Autoimmune Lymphoproliferative Syndrome with Somatic Fas Mutations

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BACKGROUND
Impaired Fas-induced apoptosis of lymphocytes in vitro is a principal feature of the autoimmune lymphoproliferative syndrome (ALPS). We studied six children with ALPS whose lymphocytes had normal sensitivity to Fas-induced apoptosis in vitro.

METHODS
Susceptibility to Fas-mediated apoptosis and the Fas gene were analyzed in purified subgroups of T cells and other mononuclear cells from six patients with ALPS type III.

RESULTS
Heterozygous dominant Fas mutations were detected in the polyclonal double-negative T cells from all six patients. In two patients, these mutations were found in a fraction of CD4+ and CD8+ T cells, monocytes, and CD34+ hematopoietic precursors, but not in hair or mucosal epithelial cells.

CONCLUSIONS
Somatic heterozygous mutations of Fas can cause a sporadic form of ALPS by allowing lymphoid precursors to resist the normal process of cell death.
Fas (also called Apo-1 and CD95) is a cell-surface receptor belonging to the tumor necrosis factor receptor (TNFR) superfamily \(^1\) (Fas is the sixth member, TNFRSF6). Once triggered by its cognate ligand (Fas ligand), Fas initiates a cascade of events within the cell that culminates in the death of the cell (apoptosis). This process involves the formation of the death-inducing signaling complex, \(^2\) consisting mainly of the Fas-associated death domain and the caspase 8 and caspase 10 proteins. The essential role of Fas in lymphocyte homeostasis was initially recognized in MRL lpr/lpr mice, which have a germ-line autosomal recessive mutation of Fas. \(^3\) Subsequently, heterozygous dominant mutations of Fas were found in children with the autoimmune lymphoproliferative syndrome (ALPS), \(^4\), which is also known as the Canale–Smith syndrome. \(^7\) The main features of this disease are splenomegaly, lymphadenopathy, hypergammaglobulinemia (IgG and IgA), and autoimmunity. \(^6,9\) ALPS is characterized by the accumulation of a polyclonal population of T cells called double-negative T cells. These lymphocytes display the marker common to mature T cells, CD3, and \(\alpha/\beta\) T-cell–antigen receptors, but neither the CD4 nor the CD8 coreceptors (CD3+ T-cell receptor \(\alpha/\beta+\) CD4–CD8–). They normally account for less than 2 percent of peripheral \(\alpha/\beta+\) T cells \(^8\) and are distinct from the double-negative thymocytes in the cortex of the thymus, which lack CD3 and T-cell receptors for antigen. The double-negative T cells in patients with ALPS are poorly responsive to mitogens and antigens and fail to produce growth and survival factors such as interleukin-2. \(^10\) In Fas-deficient MRL lpr/lpr mice, the large population of double-negative T cells appears to originate from chronically activated CD8+ T cells that down-regulate the expression of CD8 and fail to undergo apoptosis. \(^3\) In humans, double-negative T cells also seem to be antigen-exposed T cells that have escaped apoptosis.

ALPS is classified according to the underlying genetic defect. \(^11\) In type 0 disease, homozygous Fas mutations usually cause a complete deficiency of the Fas protein and a severe form of the disease. \(^4,12,13\) In ALPS type I, heterozygous Fas mutations (ALPS type 1a) \(^14-16\) or, more rarely, heterozygous mutations in the gene for Fas ligand (ALPS type 1b) \(^17\) are usually associated with a partial defect in apoptosis mediated by Fas and its ligand. ALPS type II, which is characterized by resistance to Fas-mediated apoptosis despite the presence of normal Fas ligand and Fas, has been found in two patients with caspase 10 mutations. \(^18\) In ALPS type III, Fas-mediated apoptosis is also normal in vitro, \(^19\) and the genetic defect is unclear. Patients with ALPS type III may not have all four classic features of the syndrome — lymphoproliferation, excessive numbers of double-negative T cells, hypergammaglobulinemia, and autoimmune manifestations. Many cases of ALPS type III are sporadic, precluding the use of a genetic approach to identify the molecular defect. In the present study, we obtained lymphocytes from six children with ALPS type III for an in-depth analysis.

**METHODS**

**PATIENTS**

Blood samples were obtained from the six patients, their parents, and five healthy controls. All subjects or their parents or guardians provided written informed consent, validated by the Comité Consultatif pour la Protection des Personnes en Recherche Biomédicale. Table 1 summarizes the clinical features of the six patients.

**CELL CULTURE, APOPTOSIS ASSAY, AND ANALYSIS OF THE T-CELL–RECEPTOR REPertoire**

Peripheral-blood mononuclear cells were isolated from freshly drawn heparin-treated blood by means of Ficoll–Hypaque density gradient centrifugation. Apoptosis assays and repertoire analysis were performed on activated T cells and whole blood, respectively, as previously described. \(^16,20\)

**NUCLEIC ACID PREPARATION, AMPLIFICATION, AND DETECTION OF Fas MUTATIONS**

Total RNA was isolated from freshly isolated peripheral-blood mononuclear cells and T cells that had been activated by nine days of in vitro exposure to phytohemagglutinin. The reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay was performed as previously described. \(^4,16\) DNA extracted from phytohemagglutinin-activated lymphocytes or purified double-negative T cells was amplified with oligonucleotides spanning the nine Fas exons with the use of PCR conditions described elsewhere, \(^16\) except that the annealing temperatures for exons 4 and 7 were 58°C and 60°C, respectively. Leukocyte subgroups were purified by cell sorting (purification always exceeded 95 percent) with a fluorescence-activated cell sorter (FACS) (FACStarPLUS, Becton Dickinson) from the peripheral-blood mononuclear cells as described previously. \(^23\) DNA from these subgroups was amplified by nested PCR. Sequenc-
ing was performed directly on PCR products with the use of the Big Dye DNA Sequencing Kit (Perkin-Elmer) and an ABI PRISM 377 automated sequencer (Applied Biosystems). For quantification, PCR products corresponding to Fas exon 8 were ligated into the TOPO vector with the use of the TOPO-TA Cloning Instruction Manual (Invitrogen), and at least 60 clones were individually sequenced. Allele-specific PCR was performed with the successive use of two sets of primers. The first step amplified Fas exon 8 on both alleles; the second step amplified either the mutant or the wild-type allele. PCR products were separated on 1.5 percent agarose gel and then transferred to a Hybridization Transfer Membrane (NEN Life Science Products). Oligonucleotide hybridization was performed as described previously at 3°C below the melting temperature of the oligonucleotide. Descriptions of all oligonucleotide sequences and PCR conditions are available on request.

**RESULTS**

**IDENTIFICATION OF Fas MUTATIONS**

We studied six patients with phenotypic features of ALPS (Table 1) but with normal levels of Fas-mediated apoptosis of phytohemagglutinin-activated T-cell blasts (Table 2) and no family history of ALPS. However, because of the strong association between an excess of double-negative T cells and a defect in Fas-mediated apoptosis, we looked for Fas gene mutations in FACS-sorted double-negative T cells from these patients and found heterozygous Fas mutations in all six (Table 2). Donor and acceptor splice-site mutations of exon 8 were identified in Patient 1 and Patient 6, respectively, and a donor splice-site mutation of exon 7 was identified in Patient 5 (Table 2). These mutations are predicted to lead to the splicing out of the corresponding exon on RNA. Patient 2 had a nonsense mutation in exon 8, and Patient 3 had a missense mutation in exon 9 (D244V); Patient 4 had a deletion of 8 bp in exon 9 leading to a premature stop codon at position 227 (Table 2). Identical Fas mutations or mutations leading to identical changes in the structure of Fas have been described in patients with ALPS type Ia (Table 2) and have been shown to be dominant. We also looked for Fas mutations in sorted double-negative T cells from five healthy, age-matched controls and found none.

In contrast to the results with purified double-negative T cells, mutant Fas products could not be
detected by PCR performed on DNA from T-cell blasts that had been activated in vitro for nine days by exposure to phytohemagglutinin.

**EXPRESSION OF MUTANT ALLELES**

The expression of the mutant alleles in T cells from Patient 1 and Patient 2 was analyzed. An aberrant product of RT-PCR amplification of Fas DNA, as well as the expected normal-sized product, was detected in cDNA prepared from resting peripheral-blood mononuclear cells (Fig. 1A). Sequencing of these RT-PCR products revealed a wild-type sequence and an abnormal product in which exon 8 was missing (data not shown). The omission of exon 8 in Fas messenger RNA could be the consequence of the nonsense and splice-site mutations in DNA of double-negative T cells from Patient 1 and Patient 2, respectively (Table 2). Indeed, such mutations were detected in genomic DNA from purified double-negative T cells (Fig. 1B) but not in that from phytohemagglutinin-stimulated T cells.

Moreover, the double-negative T cells of these patients, which were readily detectable among resting T cells (Fig. 1C), were undetectable after being incubated with phytohemagglutinin for nine days (Fig. 1C) or after stimulation of T cells with antibodies against T-cell receptors (data not shown). Thus, the absence of mutant cells after in vitro stimulation by phytohemagglutinin accounts for the normal Fas-mediated apoptosis in unfractonated lymphocytes from patients with ALPS type III.

**DISTRIBUTION OF FAS MUTATIONS**

To determine the cellular distribution of the Fas mutations in Patient 1 and Patient 2, we performed PCR analysis of genomic DNA from FACS-sorted peripheral-blood CD4+ or CD8+ T cells (hereafter called single-positive T cells), T-cell receptor γ/δ T cells, natural killer cells, B cells, monocytes, splenic CD34+ hematopoietic progenitors, hair cells, and buccal epithelial cells. To rule out cellular chimerism, the migration profiles of nine polymorphic markers on double-negative T cells and single-positive T cells were determined (data not shown) and were found to be identical.

We first used a mutation-specific PCR method to determine which type of cell or tissue bears the Fas mutation, and we quantified the level of mutant cells in these populations. By means of dilution experiments, we found that heterozygous Fas mutations could be detected by direct sequencing of PCR products only when more than 20 percent of cells carried the mutation (data not shown). With a more sensitive analysis, which entailed cloning and sequencing PCR products, we could detect cells with mutations when more than 1 percent of cells carried the mutation, a percentage compatible with the level of purity of FACS separation techniques.

The mutation in exon 8 was detected in all leukocyte subgroups from Patient 1, whichever method was used (Fig. 2). Thus, in this patient, a mutant Fas was present in more than 20 percent of lymphocytes and myeloid cells. The quantitative analysis

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Table 2: Levels of Fas-Mediated Apoptosis and Heterozygous Fas Mutations in Six Patients with Autoimmune Lymphoproliferative Syndrome (ALPS) Type III and Three Patients with ALPS Type Ia.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Fas-Mediated Apoptosis%</th>
<th>Fas Mutation Type</th>
<th>Location</th>
<th>Predicted Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALPS type III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>70</td>
<td>Somatic</td>
<td>Exon 8</td>
<td>P201fs(stop 204)</td>
</tr>
<tr>
<td>Patient 2</td>
<td>85</td>
<td>Somatic</td>
<td>Exon 8</td>
<td>P201fs(stop 204)</td>
</tr>
<tr>
<td>Patient 3</td>
<td>93</td>
<td>Somatic</td>
<td>Exon 9</td>
<td>D244V</td>
</tr>
<tr>
<td>Patient 4</td>
<td>73</td>
<td>Somatic</td>
<td>Exon 9</td>
<td>S214fs(stop 227)</td>
</tr>
<tr>
<td>Patient 5</td>
<td>86</td>
<td>Somatic</td>
<td>Exon 7</td>
<td>W173fs(stop 209)</td>
</tr>
<tr>
<td>Patient 6</td>
<td>83</td>
<td>Somatic</td>
<td>Exon 8</td>
<td>P201fs(stop 204)</td>
</tr>
<tr>
<td>ALPS type Ia†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>8</td>
<td>Germ line</td>
<td>Exon 8</td>
<td>P201fs(stop 204)</td>
</tr>
<tr>
<td>Patient 2</td>
<td>4</td>
<td>Germ line</td>
<td>Exon 9</td>
<td>S214fs(stop 224)</td>
</tr>
<tr>
<td>Patient 3</td>
<td>10</td>
<td>Germ line</td>
<td>Exon 7</td>
<td>K181fs(stop 199)</td>
</tr>
</tbody>
</table>

* The mean (±SD) value among 80 controls was 83±11 percent. Values are the percentages of phytohemagglutinin-activated T-cell blasts that underwent apoptosis in vitro.
† Data on Patients 1 and 2 are from Rieux-Laucat et al. Data on Patient 3 are from Vaishnaw et al.
SOMATIC Fas MUTATIONS IN AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME

A

Fas RNA

Resting T Cells

Control, Patient 1, Patient 2

Wild-type, Mutant

Activated T Cells

Control, Patient 1, Patient 2

Wild-type

B

Purified Double-Negative T Cells

Glu Thr Val Ala Ile Asn Leu Ser

Control

Glu Thr Val Ala Ile Asn Leu Ser

Patient 1

Glu Thr Val Ala Ile Asn Leu Ser

Patient 2

Stop

C

Resting T Cells

TCR α/β

CD4+CD8+

Activated T Cells

TCR α/β

CD4+CD8+

Control

Patient 1

Patient 2

0.4%

30%

9%

0.9%

0.6%

0.9%

100

101

102

103

104

100

101

102

103

104

100

101

102

103

104

100

101

102

103

104

TCR

a/β

Activated T Cells

TCR

a/β

CD4+CD8+
showed that 100 percent of double-negative T cells but only 20 percent of single-positive T cells and 14 percent of monocytes carried the mutation, indicating that, with the exception of double-negative T cells, these different leukocyte subgroups contained a similar proportion of mutant cells. The mutation was undetectable in hair cells and buccal epithelial cells from Patient 1 on direct sequencing.

In Patient 2, the Fas mutation was found in all leukocyte subgroups by means of the mutation-specific PCR method. In contrast, the mutation was detected in double-negative T cells by direct sequencing (Fig. 2) but not in other leukocyte subgroups, suggesting that it was carried by less than 20 percent of cells in the other leukocyte populations. The mutation-specific quantitative method indicated that 100 percent of double-negative T cells from the blood and spleen of Patient 2 were mutant cells, whereas only 10 percent of single-positive T cells carried the mutation.

A purified CD34+CD19−CD7− population, which was known to be enriched for hematopoietic progenitors, was obtained from a spleen sample from Patient 2. Mutation-specific PCR revealed mutant cells in this population (Fig. 2). We estimate, taking into account the purification efficiency of FACS, that less than 2 percent of these cells carried the mutation (Fig. 2). The Fas mutation was undetectable in nonhematopoietic cells, even when the mutation-specific PCR was used (Fig. 2). All these results suggest that in both Patient 1 and Patient 2, Fas mutations originated in hematopoietic stem cells or possibly earlier, in mesenchymal precursors. The specific accumulation of Fas mutants in the double-negative T-cell compartment underlines the essential role of Fas in the control of lymphocyte homeostasis in the periphery.

**T-CELL-RECEPTOR REPertoire AND THE ORIGIN OF DOUBLE-NEGATIVE T CELLS**

Somatic Fas mutations have been described in rare cases of a syndrome involving monoclonal or oligo-clonal double-negative T cells.24 We therefore analyzed the population of T-cell–receptor β chains in double-negative T cells from both patients using antibodies against the β chains and found polyclonal T-cell populations of double-negative and single-positive T cells in both patients (Fig. 3).

**DISCUSSION**

We found that patients with mosaicism carrying heterozygous Fas mutations in hematopoietic cells have an ALPS phenotype. In the light of our findings, the classification of ALPS requires revision, with patients such as ours possibly denoted as belonging to a subgroup with mosaic ALPS type I, or ALPS type I1. In support of this reclassification is the fact that the clinical phenotype associated with these somatic mutations is indistinguishable from that of ALPS type I.

Our study demonstrates that peripheral lymphocytes with a dominant somatic Fas mutation exhibit a selective advantage (Fig. 4). Germ-line mutations of Fas have been reported to impair Fas-induced apoptosis of lymphocytes in patients with ALPS type Ia.14,15 By resisting apoptosis, the mutant cells accumulate and become double-negative T cells. This
interpretation is consistent with data from Fas-deficient chimeric mice and can account for the lymphadenopathy and splenomegaly in all six of our patients. Indeed, a lymph node from Patient 4 showed paracortical expansion consisting of double-negative T cells, a histologic picture indistinguishable from that seen in patients with ALPS type I (data not shown). Similarly, the relatively large proportion of mutant cells in peripheral lymphocytes from Patient 2, as compared with the smaller proportion of mutant cells among hematopoietic progenitors, suggests that Fas mutations provide a selective advantage (by protecting against apoptosis) during hematopoesis, a finding consistent with observations in MRL lpr/lpr mice.

We identified Fas mutations in freshly purified double-negative T cells but not in phytohemagglutinin-activated T cells. The normal in vitro response to Fas-induced apoptosis by activated T cells is consistent with the absence of Fas mutations in such cells. This result might be due to the high death rate of double-negative T cells in vitro. Double-negative T cells are believed to originate from activated peripheral single-positive T cells that have received a death-inducing signal but cannot die, owing to a defect in Fas signaling. A similar abnormality of double-negative T cells has been described in patients with ALPS type I, suggesting that a signal required for the survival of double-negative T cells is lacking in tissue-culture medium. In vivo, this signal could be provided by self-antigens, and chronic stimulation of apoptosis-resistant cells by autoantigens could account for the autoimmune manifestations and lymphoproliferation of ALPS. However, autoimmune manifestations were observed in only four of our six patients. In addition, there is no clear correlation between the numbers of double-negative T cells and autoimmunity, and whether these cells recognize and respond to self-antigens is also unknown.

A proportion of all hematopoietic cells from our patients carried Fas mutations, whereas they were not found in hair cells or buccal epithelial cells. Several mechanisms could account for this finding. One is chimerism, which could be the consequence of transplacentual passage of maternal blood or cell fusion from an aborted dizygote twin. This possibility was excluded, since the population of both the double-negative T cells (containing mutant cells alone) and the single-positive T cells (containing an excess of wild-type cells) had similar patterns of DNA polymorphic markers (data not shown).

Figure 3. Analysis of the T-Cell–Receptor (TCR) Repertoire on Single-Positive (CD4+ or CD8+) T Cells and Double-Negative (CD4−CD8− TCR α/β+) T Cells from Patient 1, Patient 2, and 80 Controls.
Values in controls are expressed as the mean (horizontal line in each box), with the standard deviation (top and bottom of the box) and 95 percent confidence interval (I bar).
Therefore, these mutations must have resulted from a somatic mutation that occurred during embryonic or fetal development, or after birth. Although Fas mutations were not detected in cells from the mouth or hair (originating from ectoderm), their presence in germ cells (of endodermic origin), which would indicate a mutation early in embryogenesis, was not formally ruled out. Analyses of additional tissues would be required to narrow the timing of the mutational events.

The clinical features of our patients with ALPS type III resemble those of patients with ALPS type I who have identical or similar germ-line Fas mutations. In some other conditions, however, mosaicism is usually associated with a mild phenotype, because of somatic reversions to the wild type. In such cases, wild-type revertant cells can have a selective advantage, enabling their expansion and the partial restoration of the wild-type phenotype. Our findings in patients with acquired ALPS repre-
sent an example of dominant somatic mutations' conferring a selective advantage of mutant cells over normal cells. Nevertheless, a 10-year follow-up showed that the proportion of mutant lymphocytes was steady over time and that the cells did not outgrow normal cells.

Healthy relatives of patients with ALPS type I can carry an inherited dominant Fas mutation, thereby illustrating the partial clinical penetrance of some Fas mutations. Mutations affecting the intracellular domain of Fas are associated with greater clinical penetrance than mutations affecting the extracellular domain. Notably, all the mutations we found affected the intracellular domain. They are predicted to generate abnormal Fas molecules and have been associated with full penetrance of the disease in patients with ALPS type I.

In conclusion, we found that some patients with ALPS type III have somatic Fas mutations in cells of hematopoietic lineages in the absence of any malignant condition. This situation is an example of a nonmalignant, genetically acquired disease in which a selective advantage (resistance to death) is conferred by Fas mutations.

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