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Identification of New Fas Mutations in a Patient with Autoimmune Lymphoproliferative Syndrome (ALPS) and Eosinophilia

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Abstract: Autoimmune lymphoproliferative syndrome (ALPS) is a rare, newly recognized, chronic lymphoproliferative disorder in children and is characterized by lymphadenopathy, splenomegaly, pancytopenia, autoimmune phenomena and expansion of double-negative (DN) T lymphocytes (TCR$\alpha\beta^+$, CD4$^-$, CD8$^-$). Defective lymphocyte apoptosis caused by mutations of the Fas (CD95) gene has been linked in the pathogenesis of ALPS, as binding of Fas-ligand to Fas can trigger apoptosis. Of the ALPS cases reported to date, point mutations, frameshifts and silent mutations in Fas all have been identified. We report two new point mutations in Fas in a child with ALPS and eosinophilia; studies on other family members established the pattern of inheritance for these mutations. Flow cytometric analysis of blood and tissues (spleen, lymph node, bone marrow) revealed abnormally expanded populations of DN T lymphocytes. Furthermore, activated lymphocytes and IFNγ-activated eosinophils were resistant to Fas-mediated apoptosis. Eosinophil resistance to Fas-mediated apoptosis has not been previously described in ALPS. Sequencing of Fas revealed two separate mutations not previously reported. One mutation, a C to T change at base 836, was a silent mutation inherited from the mother, while the second mutation, a C to A change at base 916, caused a non-conservative amino acid substitution in the death domain of Fas, changing a threonine to a lysine. This mutation is associated with a predicted change in the structure of a part of the death domain from a β-pleated sheet to an α-helix. We speculate that the mutation in the death domain prevents the interaction of Fas with intracellular mediators of apoptosis and is responsible for the autoimmune manifestations of ALPS and the abnormal lymphocytosis and eosinophilia in this patient.

Keywords: ALPS, Fas, eosinophilia, apoptosis

INTRODUCTION

Autoimmune lymphoproliferative syndrome (ALPS), a rare and only recently recognized disease, has been defined using a combination of clinical, laboratory and molecular findings (1). Typically, the disease presents in childhood with generalized lymphadenopathy, splenomegaly and autoimmune-related phenomena such as thrombocytopenia, neutropenia and hemolytic anemia (2). At the cellular level, there is a non-malignant expansion of TCR$\alpha\beta^+$ CD4$^-$ CD8$^-$ ("double negative" (DN)) lymphocytes, which accumulate in blood and lymphoid tissues (3). At the molecular level, heterozygous, dominant-negative mutations in the cell surface molecule Fas (CD95) are associated with most (1,2,4-9), but not all (10) cases of ALPS. As described above, ALPS can cause significant morbidity. A study of a large kindred with ALPS reported that the risk of Hodgkin's disease and non-Hodgkin's lymphoma was increased with ALPS, but that individuals with Fas mutations can also have a normal life span, with a diminution of morbidity with age (1). Cell death by apoptosis is induced in Fas-expressing cells when Fas is engaged by its counter-receptor Fas ligand (FasL) (reviewed (11)), resulting in trimerization of Fas molecules and engagement of intracellular mediators of apoptosis. Therefore, the Fas mutations seen in ALPS patients are thought to prevent lymphocyte apoptosis resulting in an abnormal accumulation of lymphocytes and subsequently splenomegaly, lymphadenopathy and the autoimmune features of ALPS.

This report describes an individual with ALPS, his kindred and unique features of his

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disease, including a mutation in the death domain of Fas which has not been previously described. In addition, we present evidence suggesting a role for Fas in mediating the eosinophilia due to impaired apoptosis in ALPS.

MATERIALS AND METHODS

Case History

The proband (Figure 1; II-4), who was of Finnish and Danish descent, initially presented in 1994 at 5 years of age with generalized lymphadenopathy, massive splenomegaly, and pancytopenia. Past medical history had been unremarkable. Initial blood work revealed a WBC count of $3.3x10^9$/L, a hemoglobin of 96 g/L (MCV 85.6 fL), a platelet count of $122x10^9$/L and a differential of $0.9x10^9$/L neutrophils, $1.4x10^9$/L lymphocytes, $0.5x10^9$/L eosinophils and 7% reticulocytes. A bone marrow aspiration was hypercellular with normal maturation. Serological testing was repeatedly negative for CMV, EBV, HIV-1, HHV-6, Bordetella, Brucella, Tularemia, Ibuva, and Rubella. The patient had anti-platelet antibodies. Splenomegaly was initially responsive to oral prednisone, but not IVIG. Therapeutic splenectomy was subsequently performed at 6 years of age for severe symptomatic thrombocytopenia. Histology of the spleen and lymph nodes showed an expanded population of large atypical lymphocytes with frequent mitoses, a morphology consistent with a lymphoproliferative disorder. At age 7, the patient presented with leukocytosis ($54.8x10^9$/L), and eosinophilia ($35.1x10^9$/L). A second bone marrow aspiration demonstrated eosinophilia with no other abnormality. The eosinophilia transiently responded to a 7 day course of oral prednisone (1-2 mg/kg/day).

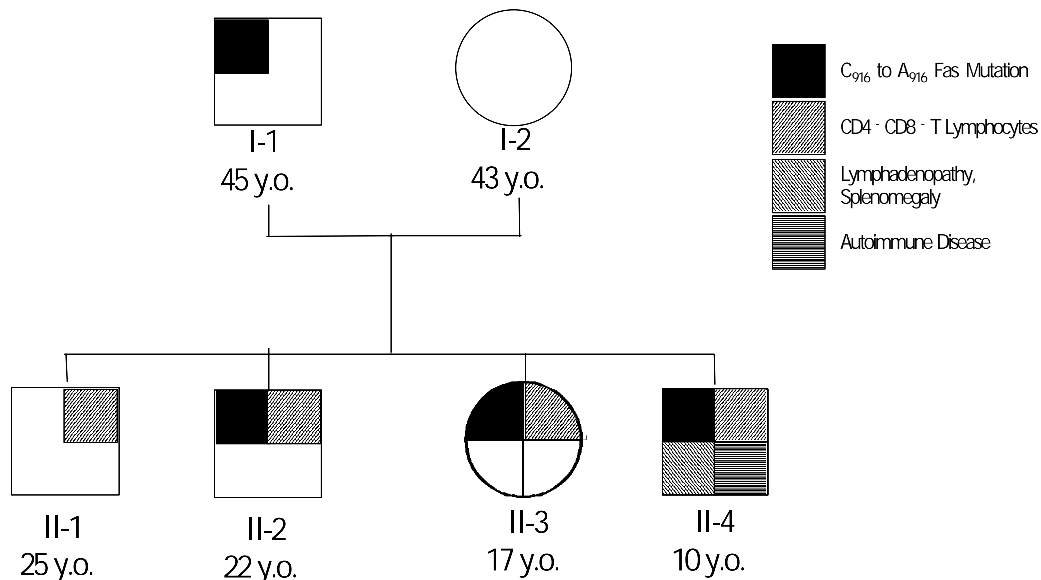

Figure 1. Pedigree of a kindred with ALPS. The proband (II-4) presented at age five with features suggestive of ALPS. Other members of the kindred did not exhibit all the classic manifestations of ALPS. The Fas mutation thought to be responsible for the ALPS in the proband was inherited from the father (I-1), and was also present in two of his siblings (II-2, II-3). The proband's siblings also demonstrated an increased presence of circulating DN lymphocytes (II-1, II-2, II-3).

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Subjects

Age-matched, healthy controls were used for the experiments evaluating the capacity of activated lymphocytes to undergo apoptosis. Other healthy controls were used for the experiments comparing the Fas DNA sequence and the capacity of activated eosinophils to undergo apoptosis.

Lymphocyte Isolation, Activation and Culture

For the functional experiments, in which lymphocyte resistance to Fas-mediated apoptosis was assessed, peripheral whole blood was incubated with CD2/2R (Becton-Dickinson, Mountain View, CA), as previously described (12), and the CH-11 anti-Fas mAb (final concentration 82 µg/mL; Beckman-Coulter, Hialeah, FL). Samples incubated without CD2/2R or anti-Fas were used as controls. After 4, 6, and 8 hours of incubation at 37 C, 50 µL aliquots were removed for staining. Lymphocyte phenotype and the percentage of apoptotic lymphocytes were determined using flow cytometry as described below.

For the experiments in which Fas cDNA was sequenced, lymphocytes were isolated from heparinized whole blood. Lymphocytes and other mononuclear cells were isolated using Histopaque 1077 (Sigma, St. Louis. MO). The mononuclear cells were cultured at a concentration of $1x10^6$ cells/mL in RPMI 1640 supplemented with 2 mM *l*-glutamine (Sigma), antibiotic/antimycotic solution (Gibco-BRL, Burlington, ONT) and 10% FBS (Gibco-BRL). Phytohemagglutinin (PHA, Sigma) was used at a final concentration of 10 µg/mL. Recombinant human IL-2 (Midwest Medical, Bridgeton, MO) was added to a final concentration of 60 IU/mL. After 24 hours of culture, the anti-CD3 mAb OKT3 (ATCC, Rockville, MD) was added as a 1/10 dilution of culture supernatant. The cells were harvested after a total of 72 hours of culture, and RNA was isolated as described below.

Eosinophil Isolation and Culture

Eosinophils were isolated from heparinized peripheral blood, as previously described (13). Briefly, granulocytes were depleted from mononuclear cells using Histopaque 1077 (Sigma). Neutrophils were separated from other granulocytes using an anti-CD16 mAb and magnetic sorting with a MACS Sorter (Miltenyi, Auburn, CA). This procedure routinely isolates eosinophils to >95% purity (13). Isolated eosinophils were cultured for 24 hours at 37 C in RPMI 1640 containing 2mM *l*-glutamine, antibiotic/antimycotic (Gibco-BRL), 10% FBS (Gibco-BRL) and 20 nM IFNγ. The anti-Fas mAb CH-11 was added (final concentration of 1 µg/mL), and the cells were cultured for an additional 12 hours. After being harvested, the cells were stained with Annexin V-PE (R&D Systems, Minneapolis, MN) to determine the percentage of apoptotic eosinophils.

RNA Isolation

Activated, cultured lymphocytes were pelleted (1200 xg for 7 min), and resuspended in Trizol (Gibco-BRL). Total RNA was isolated according to the manufacturer's directions for Trizol. RNA was quantified and its purity determined using a GeneQuant spectrophotometer (Pharmacia, Baie d'Urfe, QUE).

RT-PCR

RT-PCR was performed using a previously published procedure (4). Two sets of primers were used that spanned bases 151-1206 of the cDNA of the human Fas gene (4). The first pair of primers produced a 694 base fragment (cDNA bases 151-845) that spanned the extracellular and transmembrane domains of Fas, whereas the second pair produced a 506 base fragment (cDNA bases 700-1206) that spanned the transmembrane and intracellular domains of Fas. The RT reaction

was set up using 2 μg of Trizol-purified RNA, 100 pmol/μL of random hexamer primers (Pharmacia), 200 units of Superscript I (Gibco-BRL), 27 units RNAGuard (Pharmacia) and 10 mM of each dNTP (Gibco-BRL). The total reaction volume was 20 μL, and the reaction was performed as 10 min at 20 C, 50 min at 42 C, 10 min at 95 C and 10 min at 4 C.

The 50 μL PCR reaction was performed using 200 ng of RT product, 200 nM of each primer, and 25 μL of Qiagen's PCR MasterMix (containing Taq polymerase and dNTPs; Mississauga, ONT). The reaction was performed as 32 cycles of 1 min at 95 C, followed by 1 min at 57 C, followed by 2 min at 72 C. The PCR products were either used immediately for sequencing, or for cloning with subsequent sequencing. Cloning was done with Qiagen's TOPO-TA cloning kit, used according to the manufacturer's instructions. Prior to sequencing, the PCR products or cloned inserts were purified using Qiagen's QIAquick PCR purification kit.

Sequencing RT-PCR Products

PCR products were purified using Qiagen's mini-prep spin columns. The purified PCR products were sequencing using ABI Big Dye Terminator dyes (Perkin-Elmer Applied Biosystems, Mississauga, ONT). The sequencing reactions were set up as 25 cycles of 10 sec at 96 C, followed by 5 sec at 57 C, followed by 4 min at 60 C. Each sequencing reaction contained 11 μL of PCR product, 0.5 μM of either a forward or reverse primer, and 8 μL of the Big Dye ready reaction mixture. The labeled DNA in each sample was purified by ethanol precipitation, and then sequenced using an ABI 377 automated sequencer.

Cloning and Sequencing of Fas Fragments

Fas fragments were cloned using Qiagen's TOPO TA-cloning kit. Cycle sequencing of cloned Fas fragments was performed using primers that were supplied with the TOPO-TA kit that flanked the insertion site in the plasmid. The sequencing reaction was performed as described above for sequencing of uncloned RT-PCR products.

Phenotype Analysis and Quantifying Apoptosis

Lymphocytes were phenotyped by lysing 100 μL of peripheral blood (or 1 x 10^6 cells from tissues) using the QPrep System (Beckman-Coulter). Cells were washed with 2 mL PBS, and centrifuged at 250g for 5 minutes. The cell pellet was resuspended in 50 μL of purified goat immunoglobulin (Caltag, Burlingame, CA) to prevent binding of monoclonal antibodies via Fc receptors and after a 10 minute incubation, FITC, PE, or PC5-conjugated monoclonal antibodies (Beckman-Coulter) were added for 15 minutes at room temperature. Cells were washed as above and resuspended in 0.5 mL of PBS for analysis. The percentage of DN lymphocytes in normal individuals was determined as 2.75% ± 2.1% (n = 29).

Apoptosis was assessed in eosinophils using surface binding of PE-conjugated Annexin V (14, 15), in which 10^5 cells were labeled with Annexin V-PE, washed and stained with 7-aminoactinomycin D (7-AAD) in the dark for 15 min at room temperature. The cells were immediately analyzed with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) and early apoptotic cells were defined as those cells which bound Annexin V, but not 7-AAD (14, 15).

Apoptosis was assessed in lymphocytes by determining intracytoplasmic binding of phycoerythrin (PE)-conjugated APO2.7 mAb (Beckman-Coulter) (15, 16). T and B cell subsets were identified by staining 50 μL of activated whole blood as mentioned above with mAbs conjugated to FITC or PC5 (Beckman-Coulter). Cells were then fixed, washed, stained with APO2.7-PE (intracellular), and lysed using the IntraPrep kit (Beckman-Coulter) according to

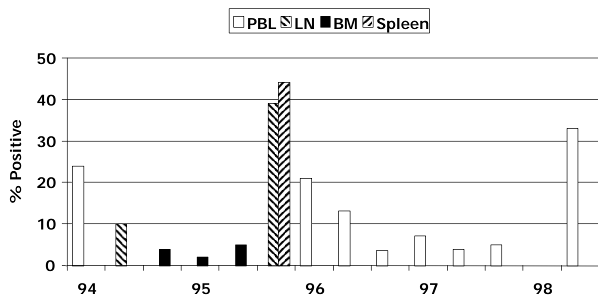

Figure 2. Flow cytometric analysis of DN lymphocytes from patient (II-4) with ALPS. Lymphocytes isolated from peripheral blood, lymph nodes, bone marrow and spleen were analyzed by flow cytometry. In normal individuals, the percentage of DN lymphocytes in peripheral blood is 2.75% ± 2.1%.

manufacturer's instructions. Cells were washed, resuspended in 2% paraformaldehyde, and analyzed using an EPICS XL-MCL (Beckman-Coulter) flow cytometer.

Prediction of Fas Secondary Structure

Secondary structure predictions of Fas were made using the PeptideStructure and PlotStructure programs of the Wisconsin Package Version 9.1 (Genetics Computer Group (GCG), Madison, WI). These programs were used with their default settings and the Chou-Fasman (17) and Robson-Garnier (18) rules regarding peptide secondary structure predictions.

RESULTS

The proband's (Figure 1, II-4) clinical presentation of splenomegaly, lymphadenopathy and thrombocytopenia was suggestive of ALPS in the absence of an infection or malignancy. To determine if the patient had ALPS, we investigated the proband and his immediate kindred for the clinical, laboratory and molecular characteristics of ALPS. The significance and potential etiology of the proband's eosinophilia was also examined.

Presence of DN Lymphocytes

Throughout the course of the proband's illness, the percentage of DN lymphocytes found in his peripheral blood has fluctuated, but has been persistently and abnormally elevated

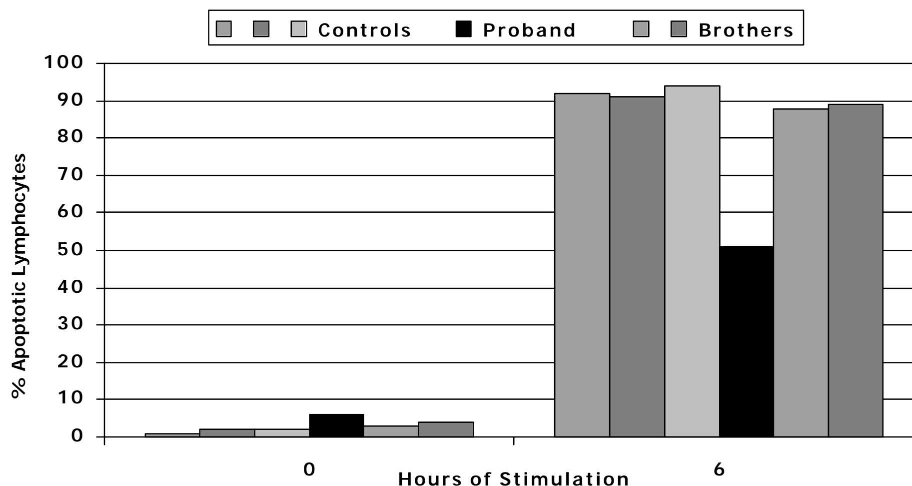

Figure 3. Resistance of lymphocytes to Fas-mediated apoptosis in a patient with ALPS. Activated peripheral blood lymphocytes were cultured for 6 hours with the anti-Fas mAb CH-11. The percentage of apoptotic cells was determined by flow cytometry as cells that bound the mAb APO2.7 intracellularly (15). Nearly all activated lymphocytes from the healthy donors and the proband's brothers underwent apoptosis following challenge with anti-Fas. Only 52% of lymphocytes from the proband underwent apoptosis under the same conditions suggesting an impairment in the proband's Fas/FasL pathway.

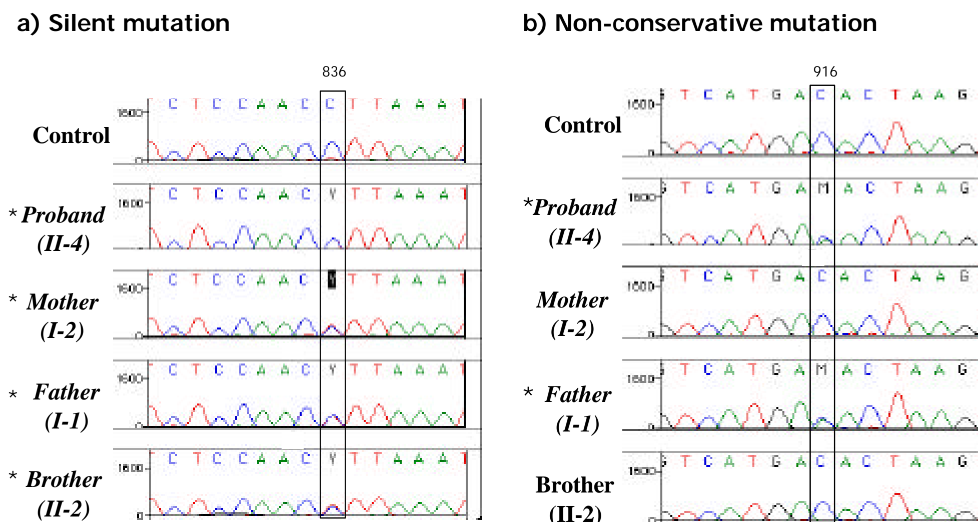

Figure 4. Fas mutations in a patient (II-4) with ALPS and his kindred. RT-PCR was performed and uncloned amplified cDNA was sequenced using cycle sequencing, ABI Big Dyes and an automated sequencer. Two Fas mutations were identified in members of this family. All individuals possessing mutations (*) were heterozygotes. Mutations were identified by the presence of two peaks at the same base. The first mutation (a) was a silent cytosine to thymine mutation at base 836 that did not lead to an amino acid substitution, and was thought to represent a silent mutation. The second mutation (b) was a cytosine to adenine point mutation at base 916 that resulted in an amino acid substitution in the death domain of Fas (see Figure 5). Neither mutation has been described previously. Mutations were confirmed by sequencing cloned cDNA (not shown).

(3.5%-33%; Figure 2). The percentage of DN lymphocytes was also elevated in lymph nodes (10-39%), spleen (44%) and bone marrow (2-5%) tissues (Figure 2). Histological sections demonstrated expansion of the interfollicular regions of lymph nodes, expansion of the marginal zone and infiltration of the red pulp of spleen (not shown). Immunohistochemical labeling with anti-CD3 demonstrated that the expanded populations of cells were T cells (not shown).

Resistance of PBL to Fas-Mediated Apoptosis

The increased presence of DN lymphocytes confirmed ALPS in the proband. After excluding malignancy, we next demonstrated that the patient's activated PBL were resistant to Fas-induced apoptosis. Whole blood was cultured with CD2/2R to activate PBL and the apoptosis-inducing anti-Fas mAb CH-11. After six hours of culture, control lymphocytes and lymphocytes from the proband's brothers (II-1 and II-2) demonstrated an appropriate response to the anti-Fas mAb, with nearly all PBL undergoing apoptosis (Figure 3). In contrast only 51% of the proband's PBL underwent apoptosis. This finding suggested an impairment in Fas-mediated apoptosis, supporting the diagnosis of ALPS. However, the exact defect in the Fas/FasL pathway remained to be elucidated.

Characterization of Fas Mutations

Using RT-PCR and Fas cDNA isolated from cultured, PBL that had been activated, we sequenced bases 151-1206 of the Fas gene in two overlapping fragments, as previously described (4). Two heterozygous mutations were identified, and cloned cDNA was sequenced to confirm the mutations (not shown). The first mutation, at base 836, was a conservative point mutation; the C to T substitution (Figure 4a) does not cause a

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                 916
                  ↘
Ctrl    | Val Met Thr Leu Ser
        | GTC ATG ACA CTA AGT

Proband | Val Met Lys Leu Ser
        | GTC ATG AAA CTA AGT
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Figure 5. Predicted structural changes in Fas associated with the cytosine to adenine mutation at base 916. In normal individuals, the codon affected by the cytosine to adenine mutation at base 916 encodes threonine (an aliphatic amino acid). Substitution of cytosine by adenine results in a predicted change from a threonine to a lysine (basic amino acid). Using peptide modeling, this amino acid substitution results in a predicted change in the secondary structure in part of the Fas death domain, which is involved in intracellular signaling (19). The mutation causes a predicted change from a β-pleated sheet to an α helix in this region of the death domain.

change in the corresponding amino acid, and was interpreted as a silent mutation. This heterozygous mutation was found in individuals I-1, I-2, II-1, II-3 and II-4 (Figures 1 and 4a). The second mutation, at base 916, was a non-conservative point mutation; the C to A substitution (Figure 4b) caused a threonine (aliphatic amino acid) to a lysine (basic amino acid) substitution (Figure 5). This mutation occurred in the death domain of Fas (19), and peptide modeling predicted that this substitution would result in a change of the peptide's secondary structure from a β-pleated sheet to an α-helix in one part of the death domain. Interestingly, the second mutation was inherited from the father (I-1) and was found in several of his children (II-2, II-3 and II-4), but only the proband (II-4) demonstrated the full clinical manifestations of ALPS.

Significance of the Eosinophilia

The proband's eosinophilia ($35.1x10^9$ cells/mL) occurred when there was a relatively low percentage of DN lymphocytes in the peripheral blood (7.2%; Figure 2). An inverse relationship was also observed in 1998 between a high percentage of DN lymphocytes (33% of lymphocytes; Figure 2) and a relatively low eosinophil count ($1.2x10^9$ cells/mL). This suggested that cytokines elaborated from DN lymphocytes may not necessarily be the only factor involved in the proband's eosinophilia. It would have been predicted that the eosinophilia would have occurred concurrently with the increased DN lymphocytes if DN-derived cytokines were the only factor promoting eosinophil production. This is in contrast to previous findings for the only other known ALPS patient with eosinophilia (7), and suggested that another mechanism directly involving the Fas/FasL pathway might be responsible for the eosinophilia in this case.

To test this hypothesis, we cultured purified eosinophils in the presence of IFNγ with or without the apoptosis-inducing anti-Fas mAb CH-11. Culturing eosinophils in the presence of IFNγ prevents apoptosis *in vitro* and increases surface expression of Fas (14). Figure 6

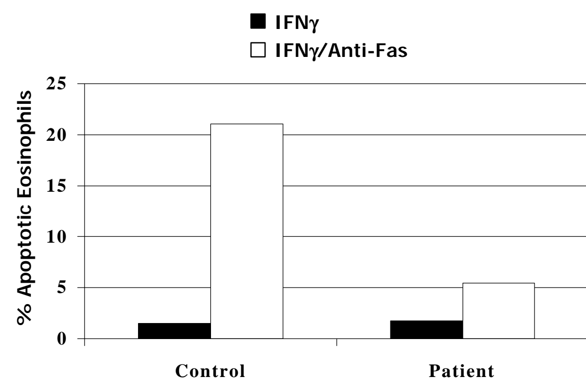

Figure 6. Resistance to Fas-mediated apoptosis in eosinophils. Eosinophils were isolated from the proband and a healthy donor. The eosinophils were activated in culture with IFNγ and then incubated with or without the anti-Fas mAb, CH-11 (14). The percentage of early apoptotic cells was assessed as cells that bound Annexin V, but excluded the viability dye 7-aminoactinomycin D (14, 15). In the absence of anti-Fas, <4% of the patient's or the control's eosinophils underwent apoptosis. However, in the presence of anti-Fas, several-fold more of the control's eosinophils underwent apoptosis than the proband's suggesting that a defect in the Fas/FasL pathway could account for the eosinophilia observed in the proband.

demonstrates that IFNγ effectively prevented spontaneous eosinophil apoptosis *in vitro*. When eosinophils from a healthy donor were cultured with IFNγ and anti-Fas, 21% of eosinophils underwent apoptosis (Figure 6), comparable to results obtained in other published studies (14). In contrast, relatively few of the eosinophils (5.4%) from the proband underwent apoptosis when cultured with IFNγ and anti-Fas (Figure 6). These findings suggest that the eosinophils from our ALPS patient had an impairment in their ability to undergo Fas-mediated apoptosis.

HLA Association with Features of ALPS in This Kindred

The demonstration of the incomplete penetrance of ALPS in this kindred despite the presence of the C to A mutation of Fas at base 916 in several family members prompted us to consider other factors which might contribute to the development of ALPS. In mice, the autoimmune phenomena associated with the homozygous recessive *lpr/lpr* mutation are much worse when the mutation is expressed in the MRL mouse (20). When the *lpr/lpr* mutation is expressed in other strains of mice, such as the C3H strain, the autoimmune features are not as severe (20). We reasoned that there might be a similar association between HLA haplotypes and the autoimmune features of ALPS in this kindred. Table 1 documents the HLA haplotype of the proband, but no conclusions could be drawn between the haplotype and features of ALPS given the small size of the comparison.

DISCUSSION

This report describes an individual with ALPS and his kindred, and emphasizes some features of the proband's disease which have not been previously reported. While the proband had features consistent with criteria proposed for establishing a diagnosis of ALPS (splenomegaly/lymphadenopathy, an abnormal population of DN lymphocytes, resistance of lymphocytes to Fas-mediated apoptosis and a mutation in Fas) (1), the patient also had some unique features. First, the mutation in Fas at base 916 (in the death domain) has not been described before. Second, the proband had a marked eosinophilia, which has been described only once before in patients with altered splicing of Fas (7), and not in any patients with mutations in Fas.

Table 1

DRB1	DRB3	DRB4	DQB1	A	B	BW	C
1104	0202	01XX	0301	A2	B44	BW4	CW5
0404			0302	A29			

Two point mutations were observed in Fas; the first was a conservative C to T substitution at base 836, and the second was a non-conservative C to A substitution at base 916. It was felt that the first mutation likely represents a silent mutation, but might have contributed to disease in the more mildly affected family members, perhaps through altered translation efficiency of this allele. However, the second Fas point mutation at base 916, is suspected to be the most likely cause of ALPS in this patient. This point mutation is located in an area of the gene that encodes the death domain, and would lead to a predicted change in the secondary structure in part of the death domain from a β-pleated sheet to an α-helix. It is likely that this would prevent the normal interaction of Fas with FADD (MORT-1) (21). FADD interacts with and activates Caspase-8, a cysteine protease that mediates the intracellular events that culminate in apoptosis (22).

The mutation identified in this patient is part of a subset of Fas mutations identified in ALPS patients that involve the death domain of Fas (21). Interestingly, a mutation has been identified in another kindred with ALPS which involves the same codon, but at base 915, causing a non-conservative amino acid substitution from threonine to proline (unlike threonine to lysine as in our case) (2). This mutation was shown to be functionally defective and prevent anti-Fas mediated apoptosis when the mutant form of Fas

was over-expressed in BW5147 cells. Our results suggest that the presence of a threonine at this site in the death domain is crucial for activating the intracellular machinery that mediates Fas-induced apoptosis.

Interestingly, the non-conservative Fas mutation was inherited from the proband's father and was also present in siblings of the proband, yet none of them exhibited the other aspects of ALPS. However, the clinical features of ALPS did not correlate with the presence of the non-conservative Fas mutation (I-1, II-2, II-3) suggesting incomplete penetrance of the mutation in causing disease. All of the siblings (II-1, II-2, II-3) had demonstrable populations of DN lymphocytes (Figure 1), suggesting that some sort of impairment gave rise to this abnormal population of cells. While it is tempting to speculate that the non-conservative Fas mutation was the cause of this abnormality, the absence of that mutation in one of the proband's siblings (II-1), despite the presence of DN lymphocytes in that individual, suggests that the Fas mutation is not the sole cause of the expanded population of DN lymphocytes. In support of this finding, it is notable that in the MRL mouse, mild autoimmune disease develops even in the absence of the *lpr* mutation (20) suggesting that the development of autoimmunity and lymphoproliferation is due to more than one gene.

The Fas mutations identified in this study are a part of a growing family of Fas mutations associated with malignancy and autoimmunity (Table 2). However, there are a number of other factors that might be involved in the pathogenesis of ALPS. Given that the genetic background of mice can determine the severity of autoimmunity in *lpr/lpr* mice (20), we determined the MHC haplotypes of family members in this kindred and compared the haplotypes to various autoimmune features of ALPS. Unfortunately, the small size of this analysis prevented any firm conclusions from being reached, but the HLA haplotype of the proband is presented in Table 1 in hopes that a comparison to other kindreds will give greater insight into the role of HLA in the etiology of ALPS.

Other factors possibly involved in the etiology of ALPS include a second defect in the Fas/FasL pathway. Indeed, patients that lack mutations in Fas, but possess defects in the Fas/FasL pathway with other clinical features of ALPS have been described (10). Whether such defects are due to abnormalities in the proteins that associate with the death domain of Fas (i.e. FADD/MORT-1) or proteins further downstream (such as the caspases) warrants further investigation.

Fuss et al (23) have observed that cytokine production in ALPS patients is skewed toward a T_h2 profile. This is another potential factor involved in the etiology of ALPS; T_h2 lymphocytes have been shown to express less FasL with a relative impairment in

Table 2. Human Diseases Associated with Defects in the Fas/FasL Pathway

Autoimmune Disease	Defect	Reference
ALPS	Mutation in Fas or Fas/FasL Pathway	(1, 2, 4-10)
SLE	FasL mutation (1 patient)	(27)
Hashimoto's Thyroiditis	Inappropriate Fas expression	(28)
Malignancy		
Multiple Myeloma	Fas mutation and undetectable Fas mRNA	(29)
T-ALL	2/81 patients with Fas mutations	(30)
NHL	16/150 patients with Fas mutations	(31)

Abbreviations: SLE, Systemic Lupus Erythematosus; T-ALL, T-Acute Lymphoblastic Leukemia; NHL, Non-Hodgkin's Lymphoma.

activation-induced cell death when compared to T_h1 lymphocytes (24, 25). Whether T_h2 cytokines alter the expression of factors downstream of Fas (i.e. FADD, FAP-1, Sentrin, DAXX, FAF-1, RIP or caspases) also warrants investigation, as these are other potential sites for defects to act in concert with Fas mutations in the pathogenesis of ALPS.

An additional factor that might give rise to ALPS might be a defect in an apoptosis pathway other than the Fas/FasL pathway. For example, mice that possess two defects in apoptosis pathways (Fas/FasL and Bcl-2) develop acute myeloblastic leukemia (26).

The marked eosinophilia was a unique feature of the proband's disease. Only one other report has noted eosinophilia associated with alternative splicing of Fas (7). The authors of that report argued that IL-3 and IL-5 production from DN lymphocytes was responsible for the observed eosinophilia. However, the functional status of Fas in the eosinophils from those patients was not examined (11). Furthermore, others have observed that DN lymphocytes produce very low amounts of cytokines (23), raising the possibility that a mutation in Fas may have a direct role in mediating the eosinophilia, especially since eosinophils express Fas and undergo Fas-mediated apoptosis (14). In the current study, we observed an inverse relationship between eosinophilia and DN lymphocyte counts, suggesting that cytokines elaborated from DN lymphocytes were not stimulating eosinophil production. We hypothesized that the same defect in Fas that contributed to the lymphoproliferation also caused the eosinophilia. Indeed, eosinophils that were activated *in vitro* were resistant to anti-Fas induced apoptosis. This finding suggests that in this patient, the eosinophilia was at least partially due to a functional defect in Fas, and was not solely cytokine-induced. In support of this finding, it has been shown that in *lpr/lpr* mice, the bone marrow contains greatly increased numbers of myeloid colony-forming cells as compared to wild-type animals (26). Our observations suggest that Fas may have a role in the regulation of myelopoiesis. Whether the magnitude of the defect in apoptosis observed in our patient caused all of the eosinophilia is unknown. It is possible that a combination of decreased apoptosis and increased production caused the eosinophilia, which is consistent with the etiology of the lymphoproliferation seen in the spleen and lymph nodes of ALPS (3).

Another important feature of the proband's disease was that only 50% of the proband's activated PBL underwent apoptosis in response to anti-Fas. This is in contrast to PBL from a sibling (II-2) who possessed the same Fas mutation as the proband; nearly all of the PBL underwent apoptosis following culture with anti-Fas. Considerable variation in anti-Fas apoptosis had been observed in other ALPS kindreds with asymptomatic family members who possess Fas mutations; some of those individuals' PBL have impaired apoptosis, whereas others do not (2). The findings of this study are consistent with the observation that there is incomplete penetrance of Fas mutations in causing ALPS (11), and that at least one factor other than a mutation in Fas is responsible for the etiology of ALPS.

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