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Constitutive activation of the Fas ligand gene in mouse lymphoproliferative disorders

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Mice homozygous for *lpr* (lymphoproliferation) or *gld* (generalized lymphoproliferative disease) develop lymphadenopathy and splenomegaly and suffer from autoimmune disease. The *lpr* mice have a defect in a cell-surface receptor, Fas, that mediates apoptosis, while *gld* mice have a mutation in the Fas ligand (FasL). Northern hybridization with the FasL cDNA as probe indicated that the cells accumulating in *lpr* and *gld* mice abundantly express the FasL mRNA without stimulation. By means of *in situ* hybridization and immunohistochemistry, we identified the cells expressing the FasL mRNA as CD4$^-$CD8$^-$ double negative T cells. The T cells from *lpr* mice were specifically cytotoxic against Fas-expressing cells. Since FasL is normally expressed in activated mature T cells these results indicate that the double negative T cells accumulating in *lpr* and *gld* mice are activated once, and support the notion that the Fas/FasL system is involved in activation-induced suicide of T cells. Furthermore, the graft-versus host disease caused by transfer of *lpr* bone marrow to wild-type mice can be explained by the constitutive expression of the FasL in *lpr*-derived T cells.

Key words: apoptosis/cytotoxicity/Fas/Fas ligand/lymphoproliferation

Introduction

T lymphocytes die at various stages of their life. Precursor T cells originate from the pluripotent stem cells in the bone marrow and enter the thymus, where they become mature T lymphocytes. During development of T cells in the thymus the immature T cells that recognize the major histocompatibility antigen (MHC) as a restriction element are positively selected, while cells that do not are left to die (positive selection; von Boehmer, 1994). Among the immature T cells that recognize the MHC, those strongly reactive to the self components in the thymus are deleted by negative selection (Nossal, 1994). In the periphery, mature T cells reactive to the autoantigen expressed in the peripheral tissues or cells reactive to foreign antigens are activated once; they then die by apoptosis (peripheral clonal deletion or the activation-induced suicide of T cells; Webb *et al.*, 1990).

Fas is a type I membrane protein of M_r 45 000 that belongs to the tumor necrosis factor (TNF)/nerve growth factor receptor family (Itoh *et al.*, 1991; Watanabe-Fukunaga *et al.*, 1992b; Nagata, 1993). The Fas ligand (FasL) is a type II membrane protein of M_r of ~40 000, and is a member of the TNF family (Suda *et al.*, 1993; Takahashi *et al.*, 1994a,b). Binding of FasL or agonistic anti-Fas antibody to Fas induces apoptosis (Itoh *et al.*, 1991; Suda *et al.*, 1993), indicating that FasL is a death factor, while Fas is its receptor mediating the apoptotic signal (Nagata, 1994). Fas is expressed not only in lymphocytes but also in the liver, heart and ovary (Watanabe-Fukunaga *et al.*, 1992b), whereas the expression of the FasL is restricted to the activated mature T lymphocytes, except in the testis (Suda *et al.*, 1993; T.Suda *et al.*, submitted). Genetic and biochemical analyses of Fas and FasL have indicated that mouse lymphoproliferation (*lpr*) and generalized lymphoproliferative disease (*gld*) mutations are loss of function mutations of Fas and FasL, respectively (Watanabe-Fukunaga *et al.*, 1992a; Adachi *et al.*, 1993; Takahashi *et al.*, 1994a). Mice carrying the classical *lpr* mutation contain an early transposable element in intron 2 of the Fas chromosomal gene, which severely reduces the expression of intact Fas mRNA (Adachi *et al.*, 1993; Kobayashi *et al.*, 1993; Wu *et al.*, 1993). The other allele of *lpr* (*lpr^{cg}*) is a point mutation in the cytoplasmic region of Fas that abolishes its ability to transduce the apoptotic signal (Watanabe-Fukunaga *et al.*, 1992a). The FasL mutation in *gld* mice is also a point mutation in the extracellular region of FasL, which inactivates the ability of FasL to bind Fas (Takahashi *et al.*, 1994a).

Mice carrying a homozygous mutation of either *lpr* or *gld* develop massive lymphadenopathy, splenomegaly, B cell activation and hypergammaglobulinemia (Cohen and Eisenberg, 1991). Lymphadenopathy in *lpr* and *gld* mice is characterized by the expansion of a non-neoplastic subset of thymus-derived cells that are TCR-α/β^+, CD3$^+$, B220$^+$, CD4$^-$ and CD8$^-$ (double negative T cells, DN T cells). These cells are first detectable in the lymph nodes between 4 and 6 weeks of age and represent 75–80% of the lymph node cells after 16–20 weeks of age. Many groups have extensively studied the mechanism of lymphadenopathy in these mutant mice. However, where and how these lymphocytes accumulate has remained uncertain. In this study, we identified abundant expression of the FasL gene in DN T cells accumulating in *lpr* and *gld* mice. These results are consistent with the notion that these cells are previously or chronically activated. It seems that the T cells which normally die after activation cannot die due to the defective Fas system and accumulate in *lpr* and *gld* mice.

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Results

Constitutive expression of the Fas ligand mRNA in the lymph nodes and spleen of lpr and gld mice

Fas is constitutively expressed in mature T cells (Drappa *et al.*, 1993; J.Ogasawara and S.Nagata, unpublished results), whereas FasL is expressed in T cells only after activation (Suda *et al.*, 1993). To correlate the expression of Fas and FasL with disease development in *lpr* and *gld* mice, we examined the expression of Fas and FasL mRNA in the lymphoid cells of aged *lpr* or *gld* mice. Total RNAs were prepared from the lymph nodes, spleen and liver of wild-type mice (MRL−+/+), or mice carrying homozygous mutations for *lpr* (MRL−*lpr/lpr*), lpr^{cg} (CBA/KlJms−lpr^{cg}/lpr^{cg}) or *gld* (C3H−*gld/gld*) at about 20 weeks of age. At this age, the *lpr* and *gld* mice showed massive lymphadenopathy and splenomegaly: weights of the spleen and lymph nodes of the mutant mice were ~10 and 100 times greater than in wild-type mice. About 85% of lymph node cells and 60% of spleen cells from mutant mice were Thy-1^+, $B220^+$, $CD4^-$ and $CD8^-$; no such T cells were found in wild-type mice.

The RNAs were analyzed by Northern hybridization using mouse FasL or Fas cDNA as probe. As shown in the top panel of Figure 1, FasL mRNA was undetectable in the lymph nodes, spleen and liver from the wild-type mice, whereas very abundant FasL mRNA was detected in the lymph nodes and spleen of *lpr*, lpr^{cg} and *gld* mice. The constitutive expression of the FasL mRNA was abundant only in aged mice, and it was barely detectable in lymphoid cells of 5-week-old mutant mice. In contrast to the lymphoid cells, liver from either wild-type or mutant mice did not express FasL at any age.

Fas mRNA was detected at moderate levels in the lymph nodes and liver, and weakly in the spleen of wild-type mice. The level of expression of Fas mRNA in mice carrying homozygous mutations of lpr^{cg} or *gld* was similar to that in wild-type mice (middle panel of Figure 1) and did not change with increasing age. *lpr* mice expressed very little Fas mRNA of normal size in lymph nodes and liver, as predicted from the transcriptional mutation of the Fas chromosomal gene in these mice (Adachi *et al.*, 1993; Kobayashi *et al.*, 1993; Wu *et al.*, 1993).

Expression of the Fas ligand mRNA in double-negative lymphocytes

To examine which cell populations in the spleen and lymph nodes express the FasL mRNA, the spleen and lymph nodes from mutant mice were analyzed by *in situ* hybridization using ^{35}S-labeled antisense or sense probe for FasL mRNA. Typical results obtained with spleen are shown in Figure 2. Very intense signals were detected with the antisense probe RNA at the marginal zones in the entire spleen of *lpr*, lpr^{cg} and *gld* mice (Figure 2a, c and e). These signals were not detected with the sense RNA probe (Figure 2b and d). When a 100-fold excess of unlabeled antisense RNA was present in the hybridization mixture, no hybridization signal was detected (data not shown), indicating that the signals detected in the spleens of mutant mice are specific to the FasL mRNA. In accord with the low level of FasL mRNA expression in wild-type mice, no specific signal was detected in the spleen of these mice by *in situ* hybridization (Figure 2f). The

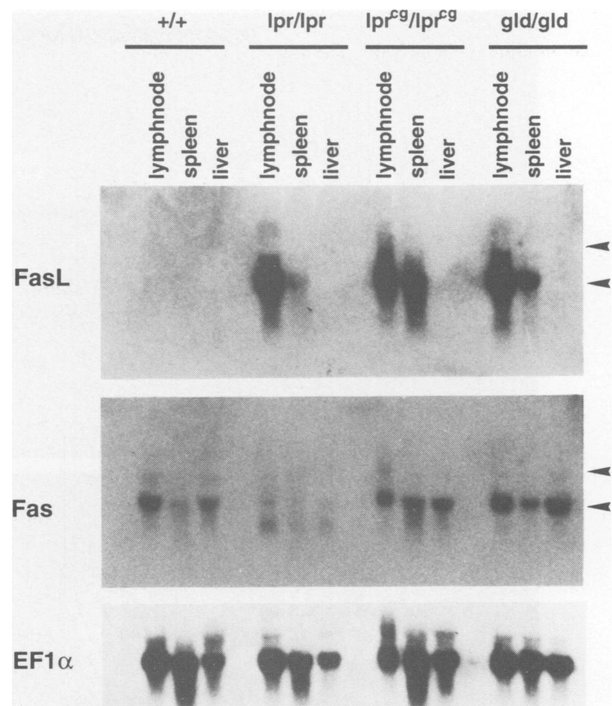

Fig. 1. The constitutive expression of FasL mRNA in lymphoid organs of aged *lpr* and *gld* mice. Total RNA was prepared from the lymph nodes, spleen and liver of wild-type, *lpr*, lpr^{cg} and *gld* mice at 20 weeks age. RNA (30 μg) was electrophoresed on a 1.0% agarose gel and subjected to Northern hybridization. As probes, mouse FasL cDNA (upper panel), mouse Fas cDNA (middle panel) or human EF-1α cDNA (lower panel) was used. The positions of 18S and 28S rRNAs are indicated on the right.

lymph nodes of aged *lpr*, lpr^{cg} and *gld* mice also gave very strong specific signals in *in situ* hybridization with the FasL probe (data not shown).

A series of cryostat sections was prepared from the spleen of *lpr* mice. Neighboring sections were analyzed by *in situ* hybridization with FasL probe, and immunohistochemically using anti-Thy-1, anti-B220 and a mixture of anti-CD4 and anti-CD8 antibodies. As shown in Figure 3, *in situ* hybridization again indicated expression of FasL mRNA in cells at the marginal zone (panel a). Immunohistochemical analysis of the spleen indicated that the Thy-1-positive cells were localized in the marginal zones and periarterial lymphatic sheaths (panel c). The cells in the periarterial lymphatic sheath were also positive for CD4 or CD8 antigen (panel d), indicating that the cells in this region are normal, mature, single-positive T cells. When the spleen was stained with anti-B220 antibody, only the cells in the marginal zone gave positive signals (panel b), and the staining profile was very similar to that obtained by *in situ* hybridization with the FasL probe (panel a). These results indicated that FasL mRNA is expressed in the cell population with the Thy-1^+, $B220^+$, $CD4^-$ and $CD8^-$ phenotype. In fact, when $CD4^-$ $CD8^-$ cells were enriched by treating the splenocytes from the aged *lpr* mice with anti-CD4, anti-CD8 and complement, they showed very intense signal for the FasL mRNA in Northern hybridization (data not shown).

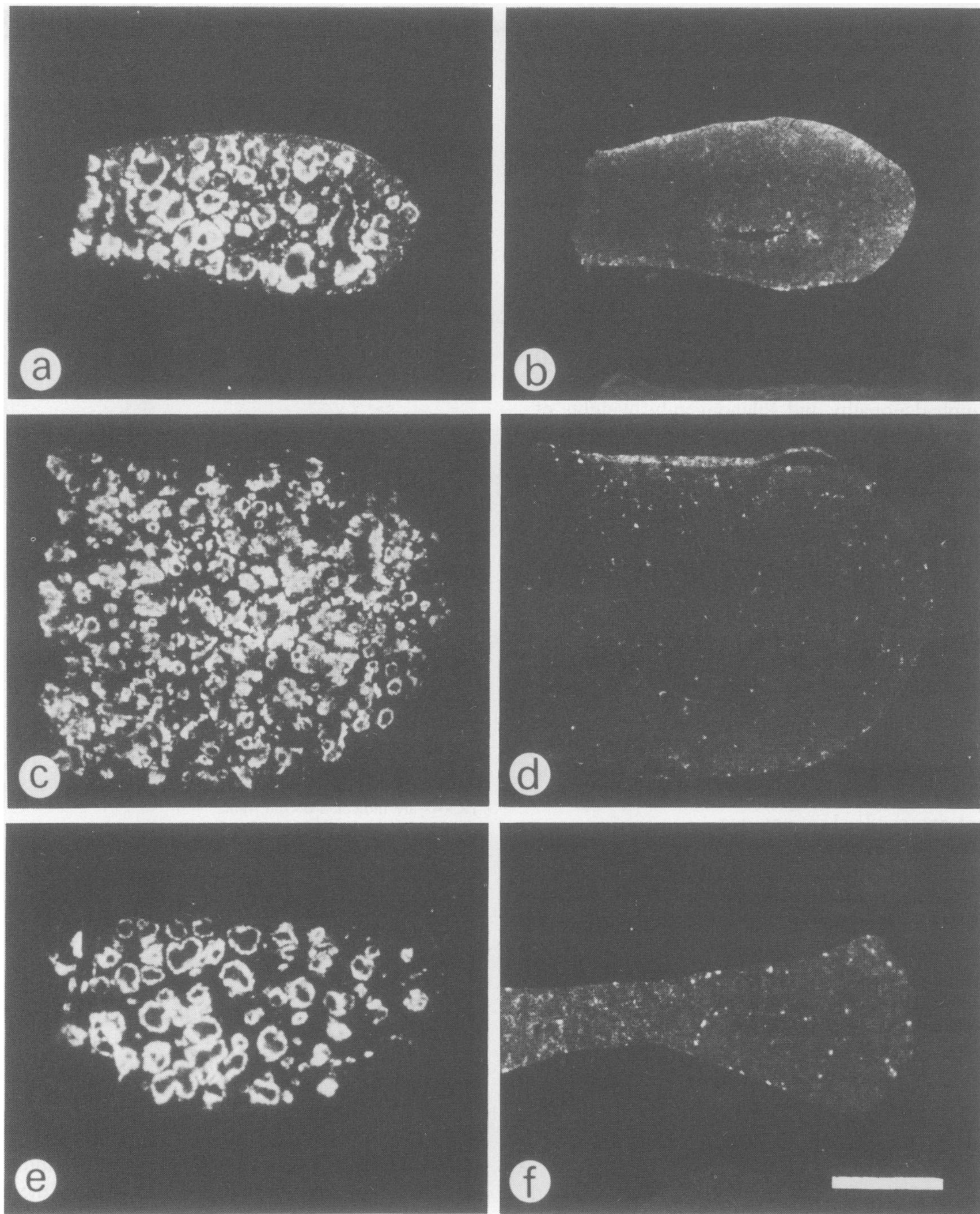

Fig. 2. *In situ* hybridization of FasL mRNA in sections of the spleen. The sections were prepared from the spleens of 20-week-old *lpr* (panels **a** and **b**), lpr^{cg} (panels **c** and **d**), *gld* (panel **e**) and wild-type (panel **f**) mice. The sections were hybridized *in situ* using ^{35}S-labeled antisense RNA for FasL (panels a, c, e and f). As controls, adjacent sections were hybridized using a ^{35}S-labeled sense RNA for FasL (panels b and d). Scale bar = 3 mm.

Cytotoxic activity of the lymphocytes accumulating in lpr mice

The FasL in *gld* mice carries a point mutation, and is non-functional (Takahashi *et al.*, 1994a), whereas the FasL mRNA in *lpr* or lpr^{cg} mice should encode a functional FasL protein. To examine whether the FasL mRNA which is abundantly expressed in the *lpr* mice codes for functional FasL, the cytotoxic activities of lymphocytes and splenocytes prepared from the lymph nodes and spleens of wild-type, *lpr* and *gld* mice were analyzed. To test the Fas-dependent cytotoxicity, mouse T cell lymphoma WR19L cells, which do not express Fas, and their transformant W4, which expresses Fas (Ogasawara *et al.*, 1993), were used as target cells. As shown in Figure 4, lymphocytes and splenocytes from wild-type mice were not cytotoxic against WR19L or W4 cells. On the other hand, both lymphocytes and splenocytes from *lpr* mice were cytotoxic against W4 but not against WR19L cells. This cytotoxic activity was inhibited by mFas-Fc, a soluble form of Fas, constructed by fusing the extracellular region of mouse

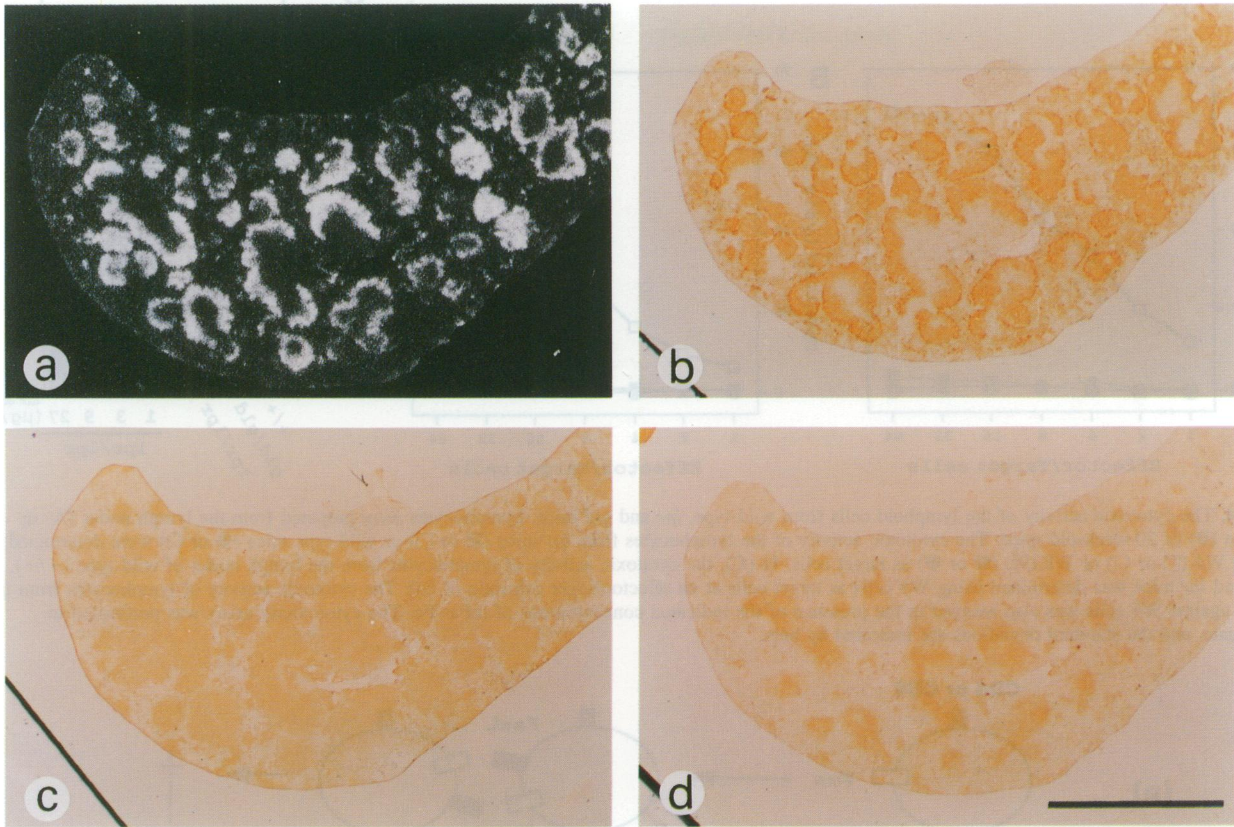

Fig. 3. Identification of FasL-expressing cells in the spleen of 20-week-old *lpr* mice. Adjacent cryostat sections were hybridized *in situ* using ^{35}S-labeled antisense RNA for FasL (panel **a**), and immunohistochemically analyzed using anti-B220 (panel **b**), anti-Thy-1 (panel **c**) or a mixture of anti-CD4 and anti-CD8 (panel **d**). Scale bar = 3 mm.

Fas to the Fc portion of human immunoglobulin (Suda and Nagata, 1994). These results indicated that the FasL constitutively expressed in the cells accumulating in *lpr* mice was functional. In contrast to the lymphocytes from *lpr* mice, lymphocytes from the lymph node of *gld* mice showed no cytotoxic activity against W4 cells (Figure 4c).

Discussion

We detected abundant constitutive expression of the FasL in the lymph nodes and spleen of aged *lpr* and *gld* mice, whereas lymphocytes and splenocytes from wild-type or young mutant mice expressed FasL very weakly. These results, together with the results of *in situ* hybridization and immunohistochemistry, indicated that the FasL gene is constitutively activated in the double-negative T cells accumulating in *lpr* or *gld* mice. The FasL in *lpr* mice is functional, whereas that in *gld* mice is non-functional, since *gld* mice carry a non-functional point mutation in the coding sequence of the FasL (Takahashi *et al.*, 1994a). The fact that the FasL in *lpr* mice has cytotoxic activity against the Fas-expressing cells agrees with the findings that *lpr* mice harbor cells with spontaneous cytotoxic activity (Muraoka and Miller, 1988; Wang *et al.*, 1993).

The expression of the FasL gene is normally induced by activation of T cells. Various groups have found abnormalities in the double negative T cells in these mutant mice (Altman, 1994). Samelson *et al.* (1986) found abnormal and constitutive tyrosine phosphorylation of a subunit of the T cell receptor in the double negative T cells of *lpr* mice, which is normally phosphorylated by activation of the T cells through the T cell receptor. The double negative T cells express large amounts of c-*myb*, c-*src* and c-*fyn* proto-oncogenes (Katagiri *et al.*, 1989a,b; Mountz *et al.*, 1984). These cells also express activation antigens such as Mel-14, CD69 and LFA-1 (Giese and Davidson, 1992). These data indicate that the double negative T cells accumulating in *lpr* and *gld* mice are previously or chronically activated, probably through the T cell receptor. Since these T cells seem to have passed the stage of CD4 or CD8 single positive cells (Cohen and Eisenberg, 1991), we propose the following model for development of lymphadenopathy in *lpr* and *gld* mice (Figure 5). Normal naive T cells express the CD4 or CD8 antigen and Fas. Interaction of the T cell receptor with the antigen complexed with MHC stimulates the T cells to proliferate and induces expression of FasL (T.Suda *et al.*, submitted). This activation may also make the cells susceptible to Fas-mediated apoptosis (Klas *et al.*, 1993; Owen-Schaub *et al.*, 1992). The activated T cells then interact, and kill each other through binding of FasL to Fas (Vignaux and Golstein, 1994). Alternatively, the cells expressing the FasL and Fas may kill themselves by an autocrine mechanism or by a suicide mechanism (Russell *et al.*, 1993; Russell and Wang, 1993). Such reactions may operate in the peripheral clonal deletion of T cells which are autoreactive to the self-antigen, or to remove mature T cells which have been activated by foreign antigens once and have completed their task. In *lpr* and *gld* mice, the T cells are activated through the T cell

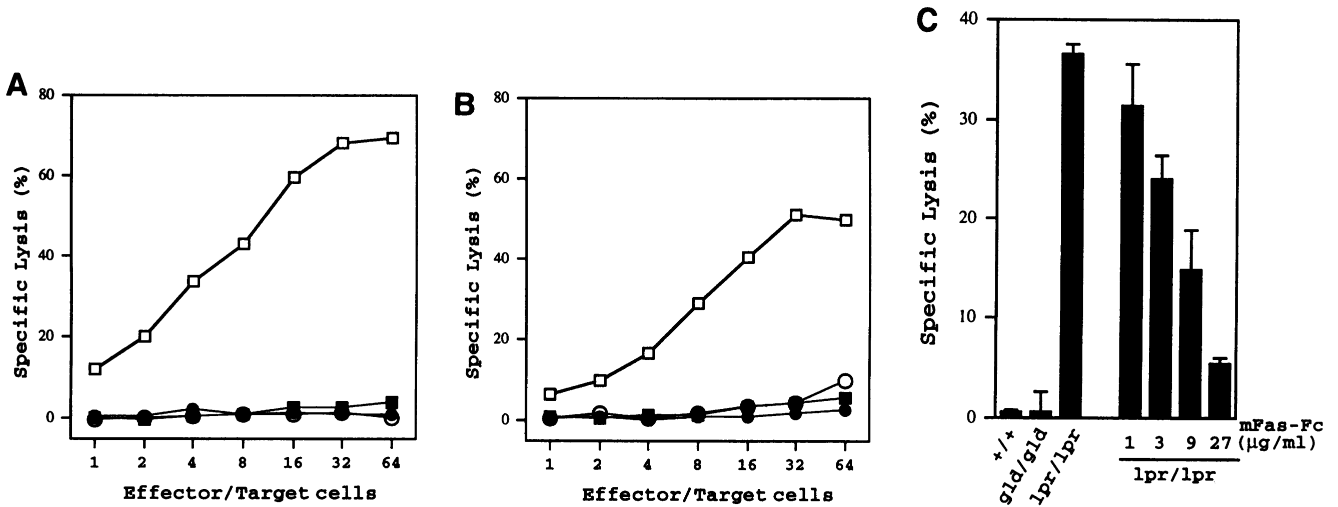

Fig. 4. The cytotoxic activity of the lymphoid cells from wild-type, *lpr* and *gld* mice. Lymphocytes were prepared from the lymph nodes (**A**) or spleen (**B**) of 20-week-old mice. The cytotoxic activity of the lymphocytes from *lpr* mice (■ or □) or wild-type mice (● or ○) were determined using W4 (□ or ○) or WR19L (■ or ●) as target cells. In (**C**), the cytotoxic activity of lymphocytes from the lymph nodes of wild-type (+/+), *gld* and *lpr* mice was determined using W4 cells as target cells at an effector/target cell ratio of 8.0. The cytotoxic activity of lymphocytes from *lpr* mice against W4 cells was also assayed in the presence of the indicated concentrations of mFas-Fc. The cytotoxicity assay was performed in triplicate, and the standard deviations are indicated by bars.

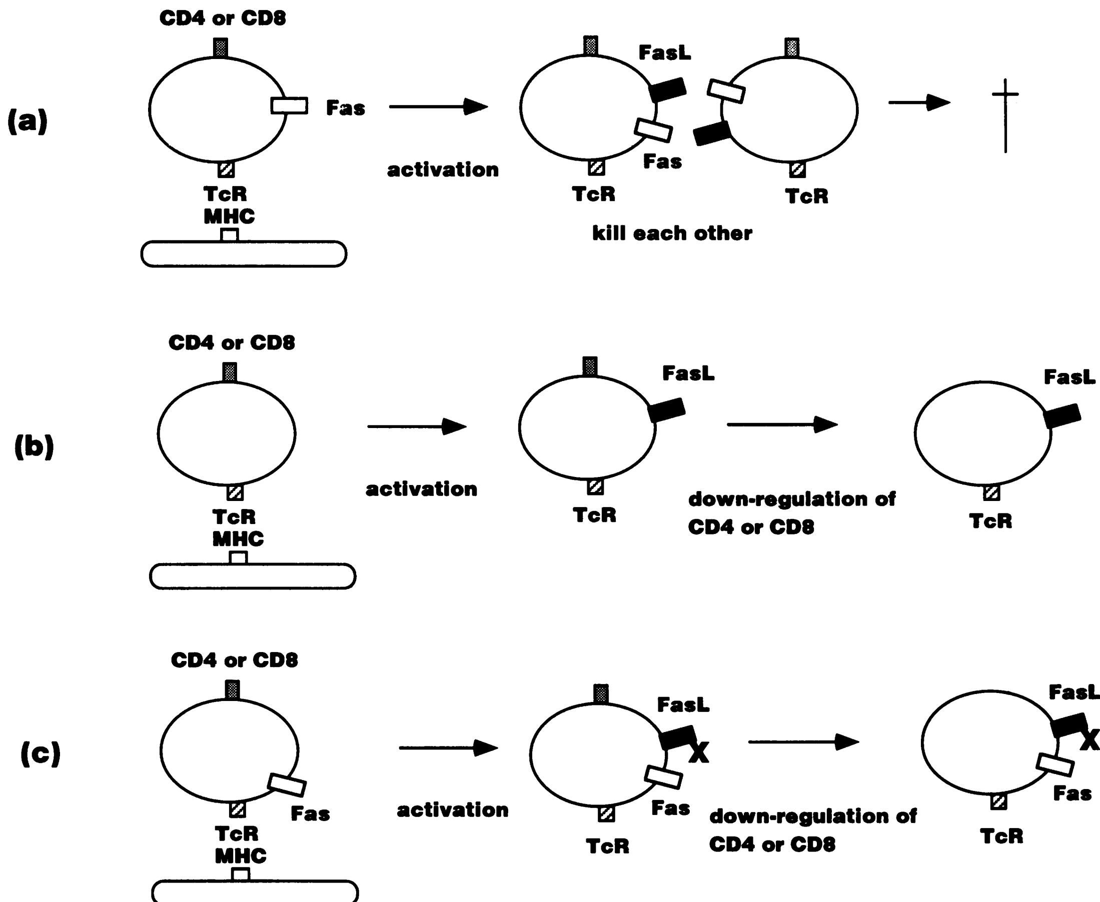

Fig. 5. Model for lymphoproliferation in *lpr* and *gld* mice. The process of deletion of activated T cells in the wild-type mice is schematically shown in (**a**). Circles indicate T cells, and long ellipses represent antigen presenting cells. In (**b**) and (**c**), the proposed processes underlying lymphoproliferation in *lpr* (b) and *gld* (c) mice are shown. In (b), *lpr* mice hardly express Fas, while lpr^{cg} mice express non-functional Fas. The 'x' on the FasL in (c) indicates a mutation of the FasL in *gld* mice.

receptor to proliferate and express FasL. However, since the T cells in these mutant mice have defects in either Fas or FasL, they cannot be killed, and remain activated. Later, these T cells may be anergized, stop cycling, and down-regulate CD4 or CD8 expression.

In addition to the massive accumulation of abnormal T

cells, *lpr* or *gld* mice produce large amounts of polyclonal IgG and IgM, indicating that B-lymphocytes are also abnormally activated (Cohen and Eisenberg, 1991; Sobel *et al.*, 1991). Since activated mature B cells express Fas (Trauth *et al.*, 1989; Drappa *et al.*, 1993), it seems that collaboration between T cells expressing FasL and activated B cells expressing Fas results in removal of the activated B cells. If this is so, B-cell-derived cells should accumulate in *lpr* or *gld* mice, but their accumulation is not pronounced compared with that of T cells. More detailed analysis of the expression of Fas and FasL and their actions upon lymphocytes would reveal not only the mechanism of disease development in these mutant mice, but also the molecular mechanisms of lymphocyte development.

When splenocytes or bone marrow cells of *lpr* mice are transferred into irradiated wild-type mice, the recipient mice develop a severe wasting syndrome (graft-versus host disease, GVHD) including liver damage, and die in 2–4 months post-transfer (Theofilopoulos *et al.*, 1985; Allen *et al.*, 1990; Fraziano *et al.*, 1994). Transfer of bone marrow cells from *gld* to irradiated wild-type mice results in the development of a *gld*-like autoimmune disease without a wasting syndrome (Allen *et al.*, 1990). These results can be explained by the constitutive expression of functional FasL in *lpr*-derived T cells. Fas, the receptor for FasL, is expressed in various tissues such as the liver, heart, lung and ovary (Watanabe-Fukunaga *et al.*, 1992b). We have shown that the administration of agonistic anti-Fas antibody into mice causes severe liver damage and rapid death (Ogasawara *et al.*, 1993). In wild-type mice receiving hemopoietic cells from *lpr* mice, the *lpr*-derived T cells express the FasL and bind to Fas in the normal tissues to cause apoptosis, which may result in a wasting syndrome. In the recipient of bone marrow cells from *gld* mice, the *gld*-derived T cells express abundant FasL, but it is non-functional. Therefore, it cannot bind to Fas in the tissues to cause the wasting syndrome. The *gld*-derived T cells express functional Fas, but functional FasL is not available in chimeric mice because it is expressed only on activated T cells and probably not in the radioresistant cells of the recipient mice. Thus, the *gld*-derived T cells accumulate in the chimeric mice to cause lymphadenopathy and splenomegaly. The co-transfer of normal bone-marrow and *gld* cells prevents the development of lymphadenopathy in the recipient (Ettinger *et al.*, 1994), which agrees with the above model. That is, the FasL expressed in co-transferred normal T cells should kill the *gld*-derived T cells that express functional Fas, preventing lymphadenopathy.

GVHD, a wasting syndrome similar to that observed by transfer of *lpr* bone marrow into wild type mice, occurs in a high proportion of humans receiving matched sibling or unrelated donor transplants. The acute phase of GVHD is initiated by mature alloreactive T cells of donor origin and results in the destruction of a number of target organs, including the skin, gut and liver (Martin and Kernan, 1990). Since the alloreactive T cells express FasL as an effector molecule (Rouvier *et al.*, 1993), it would be of interest to study whether FasL is also involved in the GVHD induced by allogenic bone marrow transplantation in humans.

Materials and methods

Mice and antibodies

MRL−+/+, MRL−*lpr*/*lpr* and C3H−*gld*/*gld* mice were purchased from Shizuoka Laboratory Animal Corporation (Hamamatsu, Japan). CBA/K1Jms−+/+ and CBA/K1Jms−lpr^{cg}/lpr^{cg} mice were provided by Dr A.Matsuzawa (Institute of Medical Science, University of Tokyo, Tokyo). Biotinylated rat monoclonal antibodies against mouse B220 (clone RA3-6B2), L3T4 (CD4; clone, RM4-5), Lyt-2 (CD8; clone 53-6.7) and Thy 1 (clone 30-H12) were purchased from PharMingen (La Jolla, CA).

RNA isolation and Northern hybridization

Total RNAs were extracted using guanidine isothiocyanate/acid phenol (Chomczynski and Sacchi, 1987). RNAs were denatured at 65°C for 5 min in 2.2 M formaldehyde and 50% deionized formamide, and electrophoresed through 1% agarose gels containing 2.2 M formaldehyde. RNA was transferred to a nitrocellulose membrane and hybridized with the probe DNA. The probe DNA was labeled with ^{32}P using [^{32}P]dCTP (Amersham) and a random primer labeling kit (Boehringer Mannheim). The probe DNAs used were a 0.94 kb *Xba*I fragment containing mouse FasL cDNA (Takahashi *et al.*, 1994a), a 1.5 kb *Eco*RI fragment carrying mouse Fas cDNA (Watanabe-Fukunaga *et al.*, 1992b), or a 1.8 kb *Bam*HI fragment containing human EF-1α cDNA (Uetsuki *et al.*, 1989). Hybridization proceeded as described (Sambrook *et al.*, 1989) under conditions of high stringency.

In situ hybridization and immunohistochemistry

Fresh mouse tissues were washed with phosphate-buffered saline (PBS) and immediately embedded in Tissue-Tek[A] OCT compound (Miles, USA), then frozen at −20°C. Cryostat sections (20 μm) were prepared in horizontal planes, and mounted on poly-L-lysine-coated slides.

In situ hybridization proceeded essentially as described (Hashimoto *et al.*, 1993). In brief, spleen sections were fixed with 3% formaldehyde for 10 min, rinsed in PBS, and treated with 0.25% acetic anhydride for 10 min at room temperature. After rinsing, the slides were dehydrated through an ascending series of alcohol and air-dried. The probe was a 0.94 kb *Xba*I fragment carrying the entire coding sequence of mouse FasL (Takahashi *et al.*, 1994) subcloned into pBluescript, and ^{35}S-labeled antisense or sense RNAs was prepared by *in vitro* transcription using T3 or T7 RNA polymerase and [$\alpha^{35}S$]CTPαS (New England Nuclear). The labeled product (specific activity ~1.0×10^9 c.p.m./μg RNA) was fragmented by incubation in hydrolysis buffer (40 mM $NaHCO_3$, 60 mM M Na_2CO_3, pH 10.2) at 60°C for 60 min, then added to the hybridization buffer (50% formamide, 2×SSC, 10 mM Tris−HCl (pH 8.0), 1× Denhardt's reagent, 10% dextran sulfate, 0.2% SDS) at a concentration of 1.0×10^5 c.p.m./μl. The slides were coversliped and incubated at 58°C for 16 h in a moist chamber. Thereafter, the slides were washed with 1×SSC, digested with 20 μg/ml RNase A (Boehringer Mannheim) at 37°C for 30 min, and rinsed with 0.1×SSC containing 10 mM β-mercaptoethanol at 60°C for 1 h. After dehydration in an ascending series of alcohol, the slides were air-dried and exposed for 2–4 weeks to Kodak T-MAT.

For immunohistochemical analysis, a series of cryostat sections was washed with PBS, then incubated with biotinylated mAbs for B220, Thy-1, CD4 or CD8 at a dilution of 1:100 for 30 min at room temperature. After washing with PBS, binding of monoclonal antibodies to the sections was visualized using peroxidase-labeled biotin/avidin detection systems (Elite, Vectastain) with diaminobenzidine as the substrate.

Cytotoxicity assays

To prepare lymphocytes, lymph nodes or spleens surgically excised from mice were pressed between glass slides, and the cells were suspended in RPMI1640 medium containing 10% fetal calf serum (FCS). The cell suspensions were then filtered through nylon mesh to remove debris. Erythrocytes of the spleen cells were lysed by treating with 17 mM Tris−HCl buffer (pH 7.2) containing 140 mM NH_4Cl. The cells were washed with PBS and finally resuspended in RPMI1640 medium containing 10% FCS. Cytotoxic activities of the cells were determined as described previously (Suda and Nagata, 1994) using mouse WR19L cells and their transformants expressing mouse Fas (W4) (Ogasawara *et al.*, 1993). In brief, 1×10^6 WR19L or W4 cells were labeled with ^{51}Cr by incubation at 37°C for 2 h in RPMI1640 medium containing 20 μCi [^{51}Cr]sodium chromate (Amersham). ^{51}Cr-labeled cells (1×10^4) were mixed at various ratios with lymphoid cells freshly isolated from the lymph node or spleens, and the specific release of ^{51}Cr was determined after incubation for 4 h at 37°C as described (Suda and Nagata, 1994).

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