"FEDERICO II"
UNIVERSITY OF NAPLES

Faculty of Medicine and Surgery

Doctorate in
"Human Reproduction, Development and Growth"

Academic Year 2004-2005

Ph. D. Thesis

"Congenital immunodeficiencies and susceptibility to viral infections: definition of novel clinical entities in their pathogenic mechanism and molecular alterations"

Tutor **Student**

Prof. Claudio Pignata **Dr. Rosanna Busiello**

Director

Prof. Claudio Pignata

Contents

Chapter 3

Technologies

Chapter 4

Human Primary Immunodeficiency syndromes

Primary immunodeficiency diseases (PIDs) represent a group of most heterogeneous syndromes characterized by development or functional defect of the Immune system. More than 100 inherited conditions described immunodeficiencies exist (Chapel, H. 2003; Fischer, A. 2001; Buckley R. H. 2000). Clinical presentation is highly variable, ranging from various patterns of microbial susceptibility to allergy, lymphoproliferation or autoimmune manifestation the most of these syndromes are monogenic and lead to defects in any aspect of the immune response. Although several form of them occur in the first time of life there are many other disorders in which the clinical manifestation develop during adulthood. A great contribution to understanding the genetic basis of PIDs came up from recent report on using tools developed to study the human genome, approximately 100 genes associated to PIDs have been identified so far (Chapel, H. 2003; Fischer, A. 2001; Buckley R. H. 2000). Along with classical form in with there are clear sign of immune defect there are often several form in which the clinical features of immune defect are not completely clear so many cases are misdiagnosed**.** In many cases, inherited diseases have led to the identification of new gene that are crucial in immune cell development or effector function, thus contributing to homeostatic mechanisms that keep the immune system in check to prevent overt autoimmune disease. In particular, in the last years the attention has been focused on the several form of ID in which an alteration of homeostatic mechanism is involved. The

process which is involved in the control of immunological homeostasis named apoptosis or programmed cell death that exert its important role trough specialized cells. These cells play their role of maintenance of the immune homeostasis through two principal mechanism: the secretory and non secretory pathway that will be illustrated below.

References

1. Chapel, H., Geha, R. & Rosen, F. (2003) Primary Immunodeficiency disease: an update. *Clin. Exp. Immunol*. 132, 9-15

2. Fisher, A. (2001) Primary immunodeficiency disease: an experimental model for molecular medicine. *Lancet* 357, 1863-1869

3. Buckley, R.H. (2000) Advances in immunology: primary immunodeficiency diseases due to defects in lymphocytes. *N. Eng. J. Med.* 343,1313-1324

Molecular basis of killing by CTLs and NK cells

CTLs and NK cells are critical for the host defense against pathogenic microorganisms and malignant cells (Russell J.H. et al 2002). When CTLs recognize target cells, the T-cell receptor (TCR) on the cell surface of CTLs engages with major histocompatibility complex (MHC) class I. Signals from the TCR activate both the non secretory pathway, known as the Fas mediated pathway, and the secretory pathway, that involves the perforin (PRF1)/granzyme mediated signaling (Kagi D. et al 1996). Genetic defects of both pathways have been described. These defect lead to completely distinct clinical and immunological phenotypes. There are 18 identified monogenic diseases that disturb the immune system homeostasis of either innate or adaptative immunity (Table 1). Both pathways play a key role in the capacity of immune system to maintain a state of dynamic equilibrium despite recurrent exposure to a diverse array of organisms and constant exposure to self-antigens. These immune cell death pathways allow the immune response to be self-limited and decline with time after antigenic stimulation. Alterations of both these mechanisms of control of the immune response may be responsible for an alteration in the homeostasis thus leading to inappropriate response to viral infections and massive lymphohistiocytic activation syndromes or hyperimmune syndromes, respectively.

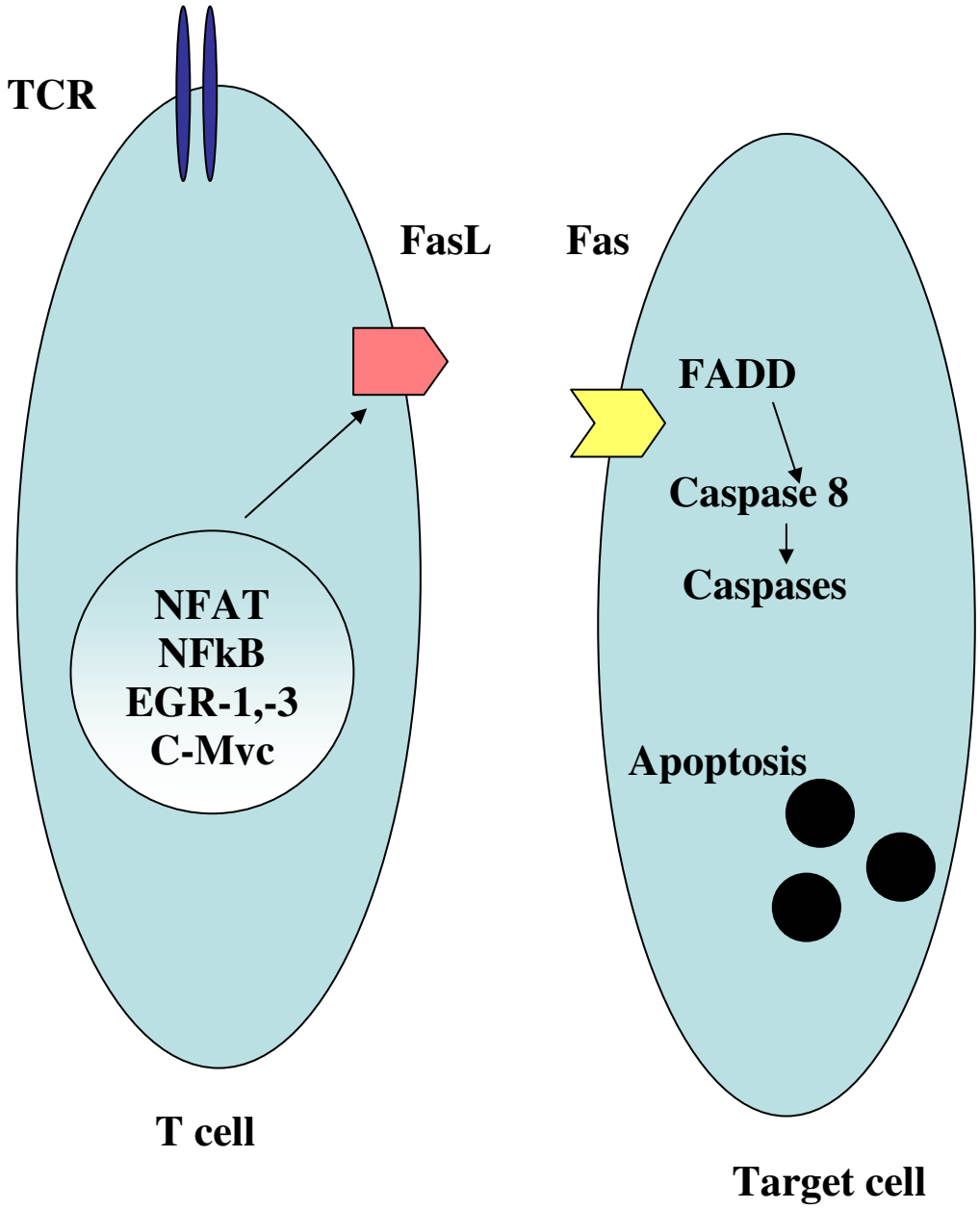
Table 1

Disease	Inheritance	Gene/Product	Mechanism	Affected cells
Haemophagocytic Syndrome				
Familial Lymphohistiocytosis	AR	PRF1 Munch-13 Snx11	Defective lymphocytes cytotoxicity	T +NK lymphocytes
Griscelli Disease	AR	Rab27		
Chediack-Higashi disese	AR	CHS/LYST		
X-linked proliferative disese	XL	SH2D1A/SAP		
Autoimmune Lymphoproliferative disease	AD (AR)	TNFRSF6/Fas Casp10/Caspase 10	Defective lymphocytes apoptosis	T+B lymphocytes
APECED	AR	AIRE	Faulty T cell central tolerance	T lymphocytes
Ipex	XL	FOXP3	Defective regulatory cells	T lymphocytes
Immunodeficiency with lymphoid infiltration	AR	IL2RA	Defective regulatory T cells activity ?	T lymphocytes

Non secretory pathway

In particular, the non secretory pathway functionally specialized membrane receptors. These receptors, called death receptors, belong to TNF-receptor (TNF-R)/NGF-receptor (NGF-R) superfamily which contain cysteine-rich domains (CRDs) in the extracytoplasmic region. The extracellular portion of these proteins is important for ligand binding. Furthermore, these proteins are characterized by the presence of a functional domain named "death domain" (DD) in the cytoplasmic region, that binds the cytoplamic signaling proteins essential to induce apoptosis (Kischkel F.C. et al 1995). Fas, also known as CD95 or Apo-1 or TNFRSF6 belongs to this family of proteins and is the most efficient inducer of apoptosis in the lymphocytes. The molecule consists of 3 extracellular CRDs and of 80 amino acid residues in the intracellular DD. Fas ligand (FasL), molecule that belong to the TNF superfamily of the type II transmembrane proteins activate this receptor. The intracellular and extracellular domains of FasL are located in the N- and C-terminal regions, respectively. The receptor-binding domain is located at the very end of the C-terminus, and deletion of at least three amino acids from this region is sufficient to interfere with interactions with its receptor, Fas (Kavurma M.M. et al 2003). After ligand binding, three molecules of Fas assemble into complexes. Fas signaling occurs through the interaction of Fas with the Fas Associated Death Domain (FADD), a cellular adaptor and, subsequently, with procaspases 8 and/or 10 in a death inducing signaling complex (DISC) (Rieux-Laucat F. et al 2003). (Figure 1)

Figure 1: Non-secretory pathway



Secretory pathway

PRF1 is present in granules of CTLs and NK cells. Cytotoxic granules gather at site of contact between the CTL and the target cell, known as the immunological synapse. The granule membrane fuses with the cell membrane and releases the contents of the granules in a process referred to as exocytosis. This results in rapid death of the target cell. This cell death mechanism also involves the secretion and a series of serine proteases called granzymes. Secreted PRF1 inserts into the lipid bilayer thorough specific receptor, while the granzymes are potent activator of the caspases. In fact they induce the cleavage of the caspases, including the caspase 3 (Darmon A. J. et al 1995). The most recent model shows that the adhesion of the cytotoxic T lymphocytes to the target cell, via the interaction between the TCR and the antigen-MHC complex, triggers a Ca^2+dependent degranulation process in the effector cells. The degranulation process, causes the release of PRF1, which recognizes the mannose-6-phosphate receptor (MPR), thus leading to a macromolecular complex, along granzymes. Eventually, the macromolecular complex results in the target cell lysis. (Figure 2)

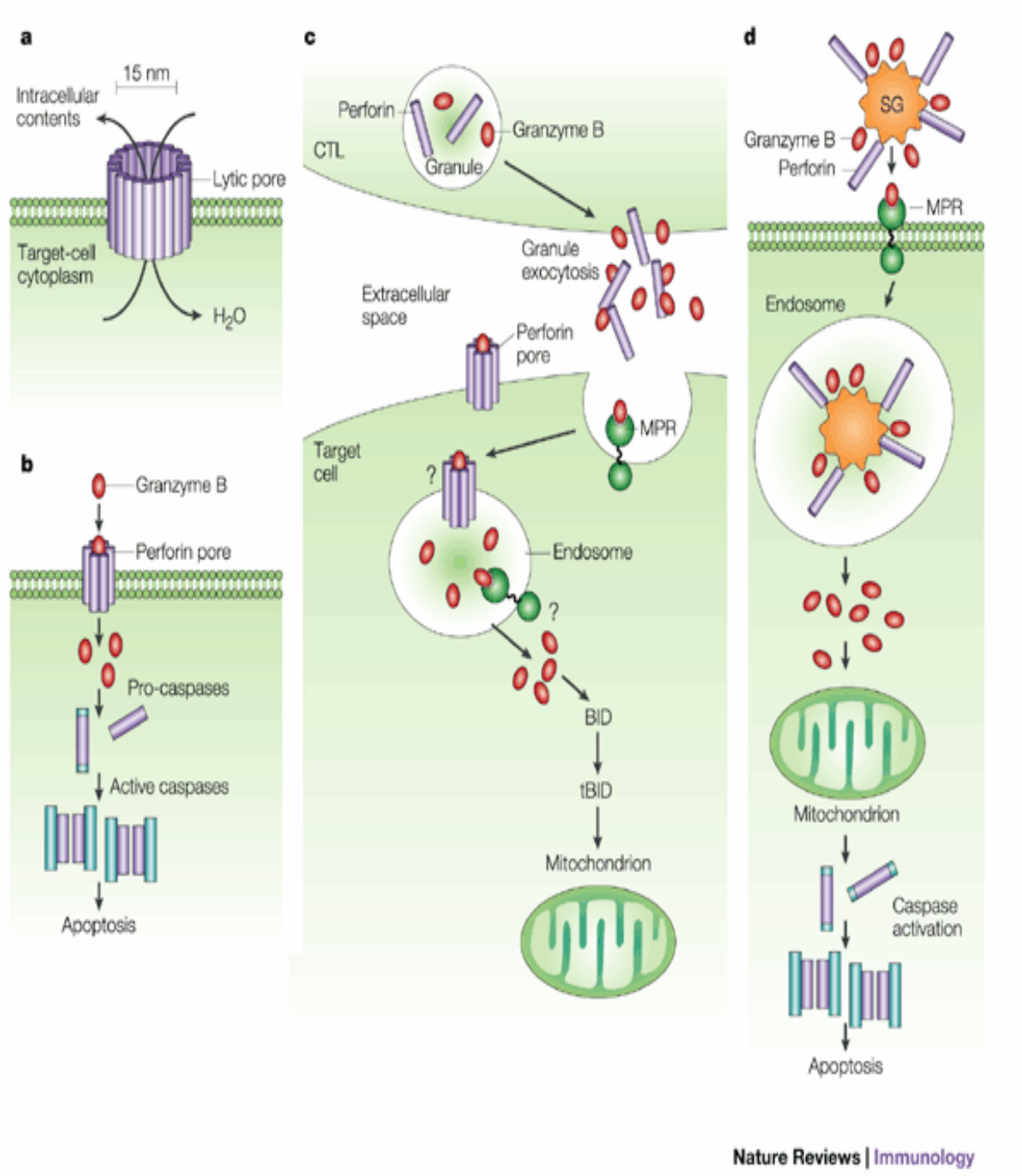
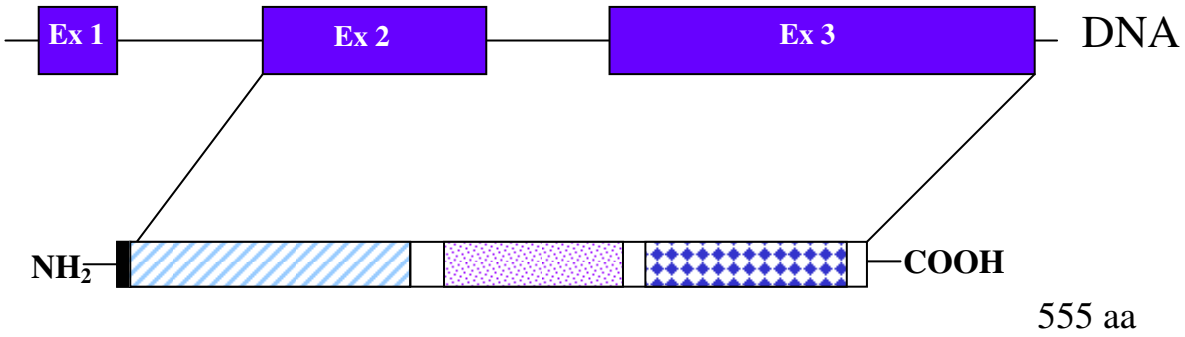

a
15 nm
Intracellular contents
Lytic pore
Target-cell cytoplasm
H_2O
b
Granzyme B
Perforin pore
Pro-caspases
Active caspases
Apoptosis
c
Perforin
Granzyme B
CTL
Granule
Granule exocytosis
Extracellular space
Perforin pore
MPR
Target cell
?
Endosome
?
BID
tBID
Mitochondrion
d
SG
Granzyme B
Perforin
MPR
Endosome
Mitochondrion
Caspase activation
Apoptosis
Nature Reviews | Immunology

Figure 2. Evolving models of CTL/NK-secretory pathway. A The initial model, which was developed in the early 1980s when perforin was first purified, emphasized the role of perforin as lytic molecule. The target cell died because of loss of plasma membrane homeostasis, with excessive uptake of water and loss of intracellular contents. **B** With the realization that granzymes are involved in the inducing cell death cooperatively with perforin,and that many cells die by apoptosis, the lytic model was adapted to accommodate the passive diffusion of granzymes into the target cell cytosol, where they could access key substrate (caspase), leading to death. **C** The next main findings to be accommodated in the model during the mid to late 1990s were that: granzymes enter target cells by endocytosis

Genetics of secretory pathway

The perforin gene (PRF1) consists of three exons (Figure 3). The coding sequence is located in the exon 2 and 3. PRF1 is 555 amino acid protein that has a 21 amino terminal signal sequence, an approximately 300 amino acid region that shares homology to the C9 complement protein, **known as Membrane Attack Complex (MAC) domain**. Moreover there are a 36 amino acid epidermal growth factor-like domain and a 132 amino acid domains homologous to the C2 domain of protein kinase C (Licthened 1988). The protein contains two N-linked glycosylation sites. PRF1 is synthesized as an inactive form, which must be cleaved at its carboxy terminus, releasing approximately 20 carboxyterminal amino acids to yield the active form of the protein (Uellner R. et al 1997).

Figure 3. perforin gene and protein structure



Signal peptide

MAC domain (28 aa-349 aa)

C2 domain(417aa-497aa)

EGF2 like domain

Mouse model

Perforin deficient mice remain generally healthy if maintained in a clean facility, but about half of the animals develop spontaneous B-cell lymphoma as they age (almost invariably beyond one year of age). Mice that are infected experimentally with lymphocytic choriomeningitis virus have a remarkably similar disease course to FHLH patients, with absent NK-cell response and anti-viral cytotoxic T-lymphocytes activity, hepotosplenomegaly and elevation of circulating cytokine levels in the setting of uncontrolled viaremia.

Perforin, immunoregulation and autoimmunity

Perforin-deficient mice have essentially normal immune homeostasis. For example, perforin deficiency impairs neither the rejection of grafted lymphocytes (Barchet W et al 2000) nor the in vivo homeostasis of DCs (Ludewig B. et al 2001). However, considerable evidence indicates a pivotal role for perforin in immunoregulation in situation of immune-system disturbance caused by microbial infection, autoimmunity or loss of other cell death pathways. For example, as in perforin-deficient *gld* (FasL defective) mice (Spielman J. et al 1998), several studies have shown recently that perforin regulates the elimination of CD8+T cells after an acute exposure to foreign antigen. Notably, perforin deficient mice have an increased clonal expansion and persistence of superantigen and virus specific T cells that could not be reproduced by inibhiting the elimination of antingen presenting cells perforin sufficient mice. (Kagi D. et al 1999) These findings and the increased clonal expansion of alloreactive perforin deficient T cells after their transfer into irradiated scid/scid mice (which lack T and B cells), strongly support a role for perforin in the activation induced cell death process. (Spaner D. 1999) In a GVHD model, perforin had an important regulatory role in the prevention of humoral autoimmunity thorouh the elimination of both autoreactive B cells and antigen specific T cells (Shustov A. 2000). Following on from previously studies by Binder et al., Maltoubian et al showed that perforin is involved in the down-regulation of T-cell response during chronic LCMV infection**.** (Binder D et al 1998, Matloubian M. et al 1999). All together, these studies support the notion of an intrinsic role of perforin in regulating the expansion, and than the contraction, of CD8+ T cell populations after infection. In summary, the data support the existence of a perforin dependent mechanism that regulate the extent of CD8+ cell clonal expansion in models as diverse as acute bacterial infections, GVHD and DC immunization. However, this regulatory process might depend on the nature of the stimulus, including pathogen virulence or strength of stimulation, and might be influenced by other genetic factors.

References

1. Russell, J.H. e Ley T.J. (2002) Lymphocyte mediated cytotoxicity. *Annual Reviews of Immunology*, 20, 323-370.
2. Kagi D., Ledermann B.., Burki K., Zinkernagel R.M., Hengartner H. (1996) Molecular mechanism of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. *Annual Reviews of Immunology*.14,207-232
3. Darmon, A.J. Nicholson D.W e Bleackley R.C. (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell derived granzyme B. *Nature,* 377, 446-448
4. Kavurma M.M and Khachigian (2003) Signaling and transcriptional control of Fas ligand gene expression. *Cell and death Differentiation,* 10, 36-34
5. Kischel F. C., Hellbardt S., Behrmann I., Germer M., Pawlita M., Krammer P.H., Peter M.E. (1995) Cytotoxic dependent APO-1 (Fas/CD95) associated form a death inducing signaling complex with receptor. *Embo Journal,* 14 (22) 5579-88
6. Barchet W., Oehen S., Klenerman P., Wodarz D., Bocharov G., Lloyd A.L., Nowak M. A., Hengartner H., Zinkernagel R.M., Ehl S. Direct quantization of rapid elimination of viral antigen-positive lymphocytes by antiviral CD8(+) T cells in vivo. *Eur J Immunol*. 2000 May;30(5):1356-63.
7. Ludewig B., Bonilla W.V., Dumerese T., Odermatt B., Zinkernagel R.M:, Hengarther H. (2001) Perforin-independent regulation of dendritic cell homeostasis by CD8+ T cells in vivo implication for adaptative immunotherapy. *Eur J Immunol* 31(6):1772-9.
8. Shustov A, Luzina I, Nguyen P, Papadimitriou JC, Handwerger B, Elkon KB, Via CS. (2000) Role of perforin in controlling B-cell hyperactivity and humoral autoimmunity *J Clin Invest*; 106(6):R39-47.

9. Binder D, van den Broek MF, Kagi D, Bluethmann H, Fehr J, Hengartner H, Zinkernagel RM. (1998) Aplastic anemia rescued by exhaustion of cytokine-secreting CD8+ T cells in persistent infection with lymphocytic choriomeningitis virus. *J Exp Med.* 1;187(11):1903-20.

10. Matloubian M, Suresh M, Glass A, Galvan M, Chow K, Whitmire JK, Walsh CM, Clark WR, Ahmed R. (1999) A role for perforin in downregulating T-cell responses during chronic viral infection. *J Virol.* 73(3):2527-36.

11. Uellner R, Zvelebil MJ, Hopkins J, Jones J, MacDougall LK, Morgan BP, Podack E, Waterfield MD, Griffiths GM. Perforin is activated by a proteolytic cleavage during biosynthesis which reveals a phospholipid-binding C2 domain. *EMBO J.* 1997 Dec 15;16(24):7287-96.

12. Rieux-Laucat F, Fischer A, Deist FL. (2003) Cell-death signaling and human disease. *Curr Opin Immunol*. 15(3):325-31. Review.

13. Spaner D, Raju K, Rabinovich B, Miller RG. (1999) A role for perforin in activation-induced T cell death in vivo: increased expansion of allogeneic perforin-deficient T cells in SCID mice. *J Immunol*. 15;162(2):1192-9.

14. Spielman J, Lee RK, Podack ER. Perforin/Fas-ligand double deficiency is associated with macrophage expansion and severe pancreatitis. *J Immunol*. 1998 Dec 15;161(12):7063-70.

Haemophagocytic Lymphohistiocytosis

The Haemophagocytic Lymphohistiocytosis (HLH) (OMIM#26770) is a rare, congenital immune deficiency that occurs in the infants and young children. The disease usually presents with unexplained fever, malaise and failure to thrive, in the context of marked hepatomegaly associated with pancytopenia, htpertrigligeridemia and hypofibrinogenemia**.** Histologically, infiltration of lymphocytes and histiocytes with haemophagocytic activity are seen in the reticuloendothelial system, bone marrow and some times in the central nervous system (Loy TS et al 1991, Janka GE, et al 1983, Henter J et al 1991). Serum levels of inflammatory cytokines derived from activated T cell and macrophages (such as IFN-γ, TNFa, IL-1 and IL-6) are markedly elevated. (Table II)

Clinical and Immunological features of patients with HLH
Hepatosplenomegaly
Fever
Lyphoadenomegaly
Edema
Neutropenia
Hypertrigliceridemia
Hyperferritinemia
Hypoalbuminemia
Hypofibrinogenemia
NK cell defect
Elevated levels IFN-γ, TNF, IL-1 and IL-6

The presentation might be preceded by overt viral infection, but there is no direct relationship with any specific pathogen. The most consistent immunologic abnormality reported in these patients has been the impairment of cytotoxic function mediated by PRF1/granzyme. It has been observed that most patients with HLH have normal numbers of B lymphocytes and normal immunoglobulin levels and a normal absolute lymphocyte counts (Egeler RM 1996). On the basis of numerous genetic studies, it has been shown that PRF1 is mutated in approximately 30% of the cases of the autosomal recessive form (Aricò M. et al 1996; Stepp SE et al 1999). More than 20 mutations have been identified in worldwide patients (Ericson K et al 2001; Clementi R et al 2001; Suga N et al 2002; Kogawa K et al 2002; Feldmann J et al 2002; McCormick J et al 2003). So far, most of the variants studied have been associated with either non detectable or extremely low levels of PRF1 expression in NK cells. These studies led to an important issue concerning on the correlation between genotype and phenotype. Although several reports greatly contributed to the understanding of the clinical features and the pathophysiology of the disease, (Henter JI et al 1998; Fujiwara F et al 1993; Janka GE 1983; Henter JI 1991; Imashuku S et al 1996) several aspects still need to be clarified. In particular, it remains to be defined which are the functional and the biochemical abnormalities in the asymptomatic phase, and what are the roles, if any, of different viruses in triggering the overt phase of the disease.

References

1. Loy TS, Diaz-Arias AA, Perry MC. (1991) Familial erythrophagocytic lymphohistiocytosis. Semin Oncol. 18(1):34-8. Review.
2. Anka GE. (1983) Familial hemophagocytic lymphohistiocytosis *Eur J Pediatr.* 1983 140(3):221-30. Review.
3. Henter JI, Elinder G, Ost A. (1991) Diagnostic guidelines for hemophagocytic lymphohistiocytosis. The FHL Study Group of the Histiocyte Society. *Semin Oncol*. 18(1):29-33.
4. Egeler RM, Shapiro R, Loechelt B, Filipovich A. Characteristic immune abnormalities in hemophagocytic lymphohistiocytosis. J Pediatr Hematol Oncol. 1996 Nov;18(4):340-5.
5. Arico M, Janka G, Fischer A, et al. Hemophagocytic lymphohistiocytosis: report of 122 children from the International Registry. FHL Study Group of the Histiocyte Society. Leukemia. 1996;10:197-
6. Caballes RL, Caballes-Ponce MG, Kim DU. (1997) Familial hemophagocytic lymphohistiocytosis (FHLH). *Pathology*. 29(1):92-5.
7. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science1999;286:1957–1959.
8. Ericson K, Fadeel B, Nilsson-Ardnor S, et al. Spectrum of perforin gene mutations in familial hemophagocytic lymphohistiocytosis.Am J Hum Genet 2001;68:590–597.
9. Clementi R, Stadt U, Savoldi G, et al. Six novel mutations in the PRF1 gene in children with haemophagocytic lymphohistiocytosis. J Med Genet 2001;38:634–646.
10. Suga N, Takada H, Nomura A, et al. Perforin defects of primary haemophagocytic lymphohistiocytosis in Japan. Br J Haematol 2002; 116:346–349.

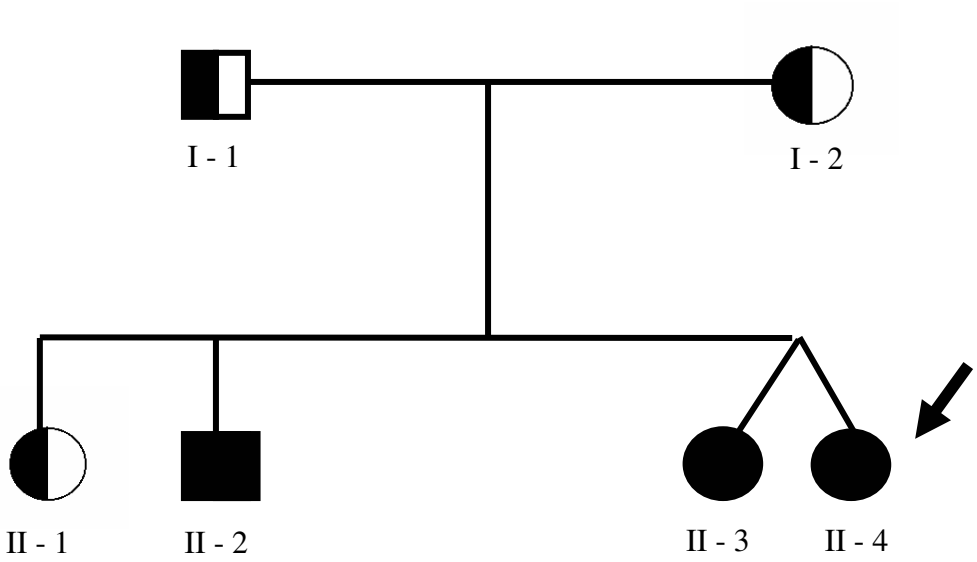
11. Kogawa K, Lee SM, Villanueva J,Marmer D, Sumegi J, Filipovich AH. Perforin expression in cytotoxic lymphocytes from patients withhemophagocytic lymphohistiocytosis and their family members. Blood 2002;99:61–66.
12. Feldmann J, Le Deist F, Ouachee-Chardin M, et al. Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. Br J Haematol 2002; 117:965–972.
13. McCormick J, Flower DR, Strobel S, Wallace DL, Beverley PC, Tchilian EZ. Novel perforin mutation in a patient with hemophagocytic lymphohistiocytosis and CD45 abnormal splicing. Am J Med Genet 2003;117A:255–260.
14. Henter JI, Arico M, Elinder G, Imashuku S, Janka G. Familial hemophagocytic lymphohistiocytosis: primary hemophagocytic lymphohistiocytosis. Hematol Oncol Clin North Am. 1998;12:417-433.
15. Fujiwara F, Hibi S, Imashuku S. Hypercytokinemia in hemophagocytic syndrome. Am J Pediatr Hematol Oncol. 1993;15:92-98.
16. Janka GE. Familial hemophagocytic lymphohistiocytosis. Eur J Pediatr. 1983;140:221-230.
17. Henter JI, Elinder G, Soder O, Hansson M, Andersson B, Andersson U. Hypercytokinemia in familial hemophagocytic lymphohistiocytosis. Blood. 1991;78:2918-2922.
18. Imashuku S, Hibi S, Fujiwara F, Todo S. Hyperinterleukin (IL)-6-naemia in haemophagocytic lymphohistiocytosis. Br J Haematol. 1996;93:803-807

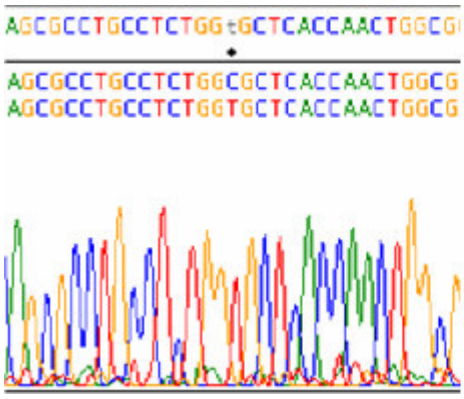
Comparison of molecular, clinical, and biochemical features in a family affected by HLH

During the first year of the activity our interest has been focused on the study of a family of six members in which one of these was affected by an overt HLH. In particular, first aim of our study was to identify any predictive clinical signs of the overt disease, to evaluate and compare the biochemical, functional and molecular features of all family members including still unaffected subjects with same genotype.

The proband presented at the age of 13 years with persistent fever, hepatosplenomegaly, cytopenia, and lymph node enlargement. Biochemical investigation revealed hypertiglyceridemia, hypofibrinogenemia and hyperferritinemia. Signs of hemophagocytosis on a liver biopsy specimen led to a temptative diagnosis of HLH. Very interesting, before the overt manifestation of disease the proband successfully cleared from a few viral infections, such as *Herpes Simplex* virus I (*HSV I*), *Rubella* virus and *Morbilli* Virus. The molecular analysis of the *PRF1* gene revealed the presence of 2 mutations. We found a C->T (C272T) homozygous substitution in exon 2 leading to an A91V amino acid change and a G->A (G695A) heterozygous substitution in exon 3 leading to an R231H change. (Figure 4)

The first mutation was already reported in similar patients with HLH (Clementi R et al 2001), while the R231H represents a novel variant. Sequence analysis of all family members revealed the presence of the A91V at the heterozygous status in both parents, in old sister and brothers. Interestingly, her asymptomatic fraternal twin sister had the mutation at homozygous status the sister whit the overt disease as depicted in figure 5 in as showed in the figure 4. The R231HG was found at the heterozygous status in the father and in healthy twin.


I - 1
I - 2
II - 1
II - 2
II - 3
II - 4



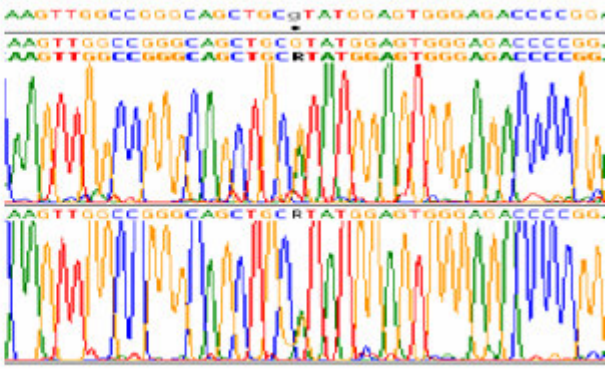
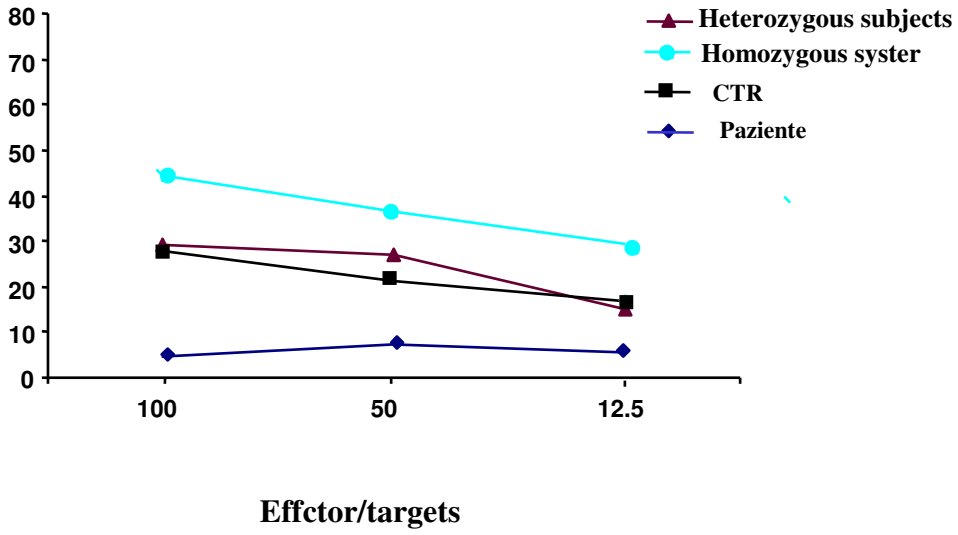


Figure 4. Family pedigree and PRF1 sequence analysis.

Pedigree of the family members indicating the subjects carrying the Ala91Val mutation of PRF1 gene at the homozygous (closed circles) or at the heterozygous (half circles) status. The proband is indicated by the arrow. The PRF1 sequence analysis revealed the presence of C3545T (causing the Ala91Val missense mutation) at the homozygous status in the proband and her twin sister and at the heterozygous status in the other family members. Furthermore, an additional G5150A mutation (causing the Arg231His missense mutation) at the heterozygous status was found in the proband and in the father.

Our subsequent step was to evaluate and compare in all family members the function of cytotoxicity through a cytotlytic assay. NK activity was normal in all family members, including the asymptomatic twin carrying the same genetic alteration of the proband (Figure 5).



Finally the laboratory findings showed that all family members are healthy and the clinical history were negative (Table II).

Table II. Clinical and laboratory features

	Proband	Tween sister	Brother	Sister	Father	Mother
Hepatosplenomegaly	+	-	-	-	-	-
Fever	+	-	-	-	-	-
Skin rash	-	-	-	-	-	-
Lyphoadenomegaly	+	-	-	-	-	-
Oedema	+	-	-	-	-	-
CNS Involvement	-	-	-	-	-	-
Platelets (x1000/mm3)	39	281	302	214	211	245
Hemoglobin (g/dl)	4.1	12.1	11.4	14.5	15.7	12.2
Neutropenia	-	-	-	-	-	-
Hypertriglyceridemia	+	-	-	-	-	-
Hypofibrinogenemia	+	-	-	-	-	-
Hyperferritinemia	+	-	-	-	-	-
Hypoalbuminemia	+	-	+	+	+	-
Hypertransaminasemia	+	-	-	-	-	-
Hyponatremia	-	-	-	-	-	-
Associated Infections	EBV,CMV, HSI,II	EBV, HSI,II	EBV, HSI,II	EBV, HSI,II	EBV, HSI,II	EBV, HSI,II

HS = Herpes Symplex virus

Conclusive remarks

In this first preliminary study we identified two mutations one of this the A91V occurs in a most important domain, the membrane attack complex that is needed for the binding of the molecule to the membrane of the cells. Remarkably, the same mutation (A91V and R231H) was found in the two twins, only in one case it being associated with delayed clinical presentation. Moreover, the comparison of the clinical history of these subjects revealed that they share several similarities with regard to the encountered viral agents. In fact, the healthy sister had a normal clinical course during several infections, including an *Epstein Barr* virus (EBV) infection, which usually considered a triggering factor (Kogawa K et al 2002). So the first conclusion is that virus-derived products are not sufficient *per se* to determine the accelerated phase of the disease. Furthermore, the functional studies in 2 twin sisters have shown that NK activity was normal in the healthy sister and severely impaired in the proband. Emerging evidence has indicates that a reduction of NK activity in some heterozygous subjects for this mutation supports the hypothesis of a partial dominant-negative effect of few mutations. Moreover, in a few patients, NK activity was initially normal and subsequently decreased, thus implying that additional genetic factors may interfere with the immune function. The molecular mechanism that underlies the different clinical and functional behavior of the disease in the 2 subjects here described remains to be clarified. It is possible that further genetic alterations may interfere in the phenotypic expression of the disease and moreover, we cannot exclude with certainty that the fraternal twin could develop the disease in the future. These results have been published on the Blood journal (see below the attached paper)

IMMUNOBIOLOGY

Brief report

Atypical features of familial hemophagocytic lymphohistiocytosis

Rosanna Busiello, Marsilio Adriani, Franco Locatelli, Mario Galgani, Giorgia Firriani, Rita Clementi, Matilde Valeria Ursini, Luigi Racioppi, and Claudio Pignata

Familial hemophagocytic lymphohistiocytosis (FHLH) is a rare, rapidly progressive disorder of early childhood characterized by uncontrolled activation of T cells and macrophages. Although perforin gene mutations have been described in a proportion of patients with FHLH, the genotype/phenotype correlation is still limited. Only a few patients with late onset clinical manifestations have been reported. The biochemical and immunologic alterations in the asymptomatic phase are not well known. We report on a family in which 2 fraternal twins both homozygous for a perforin mutation previously described as causative of the disease, markedly differed in phenotypic expression of FHLH. The twins also had a second novel heterozygous mutation. Natural killer (NK) activity was severely impaired in the patient and was normal in the asymptomatic fraternal twin. Our report highlights that FHLH may present after a long disease-free interval during which biochemical or immunologic alterations may be not evident, thus implying a role for interfering factors. (Blood. 2004;103:4810-4812)

© 2004 by The American Society of Hematology

Introduction

Familial hemophagocytic lymphohistiocytosis (FHLH) is a rare autosomal recessive disorder of the immune system whose clinical symptoms arise as a consequence of uncontrolled activation of T cells and macrophages and overproduction of inflammatory cytokines.[1] Indeed, all the clinical and biochemical signs of the disease reflect a potent inflammatory reaction triggered by still-unidentified circumstances and, presumably, infections.[2,3] In keeping with the clinical pattern of fever, hepatosplenomegaly, and pancytopenia, serum inflammatory cytokines, such as interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), and IL-6 are markedly elevated.[4,5] Massive lymphocyte and macrophage activation, leading to hemophagocytosis, are the prominent pathologic findings.[1,3] Most patients manifest symptoms of disease within the first months of life,[6] although a few cases of late onset have been reported.[7] Low natural killer (NK) and cellular cytolytic activities are generally considered a functional marker of the disease. Mutations of the perforin (*PRF1*) gene, implicated in the osmotic lysis of the target cells, have been described in some patients with FHLH.[8-12]

Although several reports greatly contributed to the understanding of the clinical features and the pathophysiology of the disease,[1-5] several aspects still need to be clarified. In particular, it remains to be defined which are the functional and the biochemical abnormalities in the asymptomatic phase, and what are the roles, if any, of different viruses in triggering the overt phase of disease. Furthermore, a clear correlation between genotype and phenotype is still lacking.

In this report, we describe a family of 6 members with 2 fraternal twins carrying alterations of the perforin gene profoundly differing in the clinical phenotype. The first subject developed a rapidly progressive disease after a disease-free interval of many years, while the second is still asymptomatic. The biochemical, immunologic, and genetic features in these 2 subjects were compared.

Study design

We investigated 6 family members. The proband presented at age 13 years with persistent fever, hepatosplenomegaly, cytopenia, and lymph node enlargement. Low white and red blood cell counts were recorded. Thereafter, moderate grade fever, jaundice, and cholestasis developed. Biochemical investigations revealed hypertriglyceridemia, hypofibrinogenemia, hypoalbuminemia, and hyperferritinemia. Signs of hemophagocytosis on a liver biopsy specimen led to a tentative diagnosis of FHLH. The patient died soon after the diagnosis because of the rapidly progressive disease. Before the overt manifestation of the disease, the patient successfully cleared a few viral infections, such as herpes simplex virus 1 (HSV 1), Rubella virus, and Morbilli virus. At the age of 11 years, she was affected with an Epstein Barr virus (EBV) infection, which was apparently self-limiting. All the remaining family members are healthy, their clinical histories are negative, and laboratory data were normal as detailed in Table 1.

Perforin mutation analysis

Genomic DNA was isolated from peripheral blood lymphocytes and the exons 2 and 3 of the *PRF1* coding region amplified using standard polymerase chain reaction (PCR) conditions. PCR products were recovered from 1.5% agarose with a size marker, purified using the QIAquick Gel extraction kit (Qiagen, Hilden, Germany) and sequenced in an automatic ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Cytotoxic assay

Infected target cells (K562) were pulsed for one hour at 37°C with Cr^{51} (30 μCi [1.11 MBq]/10^6 cells; Amersham Pharmacia, Buckinghamshire,

From the Departments of Pediatrics, Cellular and Molecular Biology and Pathology, Federico II University and the International Institute of Genetics and Biophysics, Naples, Italy; and Pediatric Hematology/Oncology, IRCCS Policlinico San Matteo, Pavia, Italy.

Submitted October 17, 2003; accepted January 4, 2004. Prepublished online as *Blood* First Edition Paper, January 22, 2004; DOI 10.1182/blood-2003-10-3551.

Supported by grants "Ministero della Salute—Roma and Regione Campania, Legge 502" and Ministero Ricerca Scientifica—Progetto Rilevante Interesse Nazionale (PRIN) 2002 (no. 200 206 4693_003).

Reprints: Claudio Pignata, Department of Pediatrics, Unit of Immunology, "Federico II" University, via S Pansini 5-80131, Naples, Italy; e-mail: pignata@unina.it.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2004 by The American Society of Hematology

Table 1. Clinical and laboratory features

	Proband	Twin sister	Brother	Sister	Father	Mother
Hepatosplenomegaly	+	–	–	–	–	–
Fever	+	–	–	–	–	–
Skin rash	–	–	–	–	–	–
Lymphadenomegaly	+	–	–	–	–	–
Edema	+	–	–	–	–	–
CNS involvement	–	–	–	–	–	–
Platelets (×1000/mm³)	39.0	281.0	362.0	214.0	211.0	245.0
Hemoglobin (g/dL)	4.1	12.1	11.4	14.5	15.7	12.2
Neutropenia	–	–	–	–	–	–
Hypertriglyceridemia	+	–	–	–	–	–
Hypofibrinogenemia	+	–	–	–	–	–
Hyperferritinemia	+	–	–	–	–	–
Hypoalbuminemia	+	–	+	+	+	–
Hypertransaminasemia	+	–	–	–	–	–
Hyponatremia	–	–	–	–	–	–
Associated infections	EBV,CMV,HSU1	EBV,HSU1	EBV,HSU1	EBV,HSU1	EBV,HSU1	EBV,HSU1

England) and washed 3 times with culture medium before the addition of effector cells. Specific lysis was measured in a triplicate assay performed with 5×10^3 target cells mixed with effector cells at different ratios (E/T = 25:1 to 3.125:1) in a total volume of 200 μL. The percentage of specific lysis was calculated as follows: 100 × (specific release − spontaneous release) /(total release − spontaneous release).

Results and discussion

The pedigree of the family is shown in Figure 1. Sequence analysis of the perforin gene revealed the presence of 2 mutations in the proband. We found a C→T (C272T) homozygous substitution in exon 2 leading to an A91V amino acid change and a G→A (G695A) heterozygous substitution in exon 3 leading to an R231H change. The A91V mutation has been already reported in some patients with FHLH,[7] while the R231H mutation represents a novel mutation. Sequence analysis of the family members revealed the presence of the A91V mutation at the heterozygous status in both parents, in the older sister and brother, and at the homozygous status in the fraternal twin sister. The R231H was found at the heterozygous status in the father and in the healthy twin. NK activity was normal in all family members, including the asymptomatic fraternal twin carrying the same genetic alterations of the proband (18%-30% of specific Cr^{51} release at the different E/T ratios) and markedly reduced in the patient, ranging between 2% and 5% of specific Cr^{51} release, in spite of a normal number of NK cells. This case report is interesting for several aspects. First, in the proband the overt clinical manifestations occurred after a disease-free interval longer than usually observed, as already reported in rare cases of adult presentation of HLH.[7] The reason why some patients remain asymptomatic until adolescence or even adulthood remains unknown. The A91V *PRF1* mutation occurs in the membrane attack complex extracellular domain of perforin, thus presumably modifying its binding to the target cell, required for pore formation. Notably, this mutation was frequently detected in association with FHLH in affected families from southern Italy.[7] Thus far, nonsense, missense, and point mutations have been described in several domains but no correlation was found between PRF1 genotype and clinical manifestations.[13] Therefore, it is unlikely that this particular mutation is associated with delayed clinical presentation. Remarkably, the same mutations were found in the 2 twins, only in one case being associated with a rapidly progressive and fatal clinical phenotype. The comparison of the clinical history of these subjects revealed that they share several similarities with regard to the encountered viral agents. The healthy sister had a normal clinical course during several infections, including an EBV infection, which is usually considered a triggering factor.[14] This would suggest that virus-derived products are not sufficient per se to determine the accelerated phase of the disease. The functional studies in the 2 sisters revealed that NK activity was absent in the patient and normal in the healthy sibling with the same mutations. Previously, a reduction of the activity has been documented in heterozygous subjects, suggesting that a few mutations may exert a partial dominant-negative effect.[15] In a large series of patients with HLH, NK activity was clearly impaired only in patients with central nervous system disease and normal in patients without brain involvement.[16] Moreover, in a few cases NK activity was initially normal and subsequently decreased, thus implying that additional genetic or environmental factors may interfere with the immune function. The molecular mechanism underlying the different functional and clinical behavior of the disease in the 2 subjects here described remains unknown. It is possible that further genetic alterations may interfere in the phenotypic expression of the disease. Also, we cannot exclude with certainty that the fraternal twin could develop the disease in the future.

Our observation would also imply that in the overall approach to these patients sequence data should be evaluated with caution in the clinical decision-making process.

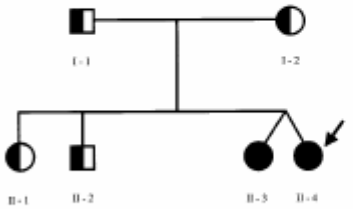


Figure 1. Family pedigree. Pedigree of the family members indicating the subjects carrying the C272T mutation of PRF1 gene at homozygous (closed circles) or at heterozygous (half circles) status. The proband is indicated by the arrow. The PRF1 sequence analysis revealed the presence of C272T (causing the A91V missense mutation) at the homozygous status in the proband and her fraternal twin and at the heterozygous status in the other family members. Furthermore, an additional G695A mutation (causing the R231H missense mutation) at the heterozygous status was found in the proband, in the healthy twin sister, and in the father.

References

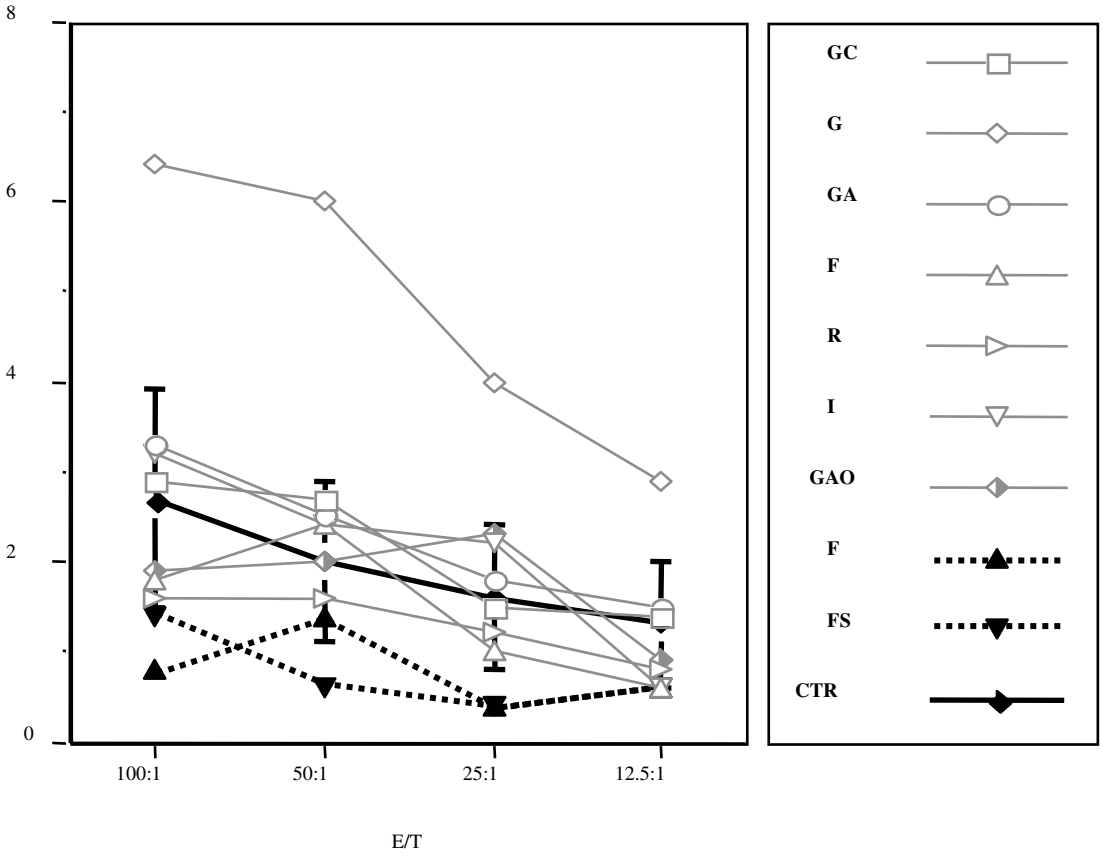
1. Henter JI, Arico M, Elinder G, Imashuku S, Janka G. Familial hemophagocytic lymphohistiocytosis: primary hemophagocytic lymphohistiocytosis. Hematol Oncol Clin North Am. 1998;12:417-433.
2. Fujiwara F, Hibi S, Imashuku S. Hypercytokinemia in hemophagocytic syndrome. Am J Pediatr Hematol Oncol. 1993;15:92-98.
3. Janka GE. Familial hemophagocytic lymphohistiocytosis. Eur J Pediatr. 1983;140:221-230.
4. Henter JI, Elinder G, Soder O, Hansson M, Andersson B, Andersson U. Hypercytokinemia in familial hemophagocytic lymphohistiocytosis. Blood. 1991;78:2918-2922.
5. Imashuku S, Hibi S, Fujiwara F, Todo S. Hyper-interleukin (IL)-6-nemia in haemophagocytic lymphohistiocytosis. Br J Haematol. 1996;93:693-697.
6. Arico M, Janka G, Fischer A, et al. Hemophagocytic lymphohistiocytosis: report of 122 children from the International Registry. FHL Study Group of the Histiocyte Society. Leukemia. 1996;10:197-203.
7. Clementi R, Emmi L, Maccario R, et al. Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying PRF1 mutations. Blood. 2002;100:2268-2267.
8. Dufourcq-Lagelouse R, Jabado N, Le Deist F, et al. Linkage of familial hemophagocytic lymphohistiocytosis to 10q21-22 and evidence for heterogeneity. Am J Hum Genet. 1999;64:172-179.
9. Liu CC, Persechini PM, Young JD. Perforin and lymphocyte-mediated cytolysis. Immunol Rev. 1995;146:145-175.
10. Lowin B, Peitsch MC, Tschopp J. Perforin and granzymes: crucial effector molecules in cytolytic T lymphocyte and natural killer cell-mediated cytotoxicity. Curr Top Microbiol Immunol. 1995;198:1-24.
11. Darmon AJ, Nicholson DW, Bleackley RC. Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B. Nature. 1995;377:446-448.
12. Darmon AJ, Bleackley RC. Proteases and cell-mediated cytotoxicity. Crit Rev Immunol. 1998;18:255-273.
13. Stinson KG, Fadeel B, Nilsson-Ardnor S, et al. Spectrum of perforin gene mutations in familial hemophagocytic lymphohistiocytosis. Am J Hum Genet. 2001;68:590-597.
14. Kogawa K, Lee SM, Villanueva J, Marmer D, Sumegi J, Filipovich AH. Perforin expression in cytotoxic lymphocytes from patients with hemophagocytic lymphohistiocytosis and their family members. Blood. 2002;99:61-66.
15. Sullivan KE, Delaat CA, Douglas SD, Filipovich AH. Defective natural killer cell function in patients with hemophagocytic lymphohistiocytosis and in first degree relatives. Ped Res. 1999;44:465-468.
16. Imashuku S, Hyakuna N, Funabiki T, et al. Low natural killer activity and central nervous system disease as a high-risk prognostic indicator in young patients with hemophagocytic lymphohistiocytosis. Cancer. 2002;94:3023-3031.

Definition of the pathogenic role of the molecular alterations in PRF1 in patients with Haemophagocytic Lymphohistiocytosis

In the second part of our project we have continued to study the important issue of difficult correlation between genotype/phenotype correlation in HLH disease. In particular our interest has been focused on the A91V and on its discussed pathogenic role in HLH. This mutation has been described either as disease causing mutation by our group and (Clementi R et al 2001) either as not associated to overt disease in some subjects not affected by HLH. Our previously published data, in which the mutation was described in 2 twin sister, one affected by FHLH and one with completely healthy phenotype, led us to hypothesize that along with the A91V mutation other factors may interfere in the clinical expression of the disease. At this phase of the study at the aim to better clarify the role of the A91V mutation in the pathogenesis of HLH we perform a biochemical and functional study of NK activity in subjects carrying the A91V mutation at the heterozygous status among the relatives of affected probands from our geographic area. All research subjects were asymptomatic and cleared normally from common viral infection. Furthermore, all subjects have normal biochemical parameters, including fibrinogen, triglycerides and ferritin values. As shown by Figure 6, all research subjects carrying the A91V alterations at the heterozygous status showed a normal NK activity. These findings have been published on the Blood journal see bellow for the attached paper.

At this state of the project we propose the A91V alteration as a molecular variant that is not per se causative of the disease, and very importantly not sufficient to impair the cytolytic activity.

This study has been published on Blood journal, see below the attached manuscript.


8
6
4
2
0
100:1
50:1
25:1
12.5:1
E/T
GC
G
GA
F
R
I
GAO
F
FS
CTR

References

1. Kogawa K, Lee SM, Villanueva J, Marmer D, Sumegi J, Filipovich AH. (2002) Perforin expression in cytotoxic lymphocytes from patients with hemophagocytic lymphohistiocytosis and their family members. *Blood* 99:61-66.

2. Clementi R, Emmi L, Maccario R, et al. (2002) Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying PRF1 mutations. *Blood* 100:2266-2267.

References

1. Gris JC, Jamin C, Benij a JL, Quere I, Madelenat P, Mares P. APC resistance and third-generation oral contraceptives: acquired resistance to activated protein C, oral contraceptives and the risk of thromboembolic disease. Hum Reprod. 2001;16:3-8.
2. Kemmeren JM, Algra A, Meijers JC, et al. Effect of second- and third-generation oral contraceptives on the protein C system in the absence or presence of the factor V_{Leiden} mutation: a randomized trial. Blood. 2004;103:927-933.
3. Curvers J, Thomassen MC, Rimmer J, et al. Effects of hereditary and acquired risk factors of venous thrombosis on a thrombin generation-based APC resistance test. Thromb Haemost. 2002;88:5-11.
4. Tans G, van Hylckama Vlieg A, Thomassen MC, et al. Activated protein C resistance determined with a thrombin generation-based test predicts for venous thrombosis in men and women. Br J Haematol. 2003;122:465-470.
5. Rosing J, Tans G, Nicolaes GA, et al. Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second- and third-generation oral contraceptives. Br J Haematol. 1997;97:233-238.

To the editor:

A91V is a polymorphism in the perforin gene not causative of an FHLH phenotype

We read with special interest the article of Busiello and colleagues in *Blood*.[1] They described atypical features of familial hemophagocytic lymphohistiocytosis (FHLH) in a patient/family presenting 2 different perforin gene alterations: an already known homozygous A91V and a novel heterozygous R231H exchange. Since the identification of this gene, which is responsible for the disease in a subgroup of patients,[2] several groups have reported on the identification of novel mutations.[3-6] Small deletions and nonsense and missense mutations were described and scattered both coding exons of the gene. In this family, the homozygous A91V and the heterozygous R231H exchange was detectable in both twins, one of them presenting typical signs of hemophagocytic lymphohistiocytosis (HLH) at the age of 11 years with a rapidly fatal course of the disease. The second twin, with the same perforin mutation pattern, had yet no signs of HLH, including a normal natural killer (NK) cell activity. The 272C>T transition was further detectable at the heterozygous level in the remaining healthy family members (father, mother, and 2 sisters). The R231H exchange was found in the father and, as mentioned before, also in the healthy twin. The authors conclude that due to the identical genetic pattern in the second twin, a late onset of the disease may still be possible in this child.

From our studies, we present evidence that the A91V exchange represents a polymorphism in the perforin gene not causative of the HLH phenotype. We analyzed exon 2 in a series of 86 control DNA samples from healthy unrelated Caucasian individuals by denaturing high-performance liquid chromatography (DHPLC) and found a heterozygous 272C>T transition in 15 cases (17.5%). Additionally, Feldmann et al reported on a homozygous A91V in a nonaffected subject.[3] Finally, Molleran Lee et al confirmed the observation of a polymorphism at this nucleotide in the perforin gene by analyzing a large cohort of controls with a heterozygosity of 3% (7 out of 202 investigated cases).[7] In contrast to these data, Clementi et al described a family including 2 brothers with late onset of the disease and a compound heterozygous pattern of mutations in the perforin gene.[8] In parallel to a W374X mutation leading to a premature stop, the heterozygous A91V exchange was found in both twins. NK cell activity and perforin expression were markedly reduced in both patients. Taken together, the A91V transition has been described either as polymorphism (Feldmann et al,[3] Molleran Lee et al,[7] and our own observations) or as disease causing mutation in 2 families including 4 patients with late onset of the disease.[1,8] The frequency of this transition differed between the geographic or ethnic origin of the samples. With the assumption of a pathologic role of A91V and an allelic frequency of about 9% in our healthy population, the incidence of HLH should be much higher than observed in Germany. However, the real disease prevalence is not yet determined exactly because of a possible underestimation of the diagnosis due to atypical phenotypic presentations. A reduced perforin expression may also occur in heterozygous carriers or may be due to additional genetic defects in the regulatory region of the gene (eg, exon 1).

The presence of a noncausative A91V polymorphism described in the paper by Busiello et al is underlined by the fact that the healthy twin, who has genetically the same mutation pattern as his affected sister, has a completely healthy phenotype, including a normal NK cell activity. This supports our findings that the described genotype is not responsible for the onset of the disease. In conclusion, we show very strong evidence that A91V represents a polymorphism rather than a relevant mutation. This should be taken into account for further genetic counseling in affected families.

Udo zur Stadt, Karin Beutel, Belinda Weber, Hartmut Kabisch, Reinhard Schneppenheim, and Gritta Janka

Correspondence: Udo zur Stadt, Department of Pediatric Hematology and Oncology, University Childrens Hospital, Martinistr 52, 20246 Hamburg, Germany; e-mail: zurstadt@uke.uni-hamburg.de

References

1. Busiello R, Adriani M, Locatelli F, et al. Atypical features of familial hemophagocytic lymphohistiocytosis. Blood. Prepublished on January 22, 2004, as DOI 10.1182/blood-2003-10-3551. (Now available as Blood. 2004;103:4610-4612).
2. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science. 1999;286:1957-1959.
3. Feldmann J, Le Deist F, Ouachee-Chardin M, et al. Functional consequences of perforin gene mutations in 22 patients with familial haemophagocytic lymphohistiocytosis. Br J Haematol. 2002;117:965-972.
4. Goransdotter Ericson K, Fadeel B, et al. Spectrum of perforin gene mutations in familial hemophagocytic lymphohistiocytosis. Am J Hum Genet. 2001;68: 590-597.
5. Clementi R, zur Stadt U, Savoldi G, et al. Six novel mutations in the PRF1 gene in children with haemophagocytic lymphohistiocytosis. J Med Genet. 2001;38: 643-646.
6. Suga N, Takada H, Nomura A, et al. Perforin defects of primary haemophagocytic lymphohistiocytosis in Japan. Br J Haematol. 2002;116:346-349.
7. Molleran Lee S, Villanueva J, Sumegi J, et al. Characterisation of diverse PRF1 mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis. J Med Genet. 2004;41: 137-144.
8. Clementi R, Emmi L, Maccario R, et al. Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying PRF1 mutations. Blood. 2002;100:2266-2267.

A91V PRF1 variation in healthy subjects and FHLH patients

Our previously published observation of a normal NK function in 9 subjects with A91V mutation at the heterozygous status seem to be consistent with a non-pathogenic role in the HLH. A summary of literature is in keeping with this observation, this mutation was described, either as a frequent polymorphism, present in 9% of healthy subjects (Feldmann 2002; zur Stadt 2004) or as a possible disease causing mutation in two families of patients with late onset disease (Clementi 2002; Busiello 2004). Additionally, Lee et al observed a 3% (7/202) frequency of this transition in a cohort of controls. Taken together, these observations leave open the discussion on the pathogenic role of the A91V mutation in FHLH. So in the effort to make a conclusive remark to this issue we examine the distribution of this mutation in populations originating from different geographic areas and compare the genetic results. In this phase of study, we evaluated the allelic frequency of A91V mutation in a group of families recruited through the "Centre d'Etude du Polymorphisme Humain" (CEPH) representative of the worldwide population. (http://www.genlink.wustl.edu/cephv8/ceph.html) The frequency was also compared with that observed in FHLH patients in association with PRF1 gene alterations, recruited through the FHLH Italian Registry. A total of 82 DNA samples from 41 unrelated healthy families of the "Centre d'Etude du

Polymorphism Humain" were obtained from the Foundation Jean Dausset, Paris. All these samples were analyzed to estimate the allelic frequency of A91V mutation in the *PRF1* gene. Moreover, molecular information of 21 subjects with FHLH and *PRF1* gene alteration was obtained from the Italian Registry of FHLH. As shown in the Table III, the sequence analysis revealed the presence of the A91V mutation at the heterozygous state in 6 out of 82 subjects belonging to CEPH families (6/164 chromosomes; 3.7%). In the patients affected with FHLH associated with a molecular alteration of *PRF1* gene recruited through the FHLH National Registry, this transition was present in 10 out 21 patients. A91V transition was present always at the heterozygous state, but in an individual patient who carried the mutation at the homozygous state (11/42 chromosomes 26.2%). This frequency was significantly higher than that observed in CEPH families ($p = 0.0002$). The observed frequency in the CEPH families is in keeping with the reported frequency in North American healthy population (Molleran Lee 2004) (3%) and is representative of the worldwide genomic variation and was also in keeping with frequency of 3.9% described in our area, reported by Trambas et al 2005 in (see the table III).

	Patients	Our Area	Italian control	CEPH
A91V frequency	26.2%	3.9%	3.7%	3.7%
A91V variation and other mutations	100%	-		-

Moreover, as illustrated in the Table III, in the patients with FHLH and *PRF1* mutation, the A91V transition is always present in association with another molecular alteration of *PRF1* gene, whereas in the CEPH families the transition is always isolated. This is also in keeping with the observation that none of the reported FHLH patients have the A91V mutation in the absence of any other *PRF1* alteration (Molleran Lee 2004). Taken together, these results support the hypothesis that A91V may represent a frequent molecular alteration, which is not causative "*per se*" of FHLH and not sufficient to abolish cytolytic activity. However, the higher frequency of the A91V substitution in FHLH patients with at least one additional *PRF1* mutation supports a role for this mutation as a strong susceptibility factor, contributing to the pathogenesis of the disease. Whether this effect is mediated by a synergic effect with other *PRF1* alterations, remains to be elucidated. These data has been submitted to International journal of Immunogenetics and are in course of revision see below for the manuscript.

References

1. Busiello, R., Adriani, M., Locatelli, F., Galgani, M., Fimiani, G., Clementi, R., Ursini, M. V., Racioppi, L. & Pignata, C. (2004a) Atypical features of familial hemophagocytic lymphohistiocytosis. *Blood* 103, 4610

2. Busiello, R., Galgani, M., De Fusco, C., Poggi, V., Adriani, M., Racioppi, L. & Pignata, C. (2004b) Role of A91V mutation in perforin gene in haemophagocytic lymphohistiocytosis. *Blood* 104, 1909

3. Clementi, R., Emmi, L., Maccario, R., Liotta, F., Moretta, L., Danesino, C. & Aricò, M. (2002) Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying *PRF1* mutations. *Blood* 100, 2266

4. Clementi, R., Zur-Stadt, U., Savoldi, G., Varotto, S., Conter, V., De Fusco, C., Notarangelo, L. D., Schneider, M., Klersy, C., Janka, G., Danesino, C. & Aricò, M. (2001) Six novel mutations in the PRF1 gene in children with haemophagocytic lymphohistiocytosis. *J Med Genet* 38, 643

5. Feldmann, J., Le Deist, F., Ouachée-Chardin, M., Certain, S., Alexander, S., Quartier, P., Haddad, E., Wulffraat, N., Casanova, J. L., Blanche, S., Fischer, A. & de Saint Basile, G. (2002) Functional consequences of perforin gene mutations in 22 patients with familial heamophagocytic lymphohistiocytosis. *Brit J Haematol* 117, 965

6. Molleran Lee, S., Villanueva, J., Sumegi, J., Zhang, K., Kogawa, K., Davis, J. & Filipovich, A. H. (2004) Characterisation of diverse *PRF1* mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis. *J Med Genet* 41, 137

7. Trambas, C., Gallo, F., Pende, D., Marcenaro, S., Moretta, L., De Fusco, C., Notarangelo, L., Arico, M. & Griffiths, G. (2005) A single amino acid change, A91V, lead to conformational changes which can impair processing to the active form of perforin. *Blood* [Epub ahead of print]

8. zur Stadt, U., Beutel, K., Weber, B., Kabisch, H., Schneppenheim, R. & Janka, G. (2004) Ala91Val is a polymorphism in the perforin gene not causative for a FHLH phenotype. *Blood* 104, 1909

International Journal of Immunogenetics

INTERNATIONAL JOURNAL OF
IMMUNOGENETICS

A91V perforin variation in healthy subjects and FHLH patients

For Review Only

Journal:	*International Journal of Immunogenetics*
Manuscript ID:	draft
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	n/a
Complete List of Authors:	Busiello, Rosanna; "Federico II" University, Pediatrics Fimiani, Giorgia; Institute of Genetics and Biophisics, A. Buzzati-Traverso Miano, Maria Giuseppina; Institute of Genetics and Biophisics, A. Buzzati-Traverso Aricò, Maurizio; , Pediatric Hematology and Oncology, «Ospedale dei Bambini G. Di Cristina» Pignata, Claudio; "Federico II" University, Pediatrics
Keywords:	Perforin, Haemophagocytic Lymphohistiocytosis, CEPH family

powered by ScholarOne
Manuscript Central™

Title: A91V perforin variation in healthy subjects and FHLH patients

Authors: Rosanna Busiello[1*], Giorgia Fimiani[2*], Maria Giuseppina Miano[2], Maurizio Aricò[3], Alessandra Santoro[3], Matilde Valeria Ursini[2] and Claudio Pignata[1]

*** these authors equally contributed**

[1]Department of Pediatrics, "Federico II" University, Naples, Italy, [2]Institute of Genetics and Biophisics, A. Buzzati-Traverso, Naples, Italy and [3]Pediatric Hematology and Oncology, «Ospedale dei Bambini G. Di Cristina», Palermo, Italy.

Running head A91V mutation in controls and FHLH patients

Corresponding Author:

Claudio Pignata, M.D., Ph.D.
Professor of Pediatrics
Department of Pediatrics, Unit of Immunology,
"Federico II" University,
via S. Pansini 5-80131, Naples, Italy
Fax # +39-081-5451278 Phone # +39-081-7464340 E-mail: pignata@unina.it

For Peer Review

Summary

Familial Haemophagocytic Lymphohistiocytosis (FHLH) is a heterogeneous autosomal recessive disorder characterized by hyperactivation of T lymphocytes and macrophages. Perforin (*PRF1*) gene alterations have been documented in 40% of patients with FHLH. Although several mutations have been identified, a clear correlation between the individual molecular alteration and the phenotypic expression of the disease is still unclear. In particular, the role that the A91V substitution plays in the pathogenesis of the disease is still controversial. In the effort to make a conclusive remark to this issue, we here report on the frequency of the A91V mutation in a group of unrelated healthy families obtained from the "Centre d'Etude du Polymorphisme Humain" (CEPH), which are considered representative of the worldwide population. This frequency was compared to that observed in FHLH patients recruited through the Italian National Registry. The frequency in CEPH healthy subjects is 3.7%, thus indicating that the alteration represents a polymorphism. However, the frequency of this alteration in FHLH patients associated with *PRF1* mutation is much higher than that observed in controls (26.2%, $p = 0.0002$), suggesting that the alteration is an important genetic susceptibility factor.

For Peer Review

Introduction

Familial Haemophagocytic Lymphohistiocytosis (FHLH) is a rare, lethal, recessive disorder characterized by fever, hepatosplenomegaly and pancytopenia. Abnormal function of cytotoxic lytic activities of natural killer (NK) cells and T cells have been well documented in these patients. As for the genetic mechanism which underlies FHLH, a number of patients have molecular alterations of the perforin (*PRF1*) gene, which plays a key role in the secretory cytotoxic pathway of apoptotic death. Mutations of the coding sequence of the *PRF1* gene have been reported in approximately 40% of FHLH cases (Ericson *et al.,* 2001; Stepp *et al.,* 1999). Small deletion, non-sense, missense and point mutations have been described in several domains although the mechanism by which the mutation results in FHLH phenotype still needs to be clarified (Clementi *et al.,* 2001; Ericson *et al.,* 2001; Feldmann *et al.,* 2002; Suga *et al.,* 2002; Ueda *et al.,* 2003). Recently, the interest has been focused, in particular, on the pathogenicity of the C272T mutation in the exon 2 of *PRF1*, which leads to A91V amino acid substitution. In particular, this mutation has been described either as a frequent polymorphism, present in 9% of healthy subjects (Feldmann *et al.,* 2002; zur Stadt *et al.,*) or as a possible disease causing mutation in two families of patients with late onset disease (Busiello *et al.,* 2004a; Clementi *et al.,* 2002). Recent evidence indicates that the A91V *PRF1* mutation induces conformational changes and impaired cleavage, thus leading to reduced activity of the molecule (Trambas *et al.,* 2005). Taken together, these observations leave open the discussion on the pathogenic role of the A91V mutation in FHLH.

For Peer Review

In the present study, we evaluated the allelic frequency of A91V mutation in a group of families recruited through the "Centre d'Etude du Polymorphisme Humain" (CEPH) representative of the worldwide population. (http://www.genlink.wustl.edu/cephv8/ceph.html) The frequency was also compared with that observed in FHLH patients in association with perforin gene alterations, recruited through the FHLH National Registry.

For Peer Review

Materials and Methods

Subjects

A total of 82 DNA samples from 41 unrelated healthy families of the "Centre d'Etude du Polymorphism Humain" were obtained from the Foundation Jean Dausset, Paris. All these samples were analyzed to estimate the allelic frequency of A91V mutation in the *PRF1* gene. Moreover, molecular information of 21 subjects with FHLH and *PRF1* gene alteration was obtained from the Italian Registry of FHLH (Arico' *et al.,* 1996).

PRF1 gene sequence

Mutation analysis of the *PRF1* gene was performed by Denaturing High-Performance Liquid Chromatography (DHPLC) and DNA genomic sequencing. The coding exon and exon-intron boundaries of *PRF1* were amplified by PCR reaction. DHPLC analysis was performed using the WAVE DNA fragment analysis system. Purified PCR products were sequenced and the reaction was performed with Perkin Elmer Big Dye Ready Reaction Mix. The oligonucleotide sequences and conditions are available on request.

Statistical analysis

Fisher's exact test was used to compare the allelic frequency in the two groups.

For Peer Review

Result and Discussion

Heterozygous A91V was found, as shown in the Table, in 6 out of 82 subjects belonging to CEPH families (6/164 chromosomes; 3.7%). This frequency is in keeping with the 3.9 % value reported in the healthy control subjects of Southern Italy (Trambas *et al.,* 2005). Among 21 patients affected enrolled in the FHLH Italian National Registry in whom a *PRF1* mutation was identified, A91V was present in 10 out 21 patients. and always at the heterozygous state, but in an individual patient who carried the mutation at the homozygous state (11/42 chromosomes 26.2%). This frequency was significantly higher than that observed in CEPH families ($p = 0.0002$). The observed frequency in the CEPH families is in keeping with the reported frequency in North American healthy population (Molleran Lee *et al.,* 2004) (3%) and is representative of the worldwide genomic variation. However, it is lower than the frequency of 9% reported by Zur Stadt and coworkers, which reflects the distribution of the variation in a limited geographic area. Moreover, as illustrated in the Table, in the patients with FHLH and *PRF1* mutation, the A91V transition is always present in association with another molecular alteration of *PRF1* gene, whereas in the CEPH families the transition is always isolated. This is also in keeping with the observation that none of the reported FHLH patients have the A91V mutation in the absence of any other *PRF1* alteration (Molleran Lee *et al.,* 2004). We have previously reported on 9 healthy subjects heterozygous for A91V, with no alteration of the usual FHLH associated biochemical and serological parameters, and normal NK cytotoxic activity, but in 2 subjects who had lower NK

For Peer Review

activity or NK cell number, respectively (Busiello *et al.,* 2004b). Taken together, these results support the hypothesis that A91V may represent a frequent molecular alteration, which is not causative "*per se*" of FHLH and not sufficient to abolish cytolytic activity. However, the higher frequency of the A91V substitution in FHLH patients with at least one additional *PRF1* mutation supports a role for this mutation as a strong susceptibility factor, contributing to the pathogenesis of the disease. Our finding supports what recently reported on a biochemical consequence of the A91V alteration (Trambas *et al.,* 2005). Whether this effect is mediated by a synergic effect with other *PRF1* alterations, remains to be elucidated.

For Peer Review

Acknowledgments

This work was supported by the Grants «Ministero della Salute-Regione Campania, Legge «502», MURST-PRIN 2002.

We are indebted to Emanuela Minopoli for editorial assistance

For Peer Review

References

Arico', M., Janka, G., Fischer, A., Henter, J. I., Blanche, S., Elinder, G., Martinetti, M. & Rusca, M. P. (1996) Hemophagocytic lymphohistiocytosis. Report of 122 children from the International Registry. FHL Study Group of the Histiocyte Society. *Leukemia* **10**, 197

Busiello, R., Adriani, M., Locatelli, F., Galgani, M., Fimiani, G., Clementi, R., Ursini, M. V., Racioppi, L. & Pignata, C. (2004a) Atypical features of familial hemophagocytic lymphohistiocytosis. *Blood* **103**, 4610

Busiello, R., Galgani, M., De Fusco, C., Poggi, V., Adriani, M., Racioppi, L. & Pignata, C. (2004b) Role of A91V mutation in perforin gene in haemophagocytic lymphohistiocytosis. *Blood* **104**, 1909

Clementi, R., Emmi, L., Maccario, R., Liotta, F., Moretta, L., Danesino, C. & Aricò, M. (2002) Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying *PRF1* mutations. *Blood* **100**, 2266

Clementi, R., Zur-Stadt, U., Savoldi, G., Varotto, S., Conter, V., De Fusco, C., Notarangelo, L. D., Schneider, M., Klersy, C., Janka, G., Danesino, C. & Aricò, M. (2001) Six novel mutations in the PRF1 gene in children with haemophagocytic lymphohistiocytosis. *J Med Genet* **38**, 643

Ericson, K. G., Fadeel, B., Nilsson-Ardnor, S., Soderhall, C., Samuelsson, A., Janka, G., Schneider, M., Gurgey, A., Yalman, N., Révész, T., Egeler, R. M., Jahnukainen, K., Storm-Mathiesen, I., Haraldsson, A., Poole, J., de Saint Basile, G., Nordenskjold, M. & Henter, J. (2001) Spectrum of perforin gene

For Peer Review

mutations in familial hemophagocytic lymphohistiocytosis. *Am J Hum Genet* **68**, 590

Feldmann, J., Le Deist, F., Ouachée-Chardin, M., Certain, S., Alexander, S., Quartier, P., Haddad, E., Wulffraat, N., Casanova, J. L., Blanche, S., Fischer, A. & de Saint Basile, G. (2002) Functional consequences of perforin gene mutations in 22 patients with familial heamophagocytic lymphohistiocytosis. *Brit J Haematol* **117**, 965

Molleran Lee, S., Villanueva, J., Sumegi, J., Zhang, K., Kogawa, K., Davis, J. & Filipovich, A. H. (2004) Characterisation of diverse *PRF1* mutations leading to decreased natural killer cell activity in North American families with haemophagocytic lymphohistiocytosis. *J Med Genet* **41**, 137

Stepp, S. E., Dufourcq-Lagelouse, R., Le Deist, F., Bhawan, S., Certain, S., Mathew, P. A., Henter, J. I., Bennet, M., Fischer, A., De Saint Basile, G. & Kumar, V. (1999) Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* **286**, 1957

Suga, N., Takada, H., Nomura, A., Ohga, S., Ishii, E., Ihara, K., Ohshima, K. & Hara, T. (2002) Perforin defects of primary haemophagocytic lymphohistiocytosis in Japan. *Brit J Haematol* **116**, 346

Trambas, C., Gallo, F., Pende, D., Marcenaro, S., Moretta, L., De Fusco, C., Notarangelo, L., Arico, M. & Griffiths, G. (2005) A single amino acid change, A91V, lead to conformational changes which can impair processing to the active form of perforin. *Blood* **[Epub ahead of print]**,

For Peer Review

Ueda, I., Morimoto, A., Inaba, T., Yagi, T., Hibi, S., Sugimoto, T., Sako, M., Yanai, F., Fukushima, T., Nakayama, M., Ishii, E. & Imashuku, S. (2003) Characteristic perforin gene mutations of haemophagocytic lymphohistiocytosis patients in Japan. *Brit J Haematol* **121**, 503

zur Stadt, U., Beutel, K., Weber, B., Kabisch, H., Schneppenheim, R. & Janka, G. (2004) Ala91Val is a polymorphism in the perforin gene not causative for a FHLH phenotype. *Blood* **104**, 1909

For Peer Review

Table Prevalence of A91V substitution in PRF1 gene in CEPH control subjects in FHLH patients and Italian controls

	CEPH (%)	**Patients (%)**	**Italian controls (%)**
A91V frequency	3.7	26.2	3.9
A91V + other *PRF1* alteration	0	100	0

For Peer Review

Molecular defects in human Severe Combined Immuodeficiency

Severe Combined Immunodeficiencies (SCID) are a genotypically and phenotypycally heterogeneous group of disorders characterized by severe defect of both T- and B-cell immunity. These form are in generally fatal within the first years of life, although a few typical cases may be characterized by a milder phenotype. These set of different disease share common clinical features, such as high susceptibility to develop severe and, sometimes, fatal infections (Fisher 1997; Kokron 1997). Thus far, all the different forms with a known molecular basis are caused by mutations in genes expressed in the hematopoietic system, which lead to intrinsic abnormalities of lymphocytic function. Our group in 1999 described a novel form of SCID (MIM 601705) which represents the human equivalent of the well known murine phenotype defined Nude/SCID (Pignata et al 1996). This form is the only human SCID caused by intrinsic abnormality of the epithelial component of the thymus (Pignata et al 1996). The gene responsible for the disease in both mice and humans encodes the forkhead/winged helix (WHN) FOXN1 transcription factor selectively expressed in thymic epithelia and skin (Nelse et al 1994, Segre et al 1995 Frank et al 1999). The most prominent clinical features is a congenital alopecia, from which the term “Nude” for the murine equivalent derived (Flanagann 1996) furthermore the disease is associated to a profound T cell defect. The first human molecular alteration was a C792T transition in exon 5 resulting in the

nonsense mutation R255X (Frank 1999). To date, the first 2 patients investigated, carrying the R2554X mutation in the homozygous status, were born to consanguineous parents originating from small community in southern Italy (Pignata et al 1996).

Molecular screening for the R255X alteration in isolated population

In this phase of research based on the presence of several cases in the community, genetic screening for the presence of the FOXN1 R255X mutation was performed in an attempt to identify an ancestral founder effect. In fact due to the geographical location of the village, lying in the mountains between Naples and Salerno, and the elevated rate of endogamy, the population may be considered isolated. Moreover, we have genotyped 2 microsatellite markers, D17S2187 and D17S1880 flanking the FOXN1 gene on chromosome 17 to define whether a founder chromosome is shared by individuals carrying the R255X mutation. Molecular analysis revealed that 55 subjects, corresponding to 6.52% of the population were found heterozygous carriers of the mutation. In these 55 subjects, originated from 39 families, we perform a genealogical study. Through the archival database, common ancestors were identified for the 55 heterozygous carriers. They are linked in an extended 7 generational pedigree comprising 483 (Figure 8) individuals founded by a single ancestral couple born in 19th century.

This work has been very important also to give a prenatal diagnosis support to this population. These results has been published on the Annals of Human Genetics see below for the manuscript.

References

1. Festing, M. F. W., May, D., Connors, T. A. *et al.* (1978) An athymic nude mutation in the rat. *Nature* 274, 365-366.
2. Fischer, A., Cavazzana-Calvo, M., De Saint Basile, G. *et al.* (1997) Naturally occurring primary deficiencies of the immune system. *Annu Rev Immunol* 15, 93-124.
3. lanagan, S. P. (1966) 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genet Res* 8, 295-309.
4. Frank, J., Pignata, C., Panteleyev, A. A. *et al.* (1999) Exposing the human nude phenotype. *Nature* 398, 473-474.
5. Hofmann, M., Harris, M., Juriloff, D. *et al.* (1998) Spontaneous mutations in SELH/Bc mice due to insertions of early transposons: molecular characterization of null alleles at the nude and albino loci. *Genomics* 52, 107-109.
6. Huth, M., Schlake, T. & Boehm, T. (1997) Transposon-induced splicing defect in the rat nude gene. *Immunogenetics* 45, 282-283.
7. Kokron, C. M., Bonilla, F. A., Oettgen, H. C. *et al.* (1997) Searching for genes involved in the pathogenesis of primary immunodeficiency diseases: lessons from mouse knockouts. *J Clin Immunol* 17, 109-126.
8. Miller, S. A., Dykes, D. D. & Polesky, H. F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Res* 16, 1215.
9. Nehls, M., Pfeifer, D., Schorpp, M. *et al.* (1994) New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* 372, 103-107.

10. Pignata, C., Fiore, M., Guzzetta, V. *et al.* (1996) Congenital Alopecia and nail dystrophy associated with severe functional T-cell immunodeficiency in two sibs. *Am J Med Genet* 65, 167-170.

11. Segre, J. A., Nemhauser, J. L., Taylor, B. A. *et al.* (1995) Positional cloning of the nude locus: genetic, physical, and transcription maps of the region and mutations in the mouse and rat. *Genomics* 28, 549-555

Short Communication

doi: 10.1046/j.1529-8817.2004.00091.x

Ancestral Founder Mutation of the *Nude* (*FOXN1*) Gene in Congenital Severe Combined Immunodeficiency Associated with Alopecia in Southern Italy Population

M. Adriani[1], A. Martinez-Mir[2], F. Fusco[1], R. Busiello[1], J. Frank[3], S. Telese[1], E. Matrecano[1], M. V. Ursini[4], A. M. Christiano[2] and C. Pignata[1,4]

[1]*Department of Pediatrics, "Federico II" University, Naples, Italy;* [2]*Department of Dermatology & Genetics and Development, Columbia University, New York, NY;* [3]*Department of Dermatology and Allergy, University Clinic of the RWTH Aachen, Germany;* [4]*International Institute of Genetics and Biophysics, CNR, Naples, Italy*

Summary

Genetic alterations of the FOXN1 transcription factor, selectively expressed in thymic epithelia and skin, are responsible in both mice and humans for the Nude/SCID phenotype. The first described human *FOXN1* mutation was a C792T transition in exon 5 resulting in the nonsense mutation R255X, and was detected in two probands originated from a small community in southern Italy. In this community, four additional children affected with congenital alopecia died in early childhood because of severe infections. In this study, we report on the screening for this mutation in 30% of the village population. This analysis led us to identify 55 heterozygous carriers (6.52%) of the R255X mutation out of 843 inhabitants screened. A genealogical study revealed that these subjects, belonging to 39 families, were linked in an extended 7-generational pedigree comprising 483 individuals. Through the archival database a single ancestral couple, born at the beginning of the 19th century, was identified. To confirm the ancestral origin of the mutation we genotyped two microsatellite markers, D17S2187 and D17S1880, flanking the *FOXN1* gene on chromosome 17. The three haplotypes identified, 3/R255X/3, 3/R255X/2 and 3/R255X/1, are consistent with a single ancestral origin for the mutation R255X.

Introduction

The inherited severe combined immunodeficiencies (SCIDs) are a group of diverse genetic diseases affecting either the T- or B-cell compartment of the immune system that share common clinical features, such as high susceptibility to develop severe and, sometimes, fatal infections (Fischer *et al.* 1997; Kokron *et al.* 1997). Thus far, all the different forms with a known molecular basis are caused by mutations in genes expressed in the hematopoietic system, which lead to intrinsic abnormalities of lymphocytic function. A recently described novel form of SCID (MIM 601705) represents the human equivalent of the well known murine phenotype defined as Nude/SCID, and is the only human SCID caused by an intrinsic abnormality of the epithelial component of the thymus (Pignata *et al.* 1996). The thymus is the lymphoid organ where T-cell precursors mature. The gene responsible for the disease in both mice and humans encodes the forkhead/winged helix (WHN) FOXN1 transcription factor selectively expressed in thymic epithelia and skin (Nehls *et al.* 1994; Segre *et al.* 1995; Frank *et al.* 1999). The most prominent characteristic of the clinical phenotype is congenital alopecia, from which the term "Nude" for the murine equivalent derived (Flanagan, 1966). The disease is always associated with a profound T-cell defect. The first described human *FOXN1* mutation was a C792T transition in exon 5 resulting in the nonsense

*Correspondence: Claudio Pignata, M.D., Ph.D., Professor of Pediatrics, Department of Pediatrics, Unit of Immunology, "Federico II" University, via S. Pansini 5-80131, Naples, Italy. Phone # +39-081-7464340; Fax # +39-081-5451278; E-mail:pignata@unina.it

© University College London 2004

mutation R255X (Frank *et al.* 1999). This mutation lies upstream of the DNA binding and transactivation domain of this transcription factor, so that the translated protein, if any, would be completely non-functional, similar to the previously described rat and mouse *Foxn1* mutations (Festing *et al.* 1978; Huth *et al.* 1997; Hofmann *et al.* 1998). To date, the first two patients investigated, carrying the R255X mutation in the homozygous state, were born to consanguineous parents originating from a small community in southern Italy (Pignata *et al.* 1996). Furthermore, from the community records four additional patients of previous generations were affected with congenital alopecia and died in early childhood because of severe infections. Due to the geographical location of the village, lying in the mountains between Naples and Salerno, and the elevated rate of endogamy, the population may be considered isolated. In this study, based on the presence of several cases in the same community, a genetic screening for the presence of the *FOXN1* R255X mutation was performed in an attempt to identify an ancestral founder effect. Moreover, we genotyped two microsatellite markers, D17S2187 and D17S1880 flanking the *FOXN1* gene on chromosome 17, to define whether a founder chromosome is shared by individuals carrying the R255X mutation.

Subjects and Methods

All the living individuals still resident in the Acerno village and related to the probands' family were interviewed. Information obtained from this survey was verified and integrated using the local church and community records dating back to 1800. Subsequently, all data were recorded and analysed using the Cyrillic 3 software (Cherwell Scientific 2000, www.cyrillicsoftware.com V3.0.400). After written informed consent, and upon approval of the Institutional Ethical Committee, genomic DNA was extracted following standard procedures (Miller *et al.* 1988) from 843 subjects of the Acerno community, corresponding to approximtely 30% of the population, and processed anonymously. Samples were coded by a third party to allow re-testing of heterozygotes to confirm the result of the first determination. A PCR fragment containing exon 5 of the *FOXN1* gene was amplified using the following primers: Exon 5F: 5′-CTTCTGGAGCGCAGGTTGTC-3′ and Exon 5R: 5′-TAAATGAAGCTCCCTCTGGC-3′. Aliquots of the samples to be studied were digested with the restriction enzyme BsrI and the product was electrophoresed on 1.5% agarose gel. PCR fragments positive to the digestion with BsrI were then purified on Edge Centriflex columns (Edge BioSystems) and sequenced directly with POP-6 polymer using an ABI Prism 310 Genetic Analyzer from Applied Biosystems Inc. (Perkin Elmer).

Haplotype analysis was performed on 47 chromosomes carrying the mutation. According to the draft human genome sequence at UCSC (http://genome.cse.ucsc.edu/; December 2001 freeze), we chose microsatellite markers D17S2187 (GATA70H05) and D17S1880, located at 3.4 and 4.1 Mb centromeric and telomeric from the *FOXN1* gene, respectively.

Results and Discussion

Fifty-five subjects, corresponding to 6.52% of the studied population, were found to be heterozygous carriers of the mutation. Twenty eight of them, corresponding to 50.9%, were female. These 55 subjects originated from 39 families, which were subjected to a genealogical study. Through the archival database, common ancestors were identified for the 55 heterozygous subjects. They are linked in an extended 7-generational-pedigree comprising 483 individuals (Figure 1), founded by a single ancestral couple born at the beginning of the 19th century. In one family, heterozygotes were born to a mother who belonged to the pedigree but was found to be genotypically normal in two independent determinations. The family name of the deceased father, who presumably transmitted the mutation, was one of the six recurrent surnames in the pedigree of the probands.

From the founding ancestral couple in the first generation, four family groups originated. All the affected cases belonged to this pedigree. On the basis of our pedigree analysis, 33.3% of the mutation carriers inherited the mutated gene from their mother, whereas 66.7% inherited the mutant allele from their father. The pedigree also shows a high rate of past and present consanguineous and endogamous matings, typical of small communities. In particular, 14 out of 151 marriages were found to be consanguineous.

© University College London 2004

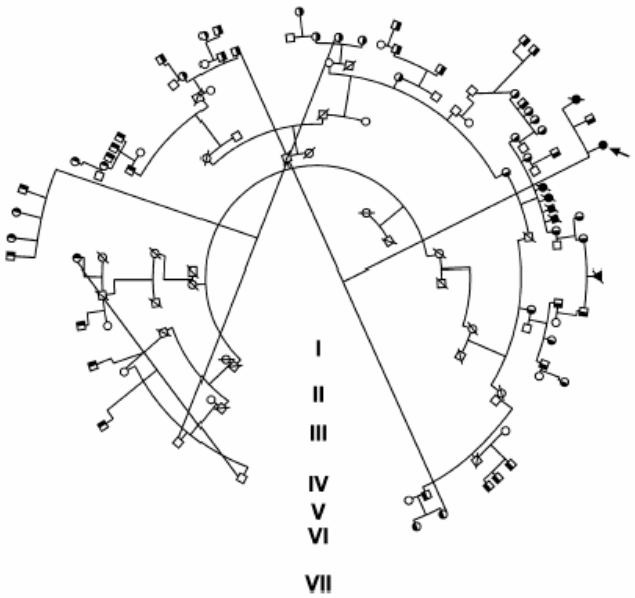


Figure 1 Seven generations pedigree including the Acerno families linked to the index family. Not all relatives are shown, but only the subjects necessary to demostrate the founder effect. Filled circles indicate affected individuals. Half-filled symbols indicate heterozygous carriers of the mutation R255X. The arrow indicates the proband.

To confirm that the R255X mutation represents a single ancestral event, we constructed the haplotype for the *FOXN1* locus for 47 chromosomes carrying the mutation R255X. The haplotype for D17S2187-*FOXN1*-D17S1880 could be established for 27 carrier chromosomes and three different haplotypes were identified: 3-R255X-3 (14/27), 3-R255X-2 (11/27) and 3-R255X-1 (2/27) (see the Table). The same haplotypes for the microsatellite markers (D17S2187-D17S1880) were identified in just 1 (3–3), 5 (3–2) and 6 (3–1) non-carrier chromosomes (data not shown). Among the carrier individuals for whom the phase could not be established (20/47), 3 and 2 were consistent with haplotypes 3–3 and 3–1, respectively, whereas 10, 2 and 3 were consistent with two of the previous haplotypes, 3–3/2, 3–3/1 and 3–2/1, respectively. Assuming recombination over the generations, these haplotypes could be consistent with a single ancestral origin of the R255X mutation in the Acerno population.

Table 1 Haplotypes analysis

	R255X carrier chromosomes	
Haplotype	Known fase	Unknown fase
3-R255X-3	14/27	3/20
3-R255X-2	11/27	–
3-R255X-1	2/27	2/20
3-R255X-3/2	–	10/20
3-R255X-3/1	–	2/20
3-R255X-2/1	–	3/20

Our study demonstrates that the *FOXN1* mutation R255X, which underlies the human equivalent of the murine Nude/SCID phenotype, is present in a heterozygous state in the Acerno population, and seems to have a single ancestral origin, dating back to the early 19th century. The prevalence of carriers in this community was 6.52%, thus leading to a presumptive estimate of expected new cases, in the absence of prenatal diagnosis, of 1:1000 live births. This estimate does not

© University College London 2004

take into account the elevated frequency of carrier status within the extended pedigree, which corresponded to 36.9% and, more importantly, the elevated rate of consanguineous matings among the families, which have occurred on several occasions in the last century.

As previously reported, this form of SCID is particularly severe due to the absence of the thymus. Thus, the importance of genetic counselling for couples at risk and of first trimester prenatal diagnosis by direct genetic analysis, given the high frequency of the *FOXN1* mutation in the Acerno population, cannot be overemphasized. It is remarkable that although a screening program for prenatal diagnosis has only been introduced for a short period of time in Acerno, an affected female fetus has already been identified, thus indicating the usefulness of this effort in an isolated community.

Acknowledgment

We especially thank the Acerno population, who enabled this study to be carried out. We also thank Dr Giuseppe Cappetta and Vito Sansone for the collection of samples. This work was supported by the Grants "Ministero della Salute - Roma and Regione Campania, Legge 502", MIUR-PRIN 2002 and Telethon #E0934

References

Festing, M. F. W., May, D., Connors, T. A. *et al.* (1978) An athymic nude mutation in the rat. *Nature* **274**, 365–366.

Fischer, A., Cavazzana-Calvo, M., De Saint Basile, G. *et al.* (1997) Naturally occurring primary deficiencies of the immune system. *Annu Rev Immunol* **15**, 93–124.

Flanagan, S. P. (1966) 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genet Res* **8**, 295–309.

Frank, J., Pignata, C., Panteleyev, A. A. *et al.* (1999) Exposing the human nude phenotype. *Nature* **398**, 473–474.

Hofmann, M., Harris, M., Juriloff, D. *et al.* (1998) Spontaneous mutations in SELH/Bc mice due to insertions of early transposons: molecular characterization of null alleles at the nude and albino loci. *Genomics* **52**, 107–109.

Huth, M., Schlake, T. & Boehm, T. (1997) Transposon-induced splicing defect in the rat nude gene. *Immunogenetics* **45**, 282–283.

Kokron, C. M., Bonilla, F. A., Oettgen, H. C. *et al.* (1997) Searching for genes involved in the pathogenesis of primary immunodeficiency diseases: lessons from mouse knockouts. *J Clin Immunol* **17**, 109–126.

Miller, S. A., Dykes, D. D. & Polesky, H. F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acid Res* **16**, 1215.

Nehls, M., Pfeifer, D., Schorpp, M. *et al.* (1994) New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* **372**, 103–107.

Pignata, C., Fiore, M., Guzzetta, V. *et al.* (1996) Congenital Alopecia and nail dystrophy associated with severe functional T-cell immunodeficiency in two sibs. *Am J Med Genet* **65**, 167–170.

Segre, J. A., Nemhauser, J. L., Taylor, B. A. *et al.* (1995) Positional cloning of the nude locus: genetic, physical, and transcription maps of the region and mutations in the mouse and rat. *Genomics* **28**, 549–559.

Received: 17 October 2003
Accepted: 7 January 2004

 © University College London 2004

Nail dystrophy associated to heterozygous mutation of the Nude/SCID FOXN1 gene.

In this phase of the project our group focusing the attention on several clinical features such as ectodermal defects. In particular, alopecia and nail dystrophy and thymic aplasia which are main features of the Nude/SCID. The program led us to physical examination of the 55heterozygous subjects with a special attention to ectodemal abnormalities (hair and nails alterations). Since FOXN1 seems to play a key role in the regulation of the differentiation process of both cutaneous and thymic epithelial tissue (Lee D, et al 1999). The cutaneous annexa were analysed at least 2 of us in the 55 heterozygotes, in 23 non heterozygous healthy married-in subjects, and 50 non heterozygous subjects belonging to the extend pedigree. Our analysis reveal that in 39 out of 55 heterozygous subjects showed a nail dystrophy. This association between nail dystrophy and heterozygous mutation of FOXN1 gene is for the first time reported. Moreover these findings is in keeping with the alteration of the digits and nails reported in a few strains of nude mice (Lee D, et al 1999) Our findings confirm that this transcriptions factor is involved in the maturation process of nails. These data has been published on Archives of Dermatology see below for the attached paper.

References

1. Pignata C, Fiore M, Guzzetta V, et al. Congenital Alopecia and nail dystrophy associated with severe functional T-cell immunodeficiency in two sibs. *Am J Med Genet.* 1996;65:167-170.

2. Frank J, Pignata C, Panteleyev AA, et al. Exposing the human nude phenotype. *Nature.* 1999;398:473-474.

3. Adriani M, Martinez-Mir A, Fusco F, et al. Ancestral founder mutation of the nude (FOXN1) gene in congenital severe combined immunodeficiency associated with alopecia in southern Italy population. *Ann Hum Genet.* 2004;68:265-268.

4. Lee D, Prowse DM, Brissette JL. Association between mouse nude gene expression and the initiation of epithelial terminal differentiation. *Dev Biol.*1999;208:362-374.

Title: Nail dystrophy associated with heterozygous mutation of the Nude/SCID FOXN1 (WHN) gene

Authors: [1*]Luigi Auricchio, MD, [2*]Marsilio Adriani, PhD, [3]Jorge Frank, MD, [2]Rosanna Busiello, PhD, [4]Angela Christiano, MD, [2]Claudio Pignata, MD, PhD

* = These authors equally contributed to the paper

Affiliations: [1]Department of Dermatology "Federico II" University, Naples; [2]Department of Pediatrics, "Federico II" University, Naples; [3]Department of Dermatology and Allergy, University Clinic of the RWTH, Aachen; [4]Department of Dermatology & Genetics and Development Columbia University, New York.

Word count: 571

Correspondence to:

Claudio Pignata MD, PhD

Professor of Pediatrics, Department of Pediatrics, Unit of Immunology,

"Federico II" University, via S. Pansini 5-80131, Naples, Italy

Fax # +39-081-5451278; Phone # +39-081-7464340; E-mail: pignata@unina.it

Ectodemal defects, such as alopecia and nail dystrophy, and thymic aplasia are the main features of the spontaneous immunodeficient mouse strain, referred as Nude/SCID. The human equivalent of the Nude/SCID phenotype, has been recently described in 2 children affected with a predominant T-cell defect associated with congenital alopecia and nail dystrophy.[1] In humans, as already reported in mice, the disease is due to a molecular alteration of the transcription factor FOXN1 (previously WHN),[2] which is selectively expressed in thymic and cutaneous epithelia. In 1999, a screening search for *FOXN1* mutation was undertaken in the community where the probands originated from to ensure to the population genetic counseling and prenatal diagnosis support. This program led to the identify 55 subjects carrying the *FOXN1* mutation at the heterozygous status all belonging to the same extended pedigree as above reported.[3]

Physical examination of the identified heterozygous subjects was conducted paying a special attention to ectodermal abnormalities and, in particular, to alterations of hair and nails in order to define whether the heterozygousity was associated with mild clinical signs. Since *Foxn1* seems to play a role in the regulation of the differentiation process of both cutaneous and thymic epithelial tissues,[4] these annexa were analysed by at least 2 of us in the

55 heterozygotes, in 23 non heterozygous otherwise healthy married-in subjects, and 50 non heterozygous subjects belonging to the extended pedigree.

No gross alteration of the hair was noted in association with the heterozygousity. Differently, nail examination revealed that 39 out of the 55 heterozygous subjects showed a nail dystrophy. This finding was not observed in any of the other control groups and was not related to an acquired form of nail dystrophy. Whenever necessary, cultures were performed to rule out mycotic infections. An accurate anamnesis was performed for each individual to evaluate any systemic disease able to induce nail dystrophy. Overt psoriasis was also excluded. However, the pattern of the phenotypic alteration of the nails was variable as shown in the Figure 1A-C. The koilonichia or "spoon nail" characterised by a concave surface and raised edges of the nail plate associated with a significant thinning of the plate itself was the more frequent alteration. As summarised in the Table, it was found in 28 subjects, whereas the canaliform dystrophy and a transverse groove of nail plate (Beau's line) were found in 13 and 11 subjects, respectively. It is note worthy that the more specific phenotypic alteration was the leukonychia characterised by a typical arcifom pattern, involving the proximal part of the nail plate reminiscent of the half-moon. This is not surprising since it reflects an abnormal differentiation process of the cells of the matrix. Seventeen out of the 39 heterozygotes with nail dystrophy had 2 or more features. It should be noted that in 18 cases fingernails

and toenails were both affected, whereas in 4 subjects the dystrophy was only observed in the feet.

Herein, is for the first time reported in humans on the association between nail dystrophy and heterozygous mutation in the *FOXN1* gene. This finding is in keeping with the alterations of digits and nails reported in a few strains of nude mice.[4] Since the nail plate originates mainly from the nail matrix and *FOXN1* is selectively expressed in such structure, our observation confirms that this transcription factor is involved in the maturation process of nails, and suggests that this sign may be indicative of heterozygousity for this molecular alteration.

References

1. Pignata C, Fiore M, Guzzetta V, et al. Congenital Alopecia and nail dystrophy associated with severe functional T-cell immunodeficiency in two sibs. *Am J Med Genet.* 1996;65:167-170.

2. Frank J, Pignata C, Panteleyev AA, et al. Exposing the human nude phenotype. *Nature.* 1999;398:473-474.

3. Adriani M, Martinez-Mir A, Fusco F, et al. Ancestral founder mutation of the nude (FOXN1) gene in congenital severe combined immunodeficiency associated with alopecia in southern Italy population. *Ann Hum Genet.* 2004;68:265-268.

4. Lee D, Prowse DM, Brissette JL. Association between mouse nude gene expression and the initiation of epithelial terminal differentiation. *Dev Biol.* 1999;208:362-374.

Table. Pattern of nail dystrophy in R255X heterozygous subjects

Pattern of Nail dystrophy	**N° of Patients**	***Nail alteration localizzation***	
		Hand	**Foots**
Leukonychia	6	5	3
Koilonychia	28	23	18
Canaliform Onycodystrophy	13	12	2
Beau's line	11	10	6

Clustering of Autoimmune Disease (CAD): identification of a novel clinical entity

The exisistance of novel clinical phenotype characterized by a Clustering of Autoimmune Disease in an individual has been recently documented. In particular, was been identified several subjects characterized by the presence of distinct autoimmune disorders which are indicative of a common polyreactive autoimmune process (Rieux-Laucat et al 2003). The abnormal function of one of two distinct pathway of apoptosis, the secretory and non-secretory pathway represents the pathogenic mechanism by which these subjects may develop hyperimmune diseases and syndromes with severe and complex clinical phenotype. A Mendelian transmission for some of these complex phenotype has been documented, in particular, for Autoimmune Polyendocrinopathy Candidiasis-Ectodermal Dystrophy (APECED) (Neufeld et al 1980), (APECED) Immunodysregulation, Polyendocrinopathy, Enteropathy, X linked syndrome (IPEX) (Wildin et al 2002) and Autoimmune Lymphoproliferative Syndrome (ALPS) (Canale et al 1967). The last one represents a prototype of CAD. Recent evidence indicates that, in a few cases, CAD may represent an unique model of monogenic autoimmune disorder (Fischer et al 2004). Hematologic autoimmune disorders associated with nonmalignant lymphadenopathy are the prominent clinical features of the ALPS, whose molecular characterization led to define five distinct entities on the basis of the location of the defect in the Fas

signaling cascade (Rieux-Laucat et al 2003). The ALPS0 represents the human analog of the *lpr* mice (Fas-/-) and is associated to a complete absence of Fas molecule. The ALPSIa, represents the most frequent form of the syndrome and is associated to heterozygous mutation of Fas gene. The ALPSIb is associated to a molecular alteration of the ligand of Fas, FasL while the ALPSII is associated to an alteration of the caspase 10 gene. Moreover, ALPSIII represents a large group of ALPS patients in which the molecular defect still remains to be identified. Along with typical form of this syndromes in several form, ALPS-like the autoimmune process is associated to an immunodeficit which leads to an increase susceptibility to viral infection. In a recent study, a novel form of ALPS has been identified. This form is characterized by a specific immunodeficit of T, B and NK lymphocytes and is associated to an alteration of caspase 8 gene. (Lenardo et al 2002) Aim of this phase of the project was been to characterize the phenotypic and immunological features of consecutive patients affected with CAD and to contribute to define the familial pattern of inheritance and clinical expression. In particular, we have focused on the evaluation of the functional integrity of Fas/FasL pathway, on the evaluation of the presence of immune defect and finally on the molecular analysis of caspase 8 gene. In this study has been identified 23 patients, affected by three or more autoimmune disorders. In the overall group of patients, 18 out of 23 CAD patients (78%) had a positive familial history. Within the probands' family

members group, 56 subjects were affected with at least one autoimmune disorder. The analysis of the pedigrees of the familial cases showed that the character of autoimmunity was inherited through the maternal lineage in the 22.2 % and through the paternal lineage in the 38.8 % of cases. In the 38.8 % of families an autoimmune phenotype was present in both lineages. Analysis of Fas-induced cell death in PHA-derived T cell lines detected defective apoptosis in 14 out of the 23 CAD patients (60%). Fas expression was evaluated in the long-term T cell lines by direct immunofluorescence on the same day in which Fas function was assessed and was expressed always at comparable levels than in controls. No relationship between abnormal Fas-induced cell death and either anticipation or the higher severity in the probands'generation was found. In the 14 patients with defective Fas-induced apoptosis, we searched for mutations of the Fas gene. Direct sequencing revealed a 2 base deletion in exon 4 (g410-411delCT) in one patient (Pt # 14). In other patients, we found several DNA changes which did not result in amino acid substitution. In particular, we found 6 different polymorphisms, 2 of them falling in the 5' UTR region, 2 mapping in the coding region, and 2 in the intronic region (IVSIII nt 46, IVSV nt 82). All these polymorphisms were already described. Direct sequencing of caspase-8 gene failed to identify causative mutations and revealed 3 already described polymorphisms: 2 mapping in the exon 9, and 1 in the intronic region (IVSIX nt

1123). This study has been submitted for publication to Journal of Clinical Immunology, see below for the manuscript.

References

1. Rieux-Laucat F, Le Deist F, Fischer A. Autoimmune lymphoproliferative syndromes: genetic defects of apoptosis pathways. Cell Death Differ 2003; 10:124-133.
2. Neufeld M, Maclaren N, Blizzard R. Autoimmune polyglandular syndromes. Pediatr Ann 1980;9:154-62.
3. Canale VC, Smith CH. Chronic lymphodenopathy simulating malignant lymphoma. J Pediatr 1967;70:891-9.
4. Fischer A. Human primary immunodeficiency diseases: a perspective. Nat Immunol 2004; 5:23-30.

- Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet 2002;39:537-45.

Title: Pattern of phenotypic expression and familial occurrence of clu autoimmune disorders in childhood

Short Title: Clinical phenotype and familiarity of CAD

Authors: P. Ferri[1*], F. Lombardi[1*], A. Franzese[1], R. Busiello[1], A. Chiocche U. Ramenghi[3], U. Dianzani [2], C. Pignata[1]

[1]Department of Pediatrics, "Federico II" University, Naples, [2]Interdisciplı Research Center of Autoimmune Diseases (IRCAD) and Department of Me Science, "A. Avogadro" University, Eastern Piedmont, Novara, [3]Departmer Pediatrics, University of Turin, Italy.

* These Authors equally contributed to the study

Corresponding to:

Claudio Pîgnata, M.D.

Professor of Pediatrics, Department of Pediatrics,

"Federico II" University,

via S. Pansini 5, 80131, Naples, Italy

phone +39-081-7464340 fax +39-081-5451278 e-mail pignata@unina.it

Keywords: Apoptosis, ALPS, Clustering of autoimmune diseases, Fas.

Summary

Clustering of Autoimmune Diseases is now emerging as a novel clinical entity within monogenic immune defects with a high familial occurrence. Aim of this study is to characterize the clinical and immunological features and to define the pattern of inheritance and intergenerational phenotypic variability within individual families. Twenty-three patients and 56 affected relatives were enrolled. Autoimmunity was inherited in the 22.2 and 38.8% of cases through maternal or paternal lineage, respectively. A partial phenotypic intergenerational correspondence was noted in the 72% of cases. Relatives showed a higher clinical heterogeneity. The younger generation always had a lower age at onset of selected disorders and frequently a more severe phenotype. In 14 patients, Fas stimulation failed to induce cell apoptosis, and in 1 case it was associated with Fas gene mutation. The remarkable prevalence of familiarity and the clear disease onset anticipation in younger generations indicate the presence of strong genetic interfering factors.

Introduction

Even though distinct autoimmune disorders may be associated in the same individual [1,2], only rare patients exhibit a clear clustering of distinct diseases which are indicative of a common polyreactive autoimmune process [3]. Along with environmental factors, a genetic susceptibility represents a well established feature in the predisposition of individuals to certain autoimmune diseases. An association with certain specific HLA and complement polymorphic variants has been well documented in several diseases, such as Juvenile Rheumatoid Arthritis (JRA) [4], type 1 Diabetes (T1D) [5,6], Autoimmune Thyroiditis (TAI) [7] and Coeliac Disease (CD) [8]. However, the intimate pathogenic mechanism of autoimmunity still remains to be unravelled. Alterations of homeostatic mechanism resulting in an abnormal lymphocyte accumulation, autoimmunity or lymphoid malignancies, have now emerged as a novel pathogenic mechanism underlying intense polyreactive autoreactions [9,10]. Recent evidence indicates that, in a few cases, Clustering of Autoimmune Disorders (CAD) may represent an unique model of monogenic autoimmune disorder [11]. Hematologic autoimmune disorders associated with non-malignant lymphadenopathy are the prominent clinical features of the Autoimmune LymphoProliferative Syndrome (ALPS), whose molecular characterisation led to define five distinct entities on the basis of the location of the defect in the Fas

signaling cascade [3]. However, in a large group of ALPS patients the molecular defect still remains to be identified.

Although several clinical studies have focused on the pattern of the clinical features of these polyreactive autoimmune syndromes, the phenotypic spectrum and familial occurrence of CAD still need to be clarified. Moreover, due to the scarce definition of the presenting signs and of the complexity of phenotypes, to date, the diagnosis of these clinical entities may be delayed and underestimated.

Aim of this monocentric study is to characterise the phenotypic and immunological features of consecutive patients affected with CAD and to contribute to define the familial pattern of inheritance and clinical expression.

Materials and methods

Subjects

Patients were defined as affected with CAD by the presence of at least three distinct organ-specific or systemic immune disorders in the proband or, alternatively, by the presence of two distinct diseases in the proband plus at least one first- or second-degree relative affected with an autoimmune disease. Twenty-three consecutive CAD patients were enrolled: 14 females, mean age 13.9 years, range 2-42 years. Juvenile Rheumatoid Arthritis was defined according to the American College of Rheumatology criteria [12]. Type 1 Diabetes (T1D) was defined on the basis of permanent fasting hyperglycemia (> 126 mg/dl), ketosis and anti-beta cell autoantibodies (Aab) [13]. Autoimmune Thyroiditis (TAI) was defined by the presence of specific Aab in patients with ultrasonographic disomogeneity (with or without impaired thyroid function). Coeliac disease (CD) was diagnosed according to ESPGHAN criteria [14]. Three patients fulfilled the accepted criteria for ALPS (a) autoimmunity, b) lymphadenomegaly or splenomegaly, c) defective Fas-mediated lymphocyte apoptosis with or without increase of double negative (DN) TCR $\alpha/\beta+$ T cells in the peripheral blood [3,9,15]. In order to define the weight of familial inheritance in the development of CAD, patients were investigated about the presence of first- and second-degree relatives affected with autoimmune disorders, including connective tissue diseases, cytopenia and endocrine autoimmune disorders. Age at

onset of each disorder was recorded in the probands and their affected relatives. Informed consent was obtained from patients and/or their parents.

Evaluation of Fas-induced cell death

Fas-induced cell death was evaluated as previously reported [16] on activated T-cell lines obtained by treating peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA) at days 0 (1 μg/mL) and 12 (0.1 μg/mL). Cells were then cultured in RPMI 1640 + 10% FCS + recombinant IL-2 (2 U/mL) (Biogen, Geneva, Switzerland). Fas function was assessed 6 days after the second stimulation (day-18 T cells), by incubating with control medium or 1 μg/mL anti-Fas mAb (CH11, IgM isotype, UBI, Lake Placid, NY) in the presence of 1 U/mL recombinant interleukin 2 (rIL-2) and 5% FCS to minimize spontaneous cell death. Cell survival was evaluated after 18 hours by counting alive cells in each well by the trypan blue exclusion test. Assays were performed in triplicate and analysed by a blind observer. Cells from 2 normal donors were included in each experiment as a positive control. Results were expressed as relative cell survival percentage, calculated as follows: (total live cell count in the assay wells/total live cell count in the untreated samples) x 100. Spontaneous cell loss in the control well was always less than 10% of the seeded cells and similar in cultures from the patients and normal donors. Cell death was evaluated both indirectly, by counting total surviving cells by the trypan blue exclusion test, or directly, by cytometric determination of the proportion displaying DNA

fragmentation after staining with propidium iodide or those stained by annexin to confirm Fas resistance. Fas function was defined defective when cell survival was higher than 78 %, which was the 95th percentile of the response displayed by normal controls.

Molecular analysis of the Fas gene

Mutation analysis of the Fas gene (TNFRSF6) was performed by DNA genomic sequencing. DNA was extracted from PBMC with standard methods. Eight fragments spanning the 5'UTR and the 9 exons of the gene were amplified in 20 µl final volume containing 12 pmol of each primer, 50 ng of genomic DNA and 0,5 units of AmpliTaq (Applied Biosystems, Warrington, UK). Amplification was performed in a Gene Amp PCR System 9700 (PE Applied Biosystems). The PCR products were sequenced using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Results

Clinical features

The twenty-three patients were diagnosed as affected with CAD based on the above mentioned criteria. Table 1 illustrates the major clinical and laboratory features of the patients. Male-to-female ratio was 1:1.6. Nine out of 23 subjects were affected with 3 or more distinct disorders. Twenty-one out of 23 patients had an organ-specific disease, mostly involving endocrine system or gut. The more frequent endocrine disorder was T1D. Ten patients had skin involvement. Five patients presented with a chronic blood autoimmune disorder. All patients but 2 had one or more Aab, as illustrated in the Table 1. Three out of the 23 patients had lymph node and/or spleen enlargement.

Within the subgroup of patients selected for the presence of 3 or more autoimmune diseases, 39% of the cases had a positive familial history for autoimmune disorders. In the overall group of patients, 18 out of 23 CAD patients (78%) had a positive familial history. Within the probands' family members group, 56 subjects were affected with at least one autoimmune disorder. Table 2 illustrates the comparison of the major clinical features between probands and affected family members. In the latter group, male-to-female ratio was 1:2.0 vs 1:1.6 in the probands. In approximately 27% of the affected relatives, the onset of the first autoimmune disease occurred in childhood. The comparison between the 2 groups

revealed a striking lower prevalence in the relatives' group of the more frequent features observed in the probands' group, such as T1D, TAI, CD and hemocytopenia. As a consequence, in the relatives' group a higher phenotypic heterogeneity was noted.

The analysis of the pedigrees of the familial cases showed that the character of autoimmunity was inherited through the maternal lineage in the 22.2 % and through the paternal lineage in the 38.8 % of cases. In the 38.8 % of families an autoimmune phenotype was present in both lineages.

In these families we next analyzed the phenotypic correspondence of autoimmunity between different generations. A total correspondence (identical pattern of clinical outcomes) was never found, whereas in the 72 % of cases a partial correspondence, defined as the presence of at least one disease, was noted in the intergenerational comparison. In the 28 % of cases, no correspondence (distinct autoimmune disorders) at all was found.

To compare the complexity of the CAD phenotype between different generations, the number of distinct disorders was evaluated in each affected member within the individual family. Surprisingly, in the spite of the lower age, a higher number of autoimmune disorders was observed in the proband generation in the most families (13 out of 18), unrespectively of the disease and the maternal or paternal inheritance (Fig. 1).

To evaluate the clinical course and features of each disorder in different family members, we then selected the families in which there was intergenerational correspondence for T1D, CD, TAI or AT. These diseases were chosen because of the low prevalence of undiagnosed cases, and the usual short interval between the onset of symptoms and the diagnosis. Twelve diseases were identified in 10 families. Interestingly, in these families all the diseases presented always much earlier in the proband generation as compared to the previous (Fig. 2).

Functional and genetic analysis of Fas

Analysis of Fas-induced cell death in PHA-derived T cell lines detected defective apoptosis in 14 out of the 23 CAD patients (60%) (Fig. 3). Fas expression was evaluated in the long-term T cell lines by direct immunofluorescence on the same day in which Fas function was assessed and was expressed always at comparable levels than in controls. No relationship between abnormal Fas-induced cell death and either anticipation or the higher severity in the probands'generation was found.

In the 14 patients with defective Fas-induced apoptosis, we searched for mutations of the Fas gene. Direct sequencing revealed a 2 base deletion in exon 4 (g410-411delCT) in one patient (Pt # 14). In other patients, we found several DNA changes which did not result in amino acid substitution. In particular, we found 5 different polymorphisms, 2 of them falling in the 5' UTR region, 2 mapping in the

coding region, and 2 in the intronic region (IVSIII nt 46, IVSV nt 82). All these polymorphisms were already described.

Discussion

This study reports on the clinical features of a large cohort of patients diagnosed as having CAD followed at an individual Center. As above mentioned, the definition of CAD was the presence of at least three distinct organ-specific or systemic immune disorders in the proband or, alternatively, the presence of two distinct diseases in the proband and at least one first- or second-degree relative affected with an autoimmune syndrome. An extensive clinical evaluation was performed in the families to define the pattern of inheritance in familial cases and the intergenerational variability of the clinical expression of the disease within each individual family. It was noted that approximately 40% of patients selected for the presence of 3 or more autoimmune diseases displayed a positive family history. Familial predisposition to autoimmune disorders is a well established feature, validated by the observations of the association between distinct entities and several highly polymorphic genomic regions, such as HLA and complement [5]. However in CAD, the occurrence of familial cases at such an extent suggests the presence of a strong genetic susceptibility factor for autoimmunity, inherited as an autosomal dominant trait rather than a generic predisposition to a certain disease. A large variety of autoimmune disorders were noted either in the probands or in the relatives, even though a wider heterogeneity of disorders was appreciable in the latter group. As for the intergenerational comparison of the phenotypic expressivity, the lack of correspondence between different generations of affected individuals in approximately 1/3 of the cases was noted. This

would imply that the participation of an individual organ to the pathologic process is not specific and, virtually, all organs may be targeted in the autoreactive process, thus suggesting that the presence of an alteration in the general mechanisms that govern the central or peripheral tolerance.

It is noteworthy that an anticipation of the age at onset in the last generation as compared to the older one was observed. In addition, in the younger generation, CAD is more severe and characterized by a higher number of autoimmune disorders in the same individual than in the older. This is unexpected and seems to be a paradox in that several of these disorders usually develop later in life and, therefore, the clinical phenotype should be still incomplete at a younger age. At present, it is not possible to explain the reason of the anticipation of the onset and the higher number of distinct diseases in the probands, and this is hardly to be attributed to an increasing role of environmental factors. It is conceivable to hypothesize that worsening of the clinical phenotype generation by generation may be due to an accumulation of alterations of distinct genes coding for molecules implicated in the process.

Genetic alterations of homeostatic mechanisms involved in the tolerance are now emerging as a possible cause of monogenic autoimmune disease. ALPS is characterized by autoimmunity associated with defective Fas-induced apooptosis [3]. A functional impairment of cell death induced through Fas triggering was found in the majority of these patients thus supporting some overlap with ALPS [3,9].

However, in our cohort of patients, only 3 patients fulfilled the accepted diagnostic criteria for ALPS. In keeping with this, the analysis of the Fas gene, which is mutated in most typical ALPS patients, revealed a mutation only in one of our CAD patients. A defective Fas induced apoptosis was also found in 11 out of 23 patients with CAD, who did not fulfil criteria for ALPS, thus indicating that abnormal Fas signaling may be implicated also in CAD. It should also be noted, however, that in 9 out of 23 patients cell survival following Fas triggering was normal, thus implying that additional Fas-independent mechanisms, not analysed in this study, may be altered causing an indistinguishable phenotype. Apoptosis is a complex process that plays a central mechanism in the homeostasis of immune response and in the regulation of the cellular differentiation [17]. It is triggered through two major signaling pathways [18,19,20]. The first involves several death receptor family members, such as CD95/Fas, TRAILR1-2, TNF-R1, that participate to the process by means of a homologous death domain intracellular region [21]. Death receptor triggering leads to the subsequent involvement of the caspases cascade, eventually resulting in caspase 3 activation [22]. This process ultimately results in the proteolytic cleaveage of nuclear and cytoplasmic substrates, and the subsequent cellular disassembly [23,24]. Along with these Fas-dependent pathways, several stimuli, such as DNA damage, metabolic imbalance, growth factor deprivation, or cell cycle perturbation activates the alternative mitochondrial apoptotic pathway [20]. The homodimerization of proapoptotic members of the Bcl-2 family, and the

subsequent cytochrome c release into the cytosol eventually result in the activation of caspase 9 that in turn activates caspase 3 [22,24,25]. This implies that a high number of signaling molecules involved in the process may be alterated thus causing an ALPS-like phenotype.

Further examples of severe and complex autoimmmune disorders are the Autoimmune PolyEndocrinopathy, Candidiasis and Ectodermal Dystrophy (APECED) [26,27] and the X-linked form of Hyperimmunity Polyendocrinophaty and Enteropathy (IPEX) [26,28]. APECED is caused by alterations of the gene encoding for the Autoimmune Regulator (Aire) molecule, which plays a crucial role in the central tolerance being expressed within the thymus [29,30]. Differently, IPEX is related to alterations of the transcription factor FOXP3, which is selectively expressed in the regulatory CD4+CD25+ cells responsible for counteracting self-reactive clones in the periphery [31,32]. However, even though such novel entities may have overlapping clinical signs and similar widespread autoimmune attack, our patients did not fulfil diagnostic criteria of APECED or IPEX.

In conclusion, our study highlights that CAD is a distinct clinical entity often associated with altered mechanisms that govern cell survival. The remarkable prevalence of familiarity in such cases in different generations strongly supports the inherited origin of the disease. Moreover, the anticipation of the disease onset in the younger generation suggests the hypothesis that additional molecular alterations of

interfering genes may accumulate, thus worsening the phenotype in younger generations.

Table 1. Major clinical and laboratory features of CAD patients

Patient	Age (years)	Sex	Associated diseases	Auto-ab
1	16	f	CD, T1D, TAI, SS	EMA, TPO-TG, ANA, DSDNA
2	9	f	CD, T1D, TAI	EMA, TPO-TG
3	21	f	CD, JRA, T1D, TAI	EMA, TPO-TG
4	20	f	MC, T1D, OF	-
5	23	f	HypoPT, Candidiasis, Addison, GHD, Skin adnexa dystrophy, TAI, OF, CP	ICA, TPO-TG
6	16	f	T1D, CD, TAI	EMA, ANA, TPO-TG
7	23	m	IgAD, T1D, Vitiligo, Psoriasis	-
8	13	m	T1D, TAI	ANA, DSDNA, TPO-TG
9	14	m	T1D, TAI, Cytopenia	TPO-TG
10	6	f	Cytopenia, AT	COOMBS, ANTI NEUTROPHILS
11	8	f	TAI, T1D	TPO, TTG
12	14	m	CD, T1D, AH	EMA, ANA, DSDNA
13	15	f	T1D, TAI	TPO-TG
14	3	f	Vasculitis, Asthma, Psoriasis	ANA, DSDNA
15	42	f	MC, TAI	ANA, P-ANCA
16	12	m	Cytopenia, AT, Lymphoadenopathy	ANTI NEUTROPHILS
17	2	f	Vasculitis, Hypergammaglobulinemia, Splenomegaly	ANA
18	12	m	Cytopenia, AT, Lymphoadenopathy	ANTIPLATELETS, ANTI NEUTROPHILS
19	6	m	AT, IgAD, Uveitis	ANTI PLATELETS
20	9	m	T1D, CD	EMA
21	22	m	JRA, CD, Uveitis, Vasculitis, Hypergammaglobulinemia	EMA
22	7	f	T1D, CD	EMA, TTG
23	18	f	T1D, CD	EMA

Note. CD = Coeliac Disease; T1D = Type 1 Diabetes Mellitus ; TAI = Autoimmune Thyroiditis; SS = Sjogren Syndrome ; JRA = Juvenile Rheumathoid Arthritis ; MC = Mixed Connectivitis ; HypoPT = HypoParaThyroidism ; GHD= Growth Hormone deficiency ; OF = Ovary Failure ; CP = Chronic Pancreatitis ; IgAD = IgA deficiency ; AT = Autoimmune Trombocytopenia ; AH = Autoimmune Hepatitis ; AF = adrenal failure. Autoimmune diseases are showed in order for each patient

Table 2. Comparison of clinical features between probands and affected family members

		Probands (N=23)	%	Affected relatives (N=56)	%
Male-to-female ratio		1:1.6		1:2.0	
Onset in childhood		23/23	100	15/56	26.8
Clinical features					
Autoimmune endocrinopathies	Thyroiditis		43.5		14.3
	Addison Disease		4.3		
	Type 1 Diabetes		60.8		16.1
	Ovary Failure		8.7		
	Hypoparathyroidism		4.3		1.8
Non-Endocrine autoimmune disorders	Vitiligo		4.3		1.8
	Psoriasis		8,7		8.9
	Vasculitis		13,0		1.8
	Juvenile Chronic Arthritis		8.7		
	Spondilarthritis/Chronic Arthritis				3.6
	Celiac Disease/Enteropathy		39.1		16.1
	Mixed Connectivitis		8.7		1.8
	Hepatitis		4.3		
	Sjogren's Syndrome		4.3		
	Sclerodermia		4.3		1.8
	Crohn Disease				1.8
	Systemic Lupus Eritematous				3.6
	Retinitis Pigmentosae				3.6
	Chronic Pancreatitis		4.3		3.6
Minor features	Uveitits		8,7		1.8
	Cytopenia/Trombocytopenia		34.8		10.7
	Candidiasis		4.3		
	Skin Adnexa Dystrophy		4.3		
	Esophageal Achalasia				1.8
	Deficit Ig A		8.7		1.8
	Lymphoadenopathy		8.7		3.6
	Hypergammaglobulinemia		8.7		

Legend to the Figures

Fig. 1. Intergenerational comparison of the severity of the CAD phenotype. A higher number of distinct disorders was observed in the 13 out of the 18 familial cases .

Fig. 2. Comparison of age at onset of selected disorders between different generations. The younger generation always had a lower age at onset.

Fig. 3. T cell sensitivity to Fas induced cell death in CAD patients. Peripheral blood T cells were stimulated by PHA and cultured in the presence of rIL-2 prior to further stimulation through Fas receptor. Results are expressed as the relative percentage of cell survival. Shaded area indicates the range of normal values (i.e. the range between the 5^{th} and the 95^{th} percentile of normal controls values).

References

1 Cronin CC, Shanahan F. Insulin-dependent diabetes mellitus and coeliac disease. Lancet 1997; **349**:1096-1097.

2 Pignata C, Alessio M, Ramenghi U et al. Clustering of distinct autoimmune diseases associated with functional abnormalities of T cell survival in children. Clin Exp Immunol 2000; **121**:53-58.

3 Rieux-Laucat F, Fischer A, Le Deist F. Cell-death signaling and human disease. Curr Opin Immunol 2003; **15**:325-331.

4 Smerdel A, Lie BA, Finholt C et al. An additional susceptibility gene for juvenile idiopathic arthritis in the HLA class I region on several DR-DQ haplotypes. Tissue Antigens 2003; **61**:80-84.

5 Levin L, Tomer Y. The etiology of autoimmune diabetes and thyroiditis: evidence for common genetic susceptibility. Autoimmunity Rev 2003; **2**:337-386.

6 Pociot F, McDermot MF. Genetics of type 1 diabetes mellitus. Genes Immun 2002; **3**:235-249.

7 Tomer Y, Davies TF. Searching for the autoimmune thyroid disease susceptibility genes: from gene mapping to gene function. Endocr Rev 2003; **24**:694-717.

8 Sollid LM. Molecular basis of coeliac disease. Ann Rev Immunol 2000; **18**:53-81.

9 Bleesing JJH, Brown MR, Straus SE et al. Immunophenotypic profiles in

families with autoimmune lymphoproliferative syndrome. Blood 2001; **98**:2466-2473.

10 Rieux-Laucat F, Le Deist F, Fischer A. Autoimmune lymphoproliferative syndromes: genetic defects of apoptosis pathways. Cell Death Differ 2003; **10**:124-133.

11 Fischer A. Human primary immunodeficiency diseases: a perspective. Nat Immunol 2004; **5**:23-30.

12 Petty RE, Southwood TR, Manners Pet al. International league of Association for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. J Rheumatol 2004; **31**:390-392.

13 Mellitus TECotDaCoD. Diagnosis and classification of diabetes mellitus. Diabetes Care 2004; **27**:S5-S10.

14 Walker-Smith JA, Guandalini S, Schmitz J, Shmerling DH,Visakorpi JK. Revised criteria for diagnosis of coeliac disease. Arch Dis Child 1990; **65**:909-911.

15 Ramenghi U, Bonissoni S, Migliaretti Get al. Deficiency of the Fas apoptosis pathway without Fas gene mutation is a familial trait predisposing to development of autoimmune diseases and cancer. Blood 2000; **95**:3176-3182.

16 Dianzani U, Bragardo M, DiFranco Det al. Deficiency of the Fas apoptosis pathway without Fas genemutations in pediatric patients with autoimmunity/lymphoproliferation. Blood 1997; **89**:2871-2879.

17 Tibbetts MD, Zheng L, Lenardo MJ. The death effector domain protein family:

regulators of cellular homeostasis. Nat Immunol 2003; **4**:404-409.

18 Li-Weber M, Krammer PH. The death of a T-cell: expression of the CD95 ligand. Cell Death Differ 2002; **9**:101-103.

19 Todaro M, Zeuner A,Stassi G. Role of apoptosis in autoimmunity. J Clin Immunol 2004; **24**:1-8.

20 Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004; **305**:626-629.

21 Zangemeister-Wittke U, Simon HU. Apoptosis - Regulation and clinical implications. Cell Death Differ 2001; **8**:537-544.

22 Adams JM. Ways of dying: multiple pathways to apoptosis. Gene Dev 2003; **17**:2481-2495.

23 Hengartner MO. The biochemistry of apoptosis. Nature 2000; **407**:770-776.

24 Bhardwaj A,Aggarwal BB. Receptor-mediated choreography of life and death. J Clin Immunol 2003; **23**:317-327.

25 Marsden VS, Ekert PG, Van Delft M, Vaux DL, J.M. A, Strasser A. Bcl-2-regulated apoptosis and cytochrome c release can occur independently of both caspase-2 and caspase-9. J Cell Biol 2004; **165**:775-780.

26 Eisenbarth G, Gottiled PA. Autoimmune polyendocrine syndromes. N Engl J Med 2004; **350**:2068-2079.

27 Perheentupa J. APS-I/APECED: the clinical disease and therapy. Endocrinol Metab Clin North Am 2002; **31**:295-320.

28 Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of

27

the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet 2002; **39**:537-545.

29 Ramsey C, Winqvist O, Puhakka Let al. Aire deficient mice develop multiple features of APECED phenotype and show altered immune response. Hum Mol Genet 2002; **11**:397-409.

30 Anderson MS, Veneziani ES, Klein Let al. Projection of an immunological self shadow within the thymus by the aire protein. Science 2002; **298**:1395-1401.

31 Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. J Biol Chem 2001; **276**:37672-37679.

32 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003; **299**:1057-1061.

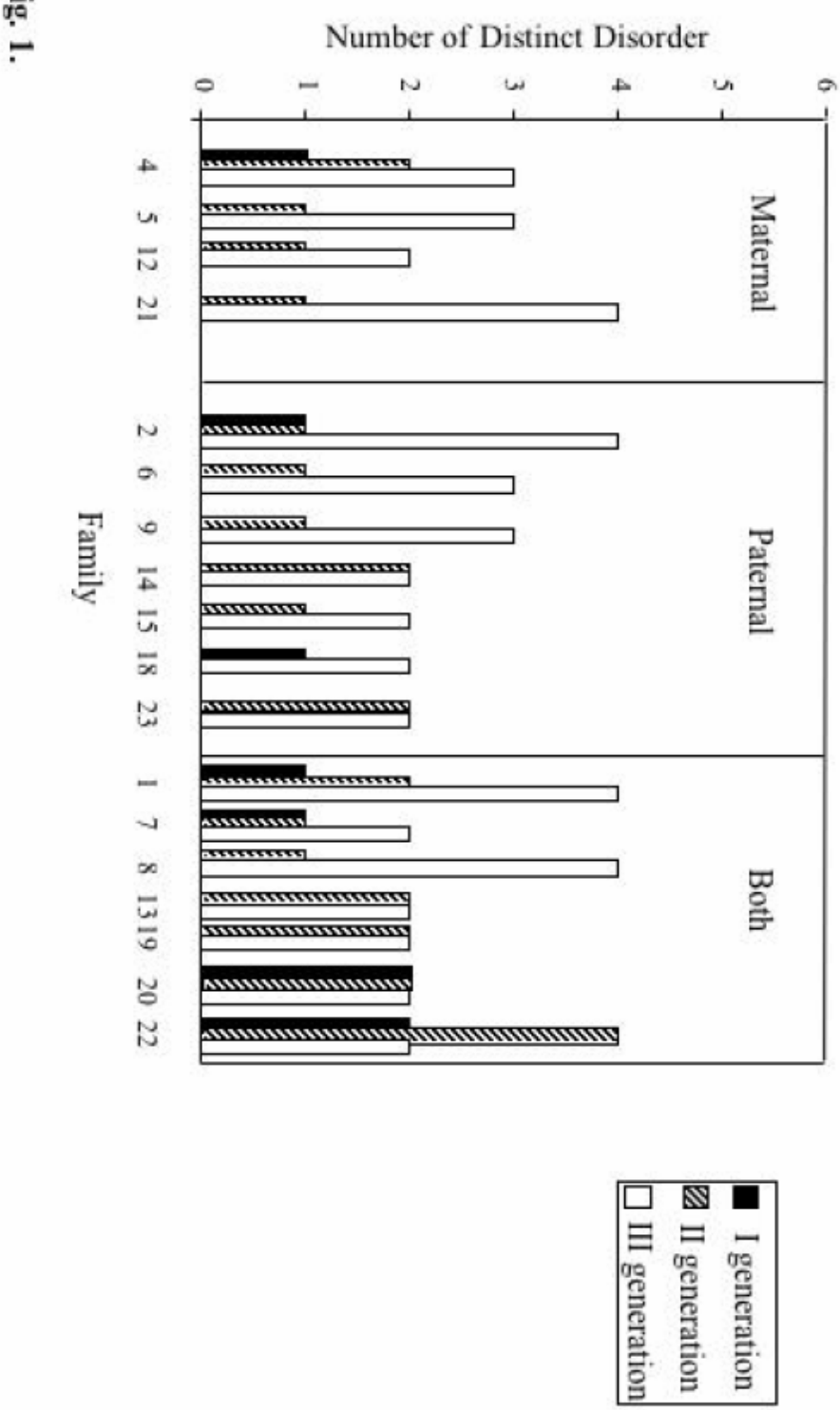

Number of Distinct Disorder
0
1
2
3
4
5
6
Maternal
Paternal
Both
4 5 12 21
2 6 9 14 15 18 23
1 7 8 13 19 20 22
Family
I generation
II generation
III generation

Fig. 1.

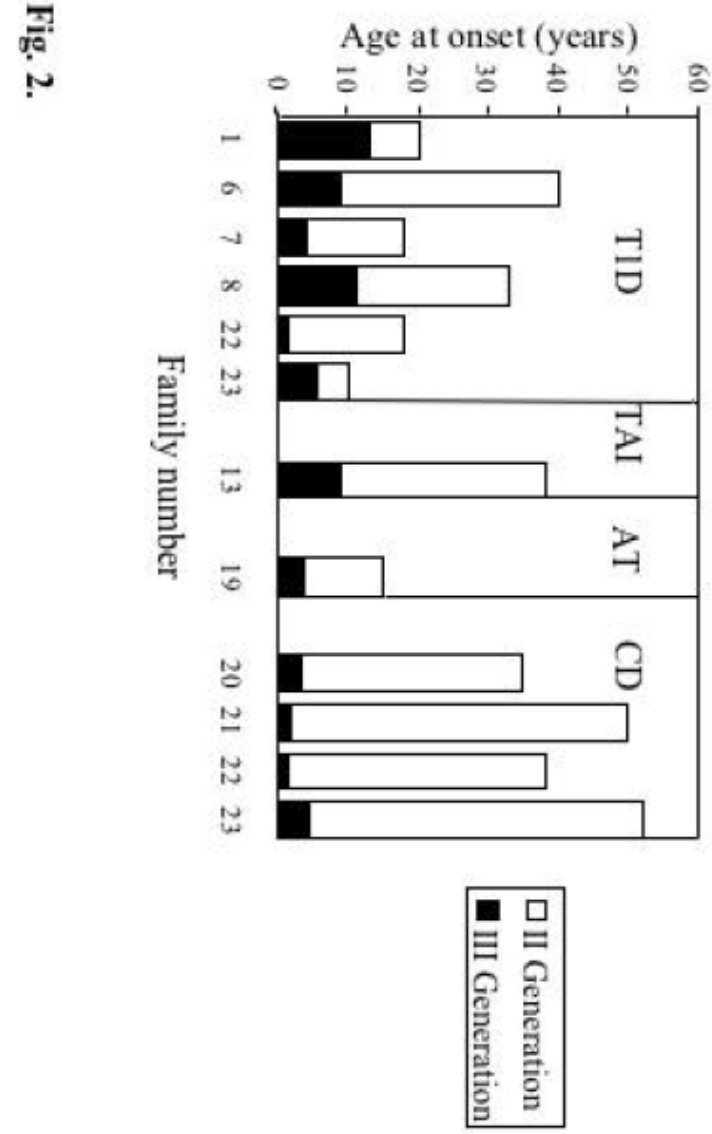

Age at onset (years)
0
10
20
30
40
50
60
TID
TAI
AT
CD
1 6 7 8 22 23
13
19
20 21 22 23
Family number
II Generation
III Generation

Fig. 2.

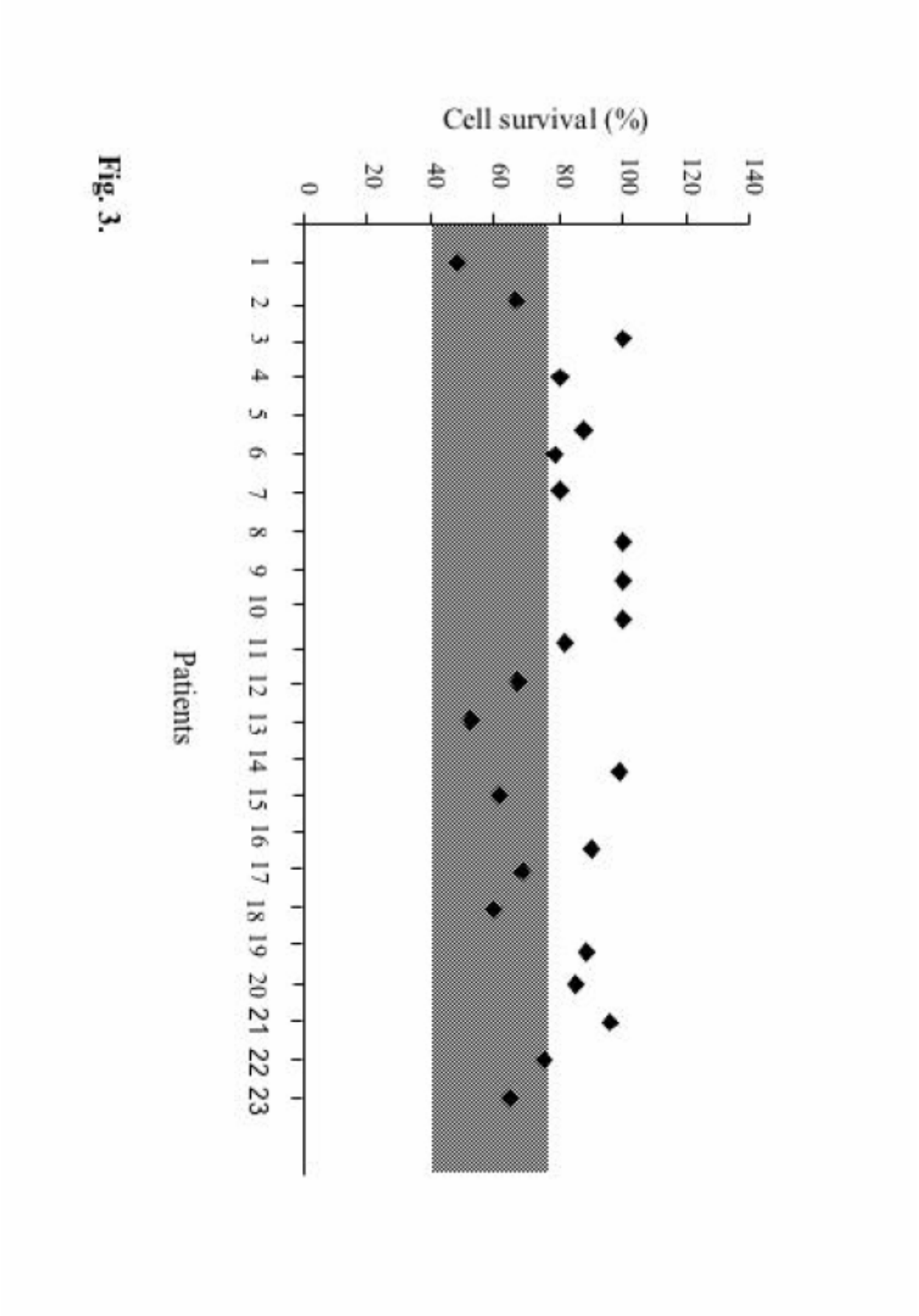

Cell survival (%)
0
20
40
60
80
100
120
140
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Patients

Fig. 3.

Primary Immunodeficiency Disease as models of immune system homeostasis: a literature survey

In the final part of our project we have considered several immune deficiency characterized by an alteration of the homeosataic mechanism caused by an aalteration of central or peripheral tolerance. In particular, we have focused our attention to syndromes such as Autoimmune Polyendocrinopathy Candidiasis-Ectodermal Dystrophy (APECED) (Neufeld 1980), (APECED) Immunodysregulation, Polyendocrinopathy, Enteropathy, X linked syndrome (IPEX) (Wildin 2002) and Autoimmune Lymphoproliferative Syndrome (ALPS) (Canale 1967).

APECED

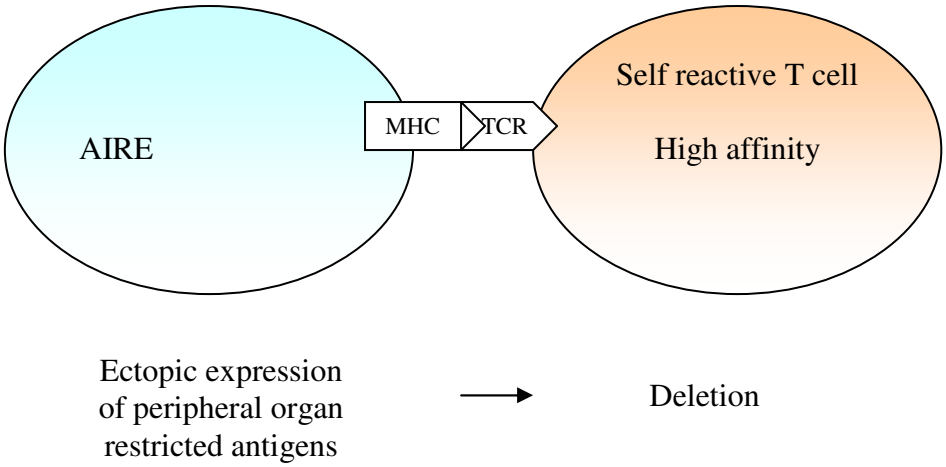
APECED is characterized by the variable occurrence of T cell and B cell mediated autoimmune diseases targeting various endocrine glands (parathyroid, thyroid, adrenal glands, gonads, pancreas, liver, skin and erythrocytes). It is often associated with chronic mucocutaneous candidiasis and ectodermal dysplasia (Fisher 2004). It is inherited as a Mendelian disease with an autosomal recessive mode. Loss of function mutations in Autoimmune Regulator gene (AIRE) cause the disease (Fisher 2004). A similar phenotype has been noted in Aire-/- mice (Fisher 2004). Considerable interest was sparked by the fact the AIRE is expressed mainly in medullary thymic epithelial cells and that the gene encodes for a protein with a DNA binding activity and gene

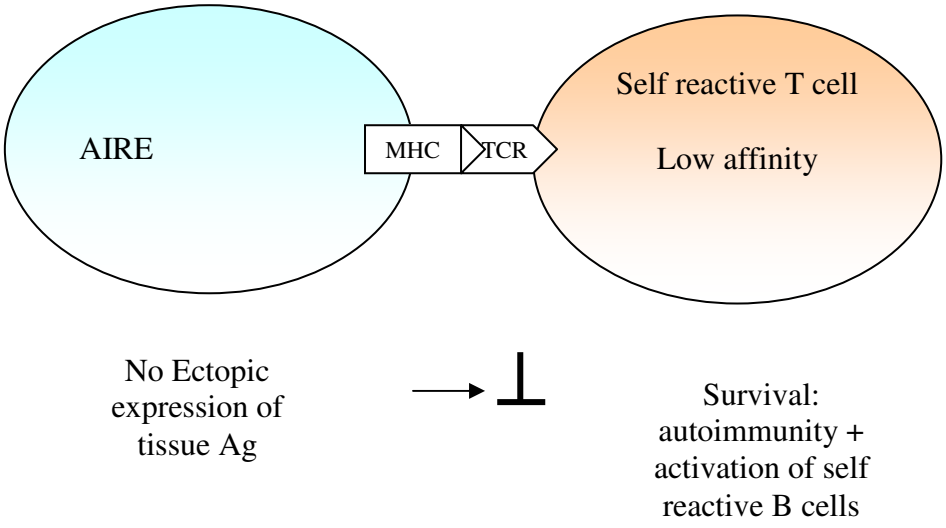
transactivation capacity (Fisher 2004). Recently, several reports have demonstrated that AIRE actually regulates the ectopic expression in medullary thymic epithelial cells of tissue specific proteins normally found in terminally differentiated organs, such as preproinsulin and zona pellucida glycoprotein 3 (Fisher 2004). These results strongly indicate that AIRE expression is key in central tolerance to a set of self proteins, although the mechanism by which AIRE triggers their gene expression is still unknown.

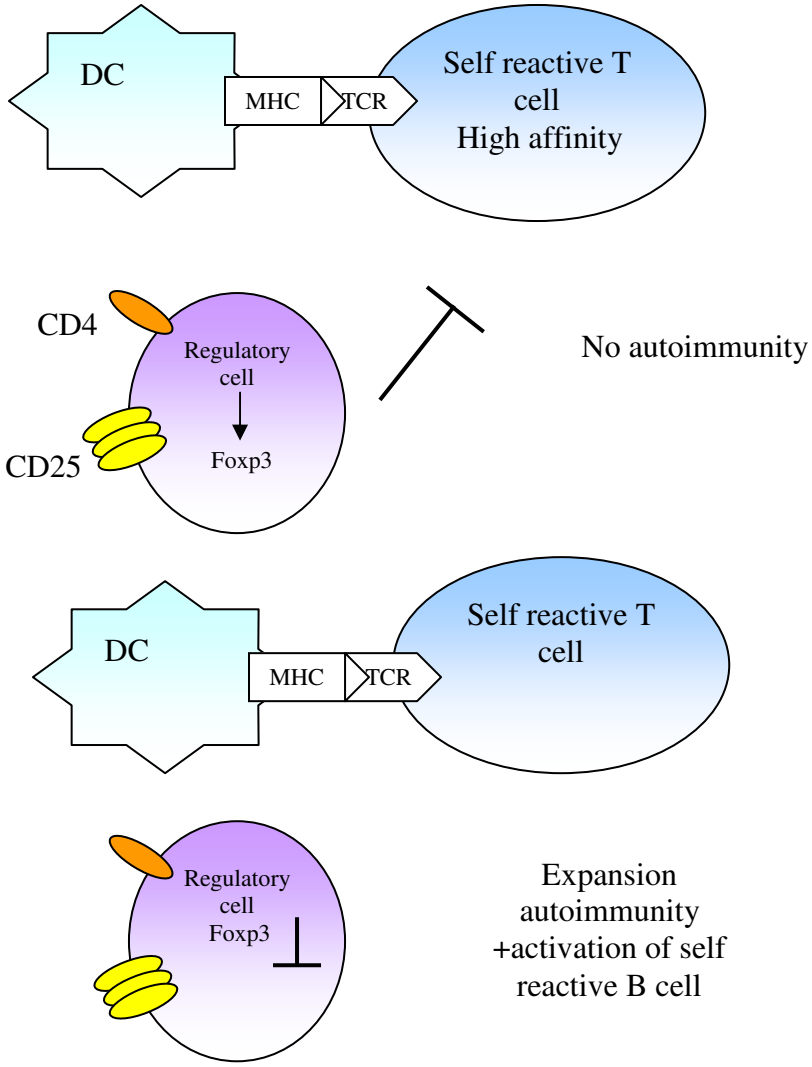
IPEX

The IPEX syndrome is a multisystemic autoimmune disease with an early onset and often fatal outcome (Fisher 2004). Severe enteropathy with diffuse and massive T cell infiltration and mucosal destruction variably associated with diabetes, eczema and autoimmunity toward blood cells are hallmarks of this disease. Loss of function mutations in the gene Foxp3 are causal (Fisher 2004). Foxp3 encodes a DNA binding protein of the forkhead family with a winged helix domain. It is expressed mainly by a subset of regulatory CD4 T cells that also express CD25 (Fisher 2004). There is a similar natural mutant in mice : the scurfy mice (Fisher 2004). As foxp3 is essential for regulatory T cell induction, this phenotype seems to be the consequence of a deficiency in regulatory T cells. The dominance of normal over mutated cells in obligate carriers and after bone marrow transplantation, respectively, results in a mixed chimerism (Fisher 2004). The IPEX phenotype differs from APECED and these differences

demonstrate the nonredundancy of the mechanism controlling autoimmune disease. (Figure 7)






DC
MHC
TCR
Self reactive T cell
High affinity
CD4
Regulatory cell
Foxp3
CD25
No autoimmunity
DC
MHC
TCR
Self reactive T cell
Regulatory cell
Foxp3
Expansion autoimmunity +activation of self reactive B cell

References

1. Canale VC, Smith CH. Chronic lymphodenopathy simulating malignant lymphoma. J Pediatr 1967;70:891-9

2. Wildin RS, Smyk-Pearson S, Filipovich AH. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. J Med Genet 2002;39:537-45.

3. Neufeld M, Maclaren N, Blizzard R. Autoimmune polyglandular syndromes. Pediatr Ann 1980;9:154-62.

4. Fischer A. Human primary immunodeficiency diseases: a perspective. Nat Immunol 2004; 5:23-30.

Novel immunodeficiencies: clustering of distinct autoimmune disorders associated to monogenic alterations

Nuove immunodeficienze: cluster di distinti disordini autoimmuni associati ad alterazioni monogeniche

R. BUSIELLO, G. GRECO, C. PIGNATA
Department of Pediatrics, "Federico II" University, Naples, Italy

Summary

The recent identification of new immunodeficiency syndromes characterized by clustering of distinct autoimmune diseases in the same individual greatly contributed to our understanding of genetic aspects of autoimmune diseases. Recent evidence also suggests that an autoimmune reaction may be associated with immunodeficiency. In this review, we will examine the clinical and functional hallmarks of these novel syndromes to focus on the diagnostic criteria to ameliorate patient detection.

Riassunto

La recente identificazione di nuove sindromi da immunodeficienza, il cui quadro clinico è caratterizzato da "clustering" di distinte malattie autoimmuni nello stesso individuo, ha permesso di fornire un considerevole contributo alla comprensione degli aspetti genetici delle malattie autoimmuni. Recenti evidenze, hanno inoltre permesso di comprendere come una risposta autoreattiva possa essa stessa rappresentare paradossalmente un segno clinico di immunodeficienza. In questo articolo di aggiornamento esaminiamo le principali caratteristiche cliniche e funzionali di queste diverse malattie allo scopo di semplificare l'iter diagnostico da seguire.

Key words

Apoptosis • APECED • AIRE • IPEX • ALPS

Parole chiave

Apoptosi • APECED • AIRE • IPEX • ALPS

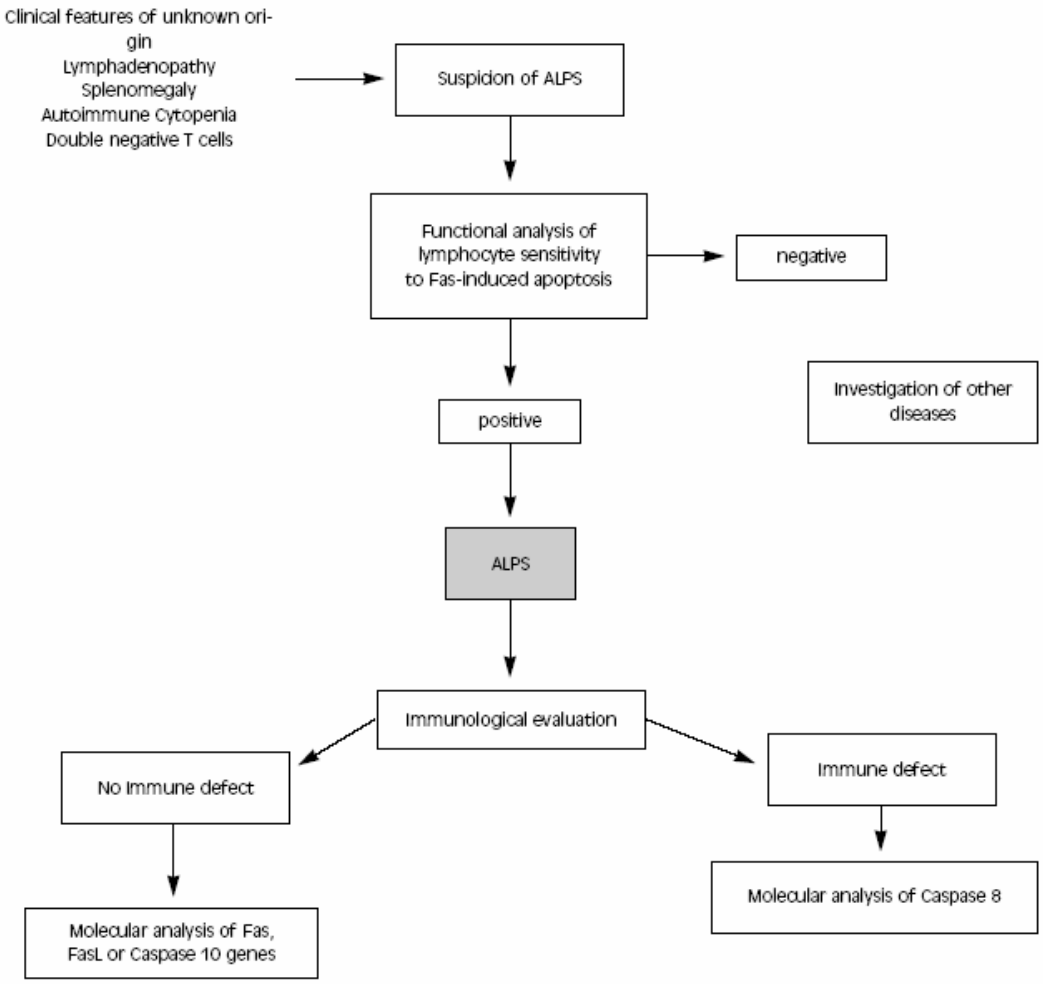
Submitted: January 28, 2004

Correspondence:
Prof. C. Pignata
Department of Pediatrics
Unit of Immunology
"Federico II" University
Via S. Pansini 5
80131, Naples, Italy
Tel. +39 081 7464340
Fax +39 081 5451278
E-mail: pignata@unina.it

Introduction

Autoimmune disorders occur in 3-5% of the population and comprise a heterogeneous group of poorly understood diseases [1-3]. This group of diseases represents a diverse set of clinical entities so far defined on the basis of the target organ involved in the pathologic process. Overall, they are thought to arise from altered homeostasis of the immune system [3]. The majority of lymphocytes that in vivo expand and proliferate in response to foreign antigens, subsequently, will die to maintain a constant cell number. During the initial expansion phase of an immune response, growth factors and cytokines participate in the process, leading to T- and B-cell proliferation after encountering the antigens. These agents, in a highly regulated fashion, are responsible for delivering cell signals that sustain the growth and survival of T cells. At the end of an immune response, programmed cell death, or apoptosis, occurs to limit cell growth, thus restoring immunological homeostasis [4]. Hence, alterations of apoptosis play a pivotal role in developing autoimmune disorders. Virtually, all organs may be target of the autoreactive process. The site of attack is organ- or tissue-specific: examples of this organ specificity are represented by pancreatic b cells in type 1 diabetes (T1 DM) [5], myelin basic protein in multiple sclerosis (MS) [6], thyreoglobulin or thyroid peroxidase in thyroiditis [7]. Alternatively, the process may be systemic, characterized by an immune attack against self-antigens in multiple tissues (e.g. systemic lupus erythematosus) [1]. A complex array of genetic and environmental factors is re-

Fig. 1. Diagnostic flow chart of children with clustering of autoimmune diseases.



quired for the development of autoimmune diseases. Studies on animal models greatly contributed to our understanding of the interaction between these factors. As for the genetic component, it has been universally recognized for the past 30 years that most of autoimmune diseases have a polygenic basis. Therefore, a great effort of research has been directed to identify susceptibility genes [8]. Although association between HLA specificities and autoimmune conditions has been established for a number of diseases, HLA represents only part of the genetic susceptibility factors, which "per se" is not able to explain the occurrence of the disease. A great contribution to understand the genetic basis of autoreaction derives from the recent reports on rare patients affected with clustering of distinct autoimmune disorders (polyreactive autoimmunity) [9]. All these cases share the hallmark of multiple organ involvement along with a high familial occurrence. The analysis of genome-wide linkage demonstrated that these multiple autoimmune syndromes shared common susceptibility loci [10]. These findings suggested that a few functionally related genes might contribute to the development of autoimmune diseases [10-12]. In this context, the existence of rare cases with Mendelian inheritance and complete penetrance provided a unique model of an autoimmune disease associated with single-gene defect. Thus far, the existence of a Mendelian inheritance for Clustering of Autoimmune Disease (CAD) has been well documented in several syndromes such as Au-

toimmune Polyendocrinopathy Candidiasis-Ectodermal Dystrophy (APECED) [13], Immunodysregulation, Polyendocrinopathy, Enteropathy, X linked syndrome (IPEX) [14] and Autoimmune Lymphoproliferative Syndrome (ALPS) [15]. It is noteworthy that in a few forms there are clear clinical signs of immunodeficiency, such as chronic candidiasis. In other cases, a functional impairment of T lymphocytes has been well documented in vitro, even though at present the clinical consequences of such defects have not been yet elucidated. The aim of this update is to summarize clinical features of these syndromes, to provide insights on the genetic basis of the individual diseases, and to delineate possible diagnostic algorithms to help pediatricians promptly recognize patients affected with these novel clinical entities.

Autoimmune-Polyendocrinopathy-Candidiasis-Ectodermal-Dystrophy (APECED)

APECED, formerly known as autoimmune Polyglandular Syndrome Type 1 (APS-1) [13], represents the first described organ-specific autoimmune disease inherited in a Mendelian autosomal recessive mode, as a monogenic disease (MIM # 240300) [13 16 17]. Recently, in one Italian family an autosomal dominant inheritance has been documented [18]. Clinically, patients affected with APECED show a variable combination of autoimmune processes involving either endocrine or non-endocrine target organs. The incidence of the syndrome has been estimated in Finnish, Sardinian and Jewish Iranian populations and approximately corresponds to 1:25,000, 1:14,400 and 1:9000, respectively [19 20]. First linkage studies mapped the genetic alteration to chromosome 21q22.3. Further studies revealed alterations of the autoimmune regulator gene (AIRE) consisting of 14 exons [21]. AIRE gene encodes for a polypeptide chain of 545 amino acids [22]. This molecule has several functional motifs suggesting a role as transcription regulator [21]. AIRE is predominantly expressed in thymic epithelial cells. It is also expressed in monocyte-derived cells of the thymus, and in a subset of cells in lymph nodes, the spleen and fetal liver. Studies in AIRE-deficient mice showed that lack of AIRE may affect the mechanism of central tolerance, thus implying that APECED is caused by a failure of central tolerance mechanisms specialized deleting forbidden T cell clones [23]. Although several mutations have been identified [24 25], most of the molecular alterations thus far described are in a limited number of fragments of the gene (hotspots) [26 27]. In particular, the vast majority of the patients have one of 2 mutations and in both cases they are a stop codon that usually results in a truncated protein lacking a functional domain. Correlation studies thus far performed failed to reveal a correlation between genotype and phenotype. Although APECED is the first well documented example of autoimmune disorder inherited as a monogenic disease, there are several genetic and environmental factors influencing the onset of the disease and its clinical expression.

Clinical features and suspicious signs

The disease generally occurs in early childhood, but patients may develop the complete clinical phenotype as late as in the fifth decade of life [17]. APECED diagnosis usually requires at least 2 of the following disorders: Addison's disease, hypoparathyroidism and chronic mucocutaneous candidiasis. Other diseases less frequently found in this syndrome are: insulin-dependent diabetes mellitus (IDDM), gonadal failure, pernicious anemia, hypothyroidism, autoimmune hepatitis and/or malabsorption symptoms. A further common feature of the syndrome is ectodermal dystrophy, presenting as onycodystrophy, alopecia, vitiligo, abnormal pili follicles and dystrophy of dental enamel [27]. So, as summarized in Table I, the clinical phenotype depends widely on the organ involved. Clinical signs of APECED have to be sought not only in the proband but also in the relatives, although members of the same family may develop completely different phenotypes [17]. Autoimmunity screening and genetic analysis must be performed also in asymptomatic relatives. Humoral immunity studies in these patients reveal the presence of multiple organ-specific autoantibodies towards a great variety of antigens [28]. An immunological study in APECED patients revealed low IFN-γ production, high serum IgM and CD4+ lymphocyte levels [17]. Furthermore, the presence of chronic Candida infection suggests a T-cell defect toward intracellular pathogens as documented by the defective T cell response in patients with APECED.

Immunodysregulation, Polyendocrinopathy, Enteropathy X-linked syndrome (IPEX)

IPEX (MIM 304790) is a rare X-linked disorder of immune regulation resulting in the expression of multiple autoimmune diseases. It is characterized by enteropathy and disorders of the endocrine system, such as insulin dependent diabetes mellitus and thyroiditis, which develop in early infancy [14]. The scurfy

Tab. I. Clinical features of APECED

Endocrine	Hypoparathyroidism (late onset)
	IDDM
	Hypothyroidism (late onset)
	Gonadal failure
	Adrenocortical failure
Endocrine Non	Gastric parietal-cell atrophy
	Chronic mucocutaneous candidiasis
	Autoimmune hepatitis
	Dystrophy of dental enamel
	Dystrophy of nails
	Alopecia
	Vitiligo
	Keratopathy
	Abnormal pili follicles
	Intestinal malabsorption

mouse is a natural mutant resembling IPEX [29]. The animal model shows an X-linked recessive inheritance, scaly skin, progressive anemia, thorombocytopenia, leucocytosis, lymphoadenopathy, gastrointestinal bleeding, cachexia [29 30]. These mice generally die by 3-4 weeks of age [29 30]. High levels of circulating cytokines have been documented in the mouse. Especially in the skin are present IL-2, IL-4, IL-5, IL-6, IL-10, interferon-α and tumor necrosis factor-α [31]. The gene mutated in the scurfy has been recently identified and named FOXP3 [32]. This gene is a member of the forkhead family of transcription regulators and is required for T-cell function. The FOXP3 mutations already described result in truncation of the protein. Recent studies have identified the IPEX locus on the human chromosome Xp11.3-q13.3 [33]. Human FOXP3 consists of 11 exons and encodes for a polypeptide of 431 amino acids known as scurfin [32]. This protein has at the carboxy terminus a conserved domain belonging to the forkhead winged helix transcription factors, a leucine zipper motif and a zinc finger motif [31]. The function of the scurfin is not fully known, but the typical structure of the protein suggests that the protein has DNA binding properties with a regulatory function of transcription. Earlier studies indicated that the scurfin binds DNA elements in the IL-2 promoter and granulocyte macrophage colony stimulating factor enhancer near Nuclear Factor of Activated T cells (NFAT) sites and is capable of repressing the transcription of these genes, thus reducing IL-2 expression in activated CD4+T cell lines. These functions do not occur in the absence of the forkhead domain [34]. Several FOXP3 mutations are in the winged helix domain of scurfin, thus altering the DNA binding function [35]. Recent data indicate that FOXP3 is expressed primarily in the CD4+CD25+. This lymphocyte subset is now emerging as an important T-cell subset, which acts as a key modulator of T-cell fate and function with potent suppressor activity.

Clinical features and suspicious signs

The onset of IPEX usually occurs in the perinatal period or early infancy. Typical clinical features of this syndrome are enteropathy, characterized by diarrohea, gastrointestinal bleeding, ileus, and involvement of the endocrine system, such as insulin dependent diabetes mellitus, probably due to inflammatory infiltration with disappearance of islet cells, and thyroiditis, as summarized in Table II. Other signs and symptoms frequently observed in patients with IPEX are hypogonadism, atopic dermatitis, autoimmune thrombocytopenia, neutropenia or anemia, leukocytosis, lymphadenopathy, collagen-vascular phenomena, respiratory distress, sepsis or other severe infections. Overall, Enterococcus and Staphylococcus are frequently responsible for such infections. Sometimes, peritonitis, pneumonitis, arthritis, or unusually severe responses to viral illness or immunizations may be observed. In more severe cases cachexia and early death may occur [33 36]. Polyarticular

Tab. II. Clinical manifestations in the X-linked Immunedysregulation, Polyendocrinopathy, Enteropathy syndrome.

Lymphoproliferation	Lymphadenopathies
Autoimmune manifestations	Hemolytic anemia
Thrombocytopenia	
Diabetes mellitus	
Neutropenia	
Dystrophy of nails	
Laboratory findings	High IL-2, IL5, IL-4, IL-6, IL-10, IFN-γ levels
Additional clinical features	Eczema or atopy
Diarrhoea	
Failure to thrive	
Leukocytosis	
Cachexia	

arthritis, asthma, ulcerative colitis, glomerulonephropathy, interstitial nephritis, hypotonia and muscle atrophy have also been described in these patients. Sometimes, an inherited metabolic disorder may be misdiagnosed due to the presence of metabolic acidosis. Prognosis is severe and in more than half the patients a long term treatment with immunosuppressants may be useful. Since a delayed onset of the disease in adulthood has been described, a diagnosis of IPEX should not be ruled out only on the basis of age. Although functional immunological results are not univocal, lymphocyte proliferation following phytohemagglutinin or pokeweed mitogen stimulation has been found defective in a few patients, since it is often associated with increased IL-2 production from CD4+ T cells14 and elevated serum IgE levels [36]. With regard to treatment, two approaches have been attempted: bone marrow transplantation (BMT) and immunosuppression. While immunosuppression seems to be effective, unfortunately, the patients who received BMT died soon after transplantation. Another supportive measure is parenteral nutrition, which is necessary in a few cases [36].

Autoimmune Lymphoproliferative Syndrome

Autoimmune lymphoproliferative syndrome (ALPS) MIM #601859 is a disorder characterized by chronic, non-malignant lymphoproliferation and autoimmunity, most commonly involving cells of hematopoietic origin. ALPS is due to a failure of the apoptotic mechanism that helps maintain normal lymphocytes homeostasis, with a subsequent accumulation of lymphoid mass along with the persistence of autoreactive cells. Apoptosis is a mechanism of cell death triggered by specialized membrane receptors. These receptors, called death receptors, belong to TNF-receptor (TNF-R)/NGF-receptor (NGF-R) superfamily which contain cysteine-rich domains (CRDs) in the extracytoplasmic region. The extracellular portion of these proteins is

important for ligand binding. Furthermore, these proteins are characterized by the presence of a functional domain named "death domain" (DD) in the cytoplasmic region [37], which binds the cytoplamic signaling proteins essential to induce apoptosis [38]. Fas, also known as CD95 or Apo-1 or TNFRSF6 belongs to this family of proteins and is the most efficient inducer of apoptosis in lymphocytes. The molecule consists of 3 extracellular CRDs and 80 amino acid residues in the intracellular DD. Fas ligand (FasL) is a molecule that belongs to the TNF superfamily of type II transmembrane proteins and is able to activate Fas receptor. The intracellular and extracellular domains of FasL are located in the N- and C-terminal regions, respectively. The receptor-binding domain is located at the very end of the C-terminus, and deletion of at least three amino acids from this region is sufficient to interfere with interactions with its receptor [39]. After ligand binding, three molecules of Fas assemble into complexes. Fas signaling occurs through the interaction of Fas with the Fas Associated Death Domain (FADD), a cellular adaptor and, subsequently, with procaspases 8 and/or 10 in a Death Inducing Signaling Complex (DISC) [39]. Again, the genetic basis of lymphoproliferation associated with autoimmunity was firstly identified in MRL/prl mice [40]. This natural mutant develops nephritis, hypergammaglobulinemia and antinuclear antibodies together with lymphadenopathy. With age, this mouse accumulates CD4-CD8-TCR+ (double negative T cells) in the periphery. This phenotype is likely the consequence of a downregulation of CD8 expression on mature peripheral lymphocytes [40]. In the last few years other natural mutants have been identified [41]. In humans, lymphocytes from ALPS patients fail to undergo apoptosis following Fas triggering. In general, Fas is not expressed on the membrane, although in a few cases Fas may be normal or slightly reduced. As shown in Table III, several forms of ALPS have been identified, which mainly differ for the molecule of the Fas/FasL pathway that is altered.

ALPS 0

This form of the syndrome is caused by the complete absence of the protein (Fas deficiency) as a consequence of a homozygous null mutation. This form of ALPS represents the analog of the Fas ko lpr/lpr mice. Thus far, three cases of this particular form of ALPS have been reported [42]. As these mutations were inherited from healthy parents, they are presumably recessive. In this type of ALPS, lymphoproliferation involves both T and B cells and is usually associated with a mild autoimmunity phenotype, often characterized exclusively by thrombocytopenia.

ALPS Ia

This form represents the more frequent form of ALPS. More than 70 cases have been described [43-45]. The disease is due to heterozygous Fas mutations (Table 3), which exert a transdominant negative effect on the wild type molecule [46]. As many as 60% of the mutations identified fall in DD domain. These mutations may result in truncated products or in a non-functional protein. Moreover, several mutations have also been described in the extracellular domain of Fas molecule. Despite the considerable number of patients suffering from ALPS, so far a correlation between genotype and severity of either the syndrome or the immunological derangement is still lacking. Within the same family, patients with the same apoptotic defect and genetic alteration, but profoundly differing in the clinical phenotype, have been observed [43 46]. This observation supports the hypothesis that there is a further event associated with mutation that induces the overt clinical manifestation. Probably, environmental factors may influence the expression of the phenotype and cause a variable penetrance of the individual genetic alteration.

ALPS Ib

This form of the syndrome was first observed in a patient who presented with systemic lupus erythematosus associated with chronic lymphocyte proliferation. The phenotype is similar to the lpr mice (Fas-deficient) and gld mice (FasL-deficient). The genetic alteration underlying this form of ALPS is the mutation of the Ligand of Fas (Tab. III) [47]. The disorder is inherited as an autosomal dominant trait.

Tab. III. ALPS

Disease	Gene
ALPS 0	CD95/Fas
ALPS IA	CD95/Fas
ALPS IB	CD168/FasL
ALPS II	Caspase 10
ALPS III	unidentified
ALPS and Immunodeficiency	Caspase 8

ALPS II

This form includes those patients who exhibit the typical clinical features of ALPS, including the defect of Fas-induced apoptosis, but with normal Fas and Fas Ligand genes, and normal expression of the Fas molecule. Recently, Caspase 10 mutations have been described in a few patients (Tab. III) [48].

ALPS III

Finally, this form of ALPS has been described in a number of patients with the clinical features of mild ALPS, associated with hypergammaglobulinemia. These subjects exhibit a normal Fas signaling pathway, and no molecular alterations have been found so far.

Tab. IV. Clinical manifestations in Autoimmune Lymphoproliferative Syndrome.

Lymphoproliferation	Lymphoadenopathies
	Splenomegaly
	Hepatomegaly
	Lymphoma
Autoimmune manifestation	Hemolityc anemia
	Trombocytopenia
	Neutropenia
	Urticaria
	Glomerulonephritis
	Guillain-Barrè syndrome
Rare symptoms	Arthritis
	Autism
	Hydrops fetalis
	Dyserythropoiesis
Laboratory findings	Double negative T cells
	Hypergammaglobulinemia
	Autoantibodies
	Elevated serum interleukin-10
	Defective Fas mediated programmed cell death

Autoimmune Lymphoproliferation Syndrome and Immunodeficiency (Caspase 8 deficiency)

It has been documented in two siblings who showed a lymphoproliferative syndrome associated with immunodeficiencies [49]. The immunodeficiency was characterized by an activation defect of B and T lymphocytes. In these subjects, the production of IL-2 was markedly reduced when PBLs from the patients were stimulated through T cell receptor. Furthermore, these patients showed decreased T cell proliferation response to phytohemoagglutinin; lymphocytes of these patients stimulated with pokeweed mitogen showed decreased production of IgM and IgG [49]. In these patients, a homozygous Caspase 8 mutation (Tab. III) was found, thus indicating that this molecule is involved in early steps of the T-cell receptor or B-cell receptor induced activation of lymphocytes [42].

Clinical features and suspicious signs

Suspicious signs of ALPS are: splenomegaly due to polyclonal accumulation of T and B cells, massive non neoplastic lymphadenopathy, autoimmune disease, expansion of double negative T cells in the periphery. Also in this syndrome it is possible to find vitiligo, alopecia and other clinical signs and laboratory markers of autoimmunity. All clinical features are summarized in Table IV. Since there is evidence of increased frequency of lymphoma and autoimmune diseases in the relatives of the probands, the former must be investigated. It is interesting to notice that although a high occurrence of either autoimmune diseases or cancer has been documented in the relatives of ALPS patients, within the individual pedigree only one of the two clinical problems is usually found, thus suggesting an additional genetic factor for such a predisposition. Patients with IDDM and Fas defect display a more severe form of diabetes. Often, in these patients IDDM is associated with other autoimmune diseases, such as thyroiditis, juvenile rheumatoid arthritis, autoimmune hepatitis, scleroderma, vitiligo, alopecia, blood cell cytopenia. Similarly, patients with Multiple sclerosis and Fas defect display a more "progressive" form of the disease, as compared to patients without Fas defect [50].

Conclusion

In conclusion, hyperimmune syndromes represent a heterogeneous group of disorders sharing the clinical peculiarity of multiple-organ involvement in the autoimmune process. In this review, we highlighted the clinical and functional hallmarks of these novel syndromes to help pediatricians promptly recognize patients affected with these clinical phenotypes.

Acknowledgement:

This work was supported by the grant MURST-PRIN 2002; prot. 2002064893_003.

References

1 Jacobson DL, Gange SJ, Rose NR, Graham NM. *Epidemiolgy and estimated population burden of selected autoimmune diseases in the United States*. Clin Immunol Immunopathol 1997;84:223-43.

2 Marrack P, Kappler J, Kotzin BL. *Autoimmune disease: why and where it occurs*. Nat Med 2001;7:899-905.

3 Maas K, Chan S, Parker J, Slater A, Moore J, Olsen N, et al. *Cutting Edge: Molecular portrait of human autoimmune disease*. J Immunol 2002;169:5-9.

4 Kaufmann SH, Hengartner MO. *Programmed cell death: alive and well in the new millenium*. Trends Cell Biol 2001;11:526-34.

5 Kukreja A, Maclaren NK. *Current cases in which epitope mimicry is considered as a component cause of autoimmune disease: immune-mediated (type 1) diabetes*. Cell Mol Life Sci 2000;57:534-41.

6 Ufret-Vincenty RL, Quigley L, Tresser N, Pak SH, Gado A, Hausmann S, et al. *In vivo survival of viral antigen-specific T cells that induce experimental autoimmune encephalomyelitis*. J Exp Med 1998;188:1725-38.

7 Martin A, Barbesino G, Davies TF. *T-cell receptors and autoimmune thyroid disease-signposts for T-cell-antigen driven diseases*. Int Rev Immunol 1999;18:111-40.

8 Theofilopoulos AN. *The basis of autoimmunity: Part II. Genetic predisposition*. Immunol Today 1995;16:150-9.

9 Pignata C, Alessio M, Ramenghi U, Bonissoni S, Difranco D, Brusco A, et al. *Clustering of distinct autoimmune diseases associated with functional abnormalities of T cell survival in children*. Clin Exp Immunol 2000;121:53-8.

10 Bias WB. *Evidence that autoimmunity in man is a Mendelian dominant trait*. Am J Hum Genet 1988;42:178-9.

11 Becker KG, Simon RM, Bailey-Wilson JE, Freidlin B, Biddison WE, McFarland HF, et al. *Clustering of non-major histocompatibility complex susceptibility candidate loci in human autoimmune diseases*. Proc Natl Acad Sci USA 1998;95:9979-84.

12 Vyse T, Todd JA. *Genetic analysis of autoimmune disease*. Cell 1996;85:311-8.

13 Neufeld M, Maclaren N, Blizzard R. *Autoimmune polyglandular syndromes*. Pediatr Ann 1980;9:154-62.

14 Powell BR, Buist NRM, Stenzel P. *An X-linked syndrome of diarrhea, polyendocrinopathy, and fatal infection in infancy*. J Pediatr 1982;100:731-7.

15 Canale VC, Smith CH. *Chronic lymphodenopathy simulating malignant lymphoma*. J Pediatr 1967;70:891-9.

16 Perheentupa J. *Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)*. Horm Metab Res 1996;28:353-6.

17 Sediva A, Cihakova D, Lebl J. *Immunological findings in patients with Autoimmune polyendocrinopathy-Candidiasis-Ectodermal dystrophy (APECED) and their family members: Are heterozygotes subclinically affected?* J Ped Endocrinol Metab 2002;15:1491-6.

18 Cetani F, Barbesino G, Borsari S, Pardi E, Cianforti L, Pinchera A, et al. *A novel mutation of the autoimmune regulator gene in an Italian kindred with Autoimmune-Polyendocrinopaty Candidiasis-Ectodermal Dystrophy, cosegregating with hypothyroid autoimmune thyroiditis*. J Ped Endocrinol Metab 2001;86:4747-52.

19 Zlotogora J, Shapiro MS. *Polyglandular autoimmune syndrome type I among Iranian Jews*. J Med Genet 1992;29:824-6.

20 Rosatelli MC, Meloni A, Devoto M, Cao A, Scott HS, Peterson P, et al. *A common mutation in Sardinian autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients*. Hum Genet 1998;103:428-34.

21 Nagamine K, Peterson P, Scott HS, Kudoh J, Minoshima S, Heino M, et al. *Positional cloning of the APECED gene*. Nat Genet 1997;17:393-8.

22 Pitkanen J, Doucas V, Sternsdorf T, Nakajima T, Aratani S, Jensen K, et al. *The autoimmune regulator protein has transcriptional transactivating properties and interacts with the common coactivator CREB-binding protein*. J Biol Chem 2000;275:16802-9.

23 Ramsey C, Winqvist O, Puhakka L, Halonen M, Moro A, Kampe O, et al. *Aire deficient mice develop multiple features of APECED phenotype and show altered immune response*. Hum Mol Genet 2002;11:397-409.

24 Pearce SH, Cheetham T, Imrie H, Vaidya B, Barnes ND, Bilous RW, et al. *A common and recurrent 13-bp deletion in the autoimmune regulator gene in British kindreds with autoimmune polyendocrinopathy type 1*. Am J Hum Genet 1998;63:1675-84.

25 Scott HS, Heino M, Peterson P, Mittaz L, Lalioti MD, Betterle C, et al. *Common mutations in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy patients of different origins*. Mol Endocrinol 1998;12:112-9.

26 Wang CY, Davoodi-Semiromi A, Huang W, Connor E, Shi JD, She JX. *Characterization of mutations in patients with autoimmune polyglandular syndrome type 1 (APS1)*. Hum Genet 1998;103:681-5.

27 Heino M, Peterson P, Kudoh J, Shimizu N, Antonarakis SE, Scott HS, et al. *APECED mutations in the autoimmune regulator (AIRE) gene*. Hum Mutat 2001;18:205-11.

28 Bjorses P, Aaltonen J, Horelli-Kuitunen N, Yaspo ML, Peltonen L. *Gene defect behind APECED: a new clue to autoimmunity*. Hum Mol Genet 1998;7:1547-53.

29 Godfrey VL, Wilkinson JE, Russell LB. *X-linked lymphoreticular disease in the scurfy (sf) mutant mouse*. Am J Pathol 1991;138:1379-87.

30 Lyon MF, Peters J, Glenister PH, Ball S, Wright E. *The scurfy mouse mutant has previously unrecognized hematological abnormalities and resemble Wiskott-Aldrich syndrome*. Proc Natl Acad Sci USA 1990;87:2433-7.

31 Kanangat S, Blair P, Reddy R, Daheshia M, Godfrey V, Rouse BT, et al. *Disease in the scurfy (sf) mouse is associated with overexpression of cytokine genes*. Eur J Immunol 1996;26:161-5.

32 Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko S, et al. *Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse*. Nat Genet 2001;27:68-73.

33 Bennett CL, Yoshioka R, Kiyosawa H, Barker DF, Fain PR, Shigeoka AO, et al. *Chance PFX-linked syndrome of polyendocrinopathy, immune dysfunction, and diarrhea maps to Xp11.23-Xq13.3*. Am J Hum Genet 2000;66:461-8.

34 Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF. *Scurfin (FOXP3) acts as a repressor of transcription and regulates T Cell activation*. J Biol Chem 2001;276:37672-9.

35 Wildin RS, Ramsdell F, Peake J, Faravalli F, Casanova JL, Buist N, et al. *X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy*. Nat Genet 2001;27:18-20.

36 Wildin RS, Smyk-Pearson S, Filipovich AH. *Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome*. J Med Genet 2002;39:537-45.

37 Ashkenazi A, Dixit VM. *Death receptors: signaling and modulation*. Science 1998;281:1305-8.

38 Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, et al. *Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor*. EMBO J 1995;14:5579-88.

39 Rieux-Laucat F, Le Deist F, Fischer A. *Autoimmune lymphoproliferative syndromes: genetic defects of apoptosis pathways*. Cell Death Differ 2003;10:124-33.

40 Nagata S, Suda T. *Fas and fas ligand: lpr and gld mutations*. Immunol Today 1995;16:39-43.

41 Nagata S, Golstein P. *The Fas death factor*. Science

1995;267:1449-56.

[42] Rieux-Laucat F, Fischer A, Le Deist F. *Cell-death signaling and human disease*. Curr Opin Immunol 2003;15:325-31.

[43] Infante AJ, Britton HA, DeNapoli T, Middelton LA, Lenardo MJ, Jackson CE, et al. *The clinical spectrum in a large kindred with autoimmune lymphoproliferative syndrome caused by a Fas mutation that impairs lymphocyte apoptosis*. J Pediatr 1998;133:629-33.

[44] Sneller MC, Wang J, Dale JK, Strober W, Middelton LA, Choi Y, et al. *Clinical, immunologic, and genetic features of an autoimmune lymphoproliferative syndrome associated with abnormal lymphocyte apoptosis*. Blood 1997;89:1341-8.

[45] Jackson CE, Puck J. *Autoimmune lymphoproliferative syndrome, a disorder of apoptosis*. Curr Opin Pediatr 1999;4:521-7.

[46] Rieux-Laucat F, Blachere S, Danielan S, De Villartay JP, Oleastro M, Solary E, et al. *Lymphoproliferative syndrome with autoimmunity: A possible genetic basis for dominant expression of the clinical manifestations*. Blood 1999;94:2575-82.

[47] Jackson CE, Fischer RE, Hsu AP, Anderson SM, Choi Y, Wang J, et al. *Autoimmune lymphoproliferative syndrome with defective Fas: genotype influences penetrance*. Am J Hum Genet 1999;64:1002-14.

[48] Wang J, Zheng L, Lobito A, Chan FK, Dale J, Sneller M, et al. *Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II*. Cell 1999;98:47-58.

[49] Chun HJ, Zheng L, Ahmad M, Wang J, Speirs CK, Siegel RM, et al. *Pleiotropic defects in lymphocyte activation caused by caspase-8 mutation lead to human immunodeficiency*. Nature 2002;419:395-9.

[50] Ramenghi U, Bonissoni S, Migliaretti G, DeFranco S, Bottarel F, Gambaruto C, et al. *Deficiency of the Fas apoptosis pathway without Fas gene mutation is a familial trait predisposing to development of autoimmune diseases and cancer*. Blood 2000;95:3176-82.

Immune System and Endocrine System a crosstalk between complex systems

The crosstalk between Nervous System (NS), Endocrine System (ES) and Immune System (IS) has been documented. Recently, a large number of experimental evidences show a tight interaction between IS and ES. In particular, the growth hormone as GH and IGF-I which play a key role in the growth and in the tissue metabolism seems to play a key role in the maintenance and in homeostasis of IS. However the mechanism of action and the role of production by leucocytes of hormones with an endocrine function still to be clarified. The aim of this phase of this research project is to clarify the biochemical and molecular mechanism of the of the network of ES and IS and to clarify if an alteration of this complex network may be implicated in the pathogenesis of Congenital Immunodeficiency (CID). Severe combined immunodeficiencies (SCIDs) represent a wide spectrum of illnesses, which differ in either the qualitative or quantitative alterations of T-, B- and natural killer- (NK-) cell (Fischer et al 1997). Most forms of SCID are associated with molecular alterations of genes selectively expressed in hematopoietic cells and implicated in cell differentiation/activation process. Thus, classical symptoms are generally considered those related to the immunological impairment that results in increased susceptibility to infections. Since patients usually die by the

first year of age without an effective treatment, the clinical phenotype is predominated by the life-threatening problems.

Functional Interaction of Common Gamma Chain and Growth Hormone Receptor Signaling Apparatus

X-linked SCID (X-SCID) is the most common form of the disease accounting for approximately half of all cases (Noguchi et al 1993; Leonard et al 2001). The gene responsible for X-SCID is *IL2RG* that encodes for the common cytokine receptor γ chain (γc), a member of the cytokine receptor class 1 superfamily**.** The molecule represents a shared component of several receptors critical for the development and function of lymphocytes (Leonard et al 2001). To our knowledge, an extrahematopoietic role of γc has not yet been demonstrated, although the abundance of the protein in non-hematopoietic cells would imply additional functions for this element (Du et al 2005; Ozawa et al 2004). We previously reported on a patient affected with a X-linked Severe Combined Immunodeficiency (X-SCID) who received a BMT late at 5.2 years of age. In this patient, short stature became evident, and a peripheral GH hyporesponsiveness associated with abnormalities of the protein phosphorylation events that occur following GH receptor (GHR) stimulation were demonstrated. The GHR was the first identified member of the cytokine receptor class 1 superfamily, which includes receptors for Erythropoietin (EPO), Granulocyte colony stimulating factor (G-CSF), Granulocyte-macrophage

colony stimulating factor (GM-CSF), Interleukins- (IL-) 2-7, IL-9, IL-11, IL-12 and many other cytokines. Due to the lack of intrinsic kinase activity, members of the cytokine receptor superfamily recruit and/or activate cytoplasmic tyrosine kinases to relay their cellular signal. The Janus associated kinase 2 (JAK2) represents the predominant non receptor tyrosine kinase required for the initiation of GH signal transduction upon ligand binding to the receptor (Argetsinger et al 1993; Zhu et al 2001). Signal transduction through GHR also involves a wide array of molecules, such as Signal Transducers and Activators of Transcription (STATs) 1, 3 and 5, Extracellular regulated kinase 1 and 2 (ERK1 and 2) and Phosphatidylinositol-3 kinase-protein kinase B (Zhu et al 2001). Activation of STAT5b is considered a prominent event in GH signaling and is crucial for the regulation of transcription of GH-responsive genes, including the gene encoding for insulin-like growth factor-I (IGF-I), which mediates many of the GH biological functions (Clark et al 1997; Dorshkind et al 2000; Le Roith et al 2001). In our previous study, mutational screening and expressional analysis failed to reveal any molecular alteration of *GHR*, *JAK2* and *STAT5A/B* genes in the patient with X-SCID and peripheral GH hyporesponsiveness (Ursini et al 2002).

Since we hypothesized a role for the **γc** chain in GHR signaling, in this study, we evaluate the functional interaction between GHR and the common γ element in either freshly isolated or Epstein-Barr virus (EBV) transformed lymphocytes

from X-SCID patients and healthy subjects. In particular, the functional response to GH stimulation, the pattern of GHR induced protein tyrosine phosphorylation and GH induced translocation from the cytoplasm to the nucleus of STAT5 were evaluated. We demonstrate the existence of a previously unappreciated functional interaction between γc and GHR. This interaction leads to the activation and intranuclear translocation of STAT5b protein. The preliminary results showed in the control subjects a phosphorilation of STAT5B after GH stimulation while in the cells of the patients was not present. Moreover in the cellular line gc- there wasn't any nuclear migration of STAT5B after GH stimulation while in the cells of the control subjects the migration was possible after 30 min of GH stimulation. Our study demonstrates the existence of a previously unappreciated relationship between individually well studied elements, such as GHR and γc, and signaling pathways. Crosstalk between receptor signaling systems is now emerging as an important and exciting area of signaling research. These results has been submitted for publication to *Journal of Immunology*.

Refernces

1. Leonard, W. J. 2001. Cytokines and immunodeficiency diseases. *Nat Rev Immunl 1:200*.

2. Du, C., Q. Guan, Z. Yin, R. Zhong, and A. M. Jevnikar. 2005. IL-2-mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP. *Kidney Int 67:1397*.

3. Ozawa, A., H. Tada, Y. Sugawara, A. Uehara, T. Sasano, H. Shimauchi, H. Takada, and S. Suguwara. 2004. Endogenous IL-15 sustains recruitment of IL-2Rbeta and common gamma and IL-2-mediated chemokine production in normal and inflamed human gingival fibroblast. *J Immunol 173:5180*.

4. Zhu, T., E. L. K. Goh, R. Graichen, L. Ling, and P. E. Lobie. 2001. Signal transduction via the growth hormone receptor. *Cell Signal 13:599*.

5. Clark, R. 1997. The somatogenic hormones and insulin-like growth factor-1: Stimulators of lymphopoiesis and immune function. *Endocr Rev 18:157*.

6. Le Roith, D., C. Bondy, S. Yakar, J. L. Liu, and A. Butler. 2001. The somatonedin hypothesis: 2001. *Endocr Rev 22:53*.

7. Ursini, M. V., L. Gaetaniello, R. Ambrosio, E. Matrecano, A. J. Apicella, M. C. Salerno, and C. Pignata. 2002. Atypical X-linked SCID phenotype

associated with growth hormone hyporesponsiveness. *Clin Exp Immunol 129:502*.

8. Argetsinger, L. S., G. S. Campbell, X. Yang, B. A. Witthuhn, O. Silvennoinen, J. N. Ihle, and C. Carter-Su. 1993. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell 74:237*.

Title: Functional Interaction of Common Gamma Chain and Growth Hormone Receptor Signaling Apparatus[1]

Running title: Functional interaction between γc and GHR.

Authors: Marsilio Adriani,[*] Corrado Garbi,[†] Giada Amodio,[*] Rosanna Busiello,[*] Eliana Matrecano,[*] Elena Cosentini,[‡] Fabio Candotti,[§] and Claudio Pignata[22*]

Affiliations: [*]Department of Pediatrics, [†]Department of Cellular and Molecular Biology and Pathology and [‡]Immunohematology Unit, "Federico II" University, Naples, Italy and [§]Genetics and Molecular Biology Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA.

Keywords: B Cells, Immunodeficiency Diseases, Cell Activation, Cytokine Receptors, Signal Transduction

[1] This work was supported by the Grants "Ministero della Salute - Roma and Regione Campania, Legge 502" and MURST-PRIN 2002.

[2] Address correspondence and reprint request to Dr. Claudio Pignata, Department of Pediatrics, Unit of Immunology, "Federico II" University, via S. Pansini 5-80131, Naples, Italy. Phone # +39-081-7464340; Fax # +39-081-5451278; E-mail:pignata@unina.it

Abstract

We previously reported on an X-linked SCID patient, who also had peripheral growth hormone (GH) hypo-responsiveness and abnormalities of the protein phosphorylation events following GH receptor (GHR) stimulation. In the present study, we examined a potential role of common cytokine receptor γ chain (γc) in GHR signaling using EBV transformed lymphocytes from healthy subjects and γc negative X-SCID patients. We demonstrated that the proliferative response to GH stimulation of γc negative patients B-cell lines was impaired despite a comparable cellular expression of GHR molecules to controls. In patients, after GH stimulation no phosphorylation of STAT5 was observed. In addition, the molecule localization through confocal microscopy revealed that in patient's B-cell lines no nuclear translocation of STAT5b following GH stimulation occurred differently from controls. Biochemical analysis of the nuclear extracts of γc negative cell lines provided further evidence that the amount of STAT5b and its phosphorylated form did not increase following GH stimulation. Our study demonstrates the existence of a previously unappreciated relationship between GHR signaling pathway and γc, which is required for the activation of STAT5b in B cell lines. These data also confirm that growth failure in X-SCID is primarily related to the genetic alteration of the *IL2RG* gene.

Introduction

Severe combined immunodeficiencies (SCIDs)[3] represent a wide spectrum of illnesses, which differ in either the qualitative or quantitative alterations of T-, B- and natural killer- (NK-) cell (1). Most forms of SCID are associated with molecular alterations of genes selectively expressed in hematopoietic cells and implicated in cell differentiation/activation process. Thus, classical symptoms are generally considered those related to the immunological impairment that results in increased susceptibility to infections. Since patients usually die by the first year of age without an effective treatment, the clinical phenotype is predominated by the life-threatening problems.

X-linked SCID (X-SCID) is the most common form of the disease accounting for approximately half of all cases (2, 3). The gene responsible for X-SCID is *IL2RG* that encodes for the common cytokine receptor γ chain (γc), a member of the cytokine receptor class 1 superfamily**.** The molecule represents a shared component of several receptors critical for the development and function of lymphocytes (3). To our knowledge, an extrahematopoietic role of γc has not

[3] Abbreviations used in this paper: GH, growth hormone; GHR, GH receptor; γc, common cytokine receptor γ chain; EPO, erythropoietin; IGF-I, insulin-like growth factor-I; BCLs, EBV transformed lymphocytes.

yet been demonstrated, although the abundance of the protein in non-hematopoietic cells would imply additional functions for this element (4, 5).

We previously reported on a patient affected with a X-linked Severe Combined Immunodeficiency (X-SCID) who received a bone marrow transplantation (BMT) late at 5.2 years of age. In this patient, short stature became evident, and a peripheral growth hormone (GH) hyporesponsiveness associated with abnormalities of the protein phosphorylation events that occur following GH receptor (GHR) stimulation were demonstrated.

The GHR was the first identified member of the cytokine receptor class 1 superfamily, which includes receptors for Erythropoietin (EPO), Granulocyte colony stimulating factor (G-CSF), Granulocyte-macrophage colony stimulating factor (GM-CSF), Interleukins- (IL-) 2-7, IL-9, IL-11, IL-12 and many other cytokines. Due to the lack of intrinsic kinase activity, members of the cytokine receptor superfamily recruit and/or activate cytoplasmic tyrosine kinases to relay their cellular signal. The Janus associated kinase (JAK) 2 represents the predominant non receptor tyrosine kinase required for the initiation of GH signal transduction upon ligand binding to the receptor (6, 7). However, GH also stimulates tyrosine phosphorylation of JAK1 (8, 9) and JAK3 (10) in certain cell lines. Signal transduction through GHR also involves a wide array of molecules, such as Signal Transducers and Activators of Transcription (STATs) 1, 3 and 5, Extracellular regulated kinase (ERK) 1 and 2 and

Phosphatidylinositol-3 kinase-protein kinase B (7). Activation of STAT5b is considered a prominent event in GH signaling and is crucial for the regulation of transcription of GH-responsive genes, including the gene encoding for insulin-like growth factor-I (IGF-I), which mediates many of the GH biological functions (11-13). In our previous study, mutational screening and expressional analysis failed to reveal any molecular alteration of *GHR*, *JAK2* and *STAT5A/B* genes in the patient with X-SCID and peripheral GH hyporesponsiveness (14).

Since we hypothesized a role for the **γc** chain in GHR signaling, in this study, we evaluate the functional interaction between GHR and the common γ element in either freshly isolated or Epstein-Barr virus (EBV) transformed lymphocytes from X-SCID patients and healthy subjects. In particular, the functional response to GH stimulation, the pattern of GHR induced protein tyrosine phosphorylation and GH induced translocation from the cytoplasm to the nucleus of STAT5 were evaluated. We demonstrate the existence of a previously unappreciated functional interaction between γc and GHR. This interaction leads to the activation and intranuclear translocation of STAT5b protein.

Materials and Methods

Reagents

Recombinant human growth hormone (rGH) was obtained from Serono (Saizer® 4). The enhanced chemiluminescence kit (ECL) was purchased from Amersham Biosciences. The antibodies anti-STAT5b, anti-STAT5a, anti-STAT1, anti-STAT3, anti-ERK (recognizing both ERK1 and ERK2), anti-phosphotyrosine ERK, anti-GHR and anti-γc and the monoclonal-antibodies anti-phosphotyrosine were purchased from Santa Cruz Biotechnology (Santa Cruz). Acrylamide and bisacrylamide were from Gibco. Prestained molecular weight standards were from BIORAD. Except where noted, other reagents were of either reagent or molecular biological grade from Sigma.

Cells and cell cultures

Mononuclear cells (PBMC) were obtained from 4 X-SCID patients and normal donors heparinized peripheral blood by Ficoll-Hypaque (Biochrom) density gradient centrifugation. Upon informed consent, lymphoblastoid cell lines (BCLs) were generated by Epstein-Barr virus immortalization of patients and controls PBMC using standard procedures (15). In all cases, γc mutations led to the absence of protein expression. Cells were maintained in RPMI 1640 (Biochrom) supplemented with 10% fetal bovine serum (Gibco), 2 mmol/L L-

glutammine (Gibco), and 50 μg/ml gentamycine (Gibco), and cultured at 37°C, 5% CO_2.

Proliferative assay

BCLs (1 X 10^5 cell/200 μl well) were cultured triplicate in 96-well U-bottomed microtiter plates (Falcon®, Becton Dickinson) with or without rGH at reported concentrations for 4 days. The proliferative response was evaluated by thymidine uptake from cultured cells pulsed with 0.5 μCi [^{3}H]thymidine (Amersham International) 8 h before harvesting (16).

Flow cytometry

The expression of GHR was detected using specific rabbit antibodies (Santa Cruz) by indirect immunofluorescence using a second-step incubation with a FITC-conjugated donkey anti-rabbit antibodies (Pierce). After washing in PBS, cells were incubated for 20 min with the specific antibodies and 30 min with secondary antibodies. After staining, all samples were washed in PBS and acquired on the FACScan flow cytometer (Becton-Dickinson) using Lysis I software.

Cell stimulation and protein extraction

Prior to hormone treatment, the cells were made quiescent through incubation in RPMI 1640 minus serum for 8-12 h. GH was used at 37°C at a concentration of 500 ng/ml in RPMI 1640 for the reported time. Incubations were terminated by washing cells with ice cold phosphate-buffer saline (PBS; BioWhittaker) followed by solubilization in 100 µl of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mmol EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadatum (Na_3VO_4), 5 µg/ml leupeptin and 5 µg/ml aprotinin. The cell lysates were stored at -80°C for Western blot analysis. Nuclear extracts were prepared by the method of Andrews et al. (17) and were subsequently mixed with sample buffer.

Western blot

Immunoblotting using phosphotyrosine monoclonal antibody was performed as previously reported (14). Immunoblotting using specific antibody was performed according to the vendors protocols. In brief, protein samples separated by SDS-PAGE were transferred onto Mixed Cellulose Esters membranes (Immobilon-NC Mixed Cellulose Esters 0.45 µm; Millipore). The membrane was incubated at room temperature for 1 h in blocking buffer consisting of 10% Bovin Serum Albumin (BSA) in wash buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20). The membrane was then washed three times in wash buffer and incubated 1 h at room temperature or overnight at 4°C

with the specific antibody. The membrane was then washed three times and an appropriate IgG horseradish peroxidase-coniugated secondary antibody was used for the second incubation. After further washings, the membrane was developed with ECL developing reagents, and exposed to x-ray films according to the manufacturer's instructions (Amersham Biosciences).

Confocal microscopy

After appropriate stimulation, quiescent cells were rinsed in ice cold PBS and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) for 30 min at room temperature. After 4 rinses of 5 min in PBS, the cells were centrifuged in a Shandon Cytospin III (Histotronix Inc.) onto glass slide and permeabilized by incubation in a 0.2% Triton X-100 solution for 20 min. The cells were then incubated for 1 h at room temperature with rabbit antibodies against STAT5b diluted 1:100 in PBS containing 1% BSA. After 4 washings for 5 min in PBS, the cells were incubated for 1 h at room temperature with a 1:200 dilution of Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Pierce Biotechnology Inc.) in PBS. After washing in PBS, the glass slides were mounted under a coverslip in a 50% glycerol/50% PBS solution. The slides were analyzed by laser scanning Confocal microscopy, using a Zeiss LSM 510 version 2.8 SP1 Confocal System.

Results

Effect of GH on the proliferative response of EBV transformed cell lines from normal subjects and γc negative X-SCID patients

It has been reported that GH enhances EBV transformed cell lines proliferation in vitro**,** its effect being direct and not mediated by IGF-I (16). Thus, to evaluate a biological role of γc in GHR signaling, we evaluated the response of

EBV transformed lymphocytes (BCLs) from γc negative X-SCID patients and normal controls to GH stimulation. As shown in Fig. 1A, GH enhanced proliferation of BCLs of control subjects in a dose-dependent fashion. Significant enhancement of [^{3}H]thymidine uptake was observed at a GH concentration of 50 ng/ml, and the maximal effect was achieved at 200 ng/ml. In contrast, γc negative BCLs didn't respond at any GH concentration. To rule out that the observed phenomena were due to different numbers of the receptor molecules on the cell membrane, GHR expression was evaluated by flow cytometry analysis on control's and patient's cells (Fig. 1B). No difference was found in the mean fluorescence intensity (130.99 $\pm$ 28.19 vs 139.88 $\pm$ 33.49 in patients and controls, respectively; p = NS) and in the percentage of positively stained cells (99.6 vs 99.7% and 99.8 vs 99.9%, respectively).

Pattern of protein tyrosine phosphorylation induced through GHR engagement in patients and controls cells

We next investigated the overall signal transduction properties of patients and control BCLs following GHR ligation by analysing the number and the timing of the proteins phophorylated on tyrosine residues. Fig. 2 illustrates a representative immunoblot with anti-phosphotyrosine antibodies of whole cell lysates from patient's and control's BCLs following stimulation with GH for 5, 15 or 30 min. In contrast to what observed in control cells, in patients GH

stimulation failed to induce phosphorylation of proteins approximately of 90 kDa, presumably corresponding to STATs molecules involved in the signal transduction through GHR. This pattern of protein tyrosine phophorylation was also observed in freshly isolated PBMC from a healthy subject and a patient stimulated with the same concentration and for the same time, thus confirming the observation on BCLs (data not shown).

GHR signal transduction pattern

The 3 main signaling modules by which signal transduction through GHR occurs involve Mitogen-activated protein kinases/ERK1 and 2, STAT and the Phosphatidylinositol-3 kinase-protein kinase B signaling (7).

To evaluate whether the γc was involved in the GHR signaling events, we next focused on STAT5 molecule. As shown in Fig. 3A**,** in control's BCLs, tyrosine phosphorylation of STAT5 was evident, with a peak of activity observed between 5 and 15 min after GH stimulation. By contrast, in patient's BCLs no phosphorylation of STAT5 was detectable after stimulation. In all cell lines examined, STAT5b and STAT5a protein expression was comparable in patients and controls.

To define whether the blockage in GHR signaling was specific of STAT5 or involved other molecules as well, we then studied ERKs phosphorylation (Fig. 3B). No difference of ERK1 and 2 phosphorylation between patient' and

control' BCLs was appreciable. As shown, the expression of the molecules was comparable in control and patient cells

STAT5 nuclear translocation after GHR triggering

Recently it has been reported that tyrosine phosphorylation of STATs molecules was not sufficient for the activation of the protein (18, 19). Since the activated STAT5 translocates into the nuclei, confocal microscopy was initially used to test the subcellular localization of STAT5b in control and patient γc negative cells under resting conditions and after stimulation with GH.

Patients and controls BCLs were stimulated with GH for 30 min, fixed and incubated with antiserum against STAT5b. As shown in Fig. 4, under basal conditions all cells displayed fluorescent staining of the cytoplasm indicating the presence of STAT5b in this compartment, and only a negligible staining of nucleus indicating absence of STAT5b in this compartment. Stimulation with GH for the time indicated induced nuclear translocation of STAT5b in the control's BCLs, as demonstrated by the marked increase in STAT5b immunoreactivity within the nucleus and not in γc negative BCLs.

We next evaluated by immunoblot of nuclear and cytoplasmic extracts the amount of STAT5b translocation and compared it with the tyrosine phosphorylation of the molecule. As shown in Fig. 5, in controls cells GH stimulation determined a rapid increase of nuclear STAT5b amount. The

translocation occurred early being evident 5 min after GH stimulation. Moreover, it still persisted 30 min after the stimulation. The translocation paralleled the amount of the tyrosine phosphorylated form of the protein into the nuclei. This was inversely correlated with the amount of the cytoplasmic form of the molecule. However, after 30 min the reconstitution of the cytoplasmic aliquot became evident. In the patient cells no changes were observed

Discussion

In the present study, we examined a potential role of the common cytokine receptor γ chain (γc) in GHR signaling using BCLs from healthy control subjects and γc negative X-SCID patients.

At a functional level, GH enhanced proliferation of control BCLs in a dose dependent fashion. By contrast, the functional response to GH of γc negative patients BCLs was severely impaired despite a comparable cellular expression of GHR molecules.

The overall signal transduction properties of GHR in X-SCID patients and control BCLs following GH stimulation was also examined by analysing the pattern of protein tyrosine phosphorylation. In contrast to what observed in control BCLs, in patients, GH stimulation failed to induce phosphorylation of proteins of 90 kDa identified as belonging to the STAT molecules family, involved in the signal transduction through GHR. In particular, after GH stimulation no phosphorylation of STAT5 protein was observed in γc negative patients cell lines in contrast to the control cells, in which a prompt activation of STAT5 occurred**.**

STAT-dependent pathways are generally believed to be utilized in cellular events such as cell proliferation, differentiation and apoptosis (20, 21), even though the overall role of the STAT molecules in GHR signal transduction has not been fully elucidated. At least three different STAT family members

(STAT1, STAT3 and STAT5) are activated following GHR perturbation (22-27), even though STAT5 seems to play a prominent role in receptor signaling. Rodent models of STAT knockouts (28) and the recent identification of a patient with a homozygous missense mutation of STAT5b gene indicate that STAT5b is essential for a normal postnatal linear growth (29). Furthermore, the patient with STAT5b mutation also had clinical features of immune deficiency such as chronic diarrhea and severe infections, including interstitial pneumopathy. Immunologic studies showed hypergammaglobulinemia and markedly decreased IL-2Rα chain expression in response to IL-2 stimulation, suggestive of a T-Cell activation defect. Thus, a few features are similar to γc negative X-SCID patients.

Although the activation of JAKs and STATs represents a prominent biochemical event during GH-dependent proliferation of lymphoid cell lines (30), other signaling pathways also contribute to a full GHR response. GH has been shown to activate the phosphatidylinositol-3 kinase-protein kinase B signaling (31), mitogen-activated protein kinases and extracellular regulated kinases 1 and 2 (ERK1 and 2) (32-34). In both STAT5 knockout mouse and in the patient with STAT5 mutation, these pathways are fully functional. In keeping with this observation, also in our experimental model, no alteration was observed in ERK 1 and 2 expression and phosphorylation that occur following GHR triggering. An increase in total STAT1 and STAT3 concentrations was

observed in STAT5 knockout rodent models and in the fibroblast cell line originated from STAT5 mutated patient (29, 35). In our experimental condition, no other alteration of the GH-induced signaling through STAT pathways was observed (data not shown). Taken together, these observations imply that GHR, as well as other receptors, is able to integrate different pathways which are individually differentially regulated. In support of this, it has been recently shown that GHR signaling and the subsequent IGF-I transcription regulation are under different regulatory controls in hepatocytes, fibroblasts and myoblasts (36). This could lead to hypothesize differential functions of an individual receptor exerted in different tissues. A cell type-restricted STATs activation has been reported (37-39). STAT5 is not activated following GH stimulation in human fibrosarcoma cells even though these cells express the STAT5 protein (39), thus implying that a selectivity in the involvement of specific STAT subset seems to be a general feature of GHR signal transduction.

Overall, activation of STAT5b is considered a prominent event in GHR signaling and is crucial for the regulation of transcription of GH-responsive genes, including the gene encoding for IGF-I. This process relies on an appropriate phosphorylation and nuclear translocation of the molecule (7, 40). Recently, it has been proposed that STATs tyrosine phosphorylation and nuclear translocation are two events that are regulated separately (19). In particular, Giron-Michel J. and coworkers demonstrated in the hybrid receptor γc/GM-

CSFRβ that γc/JAK3 complex controls the nuclear translocation of pSTAT5 rather than STAT5 phosphorylation itself (41). Hence to address the issue of defining the functional implication of γc mutation on STAT5b activation, in our study, the subcellular localization of STAT5b was investigated by analyzing cytokine-induced translocation of STAT5b from the cytoplasm to the nucleus with confocal microscopy. Stimulation with GH induced nuclear translocation of STAT5b in the control cells, whereas no efficient nuclear translocation occurred in γc negative cells. Furthermore, immunoblot of nuclear and cytoplasmic extracts showed in control cells a rapid increase of the nuclear fraction of the STAT5 molecule after GH stimulation, which paralleled the molecule phosphorylation, differently from what observed in patient cells.

It is to note that none of the sequence motifs implicated in the STAT-receptor interaction is present in the GHR. Therefore, STAT interaction with the GHR has to be mediated through adaptor molecules as SHC proteins (42, 43), JAKs-STAT direct association or, alternatively, through additional adaptor subunits yet not identified (39).

Our data suggest that the γc chain is a required signaling subunit of the GHR complex in B cell lines. In particular, in this cell line, it is selectively required for STAT5 phosphorylation and nuclear translocation, and not for the activation of other molecules as ERKs.

Our study demonstrates the existence of a previously unappreciated relationship between individually well studied elements, such as GHR and γc, and signaling pathways. Crosstalk between receptor signaling systems is now emerging as an important and exciting area of signaling research. Whether the participation of γc to the GHR confers some additional properties to the receptor in hematopoietic cell differentiation and functioning remains to be elucidated. To note, in CD34+ progenitors, γc participates to hematopoietic cell differentiation by interacting with GM-CSFRβ. This interaction does not occur in normal natural killer cells or non hematopoietic cells (41). Hence, the complexity of receptor signaling relies not only on the possibility that individual receptors interact one with each other, but also on a differential array of distinct subunits that may represent a hallmark of that specific cell type.

Our current study also leads to explain what our group previously reported on an atypical X-SCID phenotype and severe short stature associated with growth hormone hypo-responsiveness and abnormal GHR induced protein tyrosine phosphorylation (14), and indicates that growth failure in X-SCID is directly related to the genetic alteration.

Acknowledgments

We thank Emanuela Minopoli for technical assistance.

References

1. Fischer, A., M. Cavazzana-Calvo, G. De Saint Basile, J. P. DeVillartay, J. P. Di Santo, C. Hivroz, F. Rieux-Laucat, and F. Le Deist. 1997. Naturally occurring primary deficiencies of the immune system. *Annu Rev Immunol 15:93*.

2. Noguchi, M., H. Yi, H. M. Rosenblatt, A. H. Filipovich, S. Adelstein, W. S. Modi, O. W. McBride, and W. J. Leonard. 1993. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell 73:147*.

3. Leonard, W. J. 2001. Cytokines and immunodeficiency diseases. *Nat Rev Immunl 1:200*.

4. Du, C., Q. Guan, Z. Yin, R. Zhong, and A. M. Jevnikar. 2005. IL-2-mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP. *Kidney Int 67:1397*.

5. Ozawa, A., H. Tada, Y. Sugawara, A. Uehara, T. Sasano, H. Shimauchi, H. Takada, and S. Suguwara. 2004. Endogenous IL-15 sustains recruitment of IL-2Rbeta and common gamma and IL-2-mediated chemokine production in normal and inflamed human gingival fibroblast. *J Immunol 173:5180*.

6. Argetsinger, L. S., G. S. Campbell, X. Yang, B. A. Witthuhn, O. Silvennoinen, J. N. Ihle, and C. Carter-Su. 1993. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell 74:237*.

7. Zhu, T., E. L. K. Goh, R. Graichen, L. Ling, and P. E. Lobie. 2001. Signal transduction via the growth hormone receptor. *Cell Signal 13:599*.

8. Smit, L. S., D. J. Meyer, N. Billestrup, G. Norstedt, J. Schwartz, and C. Carter-Su. 1996. The role of the growth hormone (GH) receptor and JAK1 and JAK2 kinases in the activation of Stats 1, 3, and 5 by GH. *Mol Endocrinol 10:519*.

9. Hellgren, G., K. Albertsson-Wikland, H. Billig, L. M. Carlsson, and B. Carlsson. 2001. Growth hormone receptor interaction with Jak proteins differs between tissues. *J Interferon Cytokine Res 21:75*.

10. Johnston, J. A., M. Kawamura, R. A. Kirken, Y. Q. Chen, T. B. Blake, K. Shibuya, J. R. Ortaldo, D. W. McVicar, and J. J. O'Shea. 1994. Phosphorylation and activation of the Jak-3 Janus kinase in response to interleukin-2. *Nature 370:151*.

11. Clark, R. 1997. The somatogenic hormones and insulin-like growth factor-1: Stimulators of lymphopoiesis and immune function. *Endocr Rev 18:157*.

12. Dorshkind, K., and N. D. Horseman. 2000. The roles of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones in

lymphocyte development and function: Insights from genetic models of hormone and hormone receptor deficiency. *Endocr Rev 21:292*.

13. Le Roith, D., C. Bondy, S. Yakar, J. L. Liu, and A. Butler. 2001. The somatonedin hypothesis: 2001. *Endocr Rev 22:53*.

14. Ursini, M. V., L. Gaetaniello, R. Ambrosio, E. Matrecano, A. J. Apicella, M. C. Salerno, and C. Pignata. 2002. Atypical X-linked SCID phenotype associated with growth hormone hyporesponsiveness. *Clin Exp Immunol 129:502*.

15. Moss, D. J., A. B. Rickinson, and J. H. Pope. 1978. Long-term T-cell-mediated immunity to Epstein-Barr virus in man. I. Complete regression of virus-induced transformation in cultures of seropositive donor leukocytes. *Int J Cancer 22:662*.

16. Kimata, H., and A. Yoshida. 1994. Effect of growth hormone and insulin-like growth factor-I on immunoglobulin production by and growth of human B cells. *J Clin Endocrinol Metab 78:635*.

17. Andrews, N. C., and D. V. Faller. 1991. A rapid micropreparation technique for extraction of DNA-binding proteins from limit numbers of mammalian cells. *Nucleic Acids Res 19:2499*.

18. Wery-Zennaro, S., M. Letourneur, D. M., J. Bertoglio, and J. Pierre. 1999. Binding of IL-4 to the IL-13Ralpha(1)/IL-4Ralpha receptor

complex leads to STAT3 phosphorylation but not to its nuclear translocatio. *FEBS Lett 464:91*.

19. Ali, S., and S. Ali. 1998. Prolactin receptor regulates Stat5 tyrosine phosphorylation and nuclear translocation by two separate pathways. *J Biol Chem 273:7709*.

20. Battle, T. E., and D. A. Frank. 2002. The role of STATs in apoptosis. *Curr Mol Med 2:381*.

21. Calo, V., M. Migliavacca, V. Bazan, M. Macaluso, M. Buscemi, N. Gebbia, and A. Russo. 2003. STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol 197:157*.

22. Sotiropoulos, A., S. Moutoussamy, N. Binart, P. A. Kelly, and J. Finidori. 1995. The membrane proximal region of cytoplasmic domain of the growth hormone receptor is involved in the activation od Stat 3. *FEBS Lett 369:169*.

23. Campbell, G. S., D. J. Meyer, R. Raz, D. E. Levy, J. Schwartz, and C. Carter-Su. 1995. Activation of acute phase response factor (APRF)/Stat3 transcription factor by growth hormone. *J Biol Chem 270:3974*.

24. Hansen, J. A., L. H. Hansen, X. Wang, J. J. Kopchick, F. Gouilleux, B. Groner, J. H. Nielsen, A. Moldrup, E. D. Galsgaard, and N. Billestrup. 1997. The role of GH receptor tyrosine phosphorylation in Stat5 activation. *J Mol Endocrinol 18:213*.

25. Gouilleux, F., C. Pallard, I. Dusanter-Fourt, H. Wakao, L. A. Haldosen, G. Norstedt, D. Levy, and B. Groner. 1995. Prolactin, growth hormone, erythropoietin and granulocyte-macrophage colony stimulating factor induce MGF-Stat5 DNA binding activity. *EMBo J 14:2005*.

26. Sotiropoulos, A., S. Moutoussamy, F. Renaudie, M. Clauss, C. Kayser, F. Gouilleux, P. A. Kelly, and J. Finidori. 1996. Differential activation of Stat3 and Stat5 by distinct regions of the growth hormone receptor. *Mol Endocrinol 10:998*.

27. Choi, H. K., and D. J. Waxman. 2000. Pulsatility of growth hormone (GH) signaling in liver cells: role of the JAK-STAT5b pathway in GH action. *Growth Horm IGF Res:S1*.

28. Teglund, S., C. McKay, E. Schuetz, J. M. van Deursen, D. Stravopodis, D. Wang, M. Brown, S. Bodner, G. Grosveld, and J. N. Ihle. 1998. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell 93:841*.

29. Kofoed, E. M., V. Hwa, B. Little, K. A. Woods, C. K. Buckway, J. Tsubaki, K. L. Pratt, L. Bezrodnik, H. Jasper, A. Tepper, J. J. Heinrich, and R. G. Rosenfeld. 2003. Growth hormone insensitivity associated with a STAT5b mutation. *N Engl J Med 349:1139*.

30. Wang, Y. D., and W. I. Wood. 1995. Amino acids of the human growth hormone receptor that are required for proliferation and Jak-STAT signaling. *Mol Endocrinol 9:303*.

31. Kilgour, E., I. Gout, and N. G. Anderson. 1996. Requirement for phosphoinositide 3-OH kinase in growth hormone signaling to the mitogen-activated protein kinase and $p70^{s6k}$ pathways. *Biochem J 315:517*.

32. Winston, L. A., and P. J. Bertics. 1992. Growth hormone stimulates the tyrosine phosphorylation of 42- and 45-kDa ERK-related proteins. *J Biol Chem 267:4747*.

33. Ji, S., S. J. Frank, and J. L. Messina. 2002. Growth hormone-induced differential desensitization of STAT5, ERK, and Akt phosphorylation. *J Biol Chem 277:28384*.

34. Hodge, C., J. Liao, M. Stofega, K. Guan, C. Carter-Su, and J. Schwartz. 1998. Growth hormone stimulates phosphorylation and activation of elk-1 and expression of c-fos, egr-1, and junB through activation of extracellular signal-regulated kinases 1 and 2. *J Biol Chem 273:31327*.

35. Udy, G. B., R. P. Towers, R. G. Snell, R. J. Wilkins, S. H. Park, P. A. Ram, D. J. Waxman, and H. W. Davey. 1997. Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc Natl Acad Sci USA 94:7239*.

36. Frost, R. A., G. J. Nystrom, and C. H. Lang. 2002. Regulation of IGF-I mRNA and Signal Transducers and Activators of Transcription-3 and -5 (Stat-3 and -5) by GH in C2C12 myoblasts. *Endocrinology 143:492*.

37. Finbloom, D. S., E. F. r. Petricoin, R. H. Hackett, M. David, G. M. Feldman, K. Igarashi, E. Fibach, M. J. Weber, M. O. Thorner, C. M. Silva, and A. Larner. 1994. Growth hormone and erythropoietin differentially activate DNA-binding proteins by tyrosine phosphorylation. *Mol Cell Biol 14:2113*.

38. Silva, C. M., H. Lu, M. J. Weber, and M. O. Thorner. 1994. Differential tyrosine phosphorylation of JAK1, JAK2, and STAT1 by growth hormone and interferon-gamma in IM-9 cells. *J Biol Chem 269:27532*.

39. Han, Y., D. W. Leaman, D. Watling, N. C. Rogers, B. Groner, I. M. Kerr, W. I. Wood, and G. R. Stark. 1996. Participation of JAK and STAT proteins in growth hormone-induced signaling. *J Biol Chem 271:5947*.

40. Brelje, T. C., L. E. Stout, N. V. Bhagroo, and R. L. Sorenson. 2004. Distinctive roles for prolactin and growth hormone in the activation of STAT5 in pancreatic islets of langerhans. *Endocrinology 154:4162*.

41. Giron-Michel, J., M. Fogli, A. Gaggero, S. Ferrini, A. Caignard, D. Brouty-Boyé, S. Baouz, M. C. Le Bousse-Kerdilès, B. Pèault, M. van Dijk, S. Bulfone-Paus, D. Durali, S. Chouaib, and B. Azzarone. 2003.

Detection of a functional hybrid receptor γc/GM-CSFRβ in human hematopoietic CD34+ cells. *J Exp Med 197:763*.

42. VanderKuur, J., G. Allevato, N. Billestrup, G. Norstedt, and C. Carter-Su. 1995. Growth hormone-promoted tyrosyl phosphorylation of SHC proteins and SHC association with Grb2. *J Biol Chem 270:7587*.

43. Kim, S. O., K. Loesch, X. Wang, J. Jiang, L. Mei, J. M. Cunnick, J. Wu, and S. J. Frank. 2002. A role for Grb2-associated binder-1 in growth hormone signaling. *Endocrinology 143:4856*.

Figure legends

FIGURE 1. In vitro effect of GH stimulation on proliferation of X-SCID patients' and controls' EBV cell lines. *A,* Lymphoblastoid cell lines (BCLs) were generated by Epstein-Barr virus immortalization of patients' and controls' PBMC using standard procedures (15) and cultured in the presence of various concentrations of GH for 4 days. Cultures were pulsed with [^{3}H]thymidine for the final 8 h and radioactive incorporation counted. Results are expressed as the increase of cpm from the background. Vertical bars indicates 1 SD. *B,* The flow cytometry analysis indicating that the expression levels of GHR are comparable in controls and patients.

FIGURE 2. Pattern of protein tyrosine phosphorylation induced through GHR engagement. BCLs from X-SCID patients and healthy subjects were starved of serum for 8-12 h and then stimulated with GH (500 ng/ml) at 37°C for the indicated time. Stimulation was stopped with cold PBS and BCLs were resuspended in lysis buffer. After SDS-PAGE and Western blot, membranes were incubated with anti-phosphotyrosine antibodies.

FIGURE 3. STAT5 and ERKs phosphorylation induced through GHR stimulation. rGH stimulation failed to induce STAT5 tyrosine phosphorylation but not ERKs activation in γc negative BCLs. BCLs from X-SCID patients and

healthy subjects were starved of serum for 8-12 h and then stimulated with GH (500 ng/ml) at 37°C for the indicated time. After SDS-PAGE and Western blot, membranes were incubated with (*A*) anti-pSTAT5, anti-STAT5a or anti-STAT5b or (*B*) anti-ERK (recognizing both ERK1 and ERK2) or anti-phosphotyrosyl ERK antibodies.

FIGURE 4. STAT5b subcellular localization. X-SCID patients and healthy subjects control cells were cultured in the absence or presence of 500 ng/ml rGH for 30 min at 37°C. Unstimulated or stimulated cells were analyzed by Confocal microscopy for STAT5b (green) distribution in the cell, focusing particularly on whether this protein was present in the nuclei.

FIGURE 5. Nuclear fraction of the overall STAT5b amount and of the phosphorylated form of STAT5 in resting or rGH stimulated BCLs. Patient and control BCLs were stimulated with rGH (500 ng/ml) or medium alone at 37°C for the indicated time. Stimulation was stopped with cold PBS and nuclei were isolated as described in Materials and methods. After SDS-PAGE of nuclear and cytoplasmic extracts and Western blot, membranes were incubated with anti-STAT5b or anti-pSTAT5 antibodies.

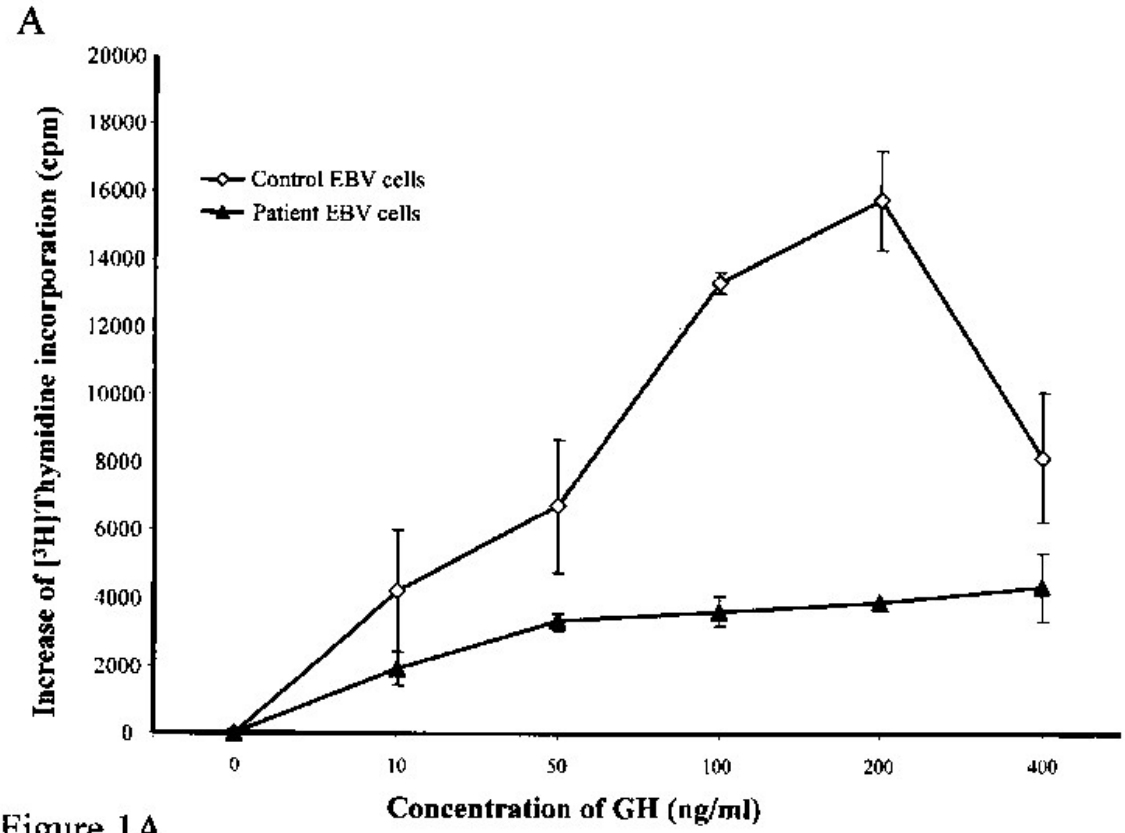

A
Increase of [3H]Thymidine incorporation (cpm)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Control EBV cells
Patient EBV cells
0
10
50
100
200
400
Concentration of GH (ng/ml)

Figure 1A

B

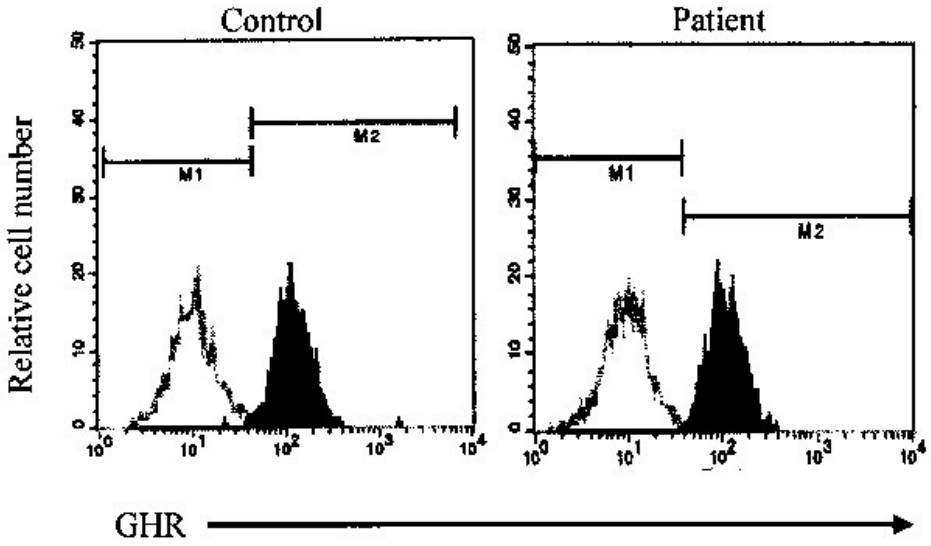


Figure 1B

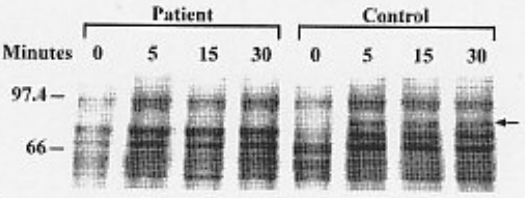


Figure 2

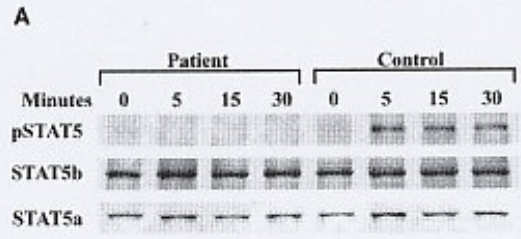

A
Patient
Control
Minutes
0
5
15
30
0
5
15
30
pSTAT5
STAT5b
STAT5a

Figure 3.4

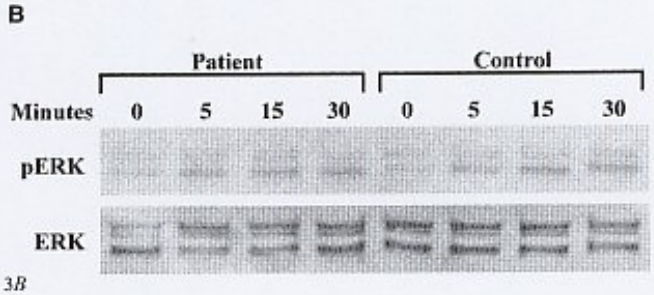


Figure 3*B*

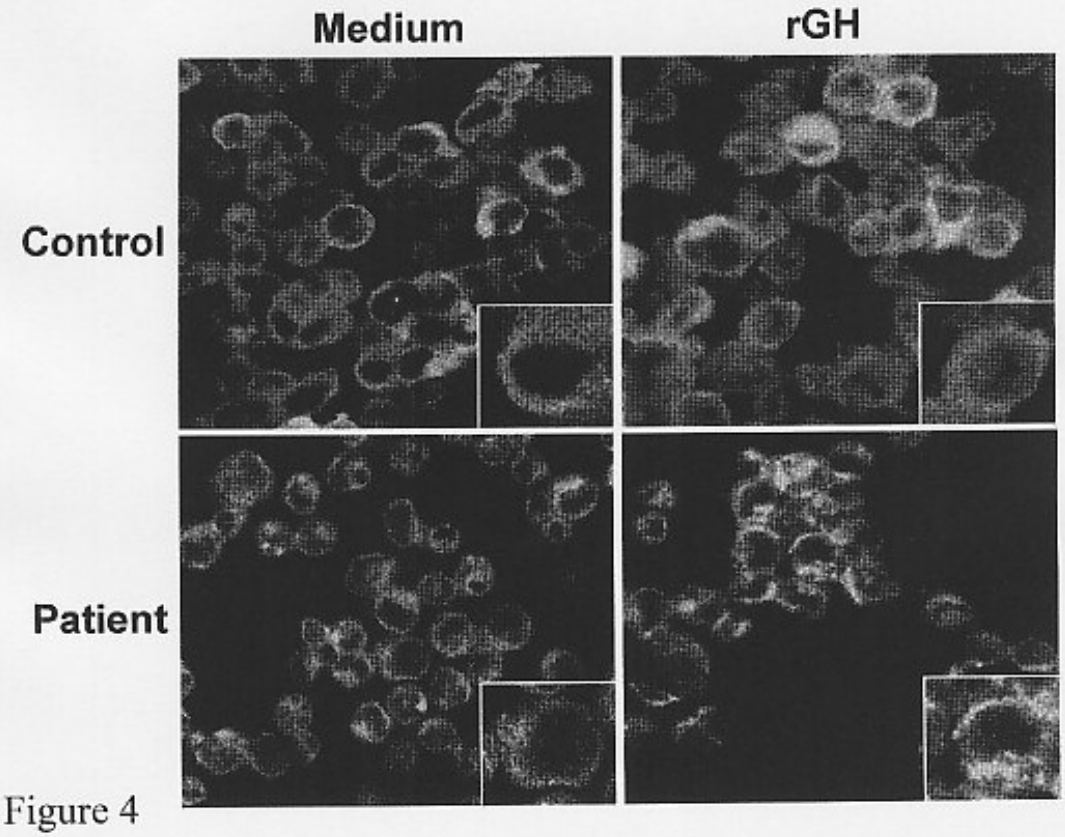


Figure 4

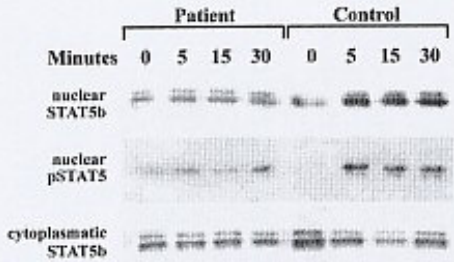

Patient
Control
Minutes 0 5 15 30 0 5 15 30
nuclear STAT5b
nuclear pSTAT5
cytoplasmatic STAT5b

Figure 5

Methods

Perforin mutation analysis

Genomic DNA was isolated from peripheral blood lymphocytes and the exons 2 and 3 of the PRF1 coding region amplified using standard PCR conditions. PCR products were recovered from 1.5% agarose with a size marker, purified using QIAquick Gel extraction kit (QIAGEN, Hilden, Germany) and sequenced in an automatic ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Natural Killer function

Infected target cells (K562) were pulsed for 1 h at 37 °C with Cr^{51}(Amersham Pharmacia, Buckinghamshire, England) (30 □Ci/10^6 cells) and washed three times with culture medium before the addition of effector cells. Specific lysis was measured in a triplicate assay performed with 5x10^3 target cells mixed with different ratios of effector cells (E:T= 25:1 to 3.125:1) in a total volume of 200 µl. After a 5 h incubation at 37 °C, 100 µl culture supernatant were collected and the amount of Cr^{51} release was counted. The percentage of specific lysis

was calculated as follows: 100 x (specific release-spontaneous release)/(total release-spontaneous release).

Evaluation of Fas-induced cell death

Fas-induced cell death was evaluated as previously reported on activated T-cell lines obtained by treating peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA) at days 0 (1 μg/mL) and 12 (0.1 μg/mL). Cells were then cultured in RPMI 1640 + 10% FCS + recombinant IL-2 (2 U/mL) (Biogen, Geneva, Switzerland). Fas function was assessed 6 days after the second stimulation (day-18 T cells), by incubating with control medium or 1 μg/mL anti-Fas mAb (CH11, IgM isotype, UBI, Lake Placid, NY) in the presence of 1 U/mL recombinant interleukin 2 (rIL-2) and 5% FCS to minimize spontaneous cell death. Cell survival was evaluated after 18 hours by counting alive cells in each well by the trypan blue exclusion test. Assays were performed in triplicate and analysed by a blind observer. Cells from 2 normal donors were included in each experiment as a positive control. Results were expressed as relative cell survival percentage, calculated as follows: (total live cell count in the assay wells/total live cell count in the untreated samples) x 100. Spontaneous cell loss in the control well was always less than 10% of the seeded cells and similar in cultures from the patients and normal donors. Cell death was evaluated both indirectly, by counting total surviving cells by the trypan blue exclusion test, or directly, by cytometric determination of the proportion displaying DNA

fragmentation after staining with propidium iodide or those stained by annexin to confirm Fas resistance. Fas function was defined defective when cell survival was higher than 78 %, which was the 95th percentile of the response displayed by normal controls.

Molecular analysis of the Fas and caspase-8 genes

Mutation analysis of the Fas (TNFRSF6) and caspase-8 genes were performed by DNA genomic sequencing. DNA was extracted from PBMC with standard methods. Eight fragments spanning the 5'UTR and the 9 exons of the Fas gene were amplified in 20 µl final volume containing 12 pmol of each primer, 50 ng of genomic DNA and 0,5 units of AmpliTaq (Applied Biosystems, Warrington, UK). PCR conditions for caspase-8 gene amplification were as follows: sixteen fragments spanning the 10 exons and intron-exon boundaries were amplified in 50 µl final volume containing 25 pmol of each primer, 100 ng of genomic DNA, 0.2 mM dNTPs and 1.25 units of AmpliTaq Gold (Applied Biosystems) in the buffer provided by the manufacturer. Amplification was performed in a Gene Amp PCR System 9700 (PE Applied Biosystems). The PCR products were sequenced using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Cells and cell cultures

Mononuclear cells (PBMC) were obtained from 4 X-SCID patients and normal donors heparinized peripheral blood by Ficoll-Hypaque (Biochrom) density gradient centrifugation. Upon informed consent, lymphoblastoid cell lines (BCLs) were generated by Epstein-Barr virus immortalization of patients and controls PBMC using standard procedures (15). In all cases, □c mutations led to the absence of protein expression. Cells were maintained in RPMI 1640 (Biochrom) supplemented with 10% fetal bovine serum (Gibco), 2 mmol/L L-glutamine (Gibco), and 50 μg/ml gentamycine (Gibco), and cultured at 37°C, 5% CO_2.

Proliferative assay

BCLs (1 X 10^5 cell/200 μl well) were cultured triplicate in 96-well U-bottomed microtiter plates (Falcon®, Becton Dickinson) with or without rGH at reported concentrations for 4 days. The proliferative response was evaluated by thymidine uptake from cultured cells pulsed with 0.5 μCi [^{3}H]thymidine (Amersham International) 8 h before harvesting .

Flow cytometry

The expression of GHR was detected using specific rabbit antibodies (Santa Cruz) by indirect immunofluorescence using a second-step incubation with a

FITC-conjugated donkey anti-rabbit antibodies (Pierce). After washing in PBS, cells were incubated for 20 min with the specific antibodies and 30 min with secondary antibodies. After staining, all samples were washed in PBS and acquired on the FACScan flow cytometer (Becton-Dickinson) using Lysis I software.

Cell stimulation and protein extraction

Prior to hormone treatment, the cells were made quiescent through incubation in RPMI 1640 minus serum for 8-12 h. GH was used at 37°C at a concentration of 500 ng/ml in RPMI 1640 for the reported time. Incubations were terminated by washing cells with ice cold phosphate-buffer saline (PBS; BioWhittaker) followed by solubilization in 100 µl of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mmol EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadatum (Na_3VO_4), 5 µg/ml leupeptin and 5 µg/ml aprotinin. The cell lysates were stored at -80°C for Western blot analysis. Nuclear extracts were prepared by the standard method and were subsequently mixed with sample buffer.

Western blot

Immunoblotting using phosphotyrosine monoclonal antibody was performed as previously reported. Immunoblotting using specific antibody was performed

according to the vendors protocols. In brief, protein samples separated by SDS-PAGE were transferred onto Mixed Cellulose Esters membranes (Immobilon-NC Mixed Cellulose Esters 0.45 µm; Millipore). The membrane was incubated at room temperature for 1 h in blocking buffer consisting of 10% Bovin Serum Albumin (BSA) in wash buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20). The membrane was then washed three times in wash buffer and incubated 1 h at room temperature or overnight at 4°C with the specific antibody. The membrane was then washed three times and an appropriate IgG horseradish peroxidase-coniugated secondary antibody was used for the second incubation. After further washings, the membrane was developed with ECL developing reagents, and exposed to x-ray films according to the manufacturer's instructions (Amersham Biosciences).

Summary

Primary immunodeficiency diseases (PIDs) represent a group of most heterogeneous syndromes characterized by development or functional defect of the Immune system. More than 100 inherited conditions described immunodeficiencies exist. Clinical presentation is highly variable, ranging from various patterns of microbial susceptibility to allergy, lymphoproliferation or autoimmune manifestation the most of these syndromes are monogenic and lead to defects in any aspect of the immune response. Although several form of them occur in the first time of life there are many other disorders in which the clinical manifestation develop during adulthood. Along with typical form of these syndromes there are several disease in which the clinical signs are less suggestive of immunodeficiency. In these form it is very difficult make the diagnosis. In the course of this PhD program we try to better clarify for some of these complex phenotypes the correlation between genotype and phenotype, identify the biochemical biomarkers that make possible the diagnosis and eventually clarify the pathogenesis mechanism of these syndromes.

Acknowledgement

I would you like to thank all the people who helped and supported me during my studies leading to the realization of this work. In particular, I would you like to thank my supervisor Prof. Claudio Pignata, for the opportunity he gave me to work in his group. He has been a great supporter because he has supported and encouraged me during all these years. Moreover, he followed me in the PhD program and in my research supporting my decision and influencing my work with stimulating advice.

Moreover I also like to acknowledge Marsilio Adriani for his help and support in the laboratory. He is not only a colleague but is also a dear friend.

Curriculum Vitae

Busiello Rosanna was born on June 24, 1977 in Portici (Naples), Italy. Her educational and professional position are listed below.

Educational/Training

- High School degree

- 2001 “Federico II” University of Naples Degree in Biology, summa cum lode
- 2002 post-graduate fellow at the Department of Pediatrics of “Federico II” University of Naples
- 2002 PhD student in “Sviluppo, Accrescimento e Riproduzione dell’uomo” at the “Federico II” University, Naples, Italy

SKILLS:

- Computer literate, knowledge of MS Windows**,** MacOS systems
- Good knowledge of scientific softwares as: Oligo 4, Gencokit, DNA strider, Cyrillic 3, NIH image (Mac and PC version), Lysis 2.
- Expertise in utilizing common online databases of proteins (Swissprot/TrEMBL, PIR, UniProt, Prosite, ect), nucleotide sequences (GeneBank.EMBL, DDBJ), mouse models (MGI, MPD, GXD, etc).
- knowledge of written and spoken English

TECHNOLOGYCAL SKILS

Direct experience in the following fields:

- Cell cultures
- DNA and RNA extractions
- PCR
- Direct Sequencing
- SSCP
- Western Blotting

AWARDS

2003- Award from "Federation of Clinical Immunology Societies"for the following abstract: *"Atypical Features of Familial Haemofagocytic Llymphoistiocitosis."* (Paris May, 2003)

2003-Award "1° Premio Città di Caserta" with the work entitled: *"Atypical Features of Familial Haemofagocytic Llymphoistiocitosis*"(Aversa November, 2003)

List of papers

I. Salerno M, Busiello R, Esposito V, Cosentini E, Adriani M, Selleri C, Rotoli B, Pignata C. Allogeneic bone marrow transplantation restores IGF-I production and linear growth in a gamma-SCID patient with abnormal growth hormone receptor signaling. Bone Marrow Transplant. 33:773-5, 2004;.

II. Busiello R., Greco G., Pignata C. Novel Immunodeficiencies: clustering of distinct autoimmune disorders associated to monogenic alteration. It J Ped. 30: 95-102, 2004

III. Adriani M, Martinez-Mir A, Fusco F, Busiello R, Frank J, Telese S, Matrecano E, Ursini MV, Christiano AM, Pignata C. Ancestral founder mutation of the nude (FOXN1) gene in congenital severe combined immunodeficiency associated with alopecia in southern Italy population. Ann Hum Genet. 68: 265-8, 2004;.

IV. Busiello R, Adriani M, Locatelli F, Galgani M, Fimiani G, Clementi R, Ursini MV, Racioppi L, Pignata C. Atypical features of familial hemophagocytic lymphohistiocytosis. Blood 103: 4610-2, 2004.

V. Busiello R., Galgani M., De Fusco C., Poggi V., Adriani M., Racioppi L., Pignata C. A91V is a polymorphism in the perforin gene not causative of an FHLH phenotype**.** Blood 104: 1909-1910, 2004

VI. Busiello R, Figiani G., Miano M.G., Aricò M., Santoro A., Ursini M. V. and Pignata C. A91V perforin variation in healthy subjects and FHLH patients. (Submitted to International Journal of Immunogenetics)

VII. Adriani M., Auricchio L., Frank J., Busiello R.,, Christiano A.M., Pignata C. Nail dystrophy associated with heterozygous mutation of the Nude/SCID FOXN1 (WHN) gene. Arch Dermatol 141: 647-648, 2005

VIII. Adriani M., Garbi C., Amodio G., Busiello R., Matrecano E., Cosentini E., Candotti F., Pignata C. Functional Interaction of Common Gamma Chain and Growth Hormone Receptor Signaling Apparatus. Journal of Immunology (submitted)

IX. Ferri P., Lombardi F., Franzese A., Busiello R., Chiocchetti A., Ramenghi U., Dianzani U., Pignata C. Pattern of phenotypic expression and familial occurrence of clustering of autoimmune disorders in childhood. Haematologica (Submitted)