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PhD Thesis

"Congenital immunodeficiencies and autoimmunity: definition of the pathogenic mechanism and identification of the molecular and genetic alteration. Identification of novel and suitable therapeutic strategies and their application"

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Chapter 1

Scientific Background

1.1   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 130 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. 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.

1.2   Apoptosis and the control of immune responses

Apoptosis is a programmed cell death mechanism involved in normal cell turnover which can be induced by a variety of stimuli to eliminate damage, infected or useless cells. Apoptosis is essential for the development of vertebrates, being involved in elimination of interdigital webs, palatogenesis, haematopoietic cell homeostasis and development of intestinal mucosa and retina (Denis F. et al 1998). Apoptosis is caracterized morphologically by condensation of nuclear chromatin, internucleosomal cleavage of DNA, compaction of cytoplasmatic organelles, decrease in cell volume and loss of plasma membrane asymmetry resulting in recognition and phagocytosis (Denis F. et al 1998). Inappropriate cell death plays a role in pathological condition such as cancer, autoimmunity and neurodegenerative diseases (Denis F. et al 1998).

Apoptosis plays a key role in the control of immune responce, infact, 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 apoptosis occurs to limit cell growth, thus restoring immunological homeostasis (Busiello R. et al 2004).

1.3 Mechanism of the apoptosis: Non secretory pathway and secretory pathway.

During the last decade, major advances were made in understanding the complex regulatory mechanism of the cell concerning the control of its own death. Several molecular pathways have been unraveled, and many proteins playing a role in these pathways have been described (Jutte van der Werff ten Bosh, Pediatr Drugs 2003). Actually, it is common knowledge that the apoptosis is activated by non secretory and secretory pathways (Rieux- Laucat F. et al 2003).

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). This complex serve to bring two or more caspase 8 precursors in close proximity, allowing for intermolecular autoproteolytic activation. The active form of caspase 8 converted the procaspase 3 in caspase 3 (active enzyme) by proteolytic processing with a cascade model. This effector caspase cleaved specific substrate inactivating or deregulating protein involved in DNA repair, mRNA splicing and DNA replication.

Therefore the caspases contribute to apoptosis through disassembly of cell structures, they disintegrate the cell into apoptotic bodies and they induce the cell to display signals that mark it for phagocytosis (Thornberry et al 1998).(Fig. 1)
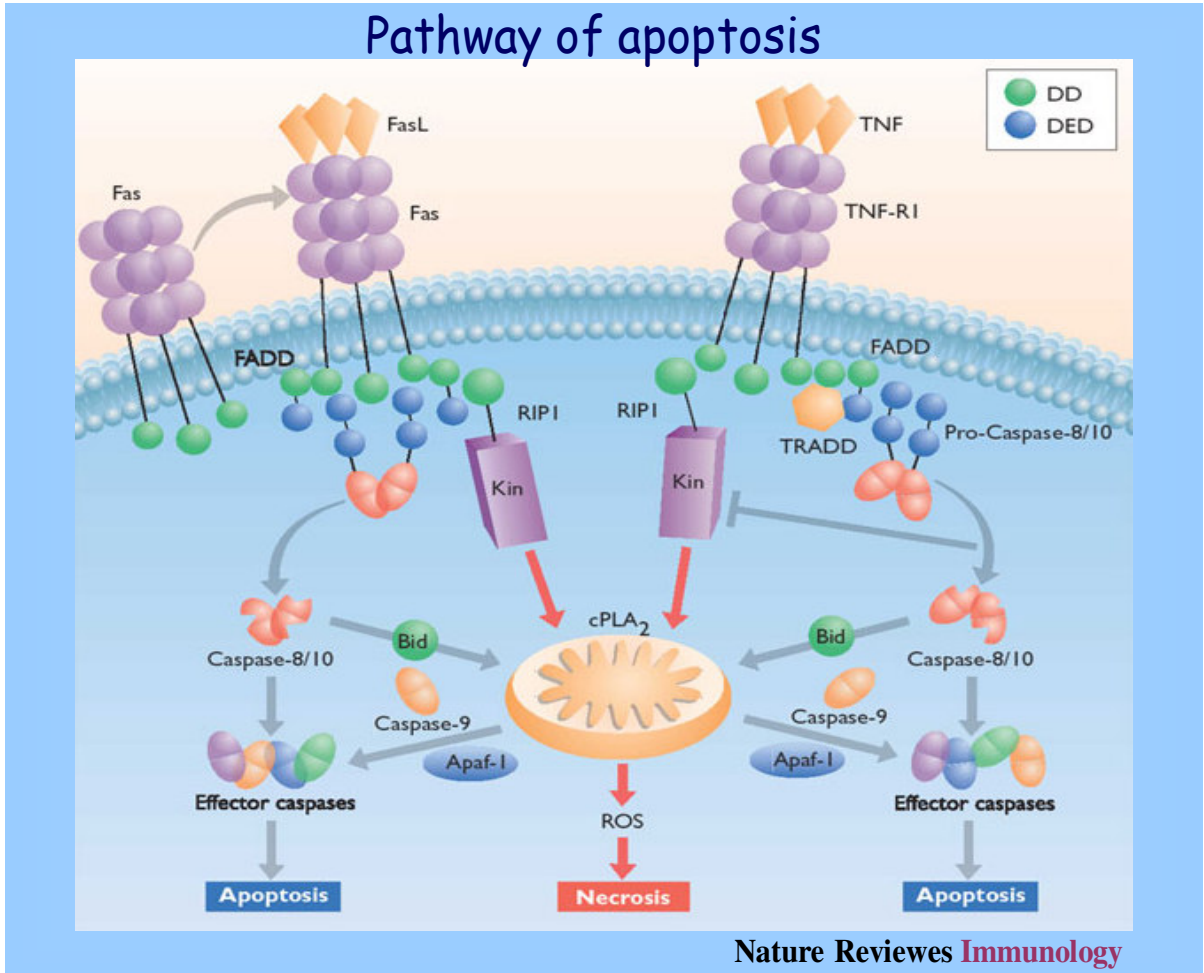
Fig. 1: Non-secretory pathway

In the secretory or calcium-dependent pathway, cytotoxic T cells (CTLs) and Natural Killer cells (NK) are key effectors for the elimination of virus-infected cells, and they deliver a lethal hit to their targets through involving a series of serine proteases called granzymes (Grz) and perforin (PRF1).

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. 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. This macromolecular complex results in the target cell lysis. (Fig. 2)

Eleven granzymes (Grz A-H, M and N) have been described (Smyth et al 1996). Ten of these are expressed in mice and five in humans (A,B,H,K and M) (Smyth and Trapani 1995, Trapani et al 2000). Among these, the granzyme B is an caspase because cleaved the substrates after

aspartic acid residues and so triggering apoptotic cell death either directly, or indirectly via the activation of cellular caspases (for example caspase 3).

Therefore, the caspases plays a critical role in the mechanism of apoptosis. They are members of a large family of proteases whose members have distinct roles in inflammation and apoptosis. In apoptosis, the caspases function in both cell disassembly (effector) and in initiating this disassembly in responce to proapoptotic signals (initiator) Thirteen caspases have been identified in the mammalian cells, the effector caspases 3, 6,7 and the initiator caspases 1, 2, 4, 5, 8, 9, 10, 14. (Figure 3). They share similarities in amino acid sequence, structure and substrate specificity. Caspases are among the most specific of proteases, with an unusual and absolute requirement for cleavage after aspartic acid. Recognition of at last four amino acids NH-terminal to the cleavage site is also a necessary requirement for efficient catalysis. The preferred tetrapeptide recognition motif differ significantly among caspases and explain the diversity of their biological function (Thornberry et al 1998).

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

Extracellular
space

Perforin
pore

Granule
exocytosis

MPR

Target
cell

?

Endosome

?

BID

tBID

Mitochondrion

d

SG

Granzyme B

Perforin

MPR

Endosome

Mitochondrion

Caspase
activation

Apoptosis
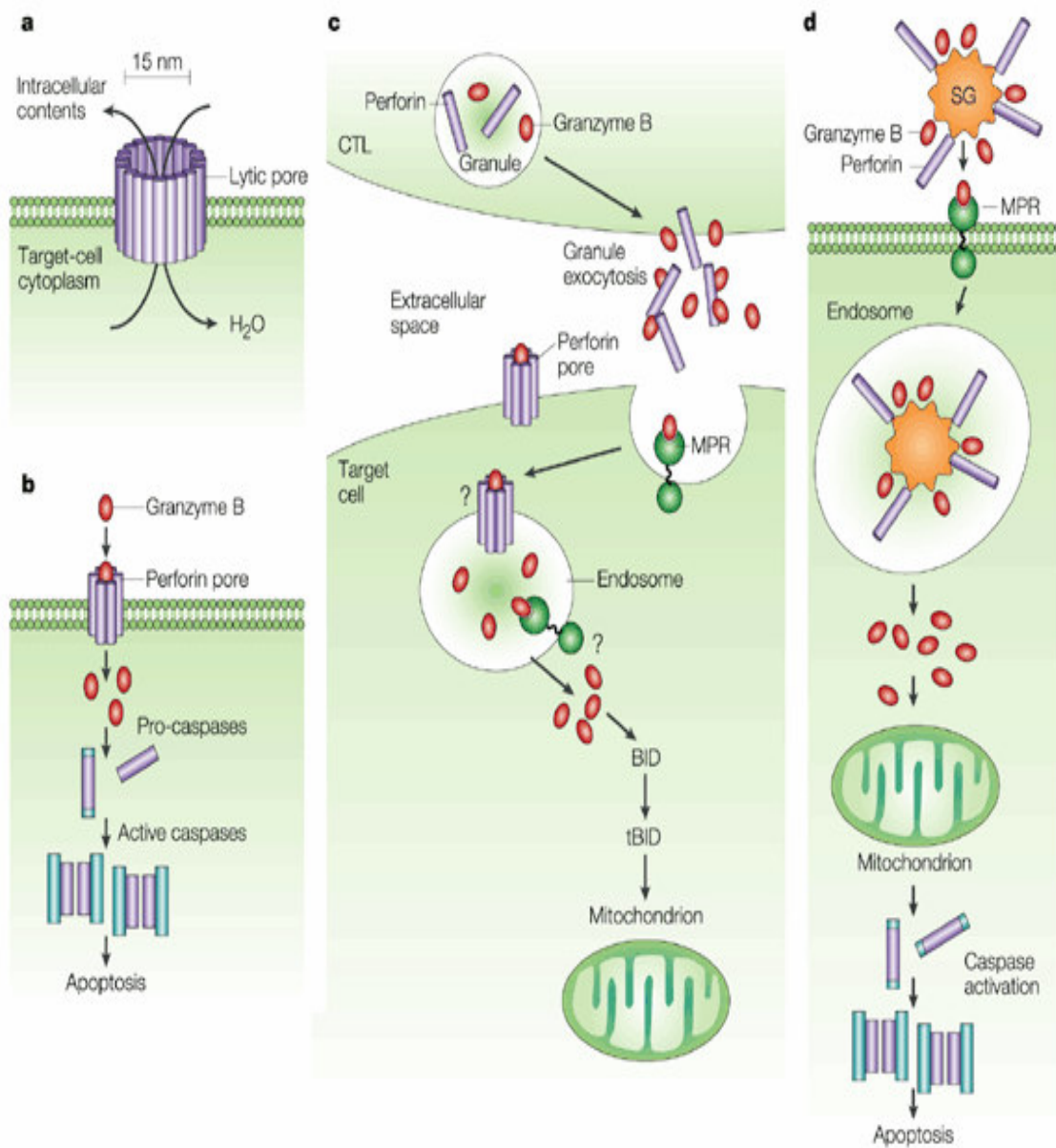
Fig. 2. Evolving models of CTL/NK-secretory pathway.

    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

1.4 The role of the caspase 8 in the apoptosis and in the cellular activation

Recently, a new clinical phenotype was found, where T, B and NK immunodeficit has been observed together with an ALPS-like phenotype. An important study shows the existence of pleiotropic defects in lymphocyte activation caused by caspase 8 mutations. Chun et al. bring that caspase 8, well known for its killer effect, plays also an important role in the lymphocytes proliferation. The authors in their study have described a group of patients with a severe functional deficit of caspase 8. In particular, they have shown that mutations in homozygosis in caspase 8 gene can produce a condition of multiple autoimmunity and immunodeficit. Clinical examinations showed that subjects carriers of this mutation in homozygosis exhibited splenomegaly, lymphadenopaty as sign of the involvement of the reticular-endothelial system, reduced Fas-induced apoptosis, recurrent respiratory infections, low levels of serum Ig without isotipic switch, Virus Herpes Simplex infections and a combined T, B and NK immunodeficit.

The existence of autoimmunity together with immunodeficit supports the hypothesis that the same genes, involved in the control of the immune response are also responsible of the activation and cellular proliferation.

1.5 Animal models

The existence of animal models and the generation of knock out mouse with mutations of single gene and analysis of related clinical phenotypes greatly contributed to ameliorating our knowledge on the pathogenesis of complex syndrome of immunodeficiency. Mice lpr and gld have mutations in Fas and FasL genes, respectively, (Nagata et al., 1995) and these mutations lead to loss of function. These mice have a clinical phenotype characterized by high levels of auto-antibodies and considerable accumulation of activated and autoreactive lymphocytes in secondary lymphoid tissues with consequent involvement of lymphoreticular system (Rieux-Laucat F. et al., 2003). Consequently, mice show autoimmune disorders, splenomegaly and lymphadenomegaly (Rieux-Laucat F. et al., 2003). Altered homeostasis of T and B cells is also been described as motive of prevalent limphoproliferative disorders. In addition, these mice show an increase of TCR α/β +, CD4- and CD8- double negative in peripheral blood, due to defective intrathymic maturation.

Homozygous targeted disruption of the mouse caspase 8 gene was found to be lethal in utero (Varfolomeev et al., 1998). The most important feature of the abnormal phenotype of the casp8$^{-/-}$ mutant was marked hyperaemia in the abdominal area, superficial capillaries and other blood vessels. Disruption of the casp8 gene appears to result in a dramatic primary or secondary depletion of the haematopoietic precursor pool. Histological examination in mutant embryos reveals a congested accumulation of erythrocytes in liver and in blood vessels areas. Normal embryonic liver tissue, with its characteristic early

haematopoietic elements, and early liver cells are visible only in the cortical area. The casp8 null embryos also exhibits impaired heart muscle development, in fact the ventricular musculature is thin and in some cases not different from early mesenchyme. In fibroblast strains derived from these embryos, the TNF receptors, Fas/Apo1 and DR3 are able to activate the Jun N-terminal kinase and to trigger IκBα phosphorylation and degradation. They failed, however, to induce cell death. These findings indicate that caspase 8 plays a necessary and non redundant role in death induction by several receptors of the TNF/NGF family and serves a vital role in embryonic development.

Because caspase 8 deletion is associated with embryonic lethality in mice has been generated a conditional knock-out mouse that have consented to study in vivo the nonapoptotic function of caspase 8. This murine model, generated with T cell specific delation of caspase 8 (tcasp8$^{-/-}$), showed T cell lymphopenia, defective activation-induced T cell proliferation, defective T cell responses to viral infection that were associated with defective T cell-death induced by CD95 (Salmena et al 2003).

Furthermore a new study on mouse tcasp8$^{-/-}$ model reported the long-term physiological consequences of caspase 8 deficiency. This study showed that tcasp8$^{-/-}$ mice develop an age-dependent lethal lymphoproliferative and lymphoinfiltrative immune disorder characterized by lymphoadenopathy, splenomegaly, and accumulation of T cell infiltrates in the lungs, liver, and kidneys. Peripheral casp8$^{-/-}$ T cells manifest activation marker up-regulation and are proliferating in the absence of any infection or stimulation (Salmena et

al 2006).   Interestingly, the condition described in tcasp8$^{-/-}$ mice manifests features consistent with the disorder described in humans with Caspase-8 deficiency and support the hypothesis that this immune disorder is different from autoimmune lymphoproliferative syndrome (Salmena et al 2006).   These findings suggest that tcasp8$^{-/-}$ mice may serve as an animal model to evaluate caspase-8–deficient patient prognosis and therapy.

1.6   Genetics of Caspase 8.

The human caspase 8 (CASP 8) gene is localized to chromosome band 2q33-34 and consists of ten exons spanning ~30Kb (Fig. 3). The structure of the human gene, corresponding to the open reading frame (ORF) portion of the mRNA, is encoded by eight exons and the coding sequence beging in exon 3. CASP 8 is expressed as proenzyme and contain three domains: an NH_2-terminal domain contain two DEDs domain, a large subunit (~18 KD) and a small subunit (~10 KD) contain two cysteine protease domain. One of the DEDs is encoded by exon 3, whereas the second is encoded by exons 4 and 5 and these are involved in the regulation of activation (see below). The linker region, located between the DEDs and cysteine protease domain, is encoded by exons 6-8. The cysteine catalitic domain are encoding by exons 9-10. The activation of the caspase 8 involves proteolytic processing between domains, followed by association of the large and small subunits to form a heterodimer. This processing, after aspartic acid, can be performed either autocatalytically or in a cascade by enzymes with similar specificity. Actually, nine different isoforms of caspase 8, all generated by alternative splicing, have been found. Analysis of human adult tissue demonstrated that caspase 8 mRNA is very little express in testis, skeletal muscle, kidney and brain. The highest level of expression was found in pheripheral blood leukocytes, consistent with caspase's 8 role in the death of peripheral T cells (Grenet et al 1999, Thornberry et al 1998).
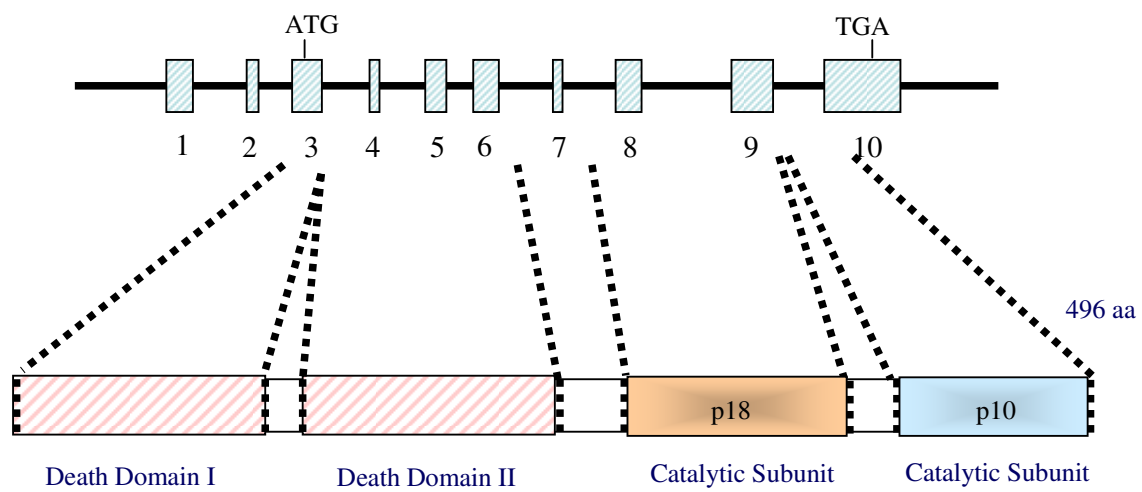
Fig. 3. Caspase 8 gene and protein structure

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Chapter 2

Research projects

2.1   Clustering of Distinct Autoimmune Disease and Apoptosis

Even though distinct autoimmune disorders may be associated in the same individual, only rare patients exhibit a clear clustering of different autoimmune diseases, which are indicative of a common polyreactive autoimmune process (Jonsson et al 1996). Several studies documented a linkage between defined HLA molecules and certain autoimmune disorders, such as rheumatoid arthritis (RA) (Brown et al 1998), insulin-dependent diabetes mellitus (IDDM) (Nepom et al 1998), and celiac disease (CoD) (Fernandez-Arquero et al 1998). Whilst HLA molecules may contribute to the predisposition to a certain disease, the mechanism responsible for clustering in a single individual is still unknown.

Programmed cell death or apoptosis is a finely regulated biological mechanism involved in tissue differentiation and homeostasis (MacDonald HR et al 1990, Raff MC 1992). In immune response, apoptosis exerts a suppressive influence by limiting clonal expansion and by participating in intrathymic negative selection of the T cell receptor (TCR) repertoire (Blakman M et al 1990). Interaction between Fas and Fas ligand induced apoptosis in several lymphoid cell lines and long-term activated peripheral T cells (Nagata S. 1997) by activating a death signaling pathway that involves several cysteine proteases

named caspases (Cohen GM et al 1997; Salvesen GS et al 1997) (see section 1.3).

An acidic sphingomyelinase-producing ceramide, that induces mithocondrial alterations probably mediated by GD3-ganglioside (De Maria R et al 1997) is also activated, even though its role in Fas signaling is still uncertain (Hsu SC et al 1998; Watts JD et al 1997). Inherited loss-of-function mutations of the Fas or FasL genes were reported in association with the autoimmune lymphoproliferative syndrome (ALPS), characterized by haematological autoimmunities and non-malignant lymphadenopathy with expansion of TCR α/β CD4-CD8- double-negative (DN) T cells (Fisher GN et al 1995; Riex-Laucat F et al 1995; Drappa J et al 1996; Bettinardi A et al 1997; Pensati P et al 1997). Functional Fas deficiency has also been reported in children with clinical findings similar to ALPS, but no DN T cell expansion and Fas mutations, indicating that alteration of other molecules in the Fas signalling pathway causes an ALPS-like syndrome (Dianzani U. et al 1997).

2.1.1 Alteration of T cell survival in children with Clustering of Autoimmune Disease phenotype

In the first phase of this study we evaluated Fas- and ceramide-induced cell death on T cells from three children affected by clustering of distinct autoimmune diseases (CAD) to assess whether a dysregulation of the T cell switching-off system may be implicated in the pathogenesis of intense polyreactive autoimmunity.

Of the three patients enrolled in this study, two patients were selected because affected by insulin-dependent diabetes mellitus (IDDM) associated with intense autoimmune disorders affecting other organs. Patient 1 showed Coeliac Disease (CoD), autoimmune thyroid disease and juvenile rheumatoid arthritis (JRA) in association with IDDM. Patient 2 showed IDDM and localized sleroderma. Patient 3 had CoD, vasculitis ,recurrent episodes of fever with arthritis and iridociclitis. All patients displayed serum autoantibodies and several features similar to ALPS. The molecular and cytofluorimetric analysis revealed that Patient 1 displayed low Fas expression both at transcript and protein level associated with expansion of DN T cells. Her resistance to Fas-induced cell death and sensitivity to ceramide were in line with a defect directly involving Fas, as that described in ALPS type Ia. However, no causal mutation has been identified in Fas cDNA. One possibility is that causal mutation may be located in unscreened gene regions, such as the promoter region, intronic splite sites, or generally in genomic DNA.

In patients 2 and 3 no expansion of DN T cells was found and Fas expression was normal. In patient 3 both Fas- and ceramide-induced cell death was abnormal, while in patient 2 only ceramide failed to induce a proper cell death. In these two patients, a further Fas transcript was found that retains intron 5 expected to encode for a truncated protein lacking the transmembrane and intracellular regions. The aberrant transcript was also observed in the healthy mother and brother of patient 3. Sequence analysis did not reveal any change in cDNA, which could explain the abnormal splicing. However, we have been unable to detect this transcript in 11 normal donors. Whether the expression of this form is implicated in the pathogenesis of the polyreactive syndrome remains to be established.

Soluble forms of Fas are expected to inhibit Fas but not ceramide-induced cell death. Whereas patient 2 was sensitive to Fas trigger and resistant to ceramide, the patient 3 was resistant to both stimuli. It is conceivable that low doses of soluble Fas may not inhibit cell death induced by high doses of anti-Fas MoAb in the vitro assay, thus explaining the normal Fas function displayed by patient 2. Thus the production of the putative soluble form of Fas could inhibit Fas function in vivo and be a cofactor for development of the polyreactive autoimmune disease when associated with other genetic and environmental factors. The current opinion is that Fas triggering activates a caspase cascade leading to cell apoptosis. The generation of ceramide is an alternative pathway that seems to boost the caspase pathway (De Maria R et al 1997), even though the role played by ceramide in Fas signalling is still debated (Hsu SC et al 1998;

Watts JD et al 1997). Moreover the system is under the control of several inhibitors belonging to the bcl-2, FLIP and IAP families (Cronin CC et al 1997).

Therefore, in both patients 2 and 3, the overt disease and the decreased function of the death pathway triggered by ceramide could be explained by the concurrence of other genetic factors hitting the pathway downstream from FAS. These factors may hit other systems involved in apoptotic induction, such as TNF, trail and granzyme systems, which use apoptotic pathways partially overlapping that of Fas (Cronin CC et al 1997; Golstein P 1997).

It is noteworthy that our patient 2 was resistant to ceramide, but responded normally to Fas triggering, which shows that the Fas pathway can also function normally when the ceramide pathway is unfunctional and suggests that alteration of T cell switching-off systems that are independent of Fas may play a role in autoimmunity.

The data reported in this work indicate that polyreactive autoimmune syndromes may be associated with heterogeneous alteration of the immune responce switching-off system.

These results has been published on the Clinical and Experimental Immunology see below for the manuscript.

Clustering of distinct autoimmune diseases associated with functional abnormalities of T cell survival in children

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SUMMARY

To ascertain whether alterations of lymphocyte switching off may be associated with clustering of autoimmune diseases in children, Fas- and C2-ceramide-induced cell death was evaluated on T cell lines derived from three patients affected by clustering of autoimmune disorders. Three patterns were found: patient 3 was resistant to Fas- and C2-ceramide, patient 1 was resistant to Fas, but sensitive to C2-ceramide, patient 2 was resistant to C2-ceramide, but sensitive to Fas. By contrast, Fas- and C2-ceramide-induced cell death was normal in five children with systemic juvenile rheumatoid arthritis, five children with insulin-dependent diabetes and 10 age-matched healthy controls. Surface expression of Fas was low in patient 1, but normal in patients 2 and 3. Together with normal Fas transcripts, patients 2 and 3 displayed a transcript 152 bp longer than the normal one retaining intron 5. Our data indicate that polyreactive autoimmune syndromes may be associated with heterogeneous alteration of the immune response switching-off system.

Keywords polyreactive autoimmune diseases cell apoptosis Fas

INTRODUCTION

Even though distinct autoimmune disorders may be associated [1,2], only rare patients exhibit a clear clustering of different diseases based on a polyreactive autoimmune (PA) process [3]. Several studies documented a linkage between defined HLA molecules and certain autoimmune disorders, such as rheumatoid arthritis (RA) [4], insulin-dependent diabetes mellitus (IDDM) [5], and coeliac disease (CoD) [6]. Whilst HLA molecules may contribute to the predisposition to a certain disease, the mechanism responsible for clustering in a single individual is still unknown.

Programmed cell death or apoptosis is a finely regulated biological mechanism involved in tissue differentiation and homeostasis [7,8]. In immune response, apoptosis exerts a suppressive influence by limiting clonal expansion, and by participating in intrathymic negative selection of the T cell receptor (TCR) repertoire [9]. Fas is a transmembrane molecule belonging to the tumour necrosis factor (TNF) receptor superfamily, and interacts with the Fas ligand (FasL), a type II transmembrane molecule. Fas ligation induces programmed

cell death in several lymphoid cell lines and long-term activated peripheral T cells [10] by activating a death signalling pathway that involves several cysteine proteases named caspases [11,12]. An acidic sphingomyelinase-producing ceramide, that induces mitochondrial alterations probably mediated by GD3-ganglioside [13], is also activated, even though its role in Fas signalling is still uncertain [14,15]. Inherited loss-of-function mutations of the Fas or FasL genes were reported in association with the autoimmune lymphoproliferative syndrome (ALPS), characterized by haematological autoimmunities and non-malignant lymphadenopathy with expansion of TCR α/β $CD4^-CD8^-$ double-negative (DN) T cells [16–20]. Functional Fas deficiency has also been reported in children with clinical findings similar to ALPS, but no DN T cell expansion and Fas mutations, indicating that alteration of other molecules in the Fas signalling pathway causes an ALPS-like syndrome [21]. This possibility has been recently confirmed in two patients with an ALPS-like clinical pattern who displayed mutations of caspase-10 [22].

In this study we evaluated Fas- and ceramide-induced cell death on T cells from three children with clustering of distinct autoimmune diseases to assess whether a dysregulation of the T cell switching-off system may be implicated in the pathogenesis of intense polyreactive autoimmunity.

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PATIENTS AND METHODS

Patients

Two patients with IDDM were selected because of its association with intense autoimmune disorders affecting other organs. The diagnosis of IDDM was performed according to the following American Diabetes Association criteria: fasting hyperglycaemia ($> 7\cdot8$ mmol/l), ketonuria, and insulin dependence. Patient 1 showed CoD, autoimmune thyroid disease and juvenile rheumatoid arthritis (JRA) in association with IDDM. She displayed serum anti-thyroid autoantibodies. The diagnosis of JRA was established according to the American College of Rheumatology criteria [23]. Patient 2 showed IDDM and localized scleroderma. He displayed serum antinuclear auto-antibodies. No other signs of lymphoreticular involvement were evident. Patient 2 had a sister who developed IDDM, but not scleroderma and did not display serum autoantibodies. Their parents were not consanguineous. In the maternal lineage, two subjects suffered from RA and uveitis, and from autoimmune thyroiditis, respectively. Patient 3 had CoD, vasculitis, recurrent episodes of fever with arthritis and iridociclitis. Major features of these patients are illustrated in Table 1.

There were no serological signs of recent infectious disease, including infections from Epstein–Barr virus (EBV), cytomegalovirus, HIV, hepatitis C virus, *Borrelia bugdorferi*. All patients had hypergammaglobulinaemia, that was associated with IgA deficiency in patient 1. No patient had thrombocytopenia or haemolytic anaemia. Standard procedures were carried out to assess the involvement of other organs. As shown in Table 1, the two patients with IDDM were receiving insulin therapy, and non-steroidal anti-inflammatory drugs (patient 1), or D-penicillamine (patient 2). Patient 3 occasionally received prednisone during acute episodes of uveitis. Histological evaluation of skin biopsies from patient 2 revealed thinning of epidermis and loss of skin adnexa; the dermis was widened by an increased amount of collagen. A series of normal controls was also included after informed consent: 11 adult subjects were used as controls for cDNA sequencing analysis; 65 adult and 10 age-matched healthy subjects were used to set the normal range for the cell death assay. Moreover, five age-matched children with systemic RA and five children affected only by IDDM were also included.

Immunophenotype analysis

Expression of surface molecules was evaluated by direct immunofluorescence and cytofluorometric analysis (FACScan; Becton Dickinson, San Jose, CA). The following MoAbs were used: anti-CD3 (Leu-4), -CD4 (Leu-3a), -CD8 (Leu-2a), -CD19 (Leu-12), -CD56 (Leu-19), -TCR α/β (Becton Dickinson), and -Fas (Immunotech, Marseilles, France). CD4 and CD8 DN TCR α/β^+ cells were detected by two-colour immunofluorescence using FITC-conjugated anti-TCR α/β MoAb and PE-conjugated anti-CD4 and CD8 MoAbs. HLA-DR, CD25 and Fas were detected by two-colour immunofluorescence on resting or activated T cells using PE-conjugated anti-CD3 MoAb and FITC-conjugated MoAb to HLA-DR, CD25 (Becton Dickinson), and Fas (Chemicon, Temecula, CA). Non-specific background fluorescence was established with the appropriate isotype-matched control MoAb (Becton Dickinson). Antigenic density was expressed as median fluorescence intensity ratio (MFI-R) of total lymphocytes according to the following formula: MFI-R $=$ (MFI of sample histogram (arbitrary units)/MFI of control histogram (arbitrary units)).

Analysis of Fas- or C2-ceramide-induced apoptosis

Fas- or C2-ceramide-induced cell death was evaluated as previously reported [21] on T cell lines obtained by activating peripheral blood mononuclear cells (PBMC) with phytohaemagglutinin (PHA) at days 0 (1 μg/ml) and 15 (0·2 μg/ml) and cultured in RPMI 1640 + 10% fetal calf serum (FCS) + recombinant IL-2 (5 U/ml) (Biogen, Geneva, Switzerland). Fas function was assessed 6 days after the second stimulation (21 days of culture). Cells were incubated with control medium or anti-Fas MoAb (IgM isotype) (1 μg/ml) (UBI, Lake Placid, NY) in the presence of rIL-2 (5 U/ml) to minimize spontaneous cell death. Cell survival was evaluated after 18 h by counting live cells in each well by the trypan blue exclusion test. The same conditions were used to measure cell death induced by methyl-prednisolone (PDN) (Upjohn, Puurs, Belgium) or C2-ceramide (N-acetyl-D-sphingosine) (Sigma, St Louis, MO). Results were expressed as specific cell survival percentage, calculated as follows: (total live cell count in the assay well/total live cell count in the control well) $\times$ 100. This technique was chosen in preliminary experiments

Table 1. Clinical and laboratory features of patients

Patient	Age (years)/sex	Positive familial history	Associated diseases (age of onset, years)	ESR/CRP (mm/h) (mg/dl)	AutoAb	Hyper IgG	Leucocytes (cells/mm^3)	Therapy
1	15/F	–	CoD (1·5) IDDM(13·7) ATD (13·7) JRA (12·9)	56/12	Th-AAb	+	18 500	NSAIDs
2	17/M	+	IDDM (6) LS (15)	10/3	ANA	+	8430	Penicillamine
3	15/M	–	CoD (1·4) AU (6) V (0·5) JRA (4)	45/25	RF	+	22 800	NSAIDs Prednisone

CoD, Coeliac disease; IDDM, insulin-dependent diabetes mellitus; ATD, autoimmune thyroid disease; JRA, juvenile rheumatoid arthritis; LS, linear scleroderma; AU, autoimmune uveitis; V, vasculitis; Th-Aab, thyroid autoantibodies; ANA, antinuclear antibodies; RF, rheumatoid factor; NSAIDs, non-steroidal anti-inflammatory drugs.

comparing sensitivity of several techniques to detect cell death triggered by anti-Fas MoAb on the PHA-derived T cell lines. It evaluates the overall cell survival in the culture at each time point and was found to be more sensitive than techniques detecting the instantaneous proportion of dying cells at each time, such as cytofluorometric determination of cells displaying shrunken/hypergranular morphology, those displaying DNA fragmentation after staining with propidium iodide, or those stained by annexin V. Fas-induced cell death was always less striking in these polyclonal T cell lines than in stabilized tumour cell lines, since it was slower and more asynchronous. The normal range of T cell responses to Fas-, ceramide- and PDN-induced T cell death was defined as the mean + 2 s.d. of data obtained from 65 non-age-matched normal donors.

Analysis of the FAS gene
Mutation analysis of the Fas gene was performed by cDNA sequencing, using a dye terminator DNA sequencing kit (Applied Biosystems Perkin-Elmer, Foster City, CA) on an ABI Model 373A automated DNA sequencer (Applied Biosystems Perkin-Elmer). Total RNA was extracted from fresh PBMC using the Ultraspec-II RNA kit (Biotecx, Houston, TX). Total RNA (2 μg) was used as a template for cDNA synthesis with the Promega (Madison, WI) cDNA synthesis kit. The entire coding sequence of the FAS gene was amplified in a single (1114 bp long) and in overlapping segments 203–302 bp long, using the polymerase chain reaction (PCR) protocol and the A-N primers previously described [21]. PCR was performed with 25 pmol of each primer and 1/4 of the cDNA synthesis reaction (35 cycles per fragment). Automated sequencing was performed twice using primers from both ends.

RESULTS

Immunophenotypic analysis of fresh PBMC
The evaluation of T and B cell markers (i.e. CD3, CD4, CD8, CD19 and CD56) did not reveal significant differences between the three PA patients, control patients with systemic JRA, and the age-matched healthy controls. Evaluation of expression of Fas, CD25, and HLA-DR on CD3$^+$ cells showed that only patient 3 had a slight increase of HLA-DR$^+$ T cells (7%), whereas the other patients had values comparable to the control group (< 2%) (data not shown).

Since the expansion of DN T cells represents a feature of lpr/lpr mice and ALPS patients, we evaluated these cells in PA patients. Only patient 1 had a slight increase (5%), while the other had values comparable to the healthy controls (< 1%).

Evaluation of Fas- and ceramide-induced T cell death
Cell survival was evaluated on T cell lines obtained through a 21 days stimulation of PBMC with PHA in the presence of exogenous rIL-2. Cells were treated with anti-Fas MoAb or C2-ceramide and survival was evaluated after 18 h. Figure 1 illustrates survival in cultures from the three PA patients, systemic JRA, control with IDDM, and age-matched healthy controls. All PA patients displayed decreased cell death response to anti-Fas MoAb and/or ceramide. In patient 3, both stimuli displayed decreased capacity to induce cell death. In patient 1, the cell death response to the anti-Fas MoAb was decreased, whereas that to ceramide was in the normal range. Patient 2 displayed the opposite pattern, since cell death induced by the anti-Fas MoAb was in the normal range, whereas that induced by ceramide was decreased. In contrast, cell death response to the anti-Fas MoAb and

ceramide was in the normal range in all JRA, IDDM patients and age-matched normal donors. The cell death response to PDN was in the normal range in all subjects. Each PA patient was evaluated three times over a 1-year period. Results were always consistent and no correlation was found with the clinical status.

Fas expression was evaluated in the long-term T cell lines on the same day in which Fas function was assessed. Figure 2 shows that normal levels of Fas were expressed by the lines derived from patient 2 and patient 3, whereas in that from patient 1 the Fas expression was slightly below the normal range. These data suggest that decreased Fas expression was not responsible *per se* for the functional Fas defect, at least in patients 2 and 3.

Fas gene molecular studies
Molecular studies of the Fas cDNA revealed that patients 2 and 3 showed normal amounts of PCR products, while patient 1 had a reduced amount, in keeping with her low surface expression of Fas. Together with the normal sized cDNA and splice variants, patients 2 and 3 showed a further transcript, 152 bp longer than the normal sequence. Figure 3a illustrates the ideogram of the transcripts found in patients 2 and 3. Figure 3b shows the abnormal transcript in patients 2 and 3 and in the healthy brother of patient 3, along with the normal transcript of patient 1. This abnormal transcript was detected by using the primer F as reverse primer and the primer C or the primer E as forward primers: primers C and F gave two bands of 211 bp (normal product) and of 363 bp (aberrant product); primers E and F gave two bands of 427 bp (normal product) and 577 bp (aberrant product). Sequence analysis showed that the abnormal bands retained intron 5. The finding was not due to contamination with genomic DNA, because PCR products encompassed exons 2–6 and 3–6, respectively, but only intron 5 was transcribed. Analysis of all members of the family of patient 3 revealed the aberrant product in the mother and brother, but not in the father. By contrast, the aberrant product could not be amplified from 11 normal controls. Sequencing of the entire cDNA, including intron 5, from the patients did not reveal any DNA change, compared with the published normal sequence [24] and polymorphisms [25]. It is noteworthy that translation of the product which retains intron 5 is expected to generate a protein lacking the transmembrane region and all the 3' region downstream to exon 5, because of a stop codon TAA located 99 bases downstream the 5' splice site of intron 5. The new protein would include 32 amino acids downstream from glycine 169 and might be a soluble form of Fas. The splice sites scores were 0·98 and 0·51, respectively, for the donor and the acceptor splice sites (calculated using the human splice site prediction software Neural Networks, Lawrence Berkley National Laboratory: http://www-hcg.lbl.gov/). Thus the 3' splice site ranks suboptimal. We could not amplify a full-length transcript encompassing intron 5 from any of the patients.

DISCUSSION

Children with clusters of distinct autoimmune disorders are far from common. In this study we describe three children showing intense polyreactive autoimmunity, as revealed by the appearance of distinct autoimmune diseases, and decreased T cell death response to Fas triggering and/or ceramide, whose death pathways partially overlap. Patient 1 was diagnosed as having IDDM, JRA, CoD and thyroiditis during the 10 years follow up. While IDDM may be associated with other autoimmune diseases, and in particular with CoD [1,2], its association with multiple autoimmune diseases in the
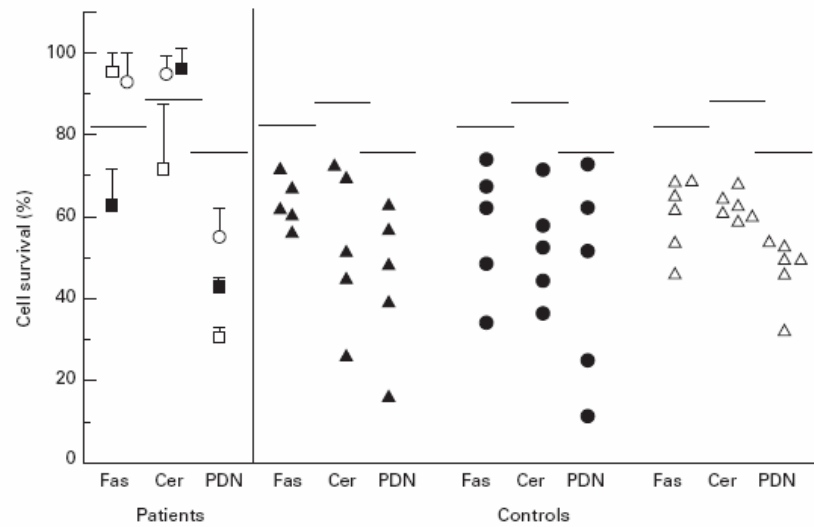
Fig. 1. Cell survival on treatment with anti-Fas MoAb, C2-ceramide, or methylprednisolone (PDN) of T cell lines derived from the three patients with polyreactive autoimmunity, five controls with systemic juvenile rheumatoid arthritis (JRA; ▲), five controls with insulin-dependent diabetes mellitus (IDDM; ●) and 10 age-matched normal controls (Δ). Long-term T cell lines were treated with the indicated reagent and survival was assessed after 18 h. Results are expressed as specific cell survival percentage. In the patients' group, each point represents the mean percentage value of three distinct experiments. Vertical bars indicate 2 s.d. The horizontal lines indicate the upper limit of the normal range calculated as the mean + 2 s.d. from data obtained from 65 non-age-matched normal donors. O, Patient 1; ■, patient 2; □, patient 3.
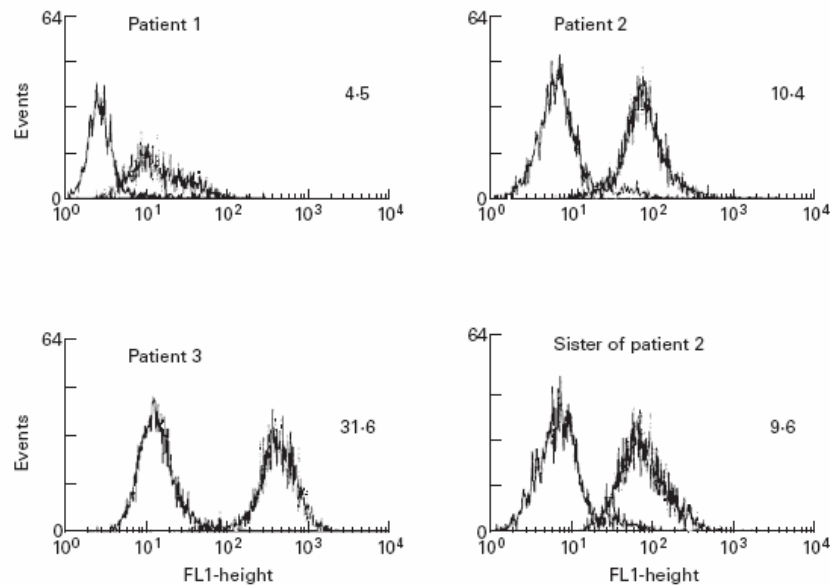
Fig. 2. Fas expression in the long-term T cell lines derived from the three patients with polyreactive autoimmunity and the sister of patient 2. Fas expression was evaluated by direct immunofluorescence and cytofluorometric analysis. Quadrants show the cytofluorometric plots (negative control and Fas staining) from the indicated subject. In each quadrant, the number indicates the median fluorescence intensity ratio (MFI-R), calculated as described in Patients and Methods. The mean ± s.d. of MFI-R obtained from T cell lines derived from 10 age-matched controls was 18 ± 8 (range 5·6–33·4).

Fig. 3. Fas transcripts identified in patients 2 and 3. (a) Schematic depiction of transcripts identified in patients 2 and 3 and the encoded protein. The insertion of intron 5 would, if translated, originate a truncated protein lacking exons 6–9, but including 32 new amino acids after glycine 169.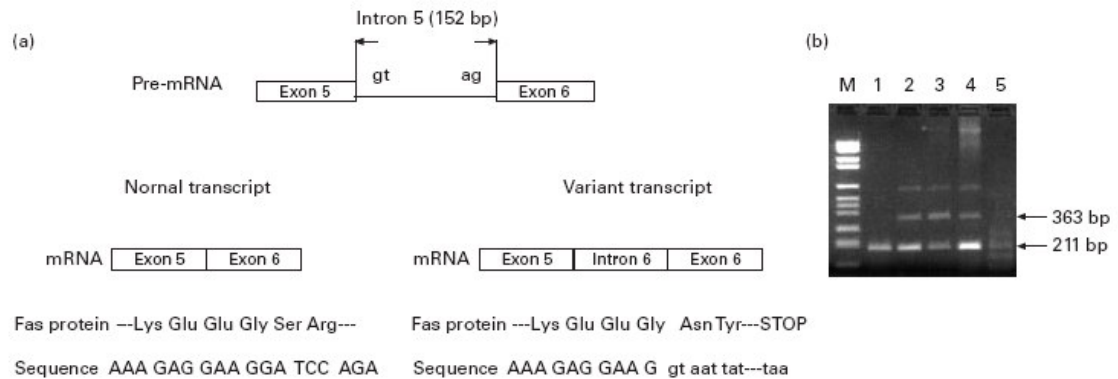 A stop codon is located at base 99 of the intronic sequence. Exonic sequence is indicated in capital letters, intronic sequence in lower case. (b) Polymerase chain reaction (PCR) products obtained using primers E and F [21] from a normal donor (lane 1), patient 2 (lane 2), patient 3 (lane 3), normal brother of patient 3 (lane 4), and patient 1 (lane 5). Patient 2, patient 3 and his healthy brother show fragments of 211 bp (normal) and 363 bp (abnormal). Sequencing showed that the 363-bp fragment includes read-through of intron 5. The PCR product from patient 1 was obtained after two rounds of PCR: nevertheless, the 211-bp band intensity is lower than the others (the multiple spurious bands in this lane are due to the two rounds of PCR). M, Marker ladder.

same individual is quite uncommon [3]. Patient 2 was diagnosed as having IDDM at age 6, and developed overt scleroderma at age 15. Patient 3 had CoD, vasculitis, recurrent episodes of fever with arthritis and uveitis. The heterogeneity of the alterations of cell survival mechanisms observed in our patients reflects the heterogeneity of the pathogenic mechanisms underlying the ALPS. A recent classification named ALPS type Ia and Ib the disease with mutations of Fas and FasL, respectively, and ALPS type II the disease without mutations of these genes [26]. Decreased Fas function has been described in ALPS type Ia with mutations of the Fas gene and in ALPS type II without these mutations. In ALPS type Ia, DN T cells are always expanded, whereas in ALPS type II these cells are often undetectable.

Our PA patients displayed several features similar to ALPS. Patient 1 displayed low Fas expression both at transcript and protein level and expansion of DN T cells. Her resistance to Fas-induced cell death and sensitivity to ceramide were in line with a defect directly involving Fas, as that described in ALPS type Ia. However, no causal mutation has been identified in Fas cDNA. One possibility is that causal mutations may be located in unscreened gene regions, such as the promoter region, intronic splice sites, or generally in genomic DNA. Most ALPS type Ia patients are heterozygous for Fas mutations and express normal levels of Fas, but some express low levels due to mutations that inhibit Fas surface expression. In contrast, Fas expression is always normal in ALPS type II patients. In some ALPS type II patients, abnormality seems to involve both the Fas signalling pathway downstream from Fas and that used by ceramide [21]. In patients 2 and 3 no expansion of DN T cells was found and Fas expression was normal. In patient 3 both Fas- and ceramide-induced cell death was abnormal, while in patient 2 only ceramide failed to induce a proper cell death. In these two patients, a further Fas transcript was found that retains intron 5 expected to encode for a truncated protein lacking the transmembrane and intracellular regions. The aberrant transcript was also observed in the healthy mother and brother of patient 3. A conclusive interpretation of this finding is not thus far possible.

Sequence analysis did not reveal any change in cDNA, which could explain the abnormal splicing. However, mechanisms which regulate pre-mRNA splicing have not been completely elucidated to date. It has been shown that silent DNA changes located far away may affect the correct splice mechanism for each splice site [27,28]. DNA changes in unscreened regions, such as introns, might be present in these patients and affect the correct splicing of intron 5. Another possible interpretation is that the longer Fas transcript is a physiologic splice variant similar to those already described [29]. However, we have been unable to detect this transcript in 11 normal donors. Whether the expression of this form is implicated in the pathogenesis of the polyreactive syndrome remains to be established. The lack of concordance between the longer transcript and the presence of the disease (relatives of patient 3 were healthy) seems to be against this possibility. However, discrepancies may be only apparent. In fact, the lack of concordance between the aberrant form and the disease status was similar to what observed in ALPS type I patients, whose parents carrying the Fas mutation are generally healthy.

Soluble forms of Fas are expected to inhibit Fas- but not ceramide-induced cell death. Whereas patient 2 was sensitive to Fas triggering and resistant to ceramide, the patient 3 was resistant to both stimuli. It is conceivable that low doses of soluble Fas may not inhibit cell death induced by high doses of anti-Fas MoAb in the *in vitro* assay, thus explaining the normal Fas function displayed by patient 2. Thus, the production of the putative soluble form of Fas could inhibit Fas function *in vivo* and be a cofactor for development of the polyreactive autoimmune disease, when associated with other genetic or environmental factors. The current opinion is that Fas triggering activates a caspase cascade leading to cell apoptosis. Hydrolysis of the phospholipid sphingomyelin and generation of ceramide is an alternative pathway that seems to boost the caspase pathway, since it is triggered by caspases and potentiates caspase activation [13], even though the role played by ceramide in Fas signalling is still debated [14,15]. Moreover, the system is under the control of

several inhibitors belonging to the bcl-2, FLIP, and IAP families [1]. Therefore, in both patients 2 and 3, the overt disease and the decreased function of the death pathway triggered by ceramide could be explained by the concurrence of other genetic factors hitting the pathway downstream from FAS. Moreover, these factors may hit other systems involved in apoptosis induction, such as TNF, trail and the granzymes systems, which use apoptotic pathways partially overlapping that of Fas [1,30].

It is noteworthy that our patient 2 was resistant to ceramide, but responded normally to Fas triggering, which shows that the Fas pathway can also function normally when the ceramide pathway is unfunctional and suggests that alteration of T cell switching-off systems that are independent of Fas may play a role in autoimmunity.

In conclusion, our data indicate that polyreactive autoimmune syndromes may be associated with heterogeneous alterations of the immune response switching-off system. However, perturbation of the T cell switching-off mechanism does not seem to be a general functional alteration common to other autoimmune diseases, since five controls with systemic JRA and five with IDDM showed a Fas- and ceramide-induced programmed cell death in the normal range. Moreover, it has been reported that PBMC isolated from patients with systemic lupus erythematosus have a high spontaneous or Fas-mediated apoptosis and increased membrane expression of Fas under resting conditions [31–34], which seems to be related to the *in vivo* constitutive activation of PBMC.

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2.1.2 Immunological characterization of patients with Clustering of Autoimmune Disease phenotype

Along with classic forms of ALPS, recently has been identified a novel phenotype ALPS-like in which the autoimmunity is associated to specific functional deficit of T, B and Natural Killer (NK) cells (Chun H.J. et al 2002). This phenotype is due to a homozygous mutation in the gene that encoded for the caspase 8 protein. Therefore on the base of these evidences we performed the second phase of this research project.

In this work, for the first time, we have performed a molecular and immunological characterization of a group of pediatric patient affected by clustering of autoimmune disease (CAD) phenotype and a functional defect in the Fas-induced cell-death. In particular aim of this study was to investigate if a clear clinical phenotype with multiple autoimmunity may be suggest of a more complex phenotype of immunodeficiency and if its pathogenesis may be caused to a single gene defect.

In the wide coorte of the subjects with CAD phenotype that referred to our Department, 22 patients, were enrolled on base of the following criteria: presence of at least two distinct autoimmune diseases in the same individual associated to the defect in the apoptosis Fas-induced.

The clinical and laboratory features of the 22 patients showed the presence of systemic and/or organ-specific autoimmune disorders (Table 1).

Table 1. Major clinical and laboratory features of CAD patients

Patient #	Autoimmune Disease	*Fas functional (%)
1	Autoimmune Thyroiditis, Type 1 Diabetes Mellitus	82
2	Type 1 Diabetes Mellitus, Autoimmune Thyroiditis, Cytopenia	100
3	Cytopenia, Autoimmune Trombocytopenia	100
4	Hypoparathyroidism, Candidiasis, Addison, Deficit of GH, Skin dystrophy, Autoimmune Thyroiditis, Ovary Failure, Pancreatitis	88
5	Type 1 Diabetes Mellitus, Coeliac Disease, Autoimmune Thyroiditis	79
6	Deficit of IgA, Type 1 Diabetes Mellitus, Vitiligo, Psoriasis	80
7	Urticaria, Atopic Dermatitis	104
8	Cytopenia, Autoimmune Trombocytopenia, Linfoadenopatia	90
9	Autoimmune Trombocytopenia, Deficit of IgA, Uveitis	106
10	Autoimmune Enteropathy, Deficit of GH	106
11	Autoimmune Trombocytopenia, Autoimmune Thyroiditis	91
12	Enteropathy, Candidiasis	82
13	Coeliac Disease, Type 1 Diabetes Mellitus, Autoimmune Thyroiditis	106
14	Vasculitis, Hypergammaglobulinemia, Splenomegaly	79
15	Type 1 Diabetes Mellitus, Autoimmune Thyroiditis	100
16	Juvenile Idiophatic Arthritis, Coeliac Disease, Uveitis, Vasculitis,	96
17	Type 1 Diabetes Mellitus, Coeliac Disease	89
18	Autoimmune Trombocytopenia, Autoimmune Thyroiditis	100
19	Coeliac Disease, Juvenile Idiophatic Arthritis, Type 1 Diabetes Mellitus	100
20	Mixed Connectivie Tissue, Type 1 Diabetes Mellitus, Ovary Failure	80
21	Splenomegaly, Lymphoadenopathy	90
22	Cytopenia, Splenomegaly	91

GH= Growth Hormone
*T cell survival after anti Fas antibody stimuli. This values are expressed in % The value > or = 79% is pathologic.

In this group of the patients was evaluated the functional immunological deficiency through the lymphocytes proliferative responce after phytoemagglutinin and/or anti-CD3 antibody stimuli. The resulted showed that 13/22 patients (59%) were also affected by a defect in T cell activation (Fig.1).
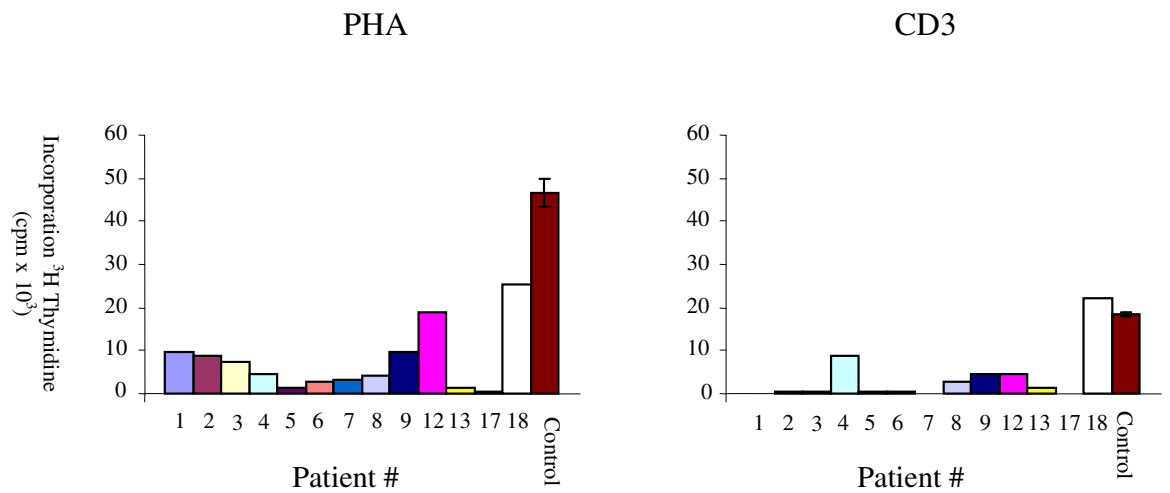
Fig. 1 Proliferative response to the PHA and CD3 in a CAD patients

The molecular analysis of caspase 8 gene performed in 12 of these 13 patients revealed the presence of three polymorphisms of which one localized in the exon 9 and the other two localized in the intron region flanking the exon 9 (Fig.2 and Table 2). At least one of three polymorphic variant was found in 10/12 subjects.
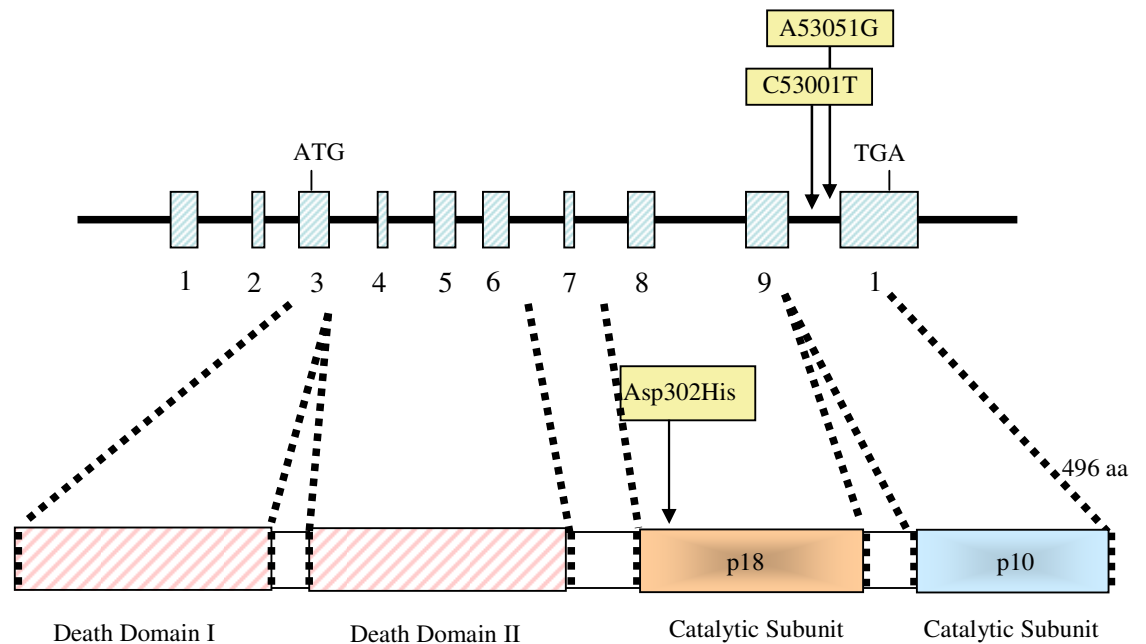
Fig.2 Alterations in exon and intron region of caspase8 gene.

Table3. Alterations of caspase8 gene in CAD patients

Pt #	Asp302His ☆	A53051G	C53001T
1	Heterozygote		
2			
3		Heterozygote	
4		Heterozygote	
5		Heterozygote	
6		Heterozygote	
7			
8			
9		Homozygote	
12		Heterozygote	
13		Heterozygote	
19		Homozygote	Heterozygote

☆ Know polymorphism

Furthermore for ruled out any alteration in perforin-dependent pathway, we evaluated the integrity of this pathway in 8/13 subjects with combined defect. In vitro, the assay of citotossicity revealed that 3 children had a decrease of cytolytic activity of the lymphocytes T (CTL) and NK cells respect to the control subject (Fig. 3).

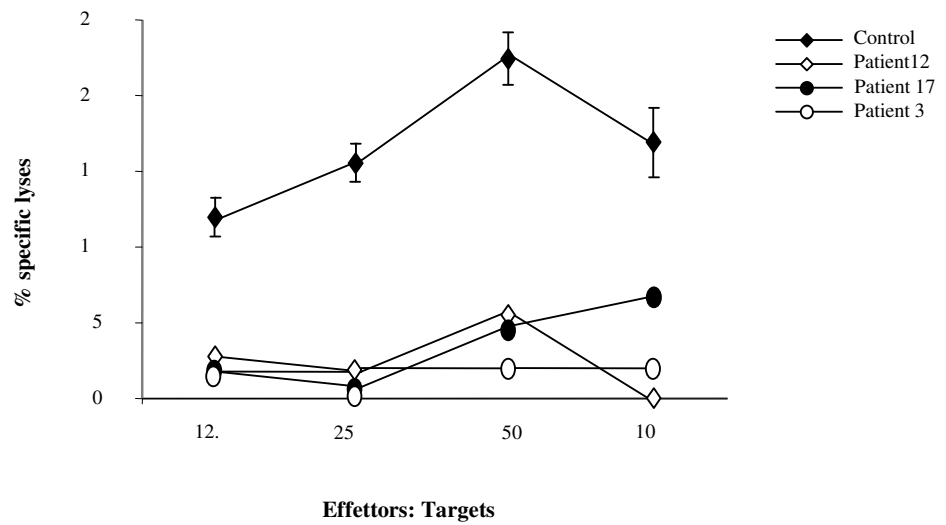
Fig. 3 Evaluation of cytolytic activity in CAD patients with combined defect

Nevertheless any causative mutation has been revealed in the gene that encode for the perforin protein, which have a crucial role in this way of the apoptotic pathway.

In conclusion, the CAD phenotype may be considered a novel clinical entities characterized to the presence in the same individual of a wide spectrum of autoimmune diseases. Even though all the patients, of this study, showed a functional defect of Fas-induced cell-death, this alteration be not ascribed to any causative mutation in Fas gene. Furthermore, these data, suggests that a lymphocyte activation defect is less uncommon than expected in patients with severe autoimmunity associated with defective Fas-induced apoptosis. The heterogeneity of the phenotype may be explained cosiderating that the alteration of different molecules shared from the apoptosis and activation cell pathways may influence the its expression.

The caspase 8 gene polymorphisms founded in our patients along does not cause the disease but its role in the pathogenesis cannot ruled out. In fact this polymorphisms may to represent a predisposing factor for this complex disorder.

These results has been published on the Journal of the "Incontri Pediatrici Normanni" International Congress. See below for the manuscript.

Identificazione di un nuovo fenotipo di malattia caratterizzato da immunodeficit T e/o NK ed autoimmunità: analisi molecolare dei geni caspasi 8 e perforina

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Le malattie autoimmuni rappresentano un gruppo di disordini estremamente eterogeneo. Attualmente, sono state descritte diverse sindromi da immunodeficienza il cui quadro clinico è caratterizzato da "clustering" di distinte malattie autoimmuni (CAD) in uno stesso individuo. Un prototipo di tali sindromi è rappresentato dalla Sindrome Autoimmune Linfoproliferativa (ALPS) di cui si conoscono diverse forme con ereditarietà monogenica. È ormai noto che un'alterazione del processo di apoptosi rappresenta una possibile, anche se rara causa di autoimmunità multipla. L'apoptosi, o morte cellulare programmata, svolge un'importante ruolo nel regolare la differenziazione cellulare e l'omeostasi delle cellule del sistema immune. Il processo di apoptosi può avvenire in due diversi modi: in seguito al legame e all'attivazione di recettori di morte (es. Fas/Fas ligando) con i loro rispettivi ligandi espressi normalmente dai linfociti T citotossici (CTL) e/o in seguito all'esocitosi di granuli intracitoplasmatici contenenti granzimi e perforina da parte delle cellule CTL e Natural Killer (NK). Entrambe queste vie causano l'attivazione a cascata di proteasi dette caspasi responsabili dell'apoptosi. Recentemente è stato dimostrato che l'alterazione del gene caspasi 8 in pazienti che mostravano un fenotipo ALPS-like era associata ad un immunodeficienza combinata (T, B ed NK) sia pure non grave.

Lo scopo del nostro studio è stato quello di caratterizzare funzionalmente e molecolarmente pazienti in età pediatrica con un fenotipo CAD e con difetto apoptotico Fas-indotto. Abbiamo identificato 22 pazienti che mostravano un fenotipo di autoimmunità associata a ridotta apoptosi indotta da FAS. Inoltre, allo scopo di valutare alterazioni della funzionalità linfocitaria veniva eseguito test di proliferazione dopo stimolo ai comuni mitogeni (PHA, CD3 X-L). Per valutare se nei soggetti con difetto funzionale apoptotico Fas-indotto e di attivazione linfocitaria vi fosse anche una compromissione funzionale dei linfociti CTL ed NK veniva eseguito un saggio di citotossicità in vitro. L'analisi molecolare dei geni caspasi 8 e perforina era valutata per sequenziamento diretto. I risultati hanno mostrato che in 13 dei 22 (59%) soggetti era presente una ridotta attivazione linfocitaria T. L'analisi di sequenza del gene della caspasi 8 effettuata in 12/13 pazienti con difetto funzionale combinato mostrava la presenza di tre polimorfismi di cui uno localizzato nell'esone 9 e gli altri due localizzati nell'introne fiancheggiante l'esone 9. Inoltre una ridotta attività citotossica era presente in 3 soggetti con difetto funzionale combinato nonostante l'analisi del gene della perforina era risultato normale.

In conclusione i nostri dati indicano che nei pazienti affetti da CAD può esserci la contemporanea presenza di ridotta apoptosi Fas-indotta e di attivazione linfocitaria. Tuttavia rimane da chiarire il ruolo dei polimorfismi identificati nel gene caspasi 8 nella patogenesi del fenotipo CAD.

INTRODUZIONE

Le sindromi autoimmuni rappresentano un gruppo di malattie clinicamente e molecolarmente eterogeneo, la cui incidenza è stimata nel 3-5% della popolazione[1]. Caratteristica comune dei processi autoimmuni è la presenza di cloni linfocitari autoreattivi che possono rappresentare la causa di malattie sistemiche o rappresentare l'effetto di processi infiammatori[2]. In alcuni casi una risposta autoimmune può derivare da un'alterata reazione immunitaria che può essere innescata da infezioni virali o batteriche[3], in altri rari casi può derivare paradossalmente da un'immunodeficit linfocitario[4].

Anche se attualmente il meccanismo patogenetico di base dell'autoimmunità è ancora poco chiaro, è ormai noto che un'alterazione del processo di apoptosi svolge un ruolo chiave nello patogenesi delle malattie autoimmuni multiple. L'apoptosi o morte cellulare programmata, è un'importante meccanismo di regolazione del sistema immune in quanto limita l'espansione clonale alla fine di una risposta immune, interviene nei processi di differenzazione cellulare e garantisce il mantenimento dell'omeostasi linfocitaria.

Il processo di apoptosi può avvenire attraverso due diversi pathway: non secretorio e secretorio. Nel primo il processo apoptotico è innescato in seguito all'interazione ed all'attivazione di specifici recettori di morte con i loro rispettivi ligandi. Il recettore Fas (CD95) è il più efficiente induttore di apoptosi linfocitaria ed è espresso sulla superficie di linfociti target. Appartiene alla superfamiglia dei recettori Tumor Necrosis Factor (TNF-R) ed è caratterizzato dalla presenza di un dominio di morte (DD) nella regione citoplasmatica. In seguito all'attivazione di tale recettore si ha la formazione di un complesso detto DISC (Death-Inducing Signaling Complex) attraverso il quale si ha la trasduzione del segnale apoptotico. Durante la formazione del DISC è reclutata ed attivata una proteina adattatrice detta FADD (Fas-Associated Death Domain) che interagendo con la procaspasi 8 (forma inattiva) ne promuove l'autoprocessamento catalitico in una proteina eterotetramerica attiva, la caspasi 8. Quest'ultima è così capace di attivare altre caspasi tra cui la caspasi 3 iniziando una cascata di reazioni proteolitiche che culminano nella morte cellulare[5]. Il ligando di Fas (FasL) è normalmente espresso sulla superficie dei linfociti T citotossici (CTL) e delle cellule Natural Killer (NK). Il secondo pathway, detto secretorio, si attua attraverso la secrezione di granuli intracitoplasmatici contenenti proteine litiche da parte delle cellule CTL ed NK, quali la perforina ed una serie di proteasi a serina chiamate granzimi. Durante l'attività litica, i granzimi A e B, contenuti nei granuli, vengono rilasciati ed entrano nella cellula bersaglio grazie ad endocitosi mediata da recettori. Il ruolo della perforina è essenziale per il rilascio endosomale del granzima B nel citosol e nel nucleo della cellula target e per la successiva attività di lisi cellulare

attuata dalla caspasi 3[6-8]. Pertanto entrambe queste vie determinano l'attivazione di una stessa molecola, la caspasi 3.

Solo recentemente è stata documentata l'esistenza di nuovi fenotipi clinici con "Clustering" di malattie autoimmuni (CAD), caratterizzati dalla presenza di disordini autoimmuni multipli in uno stesso individuo. Per tali nuovi fenotipi è stato possibile individuare il singolo difetto genetico e la modalità di trasmissione ereditaria. L'esistenza di trasmissione mendeliana di clustering di malattie autoimmuni è stata ben documentata in diversi tipi di sindromi. A tal proposito la Sindrome Autoimmune Linfoproliferativa (ALPS), di cui si conoscono diverse forme in base alla presenza di alterazioni di diverse molecole coinvolte nei vari stadi del processo apoptotico, rappresenta il prototipo delle sindromi CAD[9].

Accanto alle forme classiche di ALPS è stata recentemente identificato un nuovo fenotipo detto ALPS-like in cui all'autoimmunità si associa un immunodeficit funzionale specifico T, B, ed NK e la cui causa è dovuta ad una mutazione del gene che codifica per la caspasi 8[10].

Scopo di questo lavoro è stato quello di caratterizzare immunologicamente e molecolarmente un gruppo di pazienti con fenotipo CAD e con un chiaro difetto funzionale apoptotico Fas-indotto.

MATERIALI E METODI

Pazienti

Tra un'ampia coorte di soggetti con fenotipo CAD che afferiscono presso il nostro Dipartimento, 22 pazienti, erano selezionati in base ai seguenti criteri di inclusione: presenza di almeno 2 diversi disordini autoimmuni nello stesso individuo associati ad un difetto di apoptosi Fas-indotto. Tra le caratteristiche cliniche di tali pazienti era possibile evidenziare la presenza di disordini autoimmuni sistemici e/o la presenza di disordini autoimmuni organo specifici.

La diagnosi di Artrite Rumatoide Giovanile era eseguita in base ai criteri dell'American College of Rheumatology[11] mentre la dignosi di Diabete Mellito di tipo I era eseguita in base ai valori di iperglicemia (>126 mg/dl), alla presenza di chetosi e alla presenza di autoanticorpi specifici diretti contro le cellule beta pancreatiche. La Tiroidite Autoimmune era diagnosticata per la presenza di specifici autoanticorpi associata ad una disomogeneità ultrasonografica (in presenza o meno di una compromissione funzionale tiroidea). La Celiachia era diagnosticata in accordo ai criteri ESPGHAN[12].

Risposta proliferativa linfocitaria in vitro

Le cellule mononucleate del sangue periferico (PBMC) erano isolate mediante centrifugazione su gradiente di Ficoll-Hypaque (Biochrom, Berlino, Germania) secondo procedura standard. Il saggio di proliferazione dei linfociti veniva effettuato usando una piastra costituita da 96 pozzetti (Becton Dickinson, San Jose, CA) ponendo in coltura 2×10^5 cellule per pozzetto in assenza e/o in presenza di PHA (8 ?g/ml) (Biochrom, Berlino, Germania) e mediante cross-linking del CD3 (0.3 ng/ml) (Ortho Diagnostics Raritan, NJ),

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per 72 ore a 37°C in atmosfera umidificata al 5% di CO_2. La risposta prolifera-tiva era valutata attraverso l'incorporazione di timidina da parte delle cellule in coltura marcate con 0,5 ?Ci di timidina triziata 16 ore prima della fine della coltura. La radioattività rilasciata dalla timidina [3H] veniva misurata in conte per minuto (cpm) utilizzando uno scintillatore liquido (Packard 1600 TR Liquid Scintillation Analizer).

Saggio di citotossicità

Le cellule bersaglio (K562) venivano incubate per 1 h a 37°C con Cr[51] (Amersham Pharmacia Biotech) (30 μCi/10[6] cellule) e lavate per tre volte con mezzo di coltura prima di essere addizionate alle cellule effettrici. La lisi speci-fica veniva misurata con un saggio in triplicato effetuuato mescolando 5X10^{3} cellule bersaglio con cellule effettrici in rapporti diversi (E:T = 25:1 a 3.125:1) in un volume totale di 200 μl. Dopo un'incubazione di 5 ore della sospensione cellulare a 37°C, 100 μl di sovranatante di coltura venivano raccolti per valuta-re la quantità di Cr[51] rilasciato. La percentuale di lisi specifica veniva calcolata come segue: 100 x (rilascio specifico spontaneo)/(rilascio totale spontaneo).

Analisi molecolare dei geni caspasi 8 e perforina.

L'analisi molecolare dei geni caspasi 8 e perforina era valutata per sequenzia-mento diretto di DNA genomico. Il DNA era isolato da cellule del sangue periferi-co (PBMC) con i metodi standard. L'amplificazione dei 10 esoni del gene della caspasi 8 e degli esoni 2 e 3 del gene della perforina era eseguita mediante la rea-zione di PCR utilizzando coppie di oligonucleotidi intronici fiancheggianti cia-scun esone. Le modalità e le condizioni della reazione di amplificazione erano determinate sperimentalmente per ciascuna coppia di oligonucleotidi. La reazione di PCR veniva condotta secondo una procedura standard (35-40 cicli di 95°C per 10 min, 95°C per 1min, 58°-61°C per 1min,72°C per 1min utilizzando un GenAmp PCR System 2400 (PE Applied Biosystems). I frammenti così amplifi-cati venivano separati per elettroforesi su gel 1-2% di agarosio secondo i casi e purificati mediante protocollo "Gel Extraction Kit" (QuiaQuick, Quiagen, S.p.A., Milano, Italia). I prodotti di PCR erano sequenziati usando il "Big Dye Terminator Cycle Sequencing Kit" (Applied Biosystem) ed un sequenziatore automatico "ABI PRISM 377" della Applied Biosystem.

Risultati

Pazienti

Le principali caratteristiche cliniche e di laboratorio di 22 pazienti con fenotipo CAD e con un difetto apoptotico Fas-indotto sono riportate in Tabella 1. 7 dei 22 pazienti erano affetti da tre o più disordini autoimmuni. 17 dei 22 presentavano una patologia organo-specifica che coinvolgeva prevalentemente il sistema endo-crino o l'apparato intestinale. 7 pazienti mostravano disordini ematologici con interessamento di una o più linee cellulari mentre 4 dei 22 soggetti mostravano un interessamento del sistema linforeticolare. Due dei 22 pazienti erano affetti da una diminuzione dei livelli sierici di IgA e in altri due soggetti era stata riscontrata una ridotta produzione dell'ormone della crescita (GH).

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Risposta proliferativa

Allo scopo di valutare se nei pazienti, oggetto del nostro studio, affetti da un fenotipo CAD e da una resistenza all'apoptosi si associava anche un difetto immunologico funzionale veniva eseguito un test per mezzo del quale era possibile valutare la risposta proliferativa linfocitaria dopo stimolo con i comuni mitogeni. I risultati ottenuti mostravano che in 13 su 22 soggetti vi era una ridotta proliferazione cellulare T in risposta alla fitoemoagglutinina (PHA) e/o a seguito del cross-linking del CD3.

Analisi molecolare del gene caspasi 8

L'analisi molecolare del gene caspasi 8, eseguita in 12 dei 13 pazienti che presentavano un difetto di proliferazione dei linfociti T, rivelava la presenza di tre diversi polimorfismi noti di cui uno localizzato nell'esone 9 mentre gli altri due localizzati nella regione intronica fiancheggiante l'esone 9 (IVSIX al nucleotide 1073, C->T e IVSIX al nucleotide 1123, A -> G). Questi polimorfismi erano presenti sia allo stato eterozigote che allo stato omozigote.

Saggio di citotossicità

Allo scopo di valutare se nei pazienti CAD con difetto apoptotico e difetto funzionale T era associata anche una compromissione funzionale delle cellule citotossiche (CTL) e/o NK, in 8 di 13 soggetti era eseguito il saggio in vitro di citotossicità. Dall'analisi di tali dati è emerso che in 3 soggetti vi era un'alterata funzionalità citotossica.

Analisi molecolare del gene perforina

L'analisi molecolare del gene che codifica per la perforina era eseguito allo scopo di valutare se nei tre pazienti CAD il difetto di funzionalità CTL ad NK potesse essere dovuto ad un'alterazione genica. I dati ottenuti non mostravano la presenza di nessuna mutazione causativa.

DISCUSSIONE

In questo lavoro descriviamo le caratteristiche cliniche, funzionali e molecolari di un gruppo di 22 pazienti in età pediatrica affetti da "Clustering of Autoimmune Disease" (CAD). Questo fenotipo rappresenta una nuova entità clinica caratterizzato dalla presenza in uno stesso individuo di un ampio spettro di malattie autoimmuni. Inoltre, tutti i pazienti, oggetto del nostro studio, erano affetti da un difetto funzionale apoptotico Fas-indotto non attribuibile a nessuna mutazione genica causativa come rivelato dall'analisi molecolare del gene di Fas. È attualmente noto, che la patogenesi delle malattie autoimmuni è dovuta ad una alterazione dei meccanismi che regolano l'omeostasi e la differenziazione cellulare. Un ruolo chiave in questi meccanismi è svolto da un complesso processo apoptotico che può esplicarsi attraverso due diverse vie: secretoria e non secretoria. Entrambi i pathways condividono numerose molecole di signaling ed attivano a cascata diverse proteasi dette caspasi[5-8].

Recenti evidenze inoltre suggeriscono che l'autoimmunità può associarsi, in alcuni rari casi, ad un difetto funzionale linfocitario T, B ed NK. È stato ipotizzato che una probabile causa di tale associazione sia dovuta ad una mutazione in omo-

zigosi del gene caspasi 8[10]. Pertanto in tutti i soggetti presi in esame è stata valutata la funzionalità linfocitaria, dopo stimolo con PHA e cross-linking del CD3, che ha permesso di individuare 13 su 22 soggetti (59%) affetti anche da un difetto di attivazione linfocitaria T. L'analisi molecolare del gene casapasi 8 in 12 dei 13 soggetti con immunodeficit ha evidenziato la presenza di tre polimorfismi noti, di cui il primo localizzato nella regione dell'esone 9 e gli altri due localizzati nella regione intronica fiancheggiante l'esone 9. Almeno una delle tre varianti polimorfiche trovate è presente in 10 dei 12 soggetti.

Inoltre, allo scopo di escludere un'alterazione del pathway perforina-dipendente, abbiamo valutato l'integrità di tale pathway in 8 dei 13 soggetti in cui si presentava un difetto funzionale combinato. Dall'analisi, eseguita mediante saggio in vitro di citotossicità, è emerso che 3 soggetti mostrano una riduzione dell'attività citotossica dei linfociti T (CTL) e delle cellule NK. Infine allo scopo di escludere che tale alterazione potesse dipendere da mutazioni del gene della perforina, principale molecola effettrice in questa via di apoptosi, abbiamo effettuato l'analisi molecolare del gene. Tuttavia, l'analisi molecolare eseguita nei 3 soggetti con ridotta funzionalità litica non ha però rivelato alcuna alterazione genica.

In conclusione i nostri dati mostrano che pazienti affetti dal fenotipo CAD possono presentare difetti funzionali multipli che necessitano di ulteriori approfondimenti per la caratterizzazione patogenetica. Inoltre l'associazione di tali alterazioni è più frequente di quanto si possa sospettare. Anche se i nostri dati molecolari non hanno permesso di evidenziare alcuna mutazione causativa del gene caspasi 8, è tuttavia ipotizzabile che i polimorfismi da noi individuati possano svolgere un importante ruolo patogenetico.

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2.1.3 Apoptosis in the physiology and in the pathology of immune response

In the final part of this research project we focus our attention on rule of apoptosis in the physiology of the immune response and how alterations in molecule involved in the apoptotic process may be caused complex pathology. The majority of lymphocytes that in vivo expand and proliferate in response to foreign antigens, subsequently, will die to maintain a constant cell number (Busiello R. et al 2004). During the initial expansion phase of an immune response, also autoreactive clones are generate. Without homeostatic mechanisms controlling cell growth, this autoreactive expansion would have fatal consequences on the biological functions of the organism. At the end of an immune response, apoptosis or programmed cell-death play a key role to limit cellular proliferation, restoring immunological homeostasis, and in the deletion of autoreactive lymphocytes, controlling the self-tolerance (King et al 2004).

The apoptosis is activated by three different pathways: extrinsic, induced by death receptor triggering, intrinsic activated by extracellular stimuli and secretory (Rieux-Laucat et al 2003). All these mechanism involved the activation of the specific cysteine proteases called Caspase. Currently, 13 caspases have been identified in mice and humans. The subset of caspases that clave selected substrates to produce the changes associated with apoptosis are know as effector caspase, which in mammalian are caspase 3, 6 and 7. In most instance, these executioner caspase are activated by the initiator caspases: caspase8, 10, 2 and 9. Other initiator caspase are: caspase 1, 4, 5 and most likely caspase 14 (Martinon F et al 2004). The phylogenetic ralathionship of

caspases appears to correlated with their function (Lamkanfi et al 2002). One subfamily of caspases is constitud by caspases that have clear functions in the execution of apoptosis and includes the initiator caspase 8, 9 and 10 and the effector caspase 3, 6 and 7. However, some of the "apoptitic" caspases, in particular caspase 8, have been recently shown to have additional roles in lymphocyte proliferation and differentiation (Chun et al 2002, Salmena et al 2003). Human caspase 1, 4, and 5 and mouse caspase 11 and 12 constitute another group designated inflammatory caspases. In fact these caspases can be involved in apoptosis but their prime function is the regulation of the inflammatory processes (Martinon F et al 2004) (Fig. 4).
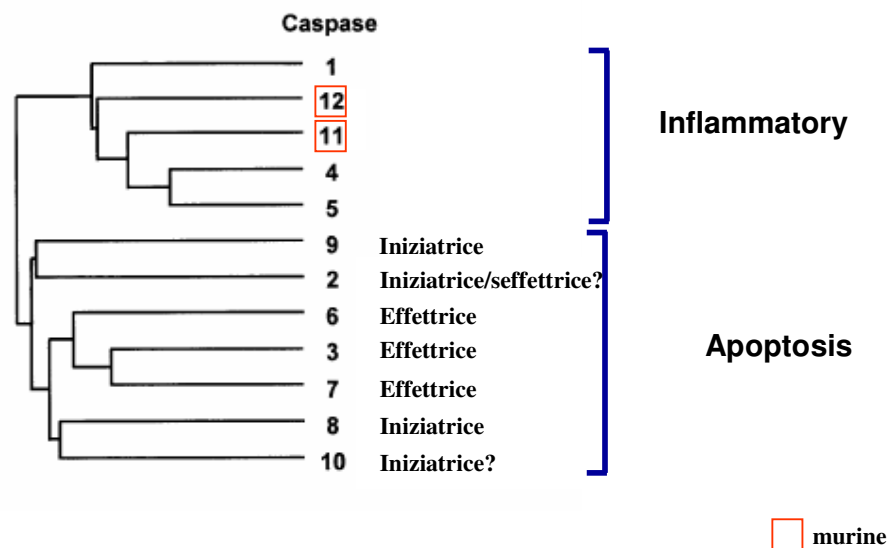
Denis F, Cell Mol Life Sci 1998

Fig.4 Phylogenies of Caspases

The identification of novel human or murine clinical phenotypes and the studies of knock out mice for a specific molecule greatly contributed to ameliorating our knowledge of such homeostatic mechanisms. In particular studies of patients affected by complex autoimmune phenotype have demonstrated a monogenic origin of same hyperimmune syndrome, such as: Autoimmune Lymphoproliferative Syndrome (ALPS) (Canale 1967), Autoimmune Polyendocrinopathy Candidiasis-Ectodermal Dystrophy (APECED) (Neufeld 1980), Immunodysregulation, Polyendocrinopathy, Enteropathy, X linked syndrome (IPEX) (Wildin 2002). These are caused to alterations of molecules involved in cell apoptosis.

ALPS

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 (Chan et al 2002) (Table 4).

Table 4. Gene mutations in Autoimmune Lymphoproliferative Syndrome.

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

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 non redundancy of the mechanism controlling autoimmune disease. This review has been published on the Journal of Prospettive in Pediatria. See below for the manuscript

L'apoptosi nella fisiologia della risposta immune e sue alterazioni nella patogenesi di quadri di patologia complessa

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Riassunto

Numerosi studi hanno permesso di definire, negli ultimi 10 anni, gli intimi meccanismi responsabili del mantenimento dell'omeostasi linfocitaria nella fisiologia della risposta immune. Le cellule del sistema immune hanno la prerogativa di espandersi proliferando a seguito dell'incontro con l'antigene specifico. Inevitabilmente, vengono anche generati cloni autoreattivi, che avrebbero conseguenze fatali se non vi fossero i meccanismi omeostatici di controllo della crescita. Un ruolo importante nel mantenimento dell'omeostasi è giocato dall'apoptosi linfocitaria, cui partecipano molecole di membrana, tra cui Fas ed il suo ligando, ed intracellulari. Il processo si realizza mediante un meccanismo estrinseco, innescato dai recettori di morte, intrinseco che si attua a seguito di stimoli esterni alla cellula o secretorio. In tutti i casi le Caspasi svolgono un ruolo centrale nella trasmissione del segnale. Peculiarità di alcune delle Caspasi è quella di partecipare sia ai processi dell'infiammazione che all'apoptosi. Nel limitare la generazione di cloni autoreattivi svolge un ruolo importante anche la tolleranza immunologica. La tolleranza centrale si realizza negli organi linfoidi primari, mentre la periferica negli organi linfoidi secondari. Contributo rilevante alla comprensione di tali meccanismi è stato dato dallo studio dei quadri di patologia sia dei modelli animali naturali o dei topi *knock out*, che di patologia umana. In particolare, lo studio dei pazienti con sindrome autoimmune linfoproliferativa ha permesso di evidenziare come quadri di iperimmunità multipla possano essere legati ad alterazioni dell'apoptosi su base genetica. Alterazioni del gene Aire, coinvolto nella tolleranza centrale, o delle cellule T regolatorie sono responsabili di due sindromi di recente definizione, l'APECED e l'IPEX. Il principale contributo di questi studi è stato quello di aver definito le basi molecolari di alcuni quadri di sindromi autoimmuni complesse trasmesse come disordini monogenici.

Summary

Recently, several studies led to define the intimate mechanisms responsible for lymphocyte homeostasis in immune response. Immune system cells have the capability to proliferate after encountering specific antigens. Within the process, autoreactive clones are generated as well. Without homeostatic mechanisms controlling cell growth, this autoreactive expansion would have fatal consequences. A major role is played by lymphocyte apoptosis, that involves intracellular or membrane molecules, as Fas and Fas ligand. The process takes place through an extrinsic, induced by death receptors triggering, intrinsic, activated by extracellular stimuli, or secretory mechanism. Caspases participate to all these mechanisms. The hallmark of some molecules of this family is the involvement in either the inflammatory response or in apoptosis. Central and peripheral tolerance plays a major role in limiting autoreactions. Central tolerance occurs in primary lymphoid organs, while the peripheral tolerance in secondary lymphoid organs. The identification of novel human or murine clinical phenotypes and the studies of knock out mice for a specific molecule greatly contributed to ameliorating our knowledge of such homeostatic mechanisms. In particular, studies of patients with autoimmune lymphoproliferative syndrome have demonstrated a monogenic origin of some hyperimmune syndromes related to alterations of molecules involved in cell apoptosis. Further examples are represented by two novel clinical entities, APECED and IPEX, caused by molecular alterations of the Aire gene, involved in central tolerance, and of T regulatory cells, respectively. These studies greatly contributed to demonstrate the molecular bases of some hyperimmune syndromes inherited as monogenic disorders.

Introduzione

Una risposta immune fisiologica è caratterizzata dall'espansione di cloni di linfociti T e B che hanno imparato a riconoscere determinati antigeni. Tale espansione clonale è tuttavia limitata da differenti fattori che determinano quel quadro di equilibrio tra crescita e morte cellulare definito meccanismo di proliferazione omeostatica (King et al., 2004). La proliferazione cellulare è soprattutto limitata dallo spazio disponibile negli organi linfoidi e dalla limitata disponibilità di specifici fattori di crescita, che non consentono l'espansione illimitata delle cellule responsive.

Apoptosi o morte cellulare programmata

L'apoptosi è un processo multifasico finemente regolato che conduce alla morte selettiva di cellule sulla base di un programma geneticamente controllato. Tale processo è essenziale per il normale sviluppo embrionale, per il mantenimento dell'omeostasi tissutale e per il rimodellamento nella rigenerazione degli organi (Gaetaniello et al., 1998; McCarthy, 2003). Al processo partecipano recettori di membrana, definiti "recettori di morte", che appartengono alla superfamiglia dei recettori del *Tumor Necrosis Factor* (TNF) e *Nerve Growth Factor* (NGF) (Kischkel et al., 1995). Tali recettori sono accomunati dalla presenza di domini omologhi nella porzione extracitoplasmatica ricchi in cisteina importanti per il legame con i ligandi e dalla presenza di un dominio funzionale intracellulare, definito dominio di morte (Ashkenazi et al., 1998), che interagisce

con le molecole del *signaling* intracellulare coinvolte nell'apoptosi. La molecola più rilevante per questo processo è rappresentata da Fas o CD95, che è il più potente induttore di apoptosi linfocitaria (Rieux-Laucat et al., 2003). La molecola consiste di tre domini extracellulari ricchi in cisteina (CRDs) e 80 residui aminoacidici che costituiscono il dominio di morte. In seguito al legame con il ligando (FasL), tre molecole di Fas si assemblano in un complesso unico, cui segue l'interazione con l'adattatore cellulare FADD (*Fas Associated Death Domain*). Successivamente, le molecole Caspasi 8 e 10 vengono ingaggiate per la formazione del DISC (*Death Inducing Signaling Complex*) (Kischkel et al., 1995).
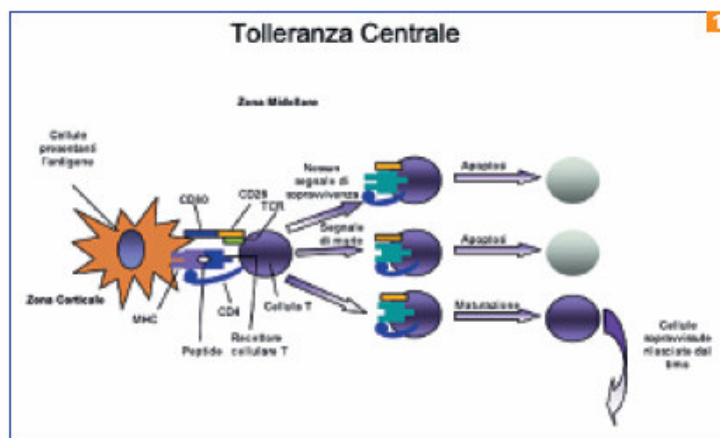
Le cellule che si avviano alla morte cellulare programmata subiscono importanti modificazioni morfologiche e molecolari: diminuiscono di grandezza, condensano il proprio DNA, che viene suddiviso in frammenti e racchiuso, insieme agli altri detriti cellulari, nei cosiddetti corpi apoptotici, in seguito fagocitati da cellule "*scavenger*".

Un potente effetto inibitorio del processo di apoptosi indotto da Fas è svolto dalle proteine virali inibitorie definite FLIPs, che contenendo un dominio effettore di morte simile alla procaspasi 8 e a FADD, competono nell'interazione con queste molecole.

Nel corso di una risposta immune produttiva è inevitabile che, durante la generazione di un repertorio di recettori specifici per gli antigeni assai diversificato, si generino anche cloni autoreattivi in conseguenza del riarrangiamento casuale dei geni che codificano per questi recettori. Evidentemente, la generazione

Fig. 1. Meccanismi della tolleranza centrale.

di cloni autoreattivi avrebbe conseguenze fatali per l'organismo biologico se non vi fossero dei meccanismi di controllo della crescita volti a mantenere la tolleranza verso il self.

Tolleranza immunologica

Esistono due meccanismi predominanti che permettono la tolleranza: il primo, tolleranza centrale, si realizza nel corso del differenziamento negli organi linfoidi primari, quali il timo ed il midollo osseo, ed è rivolto a linfociti T e B immaturi. La tolleranza centrale si realizza mediante la delezione o l'inattivazione dei linfociti autoreattivi (Mathis et al., 2004). Nel timo il precursore del linfocita T che non esprime ancora il recettore per l'antigene (TCR) non riceverà un appropriato segnale di sopravvivenza e morirà per apoptosi. Tuttavia, anche il linfocita T, che mediante il TCR interagisce con l'antigene con un legame ad alta affinità, subirà lo stesso destino di morte per apoptosi. L'unico linfocita che raggiungerà la maturazione ed espleterà la sua fun-

zione in periferia è il linfocita che interagisce con l'antigene con un legame a bassa affinità (Fig. 1). La tolleranza periferica invece si realizza nel sangue periferico e negli organi linfoidi secondari ed è rivolta ai linfociti T e B maturi (Walker et al., 2002). Anche in periferia la delezione clonale svolge un ruolo rilevante (Fig. 2). Esistono tuttavia ulteriori meccanismi di tolleranza, quali l'anergia da citochine anergizzanti, ad esempio l'IL-10, e i meccanismi di regolazione legati alle cellule CD4+ CD25+ T reg, che rappresentano circa il 10% dei linfociti CD4+. Un ulteriore meccanismo di controllo omeostatico è rappresentato dalla lisi clonale mediante meccanismo secretorio, che coinvolge le molecole di granzima A e B e la perforina (Fig. 2). È interessante notare come molte delle informazioni sulla fisiologia dei meccanismi di apoptosi e regolazione della tolleranza provengano dalle osservazioni di malattie rare dell'infanzia illustrate in seguito, che hanno permesso di chiarire il
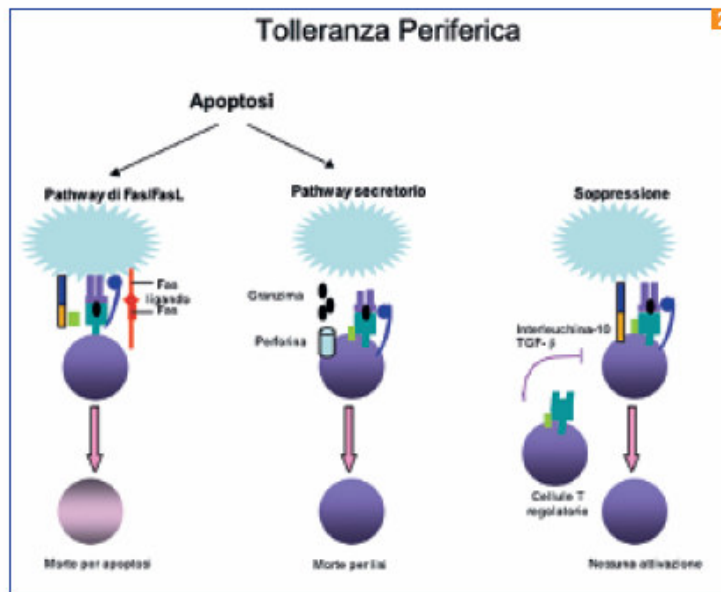
ruolo chiave che singole molecole svolgono in questi processi.

Pathways dell'apoptosi

Il processo di morte cellulare programmata può essere attivato da tre differenti *pathways*: estrinseco, intrinseco e secretorio (Rieux-Laucat et al., 2003). Nella prima via, quella estrinseca, il processo apoptotico è innescato dai "recettori di morte" già menzionati, quali il Fas che a valle attiva la cascata delle Caspasi, responsabili di reazioni proteolitiche, che infine determinano la frammentazione del DNA con conseguente morte cellulare (Fig. 3). Il taglio della matrice nucleare e delle proteine del citoscheletro produce le note alterazioni strutturali sia del nucleo sia del citoplasma che si osservano nelle cellule apoptotiche.

Un ulteriore *pathway* apoptotico è quello intrinseco, che si attua in seguito a stimoli interni alla cellula stessa, quali ad esempio quelli dovuti ad un danno genetico irreparabile, a concentrazioni di calcio citosolico estremamente alte o a gravi stress ossidativi. Si attivano così diversi fattori proapoptotici tra cui i più conosciuti sono i membri della famiglia delle proteine Bcl-2 (Petros et al., 2004). Si tratta di proteine regolatrici, alcune delle quali promuovono l'apoptosi (Bad, Bid, Bax), mentre altre (Bcl-x, Bcl-w, e la stessa Bcl-2) la prevengono. Lo stimolo di morte attiva i membri proapoptotici della famiglia Bcl-2, che traslocano dal citosol alla membrana mitocondriale esterna. Il citocromo c, debolmente associato alla superficie esterna della membrana mitocondriale interna, risiede effettivamente nello spazio in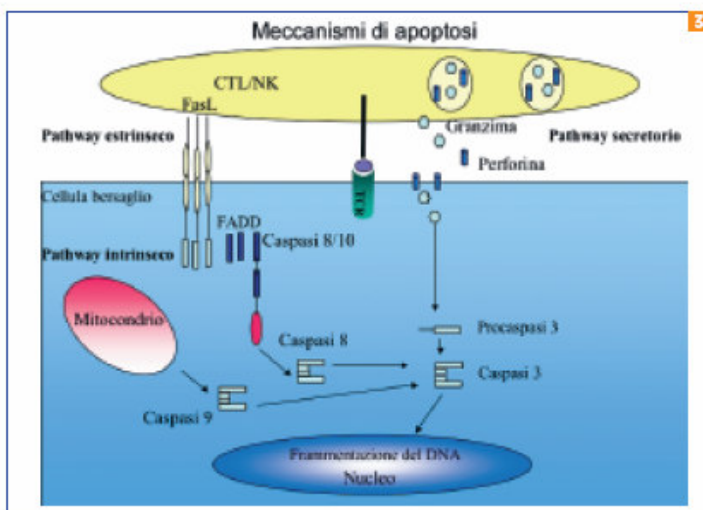termembrana. L'attacco di Bad o di altre proteine proapoptotiche alla membrana mitocondriale esterna ne altera la permeabilità (Sharpe et al., 2004) promuovendo il rilascio del citocromo c nel citosol (Nutt et al., 2002). Una volta nel citoplasma, il citocromo c forma un complesso multimerico con una proteina citosolica chiamata APAF-1 e con molecole di procaspasi 9. Le molecole di Caspasi 9 (forma attiva) attraverso specifici tagli proteolitici attivano altre Caspasi, dette effettrici, che promuovono la frammentazione del DNA e la definitiva morte cellulare. Le due vie apoptotiche, estrinseca ed intrinseca, convergono nella fase finale, attivando le stesse Caspasi effettrici, che tagliano i medesimi bersagli cellulari.

Il terzo *pathway* apoptotico, definito secretorio, si attua attraverso la secrezione di granuli contenenti proteine litiche, quali la perforina ed una serie di serina-proteasi chiamate granzimi (Darmon et al., 1995). Durante l'attività litica, i granzimi A e B, contenuti nei granuli, vengono rilasciati ed entrano nella cellula bersaglio grazie ad endocitosi mediata da recettori, in particolare grazie al recettore di recente identificazione mannosio-6 fosfato (Smyth et al., 2005). Il ruolo della perforina è essenziale per il rilascio endosomiale del granzima B nel citosol e nel nucleo della cellula target e per la successiva lisi cellulare attuata dalla Caspasi 3.

Le Caspasi

Le Caspasi giocano, quindi, un ruolo cruciale nell'apoptosi. Esse sono, tuttavia, coinvolte anche nei processi di crescita, differenziamento cellulare e maturazione citochinica (Wang et al., 2000). Esse sono delle cisteina–pro-

teasi che utilizzano l'atomo di zolfo della cisteina per compiere le reazioni di taglio proteolitico, in genere, mediante il riconoscimento di una sequenza tetrapeptidica ed il taglio specifico del legame peptidico c-terminale a livello di residui di Acido Aspartico (Boatright et al., 2003). Le proteasi hanno bersagli intracellulari specifici, quali le proteine della lamina nucleare e del citoscheletro. Esse originano da un comune precursore molecolare e nel corso dell'evoluzione filogenetica si sono differenziate in iniziatrici ed effettrici, per la presenza o meno di determinati domini funzionali (Martinon et al., 2004) (Fig. 4). In particolare, tali molecole sono provviste di ripetizioni in tandem definite *Death Effector Domain* (DEDs) e domini noti come *Caspase-Recruitment Domain* (CARDs) (Wang et al., 2000). Le Caspasi effettrici sono quelle che, mediante i tagli proteolitici su substrati selezionati, producono le modificazioni proprie

del processo apoptotico, partecipando quindi alla fase finale dell'apoptosi. Quelle iniziatrici agiscono nelle tappe iniziali del processo apoptotico, assolvendo al compito principale di attivare le Caspasi effettrici (Martinon et al., 2004). Nei mammiferi sono state identificate quali Caspasi effettrici la 3, 6, 7 e quali Caspasi iniziatrici la 1, 2, 4, 5, 8, 9, 10, 14 (Martinon et al., 2004). È importante sottolineare che le Caspasi sono attivate nella maggior parte dei programmi di morte cellulare.

In seguito alla formazione del DISC la Caspasi 8 viene attivata e viene innescata l'attivazione a cascata delle procaspasi 3, 7 e 6 nelle rispettive forme attive (Newton et al., 2003).

Nel *pathway* intrinseco, che coinvolge meccanismi mitocondrio-dipendenti l'attivazione della Caspasi 9 porta alla formazione di un complesso chiamato apoptosoma. L'attivazione di Caspasi 9 provoca a sua

volta l'attivazione delle Caspasi effettrici 3, 6 e 7 (Chen et al., 2003; Martinon et al., 2004).

Differentemente dai *pathways* estrinseco ed intrinseco, nel *pathway* secretorio, la scala gerarchica di attivazione delle Caspasi è essenzialmente invertita. Il granzima B, infatti, processa direttamente le Caspasi 3, 7, 8 e 10. In questo *pathway* le Caspasi 2, 6 e 9 sono processate direttamente dalla Caspasi 3. Inoltre, è importante notare che la Caspasi 3 svolge un ruolo fondamentale anche per la maturazione delle Caspasi 8 e 10 (Adrain et al., 2005).

Tuttavia, anche se le Caspasi svolgono un ruolo centrale nell'apoptosi, è opportuno sottolineare che alcune di queste molecole hanno un ruolo altrettanto importante nello sviluppo e nella proliferazione cellulare (Boissonnas et al., 2002). Tale coinvolgimento ha delle importanti implicazioni cliniche, come verrà di seguito evidenziato.

Dalla fisiologia alla patologia

Nell'ultimo decennio l'ampliamento delle conoscenze sui meccanismi biochimici e di regolazione dell'apoptosi è stato reso possibile grazie all'acquisizione di informazioni nei tre ambiti specifici dei modelli animali naturali, dei topi *knock-out* e dei quadri di malattie umane associate ad alterazioni di geni che codificano per molecole coinvolte nel processo biologico dell'apoptosi. Va altresì sottolineato che l'interazione di informazioni scientifiche provenienti da questi tre ambiti consente, oltre a comprendere meglio la patologia umana, anche di ipotizzare nuovi fenotipi clinici, che fino ad

Fig. 4. Albero filogenetico delle Caspasi. Le Caspasi originano da un unico precursore comune e nel corso dell'evoluzione si sono differenziate in due principali famiglie, quelle iniziatrici e quelle effettrici. Le prime sono caratterizzate dalla presenza di ripetizioni in tandem dette *Death Effector Domain* (DEDs) e domini noti come *Caspase Recruiment Domain* (CARDs). Quelle effettrici, invece, sono responsabili dei tagli proteolitici che avvengono nella fase finale del processo di apoptosi.
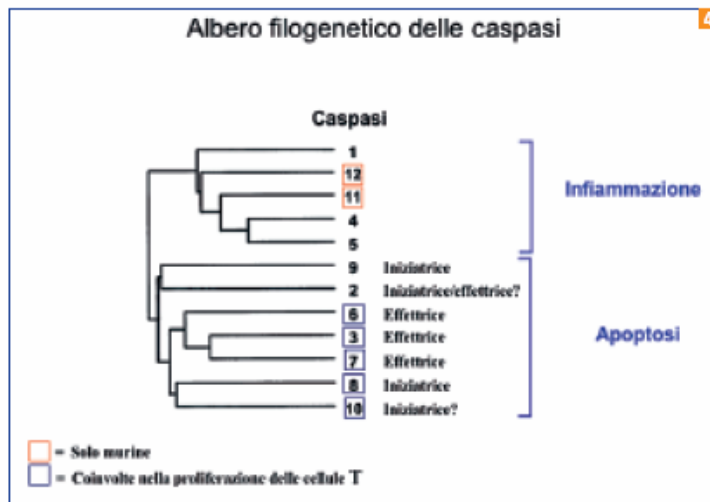
oggi non vengono identificati o non ricevono appropriato inquadramento nosografico.

Modelli animali

La presenza di modelli animali con mutazioni di singoli geni e l'analisi dei relativi fenotipi clinici ha senza dubbio dato un considerevole contributo alle conoscenze in questo settore. I topi *lpr* e *gld* sono caratterizzati da mutazioni dei geni che codificano per le proteine Fas e FasL, rispettivamente (Nagata et al., 1995). In entrambi i casi la mutazione determina perdita di funzione. Come illustrato in Tabella I, fenotipi clinici di questi topi sono essenzialmente caratterizzati dalla presenza di un alto titolo di autoanticorpi e da considerevole accumulo di linfociti attivati ed autoreattivi nei tessuti linfoidi secondari con conseguente notevole impegno del sistema linforeticolare (Rieux-Laucat et al., 2003). Ne deriva che i to-

pi presentano malattie autoimmunitarie, coinvolgenti gli organi più disparati, splenomegalia e linfoadenomegalia (Rieux-Laucat et al., 2003). Inoltre, è stata descritta una maggiore prevalenza di disordini linfoproliferativi, quale conseguenza dell'alterata omeostasi cellulare T e B. Inoltre, in questi topi è stato descritto un aumento di cellule TCR+ CD4- e CD8- doppio negative nel sangue periferico dovuto ad un difetto di maturazione intratimica. Tuttavia, numerose evidenze sperimentali, ottenute in studi genetici condotti su modelli animali ed umani, dimostrano che le mutazioni del gene Fas non sono sempre sufficienti a determinare il fenotipo ALPS. In particolare, è stato dimostrato che il background genetico dei ceppi di topo è determinante per l'espressività clinica della malattia (Choi et al., 1999). Tale background genetico può inoltre spiegare le differenze di espressione clinica tra topo ed uomo. Infatti, nel topo è par-

ticolarmente frequente la glomerulonefrite (Watanabe et al., 2002).

Modelli knock-out e ipotesi di nuovi fenotipi clinici

Attraverso tecniche di DNA ricombinante è stato possibile generare modelli di topi *knock-out* in cui venissero procurate mutazioni a carico di geni codificanti per molecole coinvolte nell'apoptosi. Ciò ha permesso una migliore definizione delle basi molecolari delle patologie associate ad alterazioni di questo processo biologico. Tali studi sono risultati fondamentali per meglio apprezzare gli eventi molecolari e cellulari che sono alla base della regolazione dell'omeostasi linfocitaria e per meglio definire le patologie associate ad alterazioni di singole molecole (Tab. I). Ad esempio, la delezione di geni, che codificano per le Caspasi murine, ha permesso di comprendere come le Caspasi siano coinvolte non solo nell'apoptosi, ma anche nella maturazione, nella crescita e nella differenziazione cellulare (Wang et al., 2000). A titolo di esempio lo studio dei topi KO caspasi 1[-/-] (Kuida et al., 1995) e caspasi 11[-/-] ha permesso di documentare che l'attivazione avviene a cascata secondo un ordine preciso che vede la Caspasi 11 agire a monte della 1 (Wang et al., 1998). Lo studio del fenotipo del topo caspasi 9[-/-] ha evidenziato che esso è più grave del topo caspasi 3[-/-] suggerendo quindi che la Caspasi 3 è coinvolta negli stadi finali dell'apoptosi e che esistono ulteriori vie effettrici a valle della 9. In altri termini, analogamente ad altri sistemi biologici, anche il sistema delle Caspasi sembra essere ridondante. Questo dato induce a ritenere che potenzialmente anche in patologia umana potrebbero

Tab. I. Modelli animali.

Genotipo	Principali alterazioni Immunologiche	Principali alterazioni immunologiche
Caspasi 1[-/-]	Assenza del "processing" di IL-1 e IL-18	Resistenza allo shock settico indotto da LPS Sviluppo normale
Caspasi 2[-/-]	B linfoblasti resistenti all'apoptosi indotta dal granzima B	Assenza di grosse anormalità Ovociti resistenti all'apoptosi farmaco indotta
Caspasi 8[-/-]	Fibroblasti resistenti ad apoptosi mediata da Fas e TNFR1	Letalità embrionale Anormale sviluppo delle cellule ematopoietiche Anormale sviluppo delle cellule muscolari cardiache
Caspasi 9[-/-]	Timociti con difetto di apoptosi	Anormale sviluppo cerebrale Letalità prenatale
Caspasi 11[-/-]	Assenza del "processing" di IL-1 e IL-18	Assenza di difetti significativi nell'apoptosi e nello sviluppo
FasL mutato (gld)	Assenza di apoptosi Fas/FasL indotta nei linfociti	Nefriti autoimmuni Linfoadenopatia Linfoproliferazione Ipergammaglobulinemia
Fas mutato (lpr)	Cellule target resistenti all'apoptosi FasL mediata	Simile a gld

esistere quadri di malattie autoimmuni monogeniche di differente gravità legate ad alterazioni di molecole che giocano un ruolo in differenti fasi della cascata.

L'attivazione delle Caspasi è considerata un meccanismo importante durante la selezione timica ed in particolare nella selezione negativa. Tuttavia, gli studi nel KO caspasi 9[-/-] hanno dimostrato come il numero totale e la composizione dei timociti sia normale in questi topi mutanti, supportando l'ipotesi che non tutte le Caspasi sono indispensabili nel processo di selezione timica.

Studi sui topi caspasi 8[-/-] hanno evidenziato che il numero dei precursori ematopoietici è drammaticamente ridotto in questi topi, suggerendo, quindi, che la Caspasi 8 è essenziale per la crescita e la differenziazione dei progenitori delle cellule ematopoietiche.

Inoltre, studi sul topo caspasi 2[-/-] hanno mostrato che la Caspasi 2 è un substrato del granzima B e pertanto coinvolta nel *pathway* secretorio di apoptosi. Tale dato indica che la Caspasi 2 è una delle probabili proteasi attivate nel corso delle attività citolitiche, mediate dai linfociti CTL e NK.

Pertanto, la conoscenza di tali fenotipi murini ha rilevanza perché permette di ipotizzare nuovi scenari di patologia umana e di individuare un certo numero di geni candidati nelle strategie d'identificazione di cause genetiche di nuovi fenotipi complessi di malattia.

Quadri di malattia umana

Le ALPS

L'esistenza di trasmissione mendeliana di *clustering* di malattie au-
toimmuni è stata ben documentata in diversi tipi di sindromi tutte caratterizzate dalla presenza di malattie autoimmunitarie multiple (Fischer, 2004). Il prototipo di tali sindromi è rappresentato dalla Sindrome Autoimmune Linfoproliferativa (ALPS) legata patogeneticamente ad alterazioni del meccanismo di apoptosi (Rieux-Laucat et al., 2003). Le principali manifestazioni cliniche sono: anemia emolitica, trombocitopenia, neutropenia, orticaria, glomerulonefrite, epatite autoimmune, cirrosi epatica biliare, sindrome di Guillan Barré, artrite reumatoide giovanile, vitiligine ed alopecia (Rieux-Laucat et al., 2003) (Tab. II). Più raramente possono essere presenti: idrope fetale, autismo, diseritropoiesi, ipereosinofilia. Anche se non vi sono sufficienti dati in letteratura è riportata un'aumentata suscettibilità

Tab. II. Segni clinici delle ALPS.

Manifestazioni autoimmuni Linfoproliferative	Glomerulonefrite
	Orticaria
	Anemia emolitica
	Trombocitopenia
	Neutropenia
	S. di Guillain-Barrè
	Linfoadenopatia
	Splenomegalia
	Epatomegalia
	Linfoma
Caratteristiche di laboratorio	Linfociti T doppio negativi
	Ipergammaglobulinemia
	Autoanticorpi
	Elevati livelli sierici di IL-10
	Difetto di apoptosi Fas mediata
	Ipereosinofilia
Caratteristiche aggiuntive	Infezioni
	Ritardo di crescita
Caratteristiche meno frequenti	Artrite reumatoide giovanile
	Autismo
	Idrope fetale
	Diseritropoiesi

a sviluppare tumori, in particolare a carico del sistema linfopoietico (Rieux-Laucat et al., 2003). Come illustrato in Tabella III, esistono varie forme di ALPS in base alla presenza di alterazioni di diverse molecole coinvolte nei vari stadi del processo apoptotico. L'ALPS di tipo 0 rappresenta l'analogo umano del topo KO per il gene di Fas (lpr-/-) ed è associato a completa assenza della molecola Fas. L'ALPS di tipo Ia, rappresenta la forma più diffusa della sindrome ed è causata da mutazioni allo stato eterozigote del gene Fas. Un'altra forma di ALPS è rappresentata, invece, dalla forma di tipo Ib, in cui il gene alterato è FasL. L'ALPS di tipo II, di recente identificazione, è causata da mutazioni del gene Caspasi 10 (Wang et al.,

1999). Va ricordato tuttavia che il ruolo patogenetico delle mutazioni riportate nel causare la malattia è ancora da definire. Generalmente, l'alta frequenza di mutazioni nella popolazione sana, indicativa che si tratta di un polimorfismo, suggerisce che quella alterazione possa avere eventualmente ruolo di co-

fattore. Infine, l'ALPS di tipo III comprende quelle forme con uguale fenotipo clinico ma in cui il difetto genetico resta ancora da identificare.

Si sta, inoltre, chiarendo un ulteriore meccanismo di malattia, in cui la somma di mutazioni di geni diversi, che codificano per molecole funzionalmente correlate e ciascuna delle quali isolatamente non è associata a segni clinici, può determinare un fenotipo di patologia complessa. Un esempio è costituito dalla doppia mutazione in eterozigosi dei geni Fas e Perforina, che determinano un quadro di autoimmunità che successivamente evolve in linfoma (Clementi et al., 2004).

Va anche menzionato che in alcuni casi la mutazione del gene Fas può essere somatica (Holzelova et al., 2004). Ciò può rendere difficile la diagnosi in quanto la mutazione può essere presente esclusivamente in un tipo cellulare.

ALPS e immunodeficit

Accanto alle forme classiche di ALPS, in cui l'autoimmunità rappresenta il marker di malattia, in alcune di queste sindromi l'iperreattività immunologica si associa ad una condizione di aumentata suscettibilità a contrarre infezioni. È stata re-

Tab. III. Eterogeneità genetica delle Sindromi Autoimmuni Linfoproliferative.

Malattia	Gene alterato	Ereditarietà
ALPS 0	CD95/Fas	AR
ALPS IA	CD95/Fas	AD
ALPS IB	CD168/FasL	AD
ALPS II	Caspasi 10	AR
ALPS III	non noto	–
ALPS e immunodeficit	Caspasi 8	AR

centemente identificata una forma di ALPS associata ad immunodeficit specifico coinvolgente sia i linfociti T che B e *Natural Killer* (NK). Tale malattia è causata da mutazioni del gene che codifica per la Caspasi 8 (Chun et al., 2002). Come illustrato in precedenza, tale Caspasi oltre a svolgere un ruolo nell'attività citolitica, gioca un ruolo determinante anche nella proliferazione linfocitaria. Pertanto, i pazienti affetti presentano associato al deficit funzionale della Caspasi 8 una condizione di autoimmunità multipla ed immunodeficienza (Chun et al., 2002). All'esame clinico i soggetti portatori di questo tipo di mutazione allo stato omozigote mostravano splenomegalia, linfoadenopatia come segno del coinvolgimento del sistema reticoloendoteliale, ed inoltre infezioni respiratorie ricorrenti e da Virus Herpes Simplex (Chun et al., 2002). Sotto il profilo funzionale immunologico, venivano documentate ridotta apoptosi Fas-indotta, bassi livelli di immunoglobuline sieriche ed assenza di *switch* isotipico (Chun et al., 2002).

L'esistenza di autoimmunità associata ad immunodeficienza conferma pertanto l'ipotesi che alcuni geni di questa famiglia siano coinvolti sia nel controllo della risposta immune sia nella fase di attivazione cellulare dopo contatto con l'antigene, e quindi anche responsabili dell'attivazione e della proliferazione cellulare.

APECED e IPEX

Singolari modelli di malattie monogeniche autoimmuni sono inoltre la Poliendocrinopatia Autoimmune con Candidiasi e Distrofia Ectodermica (APECED) (Eisenbarth et al., 2004; Perheentupa, 2002) e la Poliendocrinopatia a trasmissione recessiva legata al cromosoma X, il cui acronimo, IPEX (Schubert et al., 2001; Wildin et al., 2002), definisce le caratteristiche principali del fenotipo rappresentate da Immunodisregolazione, Poliendocrinopatia, Enteropatia, trasmissione X-recessiva. L'APECED (MIM # 240300) è causata da alterazioni del gene che codifica per la molecola di regolazione dell'autoimmunità (Aire); tale molecola gioca un ruolo chiave nella tolleranza centrale in quanto è espressa nel timo, come riferito in precedenza (Eisenbarth et al., 2004; Perheentupa, 2002).

Dal punto di vista clinico i pazienti affetti da APECED sono caratterizzati da manifestazioni autoimmuni multiple a carico di organi endocrini e non-endocrini (Tab. IV). Generalmente, la malattia si manifesta nei primi mesi di vita con candidasi mucocutanea. Successivamente, compaiono morbo di Addison, ipoparatiroidismo, diabete di tipo I e disfunzioni delle gonadi. In qualche soggetto possono anche comparire manifestazioni autoimmuni che colpiscono gli annessi cutanei determinando vitiligo o alopecia. Tuttavia, la diagnosi non è sempre agevole in età pediatrica in quanto solo alcune manifestazioni autoimmuni hanno esordio in età infantile, mentre altre possono diventare evidenti nel corso dell'età adulta. La frequenza di tale sindrome nelle popolazioni Finlandese, Sarda e Iraniana è stata stimata in 1:25000, 1:14400 ed 1:9000, rispettivamente (Rosatelli et al., 1998). Nei pazienti APECED sono state identificate 46 mutazioni a carico del gene AIRE (Rosatelli et al., 1998; Wang et al., 1998) una di queste, la R257X, è stata descritta con una frequenza dell'83% nella popolazione Finlandese e rappresenta anche la più comune nella popolazione del nord Italia (Bjorses et al., 2000; Cihakova et al., 2001).

L'IPEX è legata ad alterazioni del fattore di trascrizione FOXP3, prevalentemente espresso nei linfociti T regolatori CD4+ CD25+, responsabili della down regolazione di clo-

Tab. IV. Segni clinici dell'APECED.	
Caratteristiche endocrine	Ipoparatiroidismo
	Morbo di Addison
	Insufficienza ovarica
	IDDM
	Ipotiroidismo
Caratteristiche non endocrine	Candidiasi
	Ipoplasia dello smalto
	Alopecia
	Distrofia ungueale
	Cheratopatia
	Malassorbimento
	Vitiligine
	Anemia perniciosa
	Epatite autoimmune

Tab. V. Segni clinici dell'IPEX.	
Caratteristiche cliniche	Diabete tipo I ad esordio neonatale
	Grave enteropatia
	Eczema
	Anemia/trombocitopenia
	Ipotiroidismo
	Iper-IgE
	Positività di autoanticorpi
Caratteristiche di laboratorio	Normale numero di cellule CD3$^+$, CD4$^+$ e CD8$^+$
	Normale risposta proliferativa ai mitogeni
	Normali livelli di IgG
	Alti livelli di IgE
	Presenza di autoanticorpi

ni autoreattivi in periferia (Schubert et al., 2001; Wildin et al., 2002). Dal punto di vista clinico, la sindrome colpisce soggetti in età pediatrica ed è caratterizzata da enteropatia e disordini che colpiscono il sistema endocrino, come il Diabete Mellito insulino-dipendente. Un grosso contributo alla comprensione del fenotipo clinico è stato dato dallo studio del mutante naturale dell'IPEX, lo "*scurfy mouse*". Il gene FOXP3, la cui mutazione è causa della malattia, appartiene alla famiglia dei fattori di trascrizione "*forkhead*", che svolgono un ruolo chiave nella funzione ed attivazione delle cellule T. Tale gene è localizzato sul cromosoma X nella regione Xp11.3-q13.3 e codifica per un polipeptide di 431 amminoacidi noto come scurfina (Bennett et al., 2000). Questa proteina presenta nella regione carbossi-terminale un dominio altamente conservato nelle proteine *forkhead* il "*winged helix*", cruciale nel processo di legame al DNA, un motivo ricco di leucine e un dominio *zinc finger* necessario alla stabilizzazione del legame della scurfina al DNA.

Difetti di citolisi ed autoimmunità
Le cellule citolitiche T e NK svolgono un ruolo importante nella difesa dai microrganismi e nella sorveglianza della crescita tumorale. I segnali generati dal T-cell receptor attivano sia il *pathway* di apoptosi non secretorio mediato da Fas sia il *pathway* secretorio, che coinvolge perforina e granzima. Difetti genetici di perforina sono responsabili di una delle diverse forme di linfoistiocitosi emofagocitica, una malattia grave caratterizzata da decorso particolarmente rapido e fatale di infezioni virali e quadri morfologici di emofagocitosi (Stepp et al., 1999). La malattia è caratterizzata da massiva attivazione del sistema linfoistiocitario. I linfociti CTL e NK sono coinvolti, tuttavia, oltre che nella eliminazione di cellule infettate da virus anche nella eliminazione dei cloni autoreattivi. Non è pertanto sorprendente che una ridotta attività citolitica possa essere associata a quadri di autoimmunità. Va ricordata, per amore di completezza, anche la *Macrophage Activation Syndrome* (MAS), che talvolta complica, e ne rappresenta l'evolu-

zione fatale, quadri di infiammazione cronica di lunga durata associati a malattie autoimmunitarie di particolari gravità.

Conclusioni e prospettive

Lo studio di questi pazienti, affetti da forme inusuali di immunodeficienza la cui manifestazione clinica predominante è l'autoimmunità ha permesso di elucidare meccanismi biologici importanti, quali l'apoptosi o quelli coinvolti nella tolleranza centrale e periferica. Come spesso è avvenuto negli ultimi 15 anni grazie all'osservazione di pazienti rari si è definito il ruolo che singole molecole svolgono nella fisiologia della risposta immune. La prima implicazione, sul piano clinico è rappresentata dal fatto che l'identificazione di questi pazienti con fenotipo di malattia autoimmune complessa può portare ad identificare nuove immunodeficienze su base genetica finora non diagnosticate. Evidentemente l'identificazione di una causa genetica di una malattia del sistema ematopoietico non può non suggerire nuovi scenari terapeutici quali ad esempio terapie con cellule staminali o in un futuro non prossimo interventi terapeutici di correzione molecolare come già realizzato in alcune forme di immunodeficienza congenita classica.
Infine, lo studio molecolare e funzionale dei *pathways* dell'apoptosi potrebbe dare un considerevole contributo ad identificare alterazioni molecolari a carico di geni interferenti nell'insorgenza delle malattie autoimmuni classiche, giustamente considerate multifattoriali nella patogenesi, e nel determinare la gravità peggiorando o attenuando il fenotipo.

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2.2   Peripheral GH insensitivity is a distinct disorder in Idiophatic Short Stature

Short stature is the most important clinical problem considered the high frequency in childhood. In many case the short stature may be the sign of a wide variety of pathologic condition or inherited disorder. Indeed, decreased childhood growth resulting in short stature is caused by endocrine or chromosomal abnormalities, genetic defects in endochondral bone formation, malnutrition, chronic systemic disease, or psychosocial deprivation. In spite of that, in many children a specific etiology for the growth failure cannot be identified and this condition is referred to as Idiophatic Short Stature (ISS). The criteria used for to define this pathology are 1) a height of more than two standard deviation below the mean; (2) normal or slow height velocity; (3) normal birth weight; (4) absence of specific endocrine abnormalities (including GH deficiency) and no evidence of chronic physical or psychological illness [1-3 in Blair 2002] and idiophatic short stature is cosiderated a diagnoses of exclusion.

Actually, the teraphy of the children with ISS consist in the administered to exogenous recombinant human GH (rhGH) (Manmohan A 2005).

Before 1980, GH was administered exclusively to GH deficient (GHD) children who were unresponsive to stimulation tests. Subsequently, many investigators studied the effects of GH in very short children who were GH sufficient and were growing at a subnormal velocity for their age. Left untreated, many of these short children have failed to reach their midparental

target heights, consequently, opinions differ as to its impact on final heights (Buchlis JG et al 1998).

In these cases, the short stature may be caused to the defect in spontaneous GH secretion associated with the normal response to GH stimulation test (Spiliotis BE et al 1984) or to GH inactivity (Kowarski AAet al 1978). Nevertheless, the first mechanism has been found in a rare cases (Kerrigan JR et al 1993) while the second mechanism, also recently, has been reported (Takahashi Y et al 1997). Furthermore, it has been recognized that some children with ISS may have peripheral insensitivity to GH (GHI) at different extents of severity (Goddard Ad et al 1995). Overall, studieds in the last years have elucidated that receptor defects or molecolar alterations of downstreem elements in the signaling cascade may lead to a considerable number of diseases characterized by total or partial unresponsiveness of the target organ to appropiate stimuli (Rosenfeld RG 1994).

Laron syndrome (LS) represent the classical prototype of GH insensitivity. This is a rare autosomal recessive disorder characterized by severe peripheral resistence to the physiological action of GH (Rosenfeld RG et al 1994). Although diagnostic criteria to define patients with severe GHI are well astablished (Woods KA et al 1996), the identification of patients with milder forms is still debated (Johnston LB et al 1999).

2.2.1 Peripheral GH insensitivity: a post-receptorial defect

Considered that for understanding regulation of normal growth and metabolism, it is essential to understand the molecular basis of growth hormone (GH) action, in this first phase of research we describe the physiopathological role of GH hormone in peripheral GH insensitivity disorder. Furthermore we provide to delineate possible diagnostic algorithms to help the paediatrician promptly recognize in a heterogeneous group of ISS that forms of short stature due to a receptorial or post-receptorial defect.

The GH hormone play an important role in the cellular growth, differentiation and metabolism but its diverse effects are elicited by binding of GH to its receptor (GHR).

The human growth hormone receptor (GHR) belongs to the GH-PRL-cytokine receptor superfamily and consists of three domains: an extracellular domain responsible for hormone binding and receptor dimerization, a transmembrane domain, and an intracellular domain that initiates intracellular signaling (Postel-Vinay MC et al 1995). A soluble form of the GHR called high affinity GH binding protein (GHBP) is present in the circulation of normal individuals end acts as a reservoir and buffer for circulating GH. It is produced as a result of proteolytic cleavage of the extracellular domain from the rest of the receptor protein. GH has been shown to bind sequentially to two GHBPs, leading to the hypothesis that GH binding causes both dimerization of GHR and conformational changes important for GH function. These changes result in increased binding to GHR of JAK2, a nonreceptor tyrosine kinase, and promote

activation of JAK2. In turn, both GHR and JAK2 are phosphorylated on tyrosines by the activated JAK2. These phosphorylated tyrosines serve as docking sites for SH2 domain-containing signaling molecules. Subsequent signaling events include activation of MAP kinases, via a SHC+Grb2+Sos+ras+raf+MAPK pathway. MAP kinase substrates include other protein kinases as so as transcription factors that are phosphorylated in response to GH and are responsible for several actions of GH. A recently described family of transcription factors that serve as signal transducers and activators of transcription (Stat) participates in GH signaling between the receptor and the nucleus. Stat proteins are latent cytoplasmic factors containing SH2 and SH3 domains. Upon tyrosyl phosphorylation, often via a JAK kinase-initiated cascade, cytoplasmic Stat proteins complex with other Stat and/or non-Stat proteins, translocate to the nucleus, bind to DNA, and activate transcription of target genes (Carter-Su C. et al. 1996).

The effects of GH on growth are mostly mediated by intermediate factors, of which the insulin-like growth factor-I (IGF-I) is the most studied. The linear growth is a complex process and GH and IGF-I participate as combined factors or separately to this process. Thus, GH appears to have many cellular effects that are independent of IGF-I, and are most probably direct effects of GH mediated by its cognate receptor (Le Roith Trends in Endocrinol 2001).

Recently, has been reported that the GHR stimulation actives several pathways. The coordinate action of the proteins involved in this pathway leads to the expression of the appropriate GH functions (Winston LA 1995).

Therefore, any proteins alteration and/or protein phosphorylation events alteration in the transduction mechanism may be cause to a peripheral GH insensitivity.

Moreover studies of pathogenesis in patient with GHI showed that in same cases this condition is associated to molecular alteration of GH-R gene. In classis Laron syndrome nearly 40 distinct mutations and /or exon deletions have been identified (Rosenbloom AL et al 1999). On the contrary, molecular analysis performed in children with a milder form of GHI reveals that only in few cases this disorder is associated to mutations in GH-R gene (Goddard AD et al 1997, Sanchez JE et al 1998). Take together, this data suggest that other molecules involved in GH-R signaling might be altered and to cause a partial responsiveness to GH hormone stimulation.

Thus far the GH stimulation test is a useful tool to help to identify the patient without GH deficiency. This test is performed administrated exogenous GH for 4 consecutively days. A increase of the serum levels of IGFI I (main mediator of GH function) more than 20% of the basal value is considerate a positive test response (Blum WF 1994). This evaluation allow to leave out a structural or functional defect of the GH-receptor. Conversely, no increase of the serum levels of IGFI indicate no responsiveness of the peripheral organ to the GH and this associated to normal or elevated serum levels of GHBP suggest a defect in signaling transduction mechanism of the GH-R.

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Difetto di crescita staturale da alterata risposta periferica al GH: patologia pediatrica emergente e spesso non diagnosticata

Short stature due to peripheral GH insensitivity: emerging and often not diagnosed pediatric disorder

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PAROLE CHIAVE. — Bassa statura idiopatica - Ormone della crescita - Insensibilità periferica al GH - Trasmissione del segnale - Malattie postrecettoriali
KEY WORDS. — *Idiopathic short stature - Growth hormone - Peripheral GH insensitivity - Signal transduction - Postreceptorial diseases*

Introduzione

La bassa statura costituisce uno dei problemi clinici di maggior rilevanza in età pediatrica in considerazione della alta prevalenza di tale condizione. Mentre in molti casi la causa del ritardo è facilmente individuabile, in altri non è sempre possibile identificarne l'eziologia. In questi casi la condizione di ritardo di crescita staturale viene etichettata come bassa statura idiopatica (BSI). La Tabella I illustra i criteri generalmente utilizzati per definire una condizione di BSI. Tale diagnosi costituisce, pertanto, una diagnosi di esclusione e riflette l'impossibilità del Pediatra di porre una diagnosi eziopatogenetica. Va sottolineato come alla luce delle conoscenze attuali non vi sia consenso unanime sui criteri per porre tale diagnosi, in quanto alcuni Autori includono in questo gruppo anche il ritardo costituzionale di crescita e pubertà, e la bassa statura familiare [1], entità classicamente ritenute varianti della norma in quanto caratterizzate da una velocità di crescita normale. Tuttavia, i casi in cui ad un basso bersaglio familiare si sovrapponga anche un rallentamento della velocità di crescita possono rientrare nella BSI. A tal proposito si sottolinea come sia importante nell'approccio al bambino con bassa statura il followup auxologico che prevede almeno due misurazioni a distanza di 6 mesi, così da consentire una valutazione dinamica della crescita (velocità di crescita). Una migliore definizione di questo capitolo di patologia pediatrica ha grande rilevanza clinica. Infatti, alcuni bambini con BSI raggiungono una statura adulta inferiore al bersaglio familiare [2 3], e ciò ha indotto alcuni Autori ad effettuare un trattamento con ormone della crescita (GH) per migliorare la velocità di crescita e raggiungere un appropriato bersaglio genetico [4]. Tale trattamento, tuttavia, non è sempre efficace [4], confermando quindi che il gruppo delle BSI sia eterogeneo nel decorso clinico e negli aspetti patogenetici. Tra le possibili cause della ridotta crescita in questo gruppo di soggetti è stata suggerita una disfunzione neurosecretoria, caratterizzata da inadeguata secrezio-

Tab. I.
Criteri di definizione della bassa statura idiopatica.

— Altezza inferiore al 5° centile per l'età cronologica

— Ritardo della maturazione ossea (> media - 2DS)

— Velocià di crescita inferiore al 25° centile per l'età cronologica

— Normale lunghezza alla nascita (5° - 95° centile)

— Assenza di patologie croniche, malnutrizione, displasie ossee

— Assenza di patologie endocrine

ne spontanea di GH, sia pure associata a normale risposta ai test di stimolo [5], o la presenza di un GH inattivo [6]. Tuttavia il primo meccanismo è stata riscontrato solo in rari casi [7] ed il secondo è stato solo di recente dimostrato [8]. Un'altra possibile causa del difetto accrescitivo è rappresentata dalla ridotta sensibilità periferica all'ormone, legata ad anomalie strutturali o funzionali del recettore specifico per il GH [9]. Conoscenze recenti sui meccanismi biochimici che regolano il corretto funzionamento dei recettori stanno fornendo considerevoli contributi alla comprensione di malattie pediatriche in differenti settori della Pediatria, altrimenti non facilmente identificate. È possibile attualmente identificare e quindi diagnosticare malattie da alterazioni recettoriali o postrecettoriali. È verosimile, pertanto, che tale settore di studi possa in futuro contribuire considerevolmente a migliorare le conoscenze anche in questo gruppo di patologia pediatrica.
Lo scopo di questo articolo di aggiornamento è quello di contribuire a meglio discriminare nel gruppo eterogeneo delle BSI quelle forme da insensibilità periferica del recettore e fornire delle linee guida per un migliore inquadramento di questi pazienti.
Per facilitare la comprensione della problematica si ritiene necessario fornire degli elementi di fisiologia relativi alla costituzione del recettore per il GH e dei meccanismi che ne regolano il corretto funzionamento.

Fisiologia del recettore per il GH

Aspetti strutturali del recettore e sue funzioni

Il recettore dell'ormone della crescita umano (GHR) è una proteina di 620 amino acidi costituita da una porzione extracellulare di 246 amino acidi, che viene rilasciata come componente solubile del recettore nel siero (proteina legante il GH o GHBP), da una regione transmembrana di 24 amino acidi, ed un lungo dominio intracitoplasmatico di 350 amino acidi, come illustrato nella Figura 1A [10]. La Figura 1B rappresenta schematicamente la struttura del gene che codifica per i 10 esoni del GHR, localizzato sul cromosoma 5p 13.1-p12 [11]. Il dominio extracellulare contiene 7 residui di cisteina e 7 potenziali siti di glicosilazione [10]. Il dominio extracellulare del recettore è costituito da 2 regioni aventi una tipologia simile a quella delle immunoglobuline. Il primo evento nella trasmissione del segnale è il legame del GH con 2 molecole del GHR. Ogni molecola di GH ha 2 siti di legame e, quindi, un primo recettore lega il sito 1 mentre il secondo recettore lega il sito 2 dell'ormone. L'interazione del GH con le 2 molecole di recettore forma pertanto un omodimero (dimerizzazione del recettore). La distruzione di uno o di entrambi i siti di legame del GH o una mutazione che alteri il processo di dimerizzazione

del recettore determina perdita di funzione e quindi insensibilità al GH [10].

La regione intracitoplasmatica contiene 10 residui di tirosina, contrassegnati con la Y nella Figura 1A [12], che come si vedrà in seguito svolgono un ruolo molto importante nel processo di risposta dell'organo periferico all'ormone. La porzione extracellulare è codificata dagli esoni 2-7, mentre la porzione transmembrana dall'esone 8 e quella intracitoplasmatica dagli esoni 9 e 10 [13].

Il GH svolge un ruolo fondamentale nella crescita e nella differenziazione cellulare. La presenza del GHR è stata documentata in numerosi organi come illustrato in Figura 2. In particolare, esso è espresso sulla membrana di cellule di fegato, rene, intestino, polmone, surrene, muscolo, timo, osso, ed inoltre in neuroni, fibroblasti, adipociti e linfociti [14-18]. Oltre a promuovere la crescita longitudinale durante l'infanzia, il GH ha numerosi altri effetti biologici, che gli conferiscono un ruolo centrale anche nella fisiologia dell'adulto. Nella Tabella II sono riportati i principali effetti dell'ormone. Gli effetti sul metabolismo glucidico, lipidico e sulla sintesi proteica sono svolti direttamente dall'ormone [19], mentre per la maggior parte degli altri effetti l'azione del GH sull'organo bersaglio è mediata da un fattore periferico prodotto soprattutto a livello epatico, denominato *insulin-like growth factor-I*
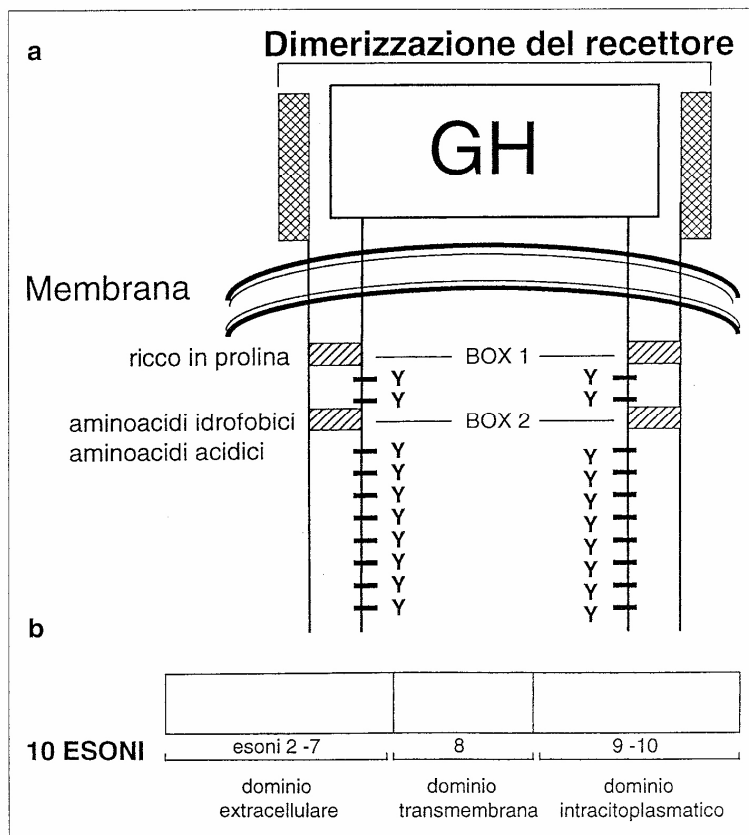

Fig. 1.
Recettore per il GH: a. schema rappresentativo del recettore nelle sue porzioni extracellulare, transmembrana ed intracellulare; b. organizzazione in esoni del gene che codifica per il recettore.
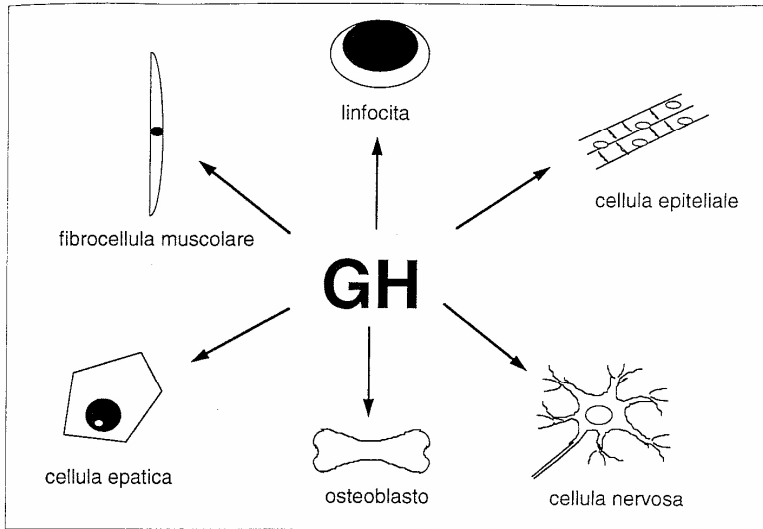
Fig. 2.
Distribuzione del recettore per il GH in differenti tipi cellulari.

(IGF-I) [20]. Anche se il recettore per il GH sembra essere ubiquitario, gli effetti biologici indotti dall'azione dell'ormone sembrano essere più evidenti in alcuni organi. Nonostante la presenza del GHR su linfociti ed organi linfoidi, il significato biologico dell'effetto modulatorio del GH sul sistema immune è ancora da definire [18]. È ipotizzabile, tenuto conto della ampia espressione del GHR su vari organi e tessuti che alcune funzioni specifiche non siano state tuttora identificate.

Messaggeri extracellulari dell'informazione
Il GH in alcuni casi agisce direttamente sull'organo bersaglio, mentre in molti casi esso agisce mediante messaggeri intermedi (Fig. 3). I principali mediatori sono rappresentati dagli *insulin-like growth factors* (IGFs), che partecipano ai meccanismi di crescita e di funzionamento di quasi tutti gli organi [20]. Le molecole IGF-I ed

IGF-II sono importanti per lo sviluppo dell'embrione e la crescita fetale, mentre dopo la nascita le informazioni sinora disponibili sulle funzioni di tali molecole indicano nell'IGF-I il principale mediatore dell'azione del GH [20]. La maggior parte dell'IGF-I viene prodotta a livello epatico per effetto dell'azione del GH e la sua concentrazione sierica correla con la concentrazione dell'ormone. Esistono evidenze che indicano come a sua volta l'IGF-I possa inibire la secrezione ipofisaria dell'ormone. Tali messaggeri circolano legati a proteine vettrici (IGFBP) di cui finora ne sono state identificate almeno 10 [21]. Tra queste l'IGFBP3, che lega più del 95% dell'IGF-I aumentandone l'emivita, sembra avere rilevanza clinica nel modulare l'effetto dell'IGF-I [21]. Le concentrazioni sieriche di IGF-I risentono di molte variabili: età, sesso, stato nutrizionale e secrezione di GH.

Tab. II.
Effetti del GH sui principali organi bersaglio.

|	
Fegato	Stimola la sintesi di IGFI
	Stimola la neoglucogenesi
Rene	Aumenta il flusso plasmatico renale
	Aumenta la filtrazione glomerulare
Osso	Stimola la proliferazione degli osteoblasti
Muscolo	Stimola la proliferazione e la differenziazione dei mioblasti
Sistema immune	Stimola la linfopoiesi
	Partecipa all'attivazione linfocitaria
S. nervoso centrale	Stimola gli oligodendrociti a produrre mielina
	Aumenta la sopravvivenza dei neuroni
Tessuto adiposo	Stimola la lipolisi
	Inibisce la differenziazione degli adipociti
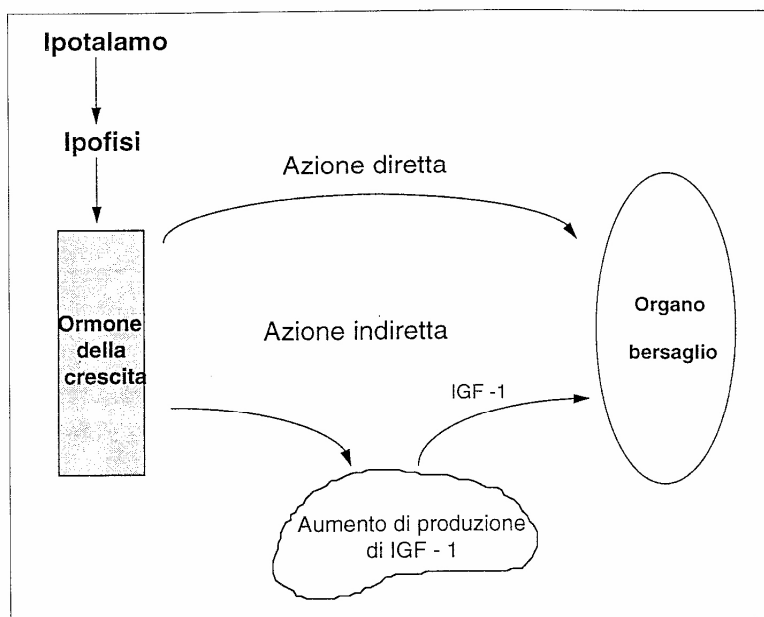
Fig. 3.
Meccanismo di azione diretta ed indiretta del GH sull'organo bersaglio. L'IGFI rappresenta il principale messaggero extracellulare nella trasmissione dell'informazione.

Trasmissione del segnale intracellulare

Dopo l'interazione dell'ormone con il recettore, è necessario che l'informazione sia trasmessa al nucleo per consentire l'attivazione della trascrizione dei geni che permettono l'espressione della funzione specifica. Questo processo di trasmissione del segnale è prevalentemente determinato da processi a catena di fosforilazione [22]. La tirosina chinasi JAK 2, appartenente alla famiglia di chinasi Janus, svolge un ruolo fondamentale in questo processo. Essa si attiva fosforilando se stessa (processo di autofosforilazione) ed in seguito fosforila il dominio intracitoplasmatico del GHR a livello dei residui di tirosina [10][23]. Questi siti di fosforilazione svolgono un ruolo fondamentale per la trasmissione del segnale in quanto rappresentano siti di legame per proteine intracellulari contenenti domini SH-2, come le proteine della famiglia delle «*Signal Transducers and Activators of Transcription*» (STATs). Queste molecole attivate mediante fosforilazione traslocano nel nucleo dove si legano a specifiche sequenze di DNA attivandone la trascrizione di appropriati geni target. Al momento 3 proteine della famiglia STAT, denominate, 1, 3, e 5, partecipano al processo; tuttavia STAT-5 sembra essere quella maggiormente coinvolta nella regolazione della risposta al GH [23]. La Figura 4 illustra uno schema semplificato della sequenza di reazioni. Va sottolineato, tuttavia, come con il rapido progredire delle conoscenze in questo settore il numero dei mediatori intracellulari sia inevitabilmente destinato ad aumentare. Alla luce di quanto esposto ben si comprende come siano importanti per un corretto funzionamento del recettore tutte le regioni intracitoplasmatiche dello stesso. Infatti, ad esempio, la regione denominata Box 1 è necessaria per l'attivazione di JAK 2 e delle chinasi attivate dai mitogeni (MAPK) [10].

Recenti studi indicano inoltre chiaramente che la stimolazione del recettore attiva *pathways* multipli, che quindi funzionano in modo integrato e consentendo l'espressione della funzione appropriata [24].

Insensibilità periferica all'ormone della crescita

La condizione più nota di insensibilità al GH è rappresentata dalla sindrome di Laron, disordine autosomico recessivo caratterizzato da una completa resistenza alla fisiologica azione del GH. Esistono anche casi di resistenza al GH secondaria a malnutrizione, malassorbimento, sepsi, insufficienza epatica o malattie infiammatorie croniche [25][26]. Le caratteristiche cliniche dei pazienti affetti da sindrome di Laron sono simili a quelle dei pazienti con grave deficit di GH. Inoltre spesso nella sindrome di Laron sono presenti alcuni segni clinici particolari, quali fronte prominente, ponte nasale ipoplasico, diminuite dimensioni verticali del viso, capelli radi, sclere blu, voce acuta, mani e piedi piccoli [26]. Tuttavia il fenotipo clinico è variabile in relazione all'eterogeneità genetica della malattia. I livelli basali di GH possono essere normali o più spesso elevati; l' IGF-I ed la IGFBP3, che rappresentano gli indicatori più diretti dell'azione del GH, sono generalmente bassi rispetto ai valori normali per sesso ed età [26]. Un ulteriore supporto alla diagnosi viene dal test di generazione somatomedinica, come illustrato in seguito nell'algoritmo diagnostico. Nei casi di insensibilità al GH non si osserva aumento significativo di IGF-I rispetto al valore basale [25]. Gli studi patogenetici sinora condotti su pazienti con insensibilità al GH hanno evidenziato come in alcuni casi tale condizione sia associata ad alterazioni molecolari del recettore per l'ormone. Fino ad oggi più di
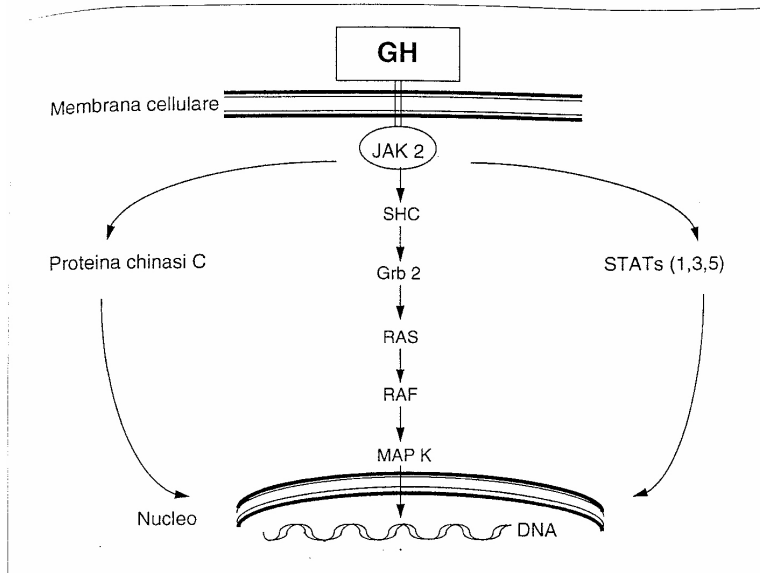
Fig. 4.
Schema rappresentativo delle principali molecole intracellulari coinvolte nella trasmissione del segnale dal recettore di membrana al nucleo. Un ruolo predominante è svolto dalla chinasi JAK 2 e dalle molecole della famiglia STAT.

30 diverse mutazioni e/o delezioni sono state descritte [11 25 27-30]. La maggior parte di esse coinvolgono gli esoni 2-7 del gene, che codificano per il dominio extracellulare del GHR. In questi casi la GHBP, che rappresenta la porzione extracellulare solubile del recettore, può essere assente [30] o comunque inferiore al 10% del normale [25]. Pertanto, il dosaggio della GHBP può essere utile per la diagnosi. In alcuni casi può essere alterato il processo di dimerizzazione del recettore [31], mentre in altri casi è stata dimostrata un'alterazione del dominio intracellulare [32], o ancora difetti nella sintesi e/o secrezione dell'IGF-I [33]. Nei casi con mutazione del dominio intracellulare, i livelli di GHBP sono normali o aumentati. La Tabella III, tuttavia, mostra come nei principali studi effettuati in pazienti con insensibilità recettoriale all'ormone o con bassa statura idiopatica, alterazioni del recettore siano state trovate solo in alcuni dei pazienti, mentre in altri non

è stato possibile identificarne il meccanismo molecolare. Ciò suggerisce che differenti meccanismi, coinvolgenti diverse molecole nei *pathways* di trasmissione del segnale, possano determinare la condizione di insensibilità recettoriale. Tale ipotesi è suffragata altresì dall'osservazione che esiste un ampio spettro clinico di GH resistenza, che va dalla parziale responsività ad un assoluta incapacità del recettore di rispondere allo stimolo ormonale [26]. Studi in questo settore potranno, quindi, consentire la diagnosi patogenetica di alcuni pazienti finora identificati come affetti da bassa statura idiopatica.

Come si riconosce un difetto recettoriale o postrecettoriale

Nonostante il progredire delle conoscenze nel settore

Tab. III.
Prevalenza di mutazioni del GH-R in pazienti con insensibilità al GH (GHI) o bassa statura idiopatica (BSI).

Autore	Classificazione dei pazienti	Numero dei pazienti	Numero dei pazienti con mutazione
Meacham (1992)	GHI	12	4 .
Blum (1994)	GHI	38	6
Goddard (1997)	BSI	100	8
Wood (1997)	GHI	82	27*

* numero di mutazioni rilevate su 31 campioni testati.

dell'Endocrinologia sperimentale, non riteniamo possibile al momento fornire delle linee guida al Pediatra per definire con certezza una condizione di difetto di crescita da malattia recettoriale o postrecettoriale. Tuttavia alcuni elementi possono essere utili nell'approccio a questa problematica clinica, come schematicamente illustrato nella Figura 5. In quei pazienti con bassa statura idiopatica, ed in cui quindi è stato in precedenza escluso un deficit di GH secondo i criteri convenzionali, il test di generazione della somatomedina rappresenta il primo strumento da utilizzare. Esso si esegue somministrando 0.1U/kg/die di ormone della crescita per 4 giorni consecutivi. Generalmente si considera un aumento di IGF-I superiore al 20% del valore basale una risposta positiva al test [25], che consente di escludere un difetto strutturale o funzionale del recettore per il GH. In questi casi, pertanto, o vi è un'altra causa della bassa statura idiopatica o è possibile che vi sia un'alterazione del recettore per l'IGF-I, evenienza tuttavia dimostrata solo in alcuni gruppi etnici [34]. Nel caso non vi sia aumento di IGFI, questa condizione è indicativa di non responsività dell'organo periferico al GH. In questo caso la valutazione della GHBP può fornire utili elementi diagnostici. Infatti, come è stato descritto in precedenza, questa proteina corrisponde alla componente extracellulare e solubile del recettore stesso. Pertanto, in caso di mutazione di tale regione del recettore frequentemente si osserva un basso valore di GHBP (< 10%). Nel caso invece si riscontri un valore normale o addirittura elevato di GHBP sierica, in riferimento ai valori normali per età [35], ciò è indicativo di un difetto intracitoplasmatico del recettore, tuttavia descritto in soli 2 soggetti della stessa famiglia, o dei meccanismi di trasmissione del segnale, che virtualmente può coinvolgere ciascuna delle numerose molecole di *signaling* come illustrato in precedenza. L'analisi molecolare del gene per il GHR trova, a nostro avviso, giustificazione esclusivamente per studi di correlazione tra

fenotipo clinico e genotipo. Va segnalato, tuttavia, che non necessariamente un valore basso di GHBP si associa a mutazione del recettore [9].
È possibile oggi, in alcuni centri altamente specializzati studiare con tecnologie innovative le fasi biochimiche che seguono all'interazione del recettore con il ligando e che permettono la trasmissione del segnale dal recettore al nucleo. Tale tecnologia consente pertanto una valutazione diretta delle capacità trasduttive del recettore e quindi può rivelarsi strumento estremamente utile nella diagnosi di questi pazienti. Un'applicazione più ampia di tali strumenti diagnostici può contribuire ad individuare pazienti, che fino ad oggi non ricevono appropriata diagnosi, e quindi a consentire una valutazione della reale prevalenza del problema, oggi non possibile in quanto i differenti gruppi di lavoro utilizzano differenti criteri di selezione dei soggetti, spesso indiretti.

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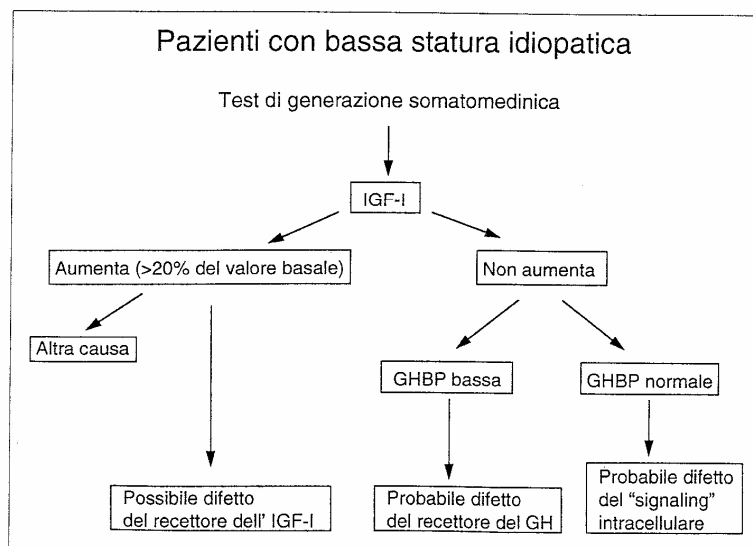J Clin Endocrinol Metab 1998;83:1075-1079.

Fig. 5.
Criteri per l'identificazione di un'eventuale insensibilità periferica all'ormone in pazienti affetti da bassa statura idiopatica. Un ruolo importante è svolto dal test di generazione somatomedinica.

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Le richieste di estratti vanno indirizzate a: dott. Claudio Pignata, Unità Specialistica di Immunologia, Dipartimento di Pediatria, Università «Federico II», via Sergio Pansini 5, 80131 Napoli - Tel. 081 7463500 int. 108 - Fax 081 5451278 - E-mail: Pignata@unina.it

2.2.2 Abnormal GH Receptor Signaling as a marker for Peripheral GH insensitivity

As GHI encompasses a wide spectrum of clinical phenotypes, ranging from classical complete forms and partial deficiencies, the identification of these patients is often difficult, and the prevalence of such cases may be underestimated (Saenger P 1999). Aim of this research were ascertain whether integrating functional provocative assays, analysis of receptor induced biochemical alterations and molecular studies may help unravel the complexity of ISS and lead to identification of more reliable markers of GHI.

The results obtained in this study revealed reduced tyrosine phosphorylation in two of 14 ISS children following GH stimulation of peripheral lymphocytes, associated with low basal and GH-stimulated serum IGF-I. In the patient 3 no tyrosine phosphorylation was observed while in the patient 12 the biochemical alteration was selective, in that it concerned exclusively the protein migrating in the 119 kDa area, whose size corresponds to that of Jak2, but immunoprecipitation experiments showed a normal JAK 2 phosphrylation thus suggesting that a different molecule migrating in the same area of JAK 2 and yet be defined was involved in the process . The molecular analysis of GHR in these patients revealed two heterozygous polymorphism: G168A in the exon 6 and I526L in the exon 10 in the patient 3 and an homozygous polymorphism I526L in the exon 10 in the patient 12. Only one heterozygous mutation Arg ^{161} Cys was found in patient 5 that showed a normal incremental response to GH. Thus, a possible mechanism to explain the wide

phenotypic variability of heterozygous mutation of GHR is that there are other genes that may interact with the signaling pathway, thus modifying the clinical phenotype. This work has been published on Clinical Endocrinology and Metabolism, see below for the manuscript.

Abnormal GH Receptor Signaling in Children with Idiopathic Short Stature

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Peripheral GH insensitivity may underlie idiopathic short stature in children. As the clinical and biochemical hallmarks of partial GH insensitivity have not yet been clearly elucidated, the identification of such patients is still difficult. We integrated functional, biochemical, and molecular studies to define the more reliable marker(s) of GH insensitivity. In particular, we measured GH receptor transducing properties through GH-induced protein tyrosine phosphorylation in patients' peripheral blood mononuclear cells and performed direct sequencing analysis of GH receptor-coding exons. Five of 14 idiopathic short stature patients with low basal IGF-I levels showed low or absent IGF-I increment after 4 d of GH administration. However, a prolonged GH stimulation induced in 3 of them an increase in IGF-I 40% above the baseline value. The

IGF-binding protein-3 behavior paralleled that of IGF-I. The 2 GH-unresponsive subjects showed an abnormal tyrosine phosphorylation pattern after GH challenge. Sequence analysis of the GH receptor gene revealed a heterozygous mutation resulting in an Arg to Cys change (R161C) in exon 6 in only 1 patient, who had normal GH receptor responsiveness. Our findings indicate that abnormal GH receptor signaling may underlie idiopathic short stature even in the absence of GH receptor mutations. Thus, combining the 4-d IGF-I generation test and the analysis of GH-induced protein tyrosine phosphorylation is a useful tool to help identify idiopathic short stature patients with partial GH insensitivity. (*J Clin Endocrinol Metab* 86: 3882–3888, 2001)

THE MANAGEMENT of patients with short stature and low growth velocity who do not meet accepted criteria for GH deficiency remains controversial. In the absence of systemic diseases or hormone deficiencies, these patients are often classified as having idiopathic short stature (ISS), which implies that the intimate pathogenic mechanism has yet to be defined (1). It has been recognized that some children with ISS may have peripheral insensitivity to GH at different extents of severity (2). Overall, studies in the last few years have elucidated that receptor defects or molecular alterations of downstream elements in the signaling cascade may lead to a considerable number of diseases characterized by total or partial unresponsiveness of the target organ to the appropriate stimuli. GH insensitivity (GHI) is characterized by the peripheral resistance to the physiological action of GH (3). Clinical features include short stature with high circulating levels of GH and low levels of IGF-I and IGF-binding protein-3 (IGFBP-3). Children with GHI exhibit significant heterogeneity in both phenotype and genotype, resulting in a wide spectrum of severity (4, 5). In classic Laron syndrome, mutations in the GH receptor (GH-R) gene have been extensively documented in the last 10 yr. Along with exon deletions, nearly 40 distinct mutations of the GH-R, including nonsense, missense, splice, or frameshift mutations, have

been identified (6). Mostly, these mutations have been found in regions coding for the extracellular domain of the GH-R, consisting of the exons 2–7 (3, 7). Only a few mutations have been documented in regions coding for intracellular domains, such as exons 9 and 10, implicated in the intracytoplasmic intermolecular connections (8–11). The possibility that GHI might also underlie ISS in those children who do not have features of Laron syndrome led to a search for alterations in the GH-R. However, mutations in the GH-R gene have been found in fewer than 5% of these patients (12, 13), thus suggesting that other molecules involved in GH-R signaling might be altered and cause short stature in those patients with ISS due to GHI (14).

As in the case of other receptors, the signal transduction induced by GH-R triggering is a complex array of biochemical events acting in a coordinated fashion and involving a large number of distinct molecules (15, 16). Protein tyrosine phosphorylation (P-Tyr) of several proteins represents a key event, ultimately resulting in nuclear factor activation and gene transcription (17).

As GHI encompasses a wide spectrum of clinical phenotypes, ranging from classical complete forms and partial deficiencies, the identification of these patients is often difficult, and the prevalence of such cases may be underestimated (18). Although diagnostic criteria to define patients with severe GHI are well established (5), the identification of patients with milder forms is still debated (19).

In this study we ascertain whether integrating functional provocative assays, analysis of receptor-induced biochemical alterations and molecular studies may help unravel the

Abbreviations: GHI, GH insensitivity; GH-R, GH receptor; IGFBP, IGF-binding protein; ISS, idiopathic short stature; JAK2, Janus kinase 2; PBMC, peripheral blood mononuclear cells; P-Tyr, protein tyrosine phosphorylation; STAT5, signal transducers and activators of transcription 5.

complexity of ISS and lead to identification of more reliable marker(s) of GHI. These patients might also be good candidates for studies aimed at identifying downstream targets in GH-R signaling.

Subjects and Methods

Study subjects

The study population consisted of 14 children (2 girls and 12 boys), aged 1.5–14.1 yr (mean age ± sd, 8.8 ± 4.3 yr) with ISS who met the following inclusion criteria: 1) height below 2 sd for chronological age, 2) bone age delay more than 2 sd for chronological age, 3) growth velocity below the 25th percentile for chronological age, 4) normal birth weight, 5) absence of known endocrine disease or skeletal dysplasia, and 6) no other reason for short stature. Data on height, growth velocity, and sex-corrected midparental height (target height) were transformed into sd scores according to the standards of Tanner (20). Growth velocity was evaluated at 6- to 12-month intervals. Bone age was assessed according to the method of Greulich and Pyle (21). GH secretion was evaluated after clonidine and arginine provocative tests by standard procedures.

Informed consent was obtained from the parents of the patients enrolled in the study. The clinical features of the 14 children with ISS are shown in Table 1. Patient 3 was initially considered to have classical GH deficiency based on low GH values after two stimulation tests performed in another hospital. However, after 1 yr of GH therapy (0.1 U/kg daily) growth velocity remained abnormal (−2.6 sd score); therefore, therapy was discontinued, and the patient was enrolled in the study.

IGF-I generation test

To assess the ability to respond to exogenous GH, each patient underwent an IGF-I generation test. This involved daily sc injections of recombinant GH (0.1 IU/kg) for 14–21 consecutive d. Blood samples were taken before the first injection and on d 5, 8, 15, and 22 for measurements of fasting IGF-I and IGFBP-3 levels. IGF-I and IGFBP-3 were measured using a two-site immunoradiometric assay kit (Diagnostics Systems Laboratories, Inc., Webster, TX). Values were expressed as absolute values (micrograms per liter) or were normalized for age and sex with the normative data provided by the manufacturer and expressed as the sd score. The increase in IGF-I and IGFBP-3 values during the generation test were also expressed as the percent increase. The IGF-I intra- and interassay coefficients of variation were, respectively, 3.4% and 8.2%, whereas the IGFBP-3 intra- and interassay coefficients of variation were 1.8% and 1.9%.

GH-induced P-Tyr

The transducing properties of the GH-R were studied through the analysis of GH-induced protein tyrosine phosphorylation on peripheral blood mononuclear cells (PBMC). PBMC were isolated by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation using standard procedure. GH stimulation (500 ng/ml) was performed

with 5- to 15-min stimulation. The pattern of P-Tyr proteins was analyzed on whole cell lysates. Cells (3×10^6) were lysed in a buffer containing 20 mm Tris (pH 8), 10% glycerol, 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. Proteins were resolved by 10% SDS-PAGE and then blocked with 3% BSA. Immunoblotting was performed by a 1- to 4-h incubation with anti-P-Tyr. In a few experiments membranes were stripped with a buffer consisting of 2% SDS, 6.25 mm Tris-HCl, and 100 mm β-mercaptoethanol and were reprobed with an anti-Janus kinase 2 (JAK2; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or antisignal transducers and activators of transcription 5 (anti-STAT5) monoclonal (Santa Cruz Biotechnology, Inc.) antibody. In immunoprecipitation experiments, JAK2 was precipitated from 7–10 $\times 10^6$ PBMC, resting or stimulated for 5 min with GH, and lysed in glycerol-free buffer by a 2-h incubation with protein A-Sepharose precoated with 5 μl anti-JAK2 (Santa Cruz Biotechnology, Inc.). The enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Little Chalfont, UK) was used after the secondary appropriate Ab incubation. Densitometric analysis of the signals was performed.

GH-R mutational analysis

Automated sequence analysis of exons 2–10 of GH-R was performed in those patients who failed to show an IGF-I increase after the standard 4-d IGF-I generation test and in three of the remaining patients with a borderline IGF-I response. Genomic DNA was isolated from peripheral blood leukocytes. Each exon and corresponding donor and acceptor splice sites were individually amplified by PCR. The coding portion of exon 10 was amplified in three overlapping fragments. Primers were derived from published sequence (22), and amplification was performed using standard PCR conditions (35 cycles of 95 C for 30 sec, 52 C for 45 sec, and 72 C for 1 min). PCR products were recovered from 1.5% agarose gel and sequenced (both coding and noncoding strands) with an ABI Prism dye terminator cycle sequencing kit using the PCR primers, and an ABI Prism 377 Automated DNA Sequencer (PE Applied Biosystems, Foster City, CA). The mutation detected in exon 6 and the polymorphism detected in exon 10 were confirmed by *Nla*III or *Fok*I restriction enzyme (New England Biolabs, Inc., Beverly, MA) digestion, respectively.

Results

IGF-I and IGFBP-3 levels during the generation test

As illustrated in Table 2, the IGF-I generation test, performed over a 4-d standard schedule, revealed an increase of 40% above the baseline value in 8 (patients 1, 2, 4, 5, 6, 7, 10, and 11) of 13 patients (patient 14 was lost to follow-up at the time of the generation test). However, in 2 subjects (patients 2 and 5) the increase in IGF-I expressed as the sd score, remained below the basal IGF-I mean value for age.

The basal IGFBP-3 value was below −1 sd score in three

TABLE 1. Clinical details of patients enrolled in the study

Patient no.	Sex	Chronological age (yr)	Bone age (yr)	Height (sd score)	Target height (sd score)	Growth velocity (sd score)	GH peak (μg/liter)
1	F	1.6	1.0	−2.7	−0.3	−3.4	18
2	M	3.3	1.5	−2.9	−0.7	−1.0	18
3	M	4.3	2.2	−3.5	−2.2	−2.6	4
4	M	4.8	2.6	−2.7	−0.6	−0.9	25
5	M	5.4	3.5	−2.0	−0.3	−1.5	15
6	M	6.0	4.1	−3.5	−0.2	−3.7	19
7	F	10.7	7.8	−3.2	−1.9	−2.9	11
8	M	11.1	9.0	−2.0	−1.4	−2.0	25
9	M	11.2	7.5	−2.4	−0.4	−1.1	13
10	M	11.9	9.5	−2.6	−1.5	−2.4	12
11	M	12.0	7.0	−2.9	−2.1	−1.0	12
12	M	12.6	10.0	−4.7	−0.9	−2.3	19
13	M	13.9	11.5	−3.1	−0.6	−3.7	13
14	M	14.1	9.0	−3.0	−0.3	−3.3	20

TABLE 2. Basal and 4-d stimulated IGF-I and IGFBP-3 levels in patients with ISS who underwent standard IGF-I generation test

Patient no.	IGF-I					IGFBP-3				
	Basal		Stimulated		Increase (%)	Basal		Stimulated		Increase (%)
	(μg/liter)	(SD score)	(μg/liter)	(SD score)		(μg/liter)	(SD score)	(μg/liter)	(SD score)	
1	58	0.5	138	3.0	172	1470	−1.0	2580	0.6	75
2	22	−1.3	33	−1.0	50	2390	0.1	2870	0.7	20
3	38	−1.2	49	−0.8	29	1460	−2.0	2148	−0.5	47
4	123	1.5	243	5.2	97	2720	0.8	4400	4.4	62
5	24	−1.5	66	−0.8	175	3130	0.8	4270	2.9	36
6	106	−0.04	286	3.2	170	2820	0.2	4280	2.9	52
7	181	−0.8	351	0.2	93	2440	−1.5	2290	−1.6	0
8	59	−1.0	58	−1.0	0	1900	−1.8	1940	−1.8	2
9	139	−0.3	154	−0.2	10	3440	−0.1	3240	−0.3	0
10	236	0.5	335	1.3	42	3700	0.2	4260	0.9	15
11	206	−0.4	410	1.2	99	3698	0.2	5315	1.5	34
12	154	−0.8	173	−0.6	12	4140	0.3	4110	0.3	0
13	113	−1.3	150	−1.1	33	3100	−1.2	4270	−0.2	38
14	120	−1.9				3690	−0.6			

Values are expressed in absolute values (μg/liter), in SD score and as percentage increase above the baseline absolute value.

of the five patients who failed to show an IGF-I increase; values were −2.0, −1.8, and −1.2 SD score (patients 3, 8, and 13). In three patients (no. 8, 9, and 12) who did not have a significant IGF-I increase, no rise in IGFBP-3 was found; the percent increase was between 0–2% above the basal values. In the remaining two patients, the increase was appreciable (47% and 38%). The stimulated IGF-I level correlated with the IGF-I basal value ($r = 0.75$; $P < 0.004$), as shown in Fig. 1A. In particular, all patients with a small IGF-I increase also had low basal IGF-I SD scores. Similarly, the stimulated IGFBP-3 level during the generation test correlated to the basal IGFBP-3 value ($r = 0.74$; $P < 0.004$; data not shown). As expected, the increase in IGF-I during the generation test paralleled the increase in IGFBP-3 ($r = 0.80$; $P = 0.001$), as depicted in Fig. 1B.

To identify late responders, the generation test was prolonged for 21 d. Figure 2 depicts the behavior of IGF-I in the five patients who failed to respond with an IGF-I increase in the 4-d test. Interestingly, in three of these five patients a late response was observed with increases in IGF-I values at 14 d of 192% and 82% in patients 9 and 13, and an increase of 137% at 21 d in patient 8, respectively. In contrast, in patients 3 and 12 no response was observed. Paternal heights in patients 3 and 12 were 158 and 168 cm, whereas maternal heights were 147 and 158 cm, respectively.

GH-R-induced P-Tyr

In control PBMC, short-term GH stimulation induced P-Tyr. In particular, two major proteins of 119 and 105 kDa were promptly phosphorylated; signals reached maximal intensity at 5 min and remained appreciable 15 min after the stimulation, as shown in Fig. 3A. As illustrated in Fig. 3, B and C, these proteins should correspond to JAK2 and STAT5, important signaling molecules in GH-R signaling. In the patient group GH-induced P-Tyr was normal in all subjects who showed a good response to IGF-I generation test, including those with a delayed response at 14 or 21 d of *in vivo* stimulation. In two patients (no. 3 and 12) who failed to respond to the sustained IGF-I generation test, *in vitro* GH stimulation of the receptor on PBMC induced abnormal P-Tyr events. Although in patient 3 no tyrosine phosphoryla-

tion was observed, in patient 12 the abnormality was selective, in that it concerned exclusively the protein migrating in the 119 kDa area, whose size corresponds to that of JAK2. Figure 3A shows the abnormal P-Tyr in this patient. This abnormality was not due to an overall low amount of the protein, in that, as depicted in Fig. 3, B and C, showing reprobing of the membranes with anti-JAK2 or anti-STAT5, both JAK2 and STAT5 molecules were expressed to an extent comparable to that in the controls. Moreover, in three experiments immunoprecipitated JAK2 from unstimulated or GH-stimulated PBMC was properly phosphorylated (data not shown). In all other patients, the number of P-Tyr proteins and the timing of the phosphorylation events were m comparable to those in the controls, as indicated by the densitometric analysis of the behavior of 105- and 119-kDa protein phosphorylation shown in Fig. 4, A and B.

GH-R analysis

To rule out a genetic alteration of the GH-R gene in patients who failed to show an IGF-I increase after GH stimulation (patients 3, 8, 9, 12, and 13) and in three of the remaining patients, chosen on the basis of a borderline IGF-I response (patients 2, 5, and 10), GH-R sequence analysis of the exons 2–10 was performed. In patient 5, a heterozygous missense mutation resulting in an Arg to Cys change (R161C) in exon 6 was found. As previously shown, this patient had a low basal value of IGF-I, which normally increased by 175% after 4 d of GH stimulation; however, the stimulated IGF-I value, expressed as the SD score, remained below the basal mean value for age. Basal and stimulated IGFBP-3 values were normal. GH stimulation induced a normal pattern of P-Tyr in PBMC. Genetic analysis of the patient's family members revealed that the mother and three older sisters of the index case were heterozygous for the same mutation, as depicted in Fig. 5, and none of them had short stature or mild features of Laron syndrome. In one of the three sisters a delay in growth and puberty (menarche at age 16 yr) was reported.

Three examined patients (no. 2, 3, and 9) were heterozygous for the known polymorphism of guanine to adenine at position 3 of codon 168 (G168) in exon 6. In addition, 3 patients (no. 2, 3, and 13) were heterozygous and 4 (no. 5, 8,
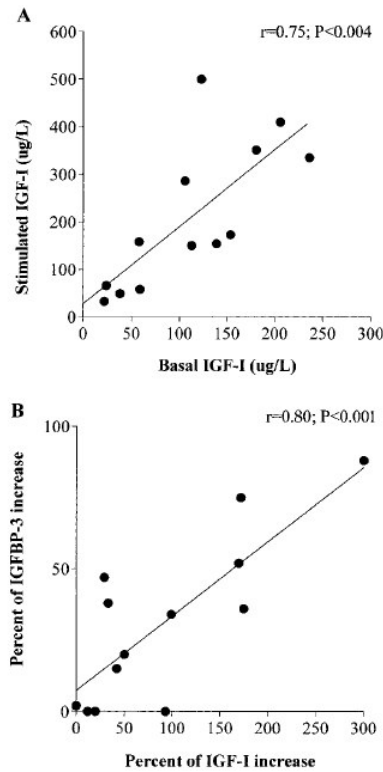
FIG. 1. A, Relationship between basal and stimulated IGF-I values. Thirteen patients with ISS underwent a standard IGF-I generation test. GH (0.1 IU/kg) was administered through daily sc injections for 4 consecutive d. Values are expressed as micrograms per liter. B, Relationship between the increase in IGF-I and IGFBP-3 during the 4-d generation test. Values are expressed as the percent increase over the basal value after 4 d of GH administration.
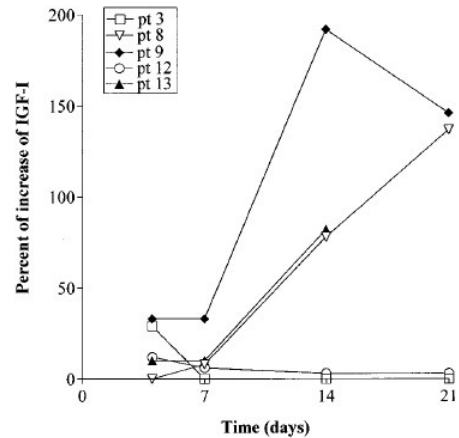

FIG. 2. Time course of the IGF-I response after a prolonged IGF-I generation test. GH (0.1 U/kg·d) was administered for 14–21 d to the five patients with ISS who failed to show an increase in IGF-I levels during the standard 4-d generation test. Values indicate the percent increase over the basal values.
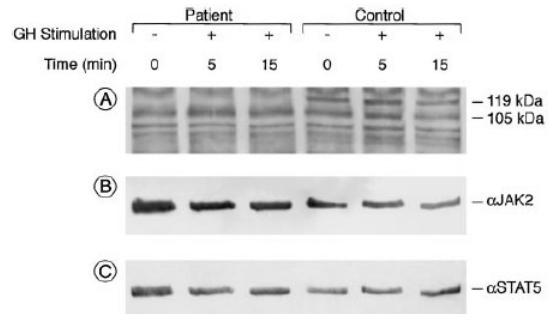

FIG. 3. A, Representative experiment showing the pattern of GH-induced P-Tyr in patient 12 and a control. PBMC were stimulated *in vitro* with GH for 5 or 15 min as indicated. In the control, two major proteins of 119 and 105 kDa were promptly phosphorylated; signals reached the maximal intensity at 5 min and were also appreciable 15 min after the stimulation. In the patient, no P-Tyr of a protein migrating in the 119 kDa area was observed. The 119- and 105-kDa proteins, corresponding to JAK2 and STAT5, respectively, are indicated. The same membrane was stripped and reprobed with anti-JAK2 (B) and anti-STAT5 (C) antibodies. Each protein in the patient was normalized to the extent comparable to the control, and no change in its amount was noted during the short stimulation, as expected.

10, and 12) were homozygous for the cytosine to adenine polymorphism at position 1 of codon 526 (I526L) in exon 10, resulting in an isoleucine/leucine change. This polymorphism was also found in 24 of 40 control chromosomes derived from individuals from the same geographic area.

Discussion

To ascertain whether GH-R hyporesponsiveness might be implicated in the pathogenesis of ISS, in this study a combination of several functional and molecular assays was used to evaluate a number of events induced by GH-R *in vivo* or *in vitro* signal transduction. This approach led to the identification of 2 of 14 ISS patients exhibiting partial GHI, thus indicating that GH-R hypo/unresponsiveness may underlie ISS in childhood.

Mutations of the GH-R gene itself in the heterozygous state have been implicated in the pathogenesis of ISS (2, 12, 13). In particular, in a large cohort of patients, 8 of 100 children affected with ISS carried mutations in the GH-R locus (12).

Nevertheless, the role of heterozygous mutations in the pathogenesis of short stature has yet to be defined, as these studies did not provide information on IGF generation or GH intracellular signaling. In our patients, heterozygosity for an Arg[161]Cys mutation of the GH-R gene was found in only 1 patient affected with ISS, who had, however, a normal incremental response to GH. Interestingly, 3 sisters and the mother of the index case, none of whom had short stature, also carried the same mutation. In 1 sister, a delay in growth and puberty, eventually resulting in regular menses, was
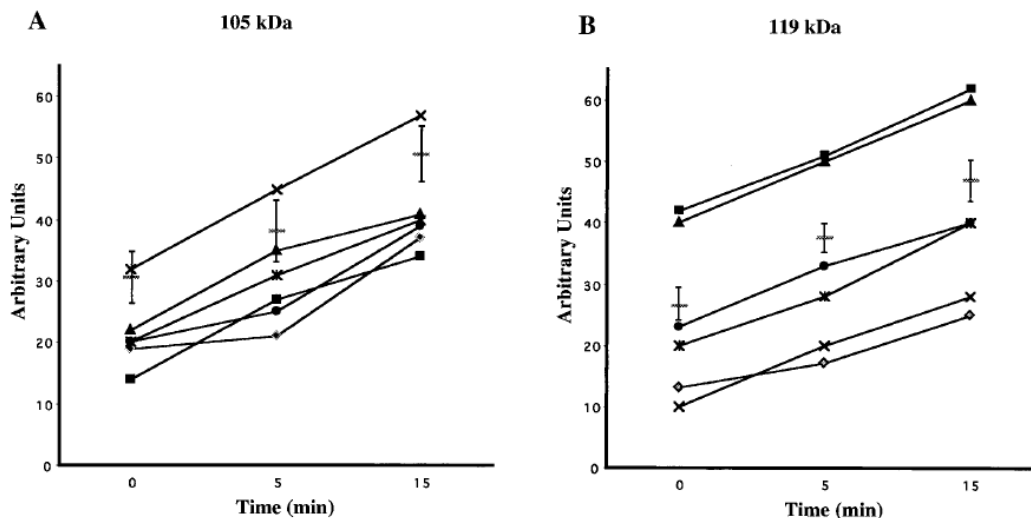
A 105 kDa

B 119 kDa

FIG. 4. Densitometric analysis of the behavior of the 105-kDa (A) and 119-kDa (B) tyrosine-phosphorylated proteins in six representative ISS patients with a normal increase in P-Tyr and a normal response to the IGF-I generation test. *In vitro* GH stimulation was performed as indicated in Fig. 3. Densitometric analysis was expressed in arbitrary units. *Vertical bars* indicate the mean ± SE of control values.
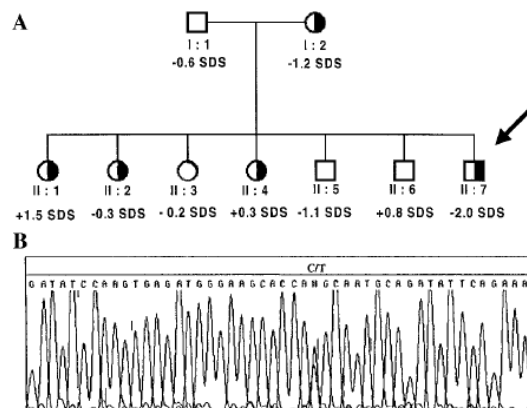

FIG. 5. A, Pedigree of patient 5 carrying the mutation of exon 6 of the GH-R gene. *Half-solid circles* and *squares* indicate subjects heterozygous for the Arg[161]Cys mutation. The *arrow* indicates the index case. The current height of the proband and his family members is given as an SD score. One sister carrying the mutation (II.I) had a history of growth and pubertal delay. B, DNA sequence analysis of exon 6 of the GH-R gene showing the mutant sequence at the heterozygous state.

reported, similar to what is frequently observed in patients with complete GHI (6). The Arg[161]Cys mutation, which is responsible in the homozygous state for complete GHI, has been previously described in the heterozygous state in 2 ISS patients originating from different geographic areas (12). However, the family data associated with the nucleotide variant reported here would argue against a pathological role of this mutation. Taken together, the low prevalence of

GH-R mutations in large cohorts of ISS patients and the lack of a clear phenotype/genotype relationship indicate that a genetic alteration of the receptor itself, although possible, is a relatively rare mechanism for ISS in childhood.

In our patients several polymorphisms of the GH-R gene have been found. In particular, the guanine to adenine polymorphism at position 3 of codon 168 (G168) was found in three patients. The I526L polymorphism in exon 10 was found at the same frequency in patients and controls originating from the same geographic area. A higher frequency of G168 polymorphism has been described in subjects with ISS compared with normal controls (12). However, the significance of this genetic alteration in influencing linear growth as well as the overall clinical phenotype remains an open and intriguing issue.

The GH-R, like all other receptors, exerts its function by delivering intracellular signals through biochemical alterations of functionally related signaling molecules (23). This complex array of biochemical events is redundant in that several genes are involved in amplifying signal transduction in the individual pathway (24). A proper functional interaction in a well coordinated fashion of several gene products is, therefore, required for fully functioning receptor signaling. Thus, a possible mechanism to explain the wide phenotypic variability of heterozygous mutations of GH-R is that there are other genes that may interact with the signaling pathway, thus modifying the clinical phenotype.

In the two patients with peripheral hyporesponsivity herein reported, an abnormal P-Tyr after *in vitro* stimulation with GH of the GH-R expressed on the surface of PBMC (25), was documented. In one of these two patients, the effect selectively involved a 119-kDa protein, a size corresponding to the JAK2 molecule, which plays a major role in GH-R

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signaling. However, the absence of tyrosine phosphorylation of the protein was not due to a reduced amount of the kinase itself, thus suggesting a possible abnormal posttranslational regulation of the molecule. Moreover, through immunoprecipitation experiments showing a normal JAK2 phosphorylation this hypothesis was ruled out, thus suggesting that a different molecule migrating in the same area of JAK2 and yet to be defined was involved in the process. Dimerization of the receptor that follows GH binding represents the first key event in the activation of target cells (24). Subsequently, tyrosine phosphorylation of JAK2 and STAT5 proteins plays a crucial role in the activation process, which ultimately results in gene transcription (16, 17). In the context of the overwhelming number of distinct molecules implicated in the process, the pivotal role of STAT5 in the signaling pathway related to GH-R, and in particular of the STAT5b protein, is also supported by the experimental evidence of reduced growth in male mice with STAT5b gene disruption (26, 27). Furthermore, the importance of the JAK-STAT pathway is also underlined by the evidence of defective GH-induced tyrosine phosphorylation and activation of STAT5 in patients affected with short stature carrying GH-R gene mutations and in patients with Laron syndrome (28–30). To our knowledge, the two cases reported here are the first patients with ISS in whom an abnormal tyrosine phosphorylation induced by GH-R signaling in PBMC has been documented in the absence of any genetic alteration of the receptor itself or classical features of Laron syndrome.

Overall, as the clinical and biochemical hallmarks of partial GHI have not yet been clearly elucidated, the identification of such patients is still difficult. Our approach was to integrate functional, biochemical, and molecular studies in an effort to identify the most reliable markers of GHI. Our findings indicate that none of the individual functional parameters, including basal and stimulated IGF-I and IGFBP-3 values, led to unequivocal identification of such patients according to previously reported observations (31). It should be noted that four patients (patients 1, 4, 7, and 10) with an ISS clinical phenotype indistinguishable from that of the two patients with the GH-R unresponsiveness had normal or close to normal basal IGF-I values, which further increased in response to GH stimulation. These patients deserve further attention, because they could represent a distinct clinical entity, such as peripheral resistance to IGF-I. The frequently used 4-d IGF-I generation test led to an overestimate of unresponsive patients. Indeed, a sustained GH stimulation induced, in thre of five unresponsive patients, a significant IGF-I increase. In three subjects (patients 5–7) a discrepancy between the increases in IGF-I and IGFBP-3 after GH administration was observed. In all of these cases only an increase in IGF-I was observed, suggesting a possible selective abnormality of the binding protein. However, arguing against this hypothesis, the amount of the binding protein was normal in two of them, and in the third patient it significantly increased after a prolonged GH stimulation. Of note, the two patients who failed to respond to the long-term IGF-I generation test also showed an abnormal pattern of P-Tyr after *in vitro* GH stimulation of PBMC, thus suggesting that different cell targets, such as hepatocytes and PBMC, share the same transducing elements. As the 21-d IGF-I generation test cannot be proposed as a diagnostic tool on a routine basis, due to the high cost and the uncomfortable nature of the test, our findings also suggest that combining the 4-d IGF-I generation test with analysis of GH-induced P-Tyr on PBMC may help identify ISS patients with partial GHI.

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2.3　Human Severe Combined Immunodeficiency (SCID) phenotype

Severe Combined Immunodeficiencies (SCID) encompasses a wide spectrum of disorders of both cell-mediated and humoral immunity. Defects in lymphoid differentiation may lead to the complete absence of T and B cells (T-B- phenotype) or, alternatively, may predominantly affect T lymphocyte development (T-B+ phenotype) (Fischer A. 1997). These form are in generally fatal within the first years of life, although a few typical cases may be characterized by a milder phenotype. Thus far, all the different forms with a known molecular basis are caused by mutations in genes that encode for proteins selectively expressed in the hematopoietic tissue, which lead to intrinsic abnormalities of lymphocyte function. This implies that in most patients, the affected cell line can be replaced by stem cells from healthy donors. However, an appropriate, productive immune response also requires appropriate intercellular connections. A relation between the hematopoietic precursor and the thymic epithelial component plays a crucial role in cell ontogeny and in negative and positive selection (Anderson G. 1994).

Our group in 1999 reported on 2 sisters affected by a novel form of SCID (MIM 601705) characterized by T cell defect associated with congenital alopecia and nail distrophy. This complex phenotype represents the human homologue of mouse Nude/SCID phenotype (Pignata et al 1996). Subsequently, the sisters were found to carry a nonsense the R255X mutation in the homozygous status in the highly conserved winged-helix-nude (WHN) gene (Frank J et al 1999). Alteration of this gene is responsable for mouse Nude

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/SCID (Nels et al 1994, Segre et al 1995). WHN is a forkhead-winged helix transcription factor whose expression is restricted to the thymic epithelium, epidermis, and hair follicle (Lai et al 1993, Kaufmann et al 1996). Human Nude/SCID is the first example of a human SCID caused by a gene not expressed in th hematopoietic cells. Before this molecular alteration was identified, the younger of the 2 WHN-/- sisters underwent allogenic bone marrow transplantation (BMT) because of her critical clinical and immunologic condition.

2.3.1 Immunologic reconstitution after bone marrow transplantation in human Nude/SCID phenotype

The current study was undertaken to evaluate the role of the thymus in immunologic reconstitution and long-term efficacy of bone marrow transplantation (BMT) in the younger of the 2 sister affected by human Nude/SCID phenotype. This is a unique model with which to investigate the role of intrathymic connections, between thymic epithelia and hematopoietic progenitors, in the regeneration and maintenance of T-cell responces after allogenic BMT.

The children was born of consanguineous parents. The clinical feather showed a complete absence of hair, distrophic nail, absence of thymus and severe functional T-cell immunodeficiency. At the age of 5 months the patient underwent HLA-identical total bone marrow transplantation from her unaffected brother. The immunophenotype performed at regular interval during post-BMT follow-up revealed that despite an increases in CD3+,CD4+,and CD8+ cells, CD4+CD45RA naive lymphocytes were not generated. Conversely, naive CD8+ cells were normal. The proliferative responses of peripheral lymphocytes to CD3 cross-linking showed an initial improvement that progressively declined 48 months after BMT. Furthermore, the proliferative responses to common mitogens of lymphocyte obtained from the WHN -/- patient revealed a remarkable deficiency compared with the genotypically identical donor cells grown in the WHN +/- environment. Analysis of T-cell receptor (TCR) repertoire of CD4+ cells revealed that only 3

of 18 Vβ families had an alterated CDR3 heterogeneity length profile. Conversely, CD8+ lymphocytes showed an abnormal distribution in most Vβ families. These data indicate that the thymus is differentially required in the reconstitution of CD4+ and CD8+ naive subsets and in the maintenance of their repertoire complexity. Despite the favorable clinical course after BMT, our data suggest that this terapeutic procedure is ineffective in the long term cure of this form of SCID. These results has been published on Blood see below for the manuscript.

Human equivalent of the mouse Nude/SCID phenotype: long-term evaluation of immunologic reconstitution after bone marrow transplantation

Claudio Pignata, Lucia Gaetaniello, Anna Maria Masci, Jorge Frank, Angela Christiano, Eliana Matrecano, and Luigi Racioppi

Human Nude/SCID (severe combined immunodeficiency) is the first severe combined immunodeficiency caused by mutation of the winged–helix–nude (WHN) gene, which is expressed in the thymus but not in the hematopoietic lineage. The disease is characterized by a T-cell defect, congenital alopecia, and nail dystrophy. A Nude/SCID patient who underwent bone marrow transplantation from the human leukocyte antigen–identical heterozygote brother was studied to investigate, in this unique model, the role of the thymus in

immunologic reconstitution. Despite an increase in CD3+, CD4+, and CD8+ cells, CD4+ CD45 RA naive lymphocytes were not regenerated. Conversely, naive CD8+ cells were normal. After an initial recovery, lymphocyte proliferation to mitogens progressively declined compared with controls and genotypically identical donor cells grown in the WHN+/− environment. Analysis of the T-cell receptor (TCR) repertoire of CD4+ cells revealed that only 3 of 18 Vβ families had an altered CDR3 heterogeneity length profile.

Conversely, CD8+ lymphocytes showed an abnormal distribution in most Vβ families. These data indicate that the thymus is differentially required in the reconstitution of CD4+ and CD8+ naive subsets and in the maintenance of their TCR repertoire complexity. Taken together, these findings suggest that bone marrow transplantation is ineffective in the long-term cure of this form of SCID. (Blood. 2001;97:880-885)

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Introduction

Severe combined immunodeficiency (SCID) encompasses a wide spectrum of disorders of both cell-mediated and humoral immunity.[1] Defects in lymphoid differentiation may lead to the complete absence of T and B cells (T−B− phenotype) or, alternatively, may predominantly affect T lymphocyte development (T-B+ phenotype).[1] In a few patients with SCID with a predominant T-cell defect, T cells are detectable in peripheral blood but are not functional because of abnormalities in the cell activation process rather than because of differentiation defects.[2]

In recent years, the identification of several molecular alterations has led to a better understanding of the mechanisms underlying SCID. Alterations in at least 8 genes have been identified and associated with a clinical phenotype of SCID. Abnormalities of signaling molecules, such as the common γ element of several cytokine receptors, the α chain of IL-7 receptor,[3] and the JAK-3 tyrosine kinase that associates with the γ chain, are responsible for the X-linked or the autosomal recessive forms of T-B+ SCID.[4,5] Mutations of RAG-1 and RAG-2 have been described in autosomal recessive forms of the disease.[6] Mutations of elements of the T-cell receptor (TCR)/CD3 multimeric complex, such as CD3ε or CD3γ chains,[7,8] or of the 70-kd kinase that associates with the ζ chain of the complex, also result in the less severe forms of activation defects.[9] It is noteworthy that all the genes so far implicated in the pathogenesis of SCID encode for proteins selectively expressed in hematopoietic tissue. This implies that in most patients, the affected cell line can be replaced by stem cells from healthy donors. However, an appropriate, productive immune response also requires appropriate intercellular connec-

tions. A relation between the hematopoietic precursor and the thymic epithelial component plays a crucial role in cell ontogeny and in negative and positive selection.[10]

We previously reported on 2 sisters affected by SCID because of a T-cell defect associated with congenital alopecia and nail dystrophy.[11] This complex phenotype was considered the human homologue of the mouse Nude/SCID phenotype.[11] Subsequently, the siblings were found to carry a nonsense mutation in the highly conserved winged–helix–nude (WHN) gene.[12] Alteration of this gene is responsible for mouse Nude/SCID.[13-15] WHN is a forkhead–winged helix transcription factor whose expression is restricted to the thymic epithelium, epidermis, and hair follicle.[16,17] Human Nude/SCID is the first example of a human SCID caused by a gene not expressed in the hematopoietic cells. Six years ago, before the molecular alteration was identified, the younger of the 2 WHN−/− siblings underwent allogeneic human leukocyte antigen (HLA)-related bone marrow transplantation (BMT) because of her critical clinical and immunologic conditions. The current study was undertaken to evaluate, in this unique model, the role of the thymus in immunologic reconstitution and the long-term efficacy of BMT in the human Nude/SCID phenotype.

Patient, materials, and methods

Patient history

The patient was born of consanguineous Italian parents with a complete absence of hair and dystrophic nails, as seen in her elder sister. Thereafter,

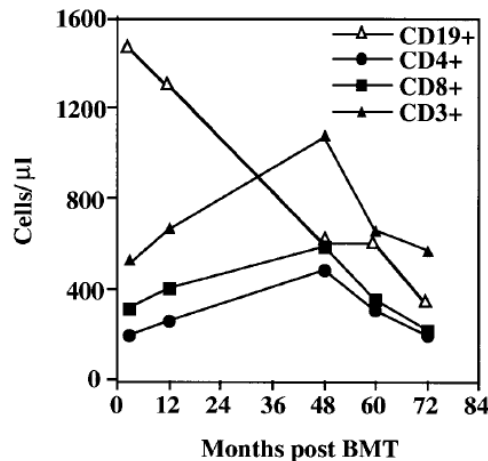© 2001 by The American Society of Hematology

Figure 1. Immunologic reconstitution in the *WHN*^{−/−} patient after allogeneic BMT. Immunophenotype analysis of CD19+, total CD3+, CD4+, and CD8+ lymphocyte subsets in the *WHN*^{−/−} patient after allogeneic BMT. Results are expressed as absolute values.

like her older sister, she was found to have severe functional T-cell immunodeficiency. No thymic shadow was evident in either child's radiologic examination. The first child died at 12 months of recurrent bronchopneumonia. The clinical and immunologic phenotype has been reported as a new human syndrome (MIM 601705).[11] At the age of 5 months, the second child underwent HLA-identical total bone marrow transplantation from her unaffected brother, after only 2 doses of antilymphocyte rabbit globulin at a dose of 2.5 mg/kg and no immunosuppressive therapy or myeloablation by irradiation. The engraftment of donor cells was established through the analysis of highly polymorphic DNA regions using the DIS80 and DQ α markers. Six years after BMT the patient is healthy, but the alopecia has persisted, indicating that it was primitive in nature. Tonsils were not evident, whereas lymph nodes were present both before and after BMT.

Immunophenotype and proliferative responses

The immunophenotype was performed at regular intervals during the post-BMT follow-up using the following monoclonal antibodies in 2-color immunofluorescence: anti-CD3 (Leu-4), anti-CD4 (Leu-3a), anti-CD8 (Leu-2a), anti-CD19 (Leu-12), anti-CD45 RA, anti-CD45 RO, anti-CD11b (Leu-15), and anti-CD28 (Leu-28) (Becton Dickinson, San Jose, CA). A B-D flow cytometer was used. For the proliferation assays, peripheral blood mononuclear cells (PBMC) (2×10^5) were obtained by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient separation and cultured at 37°C for 72 hours with medium alone or with 10 µg/mL PHA (Difco Laboratories, Detroit, MI), 8.25 µg/mL concanavalin-A (ConA) (Difco), 10 µg/mL pokeweed (PwM) (Life Technologies, Paisley, United Kingdom), 20 ng/mL phorbol-myristate acetate, and 0.5 mM ionomycin (Sigma Chemical, St. Louis, MO). CD3 cross-linking (CD3 X-L) was performed by precoating tissue culture plates with 1 or 10 ng/mL anti-CD3 monoclonal antibody (Ortho Diagnostics, Raritan, NJ); rIL-2 was used at a concentration of 100 U/mL. Cultures were pulsed with 1 µCi per well of [3H]-thymidine (Amersham International, Buckinghamshire, United Kingdom) 18 hours before harvesting.

RNA extraction and cDNA preparation

Peripheral blood mononuclear cells were isolated by density gradient centrifugation as described above. Magnetic beads coated with anti-CD8+ monoclonal antibody (Dynal Biotech, Oslo, Norway) were used for the positive and negative sorting of T cells. Total RNA was extracted from

positively or negatively sorted lymphocytes by using Tryazol (Gibco-BRL, Bethesda, MD). Reverse transcription–polymerase chain reaction (RT-PCR) was performed by using the one-step RT-PCR kit (Gibco-BRL) according to the manufacturer's instructions.

CDR3 heterogeneity length analysis

Conditions for CDR3 size distribution analysis are described elsewhere.[18] Briefly, cDNA was amplified through PCR with specific primers to 24 different Vβ families and a fluorescent Cβ primer. One microliter amplified product was mixed with 1.5 µL 100% formamide and 0.5 µL size standard (Genescan-500 ROX, ABI 373; PerkinElmer, Urayasu, Japan), heated at 90°C for 3 minutes, and electrophoresed in a 6.75% denaturing polyacrylamide gel. The distribution of CDR3 size within the amplified product of each Vβ subfamily was evaluated by using an automatic sequencer (ABI 377; PerkinElmer) equipped with a computer program that allows the determination of the fluorescence intensity of each band. Results are depicted as peaks corresponding to the intensity of the fluorescence. CDR3 size patterns that did not have a bell-shaped distribution but had prominent peaks (less than 5) were judged to be abnormal.

Results

Lymphocyte reconstitution in the *WHN*^{−/−} patient after allogeneic BMT

The patient's immunophenotype before BMT is reported elsewhere.[11] Briefly, the *WHN*^{−/−} patient had CD3+ lymphocytes of reduced number and percentage (650 cells/µL and 25%, respectively), a low number of CD4+ cells, and a low-normal number of CD8+ cells.[11] The T-cell subset and B-lymphocyte profiles during the 6-year post-BMT follow-up are shown in Figure 1. Consequent to the administration of antilymphocyte globulin, 12 days after BMT, CD3+, CD4+, and CD8+ cell levels were markedly reduced, whereas the CD19+ cell level was higher than normal (1400 cells/µL). The number of CD3+ cells started to increase progressively 3 months after BMT. Twelve months after BMT, T cells reached a level of 700/µL (27% of PBMCs). CD8+ cells increased faster than CD4+ lymphocytes. One year after BMT, chimerism analysis revealed that T cells were from the donor and B cells were from the host (data not shown). Four years after BMT, T cells remained of donor origin, and CD levels were as follows: CD3+cells, 1100/µL; CD8+ cells, 500/µL; and CD4+ cells, 400/µL (18% of PBMCs). Thereafter, CD3+, CD4+, and CD8+ levels progressively declined. The CD4+/CD8+ ratio persisted below 1.

Regeneration of the CD45 RA "naive" phenotype within CD4 and CD8 compartments in the *WHN*^{−/−} environment after BMT

Despite the increase of CD3+ cells and, albeit to a lower extent, of mature CD3+CD4+ cells, nearly all these cells showed a memory CD45 RO phenotype, whereas only 1% to 3% of lymphocytes expressed the CD4+ CD45 RA "naive" phenotype (Figure 2A). At 6 years of follow-up, no changes were observed in the CD4+ CD45 RA–CD4+ CD45 RO ratio. Because all T cells in the *WHN*^{−/−} patient were of *WHN*^{+/−} donor origin, we compared the memory and naive phenotype of CD4+ cells developed in vivo in the donor with those developed in the recipient environment. Six years after BMT, the CD4+ CD45 RA compartment was significantly reduced only in the CD4+ cells obtained from the recipient *WHN*^{−/−} environment (Figure 2B). This resulted in a very low RA:RO ratio compared with cells from the donor *WHN*^{+/−} environment (0.06
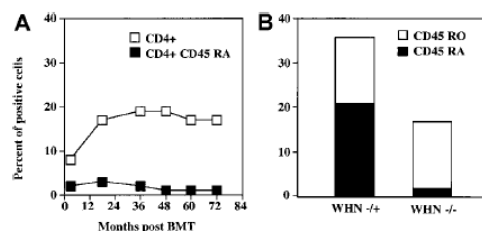
Figure 2. Reconstitution of CD4⁺ CD45 RA cells in the *WHN⁻/⁻* patient after allogeneic BMT. (A) Time-course of the reconstitution of CD4⁺ CD45 RA cells in the *WHN⁻/⁻* patient during the 72 months after allogeneic BMT. (B) Comparison of CD4⁺ CD45 RA and RO subsets between the *WHN⁻/⁻* recipient and the *WHN⁺/⁻* donor environment 6 years after BMT. Lymphocytes were double stained and analyzed by flow cytometry. Results are expressed as percentage of positive cells.

and 1.37, respectively). These findings indicate that a functional thymus is necessary to renew the naive CD4⁺ subset.

During the post-BMT period there was a marked increase in the percentage and number of CD3⁺CD8⁺ cells, which reached 27% and paralleled the increase of the whole CD3⁺ population (Figure 3A). This subset did not contain the CD3⁻CD8⁺ natural killer population, which represented 6% of PBMCs. In contrast to the CD4 compartment, the percentage of CD8⁺ CD45 RA cells was 12% and remained stable during follow-up. Six years after BMT, the CD45 RA–CD45 RO ratio was normal (1.30) (Figure 3B). This suggests that CD8⁺ CD45 RA lymphocytes are generated even in the absence of a functional thymus. Indeed, the CD8⁺ CD45 RA–CD8⁺ CD45 RO ratio was also normal (1.66) also before BMT. CD8⁺ cells were also stained for the expression of CD28 and CD11b molecules. Fifty percent of CD8⁺ cells expressed CD28, whereas only 4% of these cells bore CD11b.

Proliferative responses in the *WHN⁻/⁻* patient after allogeneic BMT

To assess the functional reconstitution of peripheral lymphocytes after BMT, we analyzed at regular intervals during the 6-year follow-up the proliferative response of PBMC from the *WHN⁻/⁻* patient to CD3 cross-linking. From the third month after BMT there was a progressive improvement in the proliferative response to CD3 cross-linking (Figure 4A). The response was completely normal 2 years after BMT. Proliferative capability began to decline 48 months after BMT. Again, because all T cells in the recipient were of donor origin, we compared the proliferative response to common mitogens of PBMC obtained from the *WHN⁻/⁻* recipient
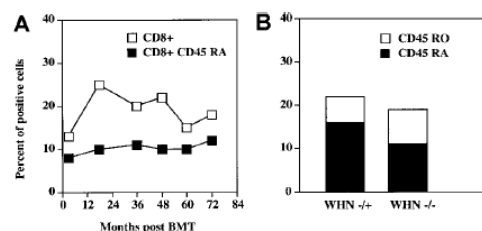

Figure 3. Reconstitution of CD8⁺ CD45 RA T cells in the *WHN⁻/⁻* patient after the BMT. (A) Time-course of the reconstitution of CD8⁺ CD45 RA cells in the *WHN⁻/⁻* patient during the 72 months after allogeneic BMT. (B) Comparison of CD8⁺ CD45 RA and RO subsets between the *WHN⁻/⁻* recipient and the *WHN⁺/⁻* donor environment 6 years after BMT. Lymphocytes were stained and analyzed as described in Figure 2 and in "Materials and methods."
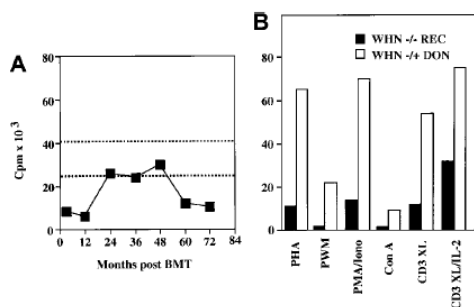

Figure 4. Proliferative responses in the *WHN⁻/⁻* patient or the *WHN⁺/⁻* healthy donor brother. (A) Proliferative response evaluated through [3H]-thymidine incorporation after stimulation of peripheral blood mononuclear cells by CD3 cross-linking. PBMCs from the *WHN⁻/⁻* patient were analyzed at regular intervals during the 6 years after allogeneic BMT. The results are expressed as cpm. Dotted lines indicate mean ± SD of control values. (B) Comparison 6 years after BMT of the proliferative response to several mitogens between cells grown in the *WHN⁻/⁻* recipient and in the *WHN⁺/⁻* donor environment. Exogenous rIL-2 was used at a concentration of 100 U/mL.

with the genotypically identical donor cells developed in vivo in the *WHN⁺/⁻* environment. Six years after BMT a remarkable deficiency of cells from the *WHN⁻/⁻* recipient was evident (Figure 4B). Even though exogenous IL-2 induced an approximately 3-fold increase of the response to CD3 cross-linking, the response never reached normal levels.

Humoral immunity studies

To evaluate whether immunologic reconstitution, as revealed by cell number and proliferative response to mitogens, was associated with normal capability to generate specific antibody production, an immunization program was scheduled in the post-BMT period. To discriminate between a response due to committed donor cells and that due to newly in vivo–primed lymphocytes, the program included immunizations both to known antigens to the donor at the time of BMT and to neoantigens such as HBsAg. Table 1 illustrates the comparison of humoral immunity in the pre- and post-BMT (36 months) periods and, in particular, antibody responses in the *WHN⁻/⁻* (recipient) versus the *WHN⁺/⁻* (donor) environment. A full immunologic reconstitution was achieved 2 years after BMT as revealed by the rise in anti-tetanus toxoid antibodies. Notably, even in the *WHN⁻/⁻* environment, B cells were able to generate a

Table 1. Humoral immunity studies

	WHN⁻/⁻ recipient	*WHN⁺/⁻* donor
Serum IgG (g/L)	4.94	6.55
Serum IgA (g/L)	0.49	0.77
Serum IgM (g/L)	0.80	0.74
Autoantibodies	abs	abs
Specific antibodies		
Allohemoagglutinins		
Pre-BMT	abs	anti-A/B (1:128)
Post-BMT	anti-A (1:8)	
Tetanus toxoid		
Pre-BMT	abs	pos
Post-BMT	pos	pos
HbsAg		
Pre-BMT	abs	abs
Post-BMT	pos	pos

abs, absent; pos, positive.

Figure 5. TCR diversity of recipient or donor T cells. Comparative analysis of TCR-Vβ CDR3 heterogeneity length in (A) CD8-depleted (CD8−) or (B) CD8-enriched (CD8+) lymphocyte suspensions derived 72 months after BMT from the *WHN*−/− recipient and the *WHN*+/− donor environment. RT-PCR was performed on RNA isolated from purified T-cell subsets. PCR products were separated on DNA sequencing polyacrylamide gel by using an automated ABI PRISM 377 apparatus (PerkinElmer). Band intensity was evaluated with the Gene Scan software. Size markers are indicated. TCR-Vβ families showing fewer than 5 major peaks were considered altered.
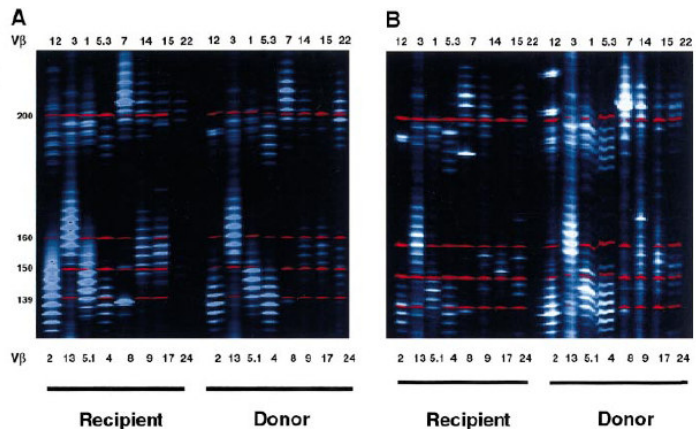
specific antibody response toward antigens unknown at the time of the transplantation. In fact, 2 boosters of HBsAg led to the appearance of specific antibodies, which demonstrates that efficient priming also occurs in the *WHN*−/− environment.

Maintenance of T-cell receptor diversity in the *WHN*−/− environment

The biologic mechanisms ensuring maintenance of T-cell repertoire diversity in mature lymphocytes are not completely known and involve central and peripheral lymphoid tissue. In this context, the analysis of TCR diversity in our *WHN*−/− patient after BMT provides a unique opportunity to analyze the role of peripheral factors in maintaining T-lymphocyte diversity. We evaluated the TCR repertoire by analyzing Vβ CDR3 heterogeneity length (spectratyping) 6 years after BMT in genotypically identical T cells obtained from the *WHN*−/− recipient and from the *WHN*+/− donor. Figure 5A shows the CDR3 length profiles of 18 Vβ families in the CD4+ cells. In cells from the *WHN*−/− recipient there was a marked alteration in the CDR3 length profile in only 3 of the 18 Vβ families.[8,12,15] Notably, in 2 of them (families 8 and 15), the altered profile overlapped in donor and recipient cells. Spectratyping analysis of CD8+ lymphocytes (Figure 5B) revealed that most of the Vβ families displayed an altered profile (11 of the 18 families). Most of them (families 5.3, 8, 9, 12, 14, 17) exhibited oligoclonal expansion. The complexity of the TCR repertoire was reduced in the remaining families. Even though all the families were altered in both *WHN*+/− and *WHN*−/− cells, within individual families the distribution pattern differed between recipient and donor, unlike what occurred in CD4+ cells. Spectratyping analysis confirmed the different behavior of CD4+ and CD8+ cells in the absence of a functional thymus.

Discussion

We used a unique model, the human counterpart of the Nude/SCID phenotype recently described by our group,[11] to explore the role of the thymus in post-BMT immunologic reconstitution. Nude/SCID is the first form of SCID whose immunodeficiency is substantially and exclusively related to an intrinsic abnormality of the thymus.[19,20] The gene involved in this disease is the *WHN* gene, which

encodes a forkhead–winged helix transcription factor and is associated with both the murine and human Nude/SCID phenotypes.[12-14] Because *WHN* is selectively expressed in the thymic epithelia and not in hematopoietic cells, this rare and novel condition in humans is a reliable model with which to investigate the role of intrathymic intercellular connections, and namely the connections between thymic epithelia and hematopoietic progenitors, in the regeneration and maintenance of T-cell responses after allogeneic BMT. The analysis of immunologic reconstitution during the 6-year follow-up after BMT in the patient described herein revealed that the lack of a functional thymus did not prevent the repopulation kinetics of CD3+, CD4+, and CD8+ cells.

In the absence of a functional thymus, in the *WHN*−/− environment no CD4+ CD45+ RA naive lymphocytes were detected in the periphery. Moreover, the long-term follow-up revealed that the defect in the generation of the naive CD4 population was not restored by BMT. Conversely, the *WHN*−/− patient was able to regenerate and maintain a normal amount of CD8+ CD45 RA T lymphocytes. Our findings are in keeping with the recent observation in cancer patients who underwent thymectomy, that after BMT the reconstitution of the CD4+ CD45 RA subset relies on a functional host thymus.[21] As did our patient, patients who undergo thymectomy retain the capacity to regenerate the naive CD8+ subset, suggesting the presence of a thymus-independent pathway for the generation of this subset.[21] Indeed, recovery of the CD4+ CD45 RO memory subset after BMT in thymus-deficient patients presumably results from expansion of the donor's mature lymphocytes. Taken together, these findings also argue against the possibility that in humans CD4+ CD45 RO cells can convert to the naive CD45 RA phenotype.[22] The prompt recovery of the CD8+ CD45 RA naive cells may indicate the involvement of extrathymic lymphopoiesis. These cells could also originate from conversion of the CD45 RO phenotype.[23] However, it should be noted that the CD8+ CD45 RA compartment is heterogeneous in that it contains unprimed and primed cells.[24] Unlike unprimed naive cells, primed cells express low levels of CD27 and CD28 and high levels of the CD11b adhesion molecule. Of note, a large fraction of CD3+CD8+ cells in our patient expressed CD28 and only a small percentage expressed CD11b, which suggests that they were unprimed naive CD8+ T cells. In patients with DiGeorge syndrome, BMT can lead

to donor T-cell engraftment,[25] though it is unclear whether reconstitution is stable over time and whether donor T cells represent an expansion of mature T lymphocytes differentiated before transplantation.[26] However, in DiGeorge syndrome, there is residual thymic function as testified by the post-BMT increase in CD4^{+} CD45 RA^{+} cells.[25]

During the 6-year post-BMT period, the proliferative responses of the recipient peripheral blood cells to common mitogens progressively declined to 20% of the genotypically identical donor cells grown in the WHN^{+/−} environment. Feasibly, in the absence of the thymus, T-lymphocyte survival and homeostasis depend principally on continuous stimulation by lymphoid tissue accessory cells.[27-29] Because all T cells were of donor origin, this progressive functional impairment could reflect accelerated accumulation of aged lymphocytes that have a low proliferative capability, somewhat similar to what occurs in the elderly.[30,31]

The thymus plays a major role in T-cell ontogeny.[32,33] In this organ, T cell precursors undergo a complex mechanism of positive and negative selection to shape the TCR repertoire of mature lymphocytes. Mature cells become capable of recognizing exogenous peptides in the context of self-major histocompatibility complex molecules. Cortical epithelial cells of the thymus are unique in their ability to mediate positive selection efficiently,[10] whereas bone marrow–derived dendritic cells are efficient mediators of negative selection; they are not involved at all in the positive selection process.[34] Patients with DiGeorge syndrome have a marked reduction in lymphopoiesis and severe alterations in the lymphocyte repertoire.[35] In this context, our analysis of T-cell repertoire complexity provides insights into the mechanisms involved in the maintenance and regeneration of T-cell repertoire diversity. The WHN^{−/−} environment did not influence the long-term maintenance of TCR repertoire diversity in the CD4^{+} population, which consisted mostly of memory cells. Furthermore, the altered Vβ families had a similar profile in host and donor lymphocytes, suggesting that the donor's repertoire is maintained in the host environment. Unlike the CD4 compartment, the complexity of the TCR CDR3 profile was reduced in most CD8^{+} cells, in a few cases indicating oligoclonal expansion. In addition, within individual Vβ families and unlike what was observed in the CD4 compartment, recipient and donor cells had different CDR3 profiles. Whether extrathymic lymphopoiesis or peripheral mechanisms may have contributed to the CD8^{+} reconstitution is an intriguing question.[36,37]

It is noteworthy that 6 years after BMT, and despite the progressive immunodeficiency status and the absence of anti-infectious prophylaxis, the patient remains free of infection, probably because of a residual immune response estimated to be 20% of donor cell proliferative potential. In addition, Nude/SCID mice can clear some viral infections. This is presumably because of a T-cell–independent antibody response and mechanisms of innate immunity.[38,39] Clearance of the polyoma virus in T-cell–deficient mice results from T-cell–independent antibody-mediated responses.[39] In our patient HBsAg immunization led to specific antibody production. The generation of specific antibody responses toward a neoantigen unknown to the donor cells at the time of BMT may indicate that donor-derived CD4^{+} cells can provide help for B cells. However, it should be noted that we observed specific antibody responses during the phase of optimal immune response; we did not test the response when immunity declined. Alternatively, the generation of a B-cell response against HBsAg may be obtained in a thymus-independent fashion.

In conclusion, our findings on T-cell reconstitution in the WHN^{−/−} environment indicate that a functional thymus is essential to regenerate the CD4^{+} naive subset. The long-term maintenance of TCR diversity of the memory–effector CD4^{+} lymphocytes may be ensured by a peripheral mechanism that does not depend mainly on antigen-driven clonal expansion.[28,29] Conversely, the regeneration of CD8^{+} naive lymphocytes after BMT is less thymus dependent and also occurs in the WHN^{−/−} environment. Lastly, despite the favorable clinical course after BMT, our data indicate that this therapeutic procedure is ineffective in the long-term cure of this form of SCID.

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2.3.2 Molecular screening for the Nude/SCID gene alteration in isolated population

Since the first 2 sisters investigated, carrying the R255X mutation in the homozygous state, born to consanguineous parents originating from small community in southern Italy (Pignata et al 1996), other cases were found in same community. So in this phase of research, the 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 1) 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.

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

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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

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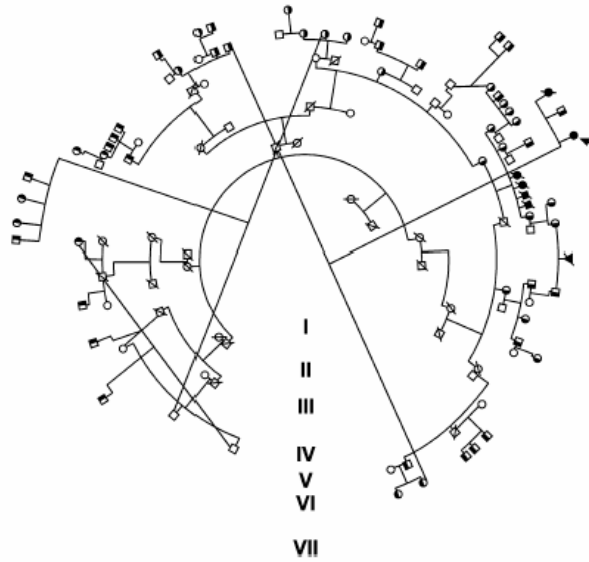
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Figure 1 Seven generations pedigree including the Acerno families linked to the index family. Not all relatives are shown, but only the subjects necessary to demostrate the founder effect. Filled circles indicate affected individuals. Half-filled symbols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

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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

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2.4   A crosstalk between Immune System and Endocrine System

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.

2.4.1 X-SCID phenotype associated with growth hormone hyporesponsiveness

X-linked Severe Combined Immunodeficiency (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 (γ_c), 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).

In this phase of research we described a patient affected with a X-linked SCID who received a BMT late at 5.2 years of age and therefore his short stature became evident. This patient showed a immunodeficiency due to a T-cell activation defect associated with peripheral GH insensitivity and abnormal haematopoiesis. The pathogenic mechanism underlying this complex phenotype involves signal transduction through both TCR and GHR. These receptors, as many other receptors, trasduce signals through a cascade of biochemical events involving phosphorylation/dephosphorylation processes (Klausener et al 1991). Even though a few signaling molecules are tissue specific, most of the signaling pathway are not restrict to a certain cell type. In this study we focuses our attention on the JAK2/STAT5 molecules involved in signaling trough several cytokine receptor even included GHR (Argetsinger et al 1996, Winston et al 1995). In this patient, the analysis of signal transduction revealed a defective

pattern of protein tyrosine phosporilation after TCR and GHR engagement. In particular, CD3 X-L failed to increase phosphorilation of protein of 42-44, 56-60,70 and 85 kD usually phosphorilated upon this activation (Del Giudice et al 2000). Similarly, after the GH stimulation there was no increase in the signal regarding the 2 protein of 105 and 119 kD generally phosphorilated upon GH stimulation, corresponding to JAK2 and STAT5 molecules.

Experimental evidence and knock out mice indicate that alteration of STAT5A and 5B result in an impaired response to a number of cytokines, including GM-CSF, IL-2, IL-3, IL-5, GH and erythropoietin (Teglund et al 1998, Socolovsky et al 1999). In particular, 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). However, in this 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.

Even though, to our knowledge, an extrahematopoietic role of γ_c 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), our data suggest the hypotesis that in this patient the γ

chain defect is the common underlying pathogenic mechanism for both endocrinological and immunological problems.

These results has been published on the Clinical and Experimental Immunology, see below for the manuscript.

Atypical X-linked SCID phenotype associated with growth hormone hyporesponsiveness

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SUMMARY

Severe combined immunodeficiency (SCID) is a heterogeneous group of disorders characterized by
defect of T- and B-cell immunity. In many cases of autosomal recessive SCID, thus far described, the
molecular alteration involves genes encoding for molecules that participate in the signal transduction.
We report on a patient affected by a combined immunodeficiency, characterized by severe T-cell func-
tional impairment, in spite of a close to normal number of circulating mature type T and B cells. NK
cells were absent. Associated with the immunodeficiency, this patient also showed short stature char-
acterized by very low growth velocity, delayed bone age and absence of increase of the plasma levels
of Insulin growth factor-I (IGF-I) after growth hormone (GH) *in vivo* stimulation indicating periph-
eral hyporesponsiveness to GH. Evaluation of the protein tyrosine phosphorylation events occurring
following either T-cell receptor (TCR) or GH receptor (GHR) triggering revealed striking abnormal-
ities. No molecular alteration of GHR gene was found, thus suggesting the presence of postrecepto-
ial blockage. Mutational screening and expression analysis failed to reveal any molecular alteration of
JAK2 and STAT 5 A/B genes thus ruling out the involvement of these genes in the pathogenesis of this
form of SCID. Mutational analysis of IL2Rγ chain gene revealed the presence of a L183S missense
mutation, thus indicating an atypical and a more complex clinical presentation of this X-linked form of
SCID. At our knowledge, this is the first report on the GH hyporesponsiveness in this disease.

Keywords Severe combined immunodeficiency janus kinase 2 signal transducer and activators of
transcription 5 idiopathic short stature

INTRODUCTION

Severe combined immunodeficiency (SCID) comprises a group of
genotipically and phenotipically heterogeneous hereditary dis-
eases characterized by profound deficiency of both T- and B- cell
immunity [1]. SCID phenotypes have been tentatively classified
according to the presence or absence of B cells in the peripheral
blood in T⁻B⁺ or T⁻B⁻ forms of SCID [2]. Of note, all the mol-
ecular alterations causing a severe combined immunodeficiency
phenotype thus far described involve genes encoding for mol-
ecules that participate in the signal transduction process [3]. This
is the case of the γchain, a common element of several receptors,
and the Janus kinase 3 (JAK3) molecule that associates with γ
element, whose alteration results in a T⁻B⁺ SCID and absence of
natural killer (NK) cells [4,5]. Mutations of the Zap-70 kinase
that associates with the ζ element of the T-cell receptor (TCR)/
CD3 complex [6,7], or decreased expression of the p56lck tyro-

sine kinase due to an alteration of the splicing pattern result in
SCIDs characterized by a selective decrease of CD8⁺ or CD4⁺ T
cells [8], respectively. Both of these forms are associated with
normal NK cell number. Even though further molecular mecha-
nisms have been recently elucidated, which involve molecules as
Rag-1, Rag-2, CD-45, IL-7Rα, CD3ε, CD3γ, in about 30% of the
cases of SCID the underlying genetic defect is still unidentified
[2,3,9].

Here, we report on a SCID patient whose immunodeficiency
is predominantly a T-cell activation defect, and is part of a multi-
systemic disorder being associated with peripheral GH insensi-
tivity and abnormal haematopoiesis. The pathogenic mechanism
underlying this complex phenotype involves signal transduction
through both TCR and GHR. These receptors, as many other
receptors, transduce signals through a cascade of biochemical
events involving phosphorylation/dephosphorylation processes
[10]. Even though a few signalling molecules are tissue specific,
most of the signalling pathway are not restricted to a certain cell
type. We first focused our attention on the JAK2/STAT5 mol-
ecules involved in signalling through several cytokine receptors
even included GHR [11,12]. JAK2 is activated following receptor

stimulation with several factors, as erythropoietin, GH, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin- (IL-)3, IL-5, IL-6, IL-12 [13,14]. It is also required for a proper interferon (INF)-γ response [15,16]. In turn, activated JAK2 phosphorylates the STAT5 thus resulting in the activation of transcription of target genes. In particular, experimental evidence and knock out mice indicate that alterations of STAT5A and 5B result in an impaired response to a number of cytokines, including GM-CSF, IL-2, IL-3, IL-5, GH, and erythropoietin (Epo) [17,18]. However, molecular studies led us to rule out alteration of GHR, JAK2 and STAT5A/B encoding genes. Remarkably, sequence analysis of the IL-2Rγchain gene revealed a L183S missense mutation, thus indicating that also this form of SCID may exhibit a more colmplex and atypical clinical presentation.

SUBJECT AND METHODS

Case report

The patient DG was born at 40 weeks of gestation to unrelated healthy parents. His birth weight was 2850 g and height was 48 cm. No morphological anomalies were appreciable. At the age of 4 months, the patient presented with severe respiratory distress due to an interstitial pneumopathy. A Pneumocystis carinii infection was diagnosed and, due to the persistence of the infectious problem, an extensive immulogical evaluation was carried out leading to a diagnosis of SCID as below described in detail. Humoral immunity studies revealed very low IgG (195 mg/dl) and IgA (9 mg/dl) serum levels at 12 months of age. IgM were I44 mg/dl. Allohemagglutinins were undetectable. A severe hyporegenerative anaemia requiring blood transfusion represented a further clinical problem until the age of 10 months. Moreover, since the age of 1 years a short stature not explained on the basis of endocrinological, nutritional or infectious causes was present as below described. Short limb dwarfism was ruled out by the measurement of body proportions which were normal. Prophylaxis against bacterial, fungal and viral infections was successfully established while the patient, at the time of the study, was pending a bone marrow transplantation.

IGF-I generation test

To assess the ability to respond to exogenous GH, DG patient underwent an IGF-I generation test through daily subcutaneous injections of recombinant GH (0·1 IU/kg) for 4 consecutive days. Blood samples were taken before the first injection and on day 5 for the measurements of IGF-I levels. IGF-I was measured by using a two-site immunoradiometric assay (IRMA) kit (Diagnostic Systems Laboratories, Webster, Texas). Values were expressed as Standard International Units (µg/l).

Phenotypic analysis and proliferative assays

The expression of surface-membrane antigens on peripheral blood mononuclear cells (PBMC) was examined by flow cytometry (Becton Dickinson, San Jose, CA, USA) using the following monoclonal antibodies: anti-CD3 (Leu-4), anti-CD4 (Leu-3a), anti-CD8 (Leu-2a), anti-CD19 (Leu-12), anti-CD56 (Leu-19). The activation marker HLA-DR, CD25 (IL-2 receptor α chain), and CD71 (transferrin receptor), were evaluated on resting or phytohemagglutinin- (PHA)-stimulated PBMC in two-colour immunofluorescence using anti-CD3 and the corresponding

monoclonal antibody from the same source (BD). The proliferative response was evaluated by [³H]thymidine (Amersham International, Buckinghamshire, UK) incorporation in PBMC cultured at a concentration of 2×10^5 cells per well for 72 h with PHA (8 µg/ml), concanavalin A (ConA; 8 µg/ml) (Difco Laboratories, Detroit, MI, USA), 20 ng/ml phorbol-myristate acetate (PMA) and ionomicin (0·5 mM). CD3 cross-linking (CD3 X-L) was performed by precoating tissue colture plates with anti-CD3 monoclonal antibody at optimal or suboptimal concentrations (10 and 1 ng/ml) (Ortho Diagnostics, Raritan, NJ, USA).

GH and TCR induced protein tyrosine phosphorylation

Transducing properties were studied through the analysis of protein tyrosine phosphorylation in PBMC after stimulation with GH, PMA or CD3 X-L. GH stimulation (500 ng/ml) was performed through 5–15 min stimulation. The pattern of tyrosine phosphorylated (Ptyr) proteins was analysed on whole cell
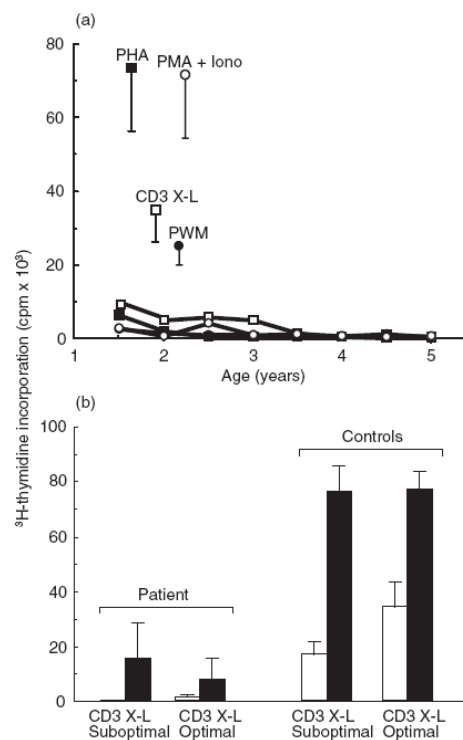

Fig. 1. Proliferative responses in the SCID patient during the 5 years follow-up. Proliferative response evaluated through[³H]-thymidine incorporation following stimulation of PBMC by PHA, PMA + Iono, PWM, and CD3 cross-linking (a). Proliferation was analysed at regular time during the 5 years follow-up. The results are expressed as cpm. Bars indicate mean value + SD of control values. (b) Proliferative response following optimal or suboptimal stimulation through CD3 cross-linking in the presence (■) or absence (□) of exogenous rIL-2 used at a concentration of 100 U/ml.

lysates. After stimulation, 3×10^6 cells were lysed in a buffer containing 20 mM Tris, pH 8, 10% glycerol, 137 mM NaCl, 1% Nonidet P-40, 10 mmol EDTA, 1 mM phenyl methane sulphonyl fluoride (PMSF), 1 mM sodium orthovanadatum (Na_3Vo_4), 5 μg/ml leupeptin and 5 μg/ml aprotinin. Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then blocked with 3% bovine serum albumin. Immunoblotting was performed by a 1–4 h incubation with anti-Ptyr. Densitometric analysis of the signals was performed.

GH receptor, STAT5A and B, JAK2 and γ chain mutational analysis

Genomic DNA and RNA were isolated from peripheral blood leucocytes [19]. Automated sequence analysis of exons 2–10 of GHR (X06562·1) was performed in DG patient. The coding portion of exon 10 was amplified in three overlapping fragments [20]. STAT5A and STAT5B mutational analysis was performed using primers designed on published sequence for a total of 18 primer sets for each gene. Each exon and corresponding donor and acceptor splice site was individually amplified by PCR. PCR conditions were as follows 35 cycles of 95°C for 30 s, 52°C for 45 s, 72°C for 1 min JAK2 cDNA was obtained by reverse transcription (RT) using 1 μg purified RNA and random examers in a buffer supplied by the manufacturer (Boehringer Roche, Mannheim, Germany). Twelve sets of primers were synthesized to amplify and sequence human JAK2 cDNA (AF005216·1) both coding and noncoding strands.

STAT5A and B cDNAs (NM012448·1 and NM003152·1, respectively) were amplified using RT-PCR performed with primers designed to amplify both cDNAs (position 1636 STAT5B/ 2130 STAT5A CCGTGCCTGACAAAGTGCTG and position 2284 STAT5B/2763 STAT5A TTCACAAACTCAGGGAC CAC). One μl of [^{32}P]-dCTP was included in the reaction. Amplified products were subsequently *Hin*f1 digested and separated by electrophoresis on 5% polyacrilamide gel. Densitometric analysis of the signals was performed.

IL-2Rγ chain mutational analysis was performed using primers designed on published sequence (L19546) for a total of 8 primer sets [21]. Each exon and corresponding donor and acceptor splice site was individually amplified by PCR. Amplified products were automatically sequenced. ABI Prism dye terminator cycle sequencing kit and an ABI 377 Automated DNA Sequencer were used.

Sequence of primers used in molecular analysis of STAT 5 A/B and γ chain are available upon request (ursini@iigbna.iigb. na.cnr.it).

RESULTS

Cell proliferation and activation markers

The proliferative response was consistently reduced during the 5 years follow-up as depicted in Fig. 1a. The addition of either suboptimal or optimal rIL-2 to the cultures of patient's PBMC stimulated through CD3 triggering, as shown in Fig. 1b, was unable to restore the proliferation. The functional impairment was associated with the presence of some circulating mature CD3+ T cells, ranging during the follow up between 23 and 85%. Table 1 shows the representative percentage values at 2 and 5 years of age indicating a spontaneous increase of CD3$^+$ cells bearing TCR α/β chains. CD4$^+$ cells were persistently reduced during the follow-up ranging from 13 and 16%, while CD8$^+$ cells

ranged between 10 and 48%. TCR γ/δ^+ cells increased over the time. However, most of these cells were CD4 and CD8 double negative. CD19$^+$ B cells were present and inversely correlated to the number of T cells, ranging between 70 and 4%. NK cells were always absent.

The evaluation of the activation markers HLA-DR, α chain of IL-2R (CD25) and transferrin receptor (CD71) on patient's T cells confirmed on a per cell basis that there was a cell activation defect, as illustrated in Table 2. PHA stimulation for 48 h of the patient's PBMC failed to induce up-regulation of the membrane expression of all 3 activation markers tested, differently from
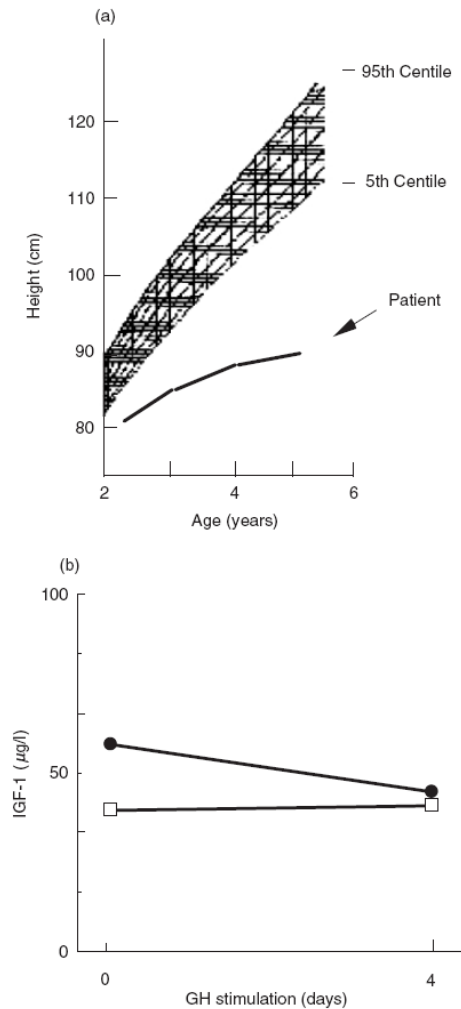

Fig. 2. (a) Growth curve over the 5 years follow-up of the patient DG compared to sex-matched percentile. (b) IGF-I response following *in vivo* generation test. GH, at a dosage of (□) 0·10 and (○) 0·16 U/kg/day, was administered for 4-days in DG. Values of IGF-I are expressed as μg/l.

Table 1. Patient's immunophenotype

	Age			
	2 years old		5 years old	
	Patient*	Control†	Patient*	Control†
Total lymphocyte count	915	3600	919	3600
Surface markers				
CD3+	23.8 (218)	64 (22232–2484)	85.3 (784)	64 (2232–2484)
CD3+TCR $\alpha\beta$	14.1 (129)	66 (1980–2592)	49.4 (459)	66 (1980–2592)
CD4+	13.4 (123)	37 (1080–1440)	16.0 (147)	37 (1080–1440)
CD8+	10.3 (94)	29 (900–1152)	48.5 (451)	29 (900–1152)
CD4-CD8-TCR $\gamma\delta$	1.0 (9.2)	abs	17.3 (158)	abs
CD19+	70.9 (652)	24 (756–1008)	4.3 (39)	24 (756–1008)
CD3+DR+	12.8 (117)	9 (216–576)	65.9 (604)	9 (216–576)
CD56+	0.6 (5)	11 (288–540)	0.1 (0.9)	11 (288–540)

Values given as *% (cells/mm³) and †% (25th–75th centile).
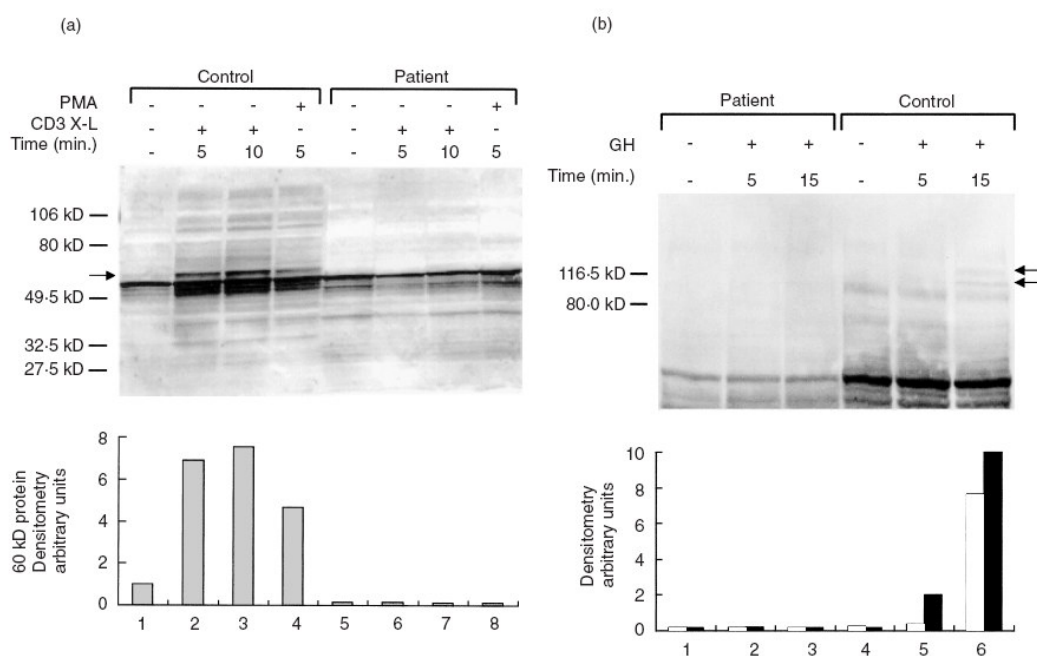

Fig. 3. Protein tyrosine phosphorylation induced by CD3 cross-linking or GH stimulation. Representative experiments showing the pattern of tyrosil phosphorylated proteins after stimulation of PBMC from DG and a control. (a) TCR triggering for 5 (lanes 2 and 6) or 10 min (lanes 3 and 7) or PMA stimulation (lanes 4 and 8) in the control's PBMC (lanes 1–4) and in the patient (lanes 5–8). Molecular markers are indicated. The lower panel illustrates the densitometric analysis of the 60 kD protein, which represents the major phosphorylated protein after TCR triggering. (b) GH stimulation for 5 or 15 min of the patient's PBMC (lanes 1–3) and of the control (lanes 4–6). The lower panel illustrated the densitometric analysis of the (■) 105 and (□) 119 kD proteins. In the patient, an abnormal pattern of protein tyrosine phosphorylation was observed following both stimulations. Western blots were performed using whole cell lysates and an antiphosphotyrosine antibody.

Table 2. T-cell activation markers in the patient DG

| | Positive cells (%) | | | |
| | Patient | | Control | |
Surface markers	T0	T48	T0	T48
HLA-DR	12·8	0·3	7·8 + 0·6	20·8 ± 7·5
CD25	0·3	0·2	0·9 + 1·0	21·7 ± 10·0
CD71	0·2	0·3	0·5 + 0·5	6·5 ± 4·4

Values are given as %. Control values indicate mean ± SD.

controls, whose cells exhibited a 3–20 fold increase of the surface expression.

Growth rate, peripheral sensitivity to GH and GHR analysis
The patient DG showed a clearly abnormal growth curve, being under the 5th centile for height, as shown in the Fig. 2a. Bone age was always markedly delayed as compared to the chronological age. The more common causes of short stature, including malnutrition, chronic diseases, and endocrinological disorders were ruled out by standard diagnostic procedures. Due to the efficacy of the anti-infectious prophylaxis, short stature could not be explained by chronic infections. In 2 out of 3 pharmacological stimulation tests, GH increase was normal, thus leading to a descriptive diagnosis of ISS.

Insulin-like growth factor-I (IGF-I) is a hepatically derived circulating mediator of growth hormone, which is regulated by GH, and mediates many of the GH biological functions, being also involved in the maintenance of lymphoid mass and functions [11]. Therefore, we sought to investigate the peripheral sensitivity to GH by examining the increase of IGF-I plasma levels following *in vivo* provocation test. As reported in Fig. 2b, peripheral hyporesponsiveness to GH was demonstrated *in vivo* on the basis of the low baseline IGF-*I*-values and the absence of plasma IGF-I increase during GH treatment performed at 2 different concentrations. An IGF-I increase below 15 μg/l during the generation test was considered a diagnostic criteria for GH insensitivity [22].

To rule out a genetic alteration of the GHR gene in the patient, a GHR sequence analysis of the exons 2–10 was performed. All 11 amplified products have sequence identical to the wild type, thus ruling out a GHR alteration. The patient was heterozygote for the already described G168 and I526L polymorphisms [23].

Defective pattern of protein tyrosine phosphorylation after TCR and GHR engagement
We next investigated the overall signal transduction properties following TCR and GHR ligation by analysing the number and the timing of the proteins phosphorylated on tyrosine residues. Figure 3a,b illustrate immunoblots with anti-Ptyr of whole cell lysates from patient's or control's PBMC following stimulation through CD3 X-L, PMA or GH. In contrast to what observed in control cells, both stimuli failed to induce substantial increase of tyrosil phosphorylated proteins in patient cells. In particular, CD3 X-L failed to increase phosphorylation of proteins of 42–44, 56–60, 70 and 85 kD usually phosphorylated upon this activation
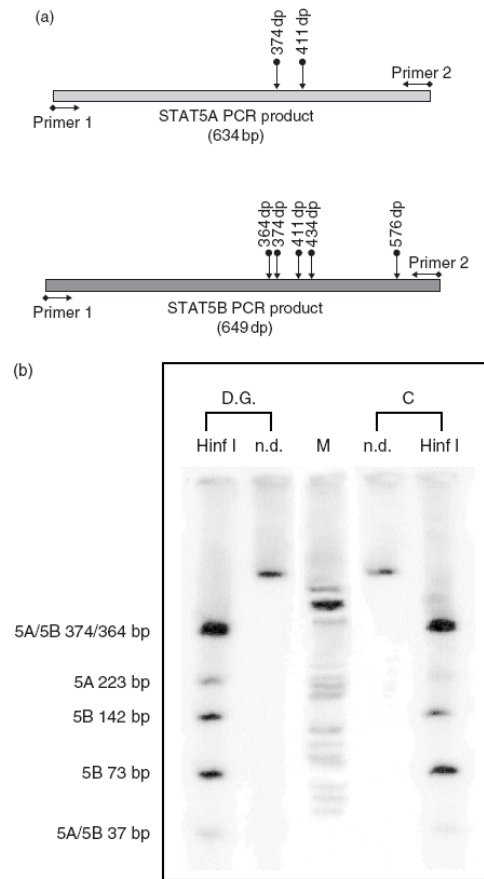

Fig. 4. Evaluation of RNA expression of STAT5A and B in the patient and a control. (a) Schematic representation of amplified products from STAT5A and B cDNA. Arrows indicate *Hin*fI restriction sites. Primers were designed on cDNA conserved regions sequence of both STAT5A and B. (b) *Hin*fI restriction pattern of patient and control STAT5A and B amplified products. Fragments were separated by 5% polyacrilamide gel electrophoresis. (C, control; D.G., proband; n.d., not digested; M, marker). Size of the fragments are indicated.

[24]. Similarly, after the GH stimulation there was no increase in the signal regarding the 2 proteins of 105 and 119 kD generally phosphorylated upon GH stimulation of PBMC, presumably corresponding to JAK2 and STAT5 molecules.

Molecular analysis of JAK2, STAT 5 A/B and IL-2Rγ chain molecules
By taking advantage of knock out mice knowledge, we focused our attention on JAK2/STAT5 signalling pathway involved in several cytokine receptors including GHR [15,16]. Since the DG PBMC contain JAK2 mRNA (data not shown), we used cDNA

produced by reverse RT-PCR and JAK2 specific oligodeoxynucleotide primers to scan the entire gene. All 12 amplified products have sequence identical to the wild type. DNA fragments appeared quantitatively and qualitatively indistinguishable from those observed in several unrelated controls.

Recently, the human genomic sequence of STAT5A and STAT5B was solved by some of us thus allowing a complete mutational analysis of each individual gene (unpublished observation). However, we found that all 18 amplified products corresponding to coding exons of each STAT gene have sequence identical to the wild type (data not shown). In addition, we analysed the expression and the relative abundance of both STAT5A and STAT5B mRNAs in the patient PBMC by RT-PCR performed with primers able to amplify both STAT 5 A/B cDNAs. Amplified products were subsequently *Hin*f1 digested and separated by electrophoresis to distinguish fragments of each gene; therefore, this analysis also reflects the STAT 5 A/5B specific mRNA ratio (Fig. 4a). Reference pattern was obtained from age-matched control. We observed that the two mRNA-derived amplified products were qualitatively and quantitatively indistinguishable from those obtained from control PBMC mRNA (Fig. 4b).

Ultimately, a mutational analysis of the 8 encoding exons of the γ chain gene was perfomed and revealed the presence of a single base pair change T548 > C (numbered from the ATG) resulting in a change in coding from leucine to serine at residue 183 in the region coding for the extracellular domain of the protein (Fig. 5). We also found that the mother and the sister of the proband are heterozigous for this mutation. Amplification of γ chain specific cDNA appeared quantitatively and qualitatively indistinguishable from those observed in several unrelated controls.

DISCUSSION

We reported here on an atypical form of SCID in a patient presenting with severe hyporegenerative anaemia, and severe functional impairment of apparently mature T cells, as shown by the absence of proliferative response to a variety of mitogens and up-regulation of activation markers. The number of B cells was normal, but they were non functional, whereas NK cells were undetectable. The patient was also affected by idiopathic short stature with a reduced serum levels of IGF-1 and peripheral insensitivity to GH. This last defect has frequently been associated with GHR mutation [20,23,25]. However, in DG we were unable to find any mutation in GHR gene. Of note, an abnormal pattern of protein tyrosine phosphorylation was found in DG
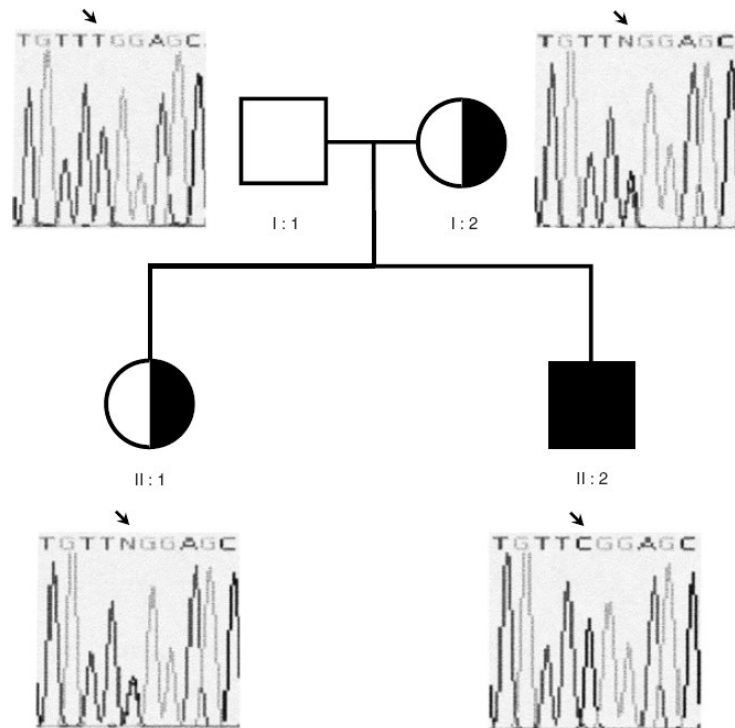

Fig. 5. Pedigree of the family and sequence analysis of the IL-2Rγ chain in family members. The proband is indicated by the arrow. The sequence analysis revealed the presence of T548C (causing the L183S missense mutation) at the hemizygous state in the proband and at the heterozigous state in the mother and sister.

PBMC either after GH stimulation or following TCR/CD3 perturbation.

Mouse mutations have become an important genetic tool for the identification of new clinical phenotypes that may help unravel complex human disorders and identify new pathogenic gene alterations [26]. The STAT5A/B mutant mice, but not the individual mutants, have a profound T-cell functional defect. In addition to prolactin, STAT5 proteins are activated by GH, Epo, GM-CSF and IL-2 [27,28]. GHR belongs to cytokine/haematopoietin receptor super family and shares with other members of this family several signal transducers even included JAK2, which, in turn, phosphorylates STAT5 [11,12]. STAT5 heterodimers are phosphorylated in response to TCR stimulation, thus indicating their involvement in T-cell proliferation [29]. Even though the JAK2 knockout in mice is embrionically lethal, this gene plays an essential non redundant role in the function of a number of cytokine receptors including Epo, GM-CSF, IL-5 and IL-3, and presumably of GHR as well [15,16]. Therefore, we first focused our attention on the JAK2/STAT5 signalling pathway. However, our molecular studies led us to rule out a direct involvement of these molecules in the pathogenesis of the disease.

The absence of NK cells is also present in SCID related to mutation of γ chain or Jak3 genes. In these forms of SCID, generally there are no T cells. These forms are generally fatal within the first years of life, but a few atypical cases are characterized by a milder phenotype [30–32]. In our patient, we documented the presence of a L183S hemyzigous mutation of the γ chain. This alteration modifies the extracellular domain of the protein, as also described for other several mutations associated with a typical phenotype of this form of SCID [21,32].

In conclusion, our finding indicates that the immunodeficiency here described is an X-linked form of SCID due to an activation deficiency associated with peripheral insensitivity to GH, resulting in a profound growth failure. We here documented that *in vitro* triggering of both TCR/CD3 complex and GHR was associated with an abnormal pattern of protein tyrosine phosphorylation events, raising the intriguing question on the mechanism by which the γ-chain defect is the common underlying pathogenic mechanism for both endocrinological and immunological problems. Of note, mutational analysis of a few additional candidate genes functionally related to short stature and T-cell activation failed to reveal any further alteration that could explain the atypical and complex phenotype. This observation is in keeping with what reported on other genetic diseases, showing that patients with an identical mutation may have different clinical and immunological features. To address the issue of the role of interfering genes in modifying the clinical expression of a genetic disease new developing technologies (e.g. biochip) are now available. We believe that unusual clinical and laboratoristic observations may be very useful to unravel complex diseases and help find novel gene-function relationship toward targeted therapeutic approaches.

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

Since, in the previously research has been hypothesized a role for the γc chain in GHR signaling, aim of this study has been evaluated, for the first time, 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.

The results showed that the proliferative response to GH stimulation of the B cell lines of γ_c-negative patients was impaired despite a comparable cellular expression of GHR molecules to controls. The functional interaction between γ_c and GHR leads to the activation and intranuclear translocation of STAT5b protein. The results showed that in patients, after GH stimulation, no phasphorylation of STAT5 was observed. In addition, the molecule localization through confocal microscopy revealed that in B cell lines of patients no nuclear traslocation of STAT5b following GH stimulation occurred differently from controls. Biochemical analysis of the nuclear extracts of γ_c-negative cell lines provided further evidence that the amount of STAT5b and its phosphorylated form did not increase following GH stimulation. In patients, cells reconstituted with wilde type γ_c abnormal biochemical and functional events were restored

resulting in nuclear traslocation of STAT5. Finally, confocal experiments revealed that GHR and γ_c were colocalized on the cell membrane.

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 published on Journal of Immunology, see belw for the manuscript.

Functional Interaction of Common γ-Chain and Growth Hormone Receptor Signaling Apparatus[1]

Marsilio Adriani,* Corrado Garbi,[†] Giada Amodio,* Ilaria Russo,* Marica Giovannini,* Stefania Amorosi,* Eliana Matrecano,* Elena Cosentini,[‡] Fabio Candotti,[§] and Claudio Pignata[2]*

We previously reported on an X-linked SCID (X-SCID) patient, who also had peripheral growth hormone (GH) hyporesponsiveness 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 (γ_c) in GHR signaling using EBV-transformed lymphocytes from healthy subjects and γ_c-negative X-SCID patients. We demonstrated that the proliferative response to GH stimulation of the B cell lines of γ_c-negative patients 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 B cell lines of patients no nuclear translocation of STAT5b following GH stimulation occurred differently from controls. Biochemical analysis of the nuclear extracts of γ_c-negative cell lines provided further evidence that the amount of STAT5b and its phosphorylated form did not increase following GH stimulation. In patients, cells reconstituted with wild-type γ_c abnormal biochemical and functional events were restored resulting in nuclear translocation of STAT5. Confocal experiments revealed that GHR and γ_c were colocalized on the cell membrane. Our study demonstrates the existence of a previously unappreciated relationship between GHR-signaling pathway and γ_c, 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. *The Journal of Immunology*, 2006, 177: 6889–6895.

S evere combined immunodeficiencies represent a wide spectrum of illnesses, which differ in either the qualitative or quantitative alterations of T, B, and NK cell (1). Most forms of SCID are associated with molecular alterations of genes selectively expressed in hemopoietic cells and implicated in the cell differentiation/activation process. Thus, classical symptoms are generally considered those related to the immunological impairment that results in increased susceptibility to infections. Because 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)[3] 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 (γ_c), 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 extrahemopoietic role of γ_c has not yet been demonstrated, although the abundance of the protein in nonhemopoietic cells would imply additional functions for this element (4, 5).

We previously reported on a patient affected with X-SCID who received a bone marrow transplantation 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 was demonstrated.

The GHR was the first identified member of the cytokine receptor class 1 superfamily, which includes receptors for erythropoietin, G-CSF, GM-CSF, 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 JAK2 represents the predominant nonreceptor 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 STATs 1, 3, and 5, ERK 1 and 2, and PI3K-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 (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).

Because we hypothesized a role for the γ_c in GHR signaling, in this study, we evaluate the functional interaction between GHR

and the common γ element in either freshly isolated or 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 γ_c and GHR. This interaction leads to the activation and intranuclear translocation of the STAT5b protein.

Materials and Methods

Reagents

Recombinant human GH (rGH) was obtained from Serono (Saizer 4). The ECL kit was purchased from Amersham Biosciences. The Abs anti-STAT5b, anti-STAT5a, anti-STAT1, anti-STAT3, anti-ERK (recognizing both ERK1 and ERK2), anti-phosphotyrosine ERK, anti-GHR and anti-γ_c and the mAbs anti-phosphotyrosine were purchased from Santa Cruz Biotechnology. The Ab anti-JAK2 was purchased from Cell Signaling Technology. The neutralizing IgG1 anti-γ_c mAb was purchased from R&D Systems. An IgG1 isotype-matched anti-CD3 mAb (Leu 3, UCHT1 clone) was purchased from BD Biosciences. Epidermal growth factor was purchased from BD Biosciences and used at the concentration of 100 ng/ml. Acrylamide and bisacrylamide were obtained from Invitrogen Life Technologies. Prestained molecular mass standards were obtained from Bio-Rad. Except where noted, other reagents were of either reagent or molecular biological grade from Sigma-Aldrich.

Cells and cell cultures

Mononuclear cells (PBMC) were obtained from four X-SCID patients and normal donors and heparinized peripheral blood by Ficoll-Hypaque (Biochrom) density gradient centrifugation. Upon informed consent, lymphoblastoid cell lines (BCLs) were generated by EBV immortalization of patients and control PBMC using standard procedures (15). In all cases, γ_c mutations led to the absence of protein expression. Cells were maintained in RPMI 1640 (Biochrom) supplemented with 10% FBS (Invitrogen Life Technologies), 2 mM/L L-glutamine (Invitrogen Life Technologies), and 50 μg/ml gentamicin (Invitrogen Life Technologies), and cultured at 37°C, 5% CO_2. In BCL transduction experiments, the pGC2Rγ retroviral vector (16) was used to transduce X-SCID BCLs with wild-type (WT) γ_c as previously described (17). Transduced cells were selected in the neomycin-analog G418 (Cellgro). NIH 3T3 fibroblasts were used in a few experiments.

Proliferative assay

BCLs (1 × 10⁵ cell/200 μl well) were cultured triplicate in 96-well U-bottom microtiter plates (Falcon; BD Biosciences) 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 of [³H]thymidine (Amersham Biosciences) 8 h before harvesting (18). In neutralization experiments, control EBV cells were preincubated with the neutralizing mAb 284 at the concentration of 6 ng/ml for 3 h or with the IgG1 isotype-matched Ab (Leu 3).

Flow cytometry

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

Cell stimulation and protein extraction

Before 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 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 mM EDTA, 1 mM PMSF, 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. (19) and were subsequently mixed with sample buffer.

Western blot

Immunoblotting using phosphotyrosine mAb was performed as previously reported (14). Immunoblotting using specific Ab was performed according to the vendor 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% BSA in wash buffer (10 mM Tris (pH 7.5), 100 mM NaCl, and 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 Ab. The membrane was then washed three times and an appropriate IgG HRP-conjugated secondary Ab 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 four rinses of 5 min in PBS, the cells were centrifuged in a Shandon Cytospin III (Histotronix) onto a 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 Abs against STAT5b diluted 1/100 in PBS containing 1% BSA. After four washings for 5 min in PBS, the cells were incubated for 1 h at room temperature with a 1/200 dilution of FITC-conjugated donkey anti-rabbit IgG (Pierce) 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 γ_c-negative X-SCID patients

It has been reported that GH enhances EBV-transformed cell line proliferation in vitro, its effect being direct and not mediated by IGF-I (18). Thus, to evaluate a biological role of γ_c in GHR signaling, we evaluated the response of EBV-transformed lymphocytes (BCLs) from γ_c-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 [³H]thymidine uptake was observed at a GH concentration of 50 ng/ml, and the maximal effect was achieved at 200 ng/ml. In contrast, γ_c-negative BCLs did not 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 of the cells of controls and patients (Fig. 1B). No difference was found in the mean fluorescence intensity (130.99 ± 28.19 vs 139.88 ± 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).

Moreover, to demonstrate a link between the γ_c and GHR, we used a neutralizing mAb in the proliferative assay. As shown in the Fig. 1C, the neutralizing mAb inhibited by 64% the proliferative response to GH. A nonspecific effect of the Ab was ruled out, because the IgG1 anti-CD3 isotype-matched Ab was ineffective in inhibiting cell proliferation.

To define whether PI3K had a role on GH-induced cell proliferation of BCLs to GH, the kinase inhibitor wortmannin was used. As shown in Fig. 1D, no inhibitory effect was appreciable. By contrast, in the positive control wortmannin was able to inhibit fibroblast proliferation to EGF by 85%.

To ascertain whether γ_c was linked to GHR, we then assessed by confocal microscopy the plasma membrane expression of these two molecules. As shown in Fig. 1D, by indirect immunofluorescence using specific Abs, as previously detailed, colocalization of γ_c and GHR was observed on the cell surface of normal BCL cells.

FIGURE 1. In vitro effect of GH stimulation on proliferation of the EBV cell lines of X-SCID patients and controls and membrane localization of GHR and γ_c. *A*, BCLs were generated by EBV immortalization of the PBMC of patients and controls 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 indicate 1 SD. *B*, Flow cytometry analysis indicating that the expression levels of GHR are comparable in controls and patients. *C*, Control BCLs were pretreated with medium alone (■) or with the neutralizing mAb 284 (□) at the concentration of 6 ng/ml for 3 h, and then cultured for 4 days in the presence of GH at the concentration of 200 ng/ml. As isotype-matched IgG1 control Ab, anti-CD3 Leu 3 was used. Cultures were processed as previously described. *D*, Control BCLs were pretreated for 1 h with medium alone or with wortmannin at the concentration of 100 nM, and then stimulated with 50, 100, 200, or 400 ng/ml GH, as indicated. As a positive control, fibroblasts were cultured in the presence of EGF. *E*, γ-chain colocalizes with GHR. Normal BCL cells were double labeled with anti-γ_c (*left*) and anti-GHR (*center*) Abs. Confocal microscopic analysis indicates a plasma membrane localization for both molecules. The yellow color in the merge (shown on the *right*) indicates areas of colocalization of the two proteins.
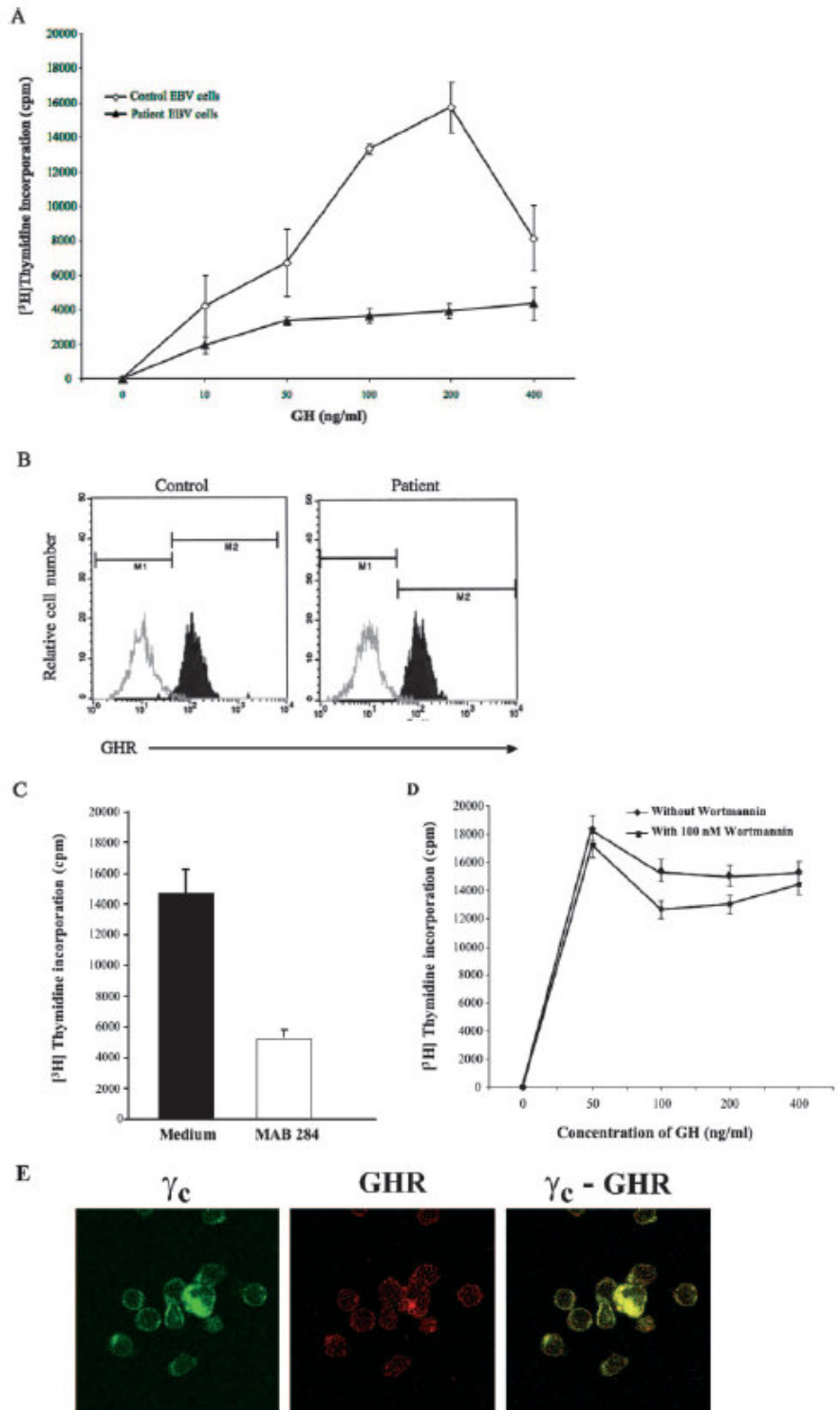
Pattern of protein tyrosine phosphorylation induced through GHR engagement in patients and controls cells

We next investigated the overall signal transduction properties of patients and control BCLs following GHR ligation by analyzing the number and the timing of the proteins phosphorylated on ty-

rosine residues. Fig. 2 illustrates a representative immunoblot with anti-phosphotyrosine Abs of whole cell lysates from BCLs of patients and controls BCLs following stimulation with GH for 5, 15, or 30 min. In contrast to what was observed in control cells, in patients, GH stimulation failed to induce phosphorylation of
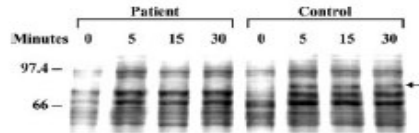
FIGURE 2. Pattern of protein tyrosine phosphorylation induced through GHR engagement. BCLs from X-SCID patients and healthy subjects were starved of serum for 8–12 h and then stimulated with GH (500 ng/ml) at 37°C for the indicated time. Stimulation was stopped with cold PBS and BCLs were resuspended in lysis buffer. After SDS-PAGE and Western blot, membranes were incubated with anti-phosphotyrosine Abs.

FIGURE 4. Phosphorylation events induced through GHR stimulation. BCLs from X-SCID patients and healthy subjects, starved of serum for 8–12 h, were stimulated for the indicated time with rGH at the concentration of 500 ng/ml. After SDS-PAGE and Western blot, membranes were incubated with (A) anti-pJAK2 or anti-JAK2, (B) anti-pSTAT1 or anti-STAT1, (C) anti-pERK or anti-ERK2, (D) anti-pSTAT3 or anti-STAT3.

proteins of ~90 kDa, presumably corresponding to STAT molecules involved in the signal transduction through GHR. This pattern of protein tyrosine phosphorylation 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 three main signal modules by which signal transduction through GHR occurs involve MAPK/ERK1 and 2, JAK2, STAT1, STAT3 and STAT5 and the PI3K-protein kinase B signaling (7).

To evaluate whether the γ_c was involved in GHR-signaling events, we first focused on STAT5 molecule. As shown in Fig. 3, in the BCLs of controls, tyrosine phosphorylation of STAT5 was evident, with a peak of activity observed between 5 and 15 min after GH stimulation. By contrast, in the BCLs of patients, 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 JAK2, STAT1, ERKs, and STAT3 phosphorylation (Fig. 4). No difference in the phosphorylation events between the BCLs of patients and controls 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 (20, 21). Because the activated STAT5 translocates into the nuclei, confocal microscopy was initially used to test the subcellular localization of STAT5b in control and patient γ_c-negative cells under resting conditions and after stimulation with GH.

BCLs of patients and controls were stimulated with GH for 30 min, fixed, and incubated with antiserum against STAT5b. As
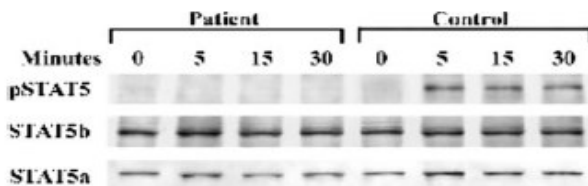
shown in Fig. 4, under basal conditions all cells displayed fluorescent staining of the cytoplasm indicating the presence of STAT5b in this compartment, and only a negligible staining of nucleus indicating absence of STAT5b in this compartment. Stimulation with GH for the time indicated induced nuclear translocation of STAT5b in the BCLs of controls, as demonstrated by the marked increase in STAT5b immunoreactivity within the nucleus and not in γ_c-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 control 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 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 (Fig. 6).

FIGURE 3. STAT5 phosphorylation induced through GHR stimulation. rGH stimulation failed to induce STAT5 tyrosine phosphorylation in γ_c-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 anti-pSTAT5, anti-STAT5b, or anti-STAT5a Abs.
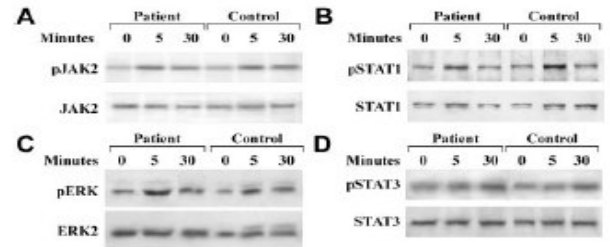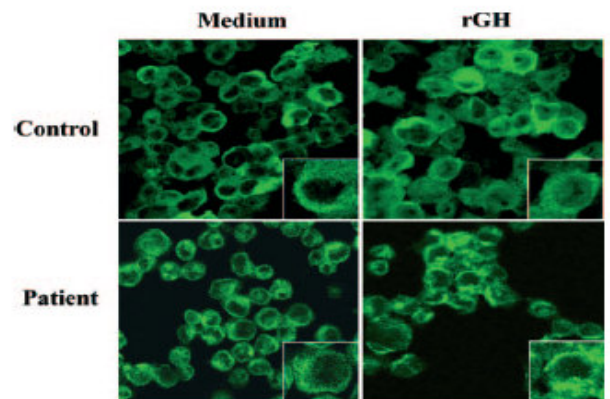

FIGURE 5. STAT5b subcellular localization. Control cells of X-SCID patients and healthy subjects were cultured in the absence or presence of 500 ng/ml rGH for 30 min at 37°C. Unstimulated or stimulated cells were analyzed by confocal microscopy for STAT5b (green) distribution in the cell, focusing particularly on whether this protein was present in the nuclei.
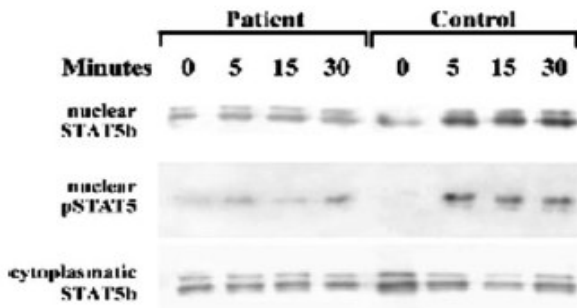
FIGURE 6. Nuclear fraction of the overall STAT5b amount and of the phosphorylated form of STAT5 in resting or rGH-stimulated BCLs. Patient and control BCLs were stimulated with rGH (500 ng/ml) or medium alone at 37°C for the indicated time. Stimulation was stopped with cold PBS and nuclei were isolated 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 Abs.

GH-induced signaling and STAT5b nuclear translocation in X-SCID EBV cells transduced with the WT γ_c gene

We next evaluated whether reconstitution of X-SCID cells with WT γ_c led to a functional recovery. As shown in Fig. 7*A*, pGC2Rγ cells expressed γ_c at a normal extent. These cells proliferated in a comparable fashion to control cells following GH stimulation (Fig. 7*B*). Moreover, in WB experiments using an anti-phospho-STAT5 Ab, a phosphorylation of the molecule was observed in pGC2Rγ cells (Fig. 7*C*). Finally, in reconstituted cells, GH stimulation induced a normal nuclear translocation of STAT5b, as shown in Fig. 7*D*.

Discussion

In the present study, we examined a potential role of the γ_c chain in GHR signaling using BCLs from healthy control subjects and γ_c-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 the BCLs of γ_c-negative patients 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 analyzing the pattern of protein tyrosine phosphorylation. In contrast to what was 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 the cell lines of γ_c-negative patients in contrast to the control cells, in which a prompt activation of STAT5 occurred. Of note, reconstitution of X-SCID cells with the WT γ_c gene corrected the functional and biochemical abnormalities resulting in an appropriate nuclear translocation of STAT5. These findings strongly support an essential role of γ_c in GHR signaling.

STAT-dependent pathways are generally believed to be used in cellular events such as cell proliferation, differentiation, and apoptosis (22, 23), 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 (24–29), even though STAT5 seems to play a prominent role in receptor signaling. Rodent models of STAT knockouts (30) and the recent identification of a patient with a homozygous missense mutation of the
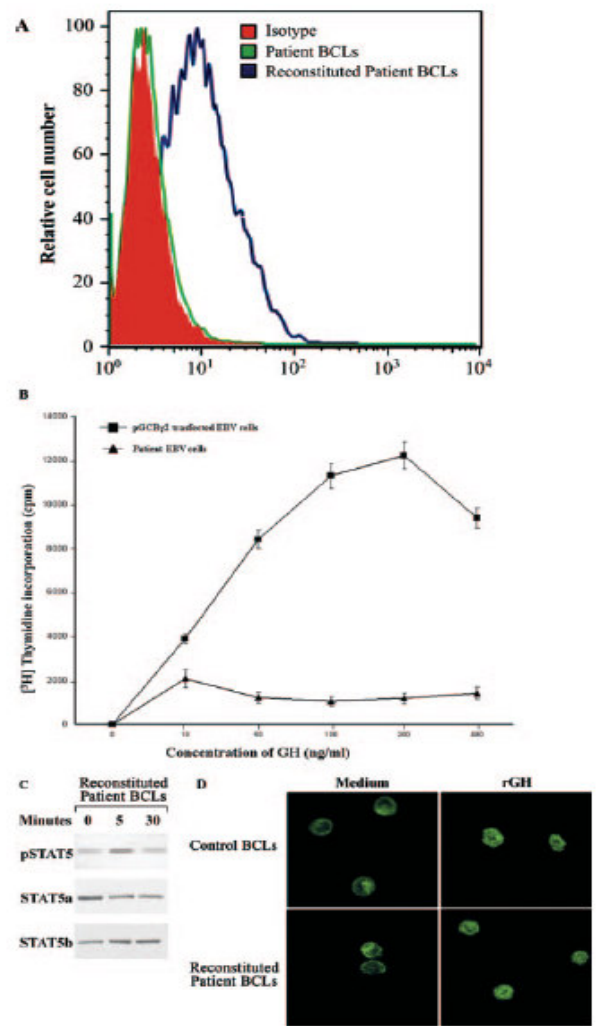

FIGURE 7. GHR signaling in patient BCLs reconstituted with WT γ_c (pGC2Rγ cells). *A*, Membrane expression of γ_c in patient or pGC2Rγ cells by flow cytometry. *B*, Proliferative response in control, patient, or reconstituted BCLs. Cells were cultured in the presence of various concentrations of GH for 4 days and pulsed with [³H]thymidine as previously described. Results are expressed as increase of cpm from the background. Vertical bars indicate 1 SD. *C*, STAT5 phosphorylation induced through GHR stimulation for the indicated time in pGC2Rγ cells. Membranes were incubated, as indicated, with anti-pSTAT5, anti-STAT5a, or anti-STAT5b Abs. *D*, STAT5b subcellular localization through confocal microscopy analysis in control or pGC2Rγ cells unstimulated or stimulated with 500 ng/ml rGH for 30 min at 37°C.

STAT5b gene indicate that STAT5b is essential for a normal postnatal linear growth (31). 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 γ_c-negative X-SCID patients.
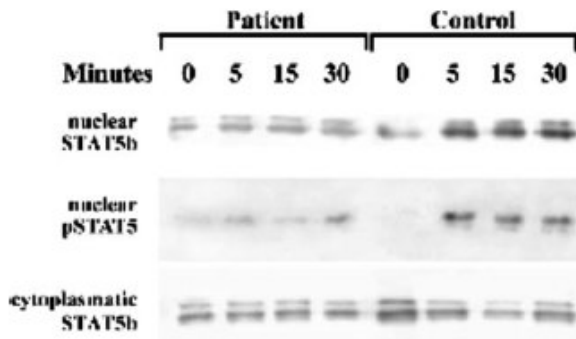
FIGURE 6. Nuclear fraction of the overall STAT5b amount and of the phosphorylated form of STAT5 in resting or rGH-stimulated BCLs. Patient and control BCLs were stimulated with rGH (500 ng/ml) or medium alone at 37°C for the indicated time. Stimulation was stopped with cold PBS and nuclei were isolated 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 Abs.

GH-induced signaling and STAT5b nuclear translocation in X-SCID EBV cells transduced with the WT γ_c gene

We next evaluated whether reconstitution of X-SCID cells with WT γ_c led to a functional recovery. As shown in Fig. 7A, pGC2Rγ cells expressed γ_c at a normal extent. These cells proliferated in a comparable fashion to control cells following GH stimulation (Fig. 7B). Moreover, in WB experiments using an anti-phospho-STAT5 Ab, a phosphorylation of the molecule was observed in pGC2Rγ cells (Fig. 7C). Finally, in reconstituted cells, GH stimulation induced a normal nuclear translocation of STAT5b, as shown in Fig. 7D.

Discussion

In the present study, we examined a potential role of the γ_c chain in GHR signaling using BCLs from healthy control subjects and γ_c-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 the BCLs of γ_c-negative patients 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 analyzing the pattern of protein tyrosine phosphorylation. In contrast to what was 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 the cell lines of γ_c-negative patients in contrast to the control cells, in which a prompt activation of STAT5 occurred. Of note, reconstitution of X-SCID cells with the WT γ_c gene corrected the functional and biochemical abnormalities resulting in an appropriate nuclear translocation of STAT5. These findings strongly support an essential role of γ_c in GHR signaling.

STAT-dependent pathways are generally believed to be used in cellular events such as cell proliferation, differentiation, and apoptosis (22, 23), 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 (24–29), even though STAT5 seems to play a prominent role in receptor signaling. Rodent models of STAT knockouts (30) and the recent identification of a patient with a homozygous missense mutation of the
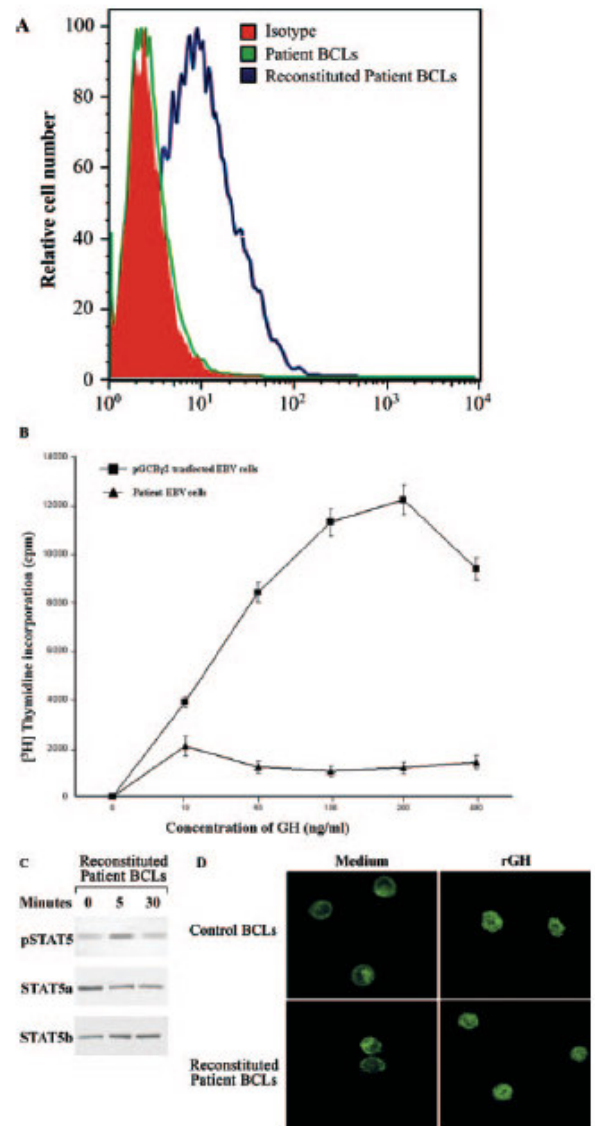

FIGURE 7. GHR signaling in patient BCLs reconstituted with WT γ_c (pGC2Rγ cells). *A*, Membrane expression of γ_c in patient or pGC2Rγ cells by flow cytometry. *B*, Proliferative response in control, patient, or reconstituted BCLs. Cells were cultured in the presence of various concentrations of GH for 4 days and pulsed with [³H]thymidine as previously described. Results are expressed as increase of cpm from the background. Vertical bars indicate 1 SD. *C*, STAT5 phosphorylation induced through GHR stimulation for the indicated time in pGC2Rγ cells. Membranes were incubated, as indicated, with anti-pSTAT5, anti-STAT5a, or anti-STAT5b Abs. *D*, STAT5b subcellular localization through confocal microscopy analysis in control or pGC2Rγ cells unstimulated or stimulated with 500 ng/ml rGH for 30 min at 37°C.

STAT5b gene indicate that STAT5b is essential for a normal postnatal linear growth (31). 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 γ_c-negative X-SCID patients.

Although the activation of JAKs and STATs represents a prominent biochemical event during GH-dependent proliferation of lymphoid cell lines (32), other signaling pathways also contribute to a full GHR response. GH has been shown to activate the PI3K-protein kinase B signaling (33), MAPKs, and ERKs 1 and 2 (34–36). 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 events involving JAK2, ERKs, STAT1 and 3 molecules that occur following GHR triggering. Moreover, in this study, the involvement of PI3K in GH-induced proliferation of BCLs was ruled out because the kinase inhibitor wortmannin was ineffective in blocking the proliferative response. Similarly, IGF-I expression has been reported to be dependent on STAT5 b, but not on the PI3K pathway (37). 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 (38). This could lead to a hypothesis of differential functions of an individual receptor exerted in different tissues. A cell type-restricted STAT activation has been reported (39–41). STAT5 is not activated following GH stimulation in human fibrosarcoma cells even though these cells express the STAT5 protein (41), 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, 42). Recently, it has been proposed that STATs tyrosine phosphorylation and nuclear translocation are two events that are regulated separately (21). In particular, Giron-Michel et al. (43) demonstrated in the hybrid receptor γ_c/GM-CSFRβ that the γ_c/JAK3 complex controls the nuclear location of pSTAT5 rather than STAT5 phosphorylation itself. Hence, to address the issue of defining the functional implication of γ_c 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 γ_c-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 was observed in patient cells. Moreover, through confocal microscopy studies, we demonstrated that GHR and γ_c colocalize, as expected in that both molecules are type I hemopoietin receptors. A physical interaction may be hypothesized as well, even though conclusive data are still lacking.

Our data suggest that the γ_c 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 γ_c, and signaling pathways. Cross-talk between receptor signaling systems is now emerging as an important and exciting area of signaling research. Whether the participation of γ_c to the GHR confers some additional properties to the receptor in

hemopoietic cell differentiation and functioning remains to be elucidated. Of note, in CD34$^+$ progenitors, γ_c participates in hemopoietic cell differentiation by interacting with GM-CSFRβ. This interaction does not occur in normal NK cells or nonhemopoietic cells (43). 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 explains what we previously reported on an atypical X-SCID phenotype and severe short stature associated with GH hyporesponsiveness and abnormal GHR-induced protein tyrosine phosphorylation (14), and indicates that growth failure in X-SCID is directly related to the genetic alteration.

Acknowledgment

Disclosures

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2.5   Brain Migration Disorder and Immune System

Neuronal migration disorders may cause mental retardation and epilepsy. Actually, several brain migration disorders are known and the lissencephaly is one of these. Classic lissencephaly (type I) is a brain malformation caused by abnormal neuronal migration at 9 to 13 weeks' gestation, resulting in a spectrum of agyria, mixed agyria/pachygyria, and pachygyria. It is characterized by an abnormally thick and poorly organized cortex with 4 primitive layers, diffuse neuronal heterotopia, enlarged and dysmorphic ventricles, and often hypoplasia of the corpus callosum (MIM 607432). The genetic basis of agyria-pachygyria-band spectrum is beginning to be elucidated and mutation in the lissencephaly gene (LIS1) or doublecortin gene (DCX also Known as XLIS) have been reported (Pilz DT et al 1998).

Nevertheless, the pathogenetic mechanism underlain these disorders still remains to be thoroughly defined.

In the immune system T-cell activation is a complex biochemical process that involves many signaling molecules, such as tyrosine kinases and phosphatases, which in turn active other proteins through phosphorylation/ dephosphorylation events (Perlmutter RM 1993). Predominant T-cell immunodeficiencies encompass a wide number of distinct entities whose pathogenetic mechanism are coming to be extensively defined (Arnaiz-Villena A et al 1992; Stiehm ER 1993). However, the mechanism of most functional T-cell activation deficiencies still remains to be elucidated.

The nervous and immune systems share several molecules that are not tissue-specific, therefore, their alteration should result in a multisystem disorder.

Even though recurrent infections in children with brain migration disorders are frequent, no extensive examination of the immune system has been performed in these patients (Dobyns WB, 1987).

2.5.1 Combined defect of neuronal migration and hyperactivity of Fyn Tyrosine Kinase

In this research, for the first time, we study the function of T- cells immune system in a patient affected by a brain migration disorder and recurrent infections.

The preliminar genetic analysis was performed and no genetic cause for the neuronal migration disorder was found.

The immunological evaluated revealed that the proliferative response to the common mitogenic stimulation was lower than that observed in age-matched controls. The PHA stimulation failed to induce a proper up-regulation of membrane expression of T-cell activation markers. Moreover this patient failed to produce specific antibodies. We also documented in lymphocytes abnormalities in the signal transduction process that follows triggering of the T-cell receptor/CD3 complex. The stimulation of the complex failed to induce protein tyrosine phosphorylation (Fig. 2a). A high constitutive autophosphorylation activity of the 59kDa Fyn tyrosine kinase was also found (Fig. 2b). This kinase participates in the T-cell activation process. In particular, it has been documented that in experimental anergy TCR stimulation leads to the activation of Fyn, differing from the productive immune response which requires the recruitment to the receptor of the different array of molecules, but no Fyn (Byrd SE et al 1989). In a similar way, the mechanism of the T-cell defect in our patient seems to involve this kinase.

This work would suggest that abnormalities in Fyn signaling may be an underlying mechanism for a combined defect of neuronal migration in agyria-pachygyria band spectrum and of T-cell functionality. These results has been published on the Neuropediatrics see below for the manuscript.

Brain Migration Disorder and T-Cell Activation Deficiency Associated with Abnormal Signaling through TCR/CD3 Complex and Hyperactivity of Fyn Tyrosine Kinase *

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In this study we report on a patient affected by a brain migration disorder and a T-cell activation deficiency presumably inherited as an autosomal recessive trait.

The immunological evaluation revealed that the mitogen stimulation failed to induce a proper up-regulation of membrane expression of T-cell activation markers, and cell proliferation. This functional impairment was associated with abnormalities of the signal transduction process that follows T-cell receptor stimulation. A constitutive hyperphosphorylation of the Fyn tyrosine kinase was documented.

This is the first report on a T-cell signaling abnormality associated with a developmental brain disorder. Whether the alteration of Fyn, which plays a role in both neurological and immunological systems, is responsible for either disorder remains to be elucidated.

■ Key words: Brain migration disorder – T-cell immunodeficiency – Signal transduction – Fyn tyrosine kinase

Abbreviations

ITAM	immunoreceptor tyrosine activation motif
ZAP	zeta-associated protein
TCR	T-cell receptor
PBMC	peripheral blood mononuclear cells
PHA	phytohemagglutinin
ConA	concanavalin A
PMA	phorbol myristate acetate
IONO	ionomycin
CD3 X–L	CD3 cross-linking
P-tyr	phosphotyrosine

Introduction

Neuronal migration disorders may cause mental retardation and epilepsy. Even though the pathogenetic mechanism still remains to be thoroughly defined, the genetic basis of agyria-pachygyria-band spectrum is beginning to be elucidated. Mutations in the lissencephaly gene (LIS1) or doublecortin gene (DCX, also known as XLIS) have been reported [12].

Recurrent infections in children with brain migration disorders are frequent, but no extensive examination of the immune system has been performed in these patients [6]. In one case, a T-cell immune defect has been documented [7]. Predominant T-cell immunodeficiencies encompass a wide number of distinct entities whose pathogenetic mechanisms are coming to be extensively defined [1,15]. However, the mechanism of most functional T-cell activation deficiencies still remains to be elucidated. T-cell activation is a complex biochemical process that involves many signaling molecules, such as tyrosine kinases and phosphatases, which in turn activate other proteins through phosphorylation/dephosphorylation events [11]. A few of these molecules are not tissue-specific and, therefore, their alteration should result in a multisystem disorder.

We report on a patient with a brain migration disorder of the agyria-pachygyria-band spectrum and a functional T-lymphocyte activation defect associated with abnormal signal transduction through the TCR/CD3 complex, and constitutive activation of the 59 kDa Fyn tyrosine kinase.

Case Report

This 3 10/12–year old boy had intractable seizures and a clinical history of recurrent infections involving the skin and the respiratory tract. Epilepsy was present in the family history. Since the 3rd day of life, he experienced daily polymorphic seizures, i.e. tonic, partial motor, myoclonic and infantile spasms, never completely controlled. Neurological evaluation revealed microcephaly, severe mental retardation, dystonic cerebral palsy and absence of visual pursuit. EEGs showed multifocal spikes and subcontinuous generalized spike-and-wave complexes both in the awake and sleep state. Connatal infections, and neurometabolic disorders were ruled out. A high resolution karyotype and fluorescence in situ hybridization (FISH) for chromosome 17 revealed no abnormalities. As shown in Fig. 1, brain MRI showed a pachygyric appearance of the parieto-occipital regions.
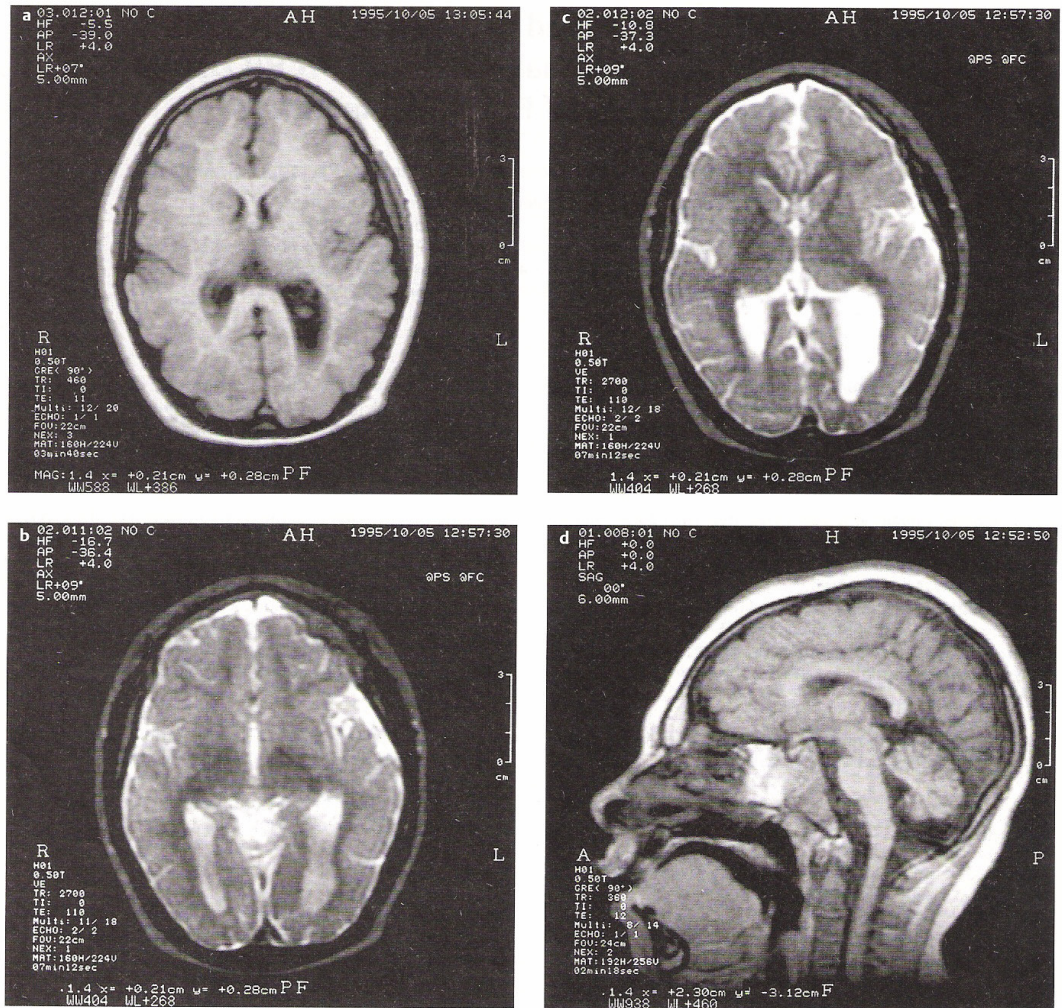
Fig. 1 a to **d** **a** Axial T_1-weighted image showing a simplified convolutional pattern with a thicker cortex in the posterior regions.
b – c Axial T_2-weighted images through the lateral ventricles showing dilatation of trigones and occipital horns.

d Sagittal T_1-weighted image showing vermian hypoplasia and a normal morphology of the corpus callosum, cerebellum and brainstem.

Due to the high number and severity of infections, the patient underwent a thorough immunological investigation, which led to a diagnosis of T-cell activation deficiency. The proliferative response, evaluated by the [3 H]-thymidine (Amersham International, Buckinghamshire, England) incorporation, was lower than that observed in age-matched controls. Mean proliferation to phytohemagglutinin (PHA; 8 μg/ml), concanavalin A (ConA; 8 μg/ml), expressed as counts per minute (cpm), was 11,153 versus 93,000 ± 24,400, and 20,248 versus 71,300 ± 19,850 in controls, respectively. The proliferation to CD3 cross-linking was 538 in the patient versus 33,660 ± 9722

in controls. Phorbol-myristate acetate (PMA; 20 ng/ml) and ionomycin (IONO; 0.5 mM) stimulation induced a higher, but still abnormal, proliferation. The activation deficiency was also confirmed by the analysis of activation markers HLA-DR, CD25, and CD71 on peripheral-blood mononuclear cells (PBMC) by flow cytometry (Becton Dickinson, San Jose, CA). An increased percentage of unstimulated cells constitutively expressed HLA class II and CD25 molecules, values being 10 and 11%, respectively. However, PHA stimulation for 48 or 72 hrs failed to induce up-regulation of these markers. The number of mature CD3 + CD4 +, and CD3 + CD8 + cells was normal, confirming a
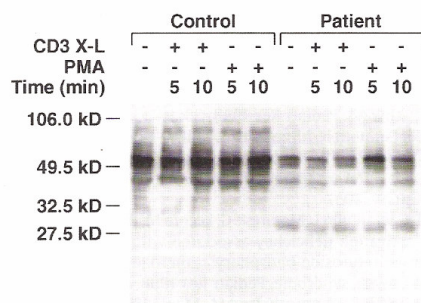
Fig. 2a Signal transduction through T-cell receptor/CD3 complex. Protein tyrosine phosphorylation in a control's and the patient's PBMC stimulated by CD3 cross-linking (X–L) or PMA for 5 or 10 min as indicated. Molecular weight markers are indicated.
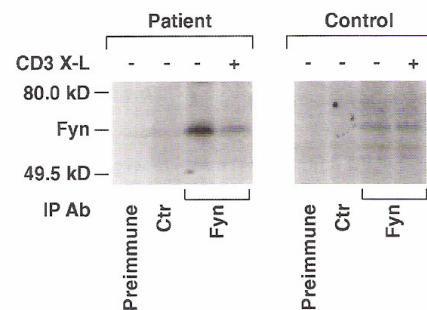
Fig. 2b Fyn kinase autophosphorylation assay. Immune complexes containing Fyn from PBMC, unstimulated or stimulated through CD3 cross-linking (X–L), were incubated for 15 min with kinase buffer containing 20 µCi [γ32 P]ATP. Phosphorylated proteins were resolved by 10% SDS-PAGE. Molecular weight markers are indicated.

functional defect. Moreover, the patient failed to produce specific antibodies.

Since the T-cell defect was functional in nature, signal transduction properties of the TCR/CD3 complex were investigated by analysing the number and timing of protein tyrosine phosphorylation (P-tyr) events occurring after receptor stimulation. Whole-cell lysates were obtained from 3×10^6 cells after appropriate stimuli and NP-40 lysis, as above described [13]. Western blots were performed using anti-P-tyr mAb, horse radish peroxidase secondary Ab and enhanced chemiluminescence (Amersham). A representative experiment is shown in Fig. 2a, which indicates that the pattern of P-tyr proteins in the patient's PBMC was aberrant, and characterized by the failure to induce a proper increase in protein phosphorylation. On the contrary, a 30–32 kDa protein was constitutively hyperphosphorylated on tyrosine. PMA stimulation slightly increased phosphorylation, but to a lower extent than in controls. In Fyn autophosphorylation kinase assays, immunoprecipitates were obtained, using protein-A Sepharose precoated anti-Fyn rabbit serum (kindly provided by Dr. C. Rudd, Dana Farber Cancer Institute, Boston, MA). Kinase buffer contained 25 mM HEPES, 10 mM MnCl2, 5 mM MgCl2 and 20 µCi [γ32 P]ATP. Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This assay revealed an intense constitutive autophosphorylation activity of Fyn. Fig. 2b illustrates a representative experiment out of the 3 performed. In one of these experiments, a phosphorylated 30–32 kDa protein was co-precipitated with Fyn. Like other kinases of the src family, Fyn contains a tyrosine in the position 531 whose phosphorylation exercises an inhibitory effect on kinase activation. Therefore, the coding region flanking the Tyr-531 of the molecule was PCR amplified from genomic DNA and sequenced directly (ABI 377 Automated DNA Sequencer). Sequence analysis of the 297 bp fragment obtained failed to reveal any molecular alteration.

Discussion

Clinical and neuroradiological features in our patient were indicative of a brain migration disorder, according to established criteria for the agyria-pachygyria band spectrum [2]. Even though we cannot provide a formal demonstration of a genetic cause for the neuronal migration disorder in our patient, a few elements are in favor of this hypothesis. In particular, the normal pattern of myelination and the absence of porencephalies or other evidence of destructive lesions support the assumption that the arrest of neuronal migration is related to a developmental anomaly.

Our patient also had a T-cell activation defect. It is worth noting that all the molecular alterations causing a combined immunodeficiency phenotype thus far described involve genes encoding for molecules that participate in the signal transduction process, such as ZAP-70, the γ chain of several receptors and the JAK 3 kinase (10–8). However, none of the clinical and immunological phenotypes associated with alterations of these molecules is similar to the overall features of the patient described here.

Recent evidence indicates that also in the case of brain migration disorders molecular alterations thus far described involve genes, such as LIS1 and XLIS, which play a role in cell signaling [12]. However, these genes are predominantly expressed in the human brain and not in hematopoietic cells [9]. Moreover, the region containing LIS1 gene was normal in this patient. As a matter of fact, a link between the failure of neurons to migrate and the failure of hematopoietic cells to undergo a full differentiation/activation process that leads to a mature lymph node architecture has already been hypothesized [7].

In this patient, we also documented in lymphocytes abnormalities in the signal transduction process that follows triggering of the T-cell receptor/CD3 complex. The stimulation of the complex failed to induce protein tyrosine phosphorylation. A high constitutive autophosphorylation activity of the 59 kDa Fyn tyrosine kinase was also documented. This kinase participates in the T-cell activation process [11]. In particular, it has

been documented that in experimental anergy TCR stimulation leads to the activation of Fyn, differing from the productive immune response which requires the recruitment to the receptor of a different array of molecules, but not Fyn [3]. In a similar way, the mechanism of the T-cell defect in our patient seems to involve this kinase.

The nervous and the immune systems share several molecules that are not tissue-specific. Fyn biological functions are diverse and include along with the T-cell activation, regulation of brain function as well, and are in particular involved in adhesion-mediated signaling [5]. A different Fyn isoform, originated by a splicing mechanism, is expressed during early neurogenesis as documented in mouse embryos [5,16]. Interestingly, in a mouse model of acquired immune deficiency syndrome, disruption of Fyn kinase-mediated signaling was responsible for encephalopathic cognitive deficits [14]. Although this is only speculative, it is conceivable to hypothesize that post-transcriptional alterations of signaling molecules expressed in different tissues might lead to multisystemic disorders.

Our observation would suggest that abnormalities in Fyn signaling may be an underlying mechanism for a combined defect of neuronal migration in agyria-pachygyria band spectrum and of T-cell functionality.

Acknowledgment

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Chapter 3

Technologies

3.1   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.

3.2 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).

3.3 Cells and cell cultures

Mononuclear cells (PBMC) were obtained from 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. 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.

3.4 Proliferative assay

BCLs or PBMC (1 X 10^5 cell/200 μl well) were cultured triplicate in 96-well U-bottomed microtiter plates (Falcon®, Becton Dickinson) with or without appropiate stimuli 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 .

3.5 Flow cytometry

The expression of specific protein was detected using specific antibodies (Santa Cruz) by indirect immunofluorescence using a second-step incubation with a FITC-conjugated secondary 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.

3.6   Natural Killer function

Infected target cells (K562) were pulsed for 1 h at 37 °C with Cr^{51}(Amersham Pharmacia, Buckinghamshire, England) (30 µCi/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).

3.7   Cell stimulation and protein extraction

Prior to appropiate stimuli treatment, the cells were made quiescent through incubation in RPMI 1640 minus serum for 8-12 h. The stimuli was used at 37°C at the reported concentration 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.

3.8   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 0.45µm; 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).

3.9   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 four rinses of 5 min in PBS, the cells were centrifuged in a Shandon Cytospin III (Histotronix) onto a 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 specific antibody diluted in PBS containing 1% BSA. After four washings for 5 min in PBS, the cells were incubated for 1 h at room temperature with FITC-conjugated secondary antibody. 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.

Chapter 4

4.1 Summary

Primary immunodeficiency diseases (PIDs) represent a group of most heterogeneous syndromes characterized by development or functional defect of the Immune System. Thus far, more than 200 distinct well-defined genetic forms have been recognized. 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. 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.

4.2   Acknowledgement

I would 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 like to thank my supervisor Prof. Claudio Pignata, whom I hold in high esteem and affection, for the opportunity he gave me to be part of his team. He has been of great support because he has encouraged me during all these years. Moreover, he followed me in the PhD program and in my research, supporting my decisions and influencing my work with stimulating advices.

I would like to thank my husband, the father of my two children, because with love and patient, he has encouraged me during these years.

4.3 Curriculum vitae

Eliana Matrecano was born on January 27, 1972 in Naples, Italy. Her education and professional position are listed below.

Education

- **1990** High School degree

- **1990-1992** School of Laboratory Technician degree

- **2003** "Federico II "University of Naples Degree in Biology

- **2003 at present** Ph.D. student in "Sviluppo, Accrescimento e Riproduzione dell'uomo" at the "Federico II" University of Naples

Employment and Experience

- **1996-2002** Technician at the Dept of Pediatrics under Prof. C. Pignata, "Federico II "University of Naples

  - during this period, I have also collaborated for the writing and to performig of the experimental protocol teraphy: "Allogenic transplantation of the thymic tissue in patients with Di Gorge syndrome and thymic aplasia". This protocol was valuated and approved to Ethic Committee of the "Federico II" University.

- **2004-2006** Integrative teaching activity, Faculty of Biotechnology, "Federico II "University of Naples

Information Technology Skills

- Computer literate, knowledge of MS Windows**,** MacOS systems

- Good knowledge of scientific softwares as: Oligo 4, Gencokit, Blast, DNA Strider 1.2, Cyrillic 3, Edit View1, Factura 2.0.1, Inherit Autoassembler 1.4.0,

- 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 Skills

Direct experience in the following fields:

- Cells culture

- Tissue culture

- Proliferation assay

- Cell death assay

- DNA and RNA extractions

- PCR

- Sequencing analysis

- Western Blotting

- Immunoprecipitation

- In vitro kinase assay

- Flow cytometer analysis

- Immunohystochemestry

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