The broad spectrum of autoimmune lymphoproliferative disease: molecular bases, clinical features and long-term follow-up in 31 patients

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Autoimmune lymphoproliferative disorders, including autoimmune lymphoproliferative syndrome (ALPS) and Dianzani autoimmune lymphoproliferative disease (DALD), are inherited defects of the Fas apoptotic pathway characterized by lymphoid accumulation and autoimmune manifestations. We report the molecular, clinical, immunologic features and the long-term progress of 31 patients. Four carried Fas gene mutations and one also displayed a caspase 10 polymorphism that probably contributed to the phenotype. Seven patients developed antibody deficiency and their clinical pictures overlapped those of subjects with common variable immunodeficiency (CVID). We postulate the existence of a disorder that involves the Fas pathway and displays the characteristics of both autoimmune lymphoproliferative disease and CVID.

Key words: autoimmune lymphoproliferative disease, apoptosis, Fas, caspase 10, common variable immunodeficiency.

Haematologica 2006; 91:538-541

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Autoimmune lymphoproliferative diseases are disorders of lymphocyte homeostasis caused by defects in the Fas/CD95 apoptotic pathway.[1,2] These disorders include autoimmune lymphoproliferative syndrome (ALPS) and Dianzani autoimmune lymphoproliferative disease (DALD). ALPS is characterized by defective function of the Fas system, autoimmunities, chronic non-malignant lymphadenopathy and splenomegaly and high levels of circulating $\alpha\beta^+CD4^-CD8^-$ lymphocytes (*double negative T cells* or DNT).[3] DALD (MIM: 605233) shows ALPS features in the absence of DNT expansion.[4] Some ALPS patients carry mutations of the *TNFRSF6* gene, which encodes Fas (ALPS type I, MIM: 601859);[3] conversely, two ALPS subjects were found to have alterations in the caspase 10 gene (*CASP10*), encoding for a protease which acts downstream from Fas, and have been classified as having ALPS type II (MIM: 603909).[5] A homozygous mutation of caspase 8 (*CASP8*) was described in an ALPS-like syndrome with immunodeficiency (ALPS type IIb; MIM: 607271), but lack of autoimmunity differentiates this from classic ALPS.[6] Patients whose genetic defect has not been identified are classified as having ALPS type III.[3] No causal genes for DALD are known.[4,7] Molecular defects of both ALPS III and DALD probably lie downstream from Fas in the apoptotic cascade. We describe the molecular, clinical and immunologic features and the long-term follow-up of 31 patients with autoimmune lymphoproliferative disorders.

Design and Methods

Thirty-one unrelated patients with autoimmune lymphoproliferative disorders (17 males, 14 females) were referred to our Center in the past 10 years. The diagnosis was based on the presence of: (i) chronic non-malignant lymphoid accumulation; (ii) autoimmune manifestations and/or laboratory markers of autoimmunity; and (iii) defective Fas-induced apoptosis (relative T-cell survival ≥82% in two independent cell death assays). DNT cell expansion enabled differentiation of ALPS (DNT cells >1% of total $TCR\alpha\beta^+$ circulating lymphocytes)[3] and DALD. Serum autoantibodies, immunoglobulin levels and lymphocyte immunophenotype were determined; infectious and neoplastic causes of lymphadenopathy were ruled out. Clinical and immunologic progression was followed and the response to treatment evaluated. Remission and response were considered stable if lasting more than 12 months. Patients who had hypogammaglobulinemia underwent further investigations for bacterial infections, isohemagglutinin titers and vaccine response. Statistical evaluations were performed with Wilcoxon's test and Fisher's exact probability test.

TNFRSF6 and *CASP10* were screened for mutations after patients had given informed consent. *CASP8* was sequenced in subjects who showed hypogammaglobulinemia. Details of the methods are reported in the online appendix.

Results and Discussion

Eight patients were classified as having ALPS (four with ALPS I and four with ALPS III) and 23 as having DALD. Two patients classified as having ALPS I due to the presence of a *TNFRSF6* mutation showed normal DNT levels. The patients' features are summarized in Table 1.

Genetics. All ALPS I patients (n. 1-4) present-

ed new heterozygous *TNFRSF6* mutations (Figure 1A-C; *Figure 2 online appendix*). Patient 1 carried a substitution of glutamic acid 194 with lysine in the intracellular portion near the cell membrane. Patient 2 had a C>T transition at nt 139, causing a stop codon and resulting in either a non-functional allele or a truncated soluble Fas fragment; the mutation was also present in the healthy mother. Patient 3 carried a substitution of glutamine 273 to histidine in the death domain; the mutation was also found in the patient's father, who had chronic immune thrombocytopenia and splenomegaly. Patient 4 had a substitution of histidine 111 for proline in the extracellular domain.

The non-conservative Y446C substitution in *CASP10* was detected in patients 2 and 10 (Figure 1B,C); patient 2 also carried a *TNFRSF6* mutation. The frequency of the allele in controls was 2%. Patients 14 and 25 were heterozygous for the V410I substitution in *CASP10*, which also proved to be a common polymorphism in our population (allelic frequency: 6%) and was found in homozygosity in one of the healthy controls and the healthy mother of patient 25. No sequence variations in the *CASP8* coding region were found.

Clinical features. The median age at diagnosis was 3 years (range 0.3-16), and the male to female ratio was 1.4. Adenopathy was present in 64% of patients, mainly located in the cervical and/or inguinal regions. Lymph node biopsy, performed in 11 subjects, showed histopathological pictures of reactive follicular hyperplasia, associated with plasmocytosis in three cases and with parafollicular DNT expansion in one. Splenomegaly was present in 87%; histopathologic examination from the splenectomized patients showed expansion of the red pulp with no increase in DNT. Hepatomegaly was present in 77% of the patients. No significant differences in the grade of lymphoid accumulation were found between the ALPS and DALD groups. Almost all patients (29/31) showed autoimmune features at diagnosis, consisting of immune cytopenias in 86% of cases (immune thrombocytopenia, ITP: 10; autoimmune hemolytic anemia, 5; neutropenia: 2; plurilinear involvement: 8). Other manifestations were immune hepatitis, arthritis, insulin-dependent diabetes mellitus, thyroiditis and rashes. Atopy was present in three patients; three showed dyserythropoietic anemia and five suffered from recurrent fevers that only responded to steroids. Hypergammaglobulinemia was found in ten patients.

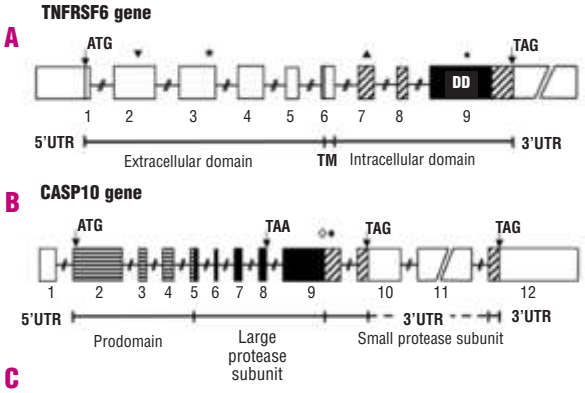
Treatment. Nineteen patients required therapy for autoimmune cytopenias. Autoimmune disorders were treated with steroids, intravenous immunoglobulins and immunosuppressants according to standard protocols. Rituximab was used to treat autoimmune hemolytic anemia in patients 11 and 31, with partial response, and severe refractory ITP in patient 27, with stable normalization of the platelet count. Patients 1 and 7 received pyrimethamine /sulfadoxine (Fansidar) therapy for severe pancytopenia and lymphoproliferation, without improvement; the first had to interrupt the treatment because of adverse reactions. Patient 28 received steroid therapy for massive lymphoproliferation without autoimmune manifestations; stable reduction of adenomegalies was obtained. Splenectomy was performed in two patients (n. 1 and 14) for massive splenomegaly and in four patients (n. 17, 19, 27 and 31) for severe ITP; patient 17 obtained stable normalization of platelet count after splenectomy. *Progression.* Patients were followed for 6 months to 23 years (median: 69 months); progression data are reported in Table 2 online. Stable decreases in lymphoid accumulation were observed in 55%, no changes in 31% and progressively increasing accumulation in 14% of the patients. No significant differences were found in the evolution of lymphoproliferation between the ALPSI, ALPS III and DALD groups, nor between subjects treated or not with corticosteroids. Stable remission of autoimmune manifestations was observed in nine patients; conversely, 12 patients developed new autoimmunities during follow-up. Seven subjects (n. 7, 10, 11, 17, 20, 23 and 31) developed persistent hypogammaglobulinemia at a median age of 13 years (range 3.4-18) and at a median time of 2.8 years (range 0.5-11) after diagnosis of an autoimmune lymphoproliferative disorder. They had initially had normal immunoglobulin levels, except for patients 11 and 31 who showed an isolated IgA defect, and fulfilled the clinical and laboratory criteria of the European Group for Immunodeficiencies (EGID) for common variable immunodeficiency (CVID).[12] Patients 7, 11 and 23 are receiving regular immunoglobulin replacement therapy. Hypo-gammaglobulinemia was also found in the 22-year old sister of patient 23 in the course of laboratory examinations for persistent otitis. She showed neither lymphoid accumulation nor autoimmune manifestations and was diagnosed as having CVID due to fulfillment of the diagnostic criteria. Her lymphocytes and those of her mother displayed defective sensitivity to Fas-induced apoptosis *in vitro*; autoimmune disorders were present in several of her maternal and paternal relatives. The mother was immunologically normal, suggesting that Fas resistance did not result from defective activation of T lymphocytes. No patients with ALPS I developed hypogammaglobulinemia.

We report the molecular, clinical and immunologic features of 31 patients with autoimmune lymphoproliferative diseases. Mutations in the gene encoding Fas were found in four, two of whom had normal DNT levels. Two mutations were inherited: the Q47X substitution in patient 2, transmitted by the healthy mother, and the Q273H substitution in patient 3, inherited from the affected father. This is consistent with literature reports that missense mutations in the death domain, which dominantly interfere with the formation of the signaling complex, are usually associated with high penetrance of the ALPS phenotype, whereas null mutations and alterations leading to truncated forms have a milder functional effect.[9] Two caspase 10 polymorphic variants were found. The V410I variant was identified in two patients and in 6% of control chromosomes. The variant was first described as a causal mutation for ALPS in a homozygous subject[5] and subsequently proved to be a common polymorphism in the Danish population;[10] since no healthy homozygotes have been found to date, the question of whether homozygous V410I causes ALPS is still open. We have now found this variant in homozygosity in two unaffected subjects, suggesting that the variant alone does not cause the disease. Nevertheless, a role for caspase 10 polymorphisms cannot be ruled out, since autoimmune lymphoproliferative disorders do not behave as classic monogenic diseases and likely result from the concurrence of more than one genetic alteration.[7] The association of *TNFRSF6* and perforin mutations has recent-

Table 1. Clinical and laboratory features of patients at disease onset.

Pt.	Sex	Classification	Age at presentation (years)	Lymphoid accumulation			Autoimmunity		IgG (mg/dL)	IgA (mg/dL)	IgM (mg/dL)	DNT cells %	Treatment
				LN§	S°	H°	autoimmune diseases	autoantibodies#					
1	M	ALPS Ia	3	+	+++	++	AHA, recurrent fever	ANA,APA	↑(2120)	n.v(78)	n.v (132)	0.8	corticosteroids, splenectomy, Fansidar®
2	M	ALPS Ia	4	++	++	+	N, ITP	APA	n.v (1250)	↑(250)	n.v (120)	0.4	none
3	F	ALPS Ia	1	-	++	-	N, ITP, DEA anemia	none	↑(1682)	n.v (100)	n.v (68)	7.3	RBC
4	M	ALPS Ia	1	++*	++++	++	ITP	none	n.v (235)	↑ (161)	n.v (114)	4.2	none
5	M	ALPS III	16	++*	-	-	N, ITP	none	↑(2430)	n.v (240)	n.v (276)	5.2	none
6	F	ALPS III	2	-	+++	++	none none	APA,ANA	↑(1870)	n.v (157)	n.v (103)	18.6	none
7	M	ALPS III	1	+	++	-	DEA, ITP	APA,ANA	↑(1450)	n.v (134)	↓ (16)	4.6	corticosteroids, Fansidar®
8	F	ALPS III	5	+++	+++	++	AHA,N, ITP recurrent fever, skin rash	APA,ANA, AMA,ASMA	↑1987)	n.v (143)	n.v (89)	3.7	corticosteroids, azathioprine, cyclosporine A
9	M	DALD	4	+	++	+ none	N	none	n.v (1080)	n.v (54)	n.v (61)	0.5	none
10	M	DALD	2	+++*	++	++	ITP	none	n.v (922)	n.v (141)	n.v (128)	0.9	corticosteroids, IVIG
11	F	DALD	5	+	++	+	AHA	none	n.v (441)	↓(7)	n.v (290)	0.7	corticosteroids, azathioprine, rituximab, RBC
12	F	DALD	3	+	++++	+	N, thyroiditis	abHTG	↑ (2922)	↑(398)	n.v (57)	0.6	none
13	M	DALD	6	++*	++	++	AHA, arthritis	APA, ANA, ASMA	n.v (1358)	n.v (200)	n.v (204)	0.5	corticosteroids
14	F	DALD	0.9	-	++++	++	recurrent fever	APA,ANA, ASMA	↑ (2600)	↑ (169)	n.v (129)	0.6	splenectomy
15	M	DALD	5	+++*	+	-	IDDM	APA	n.v (830)	n.v (20)	n.v (53)	0.4	insulin
16	M	DALD	16	+*	+	-	recurrent fever	ANA	↑ (2016)	n.v (235)	n.v (123)	0.8	none
17	M	DALD	7	++*	+	++	ITP	ANA, AMA	n.v (842)	n.v (89)	n.v (132)	0.5	corticosteroids, IVIG, anti-D, splenectomy
18	F	DALD	12	+	+	++	AHA, immune hepatitis	APA	n.v (1200)	n.v (157)	n.v (165)	0.8	corticosteroids, azathioprine
19	F	DALD	9	-	++	+	ITP	ASMA	n.v (765)	n.v (145)	n.v (79)	0.5	corticosteroids, IVIG, anti-D, splenectomy
20	M	DALD	0.5	+	++	++++	ITP, asthma	APA	n.v (356)	n.v (80)	n.v (39)	0.3	IVIG
21	M	DALD	10	-	++	+++	AHA, ITP	APA, ANA, ASMA	n.v (797)	n.v (192)	n.v (89)	0.4	corticosteroids, IVIG
22	F	DALD	5	++*	-	-	arthritis	ANA, nDNA,ASMA	↑(3060)	↑ (569)	↑ (289)	0.6	corticosteroids
23	F	DALD	2	-	++	-	AHA, ITP, eczema	APA, ASMA	n.v (1060)	n.v (44)	n.v (87)	0.8	corticosteroids, IVIG, azathioprine
24	F	DALD	1	-	++	++	AHA,N, ITP	APA, ANA, nDNA	n.v (465)	n.v (89)	n.v (56)	0.6	Corticosteroids, IVIG
25	M	DALD	7	-	-	+	ITP	ANA	n.v (1060)	n.v (138)	n.v (62)	0.6	IVIG, anti-D
26	M	DALD	2	-	++++	++++	ITP, asthma	APA, ASMA	n.v (796)	n.v (78)	n.v (74)	0.7	none
27	M	DALD	12	++*	+++	+	N, ITP	ANA	n.v (1140)	n.v (281)	n.v (132)	0.8	corticosteroids, IVIG, splenectomy, rituximab
28	M	DALD	3	+++*	-	+	none	ANA, ASMA	n.v (702)	n.v (74)	n.v (85)	0.3	corticosteroids
29	F	DALD	0.3	-	++++	++++	ITP, DEA anemia, recurrent fever	APA	n.v (592)	n.v (41)	n.v (101)	0.5	corticosteroids, IVIG, RBC
30	M	DALD	1	+*	+++	+++	ITP	APA	n.v (790)	n.v (32)	n.v (58)	0.4	corticosteroids, IVIG, anti-D, cyclosporine A
31	F	DALD	2	-	++	++	AHA	ANA	n.v (913)	↓ (5)	n.v (191)	0.7	corticosteroids, IVIG, splenectomy, cyclosporine A, MTX, rituximab

*M: male; F: female; AHA: autoimmune hemolytic anemia; ITP: immune thrombocytopenia; N: autoimmune neutropenia; APA: antiphospholipid antibodies, including lupus anticoagulant and/or anticardiolipin antibodies and/or antiβ2GPI antibodies; DEA: dyserythropoietic; ANA: anti nuclear antibodies; nDNA: anti native DNA antibodies; AMA: anti mitochondrial antibodies; ASMA: anti smooth muscle antibodies; n.v.: normal value; IVIG: intravenous high dose immunoglobulins; anti-D: anti-D immunoglobulins; RBC: red blood cell transfusions; MTX: methotrexate. Ig serum levels: ↓: <2SD from normal age levels; ↑: >2SD from normal age levels (Meites S. Pediatric Clinical Chemistry, Reference (Normal) Values. 3rd ed. Washington, D.C.: Clinical Chemistry, 1989). #Autoantibodies against blood cells not included. §Lymphoadenopathy: + 1-2cm lymph nodes; ++ 2-4cm nodes; +++ >4cm nodes. °Splenomegaly/ Hepatomegaly: + 1-2cm; ++ 3-4cm; +++ 5-6cm; ++++ >6cm below costal margin; *patients who underwent lymph node biopsy. Patients 2 and 10 had a positive direct Coombs' test with no hemolytic anemia. Patient 25 was diagnosed as having Hodgkin's lymphoma 15 months prior to the diagnosis of ALPS. The features at diagnosis of 14 patients were partly described in Chiocchetti et al, Blood 2004.*



Pt	Gene	Genomic DNA Mutation	Exon/ Intron	Expected Protein Alteration	Population Frequency	Symbol
1	FAS	580G>A	ex7	E194K	<1%	▲
2	FAS	139C>T	ex2	Q47X	<1%	▼
3	FAS	819G>C	ex9	Q273H	<1%	●
4	FAS	332A>C	ex3	H111P	<1%	■
2	CASP10	1337A>G	ex9	Y446C	2%	♦
10	CASP10	1337A>G	ex9	Y446C	2%	♦
14	CASP10	1228G>A	ex9	V410I	6%	○
25	CASP10	1228G>A	ex9	V410I	6%	○

Figure 1. Genetic alterations in patients. A. Schematic representation of *TNFRSF6* (RefSeq NM_000043.3) and localization of mutations. B. Schematic representation of *CASP10* (RefSeq NM_032977.2) and localization of polymorphic variants. C. Summarizing table TM: transmembrane domain; DD: death domain.

ly been described in a patient whose parents, carrying the *TNFRSF6* and the perforin mutation respectively, were healthy.[11] Similarly, our patient 2 had both a *TNFRSF6* mutation, and the caspase 10 Y446C substitution. His mother, who only carried the *TNFRSF6* mutation, was healthy, suggesting that the caspase variant contributed to the ALPS phenotype. A subgroup of patients developed persistent hypogammaglobulinemia, which rendered their clinical and immunologic features strikingly similar to those of CVID subjects. First, CVID is associated with autoimmune manifestations and non-malignant lymphoid accumulation[12] and relatives of patients with CVID have a higt incidence of autoimmunities and malignancies, which is also a peculiarity of autoimmune lymphoproliferative disorders.[7] Furthermore, a higher rate of spontaneous apoptosis, which characterizes CVID T lymphocytes,[13] was observed in T cells from Fas-deficient subjects,[9,14] whereas defective Fas-induced apoptosis was reported in a subset of CVID.[13] We postulate the existence of a disease, somehow involving the Fas pathway, which combines the features of both autoimmune lymphoproliferative disorders and CVID. This hypothesis is supported by our observation of a family including one sibling with DALD and hypogammaglobulinemia and the other sibling with classic CVID. Fas function was reduced in both siblings and in the healthy mother; furthermore, autoimmune manifestations recurred in the maternal and paternal lineages, pointing to inheritance of a genetic defect from each parent. This pedigree suggests that autoimmune lymphoproliferative disorders and CVID may originate from variable combinations of mutations involving two or more genes. Caspase 8, whose defect was found associated with an ALPS-like phenotype with immunodeficiency, seems to have no pathogenic role in our patients. Further molecular studies are thus needed and assessment of Fas-pathway function in CVID subjects is advisable to clarify the relationships of CVID with autoimmune lymphoproliferative disorders.

MFC and PQ drafted the manuscript, all authors contributed to the revision. UR and UD were involved in the conception and design of the experiments; UR is also the author taking primary responsibility for the paper. LG was responsible for data collection and analysis. CD and AR critically revised the paper and are responsible for important intellectual content. EG, ACa, VB, AB performed molecular analyses. ACh performed osteopontin analysis. AD performed Fas apoptosis tests. We wish to thank Drs Momcilo Jankovic, Clinica Pediatrica, Università di Milano Bicocca, Milano, Italy, Fernando M. de Benedictis, Mauro Jorini, Divisione di Pediatria, Ospedale dei Bambini "Salesi", Ancona, Italy and Prof Alberto Martini, Dipartimento di Pediatria, Università di Genova, Genova, Italy for providing data concerning their patients who entered the study. This work was supported by grants from the Ministero Italiano della Ricerca Scientifica e Tecnologica, Regione Piemonte and Telethon to UR and UD.

Manuscript received August 9, 2005. Accepted January 27, 2006.

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