzed to date contain perforin mRNA (Table 1). In addition,

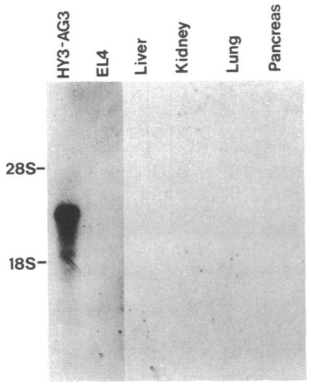

FIG. 7. Northern blot analysis of various tissues, CTL clone HY3-AG3, and control T-cell lymphoma clone EL4. The insert of P1 cDNA clone ps1.8c8 was radiolabeled by using a random priming kit (Boehringer Mannheim) and used to probe 10 μg of total RNA from each sample. Total RNA was quantitated by both absorbance at 260 nm and ethidium bromide staining.

stimulation of lymphocytes with mitogens or in mixed lymphocyte culture also results in perforin mRNA expression concomitant with the appearance of cytotoxicity (Table 1). Importantly, perforin mRNA was also expressed in CTL generated *in vivo* by a hepatotropic lymphocytic choriomeningitis virus strain (36). These CTL were directly isolated from the liver and analyzed without further culture.

Genomic Structure of Perforin. Fig. 8 shows a Southern blot of restriction endonuclease-digested genomic DNA hybridized with a full-length P1 cDNA clone. Perforin appears to be encoded in a single-copy gene of moderate size (<6 kb). So far no evidence for polymorphism has been detected in different mouse strains.

DISCUSSION

We have determined the nucleic acid sequence of cloned P1 cDNA and deduced the amino acid sequence. The original clone of perforin cDNA was obtained by antibody screening of a prokaryotic expression library contained in λgt11. That this cDNA in fact codes for perforin was verified by the production of antisera to synthetic peptides and to perforin–

Table 1. Expression of perforin mRNA in normal murine lymphocytes

Clone	Cell type and specificities*	Cytotoxic activity, LU† per 10^6 cells	Perforin mRNA,‡ %
HY3-AG3	NK-like CTL	50	100
3F9	H-2D^b allospecific CTL	35	75
5/10-20	AED-specific CTL	25	25
8/10-20	H2-K^b-restricted CTL	30	25
50.1	Three LCMV-specific,	30	75
532	H-2-restricted CTL	35	25
527	(various H-2 specificities)	35	25
Ova-7	Ovalbumin-specific, H2-IA^b-restricted T_H	0	None
EL4	T-cell lymphoma	0	None
P815	Mastocytoma	0	None
MC57G	Methylcholanthrene-induced fibrosarcoma	0	None
L929	Fibrosarcoma	0	None
	Primary MLC (BALB/c anti-B6)	ND	15
	Con A-stimulated splenocytes	ND	15
	LPS-stimulated splenocytes	ND	None
	Secondary MLC (anti-VSV splenocytes)	6.5	15
	Secondary MLC (anti-vaccinia splenocytes)	ND	15
	Primary *in vivo* (anti-LCMV splenocytes)	3	15
	Primary *in vivo* (anti-LCMV liver lymphocytes)§	25	15
	Normal splenocytes	ND	<5

*AED, *N*-iodoacetyl-*N'*-(5'-sulfonic-l-napthyl)ethylenediamine; LCMV, lymphocytic choriomeningitis virus; T_H, helper T cells; MLC, mixed lymphocyte culture; Con A, concanavalin A; LPS, lipopolysaccharide; VSV, vesicular stomatitis virus.
†One lytic unit (LU) = no. of effectors to cause 50% lysis of appropriate targets in a 4-hr ^{51}Cr release assay. ND, not determined.
‡mRNA expression was compared to the expression of P1 mRNA in HY3-AG3 (100%) cells from Northern analysis.
§Anti-LCMV liver lymphocytes were isolated as described (36).

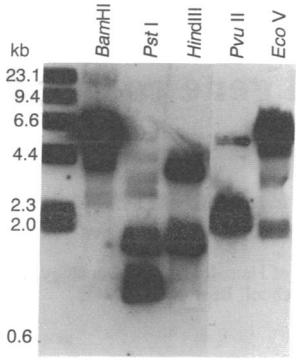

FIG. 8. Genomic Southern blot analysis of the P1 gene. Enzymes used to cut DNA isolated from mouse strain BDF_1 kidneys are shown above their lanes. The left lane contained markers of the indicated length.

galactosidase fusion proteins which reacted with authentic perforin isolated from CTL cytolytic granules.

The predicted perforin sequence shows considerable homology with the terminal complement proteins C7, C8, and C9, as expected from previously reported immunological cross-reactivity between those proteins (22, 23). The alignment of P1 with C9 shows a homology region extending from C9's C terminus to approximately amino acid 120 of C9. This region contains the putative membrane-binding site (37) and a cysteine-rich domain found in a number of proteins whose prototype is the epidermal growth factor receptor precursor. The first 120 amino acids of C9, which contain a thrombospondin-like domain, and the cysteine-rich domain of the low density lipoprotein (LDL)-receptor type are not represented in perforin, even though they are found in C7 and the subunits of C8. The previous finding that an antibody to an acidic synthetic peptide corresponding to the LDL-receptor-like domain of C9 (22) cross-reacted with perforin can be explained by an acidic region in the C-terminal part of perforin (contained in peptide 2, Fig. 2).

Perforin mRNA has been detected to date in all CTL, regardless of whether they were generated *in vivo* or cultured *in vitro*. Moreover, the expression of perforin mRNA as detectable in Northern blots preceded the onset of cytotoxicity by 12–24 hr (data not shown). Initial experiments indicate that both class I and class II major histocompatibility complex-restricted CTL express perforin. These findings suggest that perforin is intimately related to the expression of cytotoxicity by lymphocytic killer cells. This is further supported by the finding that cells devoid of cytotoxic activity and numerous tissues did not express perforin. The question whether peritoneal exudate lymphocytes (38) express perforin needs to be reexamined with these more sensitive techniques now available.

Although the perforin mRNA expression experiments show a strong correlation between perforin transcription and cytotoxicity, they do not constitute proof that perforin is essential for target lysis by lymphocytes. Indeed, the observations of CTL-mediated DNA degradation (6, 7), of soluble cytotoxic factors (8), of perforin-independent cytotoxic granule components (21), and of Ca-independent lysis of target cells (39) suggest that several pathways exist by which lymphocytes exert cytotoxic activity. The availability of cloned perforin cDNA will enable us to critically assess the role of perforin in lymphocyte-mediated cytotoxicity *in vitro* and *in vivo*.

We are grateful to Mi Kyung Lee and Laurie Fulton, who provided excellent technical support during this study. We are also grateful to Dr. Pat Grey and Sandra Soccaras, who provided their help in various parts of this work. This research was supported by National Institute of Arthritis and Infectious Disease Grant AI 21999, National Cancer Institute Grant Ca39201, and American Cancer Society Grant IM-369A to E.R.P. D.M.L. is a Research Fellow of the Cancer Research Institute (New York).

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