

Autoimmune Lymphoproliferative Syndrome with Defective Fas: Genotype Influences Penetrance

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Summary

Autoimmune lymphoproliferative syndrome (ALPS) is a disorder of lymphocyte homeostasis and immunological tolerance. Most patients have a heterozygous mutation in the *APT1* gene, which encodes Fas (CD95, APO-1), mediator of an apoptotic pathway crucial to lymphocyte homeostasis. Of 17 unique *APT1* mutations in unrelated ALPS probands, 12 (71%) occurred in exons 7–9, which encode the intracellular portion of Fas. In vitro, activated lymphocytes from all 17 patients showed apoptotic defects when exposed to an anti-Fas agonist monoclonal antibody. Similar defects were found in a Fas-negative cell line transfected with cDNAs bearing each of the mutations. In cotransfection experiments, Fas constructs with either intra- or extracellular mutations caused dominant inhibition of apoptosis mediated by wild-type Fas. Two missense Fas variants, not restricted to patients with ALPS, were identified. Variant A(–1)T at the Fas signal-sequence cleavage site, which mediates apoptosis less well than wild-type Fas and is partially inhibitory, was present in 13% of African American alleles. Among the ALPS-associated Fas mutants, dominant inhibition of apoptosis was much more pronounced in mutants affecting the intracellular, versus extracellular, portion of the Fas receptor. Mutations causing disruption of the intracellular Fas death domain also showed a higher penetrance of ALPS phenotype features in mutation-bearing relatives. Significant ALPS-related morbidity occurred in 44% of relatives with intracellular mutations, versus 0% of relatives with extracellular mutations. Thus, the location of mutations within *APT1* strongly influences the development and the severity of ALPS.

Introduction

Autoimmune lymphoproliferative syndrome (ALPS) (MIM 601859) is a rare disease characterized by chronic massive, nonmalignant lymphadenopathy and splenomegaly; expansion of a normally rare population of T cells bearing $\alpha\beta$ -antigen receptors but lacking both CD4 and CD8 coreceptors ($\alpha\beta$ double-negative T cells [$\alpha\beta$ DNTs]); and an in vitro lymphocyte apoptotic defect. Autoimmune manifestations are nearly always documented in long-term follow-up of patients with ALPS (Sneller et al. 1992; Fisher et al. 1995; Rieux-Laucat et al. 1995). We and others have described mutations in *APT1* (MIM 134637), the gene encoding the Fas/APO-1/CD95 apoptosis receptor, in patients with ALPS (Fisher et al. 1995; Rieux-Laucat et al. 1995; Drappa et al. 1996; Bettinardi et al. 1997; Pensati et al. 1997; Sneller et al. 1997; Infante et al. 1998; Kasahara et al. 1998). In two studies, homozygous mutations of *APT1* led to complete Fas deficiency and severe ALPS (Rieux-Laucat et al. 1995; Kasahara et al. 1998); in two others (Bettinardi et al. 1997; Pensati et al. 1997), compound heterozygous mutations of *APT1* were found. In contrast, all remaining ALPS-associated *APT1* mutations characterized to date have been heterozygous.

The *APT1* gene has nine exons spanning 26 kb (Behrmann et al. 1994; Cheng et al. 1995) on chromosome 10q24.1 (Inazawa et al. 1992). Exons 1–5 encode a signal sequence and three extracellular cysteine-rich domains responsible for binding Fas ligand (FasL). Exon 6 encodes the transmembrane domain of Fas, and exons 7–9 encode the intracellular portion. The Fas death domain, encoded by exon 9, is an 80–amino acid structure conserved in a subset of genes related to tumor necrosis factor receptor type 1 (TNFR1), including *TNFR1*, *APT1*, *DR3*, *DR4*, and *DR5* (Singh et al. 1998). The domain is necessary and sufficient for the death-inducing activity of the Fas receptor. The functional Fas complex is a homotrimeric receptor, which, when engaged by homotrimeric FasL, transmits an apoptosis signal via the death domain. This pathway is crucial in lymphocyte

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homeostasis, serving to delete antigen-driven and autoreactive T cell clones (Brunner et al. 1995; Dhein et al. 1995; Ju et al. 1995).

The critical role of the Fas pathway in lymphocyte homeostasis and tolerance was first shown in homozygous *lpr*, *lpr^{cg}*, and *gld* mice (Davignon et al. 1985; Davidson et al. 1986; Kimura and Matsuzawa 1994). The *lpr* and *lpr^{cg}* mice have defects in Fas, and the *gld* mice have a defect in FasL (Watanabe-Fukunaga et al. 1992; Takahashi et al. 1994). These genotypically distinct mice exhibit the same phenotype: massive lymphocyte accumulation in secondary lymphoid organs, autoimmunity, hypergammaglobulinemia, and elevated DNTs.

The disease phenotype in *lpr* and *lpr^{cg}* mice varies with the background strain, with more severe autoimmunity occurring on the MRL than the B6 background (Izui et al. 1984; Ogata et al. 1993). However, even in the same strain, penetrance is variable. Our previous studies have shown a similar effect in the human disease, in that some ALPS family members with *APT1* mutations are clinically unaffected (Fisher et al. 1995; Sneller et al. 1997). In fact, within a given kindred, a wide spectrum of lymphoproliferation and autoimmunity can be observed: Some family members with mutations manifest full-blown ALPS; some have minimal splenomegaly, adenopathy, or transient autoimmunity; others have only elevated $\alpha\beta$ DNTs; and still others are entirely well (Sneller et al. 1997; Infante et al. 1998).

In these studies, we have sought to examine the role of *APT1* mutations in the development of the ALPS phenotype. Herein we characterize *APT1* mutations in previously unreported ALPS kindreds and present the results of in vitro assays performed to determine to what extent individual Fas mutants inhibit apoptosis mediated by wild-type Fas. The large number of kindreds studied, the spectrum of disease expression within them, and the distribution of their mutations showed a strong relationship between Fas genotype and penetrance in ALPS.

Patients and Methods

Patient Diagnosis and DNA Isolation

Patients and family members were enrolled, and blood samples were obtained with informed consent under protocols approved by the National Institutes of Health (NIH). Individuals evaluated at the NIH Clinical Center underwent a review of medical history and records, physical examination, routine laboratory studies, peripheral blood lymphocyte (PBL) phenotyping, *APT1* genotyping, and in vitro lymphocyte apoptosis assay. For individuals unable to travel to the NIH, consent was obtained, samples were mailed, medical records were reviewed, and telephone interviews were conducted with both patient and referring physician. Genomic DNA was

extracted by standard methods, by use of an ABI 341 nucleic acid purification system (PE Applied Biosystems).

Mutation Analysis

The nine *APT1* exons and intron/exon boundaries were amplified from genomic DNA from each patient with the primers listed in table 1. PCR was performed for 10 cycles of 45 s at 94°C, 45 s at 68°C–59°C (i.e., decreasing 1°C each cycle), and 45 s at 72°C, followed by 25 cycles of 45 s at 94°C, 45 s at 58°C, and 45 s at 72°C. Amplicons were screened for mutations in exons 1–8 by dideoxy fingerprinting (ddF) (Sarkar et al. 1992; Puck et al. 1997) and in exon 9 by sequence determination with a *Taq* cyclist kit (Stratagene). All mutations were confirmed by reamplification of the mutant exon and sequencing. To determine the frequency of allelic variants, we used genomic DNA from a panel of unrelated whites and African Americans. The A(–1)T allele was analyzed by use of fluorescent sequencing of exon 2 on an ABI 377 DNA sequencer with a dRhodamine Terminator cycle sequencing kit (PE Applied Biosystems). Exon 4 was analyzed by use of ddF and fluorescent sequencing. Statistical analysis of the allele frequencies in white and black populations was done by use of the χ^2 test with the Yates correction or, where appropriate, by Fisher's exact test.

Penetrance Studies

A total of 37 relatives in families 1, 2, 4, 20, and 34 and a total of 73 relatives in families 3, 5, 17, 26, 27, 29–31, and 45 were screened for *APT1* mutations. Penetrance ratios for intracellular versus extracellular *APT1* mutations were analyzed by use of the χ^2 test along with the Yates correction. Significant morbidity ratios for intracellular versus extracellular *APT1* mutations were analyzed by Fisher's exact test. *P* values were reported for a two-tailed analysis, and levels $\leq .05$ were considered significant.

Expression Constructs

RNA was extracted from patient T cells that had been activated in vitro as described by Fisher et al. (1995). First-strand cDNA was generated by use of the cDNA cycle kit (Invitrogen). Full-length Fas cDNA (Genbank accession number M67454) was amplified as explained above, but with an extension interval of 1 min 15 s for 40 cycles. Primers used to generate full-length Fas cDNA are listed in table 1. Fas cDNAs were initially subcloned into pCR 2.1 (Invitrogen) or pT7Blue-3 (Novagen) and then subcloned into the pCI vector (Promega) under control of a cytomegalovirus promoter for expression studies.

Fas death-domain mutant constructs from patients 29–31 and 33 were made by replacing the 318-bp

Table 1**Primers Used in Analyses of the *APT1* Gene**

Exon ^a	Coding Strand ^b	Anticoding Strand ^b	Product Size (bp)	Sequencing Primer ^b
1	TGACTCCTTCCTCACCTGACTTC	gactaagacggggaagcctccacc	452	CCTCAGGGGCGGGCACT
2	caatthgggtgggttacactggt	ttctatttcagaggtcatgtca	496	caatthgggtgggttacactggt
3	tccccattgtatttatctcattag	tgtcctcccttctctgtgt	408	ttgtctgtcatccctataactccc
4	cctgccaccatttcatagtctgc	cctaattcttaactctcagtcagtggtactccc	276	cctgccaccatttcatagtctgc
5-6	atggcccctaatttacaagtgcc	ctgcagttgaacaagaagaac	394	atggcccctaatttacaagtgcc, atataaaatgtccaatgttc
7	catgcattctacaaggctgagacc	caaacaaaatgaaacaaagaac	252	aaaaagtaaaaggaagtaacaaaagc
8	tatttttattgtcttctctgctcc	ctgaaattggcctattactctaaaggatgc	131	ttgcctattactctaaaggatgc
9	aaataaacatggtttcactaatggg	ACAAATATGTTGGCTCTTCAGCGC	551	AAATATGTTGGCTCTTCAGCGC, AAGCTTTGGATTTCAATTTCT
9	attcccctagtcagctctcata	CATAATCTTAATCTTTCATCCTCTGC	757	GAGATCTTTAATCAATGTGTCA
cDNA	CTCAGTACGGAGTTGGGGAAGC	CAAACACTAATTGCATATACTCAGAACTG	1,107	CTGCCAAGAAGGGAAGGAG, GGTCCGGGTGCAGTTTATTT
cDNA	GGGAAGCGGTTTACGAGTGA	TGGGGTTAGCCTGTGGATAGAC	1,545	GACAAAGCCACCCCAAGTTAGA

^a cDNA denotes full-length Fas cDNA.

^b Intron nucleotides are in lowercase, exon nucleotides in uppercase.

HincIII/PflMI fragment from the wild-type Fas in pCR 2.1 plasmid with the corresponding mutant fragment. The A(-1)T construct was made by excision and replacement of the 209-bp *XhoI/StuI* fragment from the wild-type Fas pCI construct. All expression constructs were sequenced on both strands to verify fidelity. No additional mutations were observed. Mutant and wild-type sequences were equally represented in PCR products of full-length Fas from first-strand cDNA as well as PCR products of Fas genomic DNA.

Expression and Apoptosis

A total of 20 μg of DNA (20 μg of pCI vector alone, or 10 μg of pCI + 10 μg of wild-type Fas, or 10 μg of pCI + 10 μg of mutant Fas, or 10 μg of wild-type Fas + 10 μg of mutant Fas) was transfected into BW5147 murine thymoma cells by electroporation, as described elsewhere (Fisher et al. 1995). BW5147 cells do not express endogenous Fas or FasL under these culture conditions (Fisher et al. 1995). Fas mutant 3 (T225P), previously shown to interfere with apoptosis mediated by wild-type Fas (Fisher et al. 1995), was included to standardize the dominant interference assay. Viable cells were purified with Lympholyte M (Cedarlane) gradients 16 h post-transfection. An aliquot of gradient-purified cells was analyzed for Fas expression by use of flow cytometry, facilitated by staining with anti-Fas monoclonal antibody (mAb) UB2 (Immunotech). Cells (9×10^5) were incubated with or without 2 $\mu\text{g}/\text{ml}$ anti-Fas mAb CH11 (Oncor) for 3–4 h to induce apoptosis. Apoptosis was analyzed with the Apo-Direct kit (Pharmingen). The fixed cells were stained with propidium iodide to allow gating on the cycling cells, the population most susceptible to apoptosis (Lenardo 1991). Fluorescein-tagged UTP was used to label 3'OH ends of DNA (cleavage fragments generated during apoptosis). For both treated and control populations, 10,000 events were analyzed on a FACScan flow cytometer (Becton Dickinson) with Cell Quest software (Becton Dickinson). Apoptosis in each cell sample was calculated as $([\% \text{ CH11-exposed cells positive for fluorescein-UTP}] - [\% \text{ nonexposed cells positive for fluorescein-UTP}]) \div (\% \text{ of cells expressing Fas})$. To compare data from numerous killing assays, the apoptosis for each sample was expressed as a percentage of apoptosis for cells expressing wild-type Fas in that assay. The reported apoptosis represents the average of at least three assays for each mutant, with standard error of the mean.

Apoptosis assays of patient T cells were performed on phytohemagglutinin-stimulated T cells maintained in IL-2 for 21–35 days. Cells (10^6) were incubated for 5 h with or without 3 $\mu\text{g}/\text{ml}$ CH11 in 1 ml of medium to induce apoptosis. Apoptosis was analyzed as described earlier and was expressed as (% CH11-exposed cells

positive for fluorescein-UTP) – (% nonexposed cells positive for fluorescein-UTP).

Statistical analyses of the results of cotransfections with wild-type Fas construct versus constructs containing intracellular or extracellular mutations, and of T cell apoptosis of normal individuals versus ALPS probands, were done by two-tailed Student's *t* test.

Results

APT1 Mutations

Seventeen distinct *APT1* mutations were identified in affected members of unrelated families enrolled in the NIH study (fig. 1, *numbered symbols above gene diagram*). Nine of these mutations have been described elsewhere (Fisher et al. 1995; Sneller et al. 1997; Infante et al. 1998)—as was ALPS family 26, described by Infante et al. (1998) in a detailed clinical report—and are included here because of family member studies and dominant negative studies that are reported herein. Analysis of DNA from family members showed that, of the eight newly identified mutations, six were inherited; the inheritance in families 24 and 33 was uncertain because blood samples were not available from both parents. Twelve (71%) of the 17 *APT1* mutations affected the intracellular region of Fas. The 12 mutant proteins they encode are predicted to have intact and functional Fas extracellular and transmembrane domains but to lack normal death domains. All 12 were expressed equally well as wild-type Fas in transfection studies (data not shown). Figure 1 also shows the positions and types of mutations in patients with ALPS reported by other groups (*symbols below gene diagram*). The *APT1* mutations in patients whom we have studied are listed in 5' to 3' order in table 2. The mutations in ALPS families 1–6, 17, 20, and 26 are described elsewhere (Fisher et al. 1995; Sneller et al. 1997; Infante et al. 1998); previously unreported mutations are described in the next paragraphs.

The ALPS 34 mutation (T131fs) changed the invariant G residue of the 3' splice site of intron 4, leading to use of a cryptic splice site in exon 5 and deletion of the first 22 nucleotides of exon 5. The deletion predicted a frameshift and truncation of Fas after 32 missense amino acids. This predicted protein would lack both the Fas transmembrane and death domains. The ALPS 45 mutation (K181fs), an 11-bp deletion in exon 7, removed the first of what is normally a pair of direct ACAGAAA repeats, as well as four more nucleotides. This deletion caused a frameshift with a stop codon after nine missense amino acids, predicting a Fas protein with intact transmembrane and anchoring residues but truncated before the death domain. The ALPS 24 mutation (P201fs) was a G→A transition at the last base of exon 8, immediately

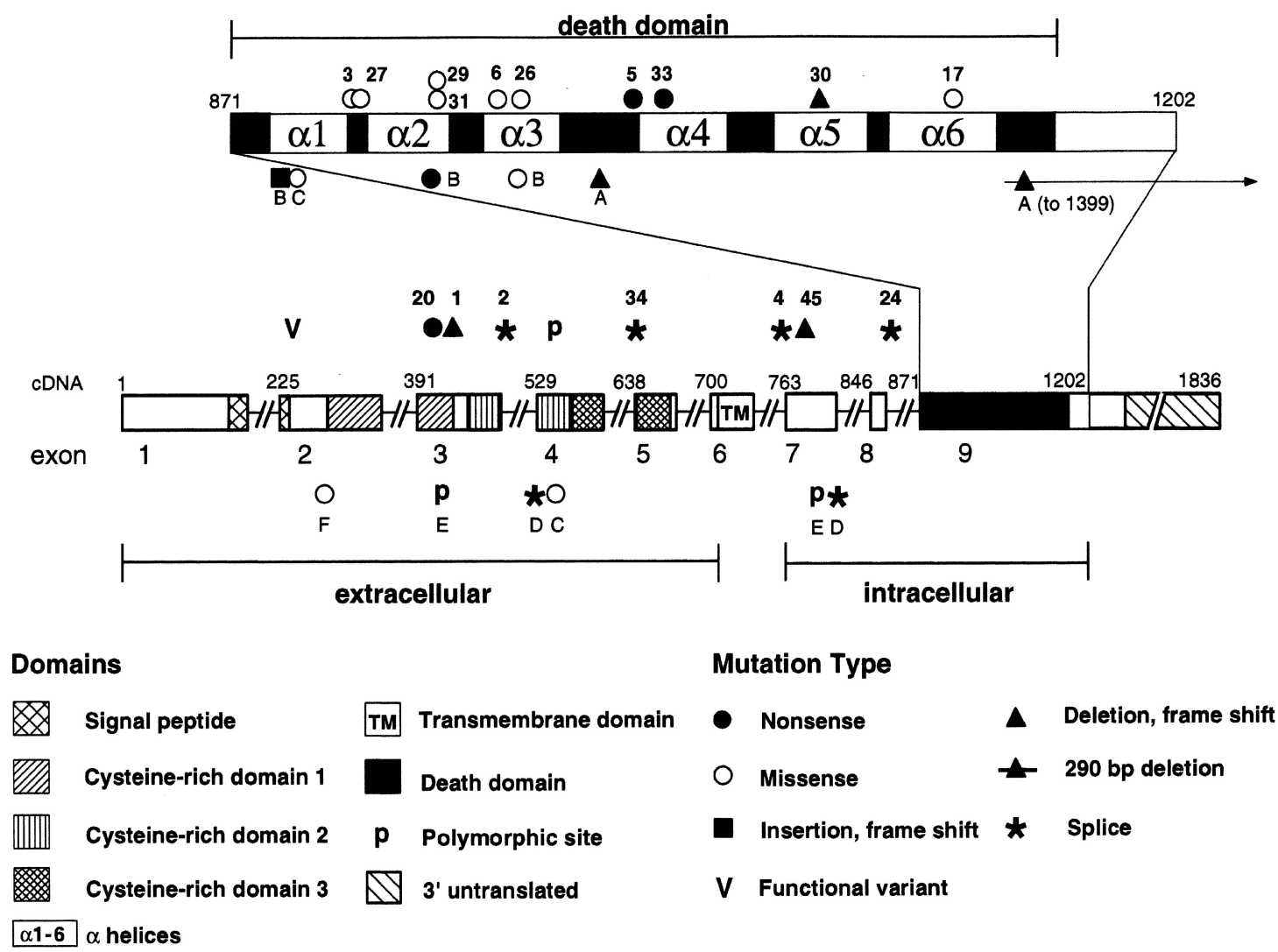


Figure 1 Structure of the *APT1* gene, showing ALPS-associated mutations, functional variants, and polymorphisms. Exon 9 is expanded to show α helical regions of the intracellular death domain (Huang et al. 1996). Numbered mutations, protein polymorphism, and functional variant above gene diagram are newly identified herein or were reported elsewhere by NIH investigators (Fisher et al. 1995; Sneller et al. 1997; Infante et al. 1998). Mutations and other DNA polymorphisms identified by other groups are shown below gene diagram: A, Rieux-Laucat et al. (1995); B, Drappa et al. (1996); C, Bettinardi et al. (1997); D, Kasahara et al. (1998); E, Fiucci and Ruberti (1994); and F, Pensati et al. (1997).

Table 2

APT1 Mutations, Phenotype, and Penetrance in 17 Families with ALPS

EXON OR INTRON	MUTATION		PROBAND NUMBER	AGE ^a	PROBAND PHENOTYPE				PENETRANCE IN RELATIVES	
	Nucleotide (mRNA) Change	Protein Change			Autoimmunity ^b	Lymphoproliferation ^c	% $\alpha\beta$ DNTs ^d	% Apoptosis ^e	Proportion with ALPS Features ^f	Number with Significant Morbidity ^g
Exon 3	413C→A	C57X	20	3 years	GBS	L, HSM	4–9	0	2/7	0
Exon 3	429delG	D62fs ^{h,i}	1	4 mo	ITP	L, HSM	14–28	6	0/3	0
Intron 3	IVS3+2insT (skip exon 3 or exons 3 and 4)	P49del46, ^j P49fs ^k	2	18 mo	GN, ITP, PBC	L, HSM	16–49	Defective ^l	0/3	0
Intron 4	IVS4(-1)G→C (cryptic splice 638del22)	T131fs ^m	34	2 years	HA	L, SM	5	1	0/1	0
Intron 6	IVS6(-2)A→C (cryptic splice ins 72)	V174fs ⁿ	4	2 years	UR	L, HSM	2–7	Defective ^l	1/3	0
Exon 7	779del11	K181fs ^o	45	4 years	ITP	L, SM	10–12	13	6/6	4
Exon 8	870G→A (skip exon 8)	P201fs ^p	24	11 mo	ITP	L, HSM	18	7	NA ^q	NA ^q
Exon 9	915A→C	T225P	3	5 years	HA, ITP, AN	L, SM	6–15	1	4/4	3 (2 with LY)
Exon 9	916C→A	T225K	27	5 years		L, HSM	8–18	0	1/3	0
Exon 9	943G→A	R234Q	29	2 years		L, HSM	2	0	2/2	1
Exon 9	943G→C	R234P	31	9 mo	HA, ITP	L, HSM	8–36	5	8/8	5
Exon 9	964C→A	A241D	6	4 mo	HA	L, SM	5–13	1	De novo mutation	De novo mutation
Exon 9	973A→T	D244V	26	Birth	HA	L, SM	1–2	0	10/10	3 (1 with LY)
Exon 9	1011C→T	Q257X	5	2 mo	HA, ITP, AN	L, SM	8–18	Defective ^l	1/3	0
Exon 9	1020C→T	Q260X	33	11 mo	HA	L, SM	6–9	0	NA ^q	NA ^q
Exon 9	1074delT	L278X ⁿ	30	1 year	GN, HA, ITP, AN	L, SM	7–19	5	4/4	1
Exon 9	1123T→G	I294S	17	4 mo	HA, AN	L, HSM	11–24	5	2/3	2

^a Age = age at presentation.

^b All had autoantibodies. Additional overt autoimmune diseases were: GBS = Guillain-Barré syndrome, ITP = immune-mediated thrombocytopenic purpura, GN = glomerulonephritis, PBC = primary biliary cirrhosis, HA = hemolytic anemia, UR = urticarial rash, and AN = autoimmune neutropenia (i.e., neutropenia persisting after splenectomy associated with normal myeloid precursors in the marrow).

^c L = lymphadenopathy, HSM = hepatosplenomegaly, and SM = splenomegaly.

^d CD3⁺ $\alpha\beta$ CD4⁻CD8⁻ cells/CD45^{bright} CD14⁻ cells in the lymphocyte gate, expressed as a range when multiple assays were performed (normal value <1%).

^e Normal range 52% \pm 12% standard deviation.

^f Features include adenopathy, abnormal autoantibodies in serum, autoimmunity, and elevated $\alpha\beta$ DNT concentrations.

^g One or more of the following: splenectomy, autoimmune disease requiring treatment, or lymphoma (LY).

^h Frameshifts; the number refers to the last normal amino acid codon.

ⁱ A total of 34 missense amino acid codons.

^j In-frame deletion of 46 amino acids.

^k A total of 38 missense amino acid codons.

^l Sneller et al. (1997); determination by assay described by Fisher et al. (1995).

^m A total of 32 missense amino acid codons.

ⁿ Frameshift to an immediate stop.

^o Nine missense amino acid codons.

^p Three missense amino acid codons.

^q Not available.

preceding the invariant GT of the 5' splice site consensus. This G residue is conserved in 77% of 5' splice sites (Cooper and Krawczak 1995, pp. 239-260). Consistent with previously analyzed mutations in other genes (Cooper and Krawczak 1995, pp. 239-260), mutant cDNA sequenced from patient 24 lacked exon 8. The resulting frameshift predicted a truncation after 3 missense amino acids, and thus no death domain was present.

Four new mutations, T225K, R234Q, R234P, and Q260X, in patients 27, 29, 31, and 33, respectively, were point mutations within the Fas death domain. The first three were missense mutations that caused nonconservative amino acid substitutions. The T225K and T225P (Fisher et al. 1995) mutations at Fas nucleotides 915 and 916 suggest that this codon is a mutational hot spot in *APT1*. In addition, R234Q and R234P were encoded by different point mutations at nucleotide 943; these mutations, together with a previously reported nucleotide 942 Fas truncation (Drappa et al. 1996), mark the 942-943 CpG dinucleotide as a second mutational hot spot in *APT1*. The ALPS 30 mutation (L278X) was a 1-bp deletion, and the deleted T was part of an ATT repeat. This frameshift predicted an immediate stop. Both Q260X and L278X predicted Fas proteins with truncated death domains.

Two *APT1* missense mutations were found in more than one individual (table 3). An alanine to threonine change, A(-1)T (fig. 1, V, above exon 2), in the last residue of the Fas signal sequence, occurred because of a G→A transition; and a threonine to isoleucine (T106I) substitution (fig. 1, p, above exon 4) was located in the second extracellular cysteine-rich domain of Fas. By patient cDNA sequencing and by sequencing exon 2 in additional members of ALPS families 29 and 33, it was determined that the A(-1)T substitution resided on the same allele as the death-domain mutations (R234Q and Q260X) in both cases. Interestingly, patient 33 had the T106I substitution on one Fas allele and A(-1)T/Q260X on the other allele. T106I was also found in an unrelated individual who did not have ALPS. All three individuals bearing these substitutions were of African

American descent. Therefore, we determined the frequency of these alleles in black and white individuals. The A(-1)T substitution was found in 13% (15/116) of black alleles but only 1% (1/90) of white alleles ($P < .005$). The T106I substitution was found in 2% (2/116) of black alleles and was not observed in 102 white alleles (not significantly different).

Aside from previously recognized silent nucleotide polymorphisms (fig. 1, p, below exons 3 and 7) (Fiucci and Ruberti 1994), no other alterations of the *APT1* sequence were found by the sensitive ddF screening method in genomic DNA from patients with ALPS or their relatives. In addition, none of these mutations was seen by single-strand conformation polymorphism analysis of *APT1* in 104 alleles from unrelated white individuals (Fisher et al. 1995; and data not shown); by ddF or sequencing of *APT1* in 18 alleles from unrelated white individuals (data not shown); or by ddF of exon 9 in 106 alleles from unrelated black individuals (data not shown).

Apoptosis

As shown in table 2, an in vitro apoptosis defect was demonstrable in T cells from every proband. The mean apoptosis induced by anti-Fas mAb in activated T cells maintained in IL-2 was $3\% \pm 4\%$, whereas cells from 11 healthy control subjects tested in the same assay averaged $52\% \pm 12\%$ ($P < .001$).

Apoptosis mediated by mutant Fas molecules was studied by transfecting BW5147 murine thymoma cells with expression constructs containing human mutant Fas cDNAs, wild-type Fas cDNA, or pCI vector alone. An example of one such apoptosis experiment is shown in figure 2. Cleavage of cellular DNA into small fragments (DNA laddering), a hallmark of apoptosis, produces an increase in free 3'OH DNA ends, detected by fluorescein-UTP incorporation into DNA termini by terminal deoxynucleotidyl transferase. The increased fluorescence induced by anti-Fas treatment in wild-type Fas transfectants (fig. 2B) was not seen in transfectants with

Table 3

***APT1* Mutation and Clinical Phenotype for Fas Protein Polymorphic Variants**

EXON	MUTATION		PROBAND NUMBER	CLINICAL PHENOTYPE			
	Nucleotide Change	Protein Change		Auto-immunity	Lympho-proliferation	% $\alpha\beta$ DNTs ^a	% Lymphocyte Apoptosis ^b
2	240G→A	A (-1)T (signal sequence)	29, 33	NA ^c	NA ^c	NA ^c	NA ^c
4	559C→T	T106I	33	None ^d	None ^d	.2 ^d	49.4 ^d

^a CD3⁺ $\alpha\beta$ CD4⁻/CD8⁻ cells /CD45^{bright} CD14⁻ cells in the lymphocyte gate (normal value <1%).

^b Normal range $52\% \pm 12\%$ standard deviation.

^c Not available (available individuals with the 240G→A change had death-domain mutations as well).

^d Phenotype of a mutation 33 negative, polymorphic variant heterozygote from ALPS family 33.

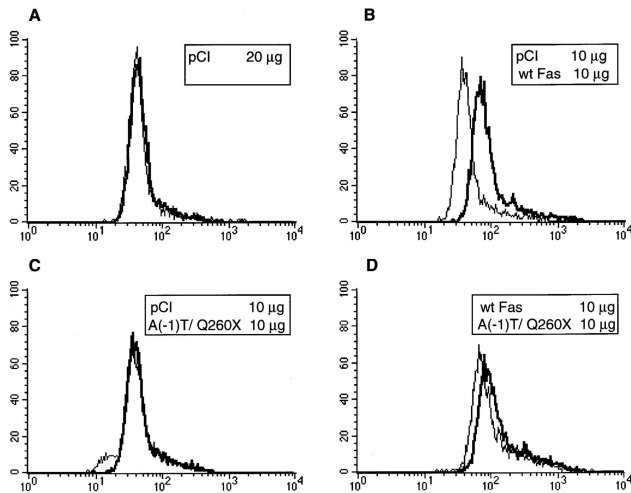


Figure 2 FACS analysis of apoptosis, using histograms that depict the shift in fluorescence as fluorescein-UTP is incorporated into DNA termini. Cell numbers are on the y-axis, fluorescence intensity on the x-axis. Control BW5147 cells without anti-Fas mAb, *light tracing*; experimental BW5147 cells with anti-Fas mAb, *bold tracing*. Transfected plasmid constructs are indicated for each experiment.

pCI alone (fig. 2A) or in transfectants with a mutant construct A(-1)T/Q260X (fig. 2C).

Apoptosis achieved in cells transfected with mutant Fas constructs was very low compared with that in cells receiving wild-type Fas (fig. 3), with a mean only 8% of that of the wild-type killing level for all 14 mutant alleles. Transfections with constructs containing alleles A(-1)T/R234Q and A(-1)T/Q260X from patients 29 and 33 did not differ significantly in apoptosis from transfections using constructs with R234Q and Q260X mutations on an otherwise wild-type Fas background. A transfected allele with the T106I variation (fig. 3, *right hatched bar*) was not significantly different from wild-type Fas in ability to mediate apoptosis. However, apoptosis mediated by an allele with the A(-1)T variant (fig. 3, *left hatched bar*) was only $52\% \pm 11\%$ that of wild-type Fas apoptosis.

To study the inhibitory effect of mutant Fas proteins on apoptosis mediated by wild-type Fas, we induced apoptosis with anti-Fas mAb in BW5147 cells cotransfected with $10 \mu\text{g}$ of wild-type and $10 \mu\text{g}$ of mutant cDNA. The assay developed by Fisher et al. (1995) was adapted to use UTP incorporation, as described. If we assume equal expression of mutant and wild-type Fas proteins, the fraction of all Fas trimers that are assembled exclusively of normal Fas protein would be $\frac{1}{8}$ ($[\frac{1}{2}]^3$). An example of dominant inhibition of apoptosis is shown in figure 2D, in which cells were cotransfected with wild-type Fas and A(-1)T/Q260X mutant Fas constructs. The addition of the mutant allele attenuated the apoptotic shift seen with wild-type Fas alone (fig. 2B).

All Fas mutants tested exerted a dominant negative effect on apoptosis by this assay (fig. 4).

Addition of the constructs with extracellular Fas mutants C57X, D62fs, and T131fs reduced apoptosis mediated by wild-type Fas to an average level 27% that of wild type alone (fig. 4, *white bars*). All but one of the intracellular mutant alleles had an even more pronounced dominant negative effect on apoptosis. The single exception was Fas mutant T225K, which, on cotransfection, diminished wild-type apoptosis by only 33% (fig. 4), a smaller decrement in apoptosis than was seen with any of the 11 other ALPS-associated deleterious Fas death-domain mutants tested. It is intriguing that the effect of this mutant was so mild, because it occurred at the same position as T225P, which had a severe effect on function. The father in this ALPS kindred, who also carries the T225K mutation, had the same degree of impairment in lymphocyte apoptosis as the proband (data not shown). Preliminary biochemical studies indicate that T225K has a milder effect on intracellular signal transduction than the other mutants (R. Siegel, D. Martin, and M. Lenardo, unpublished data). When we excluded T225K, which appeared to have a distinct molecular mechanism, the remaining intracellular mutants, on average, caused a 91% reduction in apoptosis mediated by wild-type Fas. These 11 intracellular mutants blocked Fas-mediated apoptosis significantly more than did the extracellular mutants ($P = .02$).

Dominant negative assays were also used to determine whether the Fas polymorphic variants A(-1)T and T106I noted in patients 29 and 33 caused any reductions in apoptosis when present in the heterozygous state. Cells were cotransfected with combinations of wild-type and variant Fas alleles with and without the death-domain dominant mutations R234Q and Q260X. A colinear A(-1)T substitution did not alter the dominant negative effect that R234Q or Q260X had on apoptosis (fig. 4). Cotransfections with A(-1)T/Q260X + T106I plasmids also did not differ from cotransfections with Q260X + wild-type Fas or A(-1)T/Q260X + wild-type Fas with respect to dominant inhibition of apoptosis. However, the construct containing only the A(-1)T substitution reduced apoptosis mediated by wild-type Fas by 41%. In contrast, the T106I allele did not alter apoptosis mediated by wild-type Fas.

Penetrance

All available at-risk relatives of patients with ALPS were invited to be tested for Fas defects. Fourteen of the 17 ALPS probands had relatives who shared their *APT1* mutations. Phenotypic features of ALPS, including adenopathy, significant titers of autoantibodies, overt autoimmunity, and elevated $\alpha\beta$ DNTs, were sought by his-

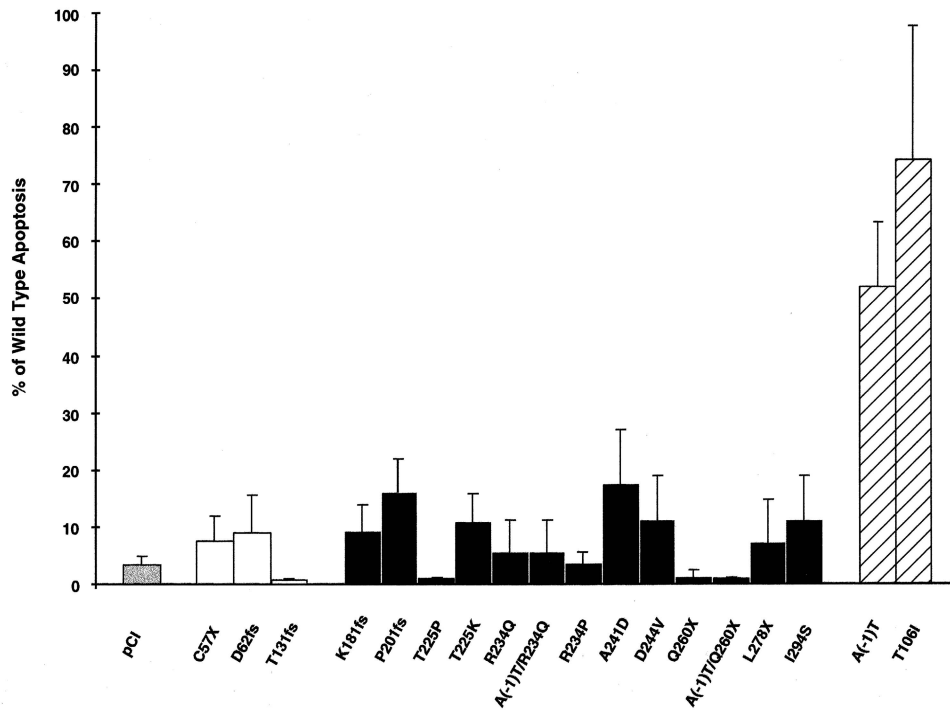


Figure 3 Apoptosis of BW5147 cells, mediated by transfected mutant Fas or Fas polymorphic variants. Twenty micrograms of DNA was used in each of at least three experiments. Gray area denotes control, white area denotes extracellular mutants, black area denotes intracellular mutants, and hatched area denotes protein polymorphic variants. Mutant Fas apoptosis was normalized to wild-type Fas apoptosis (100%) for each assay (wild-type values before adjustment were $82\% \pm 5\%$; $n = 10$). Bars show mean \pm standard error.

tory and examination, review of medical records, physician interviews, and laboratory tests. The most striking observation was that penetrance of one or more ALPS features was 88% (38/43) for individuals with intracellular mutations versus 18% (3/17) for individuals with extracellular mutations ($P < .001$; table 2). Of five families with extracellular mutations and additional members with an *APT1* mutation (1, 2, 4, 20, and 34), only two families included members other than the probands who displayed any features of ALPS. In contrast, all nine families with intracellular mutations and additional members with an *APT1* mutation (3, 5, 17, 26, 27, 29–31, and 45) included individuals besides the proband who displayed features of ALPS. Two kindreds (families 3 and 26) with 100% penetrance of ALPS features included mutation-positive members with lymphoma. In addition, ALPS-related significant morbidity (patients undergoing splenectomy, having autoimmune disease requiring treatment, or developing lymphoma) was present in 44% (19/43) of relatives with intracellular *APT1* mutations versus none of the relatives (0/17) with extracellular mutations ($P < .001$).

Discussion

A cohort of patients with ALPS and their relatives has enabled us to discover a relationship between genotype

and clinical phenotype in kindreds with *APT1* mutations. Although probands were recognized because of significant morbidity, disease expression can vary within a family (Fisher et al. 1995; Infante et al. 1998). Therefore, identification of an *APT1* mutation cannot alone precisely predict the severity of disease. However, both a greater clinical penetrance and increased rates of ALPS-related morbidity were found in relatives with intracellular versus extracellular mutations. Thus, increased dominant negative effect on apoptosis in the intracellular versus extracellular Fas mutants may contribute to increased penetrance. Our data show that, for some intracellular *APT1* mutations, penetrance is extremely high. Individuals in these families who carry *APT1* mutations but are free of significant morbidity may have genetic factors that protect against the ALPS phenotype.

We have identified and functionally characterized 17 *APT1* mutations causing ALPS, 8 of which were not previously reported. Exon 9, which encodes the Fas death domain, was found to harbor 16 (59%) of the 27 *APT1* mutations causing ALPS, both in our cohort and in reports published elsewhere (fig. 1). Mutations resulting in cell-surface expression of Fas proteins without a normal death domain account for 19 (70%) of all of the 27 published *APT1* mutations causing ALPS (fig. 1).

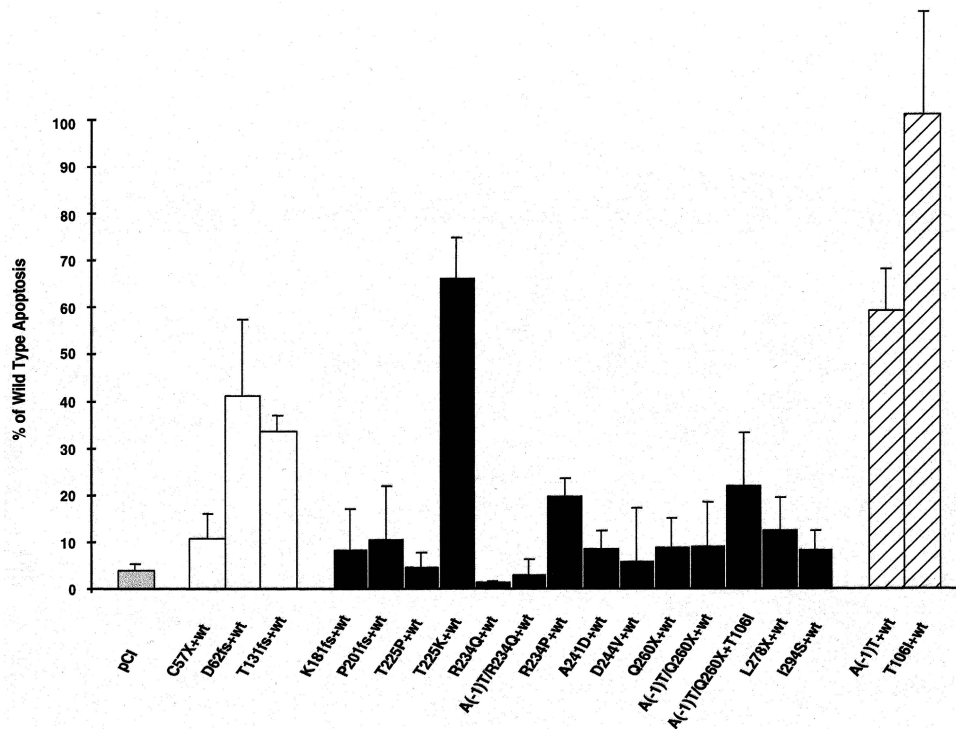


Figure 4 Apoptosis of BW5147 cells by cotransfected mutant and wild-type Fas or cotransfected Fas protein polymorphic variant and wild-type Fas. Twenty micrograms of DNA was used in each of at least three experiments. Gray area denotes control, white area denotes extracellular mutants, black area denotes intracellular mutants, and hatched area denotes protein polymorphic variants. Apoptosis mediated by mutant Fas was normalized to wild-type Fas apoptosis (100%) for each assay (wild-type values before adjustment = $83\% \pm 6\%$; $n = 14$).

All of the 17 Fas mutant proteins we studied were severely impaired in their ability to mediate apoptosis, and all interfered with apoptosis mediated by wild-type Fas in an in vitro cotransfection assay. As a group, the intracellular Fas mutants interfered with apoptosis mediated by wild-type Fas more strongly than did the extracellular mutants, suggesting different mechanisms of dominant negative interference. Three of the *APT1* mutant alleles (C57X, D62fs, and T131fs) predicted a putative soluble Fas protein. Several naturally occurring splice variants of Fas are known to produce truncated soluble Fas (Cascino et al. 1995; Liu et al. 1995; Papoff et al. 1996). Some of these proteins can interfere with apoptosis in vitro, possibly by hindering Fas-FasL binding, and may be involved in the physiologic regulation of Fas activity (Cascino et al. 1995; Liu et al. 1995). A common Fas splice variant is the deletion of exon 6, which eliminates the sequence encoding the transmembrane domain, resulting in soluble Fas. Messenger RNA encoding this Fas Δ exon 6 variant may constitute 50% of the Fas mRNA in resting lymphocytes (Liu et al. 1995). However, the mRNA encoding other soluble Fas proteins accounts for only 5% of the total Fas mRNA in peripheral blood mononuclear cells, and for <1% of the Fas mRNA in stimulated PBL (Liu et al. 1995). The

mutant Fas mRNA from our patients with ALPS who had extracellular mutations appeared stable, in that mutant cDNA clones were recovered with the same frequency as wild-type clones. The extracellular mutants may have disrupted wild-type Fas function by interference with Fas translation or intracellular processing. However, although mutants C57X, D62fs, and T131fs showed dominant interference with apoptosis mediated by wild-type Fas, they did not interfere with expression of wild-type Fas in our in vitro assays. Possible methods of apoptotic interference by extracellular mutants are incorporation into a Fas trimer that cannot transduce a signal or binding to, and blocking, anti-Fas mAb (or, in patients, FasL). The dominant effect on apoptosis seen for these putative soluble proteins implies that a simple haploinsufficiency of Fas did not cause ALPS in these patients. Interestingly, the Fas 4 mutation (V174fs), which has been shown to have a dominant negative effect on apoptosis (Fisher et al. 1995), potentially uses two methods of dominant negative interference with apoptosis: secretion of soluble protein and cell-surface expression of truncated protein. In vitro transfection of this mutant resulted in only 20%–30% of the cell-surface expression achieved by transfecting wild-type Fas (Fisher et al. 1995; and data not shown). Although the

Fas 4 mutant has a transmembrane domain, it lacks any cytoplasmic amino acids to anchor the protein in the cell membrane. Because it is likely that the cell-surface expression is transient, even *in vivo*, ALPS family 4 was considered to be functionally like families with extracellular mutations, for penetrance analyses.

The smallest dominant inhibitory effect on apoptosis exerted by any of the mutants was seen with Fas mutant T225K. Fas mutant T225P, at the same residue, is a potent dominant inhibitor of apoptosis, but proline residues are known to disrupt α helices. Whereas the T225P death domain showed global disruptions by nuclear magnetic resonance structural analysis, the structure of the T225K death domain closely resembles that of wild-type Fas (D. Martin and M. Lenardo, unpublished data). Further biochemical analysis of mutant T225K may clarify the molecular mechanism by which it impairs apoptosis.

One of the two protein polymorphic variants of Fas that we identified, A(-1)T, interfered with apoptosis mediated by wild-type Fas in transfection assays. However, unlike the ALPS-associated Fas mutants, which mediated apoptosis, on average, only 8% as well as wild-type Fas, the A(-1)T variant mediated apoptosis 52% as well as the wild-type Fas. There have been several reports of a signal-sequence mutation causing disease (Arnold et al. 1990; Watzke et al. 1991; Ito et al. 1993). In two cases—mutation of preprovasopressin leading to familial central diabetes insipidus and mutation of preproparathyroid hormone leading to familial isolated hypoparathyroidism—dominant negative effects of the mutant proteins on wild-type function were seen (Arnold et al. 1990; Ito et al. 1993). Interestingly, the preprovasopressin mutation encoded the same amino acid change as did our A(-1)T Fas variant. Processing studies of this signal-sequence mutant in preprovasopressin showed that the cleavage of its signal was reduced to 25% of the wild type's. Suggested pathologic mechanisms are accumulation of preprovasopressin in the endoplasmic reticulum or interference with protein processing (Ito et al. 1993). The Fas A(-1)T variant appears to have normal processing, in that it was expressed on the cell surface as fully as the wild-type Fas (data not shown). Cleavage of the Fas signal sequence may have occurred at an alternative site, leading to Fas trimers that are less effective at transmitting a death signal.

The effect of the Fas A(-1)T variant in individuals heterozygous or homozygous for this allele is potentially important, since several autoimmune diseases, including sarcoidosis (Arnold 1993), systemic lupus erythematosus (SLE) (Schur 1993), and Graves' disease (Yanagawa and de Groot 1996), occur more frequently in blacks than in whites. Because the A(-1)T variant allele is more common in the black population, studies are ongoing to

determine whether this allele is associated with any of these diseases. A promoter polymorphism of tumor necrosis factor α (TNF- α), which initiates the *TNFR1* apoptotic pathway, has been associated with SLE in blacks (Sullivan et al. 1997). This polymorphism is associated with increased TNF- α production and also occurs more frequently in whites with SLE, dermatitis herpetiformis, alopecia areata, and rheumatoid arthritis (Messer et al. 1994; Wilson et al. 1994; Danis et al. 1995a, 1995b; Galbraith and Pandey 1995).

Mutations in *APT1* and consequent dysfunction of the Fas-mediated apoptotic pathway may predispose individuals to lymphoma, leukemia, and myeloma, as well as other types of cancer. We have noted three cases of lymphoma in two of our ALPS kindreds (Sneller et al. 1997; Infante et al. 1998). Landowski et al. (1997) found five Fas death-domain mutations in bone marrow cells from 54 patients with multiple myeloma, and Beltinger et al. (1998) found two Fas mutations in T-lineage acute lymphoblastic leukemia blasts from 81 patients. Three patients with ALPS with other cancers have been reported (Drappa et al. 1996; Kasahara et al. 1998). Abnormal Fas function may allow transformed cells to survive and become neoplastic. However, the relative risk of neoplasia remains to be determined.

Because many family members of ALPS probands harbored an *APT1* mutation but showed no overt disease, other factors must also contribute to the pathogenesis of ALPS. Moreover, six individuals fitting the clinical definition of ALPS—lymphoproliferation, autoimmunity, >1% $\alpha\beta$ DNTs, and *in vitro* lymphocyte apoptosis defect—did not have any detectable mutations in the *APT1* gene (Sneller et al. 1997; and data not shown). The Fas pathway is initiated by engagement of trimeric Fas ligand with the extracellular portion of trimeric Fas, followed by intracellular recruitment of Fas-associating protein with death domain (PADD) and activation of a protease cascade starting with caspase 8 (FLICE, MACH 1) (Nagata 1997). Alterations in any of these proteins or in related members of the *TNFR1* apoptotic pathway or the BCL-2 family could combine with Fas mutations or act independently to contribute to the phenotype of ALPS.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Genbank, <http://www.ncbi.nlm.nih.gov/Web/Genbank> (for Fas cDNA [accession number M67454])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for ALPS [MIM 601859] and *APT1* [MIM 134637])

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