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Mice deficient for the lysosomal proteinase cathepsin D exhibit progressive atrophy of the intestinal mucosa and profound destruction of lymphoid cells

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Mice deficient for the major lysosomal aspartic proteinase cathepsin D, generated by gene targeting, develop normally during the first 2 weeks, stop thriving in the third week and die in a state of anorexia at day 26 ± 1. An atrophy of the ileal mucosa first observed in the third week progresses towards widespread intestinal necroses accompanied by thromboemboli. Thymus and spleen undergo massive destruction with fulminant loss of T and B cells. Lysosomal bulk proteolysis is maintained. These results suggest, that vital functions of cathepsin D are exerted by limited proteolysis of proteins regulating cell growth and/or tissue homeostasis, while its contribution to bulk proteolysis in lysosomes appears to be non-critical.

Keywords: cathepsin D/gene targeting/intestinal atrophy/lymphoid destruction/proteolysis

Introduction

Proteolytic processes have been detected in multiple cellular compartments. Limited proteolysis, in which only one or a few peptide bonds of a target protein are cleaved with high specificity, switches the biological activity of proteins such as enzymes and hormones on or off. Bulk proteolysis hydrolyses all peptide bonds of a target protein and generates free amino acids which are utilized as the energy source and for synthesis of new proteins. The majority of long-lived proteins with a relatively slow turnover is degraded in the lysosomal compartment. Nutritional deprivation triggers an enhanced bulk proteolysis in these organelles by autophagy, a process in which cellular structures are engulfed and degraded. Protein degradation in lysosomes is thought to be initiated by endopeptidases (proteinases) and to be continued and completed by exopeptidases (reviewed by Barrett, 1977, 1992; Hershko and Ciechanover, 1982; Bond and Butler, 1987; Kirschke and Barrett, 1987; Ciechanover, 1994).

Cathepsin D (CTSD, EC 3.4.23.5) is the major aspartic proteinase of the lysosomal compartment. It is a member of the pepsin family of proteinases (Tang, 1979). CTSD preferentially attacks peptide bonds flanked by bulky hydrophobic amino acids and its pH optimum is between pH 2.8 and 4.0 (Barrett, 1977; Offermann *et al.*, 1983). Two active site aspartic acid residues are essential for the catalytic activity of aspartic proteinases. Like other aspartic proteinases, CTSD is a bilobed molecule; the two evolutionary related lobes are mostly made up of β sheets and flank a deep active site cleft. Each of the two related lobes contributes one active site aspartic acid residue and contains a single carbohydrate group (Metcalf and Fusek, 1993).

CTSD is expressed in all tissues examined, but the level of expression varies considerably (Reid *et al.*, 1986). The enzyme has been localized in lysosomes in a variety of studies (Kirschke and Barrett, 1987). Perfusion of rat liver with pepstatin, a specific inhibitor of aspartic proteinases including CTSD, results in a significant reduction of proteolysis (Dean, 1975). This observation was the first experimental evidence for degradation of intracellular proteins in the lysosomal compartment. Genetic defects of lysosomal proteinases have not been described, which may suggest either that the defect of one proteinase can be compensated by other lysosomal proteinases, or that a proteinase deficiency may be lethal early in embryonic life (Dean, 1975). CTSD has also been detected in endosomes of macrophages and hepatocytes (Diment and Stahl, 1985; Geuze *et al.*, 1985). In macrophage endosomes CTSD is involved in limited proteolysis of parathyroid hormone, resulting in biologically active peptides (Diment *et al.*, 1989) and it has been postulated that it also participates in proteolytic processing of foreign antigens and invariant chain (Neefjes and Ploegh, 1992; Maric *et al.*, 1994; van Noort and Jacobs, 1994). Furthermore, there is evidence for secretion of CTSD (Poole *et al.*, 1974). In the extracellular space it may be implicated in pathological processes such as inflammation (Barrett, 1977), tumour progression and formation of metastases (Tandon *et al.*, 1990; Leto *et al.*, 1992; Mignatti and Rifkin, 1993).

To gain more insight into *in vivo* functions of the aspartic proteinase CTSD, mice deficient for this enzyme were generated. The present study shows that CTSD is essential for survival of mice beyond the fourth week of life under conventional as well as specified pathogen-free conditions. The mutant animals develop a progressive atrophy of the intestinal mucosa and a profound destruction of lymphoid organs, suggesting essential functions of CTSD in tissue homeostasis.

Results

Targeting of the cathepsin D gene in ES cells and generation of cathepsin D-deficient mice

The targeting construct pCDneo4 (Figure 1Ab) was used for disruption of the cathepsin D gene (*ctsd*) in embryonic stem (ES) cells. The open reading frame (ORF) of the gene is interrupted in exon 4 and the truncated ORF encodes the N-terminal quarter of mature *ctsd*, only. Since this truncated CTSD polypeptide contains one out of two active site aspartic acid residues and only half of the N-terminal lobe of the proteinase (Metcalf and Fusek, 1993), the introduced mutation is most likely a null mutation.

pCDneo4 was introduced into E-14-1 ES cells (Hooper *et al.*, 1987) and G418-resistant colonies were analysed by Southern blotting. In six out of 68 independent clones tested an additional *Eco*RI DNA restriction fragment was detected with the 5′ external probe A (Figure 1Ac), indicating a homologous recombination event in one of the *ctsd* alleles (Figure 1Ba). These results were confirmed by probing *Xba*I digested DNA with the 3′ external probe C (Figure 1Ac and Bb). The targeted ES cell clones ECD-53 and ECD-72 were microinjected into C57BL/6J blastocysts and a total of five chimeric males (no females) were generated. Of these five chimeras, three transmitted the mutant allele to their offspring; one was derived from the ES cell clone, ECD-53, and two were from the clone ECD-72. Heterozygous offspring identified by hybridization of *Bgl*II digested DNA with probe B (Figure 1Ac) were observed for more than 70 weeks. They did not show differences in phenotype or fertility as compared with wild-type litter-mates (data not shown).

Genotyping of 408 offspring from heterozygote crosses (Figure 1C) originating from both microinjected ES cell clones revealed a frequency of 23.5% for homozygous mutant mice (not shown), closely resembling the expected Mendelian frequency (25%). Hence, disruption of the *ctsd* gene does not result in embryonic lethality.

Functional inactivation of the cathepsin D gene

To test for expression of *ctsd* at mRNA and protein level, Northern blot analysis of kidney RNA and Western blotting of total cellular protein from embryonic fibroblasts were performed. A 2.0 kb *ctsd*-specific mRNA was detectable in RNA from wild-type animals, whereas no *ctsd* transcript was detectable in RNA from homozygous mutant animals (Figure 2A). Neither precursor nor the intermediate or mature form of CTSD were present in fibroblasts from CTSD-deficient mice (Figure 2B).

For determination of CTSD proteinase activity, degradation of radioactively labelled haemoglobin after incubation with kidney homogenates from wild-type and homozygous mutant mice was measured. The assays were performed in the presence and absence of pepstatin A, a potent inhibitor of the aspartic proteinase CTSD (Umezawa and Aoyagi, 1977). Pepstatin A-sensitive proteinase activity was undetectable in homogenates from homozygous mutant animals, whereas this activity was readily detectable in homogenates from wild-type animals (Figure 2C).

Northern and Western blot analyses, as well as determination of CTSD enzyme activity, show that the *ctsd* gene has been inactivated and that homozygous mutant mice are devoid of CTSD proteinase activity.

Proteolysis in cathepsin D-deficient fibroblasts

For examination of bulk proteolysis in the lysosomal compartment, cultured fibroblasts from CTSD –/– embryos were metabolically labelled and chased in the presence and absence of the cysteine proteinase inhibitors leupeptin, pepstatin A or both inhibitors. Without inhibitors no significant differences were detected between CTSD –/– and control fibroblasts (Figure 3). Pepstatin A does not impair proteolysis in either control or in CTSD –/– fibroblasts; leupeptin inhibits proteolysis in control fibroblasts and inhibition is enhanced by simultaneous addition of pepstatin A; the inhibitory effect of leupeptin is more pronounced in CTSD –/– fibroblasts than in control cells and is not enhanced by pepstatin A (Figure 3). Hence, lysosomal bulk proteolysis is not impaired in the absence of CTSD, which can be ascribed to the compensatory action of cysteine proteinases.

Phenotype of cathepsin D-deficient mice

During the first 2 weeks of life no phenotypic differences are detectable between homozygous CTSD –/– mice and wild-type litter-mates. Thereafter the weight increase of the CTSD –/– animals begins to stagnate and their weight starts to decline on entering of the fourth week. At day 25, the mean weight of the CTSD –/– mice was 5.9 ± 0.8 g, which is only ~60% of that of wild-type litter-mates (Figure 4A). Homozygous mutant animals die between day 25 and 27 (Figure 4B). During the last days of life, CTSD –/– animals exhibit reduction of spontaneous locomotion and escape reactions; progressive atactic gait disturbance was also observed. Necrosis of the small intestine was seen on autopsy of affected animals sacrificed in the final stage of the disease (Figure 5A).

Intestinal atrophy

In histological analyses major organs (liver, heart, lung, kidney, intestine, pancreas, brain, spleen and thymus) were normal until day 14, when atrophic changes of the ileal mucosa were first observed (not shown). By day 25 the atrophy of the ileal mucosa has progressed considerably (compare Figures 5B and C). The overall architecture of the mucosa is altered; the number of villi is reduced as well as the ratio between diameter and height of the villi, the limit between epithelium and central connective tissue normally formed by a well-defined basement membrane is no longer demonstrable. The lamina propria appears narrow, thus blood or lymphatic vessels are only infrequently detectable. The mean diameter of the crypts of Lieberkuhn is reduced and their secretory ducts are atrophic. In the pre-final stage of the disease, this severely compromised mucosa may be an insufficient barrier for gut bacteria, which may penetrate and cause endotoxin shock. Indications for this pathogenesis can be seen by the fact that animals in the final stage of the disease exhibited thromboses of small vessels, which in cardiac muscle concurred with myolysis (data not shown).

Destruction of lymphoid cells in spleen and thymus

A loss of lymphocytes was observed in the spleen and thymus. Already at day 14 the centres of lymphoid follicles in the white pulp of spleen from CTSD –/– mice showed reduced cell density (not shown); in the final stage, the

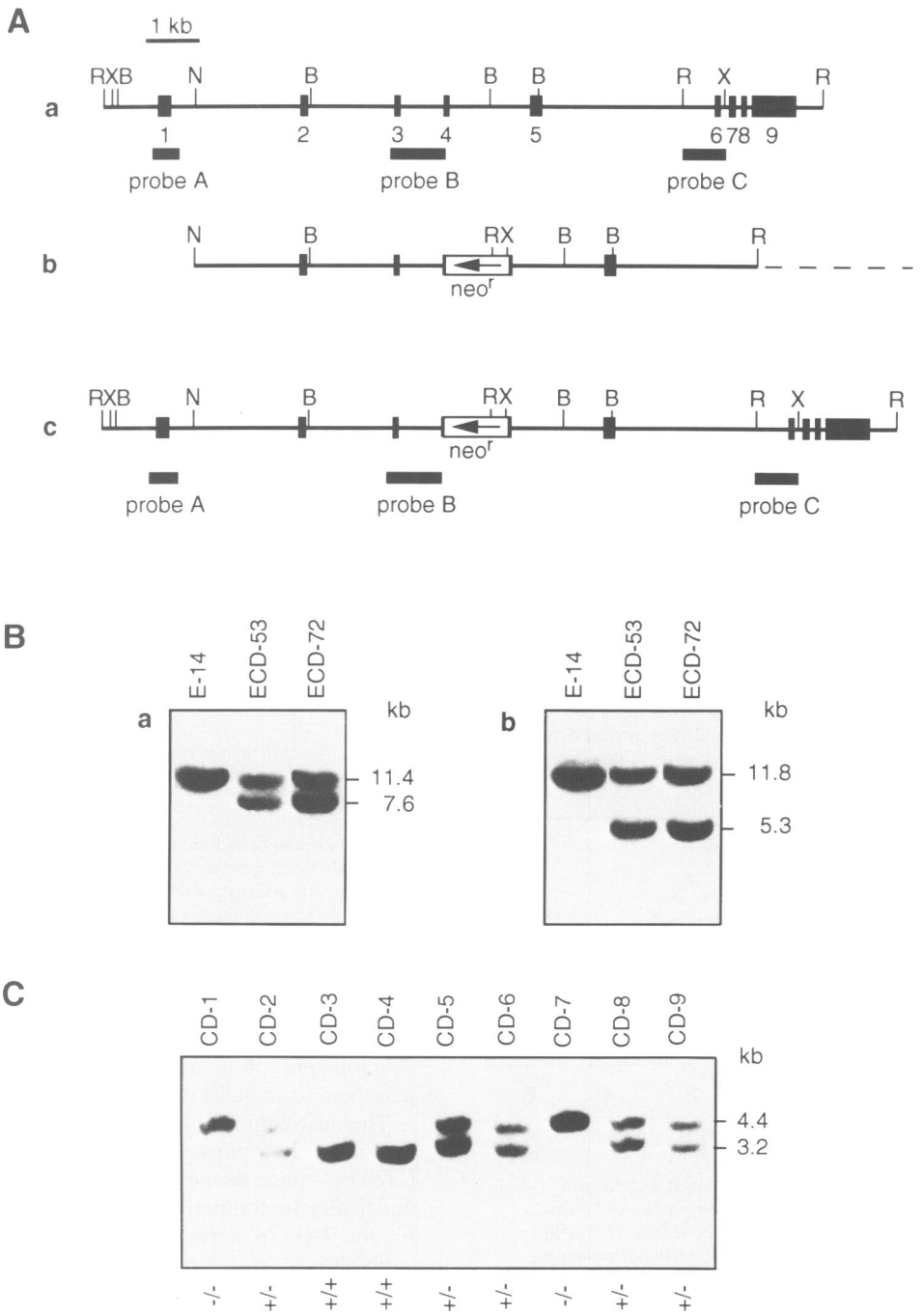

Fig. 1. Disruption of the cathepsin D gene. (**A**) Strategy for inactivation of the *ctsd* gene by homologous recombination in embryonic stem cells. (a) Genomic structure and partial restriction map of the *ctsd* gene (Hetman *et al.*, 1994). Exons are numbered and indicated by black boxes. Introns, 5′ and 3′ flanking regions are indicated by a solid line. Black bars designated probe A, B, C denote DNA probes used for Southern blot analyses. (b) Targeting vector pCDneo4 with 9.6 kb homology to the *ctsd* gene locus. The neo-cassette (open box) was inserted into a *Kpn*I restriction site located in exon 4 (not shown in partial map); arrow marks direction of transcription of the *neo* gene. A stop codon interrupting the ORF is located in the *Kpn*I–*Hin*dIII linker fragment from plasmid vector pbluescript ligated to the 5′ side of the *Kpn*I restriction site in exon 4 (for details see Materials and methods). Broken line: plasmid vector pbluescript SKII+. (c) Predicted *ctsd* gene locus after homologous recombination. 5′ external probe A detects a 7.6 and 11.4 kb *Eco*RI restriction fragment in mutated (c) and wild-type (a) alleles, respectively. 3′ external probe C hybridizes to a 5.3 and 11.8 kb *Xba*I fragment in the mutant and wild-type alleles, respectively. Probe B identifies a 4.4 kb *Bgl*II fragment in the mutated and a 3.2 kb *Bgl*II fragment in the wild-type locus. Restriction sites: R, *Eco*RI; X, *Xba*I; B, *Bgl*II; N, *Not*I. (**B**) Southern blot analysis of ES cell clones. (a) Probe A was hybridized to *Eco*RI-digested DNA from wild-type E-14–1 ES cells and two ES cell clones (ECD-53, ECD-72) with a targeted allele as indicated by an additional 7.6 kb DNA fragment. (b) Probe C was hybridized to *Xba*I-digested DNA from the same ES cells (as in a) confirming homologous recombination in ECD-53 and -72 (indicated by an additional 5.3 kb fragment). (**C**) Southern blot analysis of tail DNA from litter-mates from an intercross of *ctsd* heterozygotes. DNA was digested with *Bgl*II and analyzed with probe B (wild-type allele: 3.2 kb; mutant allele 4.4 kb; +/+: homozygous wild type; +/–: heterozygous; –/–: homozygous mutant).

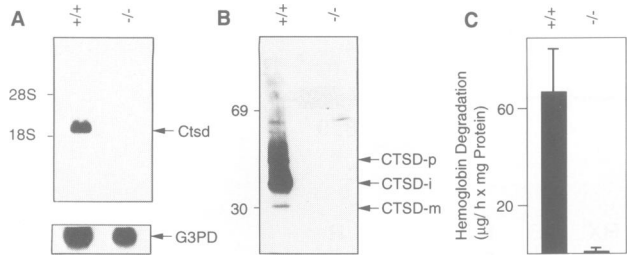

Fig. 2. The cathepsin D gene is inactivated in homozygous mutant mice. (**A**) Northern blot analysis of *ctsd* mRNA expression. Total RNA (10 µg) was hybridized to a *ctsd* mRNA specific probe covering exons 7 and 8 of the murine *ctsd* gene and a murine glyceraldehyde-3-phosphate dehydrogenase probe (G3PD, internal control), subsequently. *Ctsd* mRNA (2.0 kb) was absent in kidney and liver of homozygous mutant mice (–/–; results shown for kidney only). The positions of 28S and 18S rRNA are indicated. (**B**) CTSD protein expression in a Western blot. Total cellular protein from CTSD –/– (–/–) and normal control fibroblasts (+/+) were analysed with a purified mouse CTSD specific polyclonal antiserum. In CTSD –/– cells neither CTSD precursor (CTSD-p) nor CTSD intermediate (CTSD-i) or mature (CTSD-m) forms were detectable. Positions of standards are given in kDa. (**C**) CTSD enzyme activity. Kidney homogenates from homozygous mutant mice (–/–) and wild-type controls (+/+) were incubated with [^{14}C]haemoglobin in the presence and absence of pepstatin A (30 min, 37°C). Trichloroacetic acid (TCA)-soluble radioactivity was determined and CTSD activity calculated as pepstatin A inhibitable digestion of haemoglobin in µg/h and mg of protein in homogenates. No CTSD specific activity was detectable in homogenates from homozygous mutant mice (n = 2; range is given).

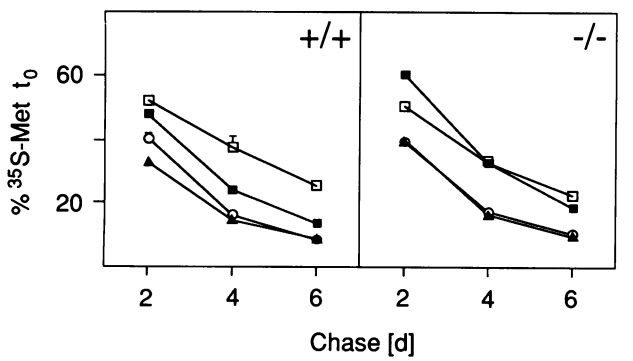

Fig. 3. Lysosomal bulk proteolysis is maintained in cathepsin D deficient fibroblasts. Fibroblasts from control mice (+/+) and CTSD –/– mice (–/–) were metabolically labelled for 12 h with [^{35}S]methionine and chased for 2, 4 or 6 days without proteinase inhibitor (○), with leupeptin (■), pepstatin A (▲) or leupeptin and pepstatin A (□). Intracellular TCA-insoluble radioactivity was determined and calculated as percent of intracellular protein-bound radioactivity at t_0 (start of chase; see also Materials and methods; n = 3; standard deviations are given when they exceeded the size of the symbols).

spleen pulp shows a diffuse homogeneous appearance due to a severe loss of lymphoid cells (Figure 6B). Although at day 14 the thymus from CTSD –/– animals is histologically indistinguishable from wild-type controls (not shown), hardly any lymphoid cells are left in its cortex by day 25. Stromal cells are prominent and necrotic cell debris is visible in the medulla (Figure 6D). To distinguish between apoptotic and necrotic cell death an *in situ* apoptosis assay (Gavrieli *et al.*, 1992) was performed. At day 14 the number of thymic cells undergoing apoptosis is increased significantly in CTSD –/– mice (Figure 6F) and is still elevated at day 25 (not shown). These observations

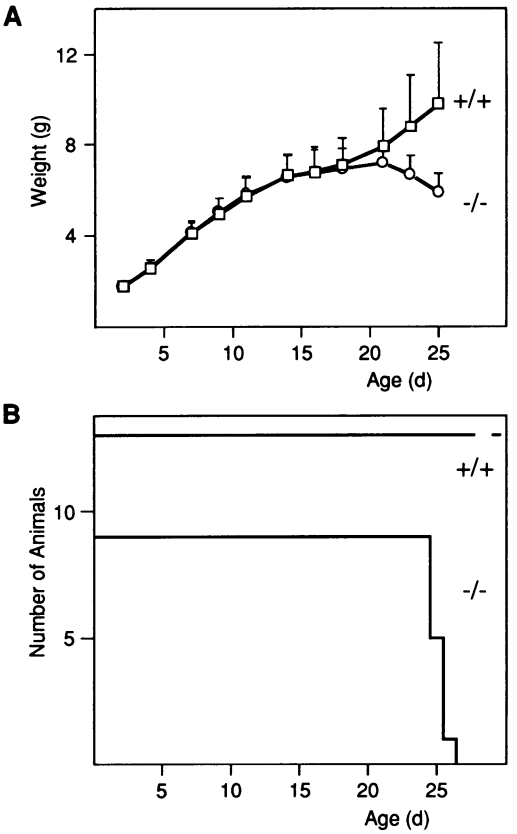

Fig. 4. Bodyweight and mortality of cathepsin D-deficient mice. (**A**) Weight development of CTSD –/– mice. The weight of CTSD –/– mice (–/–) and wild-type controls (+/+) was determined from birth to death of defective animals (n = 8 for –/– and n = 5 for +/+; standard deviations are given). (**B**) Mortality diagram of CTSD-deficient mice (–/–) and control animals (+/+).

suggest, that CTSD is essential for survival and/or regular development of T and B lymphocytes in central and peripheral lymphoid organs.

The histological observations are supported by flow cytometry. In thymocyte preparations of 24 to 26-day-old CTSD –/– mice the numbers of lymphoid cells are reduced drastically in comparison with control mice (Table I and Figure 7A). In a two-colour analysis with monoclonal antibodies against lymphocyte cell surface markers CD4 and CD8 the percentage of CD4+/CD8+ double-positive immature T cells is reduced considerably in 24 to 26-day-old affected animals (Table I and Figure 7B). Splenocytes are reduced to a comparable extent in the final stage of the disease (days 25 and 26) while the percentage of lymphocytes gated is only moderately reduced and the proportion of IgM/B220+ B cells is unaltered (Table I).

No change in course of the disease under specific pathogen-free (SPF) conditions

The observed pathology in the lymphoid system prompted us to investigate the development of CTSD –/– animals under SPF conditions. However, no differences in the course of the disease were observed. As with mice from conventional breeding, CTSD –/– animals kept under SPF conditions initially develop normally, lose weight in the fourth week and die before completing the fourth week. Extensive intestinal necroses were observed on autopsy in

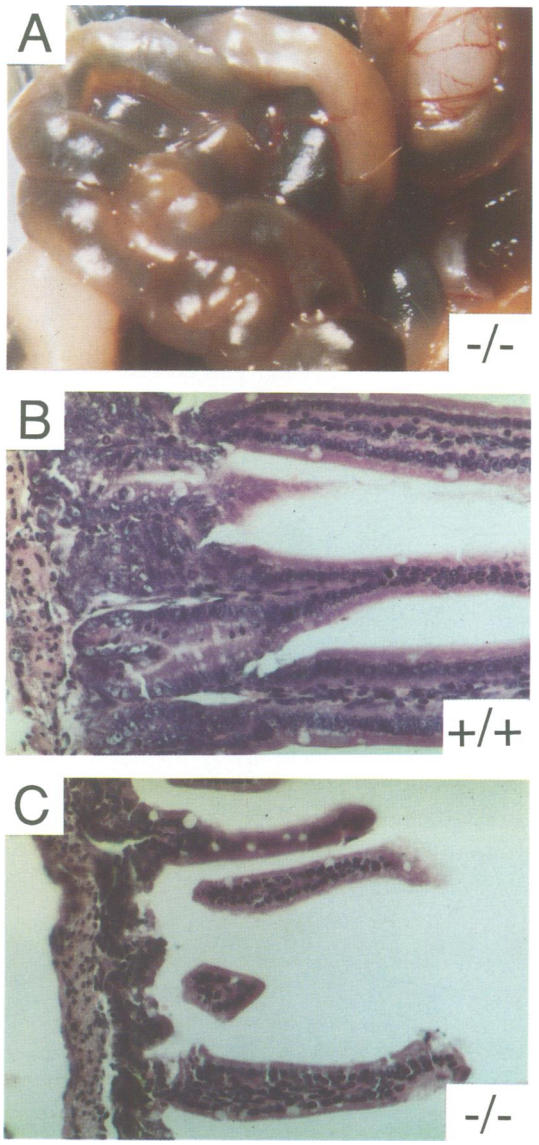

Fig. 5. Mucosal atrophy and necrosis in the small intestine. (**A**) Haemorrhagic–necrotic appearance of the small intestine in a 25-day-old CTSD –/– mouse in the final stage of the disease. Magnification ~2.75×. (**B**) Longitudinal section through ileum of a 25-day-old wild-type control mouse. Magnification 175×. (**C**) The ileal mucosa (longitudinal section) of a 25-day-old cathepsin D-deficient mouse with signs of severe atrophy: number of villi, diameter and height of villi are significantly reduced; the border between the epithelium and connective tissue is not demonstrable; narrow lamina propria, diameter of crypts of Lieberkuhn reduced. Magnification 175×. (–/–: CTSD-deficient; +/+: normal control).

the pre-final stage. Histologically, atrophy of the intestinal mucosa as well as destruction of lymphoid tissues was similar to that in animals from conventional breeding (data not shown).

Discussion

Lysosomes and endosomes are equipped with a puzzling variety of endopeptidases. In an attempt to define physiological functions for one of these proteinases, the gene coding for the lysosomal aspartyl proteinase CTSD was inactivated by homologous recombination in the mouse system.

Progressive atrophy of intestinal mucosa

Although CTSD is expressed ubiquitously (Reid *et al.*, 1986; Kirschke and Barrett, 1987) all major organs in CTSD –/– mice appear normal at birth and during the first 2 weeks of postnatal development. In the third week an atrophy of the ileal mucosa begins to develop.

Physiologically, the intestinal mucosa already displays a high level of differentiation at birth, the villi being lined with a single layer of columnar epithelial cells. Proliferation occurs at the foot of the crypts and cells migrate from there onto the villi, eventually being extruded from their tip. In the neonatal period, generation and migration of the cells is much slower than in adults, where the transit time along the villus is about 48 h (Koldovsky *et al.*, 1966). The switch towards the more rapid proliferation characteristic for adult animals occurs in the third postnatal week (Herbst and Sunshine, 1969). This switch is temporally coordinated with the onset of weaning and major changes in the profile of digestive enzymes expressed by the epithelial cells (Henning, 1981). It is achieved by replacement of neonatal-type by adult-type epithelial cells from the proliferating pool of stem cells at the base of the crypts. The molecular mechanisms underlying the changes in the pattern of expressed genes is not understood at present. At the same time a mucosal barrier to bacterial invasion is established (Henning, 1985).

The development of the mucosal atrophy in CTSD –/– mice described here coincides strikingly in time with the switch from neonate- to adult-type epithelium in the small intestine. This may indicate, that CTSD is crucial for the developmental step towards the adult-type epithelial cells. The exacerbating atrophy of the intestinal mucosa can explain the observed loss of weight due to malabsorption. Finally, this atrophy may allow penetration of gut bacteria and development of endotoxin shock associated with intravascular coagulation, thromboemboli and subsequent tissue damage (Christy, 1971; Elin and Wolff, 1976; Galanos and Freudenberg, 1993). It remains to be determined, whether the observed thromboemboli with myolysis in the cardiac muscle is secondary to the postulated endotoxin shock or represents an independent pathological development.

Profound loss of lymphoid cells

Elevated numbers of apoptotic cells in the thymus were already detected in 14-day-old CTSD –/– mice parallel to the onset of the atrophy of the intestinal mucosa. In the immune system proliferation, differentiation and also elimination by programmed cell death of lymphocytes is tightly regulated by external as well as internal signalling molecules (Raff, 1992; Schwartz and Osborne, 1993; Williams and Smith, 1993). In CTSD –/– animals the ratio between proliferation and elimination of lymphocytes is disturbed in favour of elimination by apoptosis. The present study suggests, that CTSD is not essential for differentiation of T and B cells. Single-positive CD4+ and CD8+ and double-positive CD4+/CD8+ T cells are present at day 23 and earlier. Maintenance of the more vulnerable CD4+/CD8+ double-positive T cells begins to be disturbed in the third week, resulting in a decline of these cells and a relative elevation of CD4+ and CD8+ single-positive T cells. Splenic B cells are also affected,

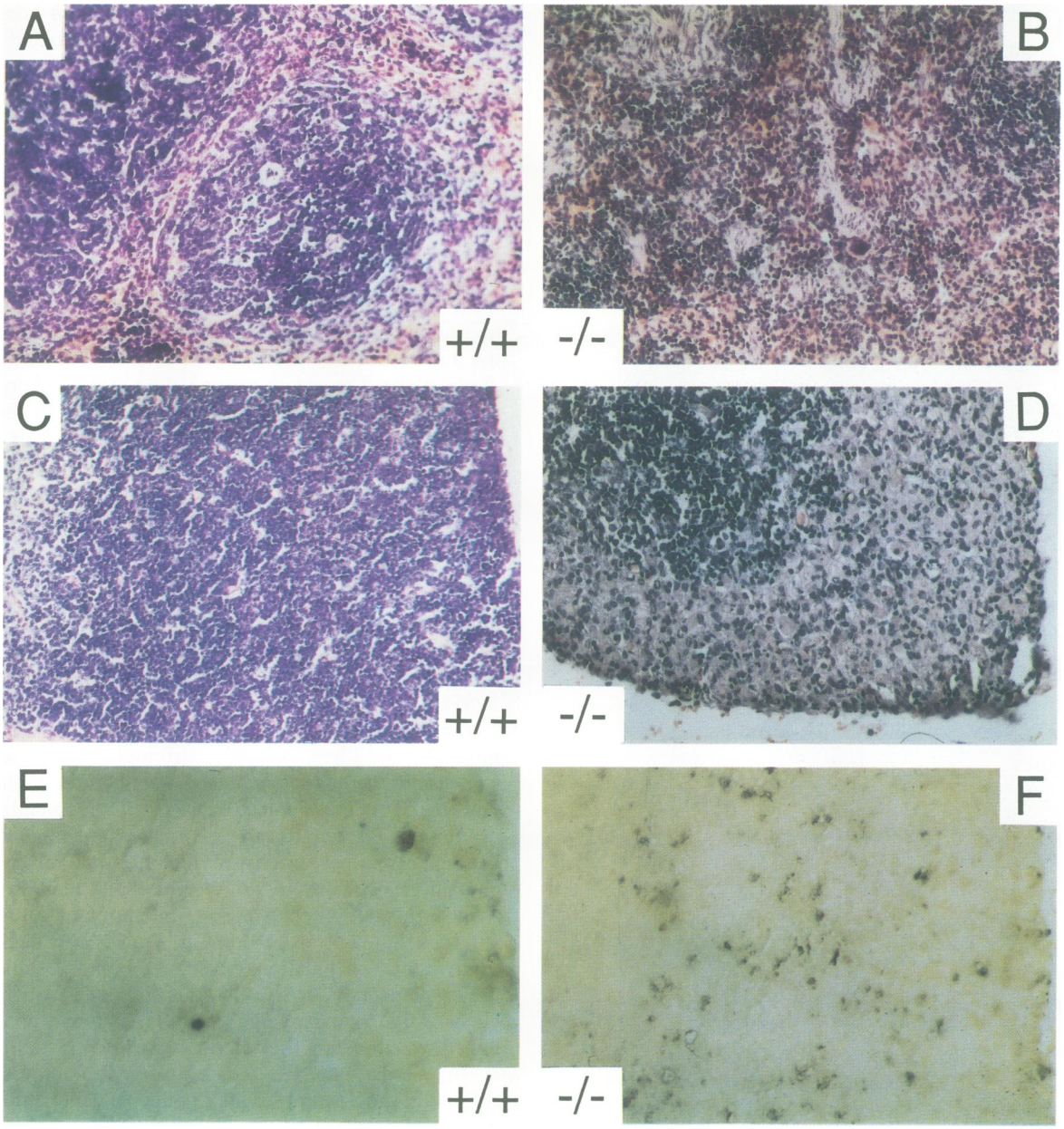

Fig. 6. Destruction of spleen and thymus in cathepsin D-deficient mice. (**A**) Section through spleen from wild-type control mouse. (**B**) Diffuse, homogenous spleen pulp without detectable lymphoid follicles in a 25-day-old CTSD –/– mouse. Magnification (A and B) 96×. (**C**) Section through thymus from wild-type control mouse. (**D**) Nearly total depletion of lymphatic cells in the cortex of a thymus from a 25-day-old CTSD –/– mouse. Magnification (C and D) 96×. Labelling of thymic sections with TdT in the TUNEL assay (Gavrieli *et al.*, 1992) shows markedly increased numbers of apoptotic cells in thymus of a 14-day-old CTSD –/– mouse (**F**) compared with a section of a thymus from a wild-type control (**E**). Magnification (E and F) 192×; (–/–: CTSD deficient; +/+: normal control).

their absolute numbers declining like those of the thymic T cells. At present it cannot be decided, whether these pathological processes in T and B lymphocytes are directly due to the absence of cathepsin D or are secondary events due to the atrophy in the ileal mucosa.

CTSD –/– mice kept under SPF conditions do not exhibit an extended life span compared with animals bred conventionally. This indicates that the observed phenotype is not caused by environmental pathogens. Since apathogenic gut bacteria, e.g. *Escherichia coli*, are still present in the intestine of SPF animals, development of endotoxin shock may also be the cause of death of CTSD –/– mice kept under SPF conditions.

Lysosomal bulk proteolysis is maintained

Severe deficiencies of one or more lysosomal hydrolases may result in intralysosomal storage of degradation intermediates and cause a lysosomal storage disease, which can be lethal. So far, more than 30 different entities of lysosomal storage disorders have been described in humans as well as animals (Scriver *et al.*, 1989). These diseases affect the metabolism of glycogen, glycosaminoglycans, glycoproteins and glycolipids. Storage disorders due to deficiencies of proteases have not been described. Bulk proteolysis in the lysosomes of fibroblasts from CTSD –/– animals is not impaired, which was shown to be due to the compensatory action of other lysosomal proteinases,

Table I. Analysis of T and B cells in CTSD –/– and control mice

Thymus								Spleen		
Mouse No.	Genotype CTSD	Age (days)	Cell No. (10^{-6})	Lymphocytes gated (%)	CD4+ (%)	CD8+ (%)	CD4+/ CD8+ (%)	Cell No. (10^{-6})	Lymphocytes gated (%)	IgM+/ B220+ (%)
1	–/–	13	155	64	15	1	70	55	20	20
2	+/+	13	155	47	11	2	72	48	19	33
3	–/–	20	130	44	7	16	42	97	28	12
4	+/+	20	100	36	8	7	59	110	21	9
5	–/–	23	90	32	8	33	43	130	20	9
6	+/+	23	130	62	9	25	59	200	23	8
7	–/–	25	17	5	58	21	5	7	19	12
8	+/–	25	130	41	16	11	70	103	24	11
9	–/–	26	13	5	51	27	8	5	13	19
10	+/+	26	102	57	13	11	73	132	39	17

Thymi and spleens from CTSD –/– and control mice of indicated ages were isolated, single cell suspensions generated, numbers of cells determined, and cells stained with monoclonal antibodies (see Materials and methods). Ten thousand events were acquired and cells present in the lymphocyte gate defined by forward and side scatter (compare Figure 7A) were analysed.

i.e. cysteine proteinases such as cathepsins B, L, S or H. It has been postulated that lysosomal degradation of proteins is initiated by endopeptidases and that this group of enzymes, rather than the exopeptidases, is rate limiting for bulk proteolysis (Kirschke and Barrett, 1987). It was shown earlier, that the aspartic proteinase inhibitor pepstatin A reduces proteolysis *in vivo* (Dean, 1975). In this study we show, however, that the deficiency of the major lysosomal aspartic proteinase cathepsin D can be compensated by the activities of the remaining endopeptidases. Proteolytic degradation intermediates generated in the presence or absence of CTSD seem to be terminally degraded by lysosomal exopeptidases with unaltered kinetics. Our results indicate, that CTSD is not essential for bulk degradation of proteins in the lysosomal compartment.

Essential *in vivo* functions of CTSD may rather concern activation – or inactivation – of signalling proteins by limited proteolysis in the endosomal and/or lysosomal compartment. An aspartic proteinase, most likely CTSD, is essential for generation of the mature form of lysosomal acid phosphatase in dense lysosomes (Gottschalk *et al.*, 1989). However, limited proteolysis catalysed by cathepsin D may also occur in a pre-lysosomal, endosomal compartment, since autocatalysis activates the zymogen procathepsin D in this compartment (Hasilik, 1992). In experiments involving cultured macrophages it has been shown that parathyroid hormone is processed proteolytically by CTSD in the endosomal compartment (Diment *et al.*, 1989). Limited proteolysis in the endosomal compartment facilitates activated proteins to exert their functions in other cellular compartments, since this compartment is connected with the plasma membrane as well as with the Golgi apparatus by vesicular traffic. Thus, CTSD may be involved in proteolytic activation or inactivation of growth factors and/or their receptors essential for cell growth or tissue maintenance. Failure to process regulatory proteins appropriately may therefore underlie the destructions observed in the intestinal mucosa and in lymphoid organs of CTSD –/– mice.

However, the possibility that the essential function of CTSD for survival of mice beyond the fourth week depends on the CTSD-propeptide rather than on proteolytically active CTSD, cannot be excluded at present.

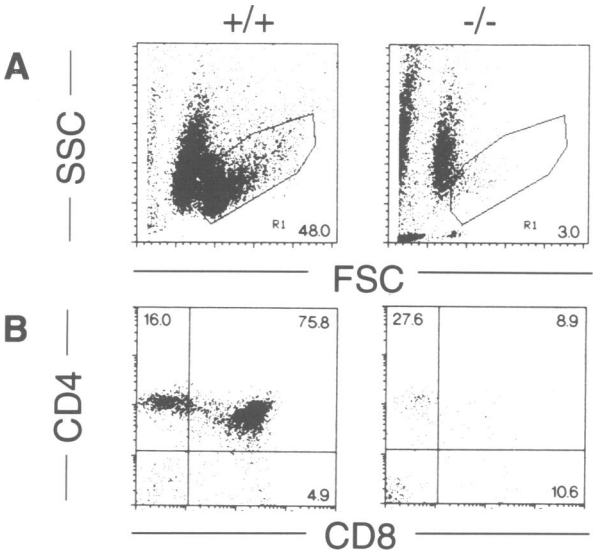

Fig. 7. Flow cytometric analysis of thymocytes. Thymi of a 24-day-old CTSD –/– (–/–) and a 24-day-old wild-type control mouse (+/+) were isolated. Single cell suspensions were generated and stained with monoclonal antibodies. 10 000 events (in the case of CTSD –/– mouse 5800 events) were acquired and cells present in the lymphocyte gate (R1) defined by forward scatter (FSC, reflecting the size of cells) and side scatter (SSC, cell granularity) were analysed. (**A**) Light scatter diagrams of thymocytes. Percentages of cells in the lymphocyte gate are given. (**B**) Analysis of thymocytes after staining with anti-CD4 and anti-CD8. Major depletion of CD4+/CD8+ double positive T-cells in the 24-day-old CTSD –/– mouse (percentages of single- and double-positive lymphocytes are given in the diagrams).

Deletion of both alleles of the gene seems to be compatible with a normal embryonal development, since homozygous deficient animals are indistinguishable from their heterozygous and homozygous wild-type litter-mates at birth; moreover, their frequency is close to 25%, which is in accord with Mendelian inheritance. It should be mentioned however, that these CTSD –/– embryos are carried to term by heterozygous mothers (homozygous defective animals cannot be bred, since their lifespan is reduced to <4 weeks). It cannot be excluded, that a certain amount of maternal enzyme reaches the fetus *in utero* via the placenta, ensuring normal fetal development and that the neonate receives CTSD with the milk, making survival

until the fourth postnatal week possible. If CTSD is provided to the deficient animal by these mechanisms, oral or parenteral enzyme substitution therapies, which currently are under investigation, may attenuate the phenotype. It seems unlikely, however, that relevant amounts of the proteinase reach the endosomal/lysosomal compartment of homozygous defective fetuses, since in comparable cases of human lysosomal storage diseases the respective enzyme activity cannot be detected in fetal tissues and lysosomal storage material is readily detectable in tissues of affected fetuses (Scriver *et al.*, 1989). Beneficial effects of breast-feeding have not been described. Furthermore, 2 h after injection of ^{125}I-labelled lysosomal enzyme arylsulphatase B into pregnant mice, radioactive enzyme was not detectable in the embryos, whereas it was readily detectable in organs such as liver, kidney and spleen of the mother (T.De Luca, unpublished results).

Theoretically, two possibilities exist: either CTSD does not cross the placenta at all, or certain amounts of the proteinase cross this barrier and are eliminated after birth due to the cut-off of supply and the turnover of the enzyme. In the first case no CTSD would be present in the organism of the deficient animal at any time and it could be concluded, that CTSD fulfils essential functions only after the fourth postnatal week. In the alternative, albeit unlikely case (see above), some CTSD would be present in the CTSD –/– animal in the prenatal and possibly also neonatal periods, but this material would be eliminated after weaning. Therefore, it cannot be excluded formally, that CTSD contributes to a normal fetal and neonatal development. After the putative postnatal decline the essential functions of CTSD for tissue homeostasis would become overt, causing the described phenotype. Unfortunately, these possibilities cannot be ruled out due to the early mortality and consequently infertility of the CTSD –/– mice.

Taken together, the data presented in this study indicate that the essential functions of CTSD depend on limited proteolysis of biologically active proteins rather than on bulk degradation of proteins in lysosomes. The limited proteolysis is proposed to activate, or inactivate, factors that regulate cell turnover in the intestinal mucosa, the lymphoid tissues and possibly elsewhere in the organism. The present study lays the basis for investigations directed towards identification of target polypeptides that are processed proteolytically by CTSD.

Materials and methods

Construction of cathepsin D gene targeting vector and production of deficient mice

A 9.6 kb *Not*I–*Eco*RI restriction fragment of cosmid clone mCD1 (Hetman *et al.*, 1994) was subcloned into pbluescript SKII+. The *neo* expression cassette from pMC1neopA (Thomas and Capecchi, 1987) was inserted into a *Kpn*I restriction site in exon 4 of the *ctsd* gene. A linker fragment from the *Kpn*I site to the *Sal*I site of the plasmid vector pUC18 had been ligated to the *Xho*I site at the 5′ end of the *neo* cassette and a linker fragment from the *Hin*dIII site to the *Kpn*I site from pbluescript SKII+ had been ligated to the *Hin*dIII site at the 3′ end of the *neo* cassette. The insertion of the *neo* cassette introduces a premature translational stop codon into the ORF of the *ctsd* gene. The recombination construct (pCDneo4, Figure 1Ab) was linearized with *Not*I and introduced into the ES cell line E-14-1 by electroporation. ES cells were cultured as described (Kühn *et al.*, 1991; Köster *et al.*, 1993). G418-resistant colonies were screened by Southern analysis of genomic DNA digested with *Eco*RI and probed with probe A (Figure 1A). ES cell clones with homologous recombination were confirmed by digesting DNA with *Xba*I and hybridization with probe C (Figure 1A). Mutated ES cells were microinjected into blastocysts of C57BL/6 mice as described (Köster *et al.*, 1993). Resulting male chimeras were mated to C57BL/6 and 129/Ola females. Mice were genotyped for the *ctsd* gene mutation by Southern analysis of *Bgl*II-digested genomic DNA, using probe B (Figure 1A). The mice were kept in a conventional animal facility at the Zentrum für Biochemie und Molekulare Zellbiologie, Universität Göttingen, Germany or under SPF conditions at the MPI für Immunbiologie, Freiburg, Germany.

Northern and Western blot analyses

Total RNA of kidney from 25-day-old mice was prepared as described (Chirgwin *et al.*, 1979). Total RNA (10 µg) was separated in a formaldehyde agarose gel and processed as described (Isbrandt *et al.*, 1994). Filters were hybridized with a 600 bp *Xba*I–*Bam*HI genomic DNA fragment containing exons 7 and 8 of the murine *ctsd* gene and a 280 bp cDNA fragment from glyceraldehyde-3-phosphate dehydrogenase (G3PD; Lyons *et al.*, 1989). Hybridization and washing of filters were performed as described (Peters *et al.*, 1990).

Total cellular protein (50 µg) from CTSD-deficient embryonic fibroblasts and normal controls was separated by 10% SDS–PAGE and subjected to Western blot analysis as described (Wenk *et al.*, 1991). The blot was probed with 20 µg/ml purified rabbit polyclonal antibody specific for mouse CTSD (M.Wendland, unpublished data). Analysis was performed using an ECL light-based immunodetection system (Amersham).

Cathepsin D assay

Kidney was homogenized (25%, w/v) in 10 mM Tris–HCl, 150 mM NaCl, pH 7.4 buffer at 4°C. The homogenate was adjusted to 0.1% Triton X-100, sonicated for 30 s on ice and centrifuged for 5 min at 12 000 *g* at 4°C. The protein concentration of the supernatant was determined (Lowry *et al.*, 1951). 4 µl of the supernatant were incubated with 100 µg of [^{14}C]carbamoyl-haemoglobin in the presence or absence of 10 µM pepstatin A for 30 min at 37°C. The reaction was terminated by addition of 500 µl of 1% (w/v) bovine serum albumin and 25% (w/v) trichloroacetic acid. After centrifugation, radioactivity was determined in the supernatant. CTSD activity is given as pepstatin A-inhibitable digestion of haemoglobin in µg/h and mg protein in supernatant of kidney homogenate (Horst and Hasilik, 1991).

Tissue culture, metabolic labelling and protein degradation

Fibroblasts from day 14 embryos were cultivated and propagated as described (von Figura *et al.*, 1983). Normal (control) or CTSD –/– fibroblasts (1.4×10^5 cells) were seeded into a well of 24-well tissue culture plates (1.3 cm^2) and incubated for 3 days to confluency. Wells were labelled for 12 h with 37 kBq [^{35}S]methionine (24.6 TBq/mmol) as described (Lemansky *et al.*, 1985). For chase the medium was changed against fresh medium containing 0.15 mg/ml methionine in the presence or absence of 200 µM leupeptin, pepstatin A or both inhibitors. Chase was performed for 2, 4 or 6 days and cells were given fresh medium after 2 days. Cells were washed with 3×1 ml ice-cold PBS, fixed for 3 min with 1 ml methanol, washed with 3×1 ml 10% trichloroacetic acid, solubilized in 1 ml 0.5 M NaOH and neutralized with 1 ml 0.5 M HCl. Radioactivity was determined by scintillation counting (Kopitz *et al.*, 1993).

Histological analysis and TUNEL

Organs were fixed in 3.5% formaldehyde and paraffin sections (5 µm) were prepared by standard protocols. Haematoxylin–eosin staining was performed according to standard procedures. For *in situ* detection of programmed cell death nuclear DNA fragments were labelled with TdT in paraffin sections as described (Gavrieli *et al.*, 1992).

Flow cytometry

Single cell suspensions from spleen and thymus were prepared by standard methods, and numbers of cells were determined (Neubauer chamber). Cells were stained with monoclonal antibodies and analysed on a FACScan cytometer using the Lysis II program (Becton-Dickinson). The following monoclonal antibodies were used for lymphocyte staining: phycoerythrin-conjugated GK1.5/4 (anti-CD4; Dialynas *et al.*, 1983) and fluorescein-conjugated 53-6.7 (anti-CD8; Ledbetter and Herzenberg, 1979) for double staining of thymocytes; phycoerythrin-conjugated R33-24 (anti-IgM; Grützmann, 1981) and fluorescein-conjugated RA3-6B2 (anti-CD45R/B220; Coffmann, 1982) for staining of splenocytes.

Lymphoid cells gated by forward scatter (FSC) and side scatter (SSC) were analysed.

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