The Role of Perforin Protein in Lymphocyte Mediated Cytotoxicity

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZÜRICH

for the degree of
Doctor of Natural Sciences

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1993

Man darf nie aufhören, sich die Welt vorzustellen,
wie sie am vernünftigsten wäre.

Friedrich Dürrenmatt
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1. Summary

Cytotoxic T cells and natural killer cells are able to eliminate virus infected tumorigenic and allogeneic cells. The effector mechanism by which these cells are able to lyse target cells is still controversial. From all candidate models, most evidence has been provided for the granula exocytosis model. It proposes that cytolytic lymphocytes, upon recognition and interaction with the target cell, release the cytolytic pore-forming protein perforin into the intercellular space, which subsequently leads to colloid osmotic lysis of the target cell. Since the relevance of this concept for cytotoxicity in vivo has been questioned, the expression of perforin was investigated during the infection of mice with lymphocytic choriomeningitis virus. It is well established that CD8 positive cytotoxic T cells play a pivotal role in the elimination of this non-cytopathic virus. Perforin expression was analyzed by in situ hybridisation of serial liver and brain sections from infected mice and by immunohistochemical staining with T cell marker and virus specific antibodies. A close histological association of infiltrating lymphocytes expressing perforin mRNA with virally infected cells was observed. The distribution of perforin expressing cells on serial sections compared to the distribution of CD8 positive cells was consistent with the notion, that CD8 positive T cells express perforin. In addition, the maximal frequency of perforin expressing cells on liver sections preceded by about 2 days maximal LCMV specific cytotoxicity of the lymphoid liver infiltrating cells.

Complementary investigation by RNA-PCR confirmed the upregulation of perforin expression in the spleen by a factor of at least 10 during LCMV infection. These findings, though somewhat circumstantial, are most consistent with an involvement of perforin in cell mediated cytotoxicity in vivo.

To provide more direct evidence for the role of perforin in vivo, the generation of perforin deficient mice by homologous recombination in embryonic stem cells was planned. Embryonic stem cells with a targeted mutation of the perforin gene were
isolated. A neomycin resistance gene with multiple translational stop codons in all three reading frames was introduced in exon 3 resulting in the formation of non-functional mRNA from the disrupted perforin gene. By blastocyst injection of the targeted pluripotent embryonic stem cells and surgical introduction of the embryo into the uterus of a foster mother chimeric mice were obtained. Eight chimeras transmitted the disrupted locus to 50% of their offsprings. By breeding the heterozygot offsprings with each other homozygous perforin deficient mice will be obtained. These animals will allow a direct assessment of the role of perforin for the cytotoxicity of lymphocytes and eventually provide a model system to test the role of cytotoxic T cells and natural killer cells in infections with various biological agents and in murine models for autoimmune diseases.
2. Zusammenfassung


Um einen direkten Beweis für die Relevanz von Perforin in vivo zu führen, wurde die Herstellung von Perforin defizienten Mäusen durch homologe Rekombination
### 3. Abbreviations

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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<td>CD</td>
<td>complex of differentiation</td>
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<td>con A</td>
<td>concanavaline A</td>
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<td>CTL</td>
<td>cytotoxic T cell</td>
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<td>DNA</td>
<td>desoxy-ribonucleic acid</td>
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<td>ES cell</td>
<td>embryonic stem cell</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>i.c.</td>
<td>intracerebral</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IL</td>
<td>interleukin</td>
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<tr>
<td>L.U.</td>
<td>lytic unit</td>
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<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
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<td>LCMV-WE</td>
<td>strain WE of LCMV</td>
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<td>LGL</td>
<td>large granular lymphocyte</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>NK</td>
<td>natural killer</td>
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<td>NOD</td>
<td>nonobese diabetes</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>pfu</td>
<td>plaque forming unit</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>TNF</td>
<td>tumour necrosis factor</td>
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4. General Introduction

The task of the immune system is to maintain the homeostasis of the body by defending its integrity against numerous microbial infections, parasites and foreign toxic substances. For this purpose, the immune system is capable to discriminate between self and nonself antigens. Nonself antigens generally generate a protecting immune response, while against self determinants a response is prevented by a state of nonresponsiveness or tolerance, which is established at an early time point in ontogeny when the immune system is not completely functional and mature yet. Already back at the beginning of the twentieth century, Ehrlich recognized the absence of reactivity towards the organisms own structures, the so called "horror autotoxicus", as a key property of the immune system. But from todays point of view, the notion, that the immune system never shows any autoreactivity, appears not absolutely correct; under certain physiological conditions, the immune system overrides the principle of non-reactivity against self by the elimination of virus infected or tumourigenic cells. In such situations, it is advantageous to the inflicted organism to sacrifice a small number of cells in order to prevent further damage by spreading of the virus or the tumour. The cells of the immune system able to lyse virus infected and tumour cells are the cytotoxic T cells and NK cells. Both of them are part of cell mediated immunity, in which antibodies play only a subordinate role.

Eversince the capacity of lymphocytes to lyse nucleated cells was discovered (Govaertz, 1960), speculations about the molecular mechanism of cell lysis by lymphocytes were made. But the issue has not found its definitive solution yet and is still controversial. Recent advances suggest a surprising similarity of the effector functions in the humoral and cellular branch of the immune system. It is now very probable, that cytotoxic T cells and NK cells perform cell lysis by a mechanism that is very similar to the antibody induced cell lysis by the complement system. But as it applies also for the complement system, the drastic impact of cell lysis not always remains beneficial to the host: On one side it can protect the body from
fatal virus infections and tumour growth but on the other side, its excessive and unfavorable manifestations can lead to autoimmune disease and life-threatening transplant rejection. Understanding the cells and molecules involved in the process of cytotoxicity could help to interfere one day with the course of such diseases in a more specific way.

**Discovery of the Cytotoxic Capacity of Lymphocytes**

The saying of the war being the father of all things applies also to the discovery of cytotoxic lymphocytes. World war II, with its many victims, generated an intense interest in transplant immunology. It was in England of the 40s that Medawar, Snell, Gorer, Billingham and Brent showed that lymphocytes are responsible for the vigorous rejection reaction after transplantation of allogeneic tissue. This was just after the central role of lymphocytes in allergy had been recognized (Landsteiner and Chase, 1942). That a direct contact between grafted and effector cells is essential for rejection of the grafted tissue was concluded from experiments in which tumour fragments enclosed in diffusion chambers were not rejected after transplantation (Algire, 1957). The transfer of allograft rejection by primed lymphoid cells but not by antisera established the cellular basis of allograft rejection (Mitchison, 1954). That indeed a lytic effect is responsible for allograft rejection was demonstrated by the cytopathic effect of thoracic duct lymphocytes, procured after kidney allograft rejection, on donor renal cells cultured in vitro. (Govaertz, 1960). Miller established the importance of the thymus in transplant rejection by demonstrating the absence of skin transplant rejection in neonatally thymectomized mice (Miller, 1961). Thymus dependent T cells were postulated and identified as the main cytolytic lymphocyte population (Cerrotini et al., 1970). Later, they were further characterized as a T cell subpopulation carrying the CD8 surface marker (Cantor and Boyse, 1975).
Cytotoxic T Cells

Cytotoxic T cells defend the host mainly against intracellular agents like viruses, tumour cells and intracellular bacteria. The capacity to lyse virus infected cells before new infectious agents have formed in an infected cell renders cytotoxic T cells mainly effective against viruses. Beyond these natural functions, cytotoxic T cells are the main effector cells in rejection of allogeneic tissue grafts as well.

T cells can be divided into the two functional subclasses by the presence of the CD4 and CD8 surface markers. Cytotoxic T cells belong to the CD8 positive subset and require, like all T cells, the thymus for development. Activated, functional cytotoxic T cells develop from resting CTL precursor cells by triggering via their receptor and by additional Interleukin 2 mediated stimulation.

In contrast to B cells, T-cells do not recognize native protein antigen, but instead short octa- or nonameric peptides bound to MHC class I molecules (Townsend et al., 1986; Falk et al., 1991). Peptides derived from intracellular proteins by a limited proteolysis called processing are mainly presented on MHC class I molecules on the cell surface (HLA-A, B, C loci in humans, H-2 K, H-2 D and H-2-L loci in mouse) while extracellular antigenic proteins are endocytosed via a phagolysosome, processed and presented on MHC class II molecules (Townsend et al., 1986; Morrison et al., 1986; Germain, 1986). The T cell does not recognizes the free peptide but rather the association of peptide with a matched MHC molecule, a property of T cells called restriction (Zinkernagel and Doherty, 1974).

The MHC class I molecule consists of the association of an α chain with an β2 microglobulin chain. On the surface of this heterodimer, two α helices confine the peptide binding groove (Bjorkman et al., 1987b; Bjorkman et al., 1987a; Garrett et al., 1989; Jardetzky et al., 1991; Madden et al., 1991). This peptide-MHC class I complex is specifically recognized by the polymorphic T cell receptor (TCR) of the cytotoxic T cell (Yoshikai et al., 1984; Allison and Lanier, 1985; Hedrick et al., 1984; Sim et al., 1984). The TCR is a α/β heterodimeric membrane protein expressed on all mature T cells. The polymorphism of both chains is generated by
recombination of V, D (only β chain) and J elements similar to the recombination mechanism functional in B cells (Bjorkman et al., 1987b).

In addition to the specific interaction conferred by the trimolecular MHC-peptide-TCR complex, cytotoxic T cells bind to their target cells via a number of unspecific accessory molecular interactions. These receptor ligand pairs involve CD8-MHC class I molecule (via a non polymorphic determinant), LFA-1 -ICAM-1, CD2 -LFA-3, CD28 - B7, CD5 - CD27 and CD45RO - CD22 (reviewed by Springer, 1990). The function of these accessory interactions is not completely clear yet but probably helps to increase the relatively low affinity of the TCR-peptide-MHC binding (Weber et al., 1992).

Also, the precise molecular events that link receptor engagement to response of the cytotoxic T cell remain unresolved. Nonetheless, it is thought that certain transduction mechanisms exist in T cells: Phosphorylation of protein kinase C and of the ζ chain of the CD3 complex by the tyrosine kinases p56<sup>lek</sup> and p59<sup>fyn</sup> of the src family, dephosphorylation by CD45RO and subsequent G protein dependent breakdown of phosphatidylinositol leading to an increase of intracellular Ca<sup>2+</sup> and activation of protein kinase C have been observed during T cell activation.
Fig.1. *Molecules involved in T cell interaction with antigen presenting cells (APC).* Interaction of CD8 positive and CD4 positive T cell with an APC combined in this drawing for illustrative purposes only. Human CD11a correspond to LFA-1, CD54 to ICAM-1 and CD58 to LFA-3. Lck and fyn are the tyrosine kinases p56\textsuperscript{Lck} and p59\textsuperscript{Fyn}. CD45RO is a tyrosine phosphatase.

**NK Cells**

NK cells appear with large granular lymphocyte (LGL) morphology and are thought to play a role in tumour resistance, host immunity to viral and perhaps other microbial agents, especially as a first line of defense against virus. In nude mice an increased NK activity, at least partly, compensates for the absence of cytotoxic T cells. NK cells do not show rearrangement of TCR genes and do not express the CD3 surface molecule. However, they usually express the asialo GM1, the NK1 and the CD56 (Leu 19) surface marker molecules. A consistent feature of NK cells is their association with a subpopulation of cells, the LGLs. LGLs can be
isolated from blood and spleens of unprimed animals by centrifugation on Percoll gradients and elimination of cells that form high affinity rosettes with sheep red blood cells.

Some target cell lines are highly susceptible for target cell lysis (human K562 and murine YAC-1 cells) and serve as prototype NK activity indicator cell lines. It is unclear why these cell lines are so sensitive to NK cells, but recent publications support the interesting idea, that NK cells preferentially lyse cells lacking a protective peptide associated to class I (reviewed by Versteeg, 1992). Following these reports, NK sensitivity would be a consequence of either downregulation of MHC class I molecule in tumour cells, which is an immunological trick to circumvent recognition by tumour antigen specific T cells, or alternatively results because a conserved peptide of an intracellular parasite competes with a related endogenous protective peptide.

The Granule Exocytosis Model of Target Cell Destruction by Lymphocytes

Already back in 1968, it was suggested that lymphocytes lyse target cells by a mechanism involving an active release of a cytolytic factor by the cytotoxic cell (Granger and Kolb, 1968). Since then, this suggestion has been confirmed by numerous observations, which are in detail listed below:

- The presence of cytoplasmic granules has been noted in most types of cytolytic lymphocytes.
- Localisation of granules become localized between nucleus and target cell by a rearrangement of the cytoskeleton after target cell binding (Bykovskaja et al., 1978a; Bykovskaja et al., 1978b).
- The observation of acid phosphatase deposition, originally found in the lysosomal granula of cytotoxic T cells, at the effector-target cell junctions during cytolytic-target cell interaction.

The isolation of a protein named Perforin from the granula of cytolytic lymphocytes with potent cytolytic properties when added to cells in vitro (Masson and Tschopp, 1985; Podack et al., 1985; Liu et al., 1986).
The direct demonstration of complement like pore structures on target cell membranes of an internal diameter of 15 nm (complement pores: 10 nm) (Dourmashkin et al., 1980; Podack, 1983; Bykovskaja et al., 1978a)

All these observations were subsumed in the granule exocytosis model of target cell lysis (Fig. 2) (Henkart, 1985). It consists of the following discrete steps: (i) Receptor and adhesion molecule mediated binding of the target cell to the lymphocyte. (ii) An increase of intracellular Ca\(^{2+}\) in the cytotoxic cell and a subsequent polarization of the Golgi apparatus, the granules and the cytoskeleton towards the target cell. (iii) Vectorial granule exocytosis and the release of pore forming protein (Perforin) monomers in the intracellular space between cytolytic and target cell. (iv) Calcium dependent assembly of membrane lesions with polymerization of the monomer into a functional channel in the target cell membrane.

The granule exocytosis model includes the notion that the formation of a transmembrane polyperforin pore of 16 - 20 nm diameter allows the equilibration of ion concentration gradients over the membrane. The passive flow of water into the injured cell from the outside in response to the colloid osmotic pressure difference generated by the proteins causes the cell to swell and eventually to burst.
Fig. 2. Granule exocytosis model of lymphocyte mediated cytotoxicity as it was suggested by (Henkart, 1985). This model shows the adhesion and recognition phase mediated by the T cell receptor and accessory molecules, followed by the cytoplasmic rearrangement bringing the granula in close proximity to the target cell and the granule exocytosis step. Today, the granule exocytosis model includes the notion, that target cell lysis is caused by the polymerization of secreted perforin monomers into a membrane pore causing colloid osmotic lysis of the target cell. After target cell lysis, the effector can recycle and, probably after replenishment of the granules by resynthesis of granula proteins, kill another target cell.
Alternative Mechanisms of Target Cell Lysis

The granule exocytosis model has been challenged mainly because target cell lysis was not completely abolished in Ca\(^{2+}\) depleted medium (Trenn et al., 1987). The complete absence of intracellular and extracellular Ca\(^{2+}\) is thought to inhibit exocytosis and to prevent the polymerization of perforin monomers.

Another challenge came from the difficulties to detect cytolytic granules in peritoneal exudate lymphocytes (PEL), potent alloreactive cytolytic cells, by light microscopy and by the hemolytic bioassay (Berke and Rosen, 1987). But subsequent more sensitive analysis with Northern blotting demonstrated considerable amounts of Perforin mRNA in these cells (Nagler Anderson et al., 1989).

The main alternative to the granule exocytosis model is the notion that target cells destroy themselves by a suicidal process named apoptosis (Ucker, 1987; Russell, 1983). Apoptosis is a concept originating from embryology to explain the controlled cell death without scaring during development. It is thought to be associated with extensive degradation of genomic nuclear DNA into multiples of about 200 bp. The internal disintegration model of cytolysis by lymphocytes is supported by the appearance of early nuclear condensation in target cells during the cytolytic process. However, the finding that the characteristic "laddering" of genomic DNA occurs only in some, but not all target cell lines during lysis, and that fibroblasts derived from mice transgenic for the apoptosis inhibiting bcl2 protein are still susceptible to cytolytic T cells seriously question the relevance of the apoptosis model. It will be important to verify the exact cause of DNA fragmentation, especially the question if DNA fragmentation represents the cause or the merely a consequence of cell death.

Additional candidate mediators of cytotoxicity are the several serine proteases called Granzymes that have been isolated from the granules of cytotoxic T cells (Gershenfeld and Weissman, 1986; Masson and Tschopp, 1987). The blockage of cytolytic lymphocytes by the addition of serine esterase inhibitors has been taken as evidence for their involvement in target cell lysis (Redelman and Hudig, 1980;
Hudig et al., 1981). Since they have been found subsequently also in non cytolytic lymphocyte subsets, their function in the granules of cytotoxic T cells needs further investigation. (reviewed by Jenne and Tschopp, 1988).

Several factors with homology to tumor necrosis factors (TNF) have been isolated from cytolytic granules (Granger and Kolb, 1968; Ruddle and Waksman, 1968; Traub, 1936). Some of these factors can induce slow (over several hours) target cell lysis and DNA fragmentation. Due to the slow activity, which is hard to reconcile with target cell lysis occurring in minutes, their relevance remains unclear.

**Molecular Characteristics of Perforin Protein**

So far, the granule exocytosis model remains the best characterized to account for cytotoxicity by lymphocytes. It relies on the recently identified pore forming protein perforin as the main effector molecule.

Originally, perforin was isolated from granula of cytotoxic lymphocytes with the help of a bioassay for hemolysis of sheep red blood cells (SRBC). SRBC are easily lysed by granula and purified perforin and after lysis display the characteristic polyperforin lesions on the cell membrane, reminding of the ringlike pores observed on the surface of cells lysed by the complement system (Podack et al., 1985; Lichtenheld et al., 1988). Subsequent to the isolation of perforin and the demonstration of its potent cytolytic activity, the complete cDNA from mouse and human was cloned and sequenced with the help of a degenerate oligonucleotide probe derived from the sequence of amino acids 21-29 (Shinkai et al., 1988; Lichtenheld et al., 1988).

Both mouse and human perforin are encoded by a single genomic locus located on mouse chromosome 10 (Trapani et al., 1989; Zink et al., 1992). The localization of human perforin to chromosome 17 (Shinkai et al., 1989) is contradicted by a recent publication reporting evidence for a localization to human chromosome 10 (Zink et al., 1992). Both genes are composed of only three exons (Fig.3). Exon one codes for the majority of the 5'-untranslated sequence; exon two for the remainder of 5'
untranslated sequence, the translation start signal, the signal peptide, and the N-terminal part of the molecule up to, but not including, the putative membrane binding site. Exon three encodes for the remainder of the molecule including the 3’ untranslated sequence.

**Fig 3. Genomic structure of murine perforin.** The perforin locus consists of untranslated exon 1 and the two coding exons 2 and 3, contained in 10 kb of genomic sequence. The distribution of sequence homologous to C9 complement component is indicated. The organisation of the human perforin locus is very similar.

The main inductors of perforin expression in human peripheral blood cells known so far is the stimulation of T cells via the TCR-CD3 complex or the exposure to high amount of IL-2. IL-6 synergizes with IL-2 in the induction of perforin expression and cytolytic potential. In the presence of IL-6, suboptimal doses of IL-2 can induce perforin synthesis (Smyth et al., 1990b; Smyth et al., 1990a).

The promoter of the human and murine perforin genes are highly homologous. However, the previously reported potential regulatory elements of the human promoter were not found in the mouse perforin promoter and consequently, novel transcription factors are at least partly regulating perforin transcription (Lichtenheld and Podack, 1989).
The perforin transcript is 2.6 - 2.9 kb long as determined by Northern blot analysis. Only one major transcription initiation site has been found in the human perforin gene. In the murine gene, in contrast, transcription starts from at least two different initiation sites (Youn et al., 1991). This results in two differently spliced mRNA species, with a 5'-untranslated region of either 222 or 115 bp. This alternative splicing is generated by the use of different splice acceptor sites.

Perforin is expressed in vivo in NK cells, CD8 positive cytolytic T cells and γδ TCR positive T cells (Nakata et al., 1992). It is generally not expressed in CD4 positive T cells, although it was found in synovial CD4 positive T cells from patients suffering from rheumatoid arthritis (Griffiths et al., 1992).

After cleavage of the 20 (mouse) or 21 (human) amino acid long signal peptide with an amino acid sequence typical for secretory proteins, the mature protein consists of 534 amino acids with a calculated molecular weight of about 60 kDa for the unmodified peptide core. Probably due to glycosylation, it migrates with an apparent molecular weight of 65-70 kDa under non-reducing and with 70 kDa under reducing conditions on acrylamide gels. Two potential N-linked glycosylation sites are conserved in both species; murine perforin contains an additional third potential glycosilation site. Human and mouse perforin share 68% amino acid identity. All 20 cysteine residues are completely conserved between these different perforin species. With the exception of the cysteins homologous to the LDL class B or the epidermal growth factor precursor domains, the disulfide bridges are not known.
Fig. 4. **Structural organisation of human perforin in comparison to late complement components.** (Figure taken from Tschopp and Nabholz, 1990). The various structural motives present are: TSP 1, type 1 thrombospondin module; B, LDL receptor class B module (EGF module); SCR, short consensus repeat; FIM, factor 1 module; LB, candidate for lipid binding protein. Only cysteines outside motives or differing from invariant cysteines within motives are indicated. By analogy to the complement proteins, Cys 236 and 258 of perforin are likely to be disulfide-bonded. Putative asparagine-linked glycosylation sites are shown as black hexagons. Regions in perforin which show no detectable homology to complement proteins are boxed in with thick solid lines.
Comparison of the amino acid sequences of C6, C7, C8α, C8β and C9 reveals no detectable homology between the hundred N-terminal amino acids on the C-terminus of perforin and complement components. However, Perforin and complement share a central region covering approximately 280 amino acids or two thirds of the perforin molecule. In this region, about 20% of the amino acids are shared by all of these molecules. This suggests that this segment is responsible for the structural and functional similarities between C9 and Perforin, in particular, for the capacity to polymerize into similar tubules and to insert into membranes. The region contains two structural elements that have been identified previously: (a) Amino acids 191 - 211 (LB) are the most conserved residues in the entire sequence, and the corresponding residues in C9 have been proposed to form an amphipathic α-helix that interacts with the lipid bilayer. (b) The central segment common to complement proteins and perforin contains a cysteine-rich region of LDL receptor class B (or EGF precursor) type. The function of this module, present in many other molecules such as coagulation proteins and cell surface receptors, is not known.

In the presence of Ca2+ and at 37°C perforin interacts with the phospholipid headgroups of the target cell membrane, inserts into the membrane and polymerizes in the membrane irreversibly (Yue et al., 1990; Young et al., 1987). At 4°C, insertion and polymerization do not take place and perforin can be removed from the membrane by chelation of Ca2+ or by high salt concentartions. It is thought that a conformational change takes place during membrane insertion and polymerization. This leads to the exposition of a hydrophobic surface, enabling perforin to insert into the membrane and to traverse it.

In the target cell membrane perforin is found as a tubular polymer with an apparent molecular weight of approximately 1000 kDa. Between 12 and 18 monomers can form a cylinder with a defined height of 16 nm and a variable diameter between 5 and 20 nm (Fig.5). In the presence of 1 mM Ca2+, purified perforin exhibits potent lytic activity against nucleated cells and against red blood cells.
Introduction of Targeted Germline Mutations into Mice by Homologous Recombination in Embryonic Stem Cells

Molecular cloning techniques have in the recent twenty years allowed the cloning and sequencing of a cornucopia of mammalian genes. However for many of these genes, it has proven difficult to establish a clear role and function in vivo. Naturally occurring mutations were of big help to clarify the role of some genes in vivo. But for most genes no mutant mouse strains are available and the possibilities to search for such mutations are quickly limited by restrictions in time and breeding space. Especially recessive lethal mutations are very rare in the collection of isolated spontaneous mutant mice. In the light of this situation the interest for a technique that allows the targeted introduction of desired mutations into the germline of mice is understandable. The making of mice deficient for a gene of interest allows a direct assessment of its function in vivo.

Establishment of this techniques relied on a number of progresses made in the 1980s, namely the establishment of pluripotent embryonal stem cells (ES cells) from mouse blastocysts (Evans and Kaufman, 1981), the generation of germline

Fig.5. Formation of the poly perforin pore in the membrane of a target cell. The scheme depicts the binding of perforin to the membrane, its insertion after undergoing a conformational change and the polymerization in the membrane compared to the assembly of the membrane attack complex (MAC) by the terminal complement components. The figure is taken from (Podack et al., 1991).
chimeras from ES cells (Bradley et al., 1984), successful homologous recombination in eucaryotic cells (Smithies et al., 1985) and later ES cells (Thomas and Capecchi, 1987; Doetschmann et al., 1987), the preservation of pluripotency through the electroporation procedure and finally the establishment of germline chimeras with targeted ES cells (Hooper et al., 1987; Thompson et al., 1989; Koller and Smithies, 1989).

The procedure, as it is today well established (reviewed by Capecchi, 1989a; Hogan and Lyons, 1988; Robertson, 1986; Capecchi, 1989b; Rossant and Joyner, 1989; Fung-Leung and Mak, 1992), is outlined in Fig.6.

Pluripotent embryonic stem cells are transfected with the targeting vector containing the desired mutation. This mutation is flanked by homologous sequence, which mediates the recombination at the desired genomic locus. In only a little fraction of all ES cells, which incorporate a DNA construct into their genome, the integration takes place at the targeted locus, in most other cells the vector integrates at a random site into the genome. The rare targeted cells are identified by a screening procedure, usually by polymerase chain reaction (PCR), cloned and maintained as a homogeneous population. These clonal cells are injected into the blastocoel cavity of a preimplantation mouse embryo and the blastocyst is surgically reintroduced into the uterus of a foster mother where development is progressing to term. The resulting animal is chimeric in that it is composed of cells derived from the blastocyst, here denoted by the white fur colour, and the ES cells denoted by the black fur colour (Fig.6). Breeding of the chimera to an albino mouse yields a certain proportion of black mice indicating that the ES cells contributed to the formation of the germline in the chimeras. Genomic screening of the progeny is used to determine which mice received the mutated allele and can bred in order to obtain animals homozygous for the mutation.
Fig. 6. Outline of the procedure to obtain mice with a targeted mutation in a gene of interest by homologous recombination in ES cells. ES cells with a targeted mutation (denoted by the black colour) are selected, amplified and injected into blastocysts of a white furred mouse strain. Chimeras derived from these manipulated blastocysts by transfer into the uterus of a pseudopregnant foster mother can pass their genotype indicated by the black fur colour to offsprings. To generate deficient mice, heterozygous offsprings are bred to homozygousity. Illustration taken from (Capecchi; 1989a).
There are two different approaches currently applied to induce the desired genomic alteration. Insertion constructs create a partial duplication of the gene and require only a single crossover, while replacement vectors lead to the exchange of the endogenous DNA with the construct and involve a double crossover. An interesting application of insertion type vectors is the introduction of subtle point mutations into a locus of interest by the hit and run method (Hasty et al., 1991; Nickoloff and Reynolds, 1990).

For standard gene inactivation experiments, where a coding exon of the gene is interrupted by a neomycin resistance expression cassette, usually the replacement type vector is chosen. In this case the targeting construct consists of a stretch of homologous DNA varying in length interrupted by a neomycin resistance gene that allows the selection of transfected ES cells by growing them in G418 containing medium. The neomycin resistance gene that is introduced into a coding exon of a targeted gene not only serves to provide a resistance marker for cells with an integrated construct but also interrupts the coding sequence of the targeted gene. It displays multiple stop codons in all three reading frames leading to a premature stop of translation and a noncomplete, nonfunctional protein product. In addition, transcriptional inactivation of the targeted gene by the introduced expression cassette has been observed as well (Zijlstra et al., 1990).

An enrichment for homologous recombinant cells has been achieved by the addition of one or two Herpes Simplex Virus thymidine kinase genes to the ends of the construct. (Mansour et al., 1988). Homologous recombination leads to the loss of the kinase genes and to a resistance of the transfected cells towards the selecting compound ganciclovir. In contrast, randomly targeted cells obtain one or two copies of the thymidine kinase gene and thereby get sensitive to ganciclovir.

The influence of transcription of the targeted locus in the ES cells on the efficiency of homologous recombination is not clear. Early experiments did not indicate any enhancement of homologous recombination by transcription (Johnson et al., 1989) while others later reported such an effect (Farrar et al., 1980). The considerable number of successful targeting experiments with loci not transcribed in ES cells
suggests, that transcription has only a limited, if any, influence of transcription on the recombination efficiency. If transcription of the targeted locus in ES cells is known, this can be exploited to enhance homologous recombination by applying a promoterless construct (Sedivy and Sharp, 1989; Schwartzberg et al., 1990). In this case, the resistance gene is preferentially expressed at the targeted locus while it remains silent at most random integration sites. This results in an apparent increase of the recombination frequency.

Gene targeting has had already a strong impact on many fields of biology, most notably on developmental biology and immunology. It should not be concealed, that the analysis of mutant mice is sometimes not trivial, because the complexity of the mammalian organism. Networks of gene interactions, pluripotency of action and redundant functional mechanisms can obscure the effect of a mutation and prevent the insight into the immediate function of a gene. Nevertheless, the ability to access and mutate virtually every nucleotide in the mouse genome has provided and will provide a valuable tool to carry out a comprehensive genetic analysis of a mammalian organism.

In the first part of this thesis, perforin expression during infection of mice with lymphocytic choriomeningitis virus was investigated. Kinetics and histological localization of expression provided evidence for an involvement of perforin in antiviral cytotoxicity in vivo. In part two, mice with a targeted disruption of the perforin gene were generated by homologous recombination in embryonic stem cells. Perforin deficient mice will allow a direct functional assessment of perforin protein in vivo. Especially, they will help to address the controversial question, whether several independent mechanism of cytotoxicity exist or whether perforin is the sole effector molecule of cytolytic lymphocytes.
5. Perforin Expression during Infection of Mice with Lymphocytic Choriomeningitis Virus

5.1 Detection of Perforin and Granzyme A mRNAs in Infiltrating Cells during Infection of Mice with Lymphocytic Choriomeningitis Virus

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Reprint from European Journal of Immunology (1989), 19: 1253-1259
Summary
The analysis of gene expression in cytotoxic T-cells by in situ hybridization of serial liver and brain sections from mice infected with lymphocytic choriomeningitis virus and immunostaining with T-cell-marker- and virus-specific antibodies revealed a close histological association of infiltrating lymphocytes expressing the perforin and granzyme A gene with virally infected cells. Maximal frequency of perforin and granzyme A mRNA containing cells on liver sections preceded by about two days maximal LCMV-specific cytotoxicity of the lymphoid liver infiltrating cells. These results are most consistent with an involvement of perforin and granzyme A in cell-mediated cytotoxicity in vivo.

Abbreviations: LCMV: Lymphocytic choriomeningitis virus  LU: Lytic unit
NK: Natural killer cell  CTL: Cytotoxic T-lymphocyte
Introduction

Cytotoxic lymphocytes seem to play an important role in immune defense against cell associated antigens. Specific cytotoxic CD8+ T-cells are the main effectors against virus infected cells (Doherty and Zinkernagel, 1974; Blanden, 1974). The same class of effector cells and natural killer cells are involved in anti-tumor immunity (Timonen et al., 1988). Antibody dependent cell mediated cytotoxicity is conferred by a variety of Fc-receptor positive lymphocytes including NK-cells. Their lytic mechanism has been studied extensively during the past 10 years (Möller, 1988).

Analysis of the contents of the large cytoplasmic granules, isolated from cloned cytotoxic T-cells and NK cells grown in vitro, allowed the characterization and intracellular localisation of various candidates for effector molecules in cell mediated cytotoxicity, including perforin (Dennert and Podack, 1983; Young et al., 1986b; Young et al., 1986d; Shinkai et al., 1988; Lowrey et al., 1989; Podack et al., 1988) which is also named cytolysin (Henkart et al., 1984) or pore forming protein (Young et al., 1986c) and several serine proteases (Pasternack and Eisen, 1985; Simon et al., 1986; Masson and Tschopp, 1987; Möller, 1988; Jenne et al., 1988). Immunoelectron-microscopical studies have shown, that perforin and granzyme A are stored in the same electron-dense cytoplasmic granules in cultured cytotoxic T cell clones (Groscurth et al., 1987; Jenne et al., 1988). Purified perforin was shown to be hemolytic and cytolytic for various tumor cell lines in vitro at low concentrations (Young et al., 1986b; Henkart et al., 1984). The role of perforin in cell mediated cytolysis in vivo is controversial (Clark et al., 1988; Ostergaard and Clark, 1987; Berke and Rosen, 1987; Young et al., 1987; Clark, 1988), because so far all attempts have failed to demonstrate perforin directly in primary cytotoxic cells with polyclonal antisera. Perforin was also not found in alloreactive, highly cytotoxic peritoneal exudate lymphocytes by immunohistochemistry and Western blot analysis (Berke and Rosen, 1987). Furthermore, granules of cytotoxic T-cells from primary mixed lymphocyte cultures, from in vivo poly IC induced NK or peritoneal exudate cells failed to exhibit any lytic activity when tested on sheep
erythrocytes (Dennert et al., 1987). However, Northern blot analysis using cloned murine perforin cDNA (Lowrey et al., 1989) as a probe elucidated a strict correlation between the presence of perforin mRNA and cytotoxicity in primary and cloned cytotoxic T-lymphocytes. The levels of the cytolytic activity of the various killer cell types did however not correlate strictly with the levels of perforin mRNA (Podack et al., 1988).

The involvement of serine proteases in cell-mediated cytotoxicity was implied by the blocking of cytotoxicity with protease inhibitors (Chang and Eisen, 1980; Redelman and Hudig, 1980; Acha-Orbea et al., 1983). In the meantime, at least 6 different serine proteases, which may constitute up to 1% of the total cellular protein, have been isolated from cytotoxic T-cells (Pasternack and Eisen, 1985; Masson and Tschopp, 1987; Simon et al., 1986; Young et al., 1986a; Pasternack et al., 1986; Podack and Konigsberg, 1984; Gershenfeld and Weissman, 1986; Cole et al., 1971). Recent comparison suggest that several of those may be identical. As for perforin, the role of serine proteases in cytotoxic activity in vivo is still debated (Dennert et al., 1987; Brunet et al., 1987; Ostergaard et al., 1987).

Infection with lymphocytic choriomeningitis virus (LCMV) in the mouse provides an excellent experimental model to investigate the cellular and molecular events during cell mediated cytolysis in vivo because this model infection is well studied (Lehmann-Grube et al., 1985; Lehmann-Grube, 1971; Zinkernagel et al., 1986; Hotchin, 1971) and because T-cell mediated damage of infected host cells is histologically well localized (Lehmann-Grube and Löhler, 1988). Intracerebral inoculation of LCMV in mice causes fatal choriomeningitis, characterized by a well defined mononuclear infiltrate along the virus infected cells of the meninges and of the chorioid plexus (Lehmann-Grube and Löhler, 1988; Cole et al., 1971). Intravenous injection of the hepatotropic strain LCMV-WE leads to a massive mononuclear infiltration of the liver. An increase of serum levels of liver enzymes like alanine aminotransferase or glutamate dehydrogenase signals an ongoing destruction of hepatocytes (Zinkernagel et al., 1986).
We used antisense RNA probes, which are complementary to naturally occurring mRNA, to assess the expression of the perforin and granzyme A genes in the infiltrating cytotoxic cells during a LCMV-infection of the liver and the brain by in situ RNA-hybridization. This method has already been used successfully to demonstrate the expression of the two serine protease genes granzyme A and B (HF (Gershenfeld and Weissman, 1986) and C11 (Lobe et al., 1986)) in the cytotoxic effector cells during an allograft rejection in vivo (Mueller et al., 1988). We provide direct evidence for activation of the perforin and the granzyme A genes in LCMV specific infiltrates during virus infection.
Material and Methods

Mice
Inbred C57BL/6 were obtained from the Institut für Zuchthygiene, Tierspital Zürich. Mice of either sex were used at an age of 8-16 weeks.

Virus
The LCMV isolate was originally obtained from Prof. F. Lehmann-Grube, Hamburg, Federal Republic of Germany, and was subsequently propagated on L929 cells. LCMV Armstrong was obtained from Dr. M. Buchmeier, Scripps Clinic and Research Foundation, and was propagated on BHK cells. LCMV was titrated in vivo. Mice were infected i.v. with $3 \times 10^5$ pfu of LCMV-WE or i.c. with 300 pfu of LCMV-ARM.

Cytotoxic T-cell assay
Single spleen cell suspensions from C57BL/6 mice were incubated with $10^4$ LCMV infected or uninfected MC57G (H-2b) or YAC-1 (H-2a, a NK-cell susceptible thymoma line) target cells at various ratios in round bottomed 96 well plates (Petra Plastik; Inotech, Wohlen, Switzerland) for 5 hrs at 37°C in air supplemented with 5% CO₂. Specific $^{51}$Cr-release from target cells was calculated by correcting for spontaneous $^{51}$Cr-release. Maximal lysis was determined in wells containing targets treated with 1N HCl without effector cells. Lytic units, a measure of relative cytolytic activity, were determined to be the number of lymphocytes necessary to lyse one third of the standard number of target cells ($10^4$ cells/wells) during the standard test duration. LU per spleen or liver were compared by dividing the total number of lymphocytes per organ by the number of lymphocytes for 1 LU (Cerottini and Brunner, 1974).

Immunohistochemistry
Sections were stained with the monoclonal antibodies 53.6.7 (anti-Lyt-2), GK 1.5 (anti-L3T4) (both hybridomas obtained from the American Type Culture
Collection, Rockville, MD) and VL-4 (anti-LCMV, gift from J. Bänziger, unpublished) as a first stage and alkaline phosphatase-conjugated goat-anti-rat IgG (Sigma), used at a 1:50 dilution in TBS containing 10% normal mouse serum, as a second stage reagent. Naphthol-As-Bi phosphate (Sigma) together with neufuchsin (Fluka) was used as a substrate for alkaline phosphatase. Levamisole was added to the substrate solution to inhibit endogenous phosphatases.

**Preparation of labeled RNA-probes**

A 521 bp cDNA fragment of the 5' end of the murine perforin gene (position 255 - 766) (Lowrey et al., 1989) was subcloned as an Eco RI-HindIII-fragment into the polylinker of the transcription vector pGEM-2 (Promega Biotech. Madison WI). A 750 bp cDNA fragment of the granzyme A gene (HF-gene) (Gershenfeld and Weissman, 1986; Mueller et al., 1988) was subcloned as an EcoRI - SalI fragment into pGEM-2 using standard techniques. After linearization of the plasmids with appropriate restriction enzymes, antisense RNA probes of the granzyme A and perforin gene, and sense probe of the perforin gene were prepared using the T7 RNA polymerase, and SP6 polymerase, respectively. A typical reaction (35 µl) contained 8.4 µl 35S-UTP 800 Ci/mMol (final concentration: 12 µM) (Amersham), 7 µl 5x SP6 buffer (40 mM Tris-HCl pH 7.9, 6mM MgCl₂, 2 mM spermidine); 3.5 µl 100 mM dithiothreitol; 3.5 µl ribonucleotides (CTP, ATP; and GTP, 10 mM each); 3.5 µl BSA 5mg/ml; 1 µl Rnasin 40 U/µl(Boehringer); 1 µl SP6-RNA polymerase 20 U/µl (or T7-RNA polymerase (both from Boehringer, Mannheim, FRG); 1 µl linearized DNA template (1µg/µl); 6.1 µl H₂O. SP6- and T7-reactions were incubated for 90 min at 40°C, and 37°C, respectively. DNA template was digested with Dnase I (Worthington) for 15 min at 37°C. Labeled RNA probes were precipitated twice and resuspended at a concentration of 2x10⁶ cpm/µl in TE, boiled for 2 min and stored at -70°C for up to two weeks. The labeled probe was mixed for hybridization at a concentration of 2 x 10⁵ cpm/µl with deionized formamide (final concentration 50%), dextran sulfate (10%), dithiothreitol
(100 mM), NaCl (300 mM), Tris-HCl, pH 7.5 (20 mM) EDTA pH 8.0 (5 mM), Denhardt's solution (1x).

In situ hybridization

In situ hybridizations of cryostat sections of the brain and liver from LCMV-infected C57Bl/6 mice were done as previously described in detail (Mueller et al., 1988). Hybridized tissue sections were dipped into NTB-2 nuclear track emulsion (Eastman Kodak, Rochester, NY), diluted 1:2 with 600 mM ammonium acetate. Sections hybridized with a 35S-labeled RNA antisense probe of the granzyme A gene were exposed for 15 days at 4°C in a light-tight box. Sections hybridized with 35S-labeled RNA antisense probe of the perforin gene and control slides hybridized with a 35S-labeled RNA-sense probe of the perforin gene were all exposed for 30 days in the dark. Slides were developed with Kodak developer PL-12 for 2.5 min and fixed with Kodak fixer for 5 min at room temperature. Counterstaining was done with nuclear fast red (0.05% in 5% aluminium sulfate).

Evaluation of the sections

A microscope with a grid in one of the two oculars was used for evaluation of the liver sections. Cells located on, or tangential to one line of the grid were considered for evaluation. For the assessment of the frequency of cells containing transcripts of the perforin or the granzyme A gene at least 2000 cells were considered per mouse. Cells were considered positive for gene expression when they showed at least twice as many silver grains as the cells with the highest background on control slides hybridized with a sense probe of the perforin gene. Generally, less than ten grains per cell were found on these control sections. For the assessment of the frequency of CD4+-, CD8+-cells and virally infected hepatocytes, at least 1000 nucleated cells per animal were considered for evaluation.
Results

**LCMV-induced T-cell mediated hepatitis.**

C57BL/6 mice were inoculated with $3 \times 10^5$ pfu of the hepatotropic strain LCMV-WE. Animals were sacrificed at 4, 6, 8, and 10 days after virus inoculation. For the immunohistochemical analysis and in situ-hybridization the livers of 4 animals were snap-frozen in liquid nitrogen at each time point (except on day 10 when only 2 animals were used). For the determination of the cytolytic activity of the splenocytes and the liver infiltrating cells of LCMV-WE infected mice, groups of 8 animals were sacrificed on day 4, 6, 8 and 10, respectively.

The inflammatory infiltrate of the liver increased steadily until day 8 after i.v. infection with LCMV-WE; it had decreased again by day 10, i.e. the end of the observation period (Fig.7). The histologically observed kinetics of infiltration was also reflected by the number of lymphoid cells isolated from the liver of these animals, which peaked on day 8 after inoculation (data not shown). The LCMV-specific cytolytic activity of unseparated splenocytes from infected animals tested on LCMV-infected fibroblasts was maximal on day 8 and dropped sharply thereafter (TABLE 1 and FIG.8). The NK-cell activity of isolated liver-infiltrating cells and unseparated splenocytes determined on YAC-1 target cells was low during the entire observation period and did not show the distinct kinetics observed for the specific cytotoxicity against LCMV-infected target cells (TABLE 1) (McIntyre and Welsh, 1986). The determination of the anti-LCMV-specific cytotoxicity (expressed as lytic units per liver) of the lymphoid cells isolated from the infected liver revealed a similar time course as the measured cytotoxicity of unseparated splenocytes from the same animals (FIG.8). Calculated lytic units were maximal on day 8 and decreased thereafter. The number of liver infiltrating lymphoid cells on day 0 and day 4 after LCMV-infection was too low for a determination of lytic units per liver.
The morphology of the cryostat sections after immunostaining or in situ hybridization did not allow an unambiguous distinction between the infiltrating cells and hepatocytes. Thus, we determined the percentage of cells, positive for T-cell markers, LCMV, or expression of the granzyme A or perforin gene from the total number of nucleated cells on a liver section. The phenotypic analysis of T-lymphocytes in the liver revealed a massive infiltration of mainly CD8\(^+\) cells after more than 4 days after inoculation. Based on the immunostaining of serial sections with an anti-CD4 and an anti-CD8-antibody respectively we found a CD4\(^+\) : CD8\(^+\) ratio of approximately 1.7 : 1 on day 4; 1 : 7 on day 6; 1 : 5 on day 8, and 1 : 3 on day 10 (Fig.9).
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<td>Day 10</td>
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a) C57 Bl/6 mice were infected intravenously with 3x10^5 pfu of LCMV-WE
b) 10^4 51Cr-labeled target cells were incubated at the indicated effector : target ratio for 5 hours at 37°C. Data represent means of duplicates. Spontaneous release values were below 33% of maximal release for uninfected MC57G and LCMV-infected MC57G, and less than 17% for YAC-1 cells.
c) Splenocytes of three animals were pooled and tested in duplicates at each timepoint.
d) Liver-infiltrating lymphocytes were isolated from 8 animals at each timepoint as described in Experimental Procedures.
FIG.7. Histology of the liver at different timepoints after LCMV-infection.

Cryostat sections of the liver from C57 Bl/6 mice on day 0 (uninfected) (A) and day 8 (B) after intravenous infection with $3 \times 10^5$ pfu of LCMV-WE were stained with H&E using standard techniques.
FIG. 8. Virus-infected cells and specific cytotoxicity in the liver after LCMV-infection. LCMV-infected liver cells ( ▲ ) were detected by immunostaining of cryostat sections at different timepoints after intravenous infection with $3 \times 10^5$ pfu of LCMV-WE with the LCMV-specific antibody VL-4. Lytic units per liver ( ■ ), as a relative measure of cytotoxicity were determined as described in Experimental Procedures.
FIG. 9. T lymphocyte subset analysis of cells infiltrating the liver after LCMV infection.
The numbers of CD4\(^+\) (▲) - and CD8\(^+\) (■) cells per thousand nucleated liver
cells were determined by immunostaining with the monoclonal antibodies
H129.19, and 53.6.7, respectively, on cryostat sections of the liver at different
timepoints after intravenous infection of C57Bl/6 mice with 3 x 10\(^5\) pfu of LCMV-WE.
Maximum expression of the perforin gene precedes maximum LCMV-specific cytotoxicity in infected livers.

For the detection and localization of cells expressing the perforin or the granzyme A gene on tissue sections we prepared $^{35}$S-labeled RNA-antisense probes of a 521 bp EcoRI - Hind III -fragment of the perforin gene and a 775 bp EcoRI - SalI fragment of the granzyme A (HF) gene, respectively. Cells expressing the granzyme A gene were found on all liver sections from day 4 on. The frequency of infiltrating cells containing transcripts of the granzyme A gene at a detectable level increased dramatically between day 4 and 6. Thereafter the number decreased again, and on day 10 cells with transcripts of the granzyme A gene were as frequent as they were on day 4 (Fig.10). The expression level of the granzyme A gene (determined as the number of silver grains over a positive cell) was also maximal on day 6, thereafter it decreased to a level on day 10 that was lower than on day 4 (quantitative analysis not shown). Hybridization of the liver sections with the perforin antisense probe revealed a comparable time course for the expression of this gene in the infiltrate of LCMV-infected livers. Cells possessing transcripts of the perforin gene were absent on liver sections from uninfected animals and only very few positive cells were found on day 4. The frequency of perforin positive cells peaked on day 6 and, similar to the results obtained with a probe of the granzyme A gene, declined thereafter (Fig.11). The largest amount of perforin mRNA in the infiltrating cells was also found on day 6 after LCMV-infection, i.e. two days before the number of lytic units against LCMV-infected targets was maximal in livers. The signal of the in situ hybridization with the perforin probe, was at least ten fold weaker than the signal obtained with the granzyme A probe throughout the entire observation period. Some variation in the frequency of granzyme A and perforin expressing cells was seen among the individual animals at each time point. However, in every animal examined 6 days after infection, the frequency of granzyme A and perforin expressing cells was at least twice as high as in any animal examined at day 4 or 8 after infection.
FIG. 10. Granzyme A and perforin expressing cells infiltrating the liver in LCMV-infected mice. Number of cells (per thousand nucleated cells) containing transcripts of the perforin (■) and the granzyme A gene (●) detected by in situ hybridization of liver sections with 35S-labeled RNA antisense probes at different timepoints after intravenous infection of C57Bl/6 mice with 3 x 10⁵ pfu of LCMV. As a negative control, slides were hybridized with a 35S-labeled RNA sense probe of the perforin gene (▲). At least 2000 cells were evaluated at each timepoint.
FIG. 11. Perforin expression by infiltrating cells in the liver of LCMV infected mice. Cryostat sections of the liver at day 0 (uninfected) (A), day 6 (B) and day 10 (C) after intravenous infection of C57Bl/6 mice with $3 \times 10^5$ pfu LCMV-WE were hybridized with a $^{35}$S-labeled RNA antisense probe of the perforin gene as described in Experimental Procedures. As a negative control, a liver cryostat
section of the same animal as in (B) was hybridized with a $^{35}$S-labeled sense probe of the perforin gene.

**Association of virally infected cells with cells expressing the genes for perforin and granzyme A in infected liver and brain tissue.**

For the analysis and correlation of surface markers, LCMV infection and gene expression of cells in LCMV infected livers and brains serial sections of tissue were prepared at different time points after intravenous and intracerebral infection with LCMV. The sections were subsequently used, in the following order, for in situ hybridization with an antisense RNA probe of the granzyme A gene, immunoperoxidase-staining for CD8, in situ hybridization with an antisense RNA-probe of the perforin gene, immunoperoxidase-staining for LCMV, immunoperoxidase-staining for CD4 and in situ hybridization with a sense RNA probe of the perforin gene as a negative control.

C57BL/6 mice, inoculated i.c. with 300 pfu of LCMV-Armstrong showed clinical signs of severe paresis and convulsion on day 6 and died between day 7 and 8 after infection. Histologically, the choriomeningitis was characterized by a well defined infiltration along the meninges, the ependymal cells and the choroid plexus. On days 5 and 6 in some areas up to 80%-95% of ependymal and meningeal cells were infected with LCMV. At the later time points, some animals showed already histological signs of cellular destruction of these LCMV-infected cells. In the brain of infected animals on day 5 the infiltrating T-cells were mainly of the CD8$^+$ phenotype (CD4$^+$:CD8$^+$ = 1 : 1.8), while no infiltrating CD4$^+$ or CD8$^+$ were detected in the brain of uninfected controls. The inflammatory infiltration was much more pronounced on day 6 and the CD4$^+$ : CD8$^+$ ratio further dropped to 1 : 3.2. Cells expressing the granzyme A gene at a low level were found in the infiltrate in small numbers on day 5, whereas infiltrating cells expressing the perforin gene at detectable levels were observed one day later, i.e. approximately one day before the infected animals died on day 7.
Since we were unable to obtain reliable results when we tried to combine immunostaining with monoclonal antibodies with in situ hybridization, we used serial sections of the brain to correlate the site of LCMV-infected cells with the infiltrating CD4+ and CD8+ cells, and also with the site of granzyme A and perforin expressing cells. Fig.12 shows the result of the immunostainings and in situ-hybridization of serial sections from a representative area of the choriomeningitis of an animal 6 days after intracerebral inoculation with LCMV-Armstrong.

CD4+ and CD8+ cells were always closely associated with LCMV-infected cells. The frequency of granzyme A and perforin expressing cells was considerably higher than the number of CD4+ cells in all animals on day 6 after infection and the distribution of the positive cells on sections that were hybridized with RNA-probes of the granzyme A or the perforin gene was very similar to the distribution of CD8+ cells.

Serial sections were also prepared from liver tissue at different time points after intravenous infection with 3 x 10^5 pfu LCMV-WE. In contrast to the choriomeningitis, in LCMV induced hepatitis the virus infected cells were not histologically confined to the mononuclear, perivascular infiltrate, but rather were found also in parenchymal tissue. Perforin, Granzyme A, CD8 and CD4 positive cells were scattered in the same pattern over the entire area of the liver section (Data not shown). The frequency of virus infected cells was maximal on day 6, when also the frequency of perforin mRNA containing cells was maximal in the liver infiltrate (Fig.8).
FIG. 12. (previous page) Immunostaining and in situ-hybridization showing the same histological situation on serial brain sections from LCMV-infected mice. C57Bl/6 mice were inoculated intracerebrally with 300 pfu of LCMV-Armstrong. Serial sections of the brain were prepared 6 days after infection and were used for:

(A) hybridization with $^{35}$S-labeled RNA antisense probe of the granzyme A gene

(B) immunoperoxidase-staining with the anti-CD8 mAb 53.6.7

(C) hybridization with $^{35}$S-labeled antisense probe of the perforin gene

(D) immunoperoxidase-staining with the anti-LCMV mAb VL-4

(E) immunoperoxidase-staining with the anti-CD4 mAb H129.19

(F) hybridization with $^{35}$S-labeled RNA sense probe of the perforin gene as a negative control. (See Experimental Procedure for details.)

(A') Detail from section (A).

(C') Detail from section (C), histological situation corresponds to (A').
Discussion

The present investigation of perforin gene expression is good evidence for an involvement of perforin in cell mediated cytotoxicity in vivo. The close association between perforin (and granzyme A) positive cells and LCMV-infected cells in infected livers and brains is a strong indication of a direct involvement of perforin containing cells in the elimination of virus infected cells.

The frequency and the signal intensity of perforin versus granzyme A mRNA positive cells differed considerably. This was found on all sections and cannot be explained solely by the different size of the cDNA fragments used for the preparation of the probes. Most likely, this difference reflects differing amounts of free mRNA available for hybridization and that there are more T-cells expressing serine proteases than perforin. We cannot exclude that some cells with very few mRNA copies of the perforin gene were below the detection level of our method. Therefore the frequency of perforin mRNA positive cells may be an underestimate.

The expression of the perforin gene was found to be slightly higher in infiltrating cells of the brain than in the liver. This, however, reflects either a difference in the actual copy number of perforin mRNA per cell or a more rapid degradation caused by endogenous RNA degrading enzymes in liver tissue.

The number of infiltrating lymphocytes and the lytic LCMV-specific T-cell activity expressed as LU per liver clearly peaked on day 8 (Fig.8), although the relative cytolytic activity measured on a cell to cell basis was comparable on days 6, 8 and 10 (TABLE 1). The delay between the peak of T-cell cytotoxicity on day 8 and perforin expression peaking on day 6 is most readily explained by a delay of production and storage in granules versus release. Thus after the initial activation of the CTLs and induced transcription of the perforin gene sufficient perforin was synthesized in LCMV-specific CTLs for optimal cytolytic activity probably without any new transcription of the perforin gene during the following 24-48 hours. Perforin is apparently a storage protein as was shown by immunohistochemistry and indirectly by the fact that cytotoxic activity does not require any protein de
novo synthesis (Thorn and Henney, 1988). The contribution of NK-cells to the measurement of $^{51}$Cr-release and the implied elimination of virus infected cells in the liver appeared to be minimal as indicated by the low lysis of NK-sensitive YAC-1 target cells (McIntyre and Welsh, 1986).

A key question is obviously, whether only CD8$^+$ T-cells produce and release perforin. We tried to address this question by a combination of immunostaining against T-cell surface markers and subsequent in situ hybridization. Unfortunately, cytoplasmic mRNA was not retained well enough in tissue sections used first to detect T-cell subclasses by immunohistochemistry. Based on the staining of serial sections with antibodies against T-cell markers and in situ hybridizations (Fig.12), it appears most likely that the perforin and the granzyme A gene are expressed in the CD8 positive subset during an LCMV infection of the liver and brain (Fig.12). This conclusion is supported by the finding, that the CD4:CD8 ratio of the infiltrating T-cells dropped during the LCMV-infection of the liver and the highest frequency of perforin and granzyme A mRNA positive cells was found in both organs at the time of maximal frequency of LCMV infected cells. We cannot formally exclude however the possibility, that a minor fraction of CD4 positive cells or even non-T-cells can express the perforin and/or granzyme A gene during a viral infection in vivo.

The presented evidence for perforin and granzyme A positive cells in a T-cell dependent infiltrate causing LCM differs from results obtained with an acute bacterial meningitis caused by Listeria monocytogenes, when evaluated on day 2 after infection and shortly before death. The cells in the bacterial infiltrate expressed the gene for TNF$\alpha$, but only very few granzyme A and no perforin mRNA containing cells could be detected. (K.Frei, in preparation). This difference may therefore be most readily explained by the absence of T-cell involvement at this early stage of Listeria meningitis.

In conclusion, in situ hybridizations with a radiolabelled RNA probe of the granzyme A and the perforin gene clearly demonstrated the induction of cells with transcripts of either gene in T-cell dependent inflammatory infiltrates of LCMV
infected mice. These positive cells were associated with virus infected cells in infected liver and brain, suggesting that they are involved in antiviral cytotoxicity. This study therefore provides evidence for the induction of the perforin and granzyme A gene during the course of an antiviral immune response in vivo, thus implying a role for these two proteins in cell-mediated cytotoxicity in vivo.

*Acknowledgment*

We wish to thank Dr. I.L. Weissman for providing the HF cDNA clone and T. Périnat and G. Wäłe for expert technical assistance.
5.2 Detection of Perforin During Infection of Mice with Lymphocytic Choriomeningitis Virus by RNA-PCR

Introduction

Transcription of the perforin gene during infection of mice with lymphocytic choriomeningitis virus (LCMV) is readily detected by in situ hybridisation as described in chapter 5. A strong upregulation of perforin transcription was found during the course of the immune response with maximal levels of perforin mRNA in infected organs on day 6 after infection. But detection of perforin mRNA with in situ hybridisation has several drawbacks: First, the method is very slow, especially because the low level of perforin mRNA present in transcribing cells in vivo calls for autoradiographic exposition times of up to 20 days. Secondly, quantification of gene expression by in situ hybridisation is easily biased by the choice of the plane of section and the region of observation. Because of this, a laborious analysis of a statistical number of different sections and fields of observation is required. In order to circumvent some of the problems of in situ hybridisation, the increase of perforin mRNA expression during infection with LCMV was confirmed and complemented by the method of RNA-PCR. The high sensitivity of the polymerase chain reaction (PCR) renders this method suitable to analyze the expression of perforin, a gene expressed at low levels in only a small portion of lymphocytes in certain tissues in vivo. Compared to the histological approach of in situ hybridisation, the method of RNA-PCR gene expression provides an integrated analysis of expression over the complete volume of an organ. In addition, RNA-PCR allows the characterization of mRNA transcribed from the wildtype or alternatively from a mutated perforin allele by applying a set of primer pairs amplifying different regions of the perforin cDNA molecule.
Material and Methods

Infection of mice

Inbred C57BL/6 mice were infected with 200 pfu of LCMV-WE intravenously. Mice were killed by cervical dislocation and spleens removed for RNA preparation.

Isolation of total cellular RNA

RNA was extracted from spleens of mice (Chirgwin et al., 1979). Briefly, cell suspensions were made by forcing the spleens through a steel mesh. 5x10^7 cells were lysed in 6 M Guanidiniumisothicyanate, 0.1 M Tris.HCl pH 7.0, 0.1 M β-Mercaptoethanol, 0.5% sarcosyl. RNA was pelleted in 5.7 M CsCl₂, 0.1 M EDTA, 0.01 M Tris.HCl pH 7.0 by ultracentrifugation for 21 hours at 11500 g. Proteins were removed by Phenol/Chloroform extraction and the RNA precipitated with Ethanol. RNA was dissolved and stored in TE (10 mM Tris.HCl pH 7.5, 0.1 mM EDTA).

First strand cDNA synthesis

100 μg total RNA was digested with 30 U of DNase I in the presence of 30 U RNAse inhibitor from human placenta in 10 mM Tris.HCl pH 7.5, 5 mM MgCl₂ for 60 minutes at 37°C. After phenol/chloroform extraction, precipitation with ethanol and resuspension in TE 2 μg RNA were retrotranscribed (Gubler and Hoffman, 1983) with 15 U reverse transcriptase form Moloney Murine Leukemia Virus in the presence of 30 U RNAse inhibitor, 0.2 μM oligo (dT)₁₅ and 0.5 mM of dNTPs in 50 mM Tris.HCl pH 7.6, 8 mM MgCl₂, 10 mM Dithiothreitol for 2 hours at 37°C. 30 U RNAse T1 was added and the RNA digested for 60 minutes at 37°C followed by heat inactivation for 5 minutes at 70°C.

PCR amplification of cDNA
cDNA synthesized from 0.2 μg of total RNA was amplified (Saiki et al., 1988) by 0.05 U of Super Taq Polymerase (Stehelin & Cie, Basel) in the presence of 0.4 μM of each primer, 200 μM dNTPs in 10 mM Tris.HCl pH 9.0, 500 mM KCl, 0.1 % gelatine, 1.5 mM MgCl₂, 1 % Triton X-100 for 35 temperature cycles. Each cycle was 1 minute at 94°C, 2 minutes at 60°C and 3 minutes at 72°C. Amplification was completed with an 10 minutes incubation at 72°C.

PCR products were analysed on a 1.2% agarose gel by standard techniques.

**Primers used for PCR amplification**

For designing and optimising the PCR primer pairs the Oligo Program, Version 4.0 (Wojcieck Rychlik, National Biosciences, USA) was used.

Perforin specific primer sequences were derived from the published cDNA sequences for perforin (Lowrey et al., 1989; Shinkai et al., 1988) (Genebank accession numbers J04148 and X12760) and for exon 1 from unpublished data (E. Podack, personal communication).

Upper Primer a: 5' - TGG GCT TCA GTG GCG TCT TG - 3'
Lower Primer b: 5' - TGT GTG GCG TCT CTC ATT AG - 3'
Upper Primer c: 5' - AGG CAG CTG CTA ATA TCA AT - 3'
Lower Primer d: 5' - TGT GCT GTT TCT TCT TCT CC - 3'
Upper Primer e: 5' - AGC CCC TGC ACA CAT TAC TG - 3'
Lower Primer f: 5' - CCG GGG ATT GTT ATT GTT CC - 3'
Upper Primer g: 5' - AGA TGG ACT TTG AGA ATG TG - 3'
Lower Primer h: 5' - TTTTTGAGACCCTGTAGACCCA - 3'

The expected fragment lengths were for primer set a/b 472 bp and 366 bp (differential splicing), for primer sets c/d, e/f and g/h 481 bp, 350 bp and 407 bp respectively.
β-Actin specific primer sequences were derived from the published cDNA sequence of cytoplasmatic β-actin (Alonso et al., 1986) (Genebank accession number M12481).

Upper Primer i: 5' - CCA ACC GTG AAA AGA TGA CC - 3'
Lower Primer k: 5' - TCG TTG CCA ATA GTG ATG AC - 3'
A 418 bp DNA fragment was amplified by primer pair i/k from β-actin cDNA.

Results
C57BL/6 mice were infected with 200 pfu of lymphocytic choriomeningitis virus of strain WE i.v. 8 days after infection total cellular RNA was extracted from the spleens of infected and uninfected mice. Polyadenylated mRNA was retrotranscribed to cDNA and was analyzed with three different primer pairs by PCR as shown in Fig. 13. Despite extensive treatment of total cellular RNA with DNAse during preparation, a minor contamination of the RNA preparation with genomic DNA can never be excluded completely. In order to prevent false positive bands due to residual genomic DNA in the RNA preparation, the primer pairs a/b and c/d were designed to span a complete intron from one exon boundary to the next exon boundary. In this configuration, amplification of contaminating genomic DNA yields a fragment of considerably higher molecular weight than the fragment amplified from cDNA. No contaminations with genomic DNA were detected in all PCR reactions (Fig. 14).
A fourth set of primers (i/k) was used to amplify ubiquitous β-actin sequence. The comparable intensities of the bands in lane 1 and lane 2 confirm, that equal amount of cDNA from the different samples were compared.
Amplification of a limiting amount of control plasmid sequence was used to assure the sensitivity of the polymerase chain reaction (Fig. 14, probe 4). The sequence contained in the control plasmid can be amplified with primer sets c/d and e/f, but not with primer sets a/b and i/k. The prominent band amplified from 50 pg of plasmid indicates the high sensitivity of the PCR reaction with primers c/d and e/f.
Despite this high sensitivity, perforin mRNA was not detectable in spleens of uninfected mice (Fig. 14, lane 2, primers a/b, c/d and e/f), while it was detected in splenic RNA from infected mice (Fig. 14, lane 1, primer sets a/b, c/d and e/f). This finding was verified with all three primer pairs specific for different regions of the perforin gene.

Although the exponential amplification process during the polymerase chain reaction impedes accurate quantification, the differences in band intensities between infected and uninfected mice can be estimated to correspond to an at least 10-fold increase of perforin expression during LCMV infection.

Besides the main PCR fragment, the primer set a/b amplified a second fragment of slightly higher molecular weight. This second fragment is readily explained by the reported differential splicing of perforin mRNA between exon 1 and exon 2, resulting in a 106 bp difference between the two alternatively spliced mRNA populations (Youn et al., 1991). This is in accordance with the result of RNA-PCR presented here. However, the proportion of intensities between the two PCR bands on lane 1 can not be taken as a representation of the stochiometric relation between the two mRNA populations. Most likely the longer mRNA species is present in a higher proportion than it is reflected by the band intensities, since the amplification of a longer fragment competes unfavorably with the amplification of a shorter fragment in the same PCR reaction.
Fig. 13. Locations of primers used for the analysis of perforin transcripts. The PCR primers, their relative locations and directions of amplification in the perforin gene are indicated by arrows. Note that primer sets a/b and c/d are located at neighboring ends of exons, spanning intron 1 or intron 2 respectively thus amplifying different fragments from contaminating genomic DNA than from cDNA.
Fig.14. Expression of perforin in noninfected and LCMV infected mice. RNA was extracted from spleens of LCMV infected and uninfected mice, retrotranscribed, amplified by PCR and separated on an agarose gel. 1: Mouse 8 days after infection with 200 pfu of LCMV-WE. 2: Uninfected mouse. 3: No cDNA added, blank. 4: Positive control plasmid for primer sets c/d and e/f. Primer sets a/b, c/d and e/f amplify perforin cDNA, primer pair i/k amplifies β-actin cDNA. Relative positions of primers a-f are shown in Fig.13. The observed fragment lengths corresponded to the expected lengths of 472 bp and 366 bp (differential splicing) for primer set a/b and of 481 bp, 350 bp and 418 bp for primer sets c/d, e/f and i/k respectively.
Discussion

It has been demonstrated that the overall expression of perforin mRNA in spleens of mice is induced by infection with LCMV. Two independent methods were used to confirm this finding. The observed upregulation of perforin expression was consolidated by RNA-PCR in accordance with the results obtained with in situ hybridisation. RNA-PCR circumvented some of the problems associated with the histological restraints of expression analysis by in situ hybridisation. It has been clearly confirmed that the overall amount of perforin mRNA in spleens of mice on day 8 after LCMV infection increased by an estimated factor of at least 10 compared to uninfected mice. The result of the in situ hybridisation, the described pattern of perforin expression in primary spleen cells and the low NK activity observed during LCMV infection lead us to the conclusion that perforin expression in the spleen occurs mainly during activation of resting virus specific T cells. Subsequently, the mature cytolytic effector cells probably infiltrate the infected tissues to eliminate virus infected cells by cytolysis. In addition, the observed expression of perforin in the virus infected tissues of liver and brain argues for a potential of mature activated cytotoxic T cells to replenish their cytolytic granula by resynthesizing perforin in order to lyse several target cells sequentially.

It has been objected against the granule exocytosis model of perforin action that perforin expression would be an in vitro artifact occurring in cells exposed over longer periods of time to high amounts of IL-2. Such high amounts of IL-2 are present in tissue culture media complemented with supernatant from concanavalin A stimulated rat spleen cells (Berke and Rosen, 1988; Clark, 1988), as they are widely used to cultivate T cells in vitro. The demonstration of significant perforin expression during the course of an antiviral immune response in vivo directly disproves these arguments.

A modification of this objection consists in the notion, that perforin expression would be triggered by exposure of cytotoxic T cell precursors, mature cytotoxic T cells and NK-cells to high local concentrations of IL-2 effective in a paracrine way (Clark, 1988). Following this hypothesis, perforin expression would be the
hallmark of some kind of unspecific cytotoxic activity exerted by bystander lymphocytes, similar to the unspecific activation of B-cells by certain bacterial cell wall products. The detection of perforin expression during LCMV infection with low levels of unspecific cytotoxic activity on uninfected fibroblasts and NK sensitive YAC-1 cells compared to the prominent LCMV specific activity are difficult to reconcile with this model. If indeed there had been an upregulation of this stipulated unspecific, perforin dependent cytotoxicity it was not measurable by our assays and in this case was much weaker than the specific part of the response. Although this does not formally disprove the hypothesis, it renders it very improbable.

It would be interesting to know, which lymphocyte subsets express perforin during LCMV infection in vivo. In chapter 5, evidence for expression mainly in CD8 positive T cells was obtained by in situ hybridisation and immunohistology on serial sections. To directly identify the perforin expressing lymphocyte subsets an alternative method is suggested here. It should be feasible to sort spleen cells by a fluorescence activated cell sorter (FACS) into different lymphocyte subsets characterized by the presence of markers like CD8, CD4 and asialo GM1 and test for perforin expression in the sorted cell populations by RNA-PCR. The technical problems associated with this procedure would probably be more easily surmountable than with the double staining procedure suggested above. Due to its high sensitivity, this approach appears also more promising than earlier experiments testing the cytolytic activity of granule preparation from different lymphocyte subsets on sheep red blood cells (Berke and Rosen, 1987; Garcia-Sanz et al., 1987).

The demonstration of perforin expression during infection with a virus mainly controlled by cytotoxic T cells strongly suggests an involvement of perforin in the mechanism of cell lysis by cytotoxic T cells and natural killer cells in vivo. However the possibility, that perforin expression in these physiological situations is rather an epiphenomenon than the effective principle at work, can not be ruled out completely. In order to gain more information about the physiological role of
perforin, the ability to directly interfere with the activity of perforin in vivo would allow a more direct assessment of the role of perforin in cytolysis by lymphocytes.
6. Generation of Mice with a Disruption of the Perforin Gene by 
Homologous Recombination in Embryonic Stem Cells

Introduction

Cytotoxic T-cells and Natural Killer cells are both capable of rapidly lysing target 
cells and are involved in lysis of virus-infected cells, allograft rejection and tumor 
surveillance. The killing process requires a one to one cell to cell contact (Marker 
and Volkert, 1973; Martz, 1976; Berke, 1983) conferred by the specific T-cell 
receptor in the case of cytotoxic T-cells and a yet hypothetical receptor for NK 
cells. In addition, several accessory adhesion molecules participate in the 
interaction between cytotoxic and target cell to increase the affinity of the 
interaction and/or to provide additional stimulatory signal to the lymphocyte. 
Eversince cytolytic activity of lymphocytes has first been described (Cerrotini et al., 
1970; Herberman et al., 1975), the mechanism of lysis has been the subject of great 
interest. Early attempts to generate inhibiting antibodies against surface 
determinants expressed only on conjugates between cytotoxic T-cells and target 
cells and not on cytotoxic cells per se did not reveal the presumed cytolytic effector 
molecules but rather accessory binding proteins (Pierres et al., 1982). The cloning 
of cytolytic lymphocytes and the identification of the content of their cytolytic 
granules have brought some although still limited understanding of the process. 
Several candidate effector molecules, partly isolated from these granules, and 
killing mechanisms have been suggested, among them perforin (also referred to as 
cytolysin, pore forming protein, PFP and P1), lymphotoxin and other TNF 
homologs (Granger and Kolb, 1968; Ruddle and Waksman, 1968; Liu et al., 1987), 
granzymes (Pasternack and Eisen, 1985; Masson and Tschopp, 1987; Masson et al., 
1986; Simon et al., 1986), secreted ATP (Di Virgilio et al., 1990) and the apoptosis 
model of cytotoxicity.

The best characterized of these candidate mechanism is the granula exocytosis 
model (Hendrik, 1985; Podack, 1985; Lachmann, 1983), which proposes, that
cytotoxic T-cells upon recognition of the target cell release the contents of their cytolytic granules. These granules contain the effector molecule perforin. The pore formed in the target cell membrane by the polymerizing perforin molecules renders the membrane permeable and leads to osmotic lysis of the target cell. Indeed electronmicroscopical studies have demonstrated ringlike pore structures on the membranes of lysed cells deposited by cytotoxic T-cells and NK-cells: These structures resemble those found during complement mediated lysis (Dourmashkin et al., 1980; Dennert and Podack, 1983). Subsequently, the cytolytic activity was shown to reside in the granules of cytolytic lymphocytes and from these granules the 70 kD protein perforin was isolated. The membranolytic perforin molecule is capable of polymerizing into pore like structures as they are observed on the surface of cells lysed by cytolytic lymphocytes or granule preparations. The functional analogy between complement C9 and perforin appears in the primary structures of these poreforming proteins as well, since the central third of the amino acid sequence for perforin (Shinkai et al., 1988; Young et al., 1986a) displays homology to C9. The carboxy- and aminoterminus, where a membranolytic peptide was localized (Ojcius et al., 1991), are specific to perforin.

The perforin genomic locus is contained in three exons spread over a short stretch of 10 kb of the mouse genome. Since translation starts in exon 2 and ends in exon 3, exon 1 remains untranslated. The region homologous to complement component C9 and thought to be functionally essential is distributed over the 3'-half of exon 2 and the 5'-half of exon 3.

Perforin expression was demonstrated in tissue cultured CD8 positive cytotoxic T-cells (Shinkai et al., 1988; Podack et al., 1988; Garcia-Sanz et al., 1987) and in NK cells (Kawasaki et al., 1992), but generally not in CD4 positive T-cells (Garcia-Sanz et al., 1987) although recently evidence for expression of perforin in CD4 positive T-cells isolated from patients with rheumatoid arthritis (Griffiths et al., 1992) and other autoimmune diseases has been reported. Intracellularly, perforin was localized to the granula of cytotoxic lymphocytes by immunoelectronmicroscopy (Groscurth et al., 1987; Peters et al., 1989). Perforin
expression in vivo is associated with the kinetics and histological localization of lymphocytic choriomeningitis virus specific, murine cytotoxic T-cells (Müller et al., 1989), with rejection of allogeneic transplants in mouse and man (Mueller et al., 1988; Young et al., 1990), with spontaneous diabetes in NOD mice (Young et al., 1989) and with cytolytic lymphocytes isolated from the synovial fluids of patients with rheumatoid arthritis (Griffiths et al., 1992). In addition, the expression of perforin was recently demonstrated in the reproductive tract of female mice, not only in infiltrating cells of the regressing corpus luteum but also in granular metrial gland cells of the placenta (Zheng et al., 1991). Because of the relation of granular metrial cells to NK cells, it was suggested that they prevent embryo derived cells to pass to the mother or alternatively, that they hinder virus infected maternal cells to pass to the embryo.

Evidence for an essential role of perforin at least for in vitro generated cytotoxicity was provided by the finding, that perforin expression and CD3 specific mediated cytotoxicity of primary mouse spleen cells could be significantly inhibited with perforin antisense oligonucleotides. (Acha-Orbea et al., 1990).

Despite this considerable evidence, the role of perforin in cell mediated immunity has remained controversial. In order to shed some light on the role of perforin in vivo, we applied the method of homologous recombination in ES cells (Evans and Kaufman, 1981; Bradley et al., 1984; Thomas and Capecchi, 1987; Hooper et al., 1987) to generate perforin deficient mice. Such mice would provide an in vivo system to clarify the question whether perforin is indispensable or only redundantly together with one or several additional mechanisms involved in cytolysis by lymphocytes.
Material and Methods

DNA sequencing

Perforin intron 2 was sequenced from the double stranded plasmid pTZ19R.GC with a perforin exon2 primer. (5' - CCT ATC AGG ACC AGT ACA ACT - 3') by Taq cycle sequencing (Taq Dye Deoxy™ Terminator Cycle Sequencing Kit, Applied Biosystems Inc., Foster City, CA). The reaction products were separated on one lane of a 6% acrylamide gel and analysed by an Applied Biosystems 373A DNA sequencer.

Synthesis of DNA oligonucleotides

For designing and optimising the PCR primer pairs the Oligo Program, Version 4.0 (Wojcieck Rychlik, National Biosciences, USA) was used. Perforin specific primer sequences were derived from the published cDNA sequences of perforin (Lowrey et al., 1989; Shinkai et al., 1988) (Genebank accession numbers J04148 and X12760) and for exon 1 from unpublished data (E. Podack, personal communication).

Primer a (Fig.20): 5'- TTT TTG AGA CCC TCT AGA CCC A -3'
b (Fig.20): 5'- GCA TCG CCT TCT ATC GCC TTC T -3'
c (Fig.18): 5'- GTA GAG TTG AGG CTG ATG CC -3'
d (Fig.18): 5'- ATT CGC CAA TGA CAA GAC GCT G -3'
e (Fig.18): 5'- CTG ACC GCT TCC TCG TGC TTT A -3'
f (Fig.18): 5'- GAG AGA TCA GGA TTT GGT GT -3'
g: 5'- GCC ACT CGG TCA GAA TGC AAG C -3'
h: 5'- AGG GTC ACA GCA TTA GGA AG -3'
i: 5'- CCG GTC CTG AAC TCC TGG CCA C -3'
j: 5'- CCC CTG CAC ACA TTA CTG GAA G -3'

Primers were synthesized by the phosphoramidite method on a solid support (Matteucci and Caruthers, 1981). The synthesis was performed automatically on an
Applied Biosystems Model 391 DNA synthesizer. After removal of the protecting groups, the neutralised primer solutions were precipitated with ethanol and purified by gel filtration on a Sephadex G50 column (Pharmacia, Uppsala, Sweden). The oligonucleotides were quantitated by optical density at 260 nm wavelength.

**Plasmids**

The plasmid pMCI.neoPA containing the neo gene with a point-mutation presumably decreasing its expression (Yenofsky et al., 1990) was a gift from Silvio Hemmi, Institut für Molekularbiologie I, Universität Zürich.

The plasmid pIC19R.MCI-TK containing the herpes simplex virus thymidine kinase gene under the control of a promoter optimised for expression in embryonic stem cells was obtained from Kurt Bürki, Sandoz AG, Basel.

**Hybridisation probes for genomic Southern blotting**

The locations of the probes is indicated in Fig.18 and Fig.20.

Probe A was a 620 bp PCR fragment amplified from perforin exon 2 with primer set g/h (see above). Probe B was a 600 bp restriction fragment prepared from plasmid pMCI.neoPA containing 3'-coding sequence for the neomycin-phosphotransferase. Probe C was a 298 bp PCR fragment amplified from perforin exon 3 with primer set i/k.

**Mice**

Mice were obtained from BRL (Füllinsdorf, Switzerland).

**Preparation of embryonal fibroblast layers**

To prepare G418 resistant feeder cells, CD1-MTKneo2 foeti transgenic for the neomycin-resistance gene were used (Stewart et al., 1987). About 30 13-14 days old foeti with heads and livers removed were washed with CMF-PBS, homogenized by pressing through a 18-gauge injection needle and trypsinized for one hour at 37°C
in 10 ml Trypsin/EDTA solution (Gibco/BRL, Paisley, Scotland). The cells in the supernatant were pelleted, taken up in feeder cell medium (DMEM complemented with 0.1 mM β-mercaptoethanol and 10% FCS and transferred to a 25 ml cell culture flask. Trypsinization of the carcasses was repeated four times for 30 min as above. Each time, the fibroblasts pelleted from the supernatant were transferred to a new flask. The embryonal fibroblasts were grown to confluent layers, passaged once and frozen. Thawed fibroblasts were expanded for not more than seven passages.

For the production of feeder layers, the fibroblasts were either treated for 150 min. with a 10 μg/ml mitomycin C solution or alternatively irradiated with a dose of 3000 rads from a 60Co source and seeded at 5 x 104 cells/cm2 onto gelatinized cell culture dishes.

In vitro culture of ES cells

The male D3 ES cell line was originally derived from an embryo of the 129/Sv strain (Gossler et al., 1986) and was provided by Dr. Marco Schilham (Toronto, Canada). D3H and D3M are sublines from the same line and obtained from Dr. Michel Aulet (Zürich, Switzerland) and Dr. Manfred Kopf (Freiburg i.Br., Germany) respectively. The male ES cell line BL/6-III was established from an embryo of the C57BL/6 mouse strain (Ledermann and Bürki, 1991). Its pluripotency was confirmed by the generation of germline chimeras from blastocysts injected with BL/6-III cells.

ES cells were passaged every second day by trypsinization with a Trypsin/EDTA solution (Gibco/BRL, Paisley, Scotland). Medium (DMEM complemented with 15% FCS, 0.1 mM β-mercaptoethanol and 1000 U/ml human Leukaemia Inhibiting Factor (LIF, Sandoz AG, Basel, Switzerland)) was changed daily to prevent differentiation. ES cells were grown routinely on growth arrested G418 resistant embryonic feeder cells in an incubator gassed with 10% CO2 at 37°C.
Transfection and selection of ES cells

The ES cells were split the day before electroporation. For electroporations, $5 \times 10^6$ ES cells were taken up in 800 μl ES cell medium without purification from feeder cells, electroporated in the presence of 15 μg of linearized plasmid DNA with a Bio-Rad Gene Pulser equipped with a capacitance extender set at 500 μF and 240 V. The cells were then immediately plated on two fibroblast coated 10 cm dishes and were allowed to recover for two days. G418 (Geneticin, Gibco/BRL, Paisley, Scotland) selection was performed for 10 days at 400 μg/ml. Some plates were additionally selected from day 7 of the G418 selection with 2 μM gancyclovir (Cymevene, Syntex Pharm AG, Allschwil, Switzerland).

Screening of ES colonies for homologous recombination

When colonies were grown to about 50 cells on day 10 or 11 of G418 selection, the medium was replaced with PBS and single colonies were removed by scraping with a 10 μl microtip. After trypsinisation in a drop of Trypsin/EDTA solution half of each colony was transferred onto a fibroblast layer in a 96 well plate and the other half was transferred to an Eppendorf tube. While one half of the colony grew to a confluent clone in the 96 well plate, the other half was screened for homologous recombination by PCR amplification. In detail, the cells were washed with PBS, hypotonically lysed with 30 μl of water, boiled for 10 min. at 95°C and digested for 90 min. at 55°C with 1μg of Proteinase K (Sigma Chemicals, St. Louis, USA). The proteinase was subsequently inactivated by boiling for 10 min at 95°C. 20 μl of this crude extract was then amplified in a 50 μl PCR reaction (Saiki et al., 1988) with 0.05 U of Super Taq polymerase (Stehelin & Cie, Basel) in the presence of 0.4 μM primers each, 200 μM dNTPs in 10 mM Tris.HCl pH 9.0, 500 mM KCl, 0.1 % gelatin, 1.5 mM MgCl$_2$, 1 % Triton X-100 for 35 temperature cycles. Each cycle was 1 minute at 94°C, 2 minutes at 60°C and 3 minutes at 72°C. Amplification was completed with 10 minutes incubation at 72°C. For cells electroporated with construct pICI.PHR the primer sets c/d or e/f were used, for construct c pICI.HK1
and pICL.HK2 the primer set a/b detected homologous recombination and for constructs pICL.HK3 and pICL.HK4 primer set b/d was used.

the PCR products were separated on a 1.2 % agarose gel, which was blotted with a vacuum blotter (Pharmacia, Uppsala, Sweden) to a Genescreen positively charged nylon membrane (NEN, Boston, MA). The membrane was prehybridized for at least 5 hours in a rotating glass cylinder at 65°C with 10 ml of 5x SSC, 10x Denhardt’s solution, 10% dextranesulfat, 0.1% SDS an 100 μg/ml denatured salmon sperm DNA. A DNA fragment identical to the expected PCR product was amplified before from a construction intermediate and used as a probe. The respective PCR fragment was radioactively labelled with 32P by random priming (Oligo labelling kit, Pharmacia, Uppsala, Sweden) and separated from unincorporated nucleotides by gel filtration over a Sephadex G50 column. 107 cpm of the labelled probe were added to the prehybridisation mix and allowed to hybridise for at least 10 hrs at 65°C. The filter was then washed stringently with 0.2x SSC, 0,1% SDS at 65°C, exposed to a phosphorimager screen for 4 hrs and analysed with the Digiscan system (Siemens AG, Munich, Germany). PCR positive clones were grown up, frozen and analysed by genomic Southern blotting. Ten micrograms of genomic DNA were digested with 50U of EcoRI, separated on a 0.8% agarose gel, blotted and hybridised as described above. The filters were first hybridized with a probe flanking to the construct, stripped and rehybridized with a probe internal to the targeting construct , stripped again and finally hybridized with a third probe derived from the neoresistance gene. Only when PCR and Southern hybridisation with all three probes showed the predicted result, a clone was regarded as homologously recombined.

*Generation of germline chimeras from targeted ES cell clones*

All blastocyst injections were carried out by Drs. B. Ledermann and K. Bürki at Sandoz AG, Basel.

Donor females of strain BALB/c were induced to ovulate by an intraperitoneal injection of 5 I.U. pregnant mare serum (PMS, Folligon, Intervet), followed 46 hr
later by an intraperitoneal injection of 5 I.U. human choriogonadotropin (Chorulon, Intervet). Morulae were flushed from the uterotubal junction 3 days after mating and cultured overnight in standard egg culture medium (Biggers et al., 1971). The ES cells were purified from embryonal fibroblasts by incubation for 1 hr in a non gelatinized tissue culture dish. Blastocysts were transferred together with suspended ES cells into drops of injection medium (DMEM, 10 mM Hepes pH 7.4, 10 mg/ml BSA, 300 U/ml DNAse I (Sigma)). About 10 to 20 ES cells were injected into each blastocyst (Hoguet al., 1986). After a recovery period of about 2 hours, injected blastocysts were transferred into both uterine horns of pseudopregnant CD-1 foster mothers. Chimeric offsprings were identified by eye and coat pigmentation. Chimeric males were set up to breed with C57BL/6 females at the age of 6 weeks and the black coat color of the offsprings indicated germ line chimerism.

Results

**Sequencing of the perforin genomic locus**

In order to design the targeting constructs, the cloning strategies and the primers to screen for homologous recombination it was necessary to compile the available information about the sequence of the genomic perforin locus and to complement it with sequence from intron 2. Intron 2 has not been sequenced so far, however sequence information going beyond the end of the targeting vector pICLPHR (Fig.15) was required to design an improved primer pair for screening electroporated ES cells. The sequences have been compiled from [Eckhard Podack, @personal communication, 2051, 5266, 6463]. There are considerable discrepancies in the published perforin sequences, especially in the first two exons, but the sequence presented here (Tab.2) is in agreement with all the PCR reactions and Southern blotting analyses done during this work and can be regarded as a bona fide consensus sequence.
1 GGGTGGATGTGACCATGTGGCCTGGGGTCTGTGGCTACTTATTCCCATC 50
51 ATGTACTCACATTAGGGGTGAGAACATACCACCCACCTGCGATGGTTTTA 100
101 CCCACCTCAAAAGCACAATTTTTTTTTTTTTTTTTAGCCCCAGATTGTATC 150
151 CATTGATCCTTGGCCATGGAAACCCTCCCACAGTAACTCAGGACAGAC 200
201 AGAGTGGCCACCTGTCTTTCCACATGACTAGCTGCTCAACACTGAGGTT 250
251 CAGCTGGGATGTGACCATGTGGCCTGGGGTCTGTGGCTACTTATTCCCATC 300
301 CTCCCCACTCCCCAGGGGGAGGGAACAGTAGCAGATGGATGTA 350
351 AAGATCGTGCTTGGTCACTCCAGACTGCACCCAGTCCTCAGGGGGAAC 400
401 ATGGGACACTGTGAGTACTCCACACTTTTTGGGAATGAGGCTGAGATGGAA 450
451 GGGTCTGAAACCCCTTGGCCAGGCTACATTCCAGGCAAGCCACTTTCCAGCA 500
501 ACTCTCTCTGACGGCGATCCCCATCCCAACACCTCAAATCACAGGAACAGG 550
551 AGGACTGCTGTTGGCCCTGTGCTACCCAGACGGCTTCAGGCCCACACGC 600
601 TGCTCTGCTTTGGCACAGTGACAGCGATCCTGAGGACTCCAGCCTCAGG 650
651 GCTGTCACAGCGCGTAGGAGGTGACAGGATGACAGTTCGGTGCTGGA 700
701 ACCTGTAGCAACTCTTGAGGGGCGAGGAGGAGATGTAATGATGATGACG 750
751 TTGGCCAGGATGGGGCTGTTGGCACAGATGATGGACATCGCAGCATTTTAAAGCCT 800
801 CCACTGCAACAGGCGGAGGAGCCAGATCGTGCATTGGGTGATGACATCCCT 850
851 CTCAGGTAAGGGAGGAGATGAAAGTGGCAAGAGGAAAGGAGGCTGATGACCTGCTGTGA 900
901 GGGGAAAGGAGGAGGAGAAGGGTAGGAAGCAGGGAGGAGTTGAGATTCTC 950
951 GCCAGCTTCTTAACTGCTACCTCTTCTCTTTCTTTGATGGTGGTGCAGAATCTC 1000
1001 GGGGAGTTCATGTTGTACAGTGGGACAGTCTTTCAAGATCTATAATTTCC 1050
1051 AGAAGTTTGGCAGTTAGAGTGGGAATGAGAAATGGAATGAGCAGCAGATATG 1100
1101 GACCACTATATAGCAGTCACCATCCCTTGGGAACCGGCTAACCCCCACTCTCA 1150
1151 AAGGATAGGAAGGGGAGGTGAGGGGAGGAGGACAGGAGGAGGAGGAAAG 1200
1201 GAGGGGGGAGGAAAGAGGAGAAGATCCAGATCTGTAATTAACCTCCCT 1250
Exon 2

2501  GATGAGGAGTGGATGGCTCAGTGGGTGGAACACTTGCCTACAGTGGCTG
2550
2551  AAGACTCTGGGGGATGAAGGATATATCCCTTCATCCCAGAACCGGGGAGG
2600
2601  GGAATCAGGTAGGAAAAGTGTCCCACTCTGGGTGGAAAATGACACATCTC
2650
2651  CAGAGCCGAACCTTTAACAGATGCTGTCTGCGGTCACAGCCCTCAAT
2700
2701  CTCGATTCCTGCTAGGTCTGAGATAATAAAATCTCAGCAGGTTTCTCTC
2750
2751  TTGGCTGTGCGACTCTGAGCTATATGTTTAAATCCCrCTGGATTTCTCTG
Exon 2

2801  TACAGTGGACGAGGCAAGCCAGTCCCAGTCTGGCATGAATACCTAACTAAGAAGT
2850
2851  TACACTCTCTCTGATGTTCCCCAGTCGTGAGAGGTCAGCATCCTTCATCC
2900
2901  CTGACCGCCACTGAGGAAACCTATTACAGATGCTGTGTCCAGGGTCACAGCT
2950
2951  CTCGATTCCTGCTAGGTCTGAGATAATAAAATCTCAGCAGGTTTCTCTC
3000
3001  TTGGCTGTGCGACTCTGAGCTATATGTTTAAATCCCrCTGGATTTCTCTG

2801  TACAGTGGACGAGGCAAGCCAGTCCCAGTCTGGCATGAATACCTAACTAAGAAGT
3050
3051  AAGTTCTCCAGGATGTATGGATGGGTGGGAAAGGATATGAGTTGCTG
3100
3101  CTCGACCGCCACTGAGGAAACCTATTACAGATGCTGTGTCCAGGGTCACAGCT
3150
3151  CTCGACCGCCACTGAGGAAACCTATTACAGATGCTGTGTCCAGGGTCACAGCT
3200
3201  CAGCCGCTACCTGTGGGAATCACCCACTGCGCCGCTGACAGTCTACAGCT
3250
3251  CCAGCGTATAATGGGGGAGGGAAGGTCATGGGGGATTCCTGC

Sac I

3301  BGCTGGGAATAGTGGGCTGAATTTCATCTTCAAGGACTGATCTTGCCAGG
3350
3351  AACCTTAGGACCAGAACCACCTCGGCTGCTGGGTCCTTCCACTC
3400
3401  CAAGGGCCACTGAGGAAACCTATTACAGATGCTGTGTCCAGGGTCACAGCT
3450
3451  TTAAATGGGAGACACATGGAGGTGCATGCTGACAGTCTACAGCTACAGCT

Exon 2

2801  TACAGTGGACGAGGCAAGCCAGTCCCAGTCTGGCATGAATACCTAACTAAGAAGT
3500
3501  ATGGGAGAAAGGAAACTGGAAGAATATGGGCAAACTCTAAGGATTGTTAGGA
3550
3551  GCCCTGGGAATAGTGGGCTGAATTTCATCTTCCACAGAAGACACACC
3600
3601  AAATCTCTGATCCTCTTGAATGGCTGCTGTGTCCACTTCTGAATGCTG
3650
3651  ACCCTCCTTAAACAGTTCTTCTGAATGGCTGCTGTGTCCACTTCTGAATGCTG
3700
3701  GTTGCTACTTTGAATCTTAAATGGGCTGCTACTGATGATGACCAATATGTA
Tab.2. (Previous pages) Incomplete genomic nucleotide sequence of the murine perforin locus. The sequence has been compiled from [Eckhard Podack, personal communication, 2051, 5266, 6463]. The partial sequence data from intron 2 has not been published before. Exon 1, exon 2 and exon 3 are underlined. Start and stop indicate the first and last translated codons.

Construction of the targeting vectors

Homologous recombination of the replacement type occurs when vectors with homologous sequence of at least 1 kb are introduced into embryonic stem cells. Exploiting the fact that interruption of the homologous sequence with small stretches of non-homologous sequence does not preclude homologous recombination a neomycin resistance gene can be introduced into a coding exon. The resistance gene not only serves as a positive selection marker but also contains multiple translation stop codons in all three reading frames leading to inactivation of the targeted allele.

Five different targeting constructs suitable for homologous recombination at the perforin locus were produced. For all constructs, the vector pMCLneoPA (Lowrey et al., 1989; Trapani et al., 1989; Youn et al., 1991) provided the neoR cassette. A point mutation of the neomycin resistance gene occurring in some of the circulating plasmids pMCLneoPA has been reported to decrease the G418 resistance conferred by the neomycin resistance cassette (Thomas and Capecchi, 1987). Retrospectively, the mutation in the coding sequence of the phosphotransferase was detected by a restriction digest in the neo cassette of all constructs.

Homologous recombination with the first construct pICLPHR would result in disruption of the perforin gene in exon 2. To construct pICLPHR (Fig.15 and Fig.16), a 1.2 SalI/XhoI fragment from pMCL.neoPA was cloned into the vector pTZ19R.GC, that contains a genomic DNA fragment of perforin (kindly provided by E.Podack, Miami). This construction intermediate pTZ19R.mP1neoPA was linearized with SmaI in exon 2 and the SmaI ends were converted to XhoI.
compatible ends by ligation with an XhoI linker and digestion with XhoI. The mutated perforin SacI/SacI fragment was subsequently subcloned into pICl19R.MCI-TK (Yenofsky et al., 1990) containing the HSV-TK gene under the control of the HSV-TK promoter engineered for optimal expression in ES cells. The electroporation construct pIClPHR was linearized with ScaI before use in electroporation experiments.

Since no homologous recombinant ES cells were recovered from electroporation with linearized plasmid pIClPHR, the four related constructs pIClHK1, pIClHK2, pIClHK3 and pIClHK4 (Fig.17) were established subsequently. These constructs lead to disruption of the perforin gene in exon 3. In order to clone the targeting constructs pIClHK1 -HK4 (Fig.15) the vector pTZ19R.GC containing a genomic fragment from the perforin gene was opened at the BstE2 restriction site in exon3. By a ligation of blunt ends an Sall/XhoI fragment containing the neo cassette from pMCI.neoPA was inserted into exon3. Parallel and antiparallel orientation of the neo cassette gave rise to the two construction intermediates pTZ19R.VKp and pTZ19R.VKap. The EcoRV/EcoRV fragments from the intermediates were then subcloned each into pICl19R.MCI-TK. Different orientations of the EcoR5 fragments to the HSV-TK gene of the vector yielded the four electroporation constructs pIClHK1, pIClHK2, pIClHK3 and pIClHK4.
Fig. 15. Structure of the exon 2 derived construct pICLPHR. The construct used to electroporate ES cells is contained in the plasmid pICLPHR. The perforin homologous sequence is a SacI/SacI fragment from intron 1, exon 2 and intron 2 of the genomic perforin locus. The perforin introns are indicated by a thicker line. The interruption with the neo cassette at the BglI site of exon 3 introduces several stop codons in all three reading frames. The structural neo gene from Tn5 (Mansour et al., 1988) is driven by a promoter optimized for expression in ES cells (beck et al., 1982). In detail, it consists of a tandem repeat of the enhancer region from the polyoma mutant PYF441, bases 5210 - 5274 (Thomas and Capecchi, 1987), the HSV-TK promoter from bases 92 - 218 (Fujimura et al., 1992) and a synthetic translation initiation sequence. The HSV-TK structural gene included in the construct (McKnight, 1992) is placed behind the same promoter. The construct was linearized in the bacterial plasmid sequence with ScaI before electroporation.
Fig. 16. Cloning strategy for targeting construct pICL.PHR. pICL.PHR was cloned in two steps. (a) First, the neo cassette from pMC1.neoPA was inserted as a SalI/XhoI fragment into pTZ19R.GC. (b) The SacI fragment carrying perforin sequence and the neo cassette was isolated, the ends were converted to HindIII compatible ends and ligated into the HindIII site of pICl19R.MC1-TK to yield the targeting construct pICL.PHR.
The intermediates pTZ19R.VKp and pTZ19R.VKap were constructed by insertion of the XhoI/Sall neo cassette from pMCL.neoPA into pTZ19R.GC. Insertion of the cassette in a parallel fashion in respect to the transcriptional directions of the genomic perforin sequence yielded pTZ19R.VKp. pTZ19R.VKap carries the neo cassette in the antiparallel direction.

Ligation of an EcoRV/EcoRV fragment from pTZ19R.VKp or VKap respectively into the blunt ended Sall site of pICI19.MCI-TK in both possible directions gave rise to the four electroporation constructs pICI.HK1, pICI.HK2, pICI.HK3 and pICI.HK4. Note that all four constructs differ in the relative orientation of perforin genomic sequence, neo cassette and HSV-TK gene.

The constructs were linearized with Cla1 before electroporation.

Transfection of ES cells with targeting constructs and screening for homologous recombination at the perforin locus

The targeting constructs described above were introduce into embryonic stem cells by electroporation. After growing the electroporated cells under selecting conditions, the clonal colonies were screened for homologous recombination by PCR and positive colonies were expanded for further characterization.

A constant number of ES cells were electroporated with 15 μg of linearized, exon 2 derived targeting construct pICI.PHR and grown in the presence of G418. Some experiments were performed with G418 and gancyclovir double selection hoping to enrich for homologous recombinants (Thomas and Capecchi, 1987). To provide a negative control, cells that were electroporated in the absence of plasmid DNA were exposed concomitantly to the same selecting conditions. In all control
experiments no G418 resistant colonies were observed confirming the stringency of the selection conditions.

Usually, the number of G418 resistant colonies recovered from 5 x 10^6 electroporated ES cells varied to a considerable degree with the ES cell line, the batch of ES cells, the number of in vitro passages they have gone through and their state of proliferation.

In order to establish the conditions for the PCR screening procedure, ES cells were electroporated with the construction intermediate pTZ.mP1neoPA (Fig.16). Random integration of this control construct can be used to simulate the screening procedure for homologous recombination, since it results in the same PCR fragment when tested with the screening primer set c/d (Fig.18). The suitability of the primer screening method was confirmed by the detection of the 1.2 kb fragment amplified from as little as 10 lysed intermediate electroporated ES cells in the presence of genomic DNA from 10000 cells. A fraction of the electroporation experiments were screened with the improved primer set e/f, which yielded the same sensitivity but worked more reliably because of the smaller fragment amplified by this primer pair.

Results of the electroporation of D3H, D3 and BL/6-III ES cells with pICLPHR are shown in Tab.3. The electroporation of D3H cells yielded 190 neo resistant ES cell colonies per 5 x 10^6 cells used for electroporation. The corresponding frequencies were for D3 ES cells 134 and for BL/6-III cells 124 colonies per 5x10^6 electroporated ES cells in average. The BL/6-III cells compared to cells of D3 origin consistently yielded 25-50% less G418 resistant colonies. This fact most probably reflects their slower proliferation rate and lower plating efficiency in in vitro culture.

After electroporation with construct pICLPHR over 1176 colonies (not all data shown) were screened for homologous recombination by PCR and subsequent Southern blotting. The theoretical crossovers for targeting of the perforin locus, the diagnostic Southern blotting fragments and the position of PCR primers employed are shown in Fig.18. No homologous recombinants could be recovered
from these experiments. Since there was a possibility, that the frequency of homologous recombination with this construct was too low to find a targeted clone in a screenable number of colonies, a positive and negative selection procedure was applied (Fig. 17). This double screening procedure with G418 and gancyclovir has been reported by some groups to increase the targeting frequency of constructs flanked by one, two identical or two different (TK1 and TK2) herpes simplex virus thymidine kinases (HSV-TK) by a factor of up to 2000 (Mansour et al., 1988). When double selection was applied to electroporation with the construct pICL.PHR, the frequency of resistant colonies dropped about fivefold compared to selection with only G418 indicating that the additional gancyclovir selection conditions were stringent. But still no homologous recombinant ES cell clone was isolated from 342 screened G418/gancyclovir double resistant colonies. Consequently, either the frequency of homologous recombination is not increased by double selection, an observation reported also by other groups (Mansour et al., 1988; Donehower et al., 1992; Chisaka et al., 1992; Thomas and Capecchi, 1990; DeChiara et al., 1990; McMahon and Bradley, 1990) or the screening procedure by PCR was not able to detect the mutation in Exon 2 of the perforin gene. Alternatively, despite the fivefold enrichment by double selection, the frequency of homologous recombinants might have been too low to allow the detection of homologous recombination in the limited number of screened colonies. Possibly, the failure to find homologous recombination with construct pICL.PHR was not due to an intrinsic property of the perforin locus but rather was a problem associated with this specific construct. Probably it either resulted because the length of homology was not sufficient to mediate homologous recombination or alternatively because the secondary structure of the perforin locus or proteins bound to the chromatin precluded homologous recombination or its detection by PCR. In order to circumvent these potential problems, four additional targeting constructs pICL.HK1, pICL.HK2, pICL.HK3 and pICL.HK4 were established. These vectors contain perforin homologous sequence derived from exon 3 and from the flanking introns. In addition, the length of homologous sequence was
more than doubled from 1.5 kb of the exon 2 derived construct pICLPHR to 3.3 kb. Fig.20 shows the theoretical crossovers for homologous recombination with the construct pICL.HK2, the lengths of the expected restriction fragments for the wildtype and the targeted allele and the positions of the employed PCR primer pairs. The restriction fragment lengths and PCR fragments of the other three constructs are given in Tab.4. The results of the electroporation of ES cells with constructs pICL.HK1-HK4 are listed in Tab.5. The frequency of G418 resistant colonies recovered varied again considerably with the ES cell line and the batch of cells used. No viable colonies were recovered from control electroporations without construct DNA added, showing the stringency of the G418 selection procedure. In a first experiment the same batch of D3H cells were electroporated with the four targeting constructs and the colonies resulting from 5 x 10^6 electroporated ES cells were screened for homologous recombination. The frequencies of neo resistant colonies per 5 x 10^6 ES cells varied between 30 for HK2 and 240 for HK4. Homologous recombinants were isolated from electroporation experiments with pICL.HK2 and pICL.HK4 at a frequency of 1 in 30 respectively of 1 in 200. These two constructs differing only in the orientation of the neo cassette in respect to the homologous sequence yielded surprisingly differing frequencies of homologous recombination and G418 resistant colonies per 5 x 10^6 electroporated ES cells. Since both constructs gave about the same number of homologous recombinant clones per 5 x 10^6 electroporated ES cells, pICL.HK4 comparatively produced much higher numbers of colonies with the construct integrated at a random site of the cellular genome. Thereafter, the frequencies of homologous recombination could be reproduced with ES cell lines D3, BL/6-III and D3M without any statistically significant difference in frequencies.
Fig. 18. Disruption of the perforin gene in mouse embryonic stem (ES) cells with the targeting construct pICLPHR.

a) The targeting construct pICLPHR consisted of a 1.5 kb SacI fragment, which was derived from perforin exon 2 and from parts of the flanking introns, interrupted by a neo cassette of 1.2 kb length. The neo gene is driven by a HSV-TK promoter. EcoRI (E), SacI (S) and Smal (M) sites are indicated.

b) Map of the murine perforin gene. The theoretical crossovers with the targeting construct are shown with crossed lines. The endogenous locus yields a 16 kb EcoRI band when analyzed by Southern blotting with a Exon 3 probe (B) and a neo probe (C).

c) The disrupted perforin locus can be detected by a PCR reaction with primer c and d or alternatively with primers e and f. Note the additional EcoRI site in the disrupted allele that the neo cassette introduces into the locus. Consequently, EcoRI digestion of the targeted locus will yield Southern blotting fragments of 2.1 kb and 15.2 kb depending on the indicated DNA probes.
Fig.19. The positive-negative procedure used to enrich for ES cells containing a targeted disruption of gene X (Mansour et al., 1988). 

a, A gene X replacement vector that contains an insertion of the neo<sup>r</sup> gene in an exon of gene X and a linked HSV-TK gene, is shown pairing with a chromosomal copy of gene X. Homologous recombination between the targeting vector and genomic X DNA results in the disruption of one copy of gene X and the loss of HSV-TK sequences. Such cells will be X<sup>-</sup>, neo<sup>r</sup> and HSV-TK<sup>-</sup> and will be resistant to both G418 and Gancyclovir. 

b, Because non-homologous insertion of exogenous DNA into the genome occurs through the ends of the linearized DNA, the HSV-TK remains linked to the neo<sup>r</sup> gene. Such cells will be X<sup>+</sup>, neo<sup>r</sup> and HSV-TK<sup>+</sup> and therefore resistant to G418 but sensitive to Gancyclovir. Open boxes denote introns or flanking DNA sequences, closed boxes denote exons and cross-hatch boxes denote the neo<sup>r</sup> or HSV-TK genes.
Fig. 20. Disruption of the perforin gene in mouse embryonic stem (ES) cells with the targeting construct pICLHK2.

a) The targeting construct pICLHK2 consisted of a 3.3 kb EcoRV fragment, which was derived from perforin exon 3 and from a part of the preceding intron, interrupted by a neo cassette of 1.2 kb length. The neo gene is driven by a HSV-TK promoter. EcoRI (E), EcoRV (R) and BstI (B) sites are indicated.

b) Map of the murine perforin gene. The hypothetical crossovers with the targeting construct are shown with crossed lines. The endogenous locus yields a 16 kb EcoRI band when analyzed by Southern blotting with an exon 2 probe (A), an exon 3 probe (B) and a neo probe (C).

c) The disrupted perforin locus can be detected by a PCR reaction with primer a and b. Note the additional EcoRI site in the disrupted allele introduced by the neo cassette. Consequently, EcoRI digestion of the targeted locus will yield Southern blotting fragments of 5.3 kb and 11.9 kb depending on the indicated DNA probes.
Tab. 3. Electroporation of ES cells with exon 2 derived construct pICLPHR and effect of Gancyclovir selection on the frequency of homologous recombination.

<table>
<thead>
<tr>
<th>Construct</th>
<th>ES cell line</th>
<th>Colonies per 5x10⁶ cells</th>
<th>Colonies screened</th>
<th>Targeted ES clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>pICLPHR</td>
<td>D3H</td>
<td>190</td>
<td>160</td>
<td>0</td>
</tr>
<tr>
<td>pICLPHR</td>
<td>D3</td>
<td>134</td>
<td>270</td>
<td>0</td>
</tr>
<tr>
<td>pICLPHR</td>
<td>BL/6-III *</td>
<td>165</td>
<td>496</td>
<td>0</td>
</tr>
<tr>
<td>pICLPHR + Ganc.</td>
<td>BL/6-III *</td>
<td>31</td>
<td>188</td>
<td>0</td>
</tr>
<tr>
<td>pICLPHR</td>
<td>BL/6-III #</td>
<td>83</td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td>pICLPHR + Ganc.</td>
<td>BL/6-III #</td>
<td>25</td>
<td>154</td>
<td>0</td>
</tr>
</tbody>
</table>

5x10⁶ ES cells of different lines were electroporated with linearized plasmid construct DNA. After 10 days of G418 selection the number of resistant colonies was determined and a certain number of resistant colonies assessed for homologous recombination by PCR and genomic Southern Blotting. In certain experiments, double selection with G418 and Gancyclovir was applied. Symbols (*, #) indicate that the same batch of ES cells was used for a series of electroporations and that the frequencies can be compared directly.

Tab. 4. Expected restriction fragment patterns and PCR products after homologous recombination of the perforin locus with the four exon 3 derived constructs. For the localization of the probes see Fig. 20.

<table>
<thead>
<tr>
<th>Construct</th>
<th>EcoR1 fragments</th>
<th>Probe A</th>
<th>Probe B</th>
<th>Probe C</th>
<th>Primer pair</th>
<th>PCR fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>pICLHK1</td>
<td>16.0, 6.0</td>
<td>16.11.2</td>
<td>6.0</td>
<td>6.0</td>
<td>b/d</td>
<td>735 bp</td>
</tr>
<tr>
<td>pICLHK2</td>
<td>16.0, 5.3</td>
<td>16.11.9</td>
<td>5.3</td>
<td>11.9</td>
<td>a/b</td>
<td>665 bp</td>
</tr>
<tr>
<td>pICLHK3</td>
<td>16.0, 5.3</td>
<td>16.11.9</td>
<td>5.3</td>
<td>11.9</td>
<td>b/d</td>
<td>665 bp</td>
</tr>
<tr>
<td>pICLHK4</td>
<td>16.0, 6.0</td>
<td>16.11.2</td>
<td>6.0</td>
<td>6.0</td>
<td>a/b</td>
<td>735 bp</td>
</tr>
<tr>
<td>Construct</td>
<td>ES cell line</td>
<td>Colonies per 5x10⁶ cells</td>
<td>Colonies screened</td>
<td>Targeted ES clones</td>
<td></td>
<td></td>
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<tr>
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<td>--------------------------</td>
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<td>--------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pICLHK1</td>
<td>D3H *</td>
<td>60</td>
<td>60</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pICLHK2</td>
<td>D3H *</td>
<td>30</td>
<td>30</td>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>pICLHK3</td>
<td>D3H *</td>
<td>180</td>
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</tr>
<tr>
<td>pICLHK4</td>
<td>D3H *</td>
<td>240</td>
<td>200</td>
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<td></td>
</tr>
<tr>
<td>pICLHK4</td>
<td>D3</td>
<td>105</td>
<td>200</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pICLHK2</td>
<td>BL/6-III *</td>
<td>120</td>
<td>150</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pICLHK4</td>
<td>BL/6-III #</td>
<td>420</td>
<td>150</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pICLHK2</td>
<td>BL/6-III</td>
<td>51</td>
<td>142</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pICLHK2</td>
<td>D3M</td>
<td>43</td>
<td>130</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pICLHK2</td>
<td>BL/6-III</td>
<td>21</td>
<td>62</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pICLHK2</td>
<td>BL/6-III</td>
<td>14</td>
<td>83</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5x10⁶ ES cells of the indicated ES cell lines were electroporated with linearized plasmid DNA. After 10 days of G418 selection the number of resistant colonies was determined and a certain number of resistant colonies assessed for homologous recombination by PCR and genomic Southern blotting. Symbols (*, #) indicate that the same batch of ES cells was used for a series of electroporations and that the frequencies can be compared directly.
Example for the screening of a successful electroporation experiment

Six lots of BL/6-III ES cells were electroporated with 30 µg of linearized pIC1.HK2 plasmid DNA. After selection for G418 resistance, the 83 resistant colonies were picked as single clones. The colonies were divided into two equal halves, one half passaged to a 96 well plate and the other half analyzed as crude cell lysates of about 50 ES cells for PCR. The PCR reaction products were separated on an 1.2 % agarose gel and since no bands were visible by ethidiumbromide staining, the gel was blotted to a nylon membrane and hybridized with the labelled respective PCR fragment. The result of the autoradiographic exposure of the membranes is shown in Fig.21. Besides two clearly positive clones on lane 59 and 75 ( translating to clones 134-74 and 134-96 ) eight further clones on lanes 12, 18, 39, 46, 49, 51, 64 and 79 ( translating to clones 134-17, 134-25, 134-48, 134-56, 134-60, 134-62 and 134-102 ) showed a fainter band at the expected length of 665 bp.

These ten clones were grown up, genomic DNA was isolated and further analyzed by Southern blotting (Fig.22a,b and c). Homologous recombination was confirmed in clones 134-74 and 134-96, which both had been strongly PCR positive. For none of the weakly positive ES cell clones the genomic 5.3 kb EcoRI restriction fragment diagnostic for homologous recombination was detected by hybridisation with flanking probe A (Fig.22a), thus identifying the faint PCR band amplified from these randomly integrated clones as an artifact. The hybridisation of the filter with the exon 3 probe B (Fig.22b) internal to the construct and with probe C complementary to the neomycin resistance gene (Fig.22c) confirmed homologous recombination in clones 134-74 and 134-96 by the detection of the 5.3 kb and 11.9 kb bands, respectively of only the 11.9 kb band (see also Fig.20).

In all of the 8 clones characterized by only a faint PCR band, the absence of homologous recombination was proven by the failure of flanking probe A to detect the 5.3 kb band. Hybridisation with probe C documented the presence of the randomly integrated construct DNA in those clones. In some clones the introduced DNA remained intact while in others it was partially degraded. Except for clone 134-60, integration of a single copy of the construct was demonstrated in these
clones. The complex pattern of bands with varying intensities detected by probe B and probe C in clone 134-60 probably resulted from multiple random integrations of construct fragments displaying varying patterns of exonuclear degradation.

The result of the genomic Southern blot was confirmed also by PCR analysis of 2 μg purified genomic DNA, an amount of PCR template that allows the detection of the diagnostic PCR fragment directly on the ethidiumbromide stained agarose gel (Fig.23). The targeted mutation in clones 134-74 and 134-96 was confirmed by this test.

Thus, two homologous recombinant clones were isolated from 83 neo resistant ES cell colonies and their genotype was confirmed by genomic Southern blotting and PCR amplification specific for the targeted mutation.
Fig.21. (previous page) PCR Screening of an electroporation experiment with exon 3 derived construct pICI.HK2. G418 resistant colonies electroporated with linearized pICI.HK2 were screened by PCR with primer pair a/b (Fig.20). Homologous recombinant clones are expected to yield a fragment of 665 bp length. VKp denotes amplification of the 665 bp fragment from 50 pg of the plasmid control pTZ19R.VKp (Fig.17). Two clearly positive clones (lane 59 and 75, translating to clones 134-74 and 134-96) and 8 weakly positive clones (lanes 12, 18, 39, 46, 49, 51, 64 and 79, translating to clones 134-17, 134-25, 134-48, 134-56, 134-60, 134-62, 134-81 and 134-102) were expanded for further analysis by genomic Southern blotting.
Fig. 22. (previous page) Genomic Southern blotting of PCR positive clones (compare Fig. 21). Genomic DNA was digested with EcoRI, blotted to filters and hybridized with a flanking Probe A (a), stripped and rehybridized with an internal probe B (b) and a Probe C from the neo coding region (c). For localization of the three probes, see Fig. 20. Expected fragment lengths can be taken from Fig. 20 and Tab. 4. B6 indicates wildtype C57BL/6 kidney genomic DNA and B6 + VKp means the former genomic DNA mixed with 250 pg of positive control plasmid pICL.VKp.
Fig. 23: Confirmation of the PCR screening by amplification of clean genomic DNA. Ten PCR positive clones (Fig. 21) were grown up, used for isolation of genomic DNA and 2 μg of genomic DNA checked by PCR with primers a and b for homologous recombination. The signals were strong enough to be detected without Southern Blotting of the PCR gel. B6 denotes normal genomic DNA from BL/6-III ES cells, VKap indicates 50 pg of the positive plasmid control.

Generation of mice heterozygous for the disruption of the perforin gene by injection of homologous recombinant ES cells into blastocysts

All blastocyst injections were carried out by Birgit Ledermann and Kurt Bürki, Sandoz AG, Basel.

Homologous recombinant ES cell clones were judged by the morphology of their colonies in vitro and then injected into blastocysts. The injected blastocysts were transferred to foster mothers and eventually gave rise to chimeras.

Several homologous recombinant ES cell clones were obtained from the D3 derived ES cell lines (D3H, D3M, D3). But microinjection of these clones did not yield any germ line chimeras inspite of the considerable effort made.

The further efforts concentrated on the homologous recombinant clones originating from the BL/6-III ES cell line. BL/6-III ES cell clones were injected
into BALB/c blastocysts and transferred to CD1 foster mothers. The male chimeras with a black and white spotted fur were bred with C57BL/6 females. The black fur colour of the offsprings indicated germline transmission of the ES cell genotype. Transmission of the BALB/c genotype yielded brown furred offsprings because of the presence of the dominant agouti depigmentation allele in the BALB/c mouse strain.

Of six homologous recombinant BL/6-III clones that were used for blastocyst injection only one gave rise to germline chimeras. In Tab.6 a series of four clones is shown with results of the blastocyst injection that clearly show their non-pluripotent phenotype. Hallmarks of non pluripotency were small or missing litters of the fostermothers, no or a small proportion of chimeras in the litters and chimeras born already dead or dying in the first week after birth. But from six tested clones one, 127-76, was pluripotent and 33 chimeras from 19 litters were derived from this clone (Tab.7). A high 30 to 3 ratio of male to female chimeras, a hint for the presence of ES cell derived cells in the germ line of the chimeras, was found.

9 of the 30 male chimeras descending from clone 126-76 proved to be 100% transmitting germline chimeras, i.e. chimeras derived from a female blastocyst that were converted during embryonic development by the male ES cells to a male phenotype (Fig.24). This was reflected by the black coat colour of all pups when the chimeras were bred to C57BL/6 female mice. Statistically, 50% of these offsprings were expected to be heterozygous for the targeted mutation. The presence of the mutation in the offspring was confirmed by Southern blotting of genomic DNA and hybridization with flanking probe A (Fig.25). The heterozygous animals appeared healthy and no difference to their wildtype littermates was noted.

Tab.8 shows an analysis of fur colour chimerism and the type of germline transmission of 30 male chimeras obtained from the pluripotent clone 127-76. All 100% germline transmitting chimeras displayed a relatively high degree of chimerism between 40 and 90% (Fig.24), while the fertile chimeras not
transmitting the ES cell derived phenotype showed a clear tendency towards lower
degrees of chimerism. Nonfertile chimeras occurred at all degrees of chimerism.
Genetically the genotype of the offsprings of 100% transmitting chimeras is
completely derived from C57BL/6 mouse strain except for the mutation in the
perforin locus. Therefore, from the breeding of a heterozygous male and female
mouse animals will be obtained, that are deficient for perforin while retaining the
C57BL/6 genotype. The well characterized C57BL/6 genotype will facilitate the
functional analysis in various immunological tests considerably.
Tab.6. *Result of the blastocyst injection of four non-pluripotent ES cell clones*

<table>
<thead>
<tr>
<th>Clone</th>
<th>Offsprings</th>
<th>Chimeras</th>
<th>Dead Chimeras</th>
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</thead>
<tbody>
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<td>128-13</td>
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<td>1</td>
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<tr>
<td></td>
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<td>0</td>
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</tr>
<tr>
<td></td>
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<tr>
<td>128-29</td>
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</table>

Homologous recombinant clones 128-13, 128-19, 128-29, 128-52 are not pluripotent. The clones were injected into BALB/c blastocysts. 15-21 injected blastocysts were then transferred to each fostermother. After birth, the number of litters, their chimerism and survival rate reflects the pluripotency and ability of the ES cells to cooperate with blastocyst derived cells in embryonic development.
Tab. 7. Results of the blastocyst injection of pluripotent ES cell clone 127-76

<table>
<thead>
<tr>
<th>Clone</th>
<th>Pups</th>
<th>Born</th>
<th>Dead</th>
<th>Alive</th>
<th>Male</th>
<th>Female</th>
<th>100% GL</th>
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<tbody>
<tr>
<td>127-26</td>
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</table>

Total 75 65 32 33 30 3 9

The targeted BL/6-III Clone 127-76 was injected into BALB/c blastocysts. 15-21 injected blastocysts were then transferred to each fostermother. After birth, the number of litters, their chimerism and survival rate reflects their pluripotency and ability to cooperate with blastocyst derived cells in embryonic development.
Fig. 24. Three germline transmitting chimeras derived from ES cell clone 126-76. ES cell contribution results in black spots in the white fur. From left to right: Goliath, Ben Hur and Al Sabah. Note that despite of the varying degree of coat colour chimerism all three mice passed the ES cell phenotype to 100% of their offsprings.

Fig. 25. Southern blot analysis of 9 offsprings from a 100% germline chimera. Genomic DNA was isolated from tail biopsies, digested with EcoRI and hybridized to Probe A (Fig. 20) after separation and blotting. 6 of 9 litters are heterozygous for the disruption of the perforin gene resulting in the appearance of the diagnostic 5.3 kb band.
Tab. 8. Correlation of germline transmission with fur colour chimerism of the 30 male chimeras generated from the targeted ES cell clone 127-76 (GL: Germline)

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<th>Transmission</th>
<th>0 - 10%</th>
<th>10 - 20%</th>
<th>20 - 30%</th>
<th>30 - 40%</th>
<th>40 - 50%</th>
<th>50 - 60%</th>
<th>60 - 70%</th>
<th>70 - 80%</th>
<th>80 - 90%</th>
<th>90 - 100%</th>
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<tr>
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Discussion
In order to clarify the role of perforin for cytolysis, mice with a mutation of the perforin gene were produced by gene targeting. To this end, ES cells were transfected with five different targeting constructs. The first construct was designed to disrupt the perforin gene by homologous recombination to exon 2. A second group of four related constructs targeted exon 3. In both cases, a neomycin resistance gene with translation stop codons in all three reading frames was introduced into the perforin gene, thus abrogating the production of functional perforin protein in cells with both alleles disrupted. Homologous recombination was obtained with two of the exon 3 derived constructs at different frequencies, while no homologous recombinant ES cell clone could be isolated from transfection experiments with the exon 2 derived construct. Subsequently, several male chimeras transmitting the targeted mutation to their offsprings were obtained from blastocysts injected with pluripotent targeted ES cells. By breeding these heterozygous offsprings, homozygous perforin deficient mice can be obtained. Their phenotype will then allow an assessment of the natural function of perforin in vivo.
Two targeting constructs derived from perforin exon 3 successfully and consistently induced homologous recombination in ES cells. Although they differed only in the direction of the neo resistance gene relative to the homologous sequence, the observed recombination frequencies differed considerably: The first construct recombined homologously at a frequency of 1 in 30 G418 resistant colonies, while for the second a frequency of 1 in 240 was observed. How can this significant difference be explained considering that the two constructs differ only in the orientation of the neomycin resistance gene? First, a conformational difference between these two constructs, which is caused by the orientation of the cassette, could either favour or inhibit homologous recombination. Alternatively, only one direction of the neo gene in exon 3 of perforin could allow the expression of the phosphotransferase. The construct carrying the neomycin resistance gene in the same direction as the endogenous locus yielded homologous recombination
frequencies eight times higher than the construct with the resistance gene in reverse direction. This finding is explained by an inhibition of the phosphotransferase promoter when it is placed in reverse direction to the endogenous promoter of the targeted gene. Consequently, the homologous recombinant cells are more susceptible towards the selecting agent G418 and would, at least partly, not survive the selection procedure. It is noteworthy, that the inhibition of homologous recombination with a vector carrying the neo gene in reverse direction to the homologous sequence was reported before for the $\beta_2$-microglobulin gene (Mansour et al., 1988).

A first attempt to induce homologous recombination with a construct derived from exon 2 was not successful. The failure to generate homologous recombinant ES-cell clones with construct pICLPHR could be explained by the following reasons: (i) Exon 2 might not lead to homologous recombination, either because the chromosomal DNA is in a unfavorable secondary structure, or alternatively because the access of introduced construct DNA to the genomic locus is blocked by DNA binding proteins. (ii) Only 1.5 kb homologous sequence were contained in the targeting construct pICLPHR, compared to typically more than 5 kb of homology reported in successful recombination experiments. Possibly, more than 1.5 kb sequence homology is required for recombination of perforin exon 2. (iii) Homologous recombination events actually occurred but were not detected, because the screening procedure was not sensitive enough.

All targeting vectors used for transfection experiments contained a point mutation known to decrease the frequency of G418 resistant cells (Koller and Smithies, 1989; Ledermann, 1991). Electroporation experiments with two targeting constructs containing this mutation (pICLPHR and an IL-3 construct) and with the unmutated pMCI.neoPA vector showed, that both mutated constructs yielded about ten times less G418 resistant colonies (Yenofsky et al., 1990). Thus, the presence of the point mutation in the perforin targeting constructs leads probably to a reduction of G418 resistant ES cells in these experiments. But since the homologous recombination frequency of construct pICLHK2 was relatively high,
this was not a substantial problem. However, there is a possibility that the mutation not only influenced the frequency of overall G418 resistant cells but also the frequency of homologous recombinant colonies. Two cases can be distinguished: First, the homologous genomic locus might be favorable for expression of transfected gene, either because of its open secondary structure, or alternatively because of adjacent promoters and enhancers. A less efficient resistance gene expression vector leads in this case to a reduction of non-desired, random integrants, because the expression of the neomycin resistance gene at the random genomic locus is dependent on additional endogenous transcription promoting elements. Consequently, the combination of a weak expression vector and a targeted locus favoring expression can result in an increase of the frequency of homologous recombination. Essentially, this phenomenon has been exploited to target genes, that are expressed in ES cells by applying constructs with the neomycine resistance coding sequence without a promoter (Ledermann, 1991) or alternatively by using the neo cassette without a polyadenylation signal (Schwartzberg et al., 1990; Sedivy and Sharp, 1989). On the other hand, a weakly expressing neomycin gene is disadvantageous in the case of recombination to a genomic locus unfavorable for gene expression. Homologous recombinant cells would not be G418 resistant in this case, while all the viable G418 resistant colonies would consist of randomly recombined cells, in which the expression of the resistance gene is enhanced by the environment of the random integration site. No direct evidence for such an effect was obtained, but eventually the successful constructs pICL.HK2 and pICL.HK4 profited from this mechanism while some unknown property of the non-successful constructs pICL.HK1 and pICL.HK3 inhibited a favorable interaction.

The experience of seven years of research in homologous recombination in ES cells have left the researchers with very little knowledge about the parameters influencing the recombination process, since this subject suffers from a lack of systematic investigations. Current knowledge ( or current state of ignorance) can be summarized as follows: Expression or lack of expression of a certain gene does
neither preclude nor reduce homologous recombination (Donehower et al., 1992; Joyner et al., 1989). The frequency of homologous recombination seems to be favorably influenced by the length of homology between the construct and endogenous targeted gene in the range between 0.5 and 7 kb of homology (Johnson et al., 1989). Recombination frequencies are neither decreased by a disruption of the homologous sequence by a stretch of non homologous sequence nor by one end of the construct having only little homology as long as it is more than 0.5 kb (Hasty et al., 1992; Thomas and Capecchi, 1987). In addition, it is believed that the use of isogenic genomic DNA in the targeting vectors favours homologous recombination, i.e. that the homologous genomic sequence in the construct preferentially originates from the same inbred mouse strain as the electroporated ES cell line (Hasty et al., 1992). Although these days, most approaches follow these guidelines, the reported frequencies of homologous recombination still vary in a wide range between 1 in 20 and 1 in 10000 G418 resistant colonies. However, it is not clear if some of the reported low frequencies reflect real frequencies or are merely caused by an unreliable screening scheme. That the above guidelines are not absolute "conditiones sine quae non" is illustrated by the fact, that the homologous sequence in the successful construct pICI.HK2 was shorter than the reported optimal length of homologous sequence and was not isogenic.

The exemplary screening procedure described above illustrated the isolation of 8 clones with a random integration of the targeting construct despite the, although weak, positive result from the PCR screening (Fig.21 and Fig.22). The faint, false-positive band observed after PCR-amplification of crude cell lysates can be explained by the synthesis of so called "shuffle" or hybrid templates. Hybrid templates can result from a primer that has been partially extended on a random integrated construct switching to the perforin locus in a subsequent cycle. This is possible because of the homologous sequence present in the random integrated construct, which provides the perforin specific sequence to the partially extended primer. The artificial "shuffle" template is amplified in the further course of the
PCR to a fragment identical to the fragment diagnostic for homologous recombination, as it has been reported before (Tibulewicz et al., 1992). Since it is not always easy to differentiate a true PCR band from a fainter false positive, especially in the absence of a homologous recombinant colony, only subsequent genomic Southern blotting confirms homologous recombination with high confidence.

It is known that the genomic integration of more than one construct copy is only rarely observed in electroporated eukaryotic cells (Kim and Smithies, 1992): Of 10 clones checked in this experiment only one carried more than a single copy of the construct (Fig.22b). Interestingly, the 1.5 kb EcoRI fragment of the linearized plasmid showed up in all but one randomly integrated clones, while the 4.2 kb fragment, arising from the more 5' region of the construct, was found in only 2 of the random recombinant clones. This finding could result because the 4.2 kb 5'-restriction fragment is protected by only 200 bp of flanking sequence against exonuclease activity (Fig.15). This is in contrast to the 1.5 kb fragment, which is protected by 2.9 kb of vector sequence. The absence of the 4.2 kb band is explained by the presence of intracellular DNAse activity, which might degrade transfected DNA in the cytoplasm before stable integration into the genome takes place. This intracellular DNA degradation might well be the reason for the higher frequencies of homologous recombination found with longer constructs. Higher molecular weight could protect the construct against degradation and consequently, the higher recombination frequencies with longer constructs would just reflect the higher stability of a transfected long DNA construct.

Of the six ES cell clones injected into blastocysts only two proved to have preserved pluripotency and consequently gave rise to germline chimeras. The most frequent cause for the loss of the pluripotent phenotype of ES cell lines lies in non-optimal cell culture conditions. The finding that clones from some electroporation experiments lost pluripotency while others conserved it, was correlated with a change from senile, slowly growing embryonic feeder cells to a more rapidly proliferating batch of feeder cells. Of course, this point was not investigated
systematically and the additional cell culture conditions were difficult to keep absolutely constant; but senile feeder cells seem to be at least partially responsible for the loss of pluripotency during electroporation and selection. This finding was further consolidated by the identification of another pluripotent targeted ES cell clone in a subsequent electroporation experiment, for which the same batch of fresh feeder cells was used. This additional pluripotent ES cell clone will be used to generate a second line of mice lacking the perforin gene, which can serve as a control to exclude an unlikely mutation cosegregating with the perforin gene in the first line of deficient mice.

In conclusion, mice carrying a heterozygous mutation in exon 3 of the perforin gene were produced by homologous recombination with relatively high efficiency in ES cells. The perforin deficient mice obtained by breeding the heterozygous animals will be a valuable tool to get further insight into the mechanism of cytotoxicity by lymphocytes.
8. General Discussion

In the first part of this thesis, evidence for an involvement of the cytolytic protein perforin in cytolysis of virus infected cells by specific cytolytic T cells was provided. A close kinetic correlation and histological association between perforin expression and antiviral cytolytic activity was consistent with the concept, that perforin expression indeed is required for cytolytic activity. In order to test this hypothesis further, mice with a disruption of the perforin gene were generated by gene targeting as described in chapter 2. These mice will allow a direct assessment of the role of perforin in cytolysis by lymphocytes as well as the evaluation of so far unknown aspects of perforin functions in vivo.

The analysis of perforin expression during an acute infection of mice with lymphocytic choriomeningitis virus presented in chapter 1 confirm the granule exocytosis model for cytotoxicity in four important points in vivo: First, perforin expression does occur in a physiological situation, namely during acute infection with the noncytopathic LCM virus, in which the prominent role of lytic cytotoxic T cells in mediating not only virus elimination but also immunopathological symptoms is well established. This directly contradicts previous claims (Potter et al., 1984) stating that perforin expression is an artifact observed only in in vitro cultivated cytotoxic T cells as a result of the exposition of T cells to excessive amounts of IL-2. Since tissue culture media complemented with supernatant from concanavalin A stimulated rat spleen cells as a source of IL-2 are required to maintain cloned cytotoxic T cells in culture, high expression of perforin in cultured cytolytic cells was claimed to be caused by overstimulation with excessive amounts of IL-2. Second, the close histological association of perforin expressing lymphocytes with virus infected liver and brain cells strongly suggests an involvement of perforin expressing cells in virus elimination. Third, the comparison of serial sections stained with an antibody specific for the CD8 surface molecule was consistent with the previous finding, that perforin is expressed in the CD8 positive cytolytic T cell subset; and fourth, the 48 hours delay between
cytolytic activity and anti-viral cytotoxicity after infection again is arguing for an involvement of perforin in the generation of MHC restricted anti-viral cytotoxicity. Several subsequent studies conducted by other groups confirmed the expression of perforin in vivo in other experimental systems with an involvement of cytotoxic lymphocytes. Perforin expression has been demonstrated in pancreata of NOD mice developing spontaneous diabetes (Clark, 1988), in human heart transplants (Young et al., 1989) and in lymphocytes from the synovial fluid of patients with rheumatoid arthritis (Griffiths et al., 1991). Although these observations provide strong evidence for a role of perforin in natural antiviral, autoimmune and allospecific cytotoxicity, they can not be taken as a formal direct proof. Especially, they fail to answer the intriguing question, whether perforin is the relevant effector molecule or merely expressed as a side effect of cytotoxic lymphocyte activation. Assuming that perforin is an important effector molecule able to induce lysis of target cells by itself, it would be important to know if additional redundant and/or synergistic lytic mechanisms do exist. These questions were addressed by two recent experimental approaches. In the first, CTLs were treated with antisense perforin oligonucleotides during activation in vitro with an anti-CD3 antibody to prevent perforin synthesis (Griffiths et al., 1992). The partial inhibition of perforin synthesis led to a reduction of cytolytic activity. In the second, a noncytotoxic rat mast cell tumor line, equipped with a functional secretory machinery, was transfected with a perforin expression gene construct (Acha-Orbea et al., 1990). The complementation with perforin rendered the mast cell line cytolytic. This strongly argues for perforin being the sole effector molecule for cytolysis, although both studies are compromised to some degree by the fact, that the cytotoxic activity investigated was somewhat artificial antibody mediated activity in vitro instead of natural cytotoxicity in vivo.

Consequently, the mechanism of cytotoxicity remained a controversial issue and it became clear, that perforin deficient mice would be extremely useful to test the different prevailing concepts and models for cytotoxicity. The recent establishment of gene targeting by homologous recombination in embryonic stem cells allows the
generation of mice deficient in any known gene, provided it can be isolated from genomic DNA (Shiver et al., 1992; Shiver and Henkart, 1991). The application of this new powerful technique to the perforin gene generated mice carrying one disrupted perforin allele as described in chapter 2. These heterzygot animals will then be used to breed inter se to generate homozygousity for the deficient perforin allele. First investigations of these mice will focus on the following aspects: The capability to mount an effective, antiviral, alloreactive and NK cytolytic response, the ability to eliminate the LCM virus from the organism after inoculation and the consequences of the lack of perforin on the fertility of female mice as suggested by reports of perforin expression in granulated metrial gland cells of the placenta.

The different prevailing theoretical models to account for lymphocyte mediated cytotoxicity can be used to make predictions about the consequences of the deletion of the perforin gene:

1. If it is assumed, that the granule exocytosis model is true and that perforin is the sole effector molecule, then the deficient mice should not be able to generate any cytotoxic activity against virus-infected, allogeneic or tumor cells. Concomitantly, the immunopathological symptoms during LCMV infection would fail to develop as well, i.e. intracerebrally infected perforin deficient mice would not succumb to the choriomeningitis observed in fully immunocompetent animals. However, if some of the immunopathology still develops in the absence of any detectable cytotoxic activity, these symptoms would not be the consequence of cell destruction but more likely of the lymphokines secreted during the inflammatory process in the infected tissues. Assuming, that perforin deficient mice lack cytotoxic activity in the cytotoxic T cell and the NK compartment, they could provide a new interesting model system to assess the role of both cytolytic cells during infection with viruses and in a number of other physiological situations.

2. A second concept involves the notion, that perforin and an alternative mechanism (or mechanisms) is synergizing in a redundant manner to generate cell lysis (Bradley et al., 1984; Thomas and Capecchi, 1987; Evans and Kaufman, 1981). In this case, one has to consider that complete elimination of perforin already
during development would probably lead to a stimulation of the second pathway resulting in a compensation of the perforin depletion. Such an effect has been observed in CD4 deficient mice, in which a compensating helper cell activity is able to induce antiviral immunoglobulin G after infection with vesicular stomatitis virus, while animals depleted of CD4 positive T cells with an anti-CD4 antibody do not develop immunoglobulin of this T-help dependent subclass (T.Kündig, personal communication).

In this case, one would expect cytotoxic activity still to be detectable in perforin deficient mice, although possibly at a lower level. However, the alternative pathway of lysis would result in different kinetics and morphological appearance of target cell lysis and the susceptibility patterns of different target cells would be altered as well. It is well conceivable, that some target cells are susceptible to one effector molecule, while others are resistant to this effector and sensitive to a second, or to complicate things further, succumb only to the synergistic attack of two effector molecules. Assuming that alternative mechanisms do exist, mice deficient for one main effector molecule would facilitate the identification of the additional effector mechanism significantly.

3. It was postulated that apoptosis is the sole mechanism responsible for cytolysis by lymphocytes (Baenziger et al., 1988; Lancki et al., 1991; Young et al., 1988; Taylor and Cohen, 1992; Berke, 1991; Young and Liu, 1988). In this case, the elimination of perforin would not influence at all the immunological competence of the mutated mice, i.e. they would be perfectly healthy and cytolytic activity of their CTLs and NK cells would be normal. In addition, no kinetic or morphological deviation in target cell killing compared to normal mice should be observed.

4. Recently, three proteins associated with the granula of cytotoxic cells, namely granzyme A, a rat NK granule serine protease and the polyadenylate binding protein TIA-1, have been shown to induce DNA fragmentation in permeabilized target cells (Russell, 1983; Golstein, 1987; Martz and Howell, 1989; Meyer et al., 1984). These findings suggest a slight modification of the original granule exocytosis model, in that access of these cosecreted molecules to the target cell via
the poly-perforin pores would result in the apoptosis observed during lysis of certain target cells by a yet unknown effect on the target cell nucleus. This modification would account for the lack of target cell DNA fragmentation observed in cells lysed by purified perforin protein, a finding that has been often taken as evidence against the granule exocytosis model. But since all three DNA fragmentation inducing molecules require a prior permeabilisation step to gain access to the target cell, the perforin deficient mouse is still expected to be devoid of cytolytic activity.

Depending on the outcome of the functional characterisation of perforin deficient mice, the relevance of the candidate models and hypothesis for cytolysis by lymphocytes will be evaluated. Despite the considerable evidence for a role of perforin in lymphocyte-mediated cytotoxicity the controversy about this issue is continuing to be unresolved. It seems, that with the availability of perforin deficient mice, it will be possible to shed some light on a discussion, that sometimes has been rather dogmatic than enlightened.
9. References


Folgende Personen haben alle in der einen oder anderen Art ganz entscheidenden Anteil am Gelingen dieser Arbeit gehabt. Danke möchte ich deshalb:

Hans Hengartner für die Betreuung meiner Arbeit und dafür, dass er immer ein offenes Ohr für alle Sorgen und Probleme hatte.

Rolf Zinkernagel für die intellektuellen Anregungen und die Möglichkeit diese Arbeit in seiner Arbeitsgruppe durchführen zu können.

Birgit Ledermann und Kurt Bürki für die grosse Arbeit, die sie in die Arbeit mit der BL/6-III Zelllinie und die Blastocysteninjektion investiert haben, und die langfristige Zusammenarbeit auch in Zeiten, in denen nicht alles rund lief.

Peter Seiler für die Herstellung der Rekombinationskonstrukte während seiner Diplomarbeit.

Alana Althage und Rosmarie Lang für die tatkräftige Hilfe und die geduldige Einweihung in die praktischen Aspekte der zellulären Immunologie.

Allen Mitarbeitern des Institutes für die unzähligen Gespräche nicht nur fachlicher Art und die kollegiale Arbeitsatmosphäre.

Meinen Eltern für ihre stete Unterstützung.
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