Massive Upregulation of the Fas Ligand in lpr and gld Mice: Implications for Fas Regulation and the Graft-versus-Host Disease–like Wasting Syndrome

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Summary

Fas-deficient lpr and gld mice develop lymphadenopathy due to the accumulation of T cells with an unusual double negative (DN) (CD4−CD8−) phenotype. Previous studies have shown that these abnormal cells are capable of inducing redirected lysis of certain Fc receptor–positive target cells. Since the Fas ligand (FasL) has recently been shown to be partly responsible for T cell–mediated cytotoxicity, lymph node cells from lpr and gld mice were examined for the expression of FasL mRNA. Northern blot analysis revealed that lymph node cells obtained from lpr and gld mice had a striking increase in the level of expression of FasL mRNA predominantly due to expression in the DN T cells. Furthermore, lpr, but not gld lymph node cells killed the B cell line, A20, in a Fas-dependent manner. These findings indicate that Fas mutations result in a massive up-regulation of FasL which, most likely, results from repetitive exposure to (self) antigen. This phenomenon could explain the lpr–induced wasting syndrome observed when lpr bone marrow–derived cells are adoptively transferred to wild-type recipients.

Materials and Methods

Mice with spontaneous mutations of the Fas receptor (FasR) (MRL/lpr and CBA/lpr§) or its ligand (FasL) (C3H/gld) develop lymphadenopathy and systemic autoimmunity (1, 2). While these phenotypic abnormalities can be explained by defects in Fas-mediated lymphocyte apoptosis, the pathogenesis of a chronic GVHD-like wasting syndrome induced by the adoptive transfer of lpr bone marrow into syngeneic hosts (3, 4) remains to be clarified.

Coculture of normal- and lpr–derived T cell lines results in the loss of the normal T cells in in vitro cultures (5). Since the FasL has recently been shown to have a cytotoxic effector function (6), overexpression of FasL in lpr lymphocytes could explain these in vitro findings as well as the wasting syndrome induced by lpr bone marrow transfers (3, 4). In this report we directly demonstrate that FasL is markedly increased in the lymphoid tissues derived from older lpr and gld mice. The increased FasL expression is most likely due to repeated antigenic stimulation of T cells that cannot undergo apoptosis through the Fas pathway.

Cell Culture and Subset Isolation. Single cell suspensions were prepared from the thymus, spleen, lymph nodes and, in some cases, bone marrow from different strains of mice. To induce expression of FasL, splenic T cells were activated by Con A (20 ng/ml) (8). The cells were cultured at 37°C in a humidified atmosphere in 5% CO2 at a density of 106 cells/ml in RPMI 1640 containing 10% FCS and 2-ME. After 2 d in culture, the cells were further stimulated by PMA (10 ng/ml) and ionomycin (500 ng/ml) for 4 h. A toxic shock syndrome (TSST)-reactive T cell line (>99% CD4+) isolated from splenic T cells from an MRL/++ mouse was kindly provided by Dr. S. Friedman and J. Tumang, Hospital for Special Surgery.

Cell subsets were isolated from the enlarged lymph nodes of 4-5-mo-old lpr mice by negative selection. To obtain an enriched population of CD4−, CD8− double negative (DN) T cells, CD4− and CD8− single positive (SP) T cells were depleted by incubation of total lymph node cells with a cocktail of anti-CD4 and anti-CD8 mAb for 45 min at 4°C followed by incubation with guinea pig complement (Cedarlane Laboratories, Inc., Hornby, Canada) for 1 h at 37°C. Residual SP T and B cells were removed by magnetic beads (Dynal, Oslo, Norway) coated with sheep anti-rat Ig. SP lymph node T cells were enriched by negative selection. DN T cells were coated with the anti-B220 mAb as well as B cells.

Discussion

The increased FasL expression is most likely due to repetitive exposure to (self) antigen. This phenomenon could explain the lpr–induced wasting syndrome observed when lpr bone marrow–derived cells are adoptively transferred to wild-type recipients.
were depleted by three successive rounds of panning at 4°C using polystyrene plates coated with anti-rat Ig. Residual antibody-coated cells were depleted with anti-rat Ig-coated magnetic beads as described above.

Cytotoxicity Studies. Unfractionated lymph node cells were used as effector cells and the Fas+ and Fas- B cell lines, A20 (9) and 1880 (10) were used as targets. 107 target cells were labeled with 51Cr for 2 h at 37°C. freshly isolated lymph node cells were incubated with 106 51Cr-labeled target cells in triplicate at E/T ratios of 80, 40, 20, and 10:1 in round bottom 96-well microtiter plates at 37°C. After 4-6 h, the cells were pelleted by centrifugation and the supernatant counted in a γ counter. 51Cr release was calculated from the formula: 100 x (test release – spontaneous release)/(maximum release – spontaneous release).

cDNA Probes and DNA Sequencing. A 1.5-kb cDNA probe encoding the FasR was produced from MRL+/+ thymus as described previously (11). To obtain cDNA probes encoding the FasL from rat (8) and mouse (2), splenocytes from a Sprague-Dawley rat (activated with Con A [2 μg/ml] for 24 h at 37°C) and unmanipulated lymph node cells from an MRL/lpr mouse, were obtained. Total RNA was isolated by the acid guanidinium method (12) and cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase and oligo-dT as described (11). cDNA was amplified by PCR on a 480 thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT) with Taq polymerase. The primers used for PCR amplification of the FasL were (all in 5'-3' orientation): rat, sense CAACCTTTTACTCTACAGAAGGAACT and antisense CAGCAGCCCATGAAATTAC; mouse, sense CATG-... (all from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Results and Discussion

FasL Expression Is Strikingly Increased in the Lymphoid Organs of Older lpr and gld Mice. Adoptive transfers of lpr (1, 2), but not gld (14), bone marrow induces a GVHD-like wasting syndrome in wild-type recipients. Similarly, cocultures of MRL/lpr and normal T cells, but not MRL/gld and normal T cells, result in the reduced survival of the normal T cells (5) in vitro. Both of these findings could be explained by the expression of a cytotoxic molecule on lpr, but not gld, T cells. Since the FasL has recently been shown to induce apoptosis on FasR+ targets (8) and the lymph node DN T cells of MRL/lpr mice induce redirected lysis on FcR-positive target cells (6), we examined the possibility that DN T cells express high levels of FasL.

When the freshly isolated lymphoid organs from 4-5-mo-old normal mice were analyzed for the expression of FasL mRNA by Northern blot analysis using the rat FasL probe, little or no FasL expression was observed in the thymus, spleen (Fig. 1), or lymph nodes (not shown), in agreement with the report by Takahashi et al. (2). This finding differs from FasR where the levels of receptor expression are modulated at varying stages of lymphocyte maturation (13, 15). In contrast, massive upregulation of the FasL mRNA was observed in the thymus and lymph nodes (∼50-fold) and, to a lesser extent, the spleens (∼20-fold) obtained from 4-5-mo-old lpr strains of mice (MRL and CBA) as well as gld mice (Fig. 1). Similar levels of FasL mRNA expression were observed in individual organs in all three strains at this age. The levels of FasL expression were barely above background in freshly isolated, normal splenic lymphocytes activated by Con A (Fig. 1) or Con A, PMA, and ionomycin (Fig. 2 A) or splenic...
lymphocytes obtained from untreated 6-12-mo-old MRL/++ mice (not shown) indicating that the striking levels of FasL expression in the mutant mice are unlikely to be explained by mild or transient cell activation. The levels of FasL expression in lpr and gld lymph nodes were approximately twofold higher than that observed in the superantigen-reactive, MRL/++ CD4+ T cell line triggered with PMA and ionomycin or superantigen 4 h before RNA isolation (Fig. 2 B). Since the level of FasL expression would be expected to be fairly uniform in the recently activated T cell line, this finding suggests that the FasL is expressed at high levels in the majority of lymph node cells.

Since the nucleotide sequence of the rat FasL (8) was published before that of the mouse FasL (2), Northern blots were initially performed with the rat FasL probe. To confirm the results, all the blots were stripped, reprobed with the mouse FasL probe, and washed under high stringency conditions. The size of the FasL (~2 kb) and relative intensities of the signals between samples were identical for the rat and mouse probes (Fig. 1).

**Figure 2.** FasL mRNA expression at different ages and in cell subsets. (A) CD4+ and CD8+ single positive cells (SP) (96% purity) were isolated by depletion of B220+ cells from lymph nodes obtained from a 4-mo-old MRL/lpr mouse. The lpr thymocytes were obtained from 4-wk-old MRL/lpr and CBA/lpr mice and can be compared to FasL expression in a 4-mo-old C3H/gld mouse (lane 1). Splenocytes obtained from a 3-mo-old CBA mouse were incubated with Con A (2 μg/ml) and IL2 (20 ng/ml) for 2 d followed by PMA (10 ng/ml) (P) and ionomycin (500 ng/ml) (I) for 4 h. (B) A CD4+ TSST-I-reactive T cell line derived from an MRL/++ mouse was activated by TSST-I and rested for 7 d. After 7 d, the line was either stimulated with PMA and ionomycin (TCL + P + I) (as in A), retriggered with TSST-I in the presence of irradiated splenic antigen presenting cells (TCL + SP), or used without further stimulation (TCL). Total lymph node (LN) cells and double negative (DN) lymph node cells were obtained from 4-mo-old lpr mice as described in Materials and Methods. As a control, RNA was obtained from the spleen of a 1-yr-old C3H mouse (C3H). Northern blot analysis was performed as in Fig. 1.

FasL Is Expressed on Both SP and DN lpr and gld Lymph Node T Cells. The lymph nodes of older lpr and gld mice comprise predominantly DN T cells (16). We therefore examined whether FasL expression was present exclusively in the DN T cell subset. After SP and DN T cell isolation by negative selection, flow cytometry analysis revealed that the purity of the isolated populations varied between 80 and 96% (results not shown). When cells that were >95% pure were analyzed for FasL expression by Northern blot analysis, the level of FasL expression in the DN T cell population (Fig. 2 B) was similar to, and the SP T cell population (Fig. 2 A) approximately half the level in total lymph node. It has recently been suggested that DN T cells arise from SP T cells that have been activated by self antigen and induced to downregulate the CD4 or CD8 coreceptor (17). High levels of FasL expression on DN T cells would therefore be expected if, as discussed below, FasL is upregulated after antigenic stimulation and the cells fail to die. We propose that lymph node SP T cells that also express increased FasL have encountered antigen and are destined to downmodulate their coreceptors. Since the thymus of these mouse strains become progressively infiltrated with mature T cells (18), increased FasL expression in the thymus of older mice may be explained by similar mechanisms.

**Regulation of the Fas Receptor and Ligand.** Based on the results of adoptive bone marrow transfer experiments, Allen et al. (14) first proposed that the lpr and gld phenotypes resulted from mutations in a receptor ligand pair. They also postulated that the receptor and ligand were reciprocally regulated. The increase in FasL expression observed in MRL/lpr mice is compatible with a feedback model of regulation since MRL/lpr mice express little or no FasR protein (13, 19) due to the addition of a retrotransposon in the FasR gene (11, 20, 21). It was reasoned that if there was reciprocal regulation of the receptor ligand pair, then FasL expression should also be increased in young MRL/lpr mice. However, as shown in Fig. 2 A, ligand expression was virtually undetectable in the thymus and spleen of 4-wk-old lpr mice. Furthermore, the increase in FasL expression was comparable in 4-mo-old lpr and C3H/gld mice (with a mutated FasL [2]) (Fig. 1). To explore this hypothesis more thoroughly, we rehybridized the Northern blots with the FasR cDNA probe (Fig. 1). As expected, FasR mRNA expression in tissues obtained from MRL/lpr mice was very low (Fig. 1). In contrast to the striking upregulation of the FasL observed in lpr and gld mice, the level of FasR expression was not significantly increased in the lymphoid organs of older mice (Figs. 1 and 3). Significantly, FasR mRNA expression in C3H/gld mice was not higher when compared to CBA/lpr or normal mice (Fig. 3). Taken together, these data indicate that Fas receptor and ligand are not reciprocally regulated in lpr and gld mice.

**Cytotoxic Activity of lpr Lymph Node Cells and Relevance to the lpr-induced Wasting Syndrome.** As discussed above, DN T cells have previously been shown to exhibit cytotoxic activity in a redirected cytotoxicity assay although little or no killing was observed in the absence of antibodies to the effector cells (6). Since DN cells expressed perforin mRNA, it was assumed that the cytotoxicity was mediated through the perforin
The striking increase in FasL mRNA expression and FasL-mediated cytotoxic activity of lpr-derived lymph node cells strongly suggest that expression of the ligand is a major factor responsible for the in vivo wasting syndrome and in vitro loss of normal cells in lpr cocultures discussed above. These findings do not exclude a role for other cytokines known to be secreted by lpr DN T cells (24). The relatively mild induction of wasting after the adoptive transfer of lpr bone marrow into wild-type hosts may be explained by the binding of the FasL to the defective FasR of the donor lymphocytes leaving little free cytotoxic FasL to damage host cells. If this explanation is correct, then FasL must be expressed on radiosensitive cells of the bone marrow recipients. Immunohistochemical analysis of human tissue indicate that, in addition to lymphocytes, FasL (APO-1) is expressed on various mesenchymal and histiocytic cells, especially in areas of inflammation (25). Interestingly, FasL was also detected in the skin, liver, and gastrointestinal tract (25): target organs for GVHD.

We propose that after adoptive transfers of lpr bone marrow, mature lpr-derived T cells are exposed to (self) antigen and upregulate FasL expression. Since these cells with abnormally high levels of the ligand cannot die by the Fas pathway, they persist and induce apoptosis on radiosensitive host cells or release cytokines such as γ-IFN which upregulate FasR ex-

### Figure 4

4. lpr, but not gld, lymph node cells are cytotoxic. (A) Lymph node cells from 4-mo-old CBA/lpr (lpr) and C3H/gld (gld) mice were isolated and used as effectors (E) in a cytotoxicity assay. The FasR⁺ B cell line, A20, and the FasR⁻ B cell line, 1881, were labeled with ⁵¹Cr and used as targets (T). The cells were incubated at 37°C for 4–6 h, centrifuged, and the specific lysis calculated. Cytotoxicity was also performed in the presence of 3 µg of FasR fusion protein (FP). The results shown are representative of three experiments performed. (B) A20 and 1881 target cells were prepared as in A and were incubated with the anti-FasR antibody, Jo2, for 4–6 h at 37°C. Specific lysis was calculated as in A.
pression on mesenchymal cells. Apoptosis of host cells results in the removal of supportive stromal and other mesenchymal cells necessary for survival of the immune system. Induction of apoptosis in FasR* cells in the skin, liver, and gastrointestinal tract therefore simulates GVHD and suggests that the Fas cytotoxic pathway may also be involved in GVHD.

Implications for Fas Regulation and Apoptosis of Activated Lymphocytes in Normals. The levels of expression of FasL and FasR in normal mice and the changes in expression noted in Fas mutant mice suggest the following series of events. When normal T cells are activated, they express FasR (as shown [13, 26, 27]) and FasL (8). However, since the receptor is not functional for several days after activation (28), the cells undergo clonal expansion. As the FasR becomes functional, the ligand kills activated cells. In view of the marked difference in the level of FasL expression in mitogen-activated total splenic lymphocytes and mitogen-activated T cell line noted here, repetitive stimulation or differentiation of T cells toward the Th1 subset (29) may be required for maximal ligand expression. It would be expected that most of the killing is "fratricide" although, since receptor and ligand can both be expressed on activated T cells, a "suicide" pathway cannot be excluded. In this way, the Fas pathway limits expansion of activated cells and would be expected to be important for the removal of cells with potential for self reactivity.

While the results obtained in this and a previous study (8) indicate that FasL can kill in an antigen-independent fashion, the role of antigen recognition and other costimulatory signals remain important areas for further study.

We thank A. Parnassa and X. Song for additional technical support.

This work was supported in part by grants P50 AR-425888 and P50 AR-38520 from The National Institutes of Health. J. Nikolić-Zugić is a Pew Scholar in Biomedical Sciences and P. Ramos is a recipient of the Fondo de Investigaciones Sanitarias, Ministerio de Sanidad de España.

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Received for publication 18 August 1994 and in revised form 21 September 1994.

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398  Fas Ligand Expression in lpr and gld Mice