Genetic Analysis of MRL/lpr Mice: Relationship of the Fas Apoptosis Gene to Disease Manifestations and Renal Disease-modifying Loci

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Summary

In MRL mice, the mostly recessive lpr mutation results in both the accumulation of CD4−, CD8−,CD3+ T cells in lymphoid tissue and many features of generalized autoimmune disease, including immune complex glomerulonephritis. To positionally clone the lpr mutation and analyze the effects of background genes, backcross offspring were examined from the cross: (MRL/MpJ-Ipr x CAST/Ei)F1 x MRL/MpJ-lpr. The lpr gene was found to be closely linked to a mouse chromosome 19 marker defined by a variation of a Fas gene restriction fragment. Our results identified differences in IL1α expression and differences in the genomic organization of the Fas gene between normal and lpr mice, and confirm the recent report that a mutation in the Fas apoptosis gene is the lpr mutation. However, our results also indicate that the Fas gene is expressed in spleen cells from normal mice, and spleen and lymph node cells from mice with a second mutation at the lpr locus (lpr%). Together these results suggest that altered Fas transcription results in the failure of lymphocytes to undergo programmed cell death and may lead to an altered immune cell repertoire. This mechanism may explain certain central and peripheral defects in tolerance that are present in autoimmune disease. The current study also demonstrates the profound effect of background genes on the degree of nephritis, lymphadenopathy, and anti-DNA antibody production. Of major note, our studies suggest the identification of chromosomal positions for genes that modify nephritis. Analysis of the backcross mice for markers covering most of the mouse genome suggests that over 50% of the variance in renal disease is attributable to quantitative trait loci on mouse chromosomes 7 and 12. Moreover, this study provides a model for dissecting the complex genetic interactions that result in manifestations of autoimmune disease.
were immune complex nephritis, and synovitis (reviewed in references 2 and 6). MRL mice without the lpr mutation develop a late onset autoimmune syndrome suggesting that lpr accelerates rather than causes disease (2). This suggestion is further supported by the finding that laboratory strain mice that carry the lpr mutation develop much less profound manifestations of autoimmune disease than MRL/lpr mice (7). Homozygous lpr mice are phenotypically similar to mice homozygous for the non-allelic gld (generalized lymphoproliferative disease) mutation (8), and the compound lpr+/gld+/heterozygote is phenotypically normal (2). However, a new mutation at the lpr locus, lpr\(^s\), has recently been described that complements gld (9). Although mice with the lpr, lpr\(^s\), or gld mutations may not mimic precisely a specific human autoimmune disease, they nevertheless represent excellent models of SLE with regard to serologic and immunopathologic features of disease (6).

The lymph nodes of most lpr/lpr mice are heavily populated with dull Thy-1\(^+\), dull CD5,CD4\(^-\),CD8\(^-\) cells. These cells have nonclonal rearrangements of TCR genes (8, 10). In addition, the cells have high expression of cell surface antigens CD45(B220), Ly-6, CD44, and PC-1, and absent expression of surface (s)Ig, Th B, and lα (8). Previous studies also have established that lymph node cells from lpr/lpr mice express large amounts of the myb proto-oncogene, which is ordinarily only expressed at high levels in the thymus or after mitogenic stimulation of T cells (11). In addition, lpr/lpr peripheral T cells display abnormalities in phosphorylation of part of the TCR complex (12). Thus, these cells represent a unique abnormal T cell subset not seen in easily detectable numbers in normal mice. However, these abnormalities may reflect secondary and tertiary events that are part of a cascade resulting from the primary defect. In fact, most studies have failed to show that the expanded double-negative T cell population found in lpr/lpr mice has any function (13).

Although the lpr mutation derived its name from the hypothesis that it causes lymphoproliferation, subsequent studies have failed to show increased mitotic activity of the abnormal cells in vivo (14). Rather, results have led to the suggestion that the accumulation of the abnormal T cells is derived from a defect in the thymic selection process, because most pre-T cells are destined to die by apoptosis (15). Analysis of mice transgenic for a TCR specific for the H-Y plus self-antigen also has suggested a partial abnormality in negative intrathymic selection (16). Examination of TCR VB repertoire in lpr/lpr mice similarly has suggested a partial abnormality in positive selection (17, 18).

The current study was initiated in a dual effort to positionally clone the lpr mutation and define the effects of background genes in the manifestation of disease. The analyses presented here of linkage relationships, genomic structure, and RNA expression confirm the suggestion of Watanabe-Fukunaga et al. (19) that a mutation in the Fas gene is the lpr mutation (19). Our results differ from theirs, however, in methodology and in important details concerning Fas gene expression. Moreover, our study examines the complex genetics of lpr-associated autoimmune disease, and strongly suggests the identification of renal disease modifying loci on Chr 7 and 12, explaining much of the large variation in renal disease observed in backcross mice homozygous for the MRL/Mpj-lpr allele.

### Materials and Methods

#### Mice

MRL/Mpj-lpr (MRL/lpr), MRL/Mpj-+ (MRL+), Mus musculus castaneus (CAST/Ei), and (MRL/lpr × CAST/Ei)F\(_1\) mice were bred at The Jackson Laboratory (Bar Harbor, ME). CBA/K1Jms (CBA) and CBA/K1Jms-lpr\(^s\) (CBA-lpr\(^s\)) mice were maintained at the Laboratory Animal Research Center, University of Tokyo. Reciprocal backcross mice produced from mating involving (MRL/lpr × CAST/Ei)F\(_1\), and MRL/lpr mice were produced at The Jackson Laboratory and maintained along with parental control mice at the Duke University Vivarium barrier facility.

#### Phenotypic Markers

Three measurements were performed (total lymphoid weight, degree of nephritis, anti-DNA antibody titer) as terminal studies on MRL/lpr, (MRL/lpr × CAST/Ei)F\(_1\), and the [(MRL/lpr × CAST/Ei)F\(_1\) × MRL/lpr] interspecific backcross mice at 20 wk of age. To minimize the variance in the genetically identical parental mice, both the lymphoid mass and nephritis index were expressed as log transformations. The terms LPR and non-LPR phenotype are used within the manuscript to refer to mice that have phenotypes characteristic of MRL/lpr (lpr/+) or (MRL/lpr × CAST/Ei)F\(_1\) (lpr/++) mice, respectively.

**Lymphoid Mass Index.** This value represents the log\(_10\) of the total lymphoid (lymph node plus spleen) weight (in grams) plus a constant of 0.97. The constant was added to minimize the effect of small variation in weight measurements among phenotype-negative animals. The addition of this constant before log transformation yields a minimum value of zero for this index.

**Nephritis Index.** Kidney sections were stained with hematoxylin and eosin and the histopathology assessed by a single observer. This nephritis index represents the log\(_10\) of the additive scores of three measurements (total lymphoid weight, degree of nephritis, anti-DNA antibody titer) as terminal studies used to determine the extent of renal disease. Anti-DNA Antibodies. IgG antibodies to single-stranded (ss) and double-stranded (ds) DNA were assayed by ELISA using calf thymus DNA (Sigma Chemical Co., St. Louis, MO) coated on 96-well polystyrene microtiter plates (Dynatech, Chantilly, VA) as previously described (20). Briefly, sera were added in serial dilutions in PBS-Triton X-100 (PBS-T) (Sigma Chemical Co.) followed by goat anti–mouse IgG peroxidase (Sigma Chemical Co.) diluted in PBS-T. After incubation, trimethylene-bis (4-formylpyridinium) (TMB) substrate (Sigma Chemical Co.) was added, and the absorbances at 380 nm were determined on a plate reader. ssDNA used...
as antigen was obtained by boiling for 10 min before immediate immersion in ice. dsDNA was obtained by treating the DNA preparation with S1 nuclease. The dsDNA preparation used showed no reactivity by ELISA using a mouse mAb specific for ssDNA.

Southern Blot Hybridization

DNA from backcross mice was extracted from tissues using standard techniques and digested with restriction endonucleases (Boehringer Mannheim Biochemicals, Indianapolis, IN). 10 μg of each sample was subjected to electrophoresis on 0.9% agarose gels and transferred to Nytran membranes (Schleicher & Schuell, Inc., Keene, NH). Hybridization of probes onto membranes was accomplished at 65°C and washed under stringent conditions (21) for mouse probes (0.2x SSC at 65°C), and reduced stringency (0.5x SSC at 55°C) for human probes.

Hybridization Probes

All probes were labeled by a hexanuclide technique with α-
[^32P]dCTP (3,000 Ci/mmoll; New England Nuclear, Boston, MA) using an oligolabeling kit and protocol from Pharmacia Fine Chemicals (Piscataway, NJ). 97 clones that define 108 loci were used to establish informative markers on each mouse autosome. The clones used for specifically cited cosmosomal markers are listed in Table 1. The unpublished clones included: L15 for Lyw-57 (lymphocyte antigen workshop 57, kind gift of P. Cohen, University of North Carolina, Chapel Hill, NC); BMT008 for D12SelI (DNA segment, Chr 12, Seldin-1), a clone randomly selected from a mouse thymus cDNA library (Stratagene, La Jolla, CA); and MJ55 for D19SelI (DNA segment, Chr 19, Seldin-3), a genomic clone derived from a flow sorted mouse Chr 19 library (38). In addition, several clones were derived by PCR amplification of Fas sequences from a mouse thymus cDNA library (Stratagene). Clones include: FasL1R2, a 3' PCR-amplified probe that contains bp 646-1,405 of the published cDNA sequence (39); FasL2S2, a 5' PCR-amplified probe that included bp 32-239; and FasS1S9, a PCR-amplified probe that includes bp 220-665. A γ-actin clone (40) was used as a control for Northern blot analysis.

Genomic and cDNA inserts from each clone were prepared by restriction endonuclease digestion and gel purification, or generated by PCR using oligonucleotide primers derived from plasmid sequences flanking the insert cloning site. Restriction fragment length polymorphisms (RFLPs) were defined that distinguished the two parental strains, MRL-Ipr and CAST/Ei; those used in segregation analyses are listed for specifically cited clones in Table 1.

Northern Blot Analysis

Total RNA was prepared from mouse tissues and separated on formaldehyde agarose gels according to standard techniques (21). Briefly, 20 μg of total RNA was loaded onto 1% agarose gels and separated at 1/5/cm for 14 h, stained with acridine orange, and transferred to Nytran Plus nylon membranes. Northern blots were hybridized under the same conditions as described in Southern Blot Hybridization, and washed at high stringency.

PCR Assays

The presence of the Fas gene transcript was assayed using PCR. First, polyadenylated RNA was reverse transcribed with AMV reverse transcriptase (Boehringer Mannheim Biochemicals) and oligo(dT) primers. First-strand cDNA was quantified and subsequently used as template in a 50-μl PCR reaction containing 1-20 ng of cDNA, 1.0 μM of primers, 200 μM dNTPs, 10 mM Tris-}

HCl, pH 8.3, 50 mM KCl, and 1.25 U ampliTaq polymerase (Perkin Elmer Cetus, Norwalk, CT). Primers used spanned nucleotides 646-675 (L1), 1385-1405 (R2), 32-52 (L2), 239-220 (S2), 220-239 (S1), and 470-451 (S4) of the published mouse Fas sequence (40). The standard PCR cycling profile consisted of 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR samples without template or Tag polymerase were irradiated for 3 min with short-wave UV light 5 cm from the source before temperature cycling. 10 μl of the completed PCR reaction was subjected to electrophoresis on 1.5% agarose gels for analysis.

Linkage and Quantitative Trait Locus Analysis

Gene linkage and genetic maps were determined applying the MAPMAKER computer package using the Kosambi mapping function (41). The MAPMAKER QTL computer package developed by Lander and Botstein (42) was used to analyze the backcross data for the presence of renal disease modifying loci. This computer package divides each chromosome into intervals between mapped markers and then calculates the maximum likelihood that a quantitative trait locus (QTL) is present within these intervals. This program examines the intervals between mapped loci and calculates the most likely phenotypic effect if a linked QTL is present. A logarithm of odds (LOD) score is calculated that reflects the strength of evidence for the presence of a QTL. The program also computes the proportion of variance that can be explained by the putative QTL. Several considerations are important in determining the appropriate level of significance, including map length and interval size. For the current study, which examined ~1,100 cM at a median interval of ~12 cm, a LOD of >2.5 was determined to correspond to an overall false-positive rate of <5% (see reference 42).

Results

Close Linkage of the Fas Gene and the lpr Mutation.

To confirm the chromosomal assignment of the lpr gene and further define linkage relationships necessary for positional cloning, (MRL-Ipr × CAST/Ei)F1 × MRL-Ipr backcross mice were analyzed. CAST/Ei, an inbred strain derived from Mus musculus castaneus mice, rather than an inbred laboratory strain, was chosen as the second parent to enhance the identification of variant genetic markers. The phenotype of individual mice (LPR vs. non-LPR) was determined at 20 wk of age by measurement of lymph node plus spleen weight (lymphoid mass) and anti-dsDNA autoantibody levels. These segregation analyses indicated that the expression of the lpr gene was influenced by background genes. For example, only 48 of 182 backcross mice had lymphoid mass index values within 2 SD of the mean of MRL-Ipr and CAST/Ei; those used in segregation analyses are listed for specifically cited clones in Table 1.

PCR Assays

The presence of the Fas gene transcript was assayed using PCR. First, polyadenylated RNA was reverse transcribed with AMV reverse transcriptase (Boehringer Mannheim Biochemicals) and oligo(dT) primers. First-strand cDNA was quantified and subsequently used as template in a 50-μl PCR reaction containing 1-20 ng of cDNA, 1.0 μM of primers, 200 μM dNTPs, 10 mM Tris-
| Genes                                           | Locus | Clone       | Reference | Restriction enzyme | MRL-lpr bands | CAST/Ei bands |
|------------------------------------------------|-------|-------------|-----------|--------------------|---------------|---------------|
| β-1 adrenergic receptor                        | Adrb-1| β-1         | 22        | TaqI               | 2.3           | 2.1           |
| DNA segment, mouse Chr 12, New York University 3|       |             |           |                    |               |               |
| DNA segment, mouse Chr 12, Seldin 1             |       |             |           |                    |               |               |
| DNA segment, mouse Chr 19, Seldin 3             |       |             |           |                    |               |               |
| Epidermal growth factor binding protein        | Egfl  |             | 24        | TaqI               | 9.4, 6.2      | 9.8, 8.5      |
| Fas antigen                                     | Fas   |             |           |                    | 1.4           | 2.1           |
| Glucagon                                        | Gcg   |             | 25        | TaqI               | 1.8           | 2.5           |
| High mobility group protein 14-related sequence|       |             | 26        | TaqI               | -             | 1.9*          |
| DNA segment, mouse Chr 12                       |       |             |           |                    |               |               |
| DNA segment, mouse Chr 12, Seldin 1             |       |             |           |                    |               |               |
| DNA segment, mouse Chr 19, Seldin 3             |       |             |           |                    |               |               |
| Epidermal growth factor binding protein        | Egfl  |             | 24        | TaqI               | 9.4, 6.2      | 9.8, 8.5      |
| Fas antigen                                     | Fas   |             |           |                    | 1.4           | 2.1           |
| Glucagon                                        | Gcg   |             | 25        | TaqI               | 1.8           | 2.5           |
| High mobility group protein 14-related sequence|       |             | 26        | TaqI               | -             | 1.9*          |
| DNA segment, mouse Chr 12                       |       |             |           |                    |               |               |
| DNA segment, mouse Chr 12, Seldin 1             |       |             |           |                    |               |               |
| DNA segment, mouse Chr 19, Seldin 3             |       |             |           |                    |               |               |
| Epidermal growth factor binding protein        | Egfl  |             | 24        | TaqI               | 9.4, 6.2      | 9.8, 8.5      |
| Fas antigen                                     | Fas   |             |           |                    | 1.4           | 2.1           |
| Glucagon                                        | Gcg   |             | 25        | TaqI               | 1.8           | 2.5           |
| High mobility group protein 14-related sequence|       |             | 26        | TaqI               | -             | 1.9*          |
| DNA segment, mouse Chr 12                       |       |             |           |                    |               |               |
| DNA segment, mouse Chr 12, Seldin 1             |       |             |           |                    |               |               |
| DNA segment, mouse Chr 19, Seldin 3             |       |             |           |                    |               |               |
| Epidermal growth factor binding protein        | Egfl  |             | 24        | TaqI               | 9.4, 6.2      | 9.8, 8.5      |
| Fas antigen                                     | Fas   |             |           |                    | 1.4           | 2.1           |
| Glucagon                                        | Gcg   |             | 25        | TaqI               | 1.8           | 2.5           |
| High mobility group protein 14-related sequence|       |             | 26        | TaqI               | -             | 1.9*          |
| DNA segment, mouse Chr 12                       |       |             |           |                    |               |               |
| DNA segment, mouse Chr 12, Seldin 1             |       |             |           |                    |               |               |
| DNA segment, mouse Chr 19, Seldin 3             |       |             |           |                    |               |               |
| Epidermal growth factor binding protein        | Egfl  |             | 24        | TaqI               | 9.4, 6.2      | 9.8, 8.5      |
| Fas antigen                                     | Fas   |             |           |                    | 1.4           | 2.1           |
| Glucagon                                        | Gcg   |             | 25        | TaqI               | 1.8           | 2.5           |
| High mobility group protein 14-related sequence|       |             | 26        | TaqI               | -             | 1.9*          |
| DNA segment, mouse Chr 12                       |       |             |           |                    |               |               |
| DNA segment, mouse Chr 12, Seldin 1             |       |             |           |                    |               |               |
| DNA segment, mouse Chr 19, Seldin 3             |       |             |           |                    |               |               |
| Epidermal growth factor binding protein        | Egfl  |             | 24        | TaqI               | 9.4, 6.2      | 9.8, 8.5      |
| Fas antigen                                     | Fas   |             |           |                    | 1.4           | 2.1           |
| Glucagon                                        | Gcg   |             | 25        | TaqI               | 1.8           | 2.5           |
| High mobility group protein 14-related sequence|       |             | 26        | TaqI               | -             | 1.9*          |
| DNA segment, mouse Chr 12                       |       |             |           |                    |               |               |
| DNA segment, mouse Chr 12, Seldin 1             |       |             |           |                    |               |               |
| DNA segment, mouse Chr 19, Seldin 3             |       |             |           |                    |               |               |
| Epidermal growth factor binding protein        | Egfl  |             | 24        | TaqI               | 9.4, 6.2      | 9.8, 8.5      |
| Fas antigen                                     | Fas   |             |           |                    | 1.4           | 2.1           |
| Glucagon                                        | Gcg   |             | 25        | TaqI               | 1.8           | 2.5           |
| High mobility group protein 14-related sequence|       |             | 26        | TaqI               | -             | 1.9*          |

* These clones detect multiple pseudogenes that segregate to positions on several chromosomes. The informative CAST/Ei restriction fragment is indicated.

Figure 1. Segregation of the lpr mutation with the Fas apoptosis gene in (MRL-lpr x CAST/Ei)F1 x MRL-lpr backcross mice. For each locus (indicated on the left hand side of each row) the filled boxes represent those mice typed as MRL-lpr homozygotes, and the open boxes represent those that typed as the heterozygote F1 pattern. The number of mice with a given haplotype pattern is shown at the bottom of each column. (A) Complete haplotype data for 110 interspecific backcross mice fully typed for mouse Chr 19 markers. (B and C) Segregation of those mice characterized as LPR and non-LPR phenotype using stringent criteria established by the analysis of backcross mice with nonrecombinant chromosomes. A total of 182 mice were examined, 84 of which fulfilled these criteria.
Since previous studies had localized the lpr mutation to mouse Chr 19 (5), we analyzed the segregation of lpr using markers that map to this chromosome (Osp, Pmc-2, D19S63, Fas, Ide, Lyp-57, Ins-2x1, and ADRB1 [39, 43; and J. M. Rochelle and M. F. Seldin, unpublished data]). Informative RFLPs were determined by analysis of the parental mice (Table 1). Haplotype analysis was then performed for each of the 182 backcross mice, resulting in the genetic map shown in Fig. 1A.

Since the manifestations of autoimmune disease, including lymphadenopathy and anti-dsDNA antibodies, can be influenced by background genes, criteria for LPR and non-LPR phenotype mice were established by analysis of those backcross mice that inherited a nonrecombinant Chr 19 from the F1 parent (Fig. 1A, columns 1 and 2), as follows: 32 mice that were homozygotes for all Chr 19 markers derived from the MRL parental genome defined the lower limit for the LPR phenotype (lymphoid mass, 0.1 g; and anti-dsDNA antibodies, 1,200), and 33 mice that were heterozygous for all Chr 19 markers defined the lower limit for the non-LPR phenotype (lymphoid mass, 2.5 g; and anti-dsDNA antibodies, 1,750). None of the 84 backcross mice classified as LPR or non-LPR by these criteria inherited a crossover event between lpr and the Fas genes, as shown by haplotype analysis (Fig. 1, B and C). In addition, when all backcross mice were examined, the MAPMAKER QTL program indicated maximum LOD scores at the Fas locus for the measurement of anti-ssDNA (LOD, 17.8), anti-dsDNA (LOD, 30.5), lymphoid mass (LOD, 36.7), and nephritis (LOD 17.4). We conclude that the lpr mutation is closely linked to the Fas gene.

**Analysis of Fas RNA Expression Shows Abnormal Transcription in MRL-lpr Mice.** The above mapping data together with the previously defined functional data suggested that an alteration in the Fas gene accounts for the phenotypic changes observed in lpr homozygous mice. Therefore, Fas expression was analyzed by Northern blotting of total RNA purified from several different tissues obtained from MRL+-, MRL-lpr, CBA, CBA-lpr+/-, C3H-/-, and C3H-gld mice. Using a 3' Fas cDNA probe (FasL1R2), high-level Fas expression was demonstrated in liver from MRL+ mice, CBA-+, and CBA-lpr+/- mice (Fig. 2). Moderate expression of the 2.1-kb Fas transcript was evident in lymphoid tissue from MRL+-, CBA-+, and CBA-lpr+/- and C3H-gld mice. In contrast, compared with MRL+ mice, the expression of Fas was markedly lower in liver, spleen, and lymph node tissues from lpr homozygous mice (Fig. 2). Finally, longer exposures of Northern membranes suggested that a low level of Fas gene expression occurred in tissues from lpr homozygous mice.

To further analyze the expression of Fas in normal and lpr/lpr mice, PCR assays were performed from cDNA obtained by reverse transcription of the RNA samples. The PCR amplification of several segments of the Fas gene using stringent annealing conditions demonstrated that Fas was transcribed in lpr/lpr spleen and lymph node cells (Fig. 3). Additionally, quantitative PCR experiments using titrated amounts of cDNA template and a smaller number of PCR cycles suggest that similar amounts of the Fas transcript are made in normal and lpr/lpr lymphoid tissue (not shown). However, in addition to amplifying fragments of the predicted size, abnormally sized PCR fragments were obtained with some of the primer pairs (e.g., Fig. 3B). Notably, the PCR primer pairs used to amplify nucleotides 220–470 (S1-S4, 250 bp) and 220–665 (S1-S9, 445 bp) of the published Fas cDNA sequence produced additional PCR fragments of 450 bp (Fig. 3B) and 650 bp (not shown), respectively, suggesting that the Fas tran-

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**Figure 2.** Northern blot hybridization of RNA from MRL-lpr, CBA-lpr+/-, C3H-gld, and congenic controls with a 3' Fas apoptosis gene probe (FasL1R2). Fas gene expression (top) is compared with γ-actin (Actg), (middle) and acridine orange staining of total RNA (bottom) for the following samples: (1) CBA+ spleen, (2) CBA-lpr+/- spleen, (3) CBA-lpr+/- lymph node, (4) MRL+ spleen, (5) MRL-lpr spleen, (6) MRL-lpr lymph node, (7) MRL-lpr liver, (8) CBA+ liver, (9) CBA-lpr+/- liver, (10) MRL+ liver, (11) C3H-/- liver, (12) C3H-/- thymus, (13) C3H-gld lymph node. Sizes of the transcripts were 2.1 kb (Fas) and 0.7 kb (Actg).

**Figure 3.** PCR amplification of the Fas cDNA segments. The templates were reverse transcribed RNA (20 ng of oligo(dT)-primed cDNA) from MRL+ spleen (lane 1), MRL-lpr spleen (lane 3), and MRL-lpr lymph node tissue (lane 5). Three partially overlapping segments of the Fas gene were amplified using the following primers: (A) L2 and S2 (bp 32–239 of the published sequence; reference 19); (B) S1 and S4 (bp 220–470); and (C) L1 and R2 (bp 646–1405). Alternate lanes (2 and 4) contained PCR control reactions in which no template was added. Results were verified by examination of RNA samples derived from multiple individual mice.
Figure 4. Comparison of Fas expression in MRL-+ and MRL-lpr mice. Probes used for Northern hybridizations were derived using either MRL-+ (A) or MRL-lpr (B) cDNA as template for PCR amplification. The region amplified represented a 3' segment (FasL1R2, bp 646-1405) of the Fas gene. In A, the wild-type probe detects bands in MRL-+ liver (lane 3), but not MRL-lpr spleen, lymph node, or liver (lanes 1, 2, and 4). In B, the lpr probe detects bands in MRL-+ liver (lane 3), and also hybridizes to transcripts in MRL-lpr spleen (lane 1) and lpr lymph node (lane 2), but does not detect bands in lpr liver (lane 4). Additional higher molecular weight bands are detected by the lpr-derived probe in lpr spleen and lymph node (lanes 1 and 2).

scripts in lpr/lpr mice contained additional and disparate sequences from the functional Fas transcript.

The MRL-lpr and MRL-+ derived amplified products were gel purified and used as probes for both genomic and Northern blot hybridization. The amplified fragments from both MRL-lpr and MRL-+ detected the same Fas genomic restriction bands that were previously observed using amplified products from a wild-type thymus cDNA library, indicating that the fragments amplified from transcribed sequences were derived from the same region of the genome in both strains (MRL-+ and MRL-lpr) (data not shown). In contrast, Northern blot analysis showed different expression patterns when lpr-derived probes, as compared with wild-type thymus cDNA probes and MRL-+ derived probes, were used. For example, when the lpr-derived fragment (FasL1R2) was used as a probe prominent transcripts of 2.3 and 4.5 kb were observed in MRL-lpr spleen and lymph node tissue (Fig. 4). These differences in hybridization patterns indicate that the fragments amplified from mRNA in lpr mice contain sequences not present in transcripts from wild-type mice. These results combined with the PCR results and genomic blot analysis strongly suggest that mRNA processing of the Fas gene is different in MRL-lpr mice.

Genomic Restriction Analyses Identify a Deletion within the Fas Gene of lpr/lpr mice. Genomic DNA digests were performed to examine whether a structural mutation was present in the Fas gene of lpr/lpr mice that might explain the differences noted in Fas RNA transcripts in cells from lpr/lpr mice. DNAs were digested with multiple restriction endonucleases and examined by Southern blot analysis using several Fas probes (FasL1S2, FasL2S5, and FasS1S9). The FasS1S9 probe detected multiple differences between MRL-lpr and MRL-+ DNA samples (e.g., see Fig. 5). Double digests defined a genomic restriction map in MRL-+ and MRL-lpr DNA (Fig. 5). These analyses indicate that a 1.4-kb fragment of the Fas gene is deleted in the lpr/lpr mouse and, together with the PCR analyses (see above), suggest that the deletion is probably within a Fas intron.

Complex Genetic Interactions Underlie the Manifestations of Autoimmune Disease. The identification of the Fas gene as the site of the lpr mutation also allows the separation of backcross mice into homozygotes and heterozygotes for lpr. The phenotypic characteristics of lpr/lpr and lpr/+ (MRL-lpr x CAST/Ei)F1 x MRL-lpr backcross mice were compared with those of the MRL-lpr parental and the (MRL-lpr x CAST/Ei)F1 mice (Table 2). The standard deviation for lymphoid mass and renal disease of lpr homozygous backcross

Figure 5. Genomic organization of the Fas gene. (Top) Representative genomic blot hybridized sequentially to probes derived from the 5' (FasS1S9, A) and the 3' (FasL1R2, B) regions of the Fas gene. DNA samples derived from MRL-+ (lane 1), MRL-lpr (lane 2), CBA-+ (lane 3), and CBA-lpr/+ (lane 4) were digested with EcoRI and BamHI. Molecular weight size standards (in kb) are shown to the far left (top). (Bottom) Restriction map of the genomic region detected by multiple Fas probes. Cleavage sites are shown for the restriction enzymes EcoRI (R), BamHI (B), PvuII (P), and PstI (Ps). The position of a 1.4-kb deletion associated with the lpr mutation is indicated.
Table 2. Phenotypic Characteristics of MRL-Ipr Crosses

| Mice*       | Nephritis index† | Lymphoid mass index | Anti-dsDNA    | Anti-ssDNA    |
|-------------|-----------------|---------------------|---------------|---------------|
| MRL-Ipr     | 0.97 ± 0.16 (17)  | 0.72 ± 0.14 (17)    | 1.047 ± 0.4733 (17) | 1.844 ± 0.2306 (17) |
| F1          | 0.10 ± 0.14 (18)  | 0.02 ± 0.04 (28)    | 0.060 ± 0.050 (27)       | 0.3489 ± 0.1638 (27) |
| BC          | 0.39 ± 0.35 (183) | 0.26 ± 0.30 (182)   | 0.6567 ± 0.5998 (178)   | 0.9291 ± 0.4924 (178) |
| BC-Ipr/Ipr  | 0.60 ± 0.35 (86)  | 0.49 ± 0.29 (86)    | 0.7360 ± 0.5762 (84)    | 1.254 ± 0.3948 (84)   |
| BC-Ipr/+    | 0.19 ± 0.20 (96)  | 0.06 ± 0.08 (96)    | 0.2486 ± 0.3600 (93)    | 0.6459 ± 0.3955 (93)  |

* The mice examined were the MRL-Ipr, (MRL-Ipr x CAST/Ei)F1 (F1), and the backcross (MRL-Ipr x CAST/Ei) x MRL-Ipr (BC). The interspecific backcross mice were also examined in subsets defined as lpr/+ and lpr/lpr according to their Fas locus genotype.
† Phenotypic indices are defined in Materials and Methods.
§ SD.
‖ Number of animals examined.

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mice was larger than for MRL-Ipr mice (Table 2). There was also a larger range of values and overlap between Ipr homozygous and lpr/+ heterozygous backcross mice for each parameter measured (Fig. 6). Although the distribution of values does not indicate a simple one- or two-gene model, the large variance within Ipr homozygous backcross mice nevertheless suggests that relatively few genes are responsible for the difference between these mice and parental MRL-Ipr mice (see references 42 and 44 for relevant discussion).

QTL Suggest Chromosomal Locations of lpr Renal Disease Modifiers. To determine if other loci, in addition to Fas, were associated with the inheritance of renal disease, we analyzed

Figure 6. Histogram of the distribution of parental and backcross mice for phenotypic indexes of anti-ssDNA, anti-dsDNA, lymphoid mass, and nephritis. The backcross mice (BC) were divided into lpr/+ and lpr/lpr genotypes as determined by a Fas RFLP. The height of each column represents the number of mice with the indicated value. (■) BC lpr/+; (□) BC lpr/lpr; (▲) F1; (◆) MRL-Ipr.

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Table 3. QTL Analysis of Renal Disease

| Chr | Maximum interval* | LOD* score | Markers† | Percent variance†† |
|-----|-------------------|------------|----------|-------------------|
| 1   | 74.3              | 1.0        | Gcg (M. castaneus)† | 3                  |
| 2   | 72.4              | 1.7        | Mme (M. castaneus)  | 5                  |
| 3   | 55.9              | 1.4        | Rpl18rs9 (MRL)     | 4                  |
| 4   | 70.6              | 1.3        |           |                   |
| 5   | 77.7              | 0.6        |           |                   |
| 6   | 70.7              | 0.6        |           |                   |
| 7   | 79.2              | 3.0 (3.0)** | Pkcg-Otf-2 (MRL) | 8 (24)††          |
| 8   | 39.0              | 0.0        |           |                   |
| 9   | 72.7              | 0.2        |           |                   |
| 10  | 53.5              | 0.9        |           |                   |
| 11  | 62.3              | 0.1        |           |                   |
| 12  | 61.8              | 2.9 (3.9)** | D12Nyu3-D12Sel1 (MRL) | 10 (38)†† |
| 13  | 61.1              | 0.0        |           |                   |
| 14  | 23.4              | 0.6        |           |                   |
| 15  | 62.0              | 0.1        |           |                   |
| 16  | 31.0              | 0.1        |           |                   |
| 17  | 26.8              | 0.1        |           |                   |
| 18  | 31.4              | 0.1        |           |                   |
| 19  | 43.9              | 17.4       | Fat (MRL) | 37                 |

* Interval from most proximal to most distal marker that was examined for each chromosome.
† Maximum LOD score on each chromosome when the interspecific backcross mice were examined for variance of the renal index.
§ Markers closest to the maximum LOD score are shown for those chromosomes in which a LOD score of at least 1.0 was reached.
‖ The percent variance explained by the putative QTL.
** LOD score for analysis of only backcross mice homozygous for the MRL-lpr Fas RFLP.
‡‡ The percent variance explained by the putative QTL when only those backcross mice homozygous for the MRL-lpr Fas RFLP were examined.

cosegregation with polymorphic markers on each mouse autosome. X chromosomal loci were not examined since there was no difference in the incidence of renal disease in male and female progeny (data not shown). A total of 108 loci were analyzed that covered between 23 and 79 cM on each autosome (Table 3) and spanned a total of 1,070 cM with only six intervals >25 cM. Comparison with previous genetic maps suggests that these markers cover ~75% of the genome at a resolution of <20 cM (M. F. Seldin, unpublished data). A minimum of 110 backcross mice were typed for all 108 loci, and 182 backcross mice were typed for 60 loci. The MAPMAKER QTL program was utilized to analyze these data for the identification of disease modifying loci (41; see Materials and Methods).

In addition to the region of mouse Chr 19 containing the Fas locus, two chromosomal regions, the proximal segment of mouse Chr 7 (Otf-2, Pkcg) and the middle segment of mouse Chr 12 (D12Nyu3, D12Sel1), were associated with renal disease showing LOD scores of 3.0 and 2.9, respectively (Table 3). The MRL/lpr haplotype was overrepresented in those mice with high nephritis indices and under represented in those mice with low nephritis indices. When only those backcross

Figure 7. Histogram of LOD score values for renal disease QTL analysis on mouse Chrs 7 and 12.
mice that were lpr homozygotes (as determined by Fas locus typing) were examined, the LOD score for the Chr 7 loci remained at 3.0 and that for the Chr 12 loci increased to 3.9 (even though the number of mice was reduced from 182 to 86; Table 2 and Fig. 7). Together, this analysis suggests that these two chromosomal regions account for >50% of the variance observed in the lpr homozygous backcross mice (Table 3).

Discussion

The current study confirms the conclusion of Watanabe-Fukunaga et al. (19) that the Fas gene contains the lpr mutation. The Fas gene is closely linked to the lpr mutation and RNA expression was dramatically altered in tissues from lpr/lpr mice. However, in contrast to previous studies (19), the current results indicate that Fas mRNA is produced in tissue from MRL-Ipr mice, albeit of different size transcripts. The PCR results using primers that amplify sequences detecting a Fas deletion in genomic DNA from lpr/lpr mice also amplify additional DNA fragments from cDNA derived from lpr/lpr mice, indicating that the aberrant cDNA contains an additional sequence that is probably intronic. In addition, our genomic restriction site analyses demonstrate that lpr/lpr mice have a 1.4-kb deletion within the Fas gene. Together, these data suggest that the Fas deletion results in the production of nonfunctional mRNA transcripts, possibly due to splicing errors.

The present studies also provide insight into the etiopathogenesis of generalized autoimmunity as well as critical events in T cell differentiation. The Fas gene is a type 1 membrane protein with sequence similarity to other members of a putative family of cell surface membrane receptors, including nerve growth factor receptor, CD40, and TNF receptor 1 (TNFR1) (39, 45). The cytoplasmic domain of Fas, presumably essential for signal transduction, is most similar to CD40 and TNFR1. Because antibodies directed to Fas cell surface antigen induce apoptosis (45), these studies suggest that the profound accumulation of CD4+CD8−CD3+ T cells from lpr/lpr mice results from escape of these cells from a normal apoptotic process.

A large variety of data suggest that during normal T cell development, both positive and negative selection takes place to eliminate potentially autoreactive cells (15). Although the double-negative cells in MRL-lpr mice do not appear to have a functional role (8), the accumulation of these cells suggests that other T cells escape the normal positive and/or negative selection process. Presumed defects in both positive and negative selection have been observed in analysis of lpr/lpr mice (16–18). The results of the current study suggest that the expression of the Fas gene within the thymus may allow elimination of potentially autoreactive T cells. Perhaps transcriptional control of the Fas gene is the first step downstream from the positive and/or negative selection signals.

Although T cell abnormalities have been the major focus of research on the lpr mutation, recent adoptive transfer studies indicate that lpr/lpr mice have primary defects in both B and T cells (46, 47). Thus, negative and/or positive selection at both the T and B cell levels is probably required to prevent the occurrence of self-reactive cells and autoreactive antibodies. Analysis of Fas gene expression during B cell ontogeny potentially can allow delineation of the role of Fas-mediated apoptosis in the determination of peripheral T and/or B cell tolerance.

The phenotypic analysis of interspecific backcross mice segregating for the lpr mutation indicates that the manifestations of generalized autoimmune disease in this model result from complex genetic interactions. This result is consistent with previous observations that lpr congenic mice (i.e., mice carrying lpr mutation on different genetic backgrounds) differ in manifestations of disease (2, 7). In addition, careful serologic analysis has indicated that subtle autoimmune disease can be detected in lpr/+ heterozygotes (48). Having identified the likely site of the lpr mutation, it was possible to reevaluate the backcross mice that could not be definitively phenotyped as LPR or non-LPR by either anti-DNA antibody levels or degree of lymphadenopathy. These data indicate that several additional genes are necessary for the full manifestation of lymphadenopathy in lpr homozygous mice and that lpr heterozygotes do have significant disease (up to 2.5 g lymphoid mass) provided certain background genes are present. Similar results were obtained in the analysis of nephritis, anti-ssDNA, and anti-dsDNA antibody levels.

Single gene mutations may have profound effects on the pathophysiology of many different diseases; however, most human and mouse diseases are the result of more complex hereditary factors. The ability to define markers at 10–20-cM intervals throughout the genome has allowed several groups to begin elucidating complex genetic diseases. Initial studies of plant genetic characteristics using these techniques (49, 50) have recently been extended to mammalian systems. Several loci have now been identified as probable disease modifiers in nonobese diabetic mice (51), in salt-sensitive hypertensive rats (52), and in a mouse model for epilepsy (53). The current study adds the MRL-lpr autoimmune renal disease to this list. Typing of the (MRL-lpr × CAST/Ei)F1 × MRL-lpr backcross mice for markers covering ~75% of the mouse genome indicated an association of renal disease with the MRL parental haplotypes for positions on mouse Chr 7 and 12. Analysis of only backcross mice that had the lpr/lpr genotype (MRL-lpr Fas homozygotes) showed similar results for a modifying gene being present on Chr 7 and more significant association for another modifying gene being present on Chr 12. At present the position of these QTL on these chromosomes is imprecisely defined. Additional breeding studies in which congenics are established for these regions of the genome will be necessary to further define the location of these lpr renal disease modifier loci (Lrdm-1, Chr 7; Lrdm-2, Chr 12). Although the current data do not exclude the Igh locus as a renal disease modifier on mouse Chr 12, the LOD score at this locus was 2.3 compared with the maximum LOD score of 3.9 for a gene located at a more proximal position on Chr 12 (Fig. 7). Potential renal disease modifiers, Olf-2, an Ig transcriptional regulator (34), and T cell growth factor B1 (Tgfb1), a mediator of inflammation and peripheral tolerance (54, 55), have been mapped close to Lrdm-1 on mouse Chr 7 (56). In
addition, Tgfla3 is located in the region of Lrdm-2 on Chr 12 (57).

None of the other chromosomal markers analyzed had LOD scores >2 (Table 2). Interestingly, one potentially weak renal disease modifier on mouse Chr 3 (LOD, 1.4) is coincident with the location of a very strong NOD modifier (51), raising the possibility that the renal disease observed in lpr/lpr mice and diabetes modification in NOD mice may represent a common genetic mechanism. In contrast to the NOD model (51) and previous studies of the NZB model (58, 59), in this study we observed no substantial effect of the MHC or TCR B loci on nephritis.

Finally, this study also may help define the pathogenesis of human generalized autoimmune diseases, such as SLE. Although a profound expansion of double-negative (CD4-, CD8-,CD3+) T cells has not been observed in these patients, the variable expression of the lpr homozygous and lpr heterozygous genotypes observed in the current study leaves open the possibility that a similar defect in lymphocyte apoptosis may, in part, account for the wide array of autoantibodies that characterize SLE. Furthermore, the localization of disease-modifying genes from mouse studies can be used to predict the chromosomal location of homologous human disease-modifying loci because of conservation of linkage groups between these species (reviewed in reference 60). For autoimmune renal disease, the human chromosomal segments of interest, corresponding to Lrdm-1 and Lrdm-2, are located on the proximal long arm of human Chr 19 and long arm of human Chr 14, respectively. In summary, studying the inheritance of complex diseases in the mouse may help define the complex genetics of human autoimmune disease.

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