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Dominant Interfering Fas Gene Mutations Impair Apoptosis in a Human Autoimmune Lymphoproliferative Syndrome

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Summary

Five unrelated children are described with a rare autoimmune lymphoproliferative syndrome (ALPS) characterized by massive nonmalignant lymphadenopathy, autoimmune phenomena, and expanded populations of TCR–CD3$^+$CD4$^-$CD8$^-$ lymphocytes. These findings, suggesting a genetic defect in the ability of T lymphocytes to respond to normal immunoregulatory mechanisms, prompted an evaluation of lymphocyte apoptosis. Each child had defective Fas-mediated T lymphocyte apoptosis associated with a unique, deleterious Fas gene mutation. One mutation appeared to cause a simple loss of function; however, four others had a dominant negative phenotype when coexpressed with normal Fas. Family studies demonstrated the inheritance of the mutant Fas alleles. The occurrence of Fas mutations together with abnormal T cell apoptosis in ALPS patients suggests an involvement of Fas in this recently recognized disorder of lymphocyte homeostasis and peripheral self-tolerance.

Introduction

Apoptosis (or programmed cell death) plays a decisive regulatory role not only during T cell ontogeny but also in immune responses involving mature peripheral T lymphocytes. It is well established that immature thymocytes bearing autoreactive receptors can be efficiently eliminated if they encounter ligand during thymic development (Burnet, 1969; Kappler et al., 1987; Hengartner et al., 1988; Kisielow et al., 1988). More recent studies indicate that peripheral, mature T lymphocytes also undergo antigen-stimulated apoptosis under appropriate conditions (Lenardo, 1991; Russell et al., 1991; Critchfield et al., 1994). Following immunization with superantigens or peptide antigens, specific T cell clones expand, but then are reduced or eliminated upon restimulation, particularly by high concentrations of antigen. Programmed cell death acts to preserve peripheral T cell homeostasis, regulating the tempo and duration of immune responses in vivo (Jones et al., 1990; Webb et al., 1990; Rocha and von Boehmer, 1991; Kawabe and Ochi, 1991; Kyburz et al., 1993; Moskophidis et al., 1993; Critchfield et al., 1995; McHeyzer-Williams and Davis, 1995).

Studies of mouse strains with genetic predispositions to autoimmune disease suggest that in addition to regulating responses to exogenous antigens, apoptosis of mature T cells maintains tolerance to self-antigens (Theophilopoulos and Dixon, 1968; Nagata and Golstein, 1995). Mice homozygous for the *lpr* or *gld* mutations manifest hypergammaglobulinemia, autoantibody production, glomerulonephritis, arthritis, vasculitis, and accumulation of nonmalignant TCRαβ$^+$CD4$^-$CD8$^-$ T lymphocytes in their secondary lymphoid organs. Understanding the *lpr* and *gld* defects began with the isolation of monoclonal antibodies (MAbs) that induced apoptosis when bound to the cell surface receptor Fas (also designated CD95, Apo-1, Fas antigen, and Apt) (Trauth et al., 1989; Yonehara et al., 1989). Genetic cloning and mapping studies revealed that *lpr* was a recessive mutation in Fas, while *gld* was a mutation in the gene for Fas ligand (FasL) (Watanabe-Fukunaga et al., 1992; Takahashi et al., 1994; Lynch et al., 1994). Homozygosity for either *lpr* or *gld* causes defective T cell receptor (TCR)-induced death of mature T cells (Russell et al., 1993; Gillette-Ferguson and Sidman, 1994; Alderson et al., 1995; Dhein et al., 1995). Thus, autoimmunity in mice with these gene defects seems to be predominantly due to a failure in peripheral, rather than thymic, T cell deletion (Singer and Abbas, 1994).

Fas and FasL are members of two superfamilies of complementary receptors and ligands that are important in immune regulation (Smith et al., 1994; Nagata, 1994; Nagata and Golstein, 1995). Fas belongs to the tumor necrosis factor receptor (TNFR) superfamily, which includes the two TNFRs (type I, which is also known as p55, and type II, which is also known as p75), the low affinity nerve growth factor receptor, CD27, CD30, CD40, OX40, and 4-1BB. These membrane-spanning receptors have 20%–25% amino acid identity and contain variable numbers of extracellular cysteine-rich domains (CRDs) (Banner et al., 1993), regions approximately 40 amino acids in length with six cysteine residues in conserved positions. There are four CRDs in the p55 TNFR and three in the Fas protein. The crystallographic structure of the p55 TNFR and other biochemical evidence suggest that the functional receptor is a trimeric complex with a threefold axis of symmetry perpendicular to the cell membrane. In contrast with their extracellular homology, the cytoplasmic regions of TNFRs have little amino acid conservation, except for Fas and the p55 TNFR. These two receptors share a 70 amino acid intracellular "death domain" that transduces signals for cell death (Tartaglia et al., 1993; Itoh and Nagata, 1993). FasL and other related ligand proteins also form trimers of subunits in a conical configuration with threefold sym-

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metry that complements the receptor complex structure. When ligand is bound, it is embraced on three sides by the individual receptor subunits, which adopt an extended linear conformation with their long axes nearly parallel to one another and projecting from the membrane. The CRDs maintain receptor subunit conformation by means of intracysteine disulfide bonds so that interaction with ligand can occur. The trimeric association of receptor and ligand subunits appears to be crucial for signal generation on the intracellular side of the receptors (Banner et al., 1993).

While inbred mouse strains bearing *lpr* or *gld* mutations provide convincing evidence that failure of mature T cell deletion may breach peripheral tolerance, such evidence has been lacking in humans. However, an association between lymphoproliferation and autoimmune disease was noted recently in two children with lymph node enlargement, hypergammaglobulinemia, and expansion of an otherwise minor population of $TCR\alpha\beta^+CD4^-CD8^-$ T lymphocytes (Sneller et al., 1992). Additional studies have been undertaken on the first patient in the report by Sneller et al. (1992) and on four additional individuals with the same constellation of findings, which we have designated autoimmune lymphoproliferative syndrome (ALPS). In this report we show that the $CD4^+$ and $CD8^+$ single-positive T lymphocytes from each child affected with ALPS had defective TCR-induced apoptosis in association with deleterious mutations of the human Fas gene (*APT1*). The mutant Fas alleles, in contrast with *lpr* in the murine disease, were heterozygous in affected patients, and four demonstrated a dominant interfering effect on apoptosis. The association of defects in a T lymphocyte apoptosis effector molecule with a human syndrome of autoimmunity and lymphoproliferation provides a basis for further dissection of the causes of human autoimmune disease.

Results

Clinical Features of ALPS

Five children came to medical attention because of lymph node and spleen enlargement as early as 2 months after birth (Table 1). Generalized hypercellularity of the secondary lymphoid organs, often of massive proportions, was exhibited by these children and required removal of enlarged spleens in four of them. In all cases, the increased populations of cells in the lymph nodes and blood appeared to be lymphocytic, but no malignant transformation or metastasis to nonlymphoid organs has occurred. There was no evidence of opportunistic infection (other than postsplenectomy sepsis in two children) or any form of chronic immune stimulation. In each patient, a normally minor population of mature TCR–$CD3^+$ T lymphocytes that lacked CD4 and CD8 expression (double negative) was expanded 3- to 60-fold. In four of the five children, these double-negative T cells, both in the circulation and in biopsied lymph nodes, expressed $\alpha\beta$ TCRs. In patient 4, however, the double-negative T cells in the lymph nodes expressed $\alpha\beta$ TCRs, whereas most circulating double-negative T cells expressed $\gamma\delta$ TCRs. Double-negative T cells in these patients responded poorly to TCR engage-

Table 1. Clinical and Laboratory Features of Five Children with ALPS

Patient	Age of Onset	Sex	Adenopathy[a]	Spleen[b]	Liver[b]	Urticarial Rash	Hemolytic Anemia	Thrombocytopenia	Neutropenia	Glomerulitis	Autoantibodies	$CD3^+CD4^-CD8^-$ (cells/mm^3) (%)[c]
1	6 months	M	3+	10 (r)	3	+	−	+	+	−	−	4003 (30)[c]
2[d]	18 months	F	4+	7 (r)	4	+[e]	+	+	−	+	−	6416 (40)
3	5 years	M	2+	2 (r)	4	+	+	+	+	−	+	2059 (15)
4	2 years	M	3+	9	6	+	−	−	+	−	−	304 (18)
5	2 months	M	4+	15 (r)	3	+	+	+	+	−	+	550 (16)

[a] Multiple chains of 1–2 cm nodes are indicated by 2+, multiple chains of nodes visible without palpation by 3+, and massive adenopathy distorting normal anatomical landmarks by 4+.
[b] Palpable below the costal margin or the last recorded enlargement of spleen prior to its removal (r).
[c] Normal adult value is 9–122 cells/mm^3 (<6% of total lymphocyte number). Percent is shown in parentheses.
[d] This individual was the first patient studied by Sneller et al. (1992).
[e] Biopsy diagnosis of leukocytoclastic vasculitis.

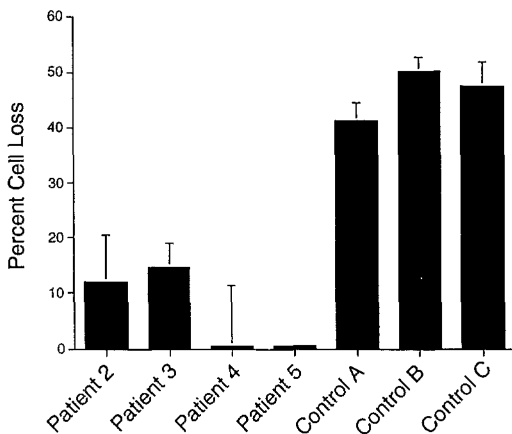


Figure 1. T Cells from ALPS Patients Show Defective Apoptosis

The percent T cell loss induced by MAb anti-CD3ε (64.1), prebound at 1 μg/ml to plastic wells, in samples from patients 2, 3, 4, and 5 and from normal individuals, controls A, B, and C. Values are the arithmetic means with standard deviations from three independent determinations.

ment and produced significantly less interleukin-2 (IL-2) and interferon-γ than did normal T cells (Sneller et al., 1992; data not shown). All patients had 5- to 20-fold elevations in B lymphocyte and natural killer cell numbers in blood and lymphoid organs, but no abnormalities in the the bone marrow.

Each of the children also manifested clinically significant autoimmune disease (Table 1). All suffered episodes of autoimmune hemolytic anemia, thrombocytopenia, neutropenia, or some combination of these. Autoantibodies against red blood cells and platelets were demonstrated in patients 3 and 5. All patients had recurrent urticarial rashes consistent with immune vasculitis. Moderate growth retardation was evident in three patients, and patient 2 required several cycles of dialysis for renal insufficiency due to glomerulonephritis. These complications could each be attributed to immune hyperreactivity and deposition of antigen–antibody complexes. Consistent with this explanation, laboratory studies showed all patients to have hypergammaglobulinemia. Taken together, the clinical picture of children with ALPS was consistent with normal lymphocyte generation, but severely impaired control of mature lymphocyte homeostasis in the periphery. Although no patient had a family history of similar illnesses, the father of patient 3 and other members of his family had Hodgkin's disease.

Defective Apoptosis via TCR Restimulation

To examine the basis for dysregulated lymphoproliferation and autoimmunity in ALPS, we considered a defect in mature T lymphocyte cell death. As a screen for impaired apoptosis, peripheral blood lymphocytes (PBLs) from patients 2–5 and controls were primed with phytohemagglutinin, anti-CD3 MAb (OKT3), and IL-2 (Pelfrey et al., 1995). Apoptosis was then induced by cross-linking the TCR–CD3 complex with anti-CD3ε MAb (64.1) immobilized in culture wells. This stimulation resulted in the apoptotic loss of 40%–55% of control lymphocytes (Figure 1). In contrast, significantly reduced destruction, 0%–15% cell loss, occurred in lymphocytes from the four ALPS patients tested. A failure of programmed cell death was confirmed by direct detection of fragmented DNA ends after anti-CD3 exposure in the control samples, but not in the samples of ALPS patients (data not shown).

The differences in ability to undergo apoptosis were not due to differences in composition of the cell populations between patient and control cultures. As previously noted by Sneller et al. (1992), the abnormal $TCR\alpha\beta^+CD4^-/CD8^-$ lymphocytes of our patients responded poorly to stimulation and were rapidly lost during in vitro cultivation. Thus, at the time of assay for apoptosis, all cultures contained >98% single-positive $CD4^+$ or $CD8^+$ mature T lymphocytes (data not shown). In addition, poor proliferation of the single-positive T cells of the patients, which would render them resistant to apoptosis (Boehme and Lenardo, 1993), was excluded by testing all cultures for proliferation at the time of assay. Finally, the lymphocytes from patients 3, 4, and 5 demonstrated intact apoptotic responses when stimulated by topoisomerase inhibitors or other antimetabolites that induce apoptosis in cycling T cells, indicating that the T cells of the patients were not innately resistant to programmed death (data not shown). We therefore concluded that patients with ALPS had a significant defect in the TCR-induced pathway to apoptosis in their mature T lymphocytes.

Fas Mutations in Affected Patients

Because Fas-mediated apoptosis has been recently implicated in T cell homeostasis and maintaining peripheral tolerance, we considered Fas as a candidate disease gene for ALPS patients. We analyzed the Fas genomic locus and Fas mRNA, cloned after reverse transcription–polymerase chain reaction (RT–PCR), from activated lymphocytes of patients and available family members. Sequence determination of cDNAs spanning the complete 1.1 kb coding region of the 2.5 kb mRNA revealed that each of the five ALPS patients expressed one or more abnormal Fas mRNAs in addition to normal Fas mRNA. The abnormal Fas allele of patient 1 had a single base deletion of G429. His mutant transcript would thus encode a normal Fas protein only up to the middle of the first CRD (CRD1), followed by 34 missense amino acids and a premature termination (Figure 2A), and therefore cause a loss of function.

Lymphocytes from patients 2 and 4 yielded Fas RT–PCR products, visualized with ethidium bromide staining, of abnormal as well as normal sizes, suggesting mutations involving mRNA splicing. Two abnormally small cDNA species from patient 2 proved upon sequence analysis to lack, in one case, exon 3 and, in the other, exons 3 and 4 (see diagram in Figure 2B). Genomic sequence determination revealed that the aberrant splicing was due to the duplication of a T residue in the 5′ splice site of intron 3, changing the normal GTAAG to GTTAAG, which is unlikely to function as a splice donor site (Krawczak et al., 1992). The relative quantity of cDNA produced from mutant allele of patient 2 after RT–PCR was comparable to that from

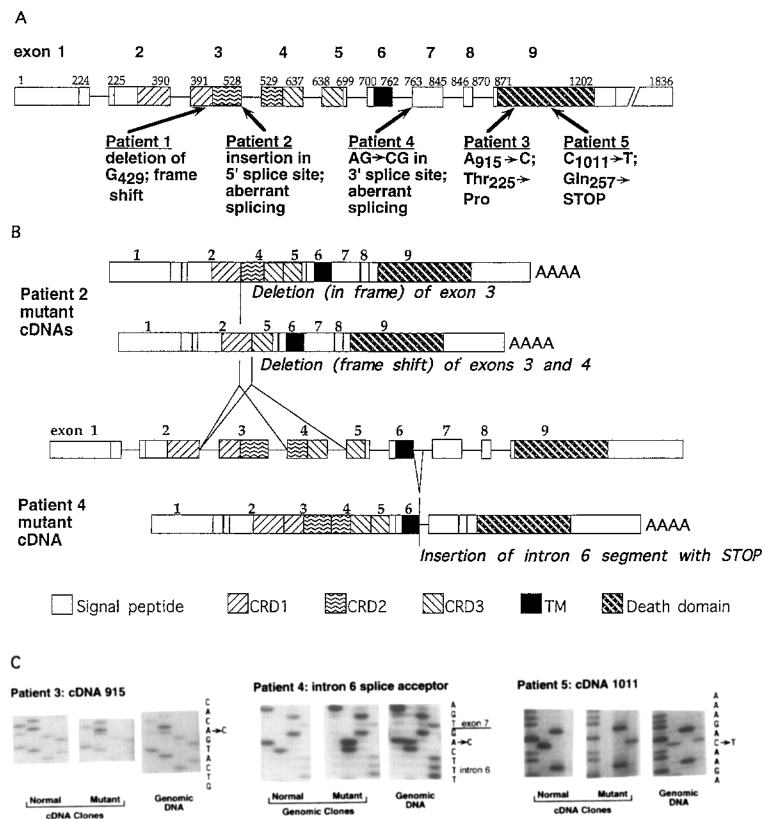


Figure 2. Fas Gene Is Mutated in ALPS Patients

(A) Structure of human Fas gene locus (*APT1*, *APO-1*), illustrating the nature and location of mutations with respect to signal peptide sequence, three CRDs, transmembrane (TM) region, and intracytoplasmic death domain involved in signal transduction. cDNA nucleotide and amino acid numbers correspond to Itoh et al. (1991); exon numbers and boundaries are as reported by Behrmann et al. (1994) and are available from GenBank (accession numbers X81334–X81342).

(B) Mechanism of the formation of abnormally spliced Fas mRNA species in lymphocytes of patients 2 and 4.

(C) Examples of sequence determination of Fas mutations from ALPS patients in individual normal and mutant cDNA clones for patients 3 and 5, genomic clones from patient 4, and directly amplified genomic DNA for each of these patients. Nucleotide base order: A, C, G, T.

her normal allele, suggesting that mRNA production and stability were not compromised by the mutation. The smallest mRNA (lacking exons 3 and 4) of patient 2 was predicted to have a frameshift and consequential premature termination after 21 missense codons, whereas the mRNA lacking exon 3 encoded an in-frame Fas protein in which the first two CRDs (CRD1 and CRD2) were foreshortened and fused into one.

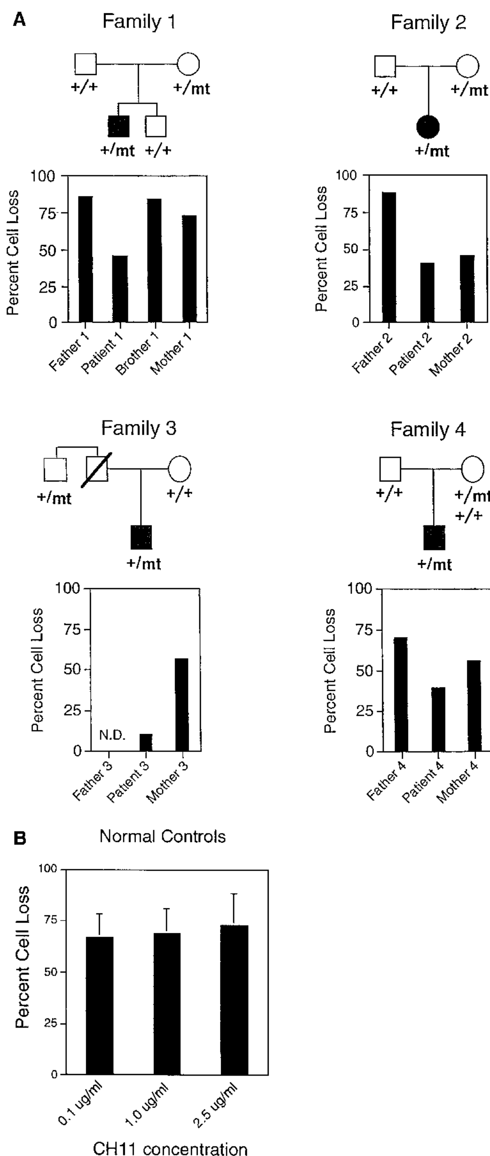
Patient 4 expressed both a normal and a slightly enlarged Fas mRNA. The large Fas mRNA was produced by the interposition of nucleotides from the distal half of intron 6 (Figure 2B). The genomic sequence of the mutant allele of patient 4 showed an A to C transversion in the 3′ splice site of intron 6, changing the invariant AG to a nonfunctional CG (Figure 2C). An alternate 3′ splice motif (TAG) that was 72 bp 5′ to exon 7 was used as the 3′ splice site for this allele, thereby causing the inclusion of the intron 6 nucleotides in the abnormally processed transcript. This aberrant mRNA thus encoded the first six Fas exons, including the transmembrane domain, followed immediately by an in-frame termination codon.

Single nucleotide substitutions found in Fas cDNA from patients 3 and 5 were confirmed by direct sequencing of genomic DNA (Figures 2A and 2C). Patient 3 had an A to C transversion at cDNA nucleotide 915 in one allele, producing a nonconservative substitution of proline for Thr-225. In patient 5, a premature termination was introduced by a C to T transition at cDNA nucleotide 1011, substituting a TAG stop codon for Glu-257. His mutant allele was noted to have the A allotype (Fiucci and Ruberti, 1994), positively distinguishing it from the normal allele, which was allotype B. Mutations in both these patients lay in the intracellular death domain of Fas (Itoh and Nagata, 1993; Tartaglia et al., 1993), as shown in Figure 2A.

Mothers of all five patients, fathers of three, and the healthy brother of patient 1 were studied. Father 3 was deceased from Hodgkin's disease. However, a DNA sample obtained from his brother (the paternal uncle of patient 3), who also had been diagnosed with Hodgkin's disease, showed heterozygosity for the same mutation as patient 3 himself. This indicated that father 3 was the source of the mutation in patient 3. Moreover, genomic DNAs from lymphocytes of mothers 1, 2, 4, and 5 each had one normal Fas allele and one with the identical Fas mutations found in their children affected with ALPS (Figure 3A). Interestingly, the lymphocytes from mother 4 yielded multiple Fas genes, including both A and C allotypes with a normal Fas coding sequence, as well as a gene of allotype C bearing the mutation found in her son. This finding, along with a normal 46XX karyotype, suggested that mother 4 was a mosaic for this Fas mutation, which would therefore be present in only a portion of her bone marrow–derived cells and germ cells. Full-length Fas cDNA sequences of at least ten RT–PCR clones or clones of different allotypes from fathers 1, 2, and 4 and from mother 3 were normal.

Lack of Fas Mutations in Normal Individuals

Unlike polymorphisms in the human Fas gene reported by Fiucci and Roberti (1994), which do not alter the amino acid sequence of the protein, the mutations found in patients with ALPS and their parents would be predicted to have detrimental effects on Fas protein function. Nonethe-



less, it was important to establish whether such mutations occur commonly in the general population. Single strand conformation polymorphism analysis of PCR-amplified Fas genomic fragments, using primers flanking or within the coding regions (Table 2), readily detected altered mobility patterns produced by each of the ALPS patient mutations. No instance of any of these alterations was seen in either Fas allele from DNA from 20 unrelated individuals in the Centre d'Etudes du Polymorphisme Humain (CEPH) genome mapping collection or from 30 additional unrelated normal subjects, comprising a total of 100 independent examples of the Fas locus on chromosome 10q23 (data not shown).

Apoptosis via Fas Cross-Linking

To determine whether the above Fas mutations were associated with specific Fas-mediated defects in apoptosis, activated and IL-2-treated lymphocytes from patients 1–4 and their family members were stimulated with 1 μg/ml of the agonistic anti-Fas MAb CH11 (Itoh et al., 1991). In all patients, there was a defect in Fas-mediated T cell death compared with family members who did not have a mutant Fas gene (Figure 3A) or to ten normal controls (Figure 3B).

Figure 3. Fas Mutations and Apoptosis Defects Are Inherited

(A) Partial pedigrees from families 1–4. Patients affected with ALPS are shown by closed symbols; unaffected individuals, open symbols; squares, males; circles, females; slash, deceased. The genotypes indicate the presence of normal (plus sign) or mutant (mt) alleles, as described in Figure 2. In all cases, complete nucleotide sequence determination proved the parental mutant Fas allele as identical to that in the affected. Graphs show percent T cell loss after exposure to 1 μg/ml anti-Fas MAb CH11 (IgM). Values are averages from duplicate determinations for the patients and indicated family members.

(B) Average percent cell loss for lymphocyte samples from ten different healthy individuals. Two independent determinations were made for each individual using the concentrations of CH11 shown. Data were pooled for each CH11 concentration and, standard deviations of all determinations were calculated. Below 0.1 μg/ml of CH11, decreased T cell apoptosis was seen (data not shown).

Table 2. Primers Used for PCR and Analysis of Fas Sequence

Primers	cDNA Location	Sequence
Primers surrounding coding region	143-164	CTCAGTACGGAGTTGGGGAAGC
	1643-1622R	ATTTAGAGGCAAAGTGGCCTGC
	1243-1221R	CTAATTGCATATACTCAGAACTG
Primers within coding region	362-385, ex2F	CCAATTCTGCCATAAGCCC
	421-440, ex3F	ATGGGGATGAACCAGACTGC
	549-569, ex4F	TGCACCCGGACCCCAGAATACC
	739-761, ex6F	TGCCAATTCCACTAATTGTTTGG
	951-975, ex9F	GGTGTCAATGAAGCCAAAATAGATG
	1243-1221, ex9R	CTAATTGCATATACTCAGAACTG
	989-962, ex9R	GTCATTCTTGATCTCATCTATTTTGGCT
	792-767, ex7R	TGCATGTTTTCTGTACTTCCTTTCTC
	562-542, ex4R	TGGGTCCGGGTGCAGTTTATT
	350-328, ex2R	CAGGCCTTCCAAGTTCTGAGTCT
Intron primers flanking exons	ex3-5′	TTGTCTGTCATCCCTCTATACTTCCC
	ex3-3′	ATTTGAGCTGATGAACCCTGTTCC
	ex7-5′	CATGCATTCTACAAGGCTGAGACC
	ex7-3′	TTTTCTTTTCAAGGAAAGCTGATACC
	ex9-5′	CTGAAGTACTATAAAAGAGAAAT

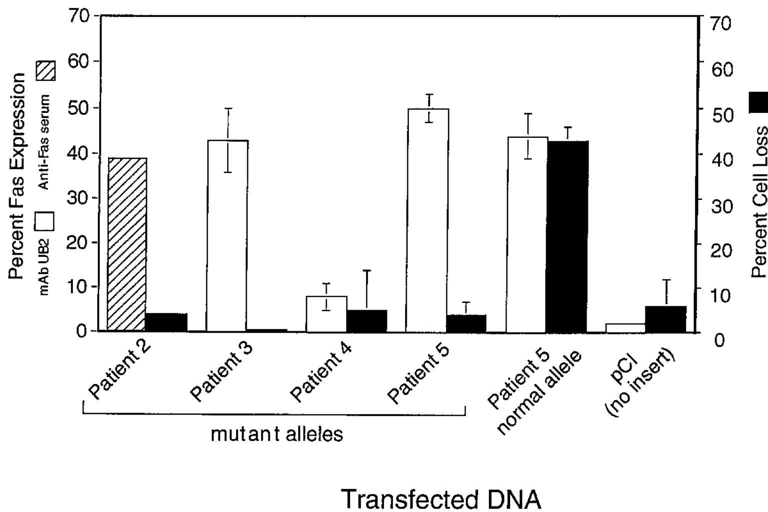


Figure 4. Fas Mutations Cause Defective Transmission of Apoptotic Signals

Left y axis (open or hatched bars) shows percent of transfected BW5147 murine thymoma cells expressing a human Fas molecule. Surface-expressed human Fas was detected using flow cytometry with MAb UB2 (open bars) or anti-rabbit (Fas amino-terminal peptide) serum (hatched bar). Right y axis (closed bars) shows percent cell loss after exposure to human anti-Fas MAb CH11. DNAs used for transfection were 10 μg of expression plasmid pCI with no insert or with either normal or mutant Fas alleles as indicated.

Doses of 0.1–2.5 μg/ml of CH11 caused the same degree of cell loss in normal controls, an indication that the antibody was present in saturating amounts (Figure 3B). Testing of >30 stimulated lymphocyte samples from healthy individuals unrelated to the ALPS patients showed robust apoptosis induction (60%–80% cell loss) by the CH11 antibody in this assay (examples in Figure 3B), demonstrating that defective lymphocyte apoptosis is not common in normal individuals.

Interestingly, mothers 2 and 4, who carried Fas mutations, but manifested no clinical abnormalities, showed impairment in T lymphocyte apoptosis, and cell loss of lymphocytes from mother 1 was at the lower end of the normal range. Mother 2 (top right in Figure 3A) exhibited cell loss comparable to that of her daughter with ALPS and significantly less than father 2, who had no Fas mutation. Mother 4 showed T cell loss that was intermediate between that of her son with ALPS and that of the nonmutant father, possibly due to her genetic mosaicism. In the family of patient 3, the Fas mutation–bearing lymphocytes of neither the paternal uncle nor the father were available for study. Interestingly, lymphocytes from mother 3, who carried no Fas mutation, exhibited greater cell loss than the affected child, but still less than normal controls, raising the question whether mother 3 could have contributed to patient 3 an abnormality in a molecule other than Fas in the apoptosis pathway.

Coexpression of Normal and Mutated Fas Alleles in ALPS Carrier Parents

It seemed paradoxical that parents could harbor Fas mutations and yet fail to manifest clinical features of ALPS. Possible explanations included instability of mutant Fas mRNA in parental lymphocytes or transcriptional silencing that could prevent mutant Fas protein expression in the unaffected parents. Because of the abnormal sizes of the aberrantly spliced mutant mRNAs in mothers 2 and 4, we were able to test whether the mutant alleles produced stable mRNA. Semiquantitative RT–PCR followed by agarose electrophoresis and ethidium bromide staining showed substantial quantities of mutant along with normal-sized cDNA from lymphocytes of both mothers 2 and 4 (data not shown). Their ratios of mutant to normal cDNA appeared comparable to those observed in ALPS patients 2 and 4, an indication that both mothers, as well as their children, expressed the abnormal Fas alleles.

Defective Apoptosis Caused by Fas Mutations

To test directly whether the mutant Fas genes in ALPS patients encoded proteins that were functionally defective, normal and mutant Fas cDNAs were transiently overexpressed under the control of the cytomegalovirus promoter and enhancer in murine BW5147 cells (White et al., 1989). Fas surface expression and the proportion of cells lost in response to exposure to anti-Fas antibody were then assessed by flow cytometry. This system measures only apoptosis signals delivered by CH11 antibody to the Fas protein encoded by the transfected allele because BW5147 cells do not express Fas or FasL under these conditions (L. Chen and M. J. L., unpublished data) and because CH11 does not recognize murine Fas (Itoh et al., 1991). Results from transfections of plasmids containing full-length mutant cDNAs from patients 2–5 and a normal cDNA (normal Fas allele of patient 5) were compared with those of control pCI plasmid with no insert (Figure 4). Each transfected Fas allele, with the exception of that from patient 4, was expressed on the surface of 40%–50% of the BW5147 cells (open and hatched bars in Figure 4). The deletion in the extracellular portion of the Fas protein encoding the mutant allele of patient 2 prevented detection with MAb UB2, CH11, or ZB4; but surface expression of this mutant protein could be detected on 39% of the transfected cells with a rabbit polyclonal antibody recognizing the 15 amino-terminal residues of Fas (hatched bar in Figure 4). The mutant allele of patient 4, truncated at the intracellular side of the membrane-spanning domain, might be predicted to have low cell surface expression because of the loss of charged intracellular anchoring residues. Indeed, expression of the mutant protein could be detected on only 8%–12% of the transfected cells using several anti-Fas antibodies, including CH11, UB2, ZB4, and the polyclonal rabbit anti-peptide described above.

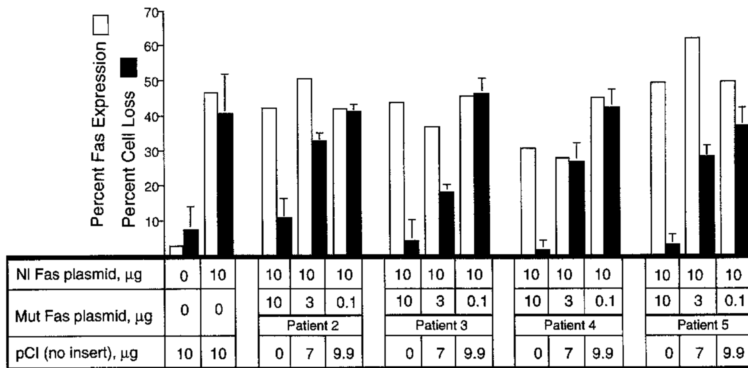


Figure 5. Mutant Fas Alleles Have a Dominant Interfering Effect on Apoptosis Mediated by a Normal Fas Allele

Cotransfections of BW5147 murine thymoma cells with pCI-based expression plasmids containing no insert, the normal Fas allele (Nl Fas plasmid), or mutant Fas alleles (Mut Fas plasmid) as indicated. Titrations of 10, 3, and 0.1 µg mutant Fas-containing plasmids with 10 µg of normal Fas-containing plasmids were tested as shown. Total DNA in the transfection was kept constant (20 µg) by the inclusion of the backbone pCI plasmid with no insert where neceassary. Y axis shows percent of BW5147 cells that express human Fas, as detected by MAb UB2 (open bars) and percent cell loss after exposure to MAb CH11 (closed bars). Cell loss values are the arithmetic means with standard deviations from three independent determinations.

The transfected pools of cells were then exposed to CH11, and the fractional cell loss due to Fas-mediated apoptosis was measured. Only the normal Fas allele conferred upon the BW5147 cells the ability to respond significantly to a CH11 apoptotic stimulus (closed bars in Figure 4). As illustrated by the equivalent height of the open and closed bars for the normal allele of patient 5 in Figure 4, expression of this normal Fas cDNA construct caused essentially all transfected cells expressing Fas to be killed within 9 hr after exposure to anti-Fas antibody. By contrast, cell loss in transfectants with mutant Fas was not different from cell loss in the transfectant with the control pCI plasmid. No transfection of the mutant allele of patient 1 was attempted because its early truncation in CRD1 would preclude surface expression of any Fas protein. We concluded that ALPS-associated mutations could abrogate Fas-mediated apoptosis signals by different mechanisms: the mutant Fas of patient 2 failed to interact extracellularly with CH11 antibody, that of patient 4 (and presumably patient 1) could not achieve substantial surface expression, and those of patients 3 and 5 failed to transmit death signals.

Dominant Interference with Signaling for Cell Death

To explain why the mutant Fas genes caused defective T cell apoptosis in heterozygous form, we investigated whether mutant Fas proteins could interfere with apoptotic signaling when coexpressed with normal Fas. Dominant negative interactions have been shown previously to impair apoptosis in the TNF/TNFR pathway (Tartaglia and Goeddel, 1992). Using a similar approach, we transfected 10, 3, or 0.1 µg of the expression constructs containing the mutant alleles for patients 2–5 together with 10 µg of plasmid containing the normal Fas allele (Figure 5). The total DNA in each transfection was adjusted to 20 µg by including compensating amounts of the control pCI plasmid. Transfection of equal amounts of plasmid containing normal and mutant Fas alleles resulted in Fas surface expression on 43%–55% of the BW5147 cells, equal to transfectants with normal Fas alone (open bars in Figure 5). However, cotransfection of any of the mutant alleles with the normal allele abrogated cell loss after Fas cross-linking (closed bars in Figure 5). The dominant interfering effect diminished in a dose-dependent fashion as the ratio of plasmid expressing the mutant allele was decreased. The reduction in cell loss we observed was approximately equal to that expected based on the probability of random assembly of trimeric complexes with no mutant subunit (for 10 µg each of normal and mutant Fas, $P = (1/2)^3 = 0.125$).

The mutant Fas alleles from patients 3 and 5 were also assessed for their ability to inhibit apoptosis mediated by endogenous Fas expressed in human Jurkat cells (Figure 6). To measure Fas expression from the transfected plasmid, we adjusted the fluorescence gate to detect only cells expressing Fas above the levels found on untransfected Jurkat cells. Significant overexpression, as measured by anti-Fas antibody staining, was achieved after transfection of 20 µg of normal or mutant plasmids (open bars in Figure 6). Nonetheless, as observed in the mouse cell system described above, mutant Fas from patients 3 and 5 substantially diminished Fas-mediated cell loss in response to CH11 binding.

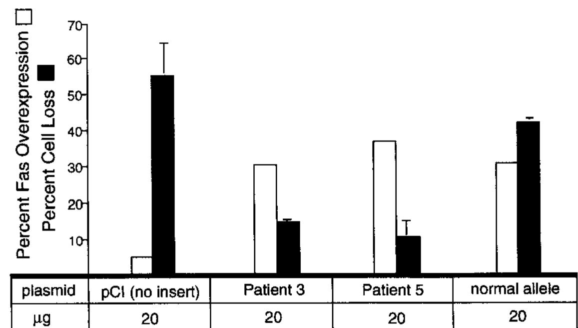


Figure 6. Mutant Fas Alleles from Two ALPS Patients Suppress Apoptosis in Human Jurkat Cells

Transfections of pCI-based plasmids containing no insert, mutant, or normal alleles. Y axis shows percent of Jurkat cells expressing Fas above the level of untransfected cells (open bars) and percent cell loss after exposure to MAb CH11 (closed bars).

Discussion

Our study associates a molecular defect in T lymphocyte apoptosis with a human disorder that we have termed ALPS. This syndrome is characterized by severe multisystem autoimmune disease and extreme overpopulation of lymph nodes, particularly with a normally rare subset of TCR–CD3$^+$CD4$^-$CD8$^-$ lymphocytes. Because these findings are reminiscent of those manifested by Fas-deficient MRL/*lpr* mice, we studied the structure of Fas as a candidate disease gene. Each child with ALPS had a unique Fas gene mutation, and these mutations had differing effects on Fas. Patient 1 had an early single base deletion and frameshift, precluding the production of a Fas protein capable of surface expression. This mutation resembled typical recessive loss-of-function mutations. All of the remaining four patients had mutations that could be predicted to produce abnormal Fas protein: two splice mutations leading to deletions either within the extracellular domain (patient 2) or spanning the intracytoplasmic anchoring region and death domain (patient 4); a point mutation causing the nonconservative substitution of proline for threonine in the death domain (patient 3); and a death domain point mutation producing a premature termination (patient 5). All children had a defect in apoptosis of phenotypically normal, mature T cells after either TCR restimulation or exposure to anti-Fas antibody. The spectrum of mutations we describe suggests that the Fas protein, specifically with intact extracellular CRDs (Banner et al., 1993; Nagata and Golstein, 1995) and a functional intracytoplasmic death domain (Tartaglia et al., 1993), is necessary for normal apoptotic signaling in mature human T lymphocytes.

Despite the clear causative relationship between Fas defects and lymphoproliferation with autoimmunity in the mouse, it was possible that the human Fas mutations we found were common in the population and coincidental to the clinical findings of ALPS in the patients we studied. Three lines of evidence argued against this hypothesis. First, the Fas genes from 100 unrelated copies of chromosome 10 in 50 individuals did not contain any of these mutations. In addition, 30 normal subjects screened for functional defects in Fas-specific lymphocyte apoptosis induced by anti-Fas MAb did not manifest the killing defects seen in lymphocytes with heterozygous Fas mutations. Finally, direct analysis of four of the mutant alleles by cell transfection showed that they were unable to transmit a death signal and had dominant interfering effects on killing mediated by nonmutant Fas. Taken together, these results indicate that the mutations we have identified are not normal polymorphisms and are closely associated with abnormal lymphocyte apoptosis and the ALPS disease state.

The mutations we have found indicate that programmed mature T cell death via the Fas molecule is a potentially critical extrathymic mechanism for maintaining self-tolerance in human (for reviews see Schwartz, 1989; Critchfield et al., 1995). Tolerance established in the periphery could prevent autoimmunity to antigens expressed only in specific tissues or only after the perinatal period. Such antigens may not be presented in the thymus prior to population of the secondary lymphoid organs. Furthermore, self-tolerance may be intimately linked to peripheral T cell homeostasis. Thus, the physiologic T cell response to a peptide antigen may comprise a dramatic increase in the numbers of specifically responding T cells followed by a nearly equal reduction over time (McHeyzer-Williams and Davis, 1995). Elimination of T cells after activation by antigen maintains a diverse repertoire and prevents accumulation of potentially harmful cells that could cross-react with self-antigens. Thus, interference with T cell death in the periphery could disrupt normal homeostasis and predispose one to autoimmune phenomena.

In our patients, the Fas defects were associated with impaired apoptosis after TCR ligation of single-positive (CD4$^+$ or CD8$^+$) activated, mature T lymphocytes. Nevertheless, the cells that accumulated in the circulation and secondary lymphoid tissue were TCR–CD3$^+$ double-negative T lymphocytes, the cell type that is also expanded in *lpr* and *gld* mice. In the mouse, the excess double-negative T cells are derived from chronically activated single-positive T cells, principally CD8$^+$ T cells, that have failed to die (Giese and Davidson, 1995). In *lpr* mice as well as in four of our ALPS patients, the double-negative cells display TCR$\alpha\beta$, but patient 4 also had increased circulating TCR$\gamma\delta$ double negatives. Thus, at least in humans, Fas-induced apoptosis may govern the homeostasis of circulating $\gamma\delta^+$ T cells. The family history of Hodgkin's disease in the father and uncle of patient 3 further suggests that abnormal lymphocyte survival may allow cells failing to be killed to accumulate mutations that lead to malignancy, as noted for Bcl-2 defects (Thompson, 1995).

As in MRL/*lpr* mice, disease in ALPS patients is primarily mediated by humoral rather than cell-mediated immunity. In both species, hypergammaglobulinemia is prominent. In MRL/*lpr* mice, autoantibodies, especially antinuclear antibodies, form immune complexes that are deposited in the kidney to cause glomerulonephritis. Autoantibodies were seen only in ALPS patients 3 and 5, and antinuclear antibodies were not observed. However, patient 2 did develop glomerulonephritis, and all children with ALPS had autoimmune cytopenias and rashes. These abnormalities of humoral immunity may be due in part to dysregulation of helper T cells (Giese and Davidson, 1995), but also may stem from the increase in absolute B cell numbers in ALPS patients. Indeed, Fas defects may impair the homeostasis of multiple immune cell types to produce a complex immunoregulatory disorder.

One of the most intriguing questions regarding ALPS is why the children with heterozygous mutations were affected while their parents were not. Several explanations were considered. The occurrence of novel Fas mutations in the affected children was ruled out by demonstrating that each was inherited from a carrier parent. Interestingly, however, parental genetic mosaicism was observed in mother 4, who had both a normal and a mutant Fas allele with the same allotype and whose apoptosis defect was less marked than that of her affected son. Also excluded in our families was the model of simple recessive inheritance, because in all five ALPS patients, one Fas allele

was mutated while the other was normal. Our findings are thus in contrast with the mouse *lpr* mutation as well as to recessive mutations of each Fas allele recently described in two human siblings with autoimmunity and lymphoproliferation (Rieux-Laucat et al., 1994, Twelfth European Immunology Meeting, meeting abstract). Except for the mutation of patient 1, most consistent with a loss of function, the Fas-associated mutations in our ALPS patients are best modeled as dominant mutations producing a cellular resistance to apoptosis and also contributing to a clinical disease phenotype. This model is supported by demonstration that patients and, in families 2 and 4, maternal carriers expressed mutant mRNA and manifested defects in the Fas-specific lymphocyte apoptosis pathway. The ability of mutant Fas alleles from patients 2–5 to prevent cell death via normal Fas is further evidence for a dominant cellular phenotype.

Our failure to find Fas defects in a control population suggests that Fas mutations, whether of a loss-of-function or dominant interfering type, are highly correlated with ALPS. However, the fact that parents with the same mutations (and even with demonstrable apoptosis defects in vitro) do not have overt clinical symptoms indicates that Fas abnormalities cannot be the sole determinant of ALPS. Concomitant environmental or genetic factors, such as mutations in other immunoregulatory genes, are required to produce ALPS. Although extensive research in the mouse has clearly established a causative role for Fas mutations in inducing cellular defects and autoimmune disease, this research has also proven that host strain differences, due to as yet undefined modifier genes, make a major contribution to disease phenotype (Theophilopoulos and Dixon, 1968). The typical *lpr* autoimmune syndrome of glomerulonephritis, vasculitis, and arthritis is regularly manifested at an early age in the MRL mouse background. By contrast, the same *lpr* mutation causes essentially no autoimmune disease when bred onto the C57BL/6 background. On other backgrounds, such as C3H, the onset is delayed and the autoimmune manifestations are milder than in MRL mice. Thus, it is not surprising that in an outbred human population, individuals bearing the same Fas gene mutation may have dramatically different phenotypes. Defining the additional segregating gene(s) that causes expression of the full manifestations of ALPS in the presence of Fas dysfunction will be important for understanding how Fas participates in immune regulation. Examples of such loci altering expressivity in mouse phenotypes include the recently defined modifier of the murine gene defect in multiple intestinal neoplasia (Dietrich et al., 1993), as well as the combinatorial autoimmune effects of heterozygous *gld* and lpr^{cg} mutations (Kimura and Matsuzawa, 1994). Human genetic loci known to affect expression of disease phenotypes are just beginning to be identified. To date these include the polymorphic minisatellite at the human insulin gene locus, now shown to be a transcription regulator that when expanded predisposes to development of diabetes (Davies et al., 1994; Kennedy et al., 1995), and the digenic form of retinitis pigmentosa, a progressive blindness due to heterozygous mutations at two loci, peripherin/RDS and *ROM1*, that encode proteins colocalizing within the retinal photoreceptor (Kajiwara et al., 1994).

In ALPS patients, defects that might convert an aysmptomatic Fas abnormality to overt disease are likely to be inherited from the parent who does not carry a Fas mutation. Recent elucidation of molecules involved in programmed death pathways suggests an abundance of potential candidate genes, including FasL, Bcl-2, IL-Iβ-converting enzyme proteases (Thompson, 1995; Enari et al., 1995; Los et al., 1995), and genes that regulate the expression of these molecules. The TNF/TNFR pathway also plays an important role in T lymphocyte apoptosis (M. J. L., unpublished data) and could be impaired in patients with ALPS. Alternatively, the unaffected carrier parents may have genes that protect against disease caused by abnormal T cell apoptosis. Further study of kindreds of ALPS patients with heterozygous Fas mutations may implicate one or even several modifier loci.

The discovery of five unique human Fas mutations in patients with lymphoproliferation and autoimmunity has provided evidence that mature lymphocyte apoptosis is important in maintaining human self-tolerance. Four of these mutants encode abnormally expressed Fas proteins that cause dominantly inherited apoptosis defects. These mutations constitute valuable tools for defining how Fas chains interact with each other within a receptor complex, with extracellular ligand, and with intracellular mediators of downstream signaling. Moreover, these mutations have provided a framework for understanding the molecular basis of a recently described human disorder, ALPS. What the human mutations reveal, which is not evident from the studies in the *lpr* mouse model, is that heterozygous dominant interfering versions of Fas exist and that these can impair apoptosis and predispose to a severe disease in humans.

Experimental Procedures

Subject Enrollment and Cell Culture and Stimulation

Participating families consented to evaluation and follow-up at the Warren Magnuson Clinical Center at the National Institutes of Health under an approved clinical study protocol. Extensive medical assessments and complete family histories were obtained. Blood donated by unrelated healthy controls was processed in parallel to patient samples in all assays. PBLs were isolated over Ficoll–Hypaque gradients (Pharmacia Biotech, Piscataway, NJ) and in some instances were cryopreserved for up to 4 years prior to the present study without substantial loss of viability. PBLs at 10^6/ml were activated with 10 μg/ml PHA on day 0 of culture in RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, and penicillin–streptomicin; on day 3 the TCR stimulator anti-CD3 (OKT3; Ortho Diagnostic Systems, Raritan, NJ) was added, and cultures were then maintained in recombinant human IL-2 (60 IU/ml) (Midwest Medical, Bridgeton, MO) with refeeding every 48–72 hr thereafter for up to 2 weeks before analysis. Some cells were cryopreserved after the initial stimulation and thawed in IL-2 at least 48 hr prior to analysis.

Detection of Patient Mutations

DNA and RNA samples were prepared from activated lymphocytes using standard methods (Sambrook et al., 1989). For cDNA analysis, RT–PCR was performed using a cDNA cycle kit (Invitrogen, San Diego, CA) and Fas-specific primers. Products were visualized in ethidium bromide–stained agarose gels and cloned in the TA plasmid pCRII (Invitrogen), propagated in Escherichia coli DH5α, and sequenced by the dideoxy method with ^{32}P end-labeled Fas primers and a PCR cyclist

kit (Stratagene, La Jolla, CA). PCR of genomic DNA with primers flanking each exon provided templates for direct sequencing. Cloned genomic PCR products were also sequenced. Primers were selected with the assistance of PRIMER (Whitehead Institute, Cambridge, MA) and DNA* programs (DNA*, Madison, WI) and the GenBank genomic sequence entries X81335–X81342 (Behrmann et al., 1994); primers used are listed in Table 2. PCR was carried out in 1 × GeneAmp PCR buffer (Perkin–Elmer, Norwalk, CT) with each primer at 0.25 μM, each dNTP at 0.2 mM, and 1.25 U of Amplitaq DNA polymerase (Perkin–Elmer) in a total volume of 50 μl. A 5 min interval at 94°C was followed by 30 cycles of 94°C for 30 s, 59°C for 60 s, and 72°C for 60 s in a 9600 Thermal Cycler (Perkin–Elmer).

Documentation that both alleles had been fully examined included complete sequence determination of normal and mutant clones as well as demonstration of previously described allotypes consisting of silent nucleotide substitutions at 416 and 836 (Fiucci and Ruberti, 1994) in families 4 and 5. For parents with homozygous allotypes, four to ten cDNA clones were sequenced.

Single strand conformation polymorphism was assayed as described elsewhere (Hyashi, 1992; Puck et al., 1995). Genomic DNA segments of 83–480 bp were amplified with primers (Table 2) surrounding the site of mutation for each patient as follows: patients 1 and 2, ex3-5′ and ex3-3′; patient 3, ex9-5′ and 989–962; patient 4, ex7-5′ and 792–767; and patient 5, ex9-5′ and 1243–1221R. Primers were end labeled with ^{32}P for autoradiographic detection of boiled single-stranded products on Hyrdolink MDE acrylamide gels (AT Biochem, Malvern, PA) with or without 10% glycerol. The 465 bp segment surrounding mutation of patient 5 was trimmed to 301 bp by digestion with TaqI to facilitate mutation detection. Templates included genomic DNA samples from 20 unrelated CEPH pedigree members (Coriell Cell Repositories, Camden, NJ) and from 30 additional unrelated normal subjects as well as genomic and cloned DNA from the five ALPS patients.

Flow Cytometry Analysis

Analysis of Fas surface expression was carried out by incubating 10^5 to 10^6 cells at 4°C for 30 min with anti-human Fas MAb UB2 or ZB4 (Kamiya Biochemical Corporation, Thousand Oaks, CA), washing, and then staining with FITC-conjugated anti-mouse immunoglobulin G1 (IgG1) (Pharmingen, San Diego, CA). When CH11 was used for staining, FITC-conjugated goat anti-mouse IgM (Pharmingen) was the second reagent. To stain the mutant Fas allele of patient 2, a rabbit polyclonal IgG anti-human Fas (peptide 21–38) (Santa Cruz Biotech, Santa Cruz, CA) was used as a primary reagent, and FITC-conjugated goat anti-rabbit IgG was used for detection. Flow cytometric quantitation of T cell death was performed by resuspending washed cells from two wells in 400 μl of FACS buffer (1 × PBS with 1% BSA and 0.01% sodium azide) plus 30 ng/ml propidium iodide. Total cells were collected for 1 min without live gating, and the viable cells were quantitated by gating on propidium iodide exclusion and forward light scatter as previously described (Boehme and Lenardo, 1993). Activated cell cultures were also stained for T cell surface markers CD3, CD4, CD8, TNFR p55 and p75, and CD25 by standard methods (Zúñiga-Pflücker et al., 1993). Flow cytometry was carried out on a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) using CellQuest software.

Induction of Apoptosis

Apoptosis was induced by incubating cells without or with an apoptotic stimulus followed by marking of nonviable cells with propidium iodide and quantitation by flow cytometry. Activated PBLs were grown in IL-2 for 48 hr prior to in vitro CD3 exposure. Flat bottom microwells (Falcon, Franklin Lakes, NJ) were untreated or coated with anti-human CD3ε MAb (64.1) at a concentration of 1 μg/ml (Wacholtz and Lipsky, 1993). Plates were washed three times with PBS, and 50,000 cells were added per well in 200 μl. The cells were incubated at 37°C and 5% CO_2 for 24 hr, and viable cells were assayed as above. The percent cell loss was calculated as follows: [(1 − cell number recovered from wells with MAb)/(cell number recovered from wells without MAb)] × 100.

Apoptosis restricted to the Fas pathway was detected by exposure of test cells to an agonist anti-Fas MAb (CH11; Kamiya Biomedical), while control cells received no antibody. At the time of apoptosis assay, an aliquot of cell population was tested for proliferation by [^{3}H]thymidine incorporation. Assay of CH11-mediated deletion was carried out using stimulated mature T cells maintained in IL-2; 50,000 cells per well were cultured in flat-bottomed microwells with 25 ng/ml actinomycin D (Sigma, St. Louis, MO). CH11 was then added to the experimental wells for 24 hr. Viable cells were quantitated by flow cytometry and percent cell loss was calculated.

Apoptosis in Transfected Cells Expressing Normal and Mutant Human Fas

BW5147 murine thymoma cells (White et al., 1989) and human-derived Jurkat cells were grown in RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, penicillin–streptomycin, and 50 mM β-mercaptoethanol (Biofluids, Rockville, MD). Full-length cDNA clones from the mutant alleles of patients 2–5 were released from pCRII with EcoRI and recloned into EcoRI-digested mamallian CMV expression vector, pCI (Promega, Madison, WI). These plasmid constructs (10–25 μg) with the correct orientation or pCI without a Fas insert were electroporated into 4 × 10^6 BW5147 cells/ml cells in 0.4 ml in an Electrocell manipulator 600 (BTX Corporation, San Diego, CA). After pulse discharge at settings 260 V, 1050 μF, and 720 Ω, the cells were immediately placed in 8 ml of medium and incubated for 12 hr. Viable cells were isolated over Lympholyte M (Cedar Lane, Hornby, Ontario, Canada). For induction of apoptosis, 50,000 cells in 200 μl were treated with 0.5 μg/ml CH11 in triplicate wells for 9 hr for BW5147 cells or with 10 or 100 ng/ml CH11 in triplicate wells for 4 hr for Jurkat cells; parallel control cultures received no antibody treatment. Viable cells excluding propidium iodide were enumerated by flow cytometry, and expression of Fas was determined as above.

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