The Defect in Fas mRNA Expression in MRL/lpr Mice Is Associated with Insertion of the Retrotransposon, ETn

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Summary

Fas is a cell surface protein of the tumor necrosis factor receptor, nerve growth factor receptor, CD40 family, and is involved in the control of lymphocyte apoptosis. A mutation in the Fas gene in MRL/lpr mice results in massive lymphoproliferation (lpr) and accelerated autoimmunity. To further study the nature of this defect, Fas mRNA expression was evaluated by reverse transcriptase polymerase chain reaction as well as by Northern blotting. These studies revealed that the wild-type Fas message was produced at ~10-fold lower levels in the lpr compared with the + + substrain of MRL mice. In addition to the wild-type transcript, lpr mice also synthesized chimeric transcripts containing an insertion of the early retrotransposon (ETn). Molecular cloning and nucleotide sequencing of a Fas-ETn chimeric cDNA suggested that the striking reduction in wild-type Fas mRNA levels and the presence of aberrant transcripts in MRL/lpr mice are most likely explained by the insertion of the ETn retrotransposon into an intron of the Fas gene and induction of alternative splicing involving the 5' ETn long terminal repeat.

MRL/lpr and MRL/++ are congenic inbred strains of mice that spontaneously develop an autoimmune disease very similar to SLE (reviewed in reference 1). The lpr gene is associated with massive infiltration of lymph nodes by double-negative (CD4−,CD8−) T cells and with accelerated autoimmunity in MRL mice. A spontaneous mutation has also arisen in CBA/K1 mice, resulting in a strain (CBA/lpr) that develops lymphoproliferation and autoantibodies (2). The lpr phenotype in these two strains of mice has recently been shown to result from abnormalities of the Fas gene (3). In MRL/lpr mice, impaired Fas expression was associated with abnormal restriction fragment patterns of Fas genomic DNA, whereas in CBA/lpr mice, a point mutation in the Fas gene (a T→A transition at nucleotide 786) was detected. The lpr mutation results in a replacement of isoleucine with asparagine in the cytoplasmic region of Fas, leading to defective signaling for apoptosis by the mutant protein (3).

The precise abnormality of the Fas gene in MRL/lpr mice has not been identified. To quantify Fas expression in MRL mice, reverse transcriptase (RT)-PCR analysis of Fas mRNA in lymphoid tissues was undertaken. These studies revealed that MRL/lpr mice produced ~10-fold lower levels of wild-type Fas mRNA as well as aberrantly sized Fas transcripts.

Materials and Methods

Mice. MRL/lpr (MRL/MpJ-lpr/lpr), MRL/++ (MRL/MpJ-++/+), NZB/W F1, and C3H (C3H/HeSnJ) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and subsequently bred at the Hospital for Special Surgery.

Northern and Southern Blotting. Single-cell suspensions were prepared from the thymus, spleen, lymph nodes, and bone marrow of MRL and C3H mice. Total RNA was isolated by the acid guanidinium method (4) and poly(A)+-enriched RNA was obtained by the method of Badley et al. (5) using oligo(dT) cellulose (Collaborative Research, Bedford, MA). RNA was blotted to nylon membranes (Gene Screen; New England Nuclear, Boston, MA) and hybridizations were performed according to the manufacturer’s instructions. PCR products were also blotted to nylon membranes and hybridized with DNA or oligonucleotide probes. DNA probes were labeled with [32P]dCTP by the random primer method (6) and oligonucleotides were biotinylated at the 3' termini using terminal deoxynucleotidyl transferase (Tdt) (7). Hybridization with oligonucleotides, blots were developed with the chemiluminescent substrate, AMPPD (Tropix, Bedford, MA), and fluorochemistry. All blots were washed under high stringency conditions.

PCR and DNA Sequencing. cDNA was synthesized from total RNA with Moloney murine leukemia virus (MoMuLV) RT and oligo(dT) as described (6). cDNA was amplified by PCR on a thermal cycler (480; Perkin Elmer Corp., Norwalk, CT) with Taq polymerase (Bethesda Research Laboratories) unless indicated otherwise. The primers used for PCR analysis are shown in Table 1. The conditions used for amplification were: denaturation at 94°C for 1 min, annealing at 5°C below the melting temperature (Tm) (calculated as described [9]) for 1 min, and extension at 72°C for 1 min except when indicated. Where appropriate, PCR products

1 Abbreviations used in this paper: ETn, early retrotransposon; RT, reverse transcriptase.
were subcloned into pGEM3 (Promega Biotech, Madison, WI) and sequenced by the dideoxy chain termination method (10) as described (8).

Quantitative PCR. For all quantitative studies, RNA was pooled from groups of three to four "young" (6–8 wk) or "old" (14–20 wk) mice to avoid individual variation. Extensive preliminary studies revealed that optimal quantitation of Fas and GAPDH were obtained by 30 cycles of PCR amplification and cDNA dilutions of 1:10 for Fas and 1:320 for GAPDH. After RT-PCR, 10 μl of the DNA from each tube was resolved on a 5% polyacrylamide gel. The gel was stained with ethidium bromide and photographed with a video imager (Appligene, Pleasanton, CA) in the reverse mode so that stained DNA appeared as black bands. The relative amount of DNA was quantified by laser densitometry (Molecular Dynamics, Sunnyvale, CA). A tube containing all of the reagents except the cDNA served as the negative control. In each experiment, a standard curve (established by serial dilution of cDNA from Con A-stimulated normal splenocytes) served as the positive control and the control for interassay variation. Results for each test sample were extrapolated from the standard curve and expressed as arbitrary units corresponding to the integrated volume calculated on the laser densitometer. A tube containing all of the reagents except the cDNA served as the negative control. In each experiment, a standard curve (established by serial dilution of cDNA from Con A-stimulated normal splenocytes) served as the positive control and the control for interassay variation. Results for each test sample were extrapolated from the standard curve and expressed as arbitrary units corresponding to the integrated volume calculated on the laser densitometer.

Results

MRL/Ipr Mice Produce Abnormal Fas mRNA Transcripts. When RT-PCR was performed with the Fas primer pair A (see Table 1) and RNA was isolated from the spleens and thymus of MRL/++ and C3H mice, a DNA product of the expected size (1 kb) was observed on ethidium bromide–stained gels (Fig. 1 A). In contrast, a low level of the 1-kb DNA product was amplified after RT-PCR of MRL/lpr RNA, and an additional band ~200 bp larger than the wild-type PCR product was noted. Similar results were observed when Fas primer pair B and either Taq or Thermophilus litoralis polymerase (New England Biolabs, Beverly, MA) were used for PCR amplification (not shown), demonstrating that this finding was not a PCR artifact. Since RNase-free DNase (10 μg/ml) had no effect, whereas DNase-free RNase (10 μg/ml) completely eliminated PCR products, the PCR products were derived from RT of RNA rather than contaminating DNA. Although a low level of Fas DNA was amplified from lpr thymus, this resulted from a lower input of total cDNA as judged by amplification of GAPDH (Fig. 1 A). As shown in Fig. 1 B, when RT-PCR of thymus RNA was performed with four different pairs (Table 1, C–F) and approximately equal amounts of DNA were loaded onto the polyacrylamide gels, DNA products of the expected sizes (wild type) were amplified in both lpr and ++ mice, but only lpr mice had a second product ~200 bp larger than the wild type.

To localize the abnormal MRL/lpr Fas transcript, RT-PCR was performed with the Fas primer pairs G (to amplify the 5' region) and H (to amplify the 3' region). RT-PCR with Fas primer pair H amplified DNA products of the expected size (550 bp) in all strains although the amount of MRL/lpr DNA was lower than that derived from the control strains (not shown). RT-PCR with the Fas primer pairs G and I revealed that two DNA products were consistently amplified

Table 1. Oligonucleotides Used for PCR and Hybridization

| PCR          | Antisense          |
|--------------|--------------------|
| Sense        |                     |
| Fas A (50)   | ATGCTGTGGATCTGGGCTGT |
| B (29)       | CTGTTTTCTCTTGCGACAGATG |
| C (29)       | CTGTTTTCTCTTGCGACAGATG |
| D (50)       | ATGCTGTGGATCTGGGCTGT |
| E (127)      | CATCTCCGAGGTAAAAAGC  |
| F (185)      | GAAGGATTATATCAAGGAGGCCA |
| G (50)       | ATGCTGTGGATCTGGGCTGT |
| H (517)      | AGCAATACAAACTGAGGAAAC |
| I (127)      | CATCTCCGAGGTAAAAAGC  |
| GAPDH GA (242) | CCATCACCCATCTTTCAGGAG |

Hybridization

| ETn8 (27) | CTTTCAGTCGAAACGTTTCAGT |

Oligonucleotides are shown as 5' → 3', with the base numbers of the most 5' nucleotide given in parentheses.

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Figure 1. (A) RT-PCR of RNA obtained from the spleens (S) and thymus (T) of MRL/Ipr(Ipr), MRL/++ (+ +), and C3H mice. DNA amplification was performed with Fas primer pair A (see Table 1), and 10 μl of the products was resolved on a 5% polyacrylamide gel. The gel was stained with ethidium bromide and photographed with a video imager in the reverse mode (the stained bands appear black). GAPDH was amplified from the same RNA samples using primer pair GA (Table 1). M, size markers (kb); C, control (RT-PCR performed with Escherichia coli from all test strains although the larger DNA product represented only a minor fraction of the amplified DNA in the control strains (Fig. 1 C). In addition, the larger PCR product amplified from the control strains was slightly smaller than that obtained from lpr mice. RT-PCR of RNA obtained from lpr liver and kidney with primer pair G also revealed two DNA products of 500 and 700 bp (not shown).

To verify that the PCR products amplified with Fas primers were specific for Fas, a Southern blot was performed with a 1-kb Fas probe (the PCR product amplified from MRL/++ thymus with the primer pair A). As shown in Fig. 2, A and B, both of the lpr-derived products amplified from spleen and thymus cDNA hybridized with the 1-kb Fas probe, whereas cDNA amplified from ++ mice showed a single band corresponding to the PCR product stained with ethidium bromide. An additional product of ~800 bp also hybridized with the Fas probe in lpr but not ++-derived PCR products (Fig. 2 B).

Northern Blot Analysis. Poly(A)+-enriched RNAs obtained from MRL/lpr, MRL/++ (+ +), and C3H thymus and liver were hybridized with the 1-kb Fas probe. As shown in Fig. 3, a transcript of ~1.8 kb hybridized with this probe in all three strains and the level of Fas mRNA expression was considerably lower in lpr thymus and liver compared with the control strains. While not distinctly resolved as a separate transcript, the lpr-derived Fas mRNA appeared to be slightly larger than the Fas mRNA in the controls. In addition, the Fas probe hybridized with a transcript ~0.8 kb in lpr thymus and liver, but with RNA derived from the control strains. Weaker hybridization with transcripts of ~13 kb were also observed in the lpr-derived thymus RNA, but similar bands were observed in the control strains after longer exposure of the autoradiograph (not shown). These bands may represent unspliced nuclear Fas RNA. A higher background was consistently observed in poly(A)+-enriched DNA derived from lpr thymus for reasons that are unknown.

Aberrant MRL/lpr Fas mRNA Contains an Insertion of the Early Retrotransposon (ETn). To determine the nature of the DNA products obtained from MRL/lpr mice after RT-PCR amplification with Fas primer pair G, the two PCR products obtained from lpr thymus were subcloned and sequenced. Nucleotide sequencing revealed that the 500-bp PCR product contained the expected sequence reported for Fas (11). The 700-bp product contained an insertion of 183 bp at position 233 of Fas DNA without altering the translational reading frame of Fas. A search of the GenBank and EMBL databases revealed that the 183-bp insertion had a 98.4% sequence iden-
tality with a region of the 5' LTR and primer binding site of the ETn retrotransposon inserted into the mouse IgG1 heavy chain switch region in the BALB/c myeloma, P3.26Bu4 (Fig. 4 A). The molecular clone isolated from P3.26Bu4 was designated γM85 (12). The 183-bp insert also had 99% sequence identity with the corresponding region of the ETn retrotransposon that has disrupted the skeletal muscle chloride channel in ADR mice (13) (Fig. 4 A). In contrast, the ETn insert detected in lpr Fas mRNA had only 51% sequence identity with the prototypic ETn sequence, MG1 (14) (Fig. 4 B). As shown in Fig. 5, where the nucleotide sequences of Fas and ETnin are known or presumed, the proposed 5' and 3' splice sites closely resembled the splice consensus sequences (15).

Southern and Northern Blot Analysis of ETnin Expression. To determine whether additional aberrant transcripts containing ETnin were amplified from lpr-derived cDNA and whether the minor DNA products amplified in the control strains (Fig. 1 C) contained ETnin sequences, Southern blots with ETnin probes were performed. When the Southern blots in Fig. 2, A and B, were stripped and rehybridized with the ETnin probe described in Fig. 2. The positions of the 18 and 28 S RNAs are shown.

Figure 3. Northern blot analysis of Fas expression in thymus and liver. 2 μg of poly(A)+-enriched RNA obtained from lpr (l), ++ (+), or C3H (C) mice was applied to each lane of a 1% agarose gel containing 2.2 M formaldehyde. After transfer to a nylon membrane, hybridization was performed with the same 32P-labeled Fas probe described in Fig. 2. The positions of the 18 and 28 S RNAs are shown.

Figure 4. Nucleotide sequence comparisons between the ETnin insertions detected in MRL/lpr Fas mRNA (LPRINS), the skeletal muscle chloride channel in ADR mice (ADRINS), and the prototype ETnin subfamily sequences. Identity between LPRINS and EthoM85 (12, 16) is 98%, and between LPRINS and ADRINS (13) is 99% (A). Identity between LPRINS and EthMGI (14) is only 51% (B). The positions of the 5' LTR and primer binding site (PBS) are shown in A. These sequence data have been submitted to Genbank under accession no. L15352.
Figure 5. Proposed mechanism of the Fas mutation in MRL/lpr mice. ETn inserts into an intron of the Fas gene. The insertion induces aberrant splicing of the Fas gene and reduced transcription or stability of the spliced messenger RNAs. It is currently unknown whether ETn is inserted as a full-length (5.7 kb) or partial retrotransposon. The nucleotide sequences, where known (Fas [3] and ETn[NS] see Fig. 4), shown as uppercase) or presumed (derived from ETnγM85 [12, 16], shown as lowercase), are provided at the proposed alternative splice sites. This is not drawn to scale.

probe 41 (a 2-kb Pst fragment of RMγ2 containing the 5' LTR and primer binding site homologous to γM85 [16], kindly provided by W. Dunnick, University of Michigan, Ann Arbor, MI), only the product 200 bp larger than wild-type cDNA from lpr thymus (Fig. 6 A) and spleen (not shown) hybridized. In addition, the ~800-bp product that hybridized with the Fas probe also hybridized with the ETn probe, indicating that this was also likely to result from a chimeric transcript. When the PCR products shown in Fig. 1 C were rehybridized with the ETn oligonucleotide 8 (Table 1), the larger DNA fragment amplified from lpr, but not the control, mice hybridized (Fig. 6 B). These findings demonstrate that only two Fas/ETn chimeric products were consistently amplified from MRL/lpr cDNA, and that the additional product amplified with the primer pair I in the control strains (Fig. 1 C) either represents a minor alternatively spliced form of Fas mRNA or is a PCR artifact.

When the Northern blot shown in Fig. 3 was stripped...
and hybridized with the ETn 41 probe (Fig. 6 C), the most intense hybridization from thymus RNA was observed at 6 kb, as has previously been reported for lymphoid cell lines and spleen (12). In contrast, a strong hybridization signal was observed at 2 kb from RNA isolated from the liver of all three test strains. Although the size of this band was close to the 1.8-kb Fas transcript, it was clearly distinguishable when the autoradiographs were superimposed.

MRL/Ipr Mice Produce ~10-fold Lower Levels of Fas mRNA. To confirm that the RT-PCR method used could quantify differences in mRNA input, thymus RNA obtained from MRL/++ mice was serially diluted, subjected to RT-PCR with primer pair B, and quantified as described in Materials and Methods. As shown in Fig. 7 A, a dose-response curve was observed under these conditions. The relative amounts of Fas mRNA amplified with primer pair G after RT-PCR of RNA obtained from bone marrow, spleen, lymph node, and thymus of MRL mice are shown in Fig. 7 B. Regardless of the source of RNA, lpr mice expressed ~10% of the wild-type Fas mRNA compared with the congenic ++ strain. Similar results were obtained when PCR performed with the Fas primer pair 1 (not shown).

**Discussion**

MRL/Ipr and MRL/++ mice are thought to be genetically identical except for a mutation in the Fas gene in the lpr substrain (1). The present study demonstrates that lpr mice have an ~10-fold reduction in the level of Fas mRNA and produce aberrant mRNA transcripts containing an insertion of a fragment of the retrotransposon, ETn. ETn is a moderately repetitive (~200 copies) retrotransposon first identified by its high level of expression in embryos and in teratocarcinoma cells (17). Although the prototypic ETn sequence, MGI, isolated from a BALB/c genomic library was originally thought to be highly conserved (14), significant sequence differences have now been identified in the U5 region and immediately downstream of the 5' LTR of many ETn retrotransposons that have disrupted cellular genes (12, 13, 16, 18, 19). Since these apparently mobile ETn retrotransposons are themselves highly homologous, they are now regarded as a separate subfamily designated ETn γM85 (16) (according to the prototypic ETn retrotransposon inserted into the mouse IgG1 heavy chain switch region in the BALB/c myeloma, P3.26Bu4 [12]). The nucleotide sequence of the 183-bp ETn fragment inserted at nucleotide 233 of the MRL/Ipr Fas cDNA is virtually identical to the corresponding γM85 ETn sequence.

In addition to transposition in lymphoid cells, ETn insertion has recently been reported to cause myotonia in ADR mice (12). Insertion of ETn in an intron of the skeletal muscle chloride channel (CIC) gene disrupts the gene by inducing an abnormal CIC/ETn chimeric transcripts through alternative splicing (13). RT-PCR and Northern blot analysis of Fas expression in MRL/Ipr mice revealed at least two aberrant Fas/ETn chimeric transcripts. Despite the large size of the full-length ETn retrotransposon (5.7 kb) (14), the position of the splice site identified in two of the three CIC/ETn transcripts is located very close to the origin of the ETn insert at nucleotide 233 of lpr Fas cDNA. While the proximity is explained, in part, by the polypyrimidine tracts (see Figs. 4 and 5), other sequence or structural features of the U5 region of the ETn LTR may facilitate alternative splicing. The splicing pattern of the smaller (~800-bp) Fas/ETn transcript has not yet been determined.

Retrotransposon insertion may either increase or decrease expression of a gene (reviewed in reference 20). The 10–20-fold reduction in the expression of C4 in H-2k mice is associated with the insertion of the B2 retrotransposon into intron 13 of the C4 gene (21). B2 contains an alternative splice site and produces an abnormally spliced B2/C4 transcript as well as low levels of the wild-type transcript (21). Transsec-
tion experiments revealed that aberrant splicing of C4 mRNA was sufficient to cause the profound defect in the expression of C4 (22). Since expression of a normal-sized Fas transcript was detected by RT-PCR and Northern blotting in lpr mice, ETn is almost certainly present in an intron of the Fas gene. We therefore propose that the inserted ETn retrotransposon provides alternative splice site(s) and, analogous to the B2/C4 gene model, reduces Fas mRNA levels either through reduced transcription or stability of the message. This interpretation differs from the study of Watson et al. (23), who have recently reported that the Fas gene in MRL/lpr mice contains a 1.4-kb deletion as determined by restriction fragment analysis and Southern blotting. However, further genomic analysis has shown results that are most consistent with a 5.7-kb insertion in the Fas gene in MRL/lpr mice (M. L. Watson and M. F. Seldin, personal communication). Attempts to amplify ETn from lpr genomic DNA by PCR using primers flanking the ETn insertion identified in lpr Fas cDNA have been unsuccessful due to the limited size that can be amplified from genomic DNA by this technique (J. L. Chu, and K. B. Elkon, unpublished observations). Similarly, the ability to identify a single copy of ETn integrated into the MRL/lpr Fas gene by Southern blot analysis of genomic DNA is complicated by the presence of ~200 copies of ETn distributed throughout the mouse genome.

It is notable that many of the reported insertions of the ETn retrotransposon have been identified in genes of immunological interest (12, 16, 18, 19). Since ETn RNA is expressed in lymphoid cell lines and in normal spleen but is not known to encode long open reading frames (12–14), trans-acting elements present in lymphoid cells may enhance expression of ETn. Interestingly, the size of the ETn transcripts derived from thymus and liver differed. Detection of a 2-kb transcript in RNA obtained from the liver of MRL and C3H mice suggests either that the ETn is constitutively expressed and the transcript is spliced in the liver, or that the ETn LTR is inserted into a normal transcript. Since an ETn insertion has been identified in the 3′ untranslated region of the Q6 and Q8 genes of the mouse MHC (19), this observation is not without precedent. Although the point mutation detected in the Fas gene of CBA/lpr mice indicates that the insertion of ETn is not the sole mechanism responsible for alteration of the Fas gene, it remains to be determined whether other background abnormalities present in MRL mice (1) facilitate transposition and/or expression of the ETn retrotransposon.

The lpr gene is responsible for acceleration of lupus in MRL mice and for the production of autoantibodies and/or lymphadenopathy when backcrossed onto normal mouse strains (24–26). While the conclusion by Watanabe-Fukunaga et al. (3) is that the absence of Fas expression results in the failure of negative selection in the thymus, the appropriate TCR Vβ deletion in unmanipulated MRL/lpr mice (27, 28) implies either that Fas does not play a role in Vβ-related negative selection in the thymus, or that the 10-fold reduction in Fas expression observed in the present study is sufficient to allow negative selection of these thymocytes to occur. Although the sequence of the full-length Fas cDNA from lpr mice has not yet been determined, a wild-type product was detected by Northern blot and PCR analyses. In addition, the ETn insert detected in the Fas mRNA did not alter the translational reading frame. These findings suggest that a small amount of Fas protein may be synthesized in lpr mice. If the defect in negative selection in the thymus is quantitative rather than absolute, this may explain why negative selection of most T cells in the thymus is apparently normal in MRL/lpr mice but is reduced in transgenic lpr/lpr H-2Dk HY* male mice where all of the thymocytes are autoreactive (29).

Failure to detect Fas mRNA expression in the spleens of normal mice by Northern blot analysis (11) suggested that the Fas mutations impair negative selection of thymocytes rather than mature T cells (3). In the present study, Fas mRNA expression was readily detectable in RNA obtained from C3H control, MRL/+ +, and NZB/W spleen cells by RT-PCR, indicating that Fas may play a role in regulating the survival of mature lymphocytes. The increase in the level of expression of Fas mRNA observed in the spleens of MRL/lpr/+ + mice with age may reflect lymphocyte activation, since Fas expression in human lymphocytes has been shown to be upregulated after stimulation (30, 31). While detection of Fas mRNA expression in NZB/W thymus and spleen at levels comparable to controls indicates that impaired Fas expression is not a universal feature of murine lupus, further studies are required to evaluate the Fas protein function in murine and human SLE.

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Note Added in Proof: Sequencing of additional Fas/ETn chimeric cDNAs after RT-PCR with primer pair 1 has revealed a transcript with a 168-bp insertion of ETn identical to the ADRINS sequence shown in Fig. 4 A (GenBank accession no. L15353). After submission of this manuscript, Adachi et al. (32) reported that a 5.7-kb ETn retrotransposon has been detected in intron 2 of the Fas gene in MRL/lpr mice.

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