GENOTYPIC AND PHENOTYPIC ASPECTS OF PRIMARY IMMUNODEFICIENCY DISEASES OF THE LYMPHOID SYSTEM

Genotypering en fenotypering van primaire immunodeficiënties van het lymfatische systeem

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CONTENTS

Chapter 1

GENERAL INTRODUCTION

Primary immunodeficiency diseases (PID) are inherited disorders of the immune system. PID can be divided into five categories, based on the component of the immune system that is affected. The recognized categories are: (1) antibody deficiencies; (2) combined or T-cell deficiencies; (3) phagocyte disorders; (4) complement deficiencies; and (5) PID with other severe symptoms.[1,2] Table 1 shows the relative distribution of the five categories. PID occur at much lower frequency than secondary immunodeficiency diseases, such as neutropenia after chemotherapy or T-cell deficiencies due to malnutrition, which are acquired disorders.

In the last decade, many genetic defects resulting in PID have been identified. Most of these genetic defects are described in more detail in Chapter 3. As the research interest in our laboratory focuses on lymphoid differentiation, and because antibody and T-cell deficiencies represent approximately 90% of all PID, this thesis focuses particularly on PID of the lymphoid system.

Antibody deficiencies

Antibody deficiencies can result from an arrest during precursor B-cell differentiation in the bone marrow (BM), leading to absent or strongly diminished numbers of B lymphocytes in the peripheral blood (PB) and agamma- or hypogammaglobulinemia. This immunological phenotype can be caused by mutations in the genes encoding Bruton's tyrosine kinase (BTK), Igμ, λ14.1, CD79a, or B-cell linker protein (BLNK).[3-8] Igμ and λ14.1 are important components of the pre-B-cell receptor (BCR), while CD79a, BTK and BLNK are involved in signal transduction from the pre-BCR. Hence, all five genetic defects result in a precursor B-cell differentiation arrest at the pre-BCR checkpoint, i.e. at the transition from CyIgμ$^-$ pre-B-I cells to CyIgμ$^+$ pre-B-II cells.[9-11] Agammaglobulinemia patients suffering from mutations in *IGH-*C_μ, *CD79a*, or *BLNK* genes generally present with a complete B-cell differentiation arrest and complete absence of B lymphocytes in the PB. In contrast, patients suffering from mutations in the *BTK* gene (causing X-linked agammaglobulinemia (XLA)) or the *λ14.1* gene can produce some mature B lymphocytes ("leaky arrest").

Some patients with antibody deficiencies present with normal numbers of PB B lymphocytes and agammaglobulinemia with or without elevated serum IgM levels. These patients suffer from the hyper IgM syndrome (HIGM) due to a defect in class-switch recom-

Table 1. Prevalence of PID in Western countries[a].

Category	The Netherlands	The United Kingdom	France	Germany	ESID Registry
Antibody deficiencies	403 (66%)	1078 (72%)	117 (28%)	128 (38%)	5022 (67%)
T-cell or combined deficiencies	137 (22%)	187 (12%)	190 (45%)	147 (44%)	1405 (19%)
Phagocyte disorders	43 (7%)	86 (6%)	96 (23%)	47 (14%)	585 (8%)
Complement deficiencies	3 (<1%)	97 (6%)	3 (1%)	10 (3%)	364 (5%)
Other PID	25 (4%) +	34 (2%) +	19 (4%) +	2 (1%) +	171 (2%) +
Total	611	1505	425	334	7547

[a] Based on the European Society for Immunodeficiencies (ESID) Registry of Primary Immunodeficiencies (May 1998), containing data of 25 European countries.

bination (CSR), generally caused by mutations in the genes encoding CD40 ligand (CD40L), activation-induced cytidine deaminase (AICDA) or CD40.[12-15]

A third group of patients suffering from antibody deficiencies present with normal numbers of PB B lymphocytes and normal serum immunoglobulin (Ig) levels. These patients appear to be unable to generate specific antibodies (Ab) due to a defect in somatic hypermutation (SHM). No causative genetic defect has been identified in this group of patients.[16,17]

T-cell or combined deficiencies

Patients suffering from T-cell deficiencies in general present with severe clinical phenotypes, characterized by opportunistic infections, failure to thrive, and protracted diarrhea. Although the number of PB B lymphocytes may be normal, B-cell function is generally severely hampered by the lack of T-cell help. Severe combined immunodeficiency diseases (SCID) can be divided into five groups based on the immunological phenotype, i.e. $T^-/B^+/NK^+$, $T^-/B^-/NK^+$, $T^-/B^+/NK^-$, $T^+/B^+/NK^-$, and $T^-/B^-/NK^-$.[18] The genetic defects responsible for these immunological phenotypes are described in Chapter 3.

In line with our studies on a subgroup of antibody deficiencies, which result from an arrest in precursor B-cell differentiation, we have focused on $T^-/B^-/NK^+$ SCID patients (approximately 20% of all SCID patients). A number of these patients suffer from mutations in the recombination activating genes (*RAG1* and *RAG2*), resulting in the complete absence of recombination activity.[19] Mutated RAG proteins that partially maintain the ability to perform gene recombination cause a variant of $T^-/B^-/NK^+$ SCID, called the Omenn syndrome (OS), characterized by oligoclonal T cells.[20] This genotype-phenotype relationship has recently become less obvious by the identification of identical *RAG* mutations in both $T^-/B^-/NK^+$ SCID and OS patients.[21] Apparently other factors, such as specific antigens, determine the immunological phenotype as well.

Not all $T^-/B^-/NK^+$ SCID patients suffer from mutations in the *RAG* genes. It was shown that the remaining group of $T^-/B^-/NK^+$ SCID patients with unmutated *RAG* genes can be subdivided based on their sensitivity to ionizing radiation.[22] A number of radiosensitive (RS) $T^-/B^-/NK^+$ SCID patients were subsequently shown to suffer from defects in DNA double strand break (dsb) repair caused by mutations in the recently discovered *Artemis* gene.[23]

Human precursor B-cell differentiation

Human precursor B-cell differentiation occurs in sequential steps in the BM, during which precursor B cells start to express B-cell specific proteins that eventually lead to the generation of mature B lymphocytes. Important B-cell specific proteins are the transcription factors E2A, early B-cell factor (EBF), and PAX5, Ig expression in the cytoplasm (Cy) or on the surface membrane (Sm), the components of the pseudo-light chain (ψL; composed of VpreB and λ14.1), and the lymphoid specific RAG1 and RAG2 proteins.[24] Using cell sorting and single-cell PCR, Ghia *et al.* have characterized several precursor B-cell differentiation stages: (1) $CyVpreB^+/CyIg\mu^-/RAG^+$ cycling pre-B-I cells; (2) $pre\text{-}BCR^+/RAG^-$ cycling pre-B-II cells; (3) $pre\text{-}BCR^-$ cycling pre-B-II cells; (4) $pre\text{-}BCR^-/RAG^+$ resting pre-B-II

cells; (5) SmIgM$^+$ immature B cells; and (6) SmIgM$^+$/SmIgD$^+$ mature B cells.[25] As cell sorting and single cell PCR are complicated techniques, which cannot be implemented routinely, we have adapted this scheme using additional markers (Figure 1).[11,26] These additional markers can be subdivided into lineage specific pan-B-cell markers (CD22, CyCD79a, and CD19), and differentiation stage-specific markers (CD34, CD10, and CD20).

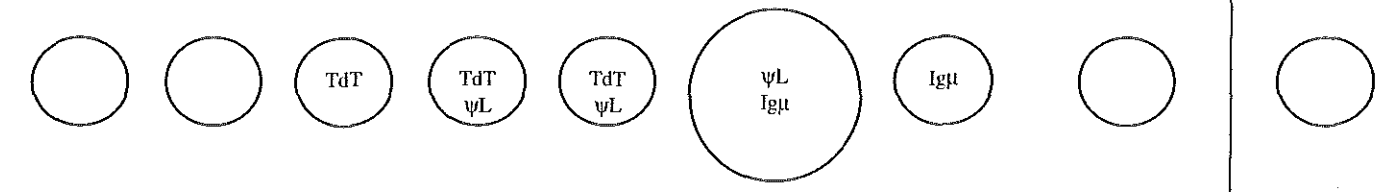
An intriguing observation is the lack of terminal deoxynucleotidyl transferase (TdT) expression in pre-B-II cells that are rearranging Ig light chain genes. This has also been reported by Ghia *et al.* and Schiff *et al.*[25,27] In contrast to mice, human Ig light chain gene rearrangements have diverse junctional regions, with both deletion and insertion of nucleotides.

During human precursor B-cell differentiation, two important checkpoints have to be surpassed, i.e. the pre-BCR checkpoint at the transition from CyIgμ$^-$ pre-B-I cells to CyIgμ$^+$ pre-B-II cells, and the BCR checkpoint from the CyIgμ$^+$ pre-B-II cells to the SmIgM$^+$ immature B cells. At the pre-BCR checkpoint, Igμ pairs with the ψL to form the pre-BCR, which signals antigen (Ag)-independently through among others CD79, BTK, and BLNK. The pre-BCR checkpoint therefore seems to check the ability of Igμ to pair with the ψL chain as surrogate alternative for the Ig light chain at a later stage. In contrast to the Ag-independent signaling by the pre-BCR, the BCR checkpoint is assumed to check for possible autoreactivity of the SmIgM molecule.[24]

Initiation of antigen receptor gene rearrangements by RAG and subsequent DNA dsb repair via non-homologous end joining (NHEJ)

The Ag binding variable domains of the Ig and T-cell receptor molecules are encoded by variable (V), diversity (D), and joining (J) gene segments, which are coupled to each other during precursor B- and T-cell differentiation, respectively.[28] This rearrangement process occurs in a hierarchical order.[29] The gene rearrangement process is initiated by the lymphoid specific RAG1 and RAG2 proteins.[30-32] The RAG proteins recognize recombination signal sequences (RSS), which are composed of conserved heptamer-nonamer sequences, separated by a spacer of 12 or 23 base pairs (bp). The RAG proteins introduce DNA dsb at the RSS, which are neutralized by the generation of a hairpin structure (top strand covalently coupled to the bottom strand) in case of coding ends, or which remain blunt and 5' phosphorylated in case of signal ends (containing the RSS).[33,34] The signal ends will fuse head-to-head to form a signal joint, which is generally present on an extrachromosomal DNA excision circle.

The hairpin-sealed coding ends need to be further processed, before they can be joined and form the coding joint or V(D)J exon. The initial phase of this process is mediated via the protein complex DNA-dependent protein kinase (DNA-PK), which consists of Ku70, Ku80 and DNA-PK catalytic subunit (DNA-PK$_{cs}$). The DNA-PK protein complex functions as a DNA damage sensor, with Ku70 and Ku80 forming a heterodimer which binds to DNA ends, while DNA-PK$_{cs}$ has Serine and Threonine protein kinase activity.[35] Although the exact function of this complex in DNA end joining is not yet clear, it might be that the Ku heterodimer binds to the broken DNA ends and brings them together. DNA-PK$_{cs}$ phosphorylates



	BONE MARROW							PERIPHERAL BLOOD
1	2	3	4	5	6	7	8	9
Pro-B			Pre-B-I		Pre-B-II		Immature B	Mature B
					large	small		
			RAG+ D_H-J_H	RAG+ V_H-J_H	pre-BCR+ RAG−	pre-BCR− RAG+ Vκ-Jκ / Vλ-Jλ	SmIgM+	SmIgM+ / SmIgD+
CD22	CD22 CyCD79a	CD22 CyCD79a	CD22 CyCD79a CD19	CD22 CyCD79a CD19	CD22 CyCD79a CD19	CD22 CyCD79a CD19	CD22 CyCD79a CD19	CD22 CyCD79a CD19
CD34	CD34	CD34	CD34 CD10	CD34 CD10	CD10	CD10 CD20	CD10 CD20	CD20
3%	5%	1%	3%	4%	26%	12%	47%	Thawed BM-MNC (n=6) [1]
9%		1%	6%	5%	34%	24%	21%	LWBM (n=18) [2]

Figure 1. Hypothetical scheme of precursor B-cell differentiation stages in BM from children younger than 16 years of age.
The distinction between large and small pre-B-II cells, expression of RAG proteins, and Ig gene rearrangement patterns were deduced from Ghia *et al.*,[25] Schiff *et al.*,[27] and Rolink *et al.*[24] According to Nomura *et al.*,[9] expression of ψL precedes CD19 expression. However, we cannot indicate in which stage ψL expression starts. LWBM = lysed whole BM; [1] Relative composition of the precursor B-cell compartment based on quadruple labelings; [2] Relative composition of the precursor B-cell compartment based on triple labelings.

Artemis. Subsequently, the activated Artemis protein can open the hairpin-sealed coding ends.[45] In the final phase, the two broken DNA ends will be ligated via the DNA NHEJ pathway, involving DNA ligase IV and X-ray cross complementation group (XRCC) 4, which form a protein complex.[36,37]

Gene-knockout mice in which the genes encoding Ku70, Ku80, DNA-PK_{cs}, XRCC4, or DNA ligase IV are disrupted suffer from $T^-B^-NK^+$ SCID, due to an inability to carry out V(D)J recombination.[38] In addition to the SCID phenotype, the *Ku70* and *Ku80* gene knockout mice are also growth retarded [39,40] and the *XRCC4* and *DNA ligase IV* gene knockout mice die during embryogenesis,[41,42] but the mechanism underlying these phenotypes is not yet clear. In the *Ku70*, *Ku80*, *XRCC4*, and *DNA ligase IV* gene knockout mice both signal and coding joint formation is severely hampered. A recent gene-knockout study showed that DNA-PK_{cs} activity is required for both coding and signal joint formation.[43] However, it has been suggested earlier that residual DNA-PK_{cs} activity might rescue signal, but not coding joint formation.[44]

Outline of this thesis

This thesis focuses on the immunological phenotype, the mutation analysis, and the residual activity of mutated proteins in patients with PID of the lymphoid system. During this project, we have investigated possible genotype-(immuno)phenotype relationships in patients with antibody deficiencies and SCID. Consequently, mutation analysis of the relevant genes formed an essential part of the study.

Part 2 focuses on the laboratory diagnosis of patients with PID of the lymphoid system. In combination with clinical data, detailed immunophenotyping of PB and BM appeared to be essential to select possible candidate genes for mutation analysis (Chapter 2). Chapter 3 describes the pre-analytical, analytical, and post-analytical phases of mutation analysis and comments on the pitfalls that might occur when trying to establish a molecular diagnosis.

Part 3 focuses on antibody deficiencies, with primary forms of agammaglobulinemia as the main topic (Chapters 6, 7 and 8). As agammaglobulinemia can result from an arrest in precursor B-cell differentiation in the BM, we have used detailed immunophenotyping of BM samples for assessment of the precise differentiation arrest, for identifying possible target genes, and for studying possible genotype-immunophenotype relationships.

Part 4 focuses on SCID, especially patients suffering from $T^-B^-NK^+$ SCID or OS caused by mutations in the *RAG* genes (Chapters 11 and 12). Whenever available, the BM samples of these B^- SCID patients were subjected to detailed immunophenotyping. Furthermore, we tried to unravel the residual recombination activity of mutated RAG proteins via several approaches. In addition to *in vitro* analyses with *RAG* gene transfection and subsequent recombination of a plasmid recombination substrate as read-out system, we used Ig gene rearrangement patterns in BM samples as an *in vivo* read-out system of RAG protein function.

Finally, Part 5 describes two patients with mycobacterial infections due to mutations in the gene encoding the interferon gamma receptor 1 chain (*IFNGR1*). Although both

patients suffered from complete signaling defects from the IFN-γR, one patient died at young age while the other patient is still alive without BM transplantation, showing the variability in clinical phenotypes in patients suffering from complete IFN-γR signaling defects.

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Chapter 2

FLOW CYTOMETRIC IMMUNOPHENOTYPING IN THE DIAGNOSIS AND FOLLOW-UP OF IMMUNODEFICIENT CHILDREN

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SUMMARY

From time to time, paediatricians are confronted with children who might suffer from a primary immunodeficiency disease. For practical purposes, these children can be divided into four main clinical categories: 1) a relatively large group of children with recurrent ear-nose-throat and lower respiratory tract infections, in some cases caused by deficiencies of antibodies or complement; 2) children with failure to thrive, intractable diarrhoea or an opportunistic infection, which can be caused by a T-lymphocyte or combined immunodeficiency; 3) children with infections with pyogenic bacteria or fungi as seen in case of granulocyte/monocyte function deficiency; and 4) a small heterogeneous group of children with recurrence of particular infections. Also, acquired immunodeficiency becomes a more common problem in paediatric practice. Flow cytometric immunophenotyping of leukocytes appears to be an efficient and rapid tool in the diagnosis and follow-up of immunodeficient patients, supporting early recognition, before serious infections have compromised the child's general condition. This technique can now be performed in many hospitals. In this review, we give directions for the use of flow cytometric immunophenotyping of leukocytes in the diagnosis and follow-up of immunodeficient children according to the four main clinical categories.

INTRODUCTION

From time to time, paediatricians are confronted with children who might suffer from a primary immunodeficiency disease.[21] For practical purposes, these children can be divided into four main clinical categories.[7, 26, 28] The first clinical category comprises the relatively large group of children with recurrent ear-nose-throat (ENT) and lower respiratory tract infections, only in some cases caused by an underlying immunodeficiency, especially if encapsulated extracellular bacteria are found as pathogens. In patients with hypo- or agammaglobulinemia, deficient opsonisation with antibodies leads to impaired phagocytosis of these micro-organisms. Complement deficiencies are rare, and in most types of complement deficiency affected children present with collagen-vascular disease.[15] However, some complement deficiencies lead to severely impaired opsonisation with recurrent serious bacterial infections, resembling agammaglobulinemia (C3 deficiency, factor D deficiency and factor I deficiency; to a lesser extent C2 deficiency). Most children with recurrent, often viral, ENT and lower respiratory tract infections do not have an antibody or complement deficiency, but are immunocompetent. Their problems are due to other more common causes such as bronchial hyperreactivity, adenoidal hypertrophy, or an allergic constitution, or they may suffer from rare non-immunological diseases like cystic fibrosis or immotile cilia syndrome.

The second clinical category comprises children with failure to thrive, intractable diarrhoea or an opportunistic infection, which can be caused by a T-lymphocyte or combined

immunodeficiency with absent or functionally deficient T-lymphocytes. Their defence against intracellular micro-organisms is inadequate. The third clinical category comprises children with superficial and systemic infections with pyogenic bacteria or fungi, who apparently have problems with their first line of defence against invading micro-organisms as in cases of granulocyte/monocyte function deficiency. Finally, the fourth clinical category comprises a small heterogeneous group of children with recurrence of particular infections such as recurrent neisserial infections in cases of late complement component deficiency, or recurrent mycobacterial infections in cases with a defect in the interferon (IFN)-γ receptor, interleukin (IL)-12 or the IL-12 receptor.[19] These last three clinical categories are only rarely encountered in general paediatric practice.

Most cases of acquired immunodeficiency are iatrogenic and can therefore be anticipated: as more and more children are being treated with immunosuppressive drugs, aggressive chemotherapy protocols, and stem cell transplantation, acquired immunodeficiency becomes a more common problem in paediatric practice.[4] Also, the prevalence of paediatric human immunodeficiency virus (HIV) infection in Europe is increasing, especially in children born to parents who originate from HIV-endemic areas.[e.g. 8]

Laboratory studies for identification and clinical follow-up of immunodeficient children can now be performed in many hospitals. In this review, we focus our discussion on the increasingly important role of flow cytometric immunophenotyping of leukocytes in the diagnosis and follow-up of immunocompromised children in paediatric practice.[12] Application of flow cytometric techniques helps to speed up the diagnostic process, thereby supporting early recognition of primary immunodeficiencies, before serious infections have compromised the child's general condition, reducing the chances of survival.[21]

FLOW CYTOMETRIC IMMUNOPHENOTYPING

Flow cytometric immunophenotyping allows precise assessment of relative frequencies of leukocyte subpopulations by detection of cell surface and intracellular markers with fluorochrome-labelled monoclonal antibodies (McAb). Immunophenotyping can be focussed on lymphocytes, monocytes, or granulocytes, because these leukocyte populations can be defined on the basis of their light scatter characteristics, such as forward scatter (FSC) as measure for size, and side scatter (SSC) as measure for cellular irregularity (Figure 1).[12] Leukocyte count and differential cell count can then be used to calculate absolute counts of the blood lymphocyte subpopulations, which should be compared with age-matched reference values.[6]

Blood sample handling, cell separation methodology and labelling techniques influence the reliability of the results obtained by flow cytometric immunophenotyping of leukocytes.[12] Dead cells show increased autofluorescence, potentially leading to incorrect interpretation of staining results. This can be minimised by using freshly collected samples, which are fully analysed within 24 hours. The immunophenotyping procedures take 4 to 5 hours,

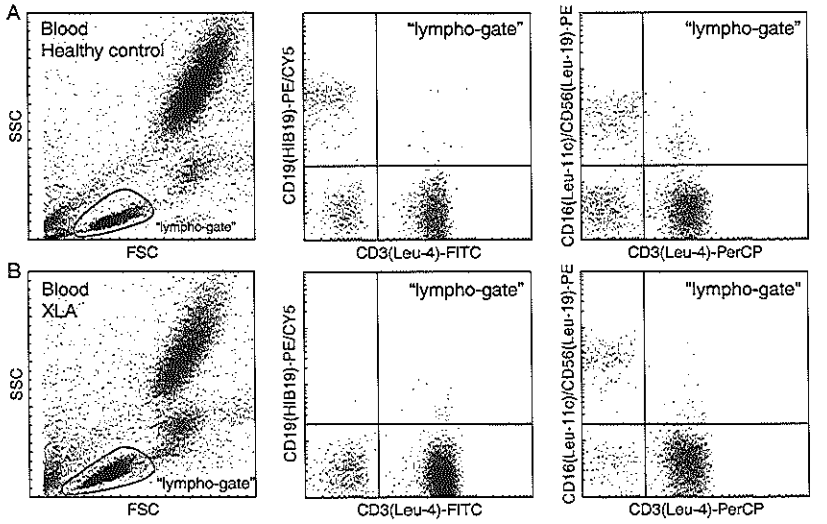

Figure 1. Flow cytometric immunophenotyping of lymphocytes in the blood of a BTK-deficient X-linked agammaglobulinemia patient as compared to a healthy control.
A, Analysis of peripheral blood of a healthy control. Dot plot with forward scatter (FSC) and side scatter (SSC) showing "lympho-gate", "mono-gate", and "granulo-gate" (left). The B-lymphocytes in the left upper quadrant express CD19 on their cell surface (middle). The T-lymphocytes in the right lower quadrant express CD3 on their cell surface (middle and right). The NK-cells in the left upper quadrant are defined as CD3-negative and CD16- and/or CD56-positive (right). *B*, Analysis of peripheral blood of a BTK-deficient X-linked agammaglobulinemia patient. This three-year-old boy presented at the age of two years with skin infections. Dot plot with FSC and SSC showing "lympho-gate " (left). Complete absence of $CD19^+$ B-lymphocytes (middle), with $CD3^+$ T-lymphocytes and $CD16^+$ and/or $CD56^+/CD3^-$ NK-cells normally present (right).

implying that the sample should arrive in the laboratory in the morning. Cell separation via density gradients may result in differential loss of specific lymphocyte subpopulations. This is avoided when the lysed whole blood (LWB) technique is used. The currently available LWB techniques require fewer preparation steps and less sample handling through direct incubation of the anticoagulated blood with McAb, in combination with red blood cell lysis. These LWB techniques also reduce aspecific binding of McAb, especially if the applied McAb are directly conjugated with fluorochromes.

DIAGNOSING CHILDREN WITH PRIMARY IMMUNODEFICIENCY

Children with recurrent ENT and lower respiratory tract infections

In some children with recurrent ENT and lower respiratory tract infections, a relatively mild antibody deficiency like IgA deficiency, IgG-subclass deficiency, or anti-polysaccharide antibody deficiency is found.[21] Lymphocyte number and immunophenotype are com-

Table 1. Primary immunodeficiency diseases

Diseases	Pathogenesis	Immunophenotyping	
Mild Antibody Deficiencies (Recurrent ENT and lower respiratory tract infections, lambliasis, sometimes asymptomatic)			
IgA deficiency IgG-subclass deficiency Anti-polysaccharide antibody deficiency	Pathogenesis unknown; in anti-polysaccharide antibody deficiency, B-lymphocytes are unable to produce antibodies against polysaccharide antigens, despite adequate responses against protein antigens	Generally not useful	Normal immunophenotyping results. Focus on serum Ig (sub) classes and antibody reactivity
Severe Antibody Deficiencies (Recurrent respiratory tract and ENT-infections, life-threatening bacterial infections, enteroviral CNS infections, lambliasis)			
BTK enzyme deficiency (X-linked agammaglobulinemia) Igμ heavy chain deficiency (autosomal recessive) Several other rare forms (autosomal recessive)	Early arrest in B-cell development	"Limited" protocol (bone marrow protocol)	Absent blood B-lymphocytes. Differentiation arrest reflected in precursor B-cell population in bone marrow (useful for directing DNA diagnostics in non-XLA patients
X-linked hyper IgM syndrome with CD40 ligand deficiency	CD40L deficiency leads to absent isotype switch of B-lymphocytes and associated T-lymphocyte defect due to absent CD40 stimulation	"Extended" protocol (CD40L)	Absent or decreased CD40L expression on activated T-lymphocytes in X-linked form; blood B-lymphocytes present
Autosomal recessive hyper IgM syndrome	Mutations in the *AID* gene disrupt isotype switching and somatic hypermutation in germinal centre B-lymphocytes	"Limited" protocol	Blood B-lymphocytes present
Common variable immunodeficiency	Probably defects in B-cell maturation and helper T-lymphocyte function	"Limited" protocol	Blood B-lymphocytes present; some times T-lymphocytopenia
Combined Immunodeficiency Diseases (Failure to thrive, intractable diarrhea, eczema, and other chronic problems, or opportunistic infections)			
Adenosine deaminase deficiency (autosomal recessive) Purine nucleotide phosphorylase deficiency (autosomal recessive)	Accumulation of toxic purine metabolites due to enzyme deficiency	"Limited" protocol	Increasing T-lymphocytopenia in the first months after birth; also B-lymphocytopenia in adenosine deaminase deficient SCID
MHC class-I expression deficiency (autosomal recessive)	Impaired antigen presentation	"Extended" protocol (β_2 micro-globulin)	Deficient MHC class-I expression
MHC class-II expression deficiency (autosomal recessive)	Impaired antigen presentation	"Extended"protocol (HLA-DR)	Deficient MHC class-II expression; low numbers of $CD4^+$ T-lymphocytes
Wiskott Aldrich syndrome (X-linked) (patients also suffer from eczema, thrombocytopenia, and malignancy)	Impaired cytoskeletal reorganization upon activation of platelets and T-lymphocytes	"Limited" protocol	Increasing T-lymphocytopenia with time; (impaired CD43 expression)

Table 1. continued

Severe Combined Immunodeficiencies (Failure to thrive, intractable diarrhea, eczema, and other chronic problems, or opportunistic infections)			
X-linked SCID (common γ-chain deficiency)	Defect in T-lymphocyte and NK-cell development	"Limited" protocol	T-lymphocytes absent, NK-cells low, and B-lymphocytes present
JAK3 deficiency (autosomal recessive)			
RAG1 or RAG2 deficiency (autosomal recessive)	Early arrest in common lymphocyte development	"Limited" protocol	T- and B-lymphocytes absent, NK-cells present
Omenn syndrome (autosomal recessive; partial RAG deficiency) (patients present with severe eczema)	Defective T-lymphocyte development with B-lymphocyte deficiency	"Limited" protocol	Oligoclonal T-lymphocytes, very low or absent B-lymphocytes
ZAP70 protein tyrosine kinase deficiency (autosomal recessive)	Defect in T-lymphocyte development	"Limited" protocol	T-lymphocytes - especially $CD8^+$ - decreased; B-lymphocytes present
CD3 γ- or ε-chain deficiency (autosomal recessive)	Defect in T-lymphocyte function	"Limited" protocol	T-lymphocytes decreased, CD3 expression decreased; B-lymphocytes present
IL-7 receptor deficiency	Defect in the α-chain of the IL-7 receptor, leading to a defect in T-lymphocyte development	"Limited" protocol	T-lymphocytes absent, NK-cells and B-lymphocytes present
Reticular dysgenesis (patients present with pancytopenia)	Stem cell defect leading to defective maturation of T- and B-cells and myeloid cells	"Limited" protocol	Pancytopenia
Milder Combined Immunodeficiencies			
DiGeorge (CATCH-22) syndrome (cardiac malformations, hypoparathyroidism, thymic insufficiency)	Disturbance of embryonic development; 22q1 deletion	"Extended" protocol (TCRαβ, TCRγδ)	Sometimes $TCR\alpha\beta^+$ T-lymphocytopenia with normal $TCR\gamma\delta^+$ T-lymphocyte counts
Ataxia teleangiectasia (autosomal recessive)	Mutation in the *ATM* gene; exact pathogenesis unknown	"Limited" protocol	Sometimes increasing T-lymphocytopenia with time
Chromosomal breakage syndromes (Nijmegen breakage syndrome, Blooms syndrome, ICF syndrome) (mental retardation, photosensitivity, abnormal physionomy)	Mutation in respectively the *NBS*, *BLM*, and *ICF* genes;	"Limited" protocol	Immunophenotyping mostly normal, but disturbances may be present; exact pathogenesis unknown.
Auto-immune lymphoproliferative syndrome	Defect in apoptosis	"Extended" protocol (CD95)	Absence of CD95 (FAS) on blood leukocytes in some patients, presence of $CD3^+/CD4^-/CD8^-$ T-lymphocytes[24]
Deficiency of IL12, IFNγ receptor, or IL12 receptor (recurrent mycobacterial infections, salmonellosis)	Disturbed communication between T-lymphocytes/NK-cells and monocytes; failure of monocyte activation by T-lymphocytes/NK-cells	"Extended" protocol (IL12, IFNγR, IL12R)	Absence of IL-12 receptor on T-lymphocytes and NK-cells, or absence of IFN-γ receptor or intracellular IL-12 in monocytes

Table 1. continued

Granulocyte/monocyte Deficiencies (Superficial and systemic infections with pyogenic bacteria and fungi)			
Chronic granulomatous disease (X-linked or autosomal recessive)	Defective microbial killing due to a defect in one of the five NADPH oxidase components	Generally not used; "Extended" protocol (b558)	Normal immunophenotyping results; focus on defective microbial killing in granulocyte function tests; (in patients with X-linked CGD the expression of cytochrome b558 may be defective)
Leukocyte adhesion deficiency type 1 (autosomal recessive)	Defective leukocyte adhesion due to a defect in CD18, the integrin β_2 chain	"Extended" protocol (CD18)	Absence of CD18 expression, otherwise normal
Leukocyte adhesion deficiency type 2 (autosomal recessive)	Defective leukocyte adhesion due to a defect in CD15s, sialyl-Lewis-X	"Extended" protocol (CD15s)	Absence of CD15s expression, otherwise normal
Complement Deficiencies (Neisserial infections, serious bacteremic infections, collagen-vascular diseases, sometimes asymptomatic)			
C2 deficiency (autosomal recessive)	Impaired opsonization due to classical pathway defect sometimes leading to serious bacteremic infections	Not useful	Normal immunophenotyping results; focus on determination of serum complement activity
C3 deficiency, factor I deficiency (autosomal recessive), factor D deficiency	Seriously impaired opsonization leading to a phenotype similar to that seen in agammaglobulinemia	Not useful	Normal immunophenotyping results; focus on determination of serum complement activity
C6, C7, C8, C9 deficiency (autosomal recessive), properdin deficiency (X-linked)	Impaired terminal complement pathway leading to predisposition to neisserial infections; exact pathogenesis not clear	Not useful	Normal immunophenotyping results; focus on determination of serum complement activity

pletely normal in these children (Table 1).

It is useful to perform immunophenotyping of lymphocytes according to the "limited" protocol in Table 2, when a rare severe antibody deficiency with hypo- or agammaglobulinemia is found. Determination of T-lymphocyte counts generally rules out severe combined immunodeficiency (SCID), and determination of B-lymphocyte counts supports the discrimination between the various causes of severe antibody deficiency (Table 1).[21] If immunophenotyping of lymphocytes shows that B-lymphocytes are virtually absent in the peripheral blood compartment (Figure 1), this strongly suggests an early stop in B-lymphocyte development. This occurs in X-linked agammaglobulinemia with Bruton's tyrosine kinase (BTK)-deficiency as well as in rare autosomal recessive forms of agammaglobulinemia, such as Igμ heavy chain deficiency. Using a McAb directed against BTK, it is now possible to detect BTK-deficiency in most patients, by analysing the monocytes with flow cytometry; normal monocytes also express BTK.[14] Furthermore, the type of B-cell differentiation arrest in bone marrow can be assessed with a special research protocol (Figure 2) and can direct the diagnostic analysis at the molecular level (Figure 3).

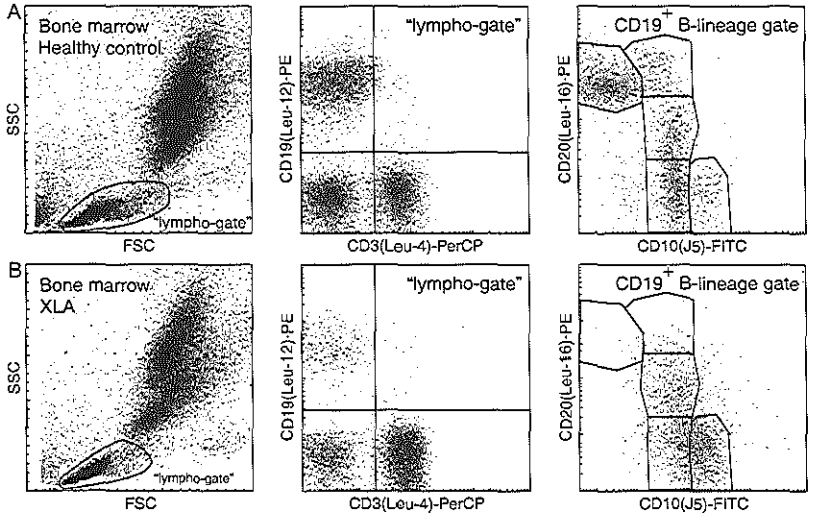

Figure 2. Flow cytometric immunophenotyping of lymphocytes in the bone marrow of a BTK-deficient X-linked agammaglobulinemia patient as compared to a healthy control.
A, Analysis of bone marrow of a healthy child (bone marrow donor). Dot plot with forward scatter (FSC) and side scatter (SSC) showing "lympho-gate" (left). $CD3^+$ T-lymphocytes from contaminating peripheral blood are present in the right lower quadrant (middle). $CD19^+$ B-lineage cells are present in the left upper quadrant (middle). CD19 is present on all B-lineage cells from the pre-B-I cell stage onwards. CD10 is expressed on immature B-lineage cells, and CD20 is expressed on mature B-lymphocytes. Immature $CD10^+/CD20^-/CD19^+$ precursor-B-cells as well as mature $CD10^-/CD20^+/CD19^+$ B-lymphocytes are present in the right lower and left upper regions, respectively (right). *B*, Analysis of bone marrow of a BTK-deficient X-linked agammaglobulinemia patient (same patient as Figure 1). Dot plot with FSC and SSC showing "lympho-gate" (left). Few $CD19^+$ B-lineage cells are present (middle), which are mostly immature $CD10^+/CD20^-/CD19^+$ precursor-B-cells (right).

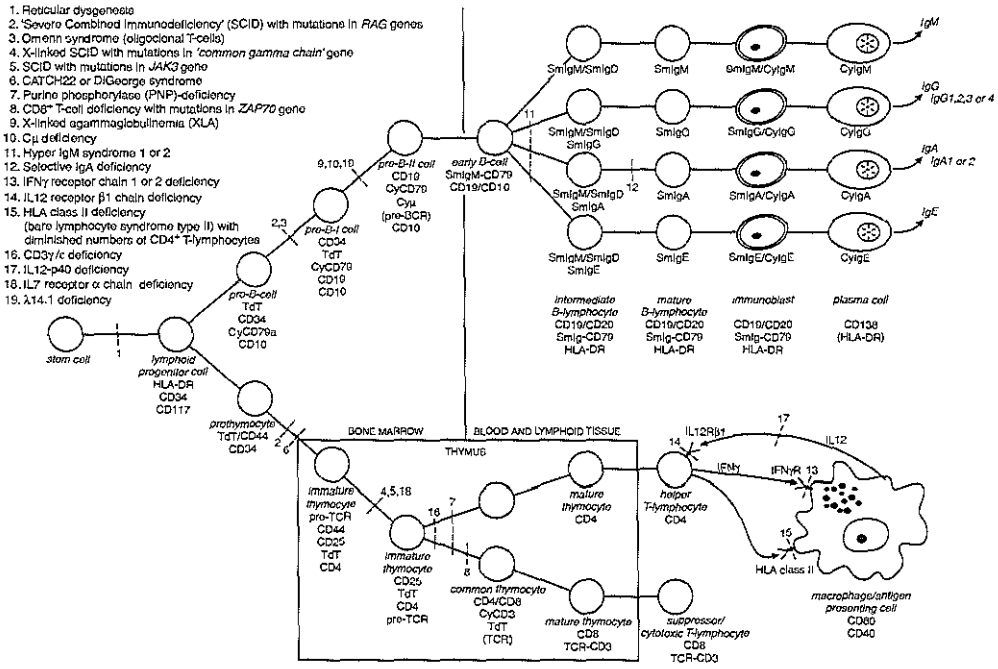

Figure 3. Scheme of lymphocyte differentiation in the bone marrow and thymus.
Relevant cell surface and intracellular markers are indicated. Dotted lines show the assumed positions of the differentiation arrests in various primary immunodeficiency diseases.
Cy = cytoplasmic, Sm = surface membrane.

If B-lymphocytes are found to be present in the peripheral blood compartment of patients with a severe antibody deficiency, common variable immunodeficiency or hyper-IgM syndrome are more likely diagnoses. In common variable immunodeficiency, progressive hypogammaglobulinemia with combinations of IgA deficiency, IgG-subclass deficiency, and/or anti-polysaccharide antibody deficiency can be found. Immunophenotyping of blood lymphocytes can show accompanying T-lymphocytopenia.[25] In X-linked hyper-IgM syndrome with a defect in CD40 ligand (CD40L), severe hypogammaglobulinemia with normal or increased IgM is found. Immunophenotyping of activated blood T-lymphocytes can show absence or decreased expression of CD40L (Figure 4; "extended" protocol in Table 2).[16] In some patients CD40L is expressed but not functional; tests for CD40-binding can identify these patients. The defect in CD40L disrupts the CD40L-CD40 mediated T-B cell communication, which is indispensable for isotype switching from IgM to IgG, IgA or IgE. Consequently, X-linked hyper-IgM syndrome is not a pure antibody deficiency, but but should be considered as a T-lymphocyte disorder, which probably explains why affected children often present with an opportunistic infection. The definitive diagnosis of X-linked hyper-IgM syndrome can be made via mutation analysis of the *CD40L* gene. The autosomal recessive form of hyper-IgM syndrome is caused by mutations in the activation-induced cyti-

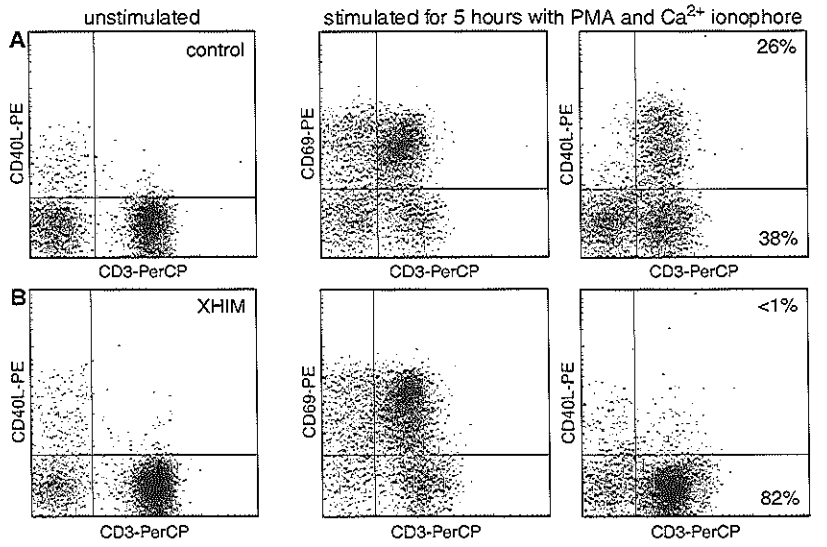

Figure 4. Flow cytometric analysis of CD40L expression on activated T-lymphocytes in the peripheral blood of an X-linked hyper-IgM syndrome patient.
A. Analysis of stimulated peripheral blood of a healthy control (PMA and Ca-ionophores for 5 hours). Unstimulated $CD3^+$ T-lymphocytes do not express CD40L (left). Expression of the early activation marker CD69 (middle) and CD40L (right) upon activated $CD3^+$ T-lymphocytes. *B.* Analysis of stimulated peripheral blood of an X-linked hyper-IgM syndrome patient. This boy presented at the age of 8 months with chronic diarrhoea, and has undergone a bone marrow transplantation at the age of 5 years. $CD3^+$ T-lymphocytes are normally present (left), and show normal expression of CD69 (middle), but absent expression of CD40L (right) upon activation.

dine deaminase (*AID*) gene. AID proteins are specifically expressed in germinal centre B-cells in secondary lymphoid organs and are required for immunoglobulin isotype switching as well.[23]

Children with failure to thrive, intractable diarrhea, or with an opportunistic infection

Many children with T-lymphocyte or combined immunodeficiency do not present with recurrent infections, but with failure to thrive or intractable diarrhoea.[26] The differential diagnosis is extensive in these cases. The combination of lymphopenia and agammaglobulinemia can direct the search towards an immunodeficiency, but these are not invariably present. Opportunistic infections almost always indicate an immunodeficiency, either acquired (see below) or congenital.

The "limited" immunophenotyping protocol as shown in Table 2 can easily detect severe combined immunodeficiency disease. Generally, T-lymphocytes will be absent or very low in number.[21] However, transplacental transfer of maternal T-lymphocytes in SCID patients, or remaining (oligoclonal) T-lymphocyte production in Omenn syndrome (see Table 1) might coincidentally lead to seemingly normal T-lymphocyte counts. The presence or

absence of B-lymphocytes differentiates between B^+ SCID and B^- SCID, as found in X-linked common γ-chain deficiency or autosomal recessive JAK3 deficiency and in RAG1 or RAG2 deficiency, respectively (Figure 5). Major changes in either the $CD4^+/CD3^+$ helper T-lymphocyte count or the $CD8^+/CD3^+$ suppressor/cytotoxic T-lymphocyte count can be suggestive for the diagnosis of MHC class-II deficiency with low $CD4^+/CD3^+$ counts,[5] or a diagnosis of ZAP70 deficiency with low $CD8^+/CD3^+$ counts, respectively (Figure 3; Table 1). However, in many cases of combined immunodeficiency immunophenotyping results are less aberrant with lymphopenia developing over time. Additional tests are then required to come to a diagnosis. Lymphopenia develops within months after birth in adenosine deaminase or purine nucleotide phosphorylase deficiency due to metabolic poisoning of the cells. Lymphopenia also gradually develops in the first years after birth in Wiskott-Aldrich syndrome and towards adolescence or even adulthood in ataxia teleangiectasia (Table 1).[10]

If immunodeficiency is highly suspected, but the "limited" immunophenotyping protocol shows no abnormalities, an "extended" immunophenotyping protocol as shown in Table

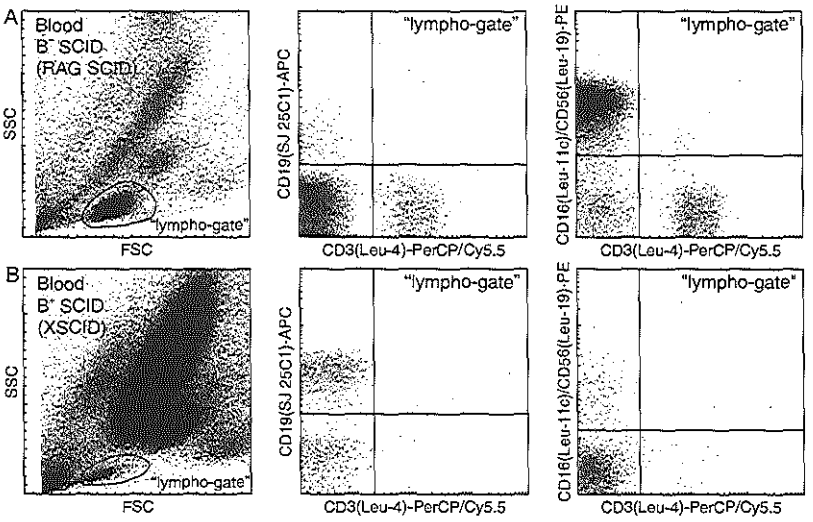

Figure 5. Flow cytometric immunophenotyping of blood lymphocytes in severe combined immunodeficiency patients.

A. Analysis of peripheral blood of a B^- severe combined immunodeficiency (SCID) patient with a mutation in the *RAG2* gene. This girl presented at the age of 12 months with recurrent otitis media and respiratory infections, and chronic diarrhoea. Dot plot with FSC and SSC shows the "lympho-gate" (left). Within the "lympho-gate", virtually no $CD19^+$ B-lymphocytes were detected (middle), and strongly reduced $CD3^+$ T-lymphocyte counts were found (right), whereas the NK-cell counts ($CD3^-/CD56^+/CD16^+$) were within the normal range for age (right). *B*. Analysis of peripheral blood of a B^+ X-linked SCID patient with a mutation in the γ_c gene. This boy presented at the age of 4 months with pneumonia, diarrhoea and sepsis. Dot plot with FSC and SSC shows the "lympho-gate" (left). Within the "lympho-gate" $CD19^+$ B-lymphocytes were detected (middle), but $CD3^+$ T-lymphocytes were absent (right), and the NK-cell counts ($CD3^-/CD56^+/CD16^+$) were decreased (right).

2 can identify additional children with specific subtypes of T-lymphocyte or combined immunodeficiency that are not accompanied by major changes in the main lymphocyte populations (Table 1). For example, in MHC class-II deficiency the absence of HLA-DR surface expression gives the clue to the diagnosis;[5] CATCH-22 (DiGeorge) syndrome is sometimes accompanied by severe T-lymphocytopenia with low to absent TCR$\alpha\beta^+$ T-lymphocytes and normal TCR$\gamma\delta^+$ T-lymphocyte counts (J.J.M. van Dongen, unpublished observation). In

Table 2. Flow cytometric immunophenotyping protocol for the diagnosis of primary immunodeficiency diseases in paediatric practice.

"Limited" protocol
(performed in many hospitals; use absolute counts and age-matched reference values[6])

T-lymphocytes	$CD3^+$	total T-lymphocytes
	$CD4^+/CD3^+$	helper subset
	$CD8^+/CD3^+$	suppressor/cytotoxic subset
B-lymphocytes	$CD19^+$ or $CD20^+$	total B-lymphocytes
NK-cells	defined as CD3 - as well as $CD16^+$ and/or $CD56^+$ population	
Quality control	the "lymphosum" should equal 92-98% (= % T + % B + % NK)	

"Extended" protocol
(performed in specialised laboratories only; select relevant items based on the information presented in Table 1; always perform the "limited" protocol as well; compare with normal age-matched controls run in parallel, if no reference values are available)

Analysis of the arrest in B-cell development in the bone marrow, and of the intracellular expression of BTK in monocytes of patients with agammaglobulinemia with absent B-lymphocytes.

Common γ-chain expression (CD132) on lymphocytes in SCID patients with B-lymphocytes.

CD40 ligand expression (CD154) on activated T-lymphocytes in hypogammaglobulinemia with normal or high IgM; CD40 ligand function is assessed by CD40 binding assays.

HLA-DR (class-II) and β_2 microglobulin (class-I) expression on leukocytes in children suspected of severe combined immunodeficiency with normal lymphocyte numbers and phenotype on initial screening.

TCR$\alpha\beta$- and TCR$\gamma\delta$-expression on T-lymphocytes in children with neonatal hypocalcemia and congenital heart disease.

Extensive specialised protocols in unclassifiable cases of combined immunodeficiency with stainings for 'naive' (CD45RA) and 'memory' (CD45RO) T-lymphocytes, for markers of activation and proliferation, for co-stimulatory and signalling molecules, and for intracellular cytokines (T-helper 1 and T-helper 2 profiles).

Extracellular cytochrome b558 expression in phagocytes if chronic granulomatous disease is suspected in children with phagocyte function deficiency.

CD18 and CD15s expression on leukocytes if leukocyte adhesion deficiency type 1 or type 2 is suspected in children with phagocyte function deficiency and extreme leukocytosis.

Analysis of the IL-12 receptor β1 chain on T-lymphocytes/NK-cells, and of intracellular IL-12 and the IFN-γ receptor chains on monocytes in children with recurrent mycobacterial infections.

unclassifiable cases, extensive specialised immunophenotyping protocols may help to unravel the immunodeficiency.[12, 13, 21] These protocols may include markers of maturation, such as CD45RA ("naive") and CD45RO ("memory") on T-lymphocytes, markers of activation and/or proliferation, co-stimulatory and/or signalling molecules, and intracellular cytokines, such as IL2 and IFNγ (T-helper 1 profile), and IL4, IL5, and IL10 (T-helper 2 profile). These tests are generally not performed in routine laboratories, and will not be further discussed here.

Children with superficial and systemic infections with pyogenic bacteria or fungi

Most children with superficial and systemic infections with pyogenic bacteria or fungi suffer from neutropenia due to haematological disorders or iatrogenic causes (see below). Flow cytometric immunophenotyping of leukocytes plays only a minor role in the diagnostic process in these children. A defect in granulocyte/monocyte function, such as in chronic granulomatous disease or leukocyte adhesion deficiency, is rare. In chronic granulomatous disease, granulocytes show deficient microbial killing due to a defect in one of the five NADPH oxidase complex components.[21] This can be investigated by function tests of granulocytes. Flow cytometric immunophenotyping with McAb directed against cytochrome b558 can show absence of this compound in many but not all patients with the most frequent (65%) X-linked form of chronic granulomatous disease ("extended" protocol in Table 2)[3]. In leukocyte adhesion deficiency type 1 (CD18 deficiency) and type 2 (CD15s deficiency) leukocyte adhesion is impaired. This leads to leukocytosis, which is even more pronounced during infections.[21] CD18 and CD15s expression can be analysed with immunophenotyping ("extended" protocol in Table 2; Figure 6).[1, 11]

Children with recurrent particular infections

Deficiencies in one of the components of the terminal complement pathway result in recurrent neisserial -mainly meningococcal - infections (C6 deficiency, C7 deficiency, C8 deficiency, and C9 deficiency; also properdin deficiency).[15] Lymphocyte number and

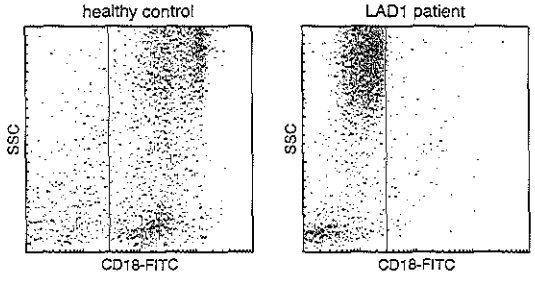

Figure 6. Flow cytometric detection of CD18 molecules in the diagnosis of leukocyte adhesion deficiency type 1.
In the healthy control (left panel), virtually all leukocytes express the CD18 molecule whereas the leukocytes of the leukocyte adhesion deficiency type 1 patient are deficient for CD18 expression (right panel).

immunophenotype are normal in these children (Table 1). Deficiencies in the IFN-γ receptor, IL-12 or the IL-12 receptor lead to recurrent mycobacterial infections.[19] The IL-12 receptor can be detected on T-lymphocytes or NK-cells, and the IFN-γ receptor can be detected on monocytes by flow cytometry. Information about the expression patterns of these molecules can direct the diagnostic analysis at the molecular level, but it is important to realise that protein expression does not necessarily imply intact protein function.

FOLLOW-UP OF CHILDREN WITH ACQUIRED IMMUNODEFICIENCY

During follow-up of children treated with immunosuppressive drugs or chemotherapy, the degree of neutropenia is easily assessed with a leukocyte count and differential. The slow reconstitution of lymphocytes after bone marrow transplantation[2] can be monitored by a simple immunophenotyping protocol to determine the numbers of the main lymphocyte populations ("limited" protocol in Table 2). It should be kept in mind, however, that functional restoration of the lymphoid system takes more time than is needed for the normalisation of absolute counts.[22]

HIV-infection is generally diagnosed by serology or PCR. Immunophenotyping has a role in the follow-up of HIV-infected children with the determination of decreasing $CD4^+/CD3^+$ helper T-lymphocyte counts as a prognostic marker for the development of AIDS: long-term risk of mortality increases from 33% to 97% with baseline $CD4^+$ percentage decreasing from ≥35% to <5%.[18] It also has a role - together with HIV RNA quantification - for the timing of antiretroviral therapy. The $CD4^+$ helper T-lymphocyte count is used for the classification of the degree of immune suppression: ≥1500/μl under 12 months of age, ≥1000/μl from 1-5 years of age, and ≥500/μl from 6-12 years of age indicate the absence of immune suppression, whereas <750/μl under 12 months of age, <500/μl from 1-5 years of age, and <200/μl from 6-12 years of age indicate severe suppression.[27] During antiretroviral therapy monitoring of $CD4^+$ helper T-lymphocyte counts is useful for evaluating the treatment effectiveness[20] in addition to HIV RNA quantification.[27] For further guidelines, the reader is referred to the literature.[27]

More elaborate immunophenotyping protocols can be reserved for research purposes, e.g. for comparison of immune reconstitution after bone marrow transplantation with normal ontogeny. Such studies are important for understanding the regeneration processes, but have no direct clinical relevance as yet.

CONCLUSION

Immunophenotyping of leukocytes forms an important part of the diagnostic work-up in children with suspected immunodeficiency. A "limited" protocol (Table 2) can be per-

formed in many hospitals. This enables the general paediatrician to determine the presence and number of the main lymphocyte populations to identify (severe) combined immunodeficiency patients at an early stage, and to direct the diagnostic work-up in agammaglobulinemic patients by determining the presence or absence of B-lymphocytes. In selected cases, a set of extra measurements can be performed in specialised laboratories ("extended" protocol in Table 2). This identifies children with immunodeficiencies that are not accompanied by major changes in the main lymphocyte populations. However, it should be noted that not all children with an immunodeficiency show alterations in immunophenotyping results (Table 1). Also, genetic mutations do not always lead to absence of protein expression, even if protein function is severely hampered.

More extensive immunophenotyping protocols used in specialised laboratories or research settings help to recognise new immunodeficiency diseases by identifying as yet undescribed staining patterns. Also, pathogenesis of known defects can be further elucidated: the absence of specific cell surface markers,[16, 21] the loss of specific epitopes defined by McAb staining patterns,[9, 17] or the determination of the B-cell differentiation arrest in bone marrow can support the precise characterisation of the defect. Therefore, it is important to use these more extensive immunophenotyping protocols in patients with an established immunodeficiency disease, as well as in patients with probable immunodeficiency disease that has not been proven with other methods.

Due to the rapid developments in the field of flow cytometry and antibody production on the one hand and the recognition of new immunodeficiency diseases on the other hand, it is likely that the "limited" and "extended" immunophenotyping protocols defined here will have to be updated in a few years time.

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Chapter 3

IMMUNOGENOTYPING IN THE DIAGNOSIS OF PRIMARY IMMUNODEFICIENCY DISEASES OF THE LYMPHOID SYSTEM

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INTRODUCTION

Although the clinical phenotype (sex, type of infection, and family history), and the immunophenotyping results of peripheral blood (PB) and/or bone marrow (BM) (absolute counts of T-, B-, and NK-cells, and expression profiles of specific proteins) provide powerful information for the diagnosis of primary immunodeficiency diseases (PID) of the lymphoid system, the definitive diagnosis can only be made after mutation analyses of suspected genes. In the last decade, a large number of genes involved in the pathogenesis of PID have been identified (Table 1), enabling the genetic diagnosis in affected patients. However, the identification of a mutated gene might not always result in a correct diagnosis of PID, as genetic polymorphisms might erroneously be interpreted as disease-causing mutations, particularly in rare forms of PID where mutation studies are scarce. Functional studies using cloned wild type (wt) and mutated genes are required, but not always available, in rare forms of PID. This chapter provides information on the diagnostic and technical strategies used for the genetic analysis of 16 different genes involved in the pathogenesis of lymphoid PID (Table 2).

The diagnostic laboratory process can be separated into pre-analytical, analytical, and post-analytical phases. In the pre-analytical phase the genetic differential diagnosis is formulated, based on clinical information and immunophenotyping results. The analytical phase mainly consists of technical strategies, while the post-analytical phase involves the correct interpretation of results and drawing of conclusions. For convenience of the readership, the analytical and post-analytical phases are combined in this chapter.

DIAGNOSTIC STRATEGIES: PRE-ANALYTICAL PHASE

Choice of target gene

The target gene is chosen based on clinical and immunophenotyping information. First, it is important to realize that most immunodeficiencies are acquired, for example by loss or deficient uptake of proteins, chemotherapy, or virus infections. When a PID is highly suspected, the family-history, sex of the patient, consanguinity of the parents, type of infections, age at clinical diagnosis, and serum immunoglobulin (Ig) titers are some of the most important clinical parameters. Based on these parameters a clinical differential diagnosis is formulated.

This clinical differential diagnosis will become more focussed by the results of the immunophenotyping analyses of peripheral blood (PB), bone marrow (BM), or lymph nodes. It is important to realize that protein expression detected by flow cytometry does not exclude a mutation in the coding gene.[1] Subtle point mutations leading to amino acid (aa) substitutions can affect the function but not the expression of the protein. Even small deletions causing frame-shifts and subsequent premature stop codons can sometimes be encompassed by the

Table 1. Immunogenetic aspects of primary immunodeficiency diseases.

Immunodeficiency [a]	Inheritance [b]	Chromosome localization of affected gene	Affected gene	Estimated frequency of live births (estimated number of patients in The Netherlands)
Combined immunodeficiencies (CID) (19%)				(5 new patients per year) Total CID: 5-10 per 10^6
- X-linked SCID (SCID-X1)	X	Xq13.1	*CD132/γ_c*	50% of CID
- JAK3 deficient SCID	AR	19p13.1	*JAK3*	rare
- RAG deficient SCID	AR	11p13	*RAG1/RAG2*	20% of CID
- Radiosensitive SCID	AR	10p	*Artemis*	rare
- IL7 receptor α chain deficient SCID	AR	5p13	*CD127/IL7R*	rare
- IL2 receptor α chain deficient SCID	AR	10p15-p14	*CD25/IL2RA*	rare
- CD45 deficient SCID	AR	1q31-q32	*CD45/PTPRC*	rare
- NK-cell deficient SCID	AR	?	–	rare
- Other autosomal recessive inherited SCID	AR	?	–	10% of CID
- ADA deficient SCID	AR	20q13.11	*ADA*	10% of CID
- PNP deficient SCID	AR	14q13.1	*PNP*	rare
- HLA-class II deficient SCID (bare lymphocyte syndrome type II)	AR	several genes	*RFX5/CIITA RFXANK/RFXAP*	rare
- HLA-class I deficient SCID (bare lymphocyte syndrome type I)	AR	6p21.3	*TAP2*	rare
- CD8 T-cell deficient SCID	AR	2q12	*ZAP70*	rare
- CD3 gamma or epsilon chain deficient SCID	AR	11q23	*CD3G/CD3E*	rare
- Reticular dysgenesis	AR	?	–	rare
Antibody deficiencies (67%)				(15 new patients per year)
- X-linked agammaglobulinemia (XLA)	X	Xq21.3-22	*BTK*	10-15 per 10^6 boys
- C_μ deficiency	AR	14q32.33	*IGHM*	rare
- λ5/14.1 deficiency	AR	22q11.23	*CD179B/IGLL1*	rare
- CD79a deficiency	AR	19q13.2	*CD79A*	rare
- B-cell linker protein (BLNK) deficiency	AR	10q23.2-q23.33	*BLNK*	rare
- X-linked hyper IgM syndrome (HIGM1)	X	Xq26	*CD154/CD40L*	rare
- Non X-linked hyper IgM syndrome (HIGM2)	AR	12p13	*AICDA*	rare
- CD40 deficiency (HIGM3)	AR	20q12-q13.2	*CD40*	rare
- *IGH* gene deletions	AR	14q32.3	*IGH*	1-3 per 10^4
- IgG subclass deficiency	?	?	–	unknown
- Common variable immunodeficiency (CVID)	AR	? (HLA association?)	–	10-20 per 10^6
- Selective IgA deficiency	AR?/AD?	? (HLA association?)	–	1 per 10^3

Table 1. continued

Immunodeficiencies with other severe symptoms (2%)				(2 new patients per year)
- Wiskott-Aldrich syndrome (WAS)	X	Xp11.22	*WASP*	1-5 per 10^6 boys
- Ataxia telangiectasia (AT)	AR	11q22.3	*ATM*	10-20 per 10^6
- DiGeorge syndrome / CATCH22 / velocardiofacial syndrome	AD	deletion 22q11	–	1-2 per 10^6 (complete DGS)
- NK-cell deficiency	AR	1q23	*CD16/FCGR3A*	rare
- Auto-immune lymphoproliferative syndrome (ALPS) type 1A	AR/AD	10q24.1	*CD95/FAS*	rare
- ALPS type 1B	AD	1q23	*CD178/FASL*	rare
- ALPS type II	AR	2q33-q34	*CASP10*	rare
- Osteopetrosis	AR	11q13.4-q13.5	*TCIRG1*	rare
- Familial hemophagocytic lympho-histiocytosis (HPLH 2)	AR	10q21-22	*PRF1*	rare
- Griscelli syndrome	AR	2 genes on 15q21	*MYO5A/RAB27A*	rare
- Chediak-Higashi syndrome	AR	1q42-43	*CHS1*	rare
- X-linked lymphoproliferative disease (XLP)	X	Xq25-q26	*SH2D1A*	rare
- anhydrotic ectodermal dysplasia with immunodeficiency	X	Xq28	*IKBKG*	rare
- immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance (IPEX)	X	Xp11. 23-Xq13.3	*FOXP3*	rare
- Nijmegen breakage syndrome	AR	8q21-q24	*NBS1*	rare
- Immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome	AR	20q11.2	*DNMT3B*	rare
Defects in the complement system (5%)				(1 new patient per year)
- C1q, C1r, C4, C2, C3, C5, C6, C7, C8 en C9 deficiencies	AR	autosomal chromosomes	+ (multiple genes)	rare, except for partial C2 deficiency: 1 per 10^3
- Properdine deficiency (type I, II, or III)	X	Xp11.3-p11.23	*PFC*	rare
- C1-inhibitor-deficiency	AD	11q12-q13.1	*C1NH*	10-20 per 10^6
Phagocyte defects (8%)				(2 new patients per year)
- Chronic granulomatous disease (CGD)				Total CGD: 1-5 per 10^6
. X-linked CGD: gp91^{phox} deficiency, 91 kDa β chain of cytochrome b (CYBB)	X	Xp21.1	*CYBB*	60-65% of CGD
. AR-CGD: p22^{phox} deficiency, 22 kDa α chain of cytochrome b (CYBA)	AR	16q24	*CYBA*	5-10% of CGD
. AR-CGD: p47^{phox} deficiency	AR	7q11.23	*NCF1*	30% of CGD
. AR-CGD: p67^{phox} deficiency	AR	1q25	*NCF2*	5% of CGD
- Leukocyte adhesion defect (LAD)				
. LAD-1: deficiency of β_2 chain (CD18 antigen) of CD11-CD18 molecules	AR	21q22.3	*CD18/ITGB2*	rare
. LAD-2: defect in fucosylation of selectin ligands, like Sialyl-Lewis X (SLeX; CD15s)	AR	?	–	rare

Table 1. continued

- Glucose-6-phosphate dehydrogenase (G6PD) deficiency in granulocytes	X	Xq28	*G6PD*	rare
- Myeloperoxidase (MPO) deficiency	AR	17q23.1	*MPO*	250-500 per 10^6
- Interferon γ receptor chain 1 deficiency	AR	6q23-q24	*CD119/IFNGR1*	rare
- Interferon γ receptor chain 2 deficiency	AR	21q22.1-q22.2	*IFNGR2*	rare
- IL12 (p40) deficiency	AR	5q31.1-q33.1	*IL12B*	rare
- IL12 receptor β1 chain deficiency	AR	19p13.1	*IL12RB1*	rare
- STAT1 deficiency	AD	2q32.2	*STAT1*	rare

a Classification according to WHO and Conley.77, 78

b X = X-linked; AR = autosomal recessive; AD = autosomal dominant.

Table 2. Molecular characteristics of genes involved in the pathogenesis of lymphoid PID.

Immunodeficiency	Involved gene	Gene structure		Protein
		size	exons	
X-linked SCID (SCID-X1)	*CD132 / γ_c*	4.6 kb	8	369 aa (42 kD)
JAK3 deficient SCID	*JAK3*	14 kb	21	1124 aa (125 kD)
Non-T non-B SCID ┐	*RAG1*	9.9 kb	2	1043 aa (119 kD)
Omenn syndrome ┘	*RAG2*	7.1 kb	3	527 aa (59 kD)
Radiosensitive non-T non-B SCID	*Artemis*	?[a]	14	692 aa (≈70 kD)
IL7R α chain deficient SCID	*CD127 / IL7R*	5.2 kb	8	459 aa (51 kD)
X-linked agammaglobulinemia (XLA)	*BTK*	38 kb	19	659 aa (76 kD)
Igμ deficient agammaglobulinemia	*IGHM*	4.4 kb	6	454 aa (49 kD)
CD79a deficient agammaglobulinemia	*CD79A*	4.4 kb	5	226 aa (25 kD)
λ14.1 deficient agammaglobulinemia	*CD179B / IGLL1*	?[a]	3	213 aa (22 kD)
B-cell linker protein deficient agammaglobulinemia	*BLNK*	65 kb	17	456 aa
X-linked hyper IgM syndrome (HIGM1)	*CD154 / CD40L*	13 kb	5	261 aa (29 kD)
Non X-linked hyper IgM syndrome (HIGM2)	*AICDA*	11 kb	5	198 aa (24 kD)
Wiskott-Aldrich syndrome (WAS)	*WASP*	9 kb	12	502 aa (52 kD)
Auto-immune lymphoproliferative syndrome (ALPS1A)	*CD95 / FAS*	25 kb	9	335 aa (37 kD)
Mendelian susceptibility to mycobacterial infections	*CDw119 / IFNGR1*	23 kb	7	489 aa (54 kD)

a Until now, only the mRNA sequence has been published.

translation machinery using a downstream UTG translation start site, resulting in an N-terminal truncation of the protein, which does not necessarily affect the protein expression.[2,3]

Cell material, DNA and RNA

As requests for mutation analysis in PID often concern young children, the amount of available cell material might be limited. Therefore, it is important to optimally use the cell material. Mutations causing PID are generally inherited from the parents and are thus present in the germline DNA of the patient, making virtually every cell source suitable for DNA isolation. For example, PB is a readily available source for genetic material, which can be separated in two fractions, using classical Ficoll density centrifugation. The mononuclear cell

(MC) fraction can be frozen in liquid nitrogen and revitalized at a later time point (for example to study the Ig or T-cell receptor (TCR) repertoire), while the granulocytes (which cannot be frozen) can be used to isolate genetic material, DNA and/or RNA.

Mutation analysis can be performed both at the DNA and the mRNA (complementary (c)DNA) level. When cDNA is used for mutation analysis, only cell populations that actually express the gene of interest can be used to obtain mRNA, while DNA can be isolated from any cell source except erythrocytes and thrombocytes.

Mutation analysis at the cDNA level is often used for the diagnosis of PID and is generally less time-consuming compared to mutation analysis at the DNA level, because mRNA does not contain genomic intron sequences and can be amplified with a single or a few PCR primer pairs. However, in case of carrier detection, mutation analysis via cDNA is not reliable as shown in Figure 1. A mutation can render the mRNA unstable, leading to the detection of mRNA from the non-affected wt allele only, thereby causing false-negative results.

TECHNICAL STRATEGIES: ANALYTICAL PHASE

Based on the arguments mentioned above, we prefer mutation analysis at the DNA level. To enhance the speed and specificity of mutation analysis at the DNA level, we have chosen to amplify large stretches of genomic DNA using long-range (LR)-PCR. The resulting PCR fragments usually span around 5,000 base pairs (bp) and can be purified using com-

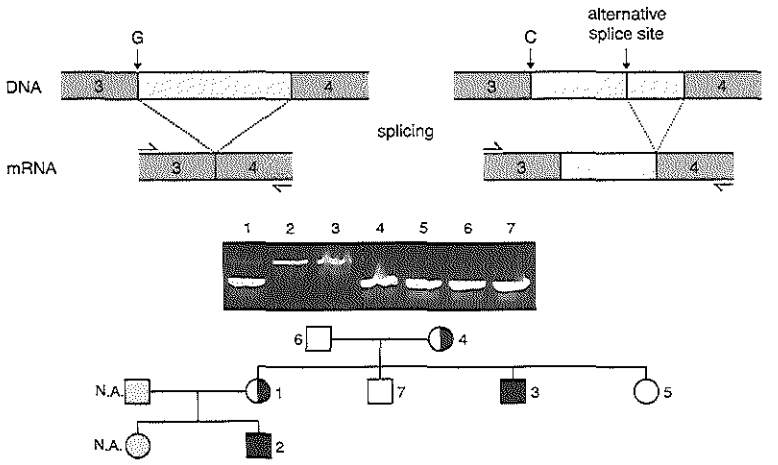

Figure 1. Carrier detection at the mRNA level can be unreliable.
Two male patients from family XLA-18 (Table 3) suffered from a splice site mutation in intron 3 of the *BTK* gene, resulting in the insertion of 106 nucleotides at the mRNA level. This larger mRNA could be detected in both patients (lanes 2 and 3), and in one female carrier (lane 1). However, the maternal grandmother was shown to be a carrier at the genomic DNA level, although the larger mRNA could not be identified (lane 4).

mercial columns (e.g. Qiagen, Chatsworth, CA, USA). The purified and concentrated PCR products are sequenced using internal sequence primers, which are positioned in the introns flanking the exons at more than 50 bp distance from the splice site, in order to detect mutations causing mRNA splicing defects.

One should realize that the PCR-based amplification of a target gene via *Taq* polymerase is not 100% error-free. Consequently, mutations might be introduced during PCR amplification. This pitfall is particularly prominent when the target gene is cloned after PCR amplification, as only one PCR fragment is cloned into a vector. However, when using the entire PCR mixture for direct sequencing, PCR mediated mutations will generally remained undetected. However, when a mutation occurs in one of the first cycles of the amplification reaction, this might lead to a false positive result. Therefore, we use two different PCR reactions for sequencing. One reaction mixture is sequenced with the forward primer, while the other is sequenced with the reverse primer. The chance that a DNA polymerase will introduce the same error at the same position in two independent amplification reactions is negligible.

Some diagnostic laboratories prefer to screen the PCR fragments of a target gene for mutations before sequencing, using for example the single strand conformation polymorphism (SSCP) technique (Figure 2). SSCP frequently shows aberrant migration patterns in case of mutated PCR fragments.[4] Subsequently, only these PCR fragments are sequenced, which saves time. However, SSCP has a sensitivity of approximately 80%, because not all mutations result in an aberrant migration pattern. Furthermore, the availability of automated fluorescent sequencing (e.g. Big-Dye terminators on the ABI Prism 377 from Applied Biosystems, Foster City, CA, USA) has made direct sequencing less time-consuming. We wish to be sure that the identified mutation is the only mutation in the analyzed gene, and therefore we prefer to sequence all exons. SSCP is used in our laboratory to confirm the identified mutation in the affected patient and to perform rapid carrier diagnostics in family members.

GENETIC ANALYSES: ANALYTICAL AND POST-ANALYTICAL PHASES

Antibody deficiencies

X-linked agammaglobulinemia (XLA)

Approximately 85% of all agammaglobulinemia cases are caused by mutations in the gene encoding Bruton's tyrosine kinase (*BTK*), which consists of 19 exons and spans 38 kb (Table 2 and Figure 3).[5] BTK is a cytoplasmic protein of 659 aa, expressed in precursor-B-cells, B-lymphocytes, monocytes and granulocytes, and is probably involved in signal transduction from the (pre-)B-cell receptor (BCR).[6] XLA patients suffer from a severe reduction of B-lymphocytes in their PB, resulting from a differentiation arrest at the pre-BCR-expressing pre-B-II cells in the BM.[7,8] XLA patients have normal numbers of monocytes and granulocytes in their PB, which strongly suggests that BTK is only required for the

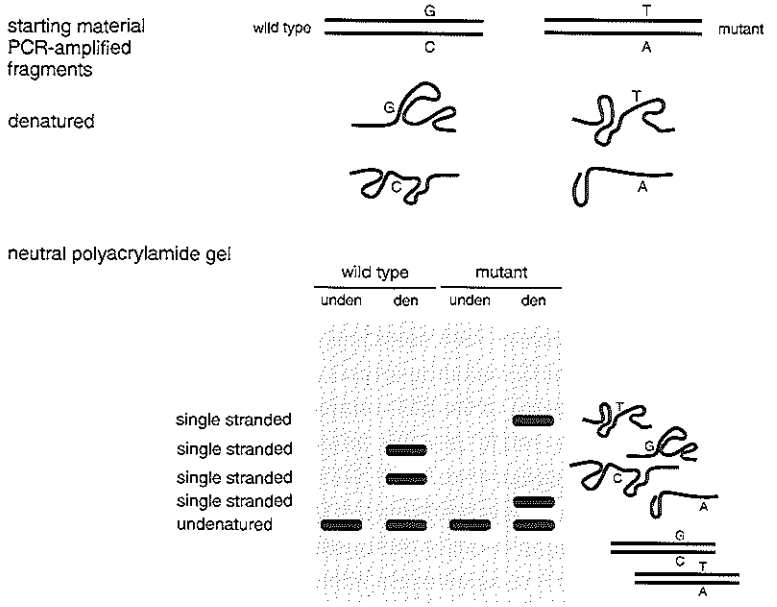

Figure 2. Single strand conformation polymorphism (SSCP) technique.
The migration pattern of single strand PCR products in a non-denaturing gel depends on the sequence of the PCR products. However, this effect also depends on the size of the PCR products (Figure adapted from Prosser).[4]

development of B-lymphocytes. Female carriers of *BTK* mutations have normal numbers of PB B-lymphocytes, showing a non-random X-chromosome inactivation pattern, while the monocytes and granulocytes have randomly inactivated X-chromosomes. This phenomenon has been used to develop a rapid technique to identify female carriers. Using flow cytometry

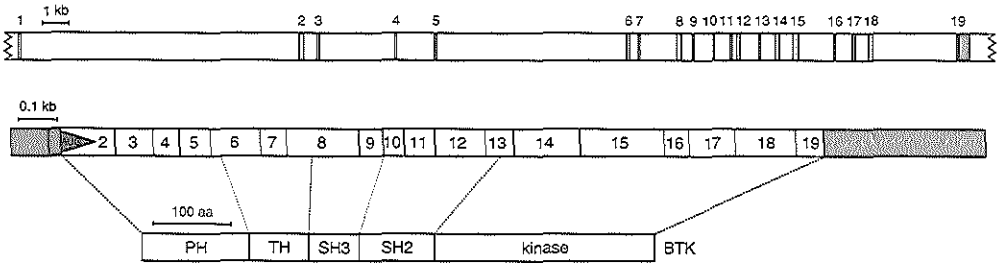

Figure 3. Schematic representation of the *BTK* gene, mRNA and protein.
The *BTK* gene spans 38 kb and consists of 19 exons. The *BTK*-mRNA is expressed in B-lymphocytes, granulocytes, and monocytes in the PB and from a certain stage during precursor B-cell differentiation in the BM. The BTK protein consists of 659 aa and five different domains (PH=pleckstrin homology, TH=Tec homology, SH=Src homology). UTR=untranslated region. The respective scales are indicated and based on Oeltjen *et al.*[5]

with a monoclonal antibody (mAb) against the BTK protein, expression is found in approximately half of PB monocytes in female carriers.[1]

Autosomal recessive (AR) inherited agammaglobulinemia

Based on the information presented above, the gene of interest in male agammaglobulinemia patients without B-lymphocytes will be *BTK*. If the *BTK* gene appears to be unmutated or if the patient is female, several other genes involved in the pathogenesis of AR inherited agammaglobulinemia have been identified, such as *IGH-*C_μ, *λ14.1* (*CD179b*), *CD79a*, and B-cell linker protein (*BLNK*) (Table 2 and Figure 4).[9-13] Interestingly, the protein products of these genes are all components of the pre-BCR or involved in the signal transduction from this receptor. Although few patients with mutations in these genes have been identified until now, it seems that the B-cell differentiation arrests are identical to XLA patients.[14] As these proteins are specifically expressed in the BM, immunophenotyping of BM samples from AR agammaglobulinemia patients might provide information concerning the defective gene. Furthermore, it has been suggested that Igμ, CD79a, and BLNK deficient patients show a complete B-cell differentiation arrest, while in λ14.1/CD179b and BTK deficient patients this arrest can be "leaky", resulting in strongly diminished but detectable numbers of B-lymphocytes in PB.[6] Approximately 5% of agammaglobulinemia cases cannot be explained by mutations in one of the five candidate genes. Therefor, it can be anticipated that new gene defects will be reported in the future, for example in the *VpreB* (*CD179a*), *CD79b*, *PAX5*, or *PLCγ2* genes.

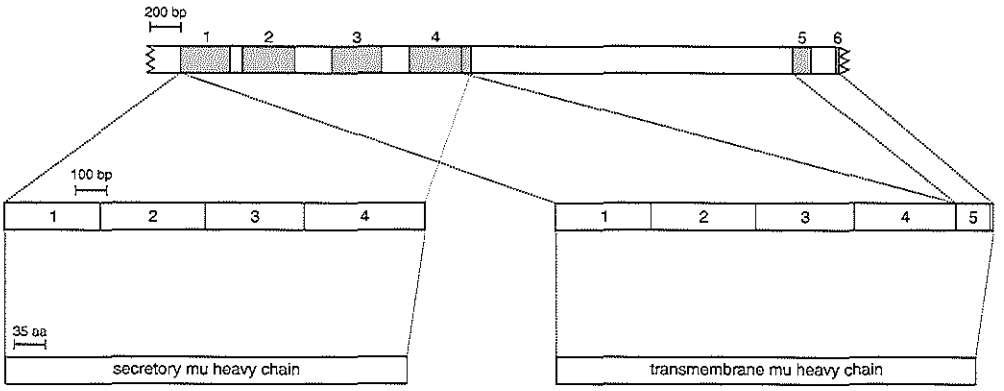

Figure 4. Schematic representation of the C_μ gene, mRNA and protein.
The C_μ gene spans 4.4 kb and consists of 6 exons. Two C_μ-mRNA splice variants can be expressed. Exons 1-4 encode the CyIgμ protein, which consists of 454 aa and is expressed in pre-B-II cells in the BM and in Ig secreting plasma cells in the PB. Exons 1-6 encode the SmIgM protein, which consists of 478 aa and is expressed on immature and mature B cells in the BM and B-lymphocytes in the PB. The respective scales are indicated and based on Friedlander *et al.*[15]

Structure of the IGH-C_μ gene

The *IGH-C_μ* gene segments are located downstream of the variable (V), diversity (D), and joining (J) segments of the Ig heavy chain (*IGH*) gene on chromosome 14q32.3. At the mRNA level, *IGH-C_μ* is spliced to the VDJ recombination product of *IGH*. The *IGH-C_μ* gene consists of 6 exons (Table 2) with an alternative splice site in exon 4. If this alternative splice site is not used, translation will stop at the end of exon 4, leading to the cytoplasmic (Cy) localization of IgM. Usage of the alternative splice site in exon 4 bypasses the termination codon at the end of exon 4, and adds exons 5 and 6 encoding the transmembrane domain to the mRNA, leading to the generation of surface membrane (Sm) IgM (Figure 4).[15]

Hyper IgM syndrome (HIGM)

If patients present with agammaglobulinemia or high levels of serum IgM but low levels of other isotypes, and normal numbers of B, T, and NK cells in the PB, it is more likely that they suffer from HIGM. Three different genetic defects resulting in HIGM have been described (Table 1). Mutations in the *CD40 ligand* gene (*CD40L*) cause the X-linked form of HIGM (HIGM1, Figure 5).[16,17] The CD40L protein (CD154) is expressed on activated CD69+/CD4+ T-lymphocytes, for example activated *in vitro* by phorbol myristate acetate (PMA) and Ca ionophores (Figure 4 in Chapter 2). Mutations in the *CD40L* gene do not only result in an antibody deficiency, but also the T-cell function is affected, as illustrated by the

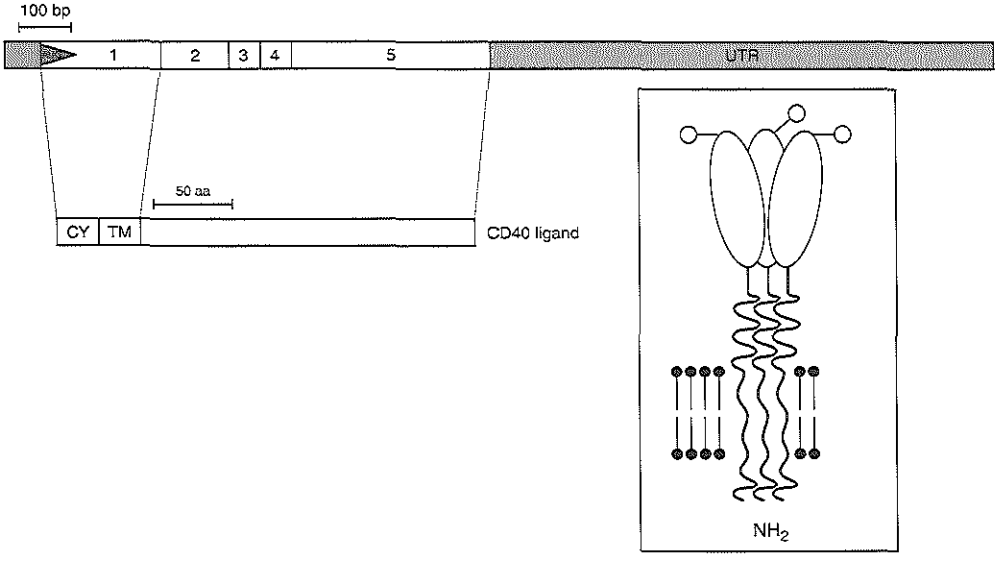

Figure 5. Schematic representation of the *CD40L*-mRNA and protein (CD154).
The *CD40L* gene spans 13 kb and consists of 5 exons. The *CD40L*-mRNA is expressed on activated (CD69+) CD4+ T-lymphocytes in the PB and encodes a protein of 261 aa, which forms a homotrimer (Figure adapted from Barclay *et al.* The Leukocyte Antigen Facts Book). UTR=untranslated region, CY=cytoplasmic region, TM=transmembrane region. The respective scales are indicated and based on Shimadzu *et al.*[71]

Table 3. Mutation analysis in patients presenting with antibody deficiencies (1997-2001).

Family	Mutation			Mother of the index patient [a]	Family members [c]	
	DNA level	RNA level	Protein level		Carrier	Non-carrier
XLA-1 [h]	Exon 17 C53T		R 562 W	Carrier	1	0
XLA-2	Exon 7 26delG		Codon 198 stop	NA	NA	NA
XLA-3	Exon 2 142delTC		Codon 40 stop	NA	NA	NA
XLA-4	Exon 15 G178A		M 509 I	Carrier [f]	2	1
XLA-5	Exon 15 C106A		Y 485 stop	Carrier	1	0
XLA-6	Exon 3 75insA		Codon 84 stop	Carrier [f]	2	0
XLA-7	Exon 14 C98A		Y 425 stop	Carrier	1	1
XLA-8	Exon 18 T10C		M 587 T	Carrier	1	0
XLA-9	Exon 3 G99A	Ins 106 nt intron 3	Codon 119 stop	Carrier	1	0
XLA-10 [d]	Intron 15 A1356C	Del exon 16	Codon 527 stop	Carrier [f]	3	1
XLA-11	Intron 18 G1A	Del last 33 nt exon 18	In frame del aa 626-636	Carrier	1	0
XLA-12	Exon 8-18 large del	Del exon 8-18	In frame del aa 197-636	Carrier [e]	1	2
XLA-13 [d]	Intron 5 G1T	Del exon 5	Codon 104 stop	Carrier [b, f]	3	0
XLA-14	Exon 3 91del 15 nt		Codon 114 stop	NA	NA	NA
XLA-15	Exon 15 T77G		Y 476 D	Carrier [f]	3	1
XLA-16	Exon 8 C175T		R 255 stop	Carrier	1	0
XLA-17	Exon 2 G33T		M 1 I	Carrier	1	0
XLA-18 [d]	Intron 3 G1C	Ins 106 nt intron 3	Codon 119 stop	Carrier	2	1
XLA-19	Exon 8 54delTG		Codon 223 stop	Carrier [b]	1	NA
XLA-20 [h]	Exon 17 C53T		R 562 W	NA	NA	NA
XLA-21	Exon 15 T177C		M 509 T	Carrier [b]	1	NA
XLA-22	Exon 18 C105T		P 619 S	Carrier	1	NA
XLA-23	Intron 4 A1552G	Ins 21 nt intron 4	In frame ins 7 aa	Carrier [b]	1	NA
XLA-24	Exon 5 G63A		W 124 stop	NA	NA	NA
XLA-25	Exon 2 C112T		R28C	NA	NA	NA
XLA-26	Exon 2 C128T		T33I	NA	NA	NA
XHIM-1 [d]	Exon 5 C782T		T 254 M	Carrier	3	1
XHIM-2	Exon 5 T208C		L 206 P	NA	NA	NA
XHIM-3	Exon 5 A253C		Q 221 P	NA	NA	NA
XHIM-4	Exon 5 C69T		Codon 160 stop	Carrier	1	1
XHIM-5 [d]	Exon 1 125del17		Codon 42 stop	Carrier [b]	1	NA
Cmu-1	Large deletion			NA	NA	NA
AICDA-1	Exon 2 G141T [g]		R50L	Carrier [b]	1	2
	Exon 3 G179A [g]		R112H			

[a] The index patient was the oldest analyzed patient in the family.

[b] Carriership in the mother of the index patient was based on family history instead of DNA analysis.

[c] In case of X-linked inheritance only the female family members were analyzed. The mother of the index patient was included as well.

[d] In these families two patients were analyzed.

[e] Maternal grandmother non-carrier: de novo mutation.

[f] Maternal grandmother carrier: origin of mutation not localized.

[g] Both heterozygous mutations were located on the same allele.

[h] Identical mutations in patients from different families.

Ins = insertion; Del = deletion; NA = not analyzed.

clinical observation that HIGM1 patients often present with *Pneumocystis carinii* pneumonia (PCP), which is an opportunistic infection indicative for T-cell dysfunction.

The AR form of HIGM can be caused by mutations in the activation-induced cytidine deaminase gene (*AICDA*) (HIGM2) or the *CD40* gene.[18-20] The *AICDA* gene consists of 5 exons and is expressed in germinal center B-lymphocytes only. CD40 protein is expressed constitutively on B-lymphocytes and antigen-presenting cells (APC).

The results of our mutation analyses (1997-2001) in patients with antibody deficiencies and their family members are shown in Table 3.

Severe combined immunodeficiency diseases (SCID)

As SCID consists of a heterogeneous group of diseases, immunophenotyping of PB from SCID patients, focussing on absolute counts of T-lymphocytes (CD3, CD4, and CD8), B-lymphocytes, and NK cells, and expression of HLA class I and II, is of utmost importance to identify the target gene. SCID patients in general present with absence or dysfunction of PB T-lymphocytes, resulting in opportunistic infections. However, transplacental T-cell engraftment from mother to fetus has been described and this phenomenon can result in confusing immunophenotyping results. HLA typing or genetic fingerprinting of PB T-lymphocytes from mother and child or additional labelings, such as CD45RA versus CD45RO, may solve this problem.[21]

$T^-/B^+/NK^+$ SCID

An isolated T-cell deficiency can be caused by defects in the interleukin (IL) 7 receptor (R) α chain (CD127),[22-24] IL2Rα chain (CD25),[25] CD45,[26,27] HLA class II expression (diminished $CD4^+$ T-lymphocyte counts, complementation groups A, B, C, and D),[28-31] HLA class I expression (absolute T-cell counts can be normal),[32] zeta-chain-associated protein kinase (ZAP70; diminished $CD8^+$ T-lymphocyte counts, $CD4^+$ T-lymphocytes are present but not functional),[33] CD3γ,[34] CD3ε,[35] or purine nucleoside phosphorylase (PNP) (Table 1).[36] The substrate for PNP, inosine, is particularly toxic to T-lymphocytes but not to B-lymphocytes.[37] Although B-lymphocytes are generally present in normal numbers in these types of $T^-/B^+/NK^+$ SCID, B-cell function is generally diminished due to lack of T-cell help. As mAb against IL7Rα (CD127), IL2Rα (CD25), CD45, HLA class II (HLA-DR), HLA class I (β_2 microglobulin), and CD3 are commercially available, flow cytometric screening of PB can lead to identification of these deficiencies in an early stage. Furthermore, genetic defects resulting in $T^-/B^+/NK^+$ SCID are rare as shown in Table 4 and Figure 6.

$T^-/B^-/NK^+$ SCID

Other types of SCID are characterized by absence of T-lymphocytes accompanied by absence of B-lymphocytes and/or NK cells. Mutations in the recombination activating genes (*RAG1* and *RAG2*, Figure 7) account for approximately 30% of $T^-/B^-/NK^+$ SCID cases (J.G. Noordzij and J.J.M. van Dongen, unpublished results). These mutations result in 'null' alleles, encoding non-functional RAG proteins.[38] However, mutations causing partial activi-

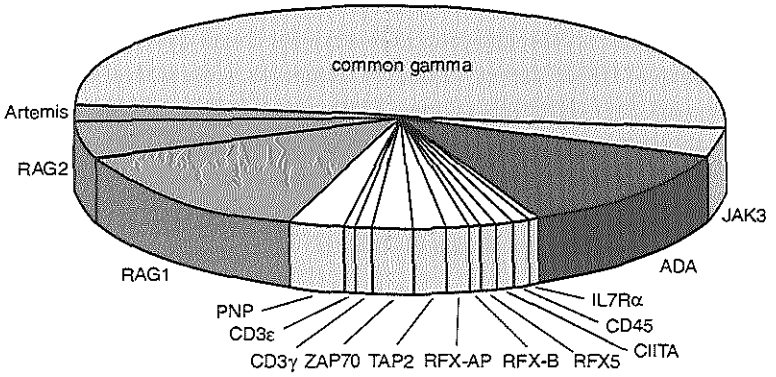

Figure 6. Relative distribution of mutations in genes causing SCID.
Based on the mean of the two numbers in each row in Table 4.

Table 4. Relative frequency of identified gene defects resulting in SCID based on information from mutation databases (September 2001).

Gene defect	Total mutations		Unique mutations	
	Number	Frequency	Number	Frequency
T⁻/B⁺/NK⁺ SCID				
IL7Rα	2	<1%	2	<1%
IL2Rα	1	<1%	1	<1%
CD45	3	<1%	3	<1%
CIITA	3	<1%	3	<1%
RFX5	3	<1%	3	<1%
RFX-B	2	<1%	2	<1%
RFX-AP	5	1%	5	2%
TAP2	6	1%	6	2%
ZAP70	11	3%	3	<1%
CD3γ	3	<1%	3	<1%
CD3ε	2	<1%	2	<1%
PNP	10	2%	10	3%
T⁻/B⁻/NK⁺ SCID				
RAG1	59	14%	44	14%
RAG2	21	5%	18	6%
Artemis	11	3%	8	2%
T⁻/B⁺/NK⁻ SCID				
γ_c [a]	220	52%	147	46%
JAK3	16	4%	16	5%
T⁻/B⁻/NK⁻ SCID				
ADA	46 +	11% +	46 +	14% +
Total	424	103%	322	102%

[a] As mutations in γ_c result in X-linked SCID, in general one mutation per patient has been identified. Patients with AR inherited SCID can be either compound heterozygotes, in which case at least two different mutations per patient have been reported, or homozygotes. This phenomenon will result in an underestimation of the relative frequency of X-linked SCID.

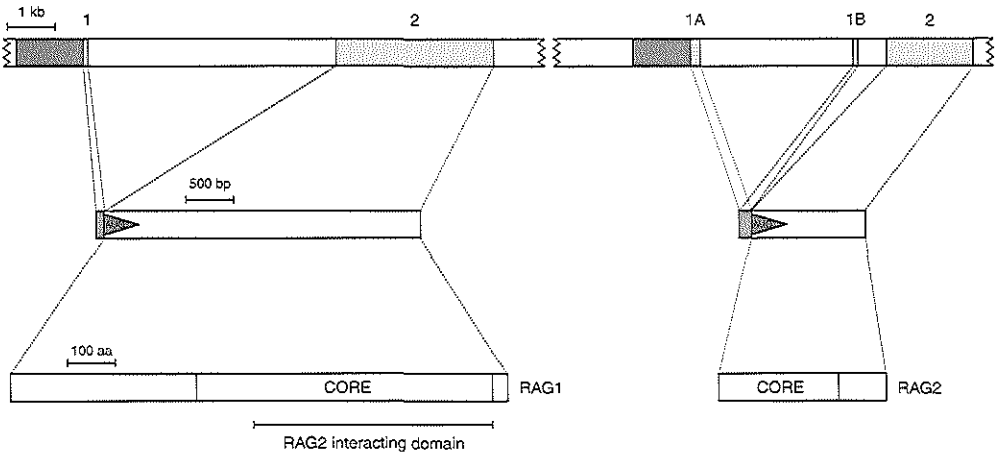

Figure 7. Schematic representation of the *RAG1* and *RAG2* gene, mRNA and protein.
The *RAG1* gene spans 9.9 kb and consists of 2 exons, while the *RAG2* gene spans 7.1 kb and consists of 3 exons, which can be coupled together in two different ways. *RAG*-mRNA is expressed in precursor B cells in the BM and precursor T cells thymocytes in the thymus. The RAG1 protein consists of 1043 aa, while the RAG2 protein consists of 527 aa. In both proteins core domains have been identified, which are essential for recombination activity. The N-terminus of the RAG1 protein and the C-terminus of the RAG2 protein are dispensable for recombination activity, but seem to harbor specificity for Ig gene rearrangement. The respective scales are indicated and based on Schatz *et al*, Ichihara *et al*, and Zarrin *et al*.[72-74]

ty of the RAG proteins result in a variant SCID phenotype (Omenn syndrome) with presence of autologous oligoclonal T-lymphocytes, which are auto-reactive and show a T-helper (Th) 2 phenotype, pushing the limited number of available B-lymphocytes to produce IgE.[39] Although flow cytometric analysis does not show the $T^-/B^-/NK^+$ SCID phenotype in case of Omenn syndrome, the specific clinical symptoms (erythrodermia, failure to thrive, eosinophilia) should provide enough information to correctly interpret the immunophenotyping results. This genotype-phenotype relationship between mutations in the *RAG* genes and the development of $T^-/B^-/NK^+$ SCID versus Omenn syndrome has become less obvious by a recent report in which patients with $T^-/B^-/NK^+$ SCID or Omenn syndrome were shown to carry identical mutations in their *RAG* genes.[40]

Patients with a $T^-/B^-/NK^+$ SCID or Omenn syndrome without mutations in the *RAG* genes (approximately 70%) can be further screened for transcriptional defects by analyzing the *RAG*-mRNA expression in BMMC. However, no *RAG* promotor or enhancer mutations have been reported until now.

Fibroblasts of some $T^-/B^-/NK^+$ SCID patients were found to be radiosensitive and therefore likely to have a defect in one of the genes involved in DNA double strand break (dsb) repair (*Ku70, Ku 80, DNA-PK$_{cs}$, DNA-ligase IV*, and *XRCC4*).[41] However, linkage analysis showed that a gene on chromosome 10p, named *Artemis*, was involved in the pathogenesis of radiosensitive SCID.[42] The Artemis protein has been suggested to be involved in

the processing of the hairpin of the coding joints.[43,79]

T⁻/B⁺/NK⁻ SCID

The largest group of SCID patients suffers from mutations in the gene encoding the common gamma chain (γ_c) (Figure 8), resulting in SCID-X1, which is characterized by absence of T and NK cells, but presence of B-lymphocytes.[44] The γ_c chain is a component of the IL2R, IL4R, IL7R, IL9R, and IL15R and can be detected using flow cytometry. Upon activation of these IL receptors, intracellular signaling takes place via tyrosine phosphorylation and activation of Janus associated kinases (JAK) 1 and 3. Female T⁻/B⁺/NK⁻ SCID patients with a clinical phenotype identical to male SCID-X1 patients have been shown to suffer from mutations in the *JAK3* gene.[45]

T⁺/B⁺/NK⁻ SCID

Recently, a patient was described with low absolute numbers of T-lymphocytes, normal absolute numbers of B-lymphocytes, but absence of NK cells.[46] Although no mutations in the γ_c or *JAK3* genes could be detected, functional studies showed decreased tyrosine phosphorylation of JAK3 through the IL-2R complex. Subsequent flow cytometric analysis showed strongly decreased expression of the IL-15Rβ chain. However, no mutation could be identified in the *IL-15Rβ* gene, suggesting that transcriptional defects were responsible for the lack of IL-15Rβ chain expression.

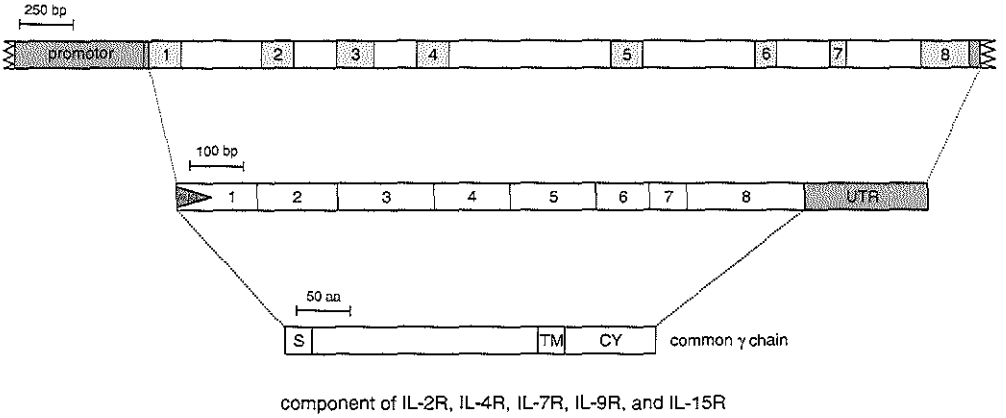

Figure 8. Schematic representation of the γ_c gene, mRNA and protein (CD132).
The γ_c gene spans 4.6 kb and consists of 8 exons. The γ_c-mRNA is expressed in T- and B-lymphocytes, NK cells, monocytes, macrophages and neutrophils in the PB and encodes a protein of 369 aa. Although the γ_c is a component of several IL receptors, their respective α and β chains confer unique functions to each receptor. UTR=untranslated region, S=signal peptide, TM=transmembrane region, CY=cytoplasmic region. The respective scales are indicated and based on Noguchi *et al.*[75]

Table 5. Mutation analysis in patients presenting with SCID (1997-2001).

Family	Diagnosis	Mutation		Mother of the index patient	Family members		Consanguinity of parents
		DNA level [a]	Protein level		Carriers	Non-carriers	
XSCID-1	T-B+ SCID	Exon 2 98insA	Codon 79 stop	Carrier	3	1	No
XSCID-2	T-B+ SCID	Exon 5 C82T	R226C	NA	NA	NA	No
XSCID-3	T-B+ SCID	Exon 2 C137A	N84K	NA	NA	NA	No
RAG-SCID 1	OS	*RAG1* 631delT [c]	Codon 199 stop	Carrier	3	0	Yes
		RAG1 G858A [c]	R249H				
RAG-SCID 2 [c, d]	T-B- SCID	*RAG1* A2571G [c]	K820R	Carrier	2	0	Yes
		RAG2 1913delG [c]	Codon 247 stop				
RAG-SCID 3	T-B- SCID	*RAG2* A2643C [f]	H481P	NA	NA	NA	Yes
RAG-SCID 4 [d]	T-B- SCID	*RAG1* A2571G [f]	K820R	NA	NA	NA	Unknown
		RAG2 1913delG [f]	Codon 247 stop				
RAG-SCID 5	OS	*RAG1* G858A [c]	R249H	Carrier	2	0	No
		RAG2 T2558A [c]	W453R				
RAG-SCID 6	T-B- SCID	*RAG2* C1247T [f]	Q16 stop	NA	NA	NA	No
RAG-SCID 7 [c]	T-B- SCID	*RAG1* G858A [f]	R249H	Carrier [b]	1 [b]	NA	No
		RAG1 G1323A [f]	R404Q				
RAG-SCID-8	OS	*RAG1* G858A [g, h]	R249H	NA	NA	NA	No
		RAG1 A1415G [g, i]	M435V				
		RAG1 A2571G [g, i]	K820R				
		RAG1 A3128G [g, h]	M1006V				
		RAG2 A2705G [g]	M502V				

[a] Numbering of *RAG1* gene according to Schatz *et al.*, *RAG2* gene according to Ichihara *et al.*; [b] Carriership in the mother was based on family history instead of DNA analysis; [c] In these families two patients were analyzed; [d] Patients from different families with identical mutations; [e] Homozygous mutation, both parents were carrier; [f] Seemingly homozygous mutation, but deletion of the other allele was not ruled out; [g] Heterozygous mutation; [h] Mutations on identical allele; [i] Mutations on different alleles; NA = not analyzed.

T-/B-/NK- SCID

Finally, patients with SCID can present immunophenotypically as T-/B-/NK-, resulting in lymphopenia. This type of SCID is caused by defects in the gene encoding adenosine deaminase (*ADA*) and generally develops after birth due to the accumulation of toxic metabolites.[47] ADA is expressed in all cells, and therefore the substrate for the enzyme, adenosine, accumulates in all cells. Immunodeficiency is the consequence of the high sensitivity of immature lymphoid cells to the toxic effects of this substrate. In addition, some patients have neurological abnormalities that may be due to ADA deficiency.

The results of our mutation analyses (1197-2001) in patients with SCID and their family members are shown in Table 5.

Immunodeficiencies with other severe symptoms

As shown in Table 1, this subgroup of PID is heterogeneous and consists among others of macrophage activation syndromes (familial hemophagocytic lymphohistiocytosis, Griscelli syndrome, and Chediak-Higashi syndrome) and several types of auto-immune lymphoproliferative syndromes (ALPS). We will only discuss ALPS and the Wiskott-Aldrich

syndrome (WAS) here.

WAS

The gene encoding WAS protein (*WASP*) is one of the few genes involved in the pathogenesis of PID, in which one genetic defect is associated with two disease phenotypes.[48] WAS patients suffer from immunodeficiency, eczema and thrombocytopenia, while patients with X-linked thrombocytopenia (XLT) have small platelets but hardly any eczema or immunodeficiency. Both patient groups have mutations in the *WASP* gene. However, it was shown that mutation analysis at the DNA level was not sufficient for predicting the clinical course and that studies at the mRNA and protein levels were needed for a better assessment.[49] Defects in WASP result in disturbance of cell signaling, polarization, motility, and phagocytosis.[50]

ALPS

ALPS is characterized by autoimmunity (hemolytic anemia and thrombocytopenia with massive lymphadenopathy and splenomegaly), hypergammaglobulinemia, and the accumulation of non-malignant lymphocytes due to the failure of FAS-mediated apoptosis. Characteristic are the CD4/CD8 double negative T-lymphocytes expressing the TCRαβ in the PB (>1% of TCRαβ+ T-lymphocytes). ALPS can be caused by mutations in *FAS/CD95* (ALPS type 1A, Figure 9),[51] *FAS ligand/CD178* (ALPS type 1B),[52] or *Caspase 10* (ALPS type II),[53] which is a signal transduction protein downstream of the intracellular FAS domain. It has been suggested that yet unknown mutations in other genes involved in FAS-mediated apoptosis result in the same clinical phenotype (ALPS type III).[54] Also in the diagnosis of ALPS the role of flow cytometry becomes increasingly important as a recent study showed that ALPS 1A patients showed statistically significant changes in the immunophenotypic profile of PB cells compared to other individuals, such as family members who did not carry the mutation.[55]

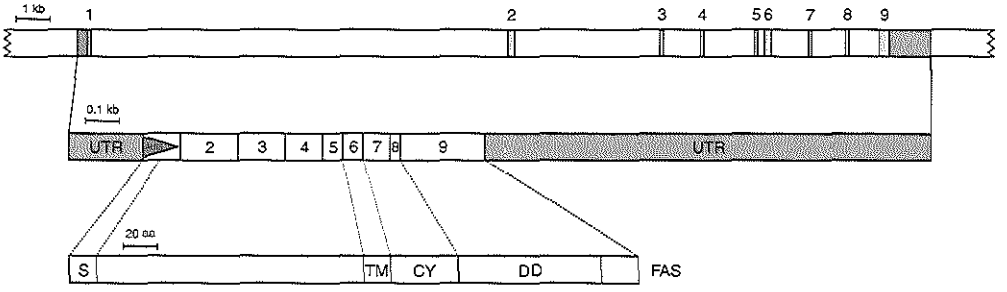

Figure 9. Schematic representation of the *FAS* gene, mRNA and protein (CD95).
The *FAS* gene spans 25 kb and consists of 9 exons. The *FAS*-mRNA is expressed typically at high levels in activated T- and B-lymphocytes, but also in granulocytes in the PB and encodes a protein of 335 aa. UTR=untranslated region, S=signal peptide, TM=transmembrane region, CY=cytoplasmic region, DD=death domain. The death domain of FAS interacts with the death domain of FADD (FAS-associated via death domain), which results in intracellular apoptotic signaling. The respective scales are indicated and based on Cheng *et al.*[76]

Mutations in *FAS* can inherit either autosomal dominant (AD, the mutated protein has a dominant negative effect) or AR.[56] This phenomenon, together with the low prevalence of ALPS, makes it difficult to determine whether a heterozygous *FAS* mutation is indeed the cause of ALPS. We identified heterozygous *FAS* mutations in four patients with the clinical presentation of ALPS (Table 6). In three of these patients, the other unmutated *FAS* allele was normally present without deletions as was demonstrated by Southern blot analysis. The father of patient FAS-2 also suffered from ALPS and was shown to be heterozygous for the same *FAS* mutation, indicating AD inheritance. However, the mother of patient FAS-3 was clinically healthy although she carried the same heterozygous *FAS* mutation as her affected child. The insertion of one bp in exon 2, resulting in a premature stopcodon cannot be a polymorphism, so the question is what caused the variation in phenotype between mother and child, who are both heterozygous for the same *FAS* mutation. It has been shown before that missense mutations in the death domain of FAS (as in patient FAS-2) resulted in high clinical penetrance of ALPS, while deletions or truncations of the death domain (as in patient FAS-3) resulted in lower clinical penetrance.[57] One explanation for this phenomenon might be that FAS proteins with an aa substitution are more likely to be incorporated into a FAS trimer and disrupt apoptotic signaling. Severely mutated FAS proteins may be instable, allowing the unmutated FAS proteins encoded by the unaffected allele to form intact trimers. Another explanation might be that patient FAS-3 has acquired additional genetic defects resulting in ALPS. These defects might occur in Fas ligand, in other proteins of the death-inducing signaling complex (DISC), such as FADD (Fas-associated via death domain) or Caspase 8, or in other factors involved in controlling cell death and/or proliferation.[56,57] Finally, it was reported that penetrance of one or more ALPS features was significantly higher for individuals with intracellular *FAS* mutations versus individuals with extracellular *FAS* mutations. This phenomenon was explained by suggesting different mechanisms of dominant negative interference.[58]

Table 6. Mutation analysis in patients presenting with PID with other severe symptoms (1997-2001).

Family	Mutation		Family members	
	DNA level	Protein level	Carriers	Non-carriers
WAS-1	Exon 1 C124T	Codon 34 stop	NA	NA
FAS-1	Exon 9 C280T [a, b]	T319I	NA	NA
FAS-2	Exon 9 G73A [a, b]	R250G	1 [c]	1
FAS-3	Exon 2 8insA [a, b]	Codon 17 stop	1 [d]	1
FAS-4	Exon 2 G16A [a]	A16T	NA	NA
FAS-5	Exon 9 ins 20 nt [e]	Ins 31 aa	5	0

[a] Heterozygous mutation.

[b] The unmutated allele was not (partially) deleted as confirmed by Southern blot analysis.

[c] Heterozygote, who was clinically affected as well (patient).

[d] Heterozygote, who was clinically healthy (carrier).

[e] Homozygous mutation.

NA = not analyzed; Ins = insertion; aa = amino acid.

Table 7. Mutation analysis in patients presenting with phagocyte defects (1997-2001).

Family	Mutation		Family members	
	DNA level	Protein level	Carriers	Non-carriers
IFNGR1-1	Intron 3 G1T [a]	Codon 73 stop	2	0
IFNGR1-2	Exon 3 G54A [a]	C85Y	3	1

[a] Homozygous mutation, both parents were carrier.

Phagocyte defects

Most phagocyte defects (and complement deficiencies) do not lead to lymphoid PID and are therefore not discussed here. However, we want to make an exception for the Mendelian susceptibility to mycobacterial infections. This disease is the result of the disturbed communication between Th1/NK cells and monocytes/macrophages via the cytokines interferon (IFN)-γ and interleukin (IL)-12. Defects have been described in the genes encoding the IFN gamma receptor (IFN-γR) 1 chain,[59,60] IFN-γR2 chain,[61] IL12-p40,[62] IL-12Rβ1 chain,[63,64] and STAT1.[65] Defects in the IFN-γR seem to cause a more severe clinical phenotype than defects in the IL-12Rβ1 chain, as Th1 and NK cells have IL-12Rβ1-independent pathways to produce IFN-γ.[66] Furthermore, defects in the IFN-γR can result in either complete or partial intracellular signaling deficiencies, with different prognoses.[67] The results of our mutation analyses (1997-2001) in patients with mycobacterial infections and their family members are shown in Table 7.

Confirmation of identified mutations

As the diagnosis of a genetic defect can have major impact on an affected family (e.g. termination of an existing pregnancy, decision to have no children), we feel that it is important to confirm the mutation by a second, independent technique.

As mentioned before, SSCP can be used to detect PCR fragments with small point mutations. The influence of the point mutation on the migration pattern decreases when the PCR product is larger. Therefore, the optimal length of PCR products to be used in SSCP is between 100 and 200 bp.

Heteroduplex analysis (HDA) uses the generation of "heteroduplexes" between strands of mutated and unmutated PCR fragments and can be used to pick up larger mutations, such as the insertion or deletion of several bp.[68]

Sometimes, a mutation results in the appearance or disappearance of a restriction site. In such cases, the use of positive and negative controls is essential, to detect failure of the restriction enzyme due to for example unidentified high salt concentrations.

INTERPRETATION OF IDENTIFIED MUTATIONS: POST-ANALYTICAL PHASE

Mutation analysis is essential for the diagnosis of PID and is becoming a more routine laboratory technique. However, certain pitfalls can make the genetic diagnosis difficult or

unreliable. We have already discussed some examples, such as protein expression despite a genomic mutation,[1] unusual immunophenotyping results (normal absolute numbers of T and/or B-lymphocytes in the PB despite a mutation in the *RAG* genes),[69] and carrier detection at the mRNA level. We will now discuss some other pitfalls.

It might be difficult to determine whether a point mutation resulting in an aa substitution is indeed the cause of the PID or represents a polymorphism. This is particularly difficult when the substituted aa is of the same subgroup (e.g. both neutral), when single nucleotide mutations occur outside a conserved or functional domain, or when only a few mutations in the target gene have been described. Sequencing a certain number of alleles from healthy individuals to screen for the occurrence of the same point mutation might provide some insight, although some polymorphisms are preferentially found in specific ethnic groups. The best test would be to clone the mutated and wt genes and perform functional analyses. However, such functional tests are not always available or are time-consuming and costly and therefore not easy to perform in a routine diagnostic setting.

A number of polymorphisms known to occur in the coding regions of genes involved in the pathogenesis of lymphoid PID are listed in Table 8. A silent mutation will almost always represent a polymorphism, although it might disrupt an exonic splicing enhancer (ESE, discussed below). Furthermore, mutations in untranslated regions (UTR) might affect the stability of the mRNA and mutations in the intron away from the splice site might disrupt intronic splicing elements or regulators.[70]

Ferrari *et al.* described absence of CD40 protein expression with a silent mutation in the coding region of the *CD40* gene.[20] In this case, we would search for a transcriptional defect of the *CD40* gene by sequencing the promoter/enhancer regions or quantitative analysis of *CD40*-mRNA expression levels using real time quantitative (RQ)-PCR. However, it was shown that this silent mutation affected an ESE, resulting in aberrantly spliced, out-of-frame *CD40*-mRNA.

Mutated mRNA might show an altered stability, but it is certainly not the case that the

Table 8. Polymorphisms identified in the coding regions of genes involved in the pathogenesis of PID of the lymphoid system.

Gene	DNA polymorphism		Protein polymorphism
BTK	Exon 11	T60C	Silent
	Exon 15	G145C	Silent
	Exon 18	T131C	Silent
	Exon 18	C149T	Silent
CD40L	Exon 1	T148C	Silent
AICDA	Exon 2	C65T	R25C
	Exon 2	A121C	Silent
	Exon 4	C38T	Silent
RAG1		C579T	A156V
		A842G	R244G
		G858A	R249H
		A2571G	K820R
		A2992G	Silent

expression levels of mutated mRNA are always lower or absent compared to the wt expression levels. Even when protein expression is absent, mutated mRNA might be detectable. Some laboratories screen for example the mRNA isolated from BMMC for the expression of several genes known to be involved in B-cell differentiation (such as *PAX5*), in order to find new gene defects that would explain agammaglobulinemia cases. Although it is a lot of work to sequence al the genes known to be involved in B-cell differentiation, given the arguments mentioned above, mRNA expression studies cannot be used to exclude genomic mutations.

Although immunophenotyping is a powerful tool for screening and focussing the genetic analyses, we want to mention one more pitfall of this technique. Infections in a certain organ, for example the lungs during PCP, might cause massive extravasation of $CD8^+$ T-lymphocytes and/or NK-cells (compartmentalization). Immunophenotyping of PB at the time of infection will show a seemingly $CD8^+$ T-cell deficiency, which would point towards mutation analysis of the *ZAP-70* gene.

CONCLUSION

In this chapter, we tried to provide information on the diagnosis of several genetic defects known to be involved in the pathogenesis of lymphoid PID. Immunophenotyping of PB and/or BM is a powerful tool to select for the candidate target gene, although its disadvantages must be kept in mind. We prefer sequencing of the entire gene at the genomic level, especially in case of carrier detection. A second, independent technique might be used to confirm the mutation.

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Chapter 4

INTRODUCTION PRIMARY ANTIBODY DEFICIENCIES

INTRODUCTION PRIMARY ANTIBODY DEFICIENCIES

Patients suffering from primary antibody (Ab) deficiencies are clinically characterized by bacterial infections. The patients particularly suffer from infections in the upper and lower respiratory tract and gastrointestinal infections, but also from bacterial skin infections, meningitis, septicemia or osteomyelitis.[1] In general, these patients are diagnosed after maternal Ab have disappeared from their circulation (usually six months after birth). However, premature infants might not have acquired maternal Ab in utero. When these children suffer from a primary Ab deficiency, symptoms will be present before the age of 6 months.

Patients suffering from Ab deficiencies can be divided into three main categories, based on their serum immunoglobulin (Ig) levels: (1) patients with no or very low serum Ig levels (agamma- or hypogammaglobulinemia);[1] (2) patients with normal or elevated levels of serum IgM while the other subclasses are absent or strongly decreased (hyper IgM syndrome (HIGM));[2] (3) patients with normal serum Ig levels, who fail to generate specific Ab after vaccination, potentially due to a defect in somatic hypermutation.[3,4] However, patients suffering from HIGM can also present with agammaglobulinemia, making the clinical diagnosis difficult, although in these cases other clinical symptoms such as *Pneumocystis carinii* pneumonia or *Cryptosporidium* infections of the liver can suggest a diagnosis of HIGM.[5]

Patients with agamma- or hypogammaglobulinemia (category 1) have no or decreased numbers of B lymphocytes in their peripheral blood (PB), while patients suffering from primary Ab deficiencies in categories 2 and 3 have normal numbers of PB B lymphocytes. 85% of agammaglobulinemia patients are males suffering from X-linked agammaglobulinemia (XLA) due to a defect in the gene encoding Bruton's tyrosine kinase (*BTK*).[6-8] 5-10% of agammaglobulinemia patients suffer from autosomal recessive inherited gene defects, such as mutations in *IGH-*C_{μ}, *λ14.1*, *CD79a*, or B-cell linker protein (*BLNK*).[8-12] 5-10% of agammaglobulinemia patients suffer from an unknown gene defect.[8]

The decreased numbers of PB B lymphocytes in agammaglobulinemia patients result from an arrest during precursor B-cell differentiation in the bone marrow (BM). This arrest is located at the pre-B-cell receptor checkpoint at the transition from CyIgμ$^-$ pre-B-I cells to CyIgμ$^+$ pre-B-II cells, and is identical for all five genetic defects.[13-15] However, the severity of the arrest ("leakiness") varies between different forms of agammaglobulinemia. XLA patients and patients with mutations in the *λ14.1* gene can show low frequencies of PB B lymphocytes due to a leaky differentiation arrest, while patients with mutations in *IGH-*C_{μ}, *CD79a*, or *BLNK* have no B lymphocytes in their PB due to a complete differentiation arrest.[8,15]

HIGM can present as an X-linked disease (HIGM1), caused by mutations in the gene encoding CD40 ligand (*CD40L*),[16,17] or as a non-X-linked inherited disease, caused by mutations in the genes encoding activation-induced cytidine deaminase (*AICDA*; HIGM2) or *CD40* (HIGM3).[18,19] Although HIGM patients show absence or strongly decreased somatic hypermutation as well, no genetic defects have been identified in patients who are unable to generate specific Ab.[3,4]

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Chapter 5

THE 782C→T (T254M) XHIM MUTATION: LACK OF A TIGHT PHENOTYPE-GENOTYPE RELATIONSHIP

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In a recent report in Blood, Seyama *et al.* described 28 unique mutations of the CD40 ligand (CD40L) gene in 45 X-linked hyper IgM syndrome (XHIM) patients from 30 unrelated families.[1] Generally, peripheral blood mononuclear cells (PBMC) are screened for CD40L expression after activation with phorbol myristate acetate (PMA) and ionomycin to diagnose XHIM. Activated PBMC of most XHIM patients did not express functional CD40L (CD154): they failed to bind a CD40-Ig fusion protein (bCD40-Ig). The investigators discerned 5 different CD40L staining patterns on cultured T cells, using a polyclonal antiserum (pAb), 4 different monoclonal antibodies (McAb), and bCD40-Ig. The type 1 pattern showed weak staining of CD40L with all reagents mentioned; the type 2 through 5 patterns successively showed loss of functional activity (bCD40-Ig binding) and protein epitope expression (loss of 1 to 4 McAb and finally pAb binding). A relationship between genotype and phenotype was suggested, with "milder genotypes" (resulting in staining pattern type 1 or 2) showing milder clinical phenotypes: in 5 of 10 patients from 9 families with staining pattern type 1 or 2, symptoms started relatively late, and none of them suffered from opportunistic infections.

Two patients with the 782C→T mutation, a missense mutation in exon 5, fit into this group. We found the 782C→T XHIM mutation in 4 patients from 2 different families. Although they showed a favorable clinical course after Ig replacement therapy was started, the 2 index patients had symptoms from an early age onwards, including 1 who presented with Pneumocystis carinii pneumonia (PCP). The other 2 children were diagnosed shortly after birth because of the XHIM index patient in the family.

The first index patient, patient no. 1 (of family A), is a young Dutch man, now 19 years of age, who was treated with Ig replacement therapy from the age of 2 years onward because of hypogammaglobulinemia and frequent respiratory tract infections. Since then, the frequency of infections has normalized. He never had an infection with an opportunistic pathogen and never received prophylaxis with Co-trimoxazole. Recently, stimulation of his PBMC with PMA and ionomycin induced expression of the early activation marker CD69 (Leu-23; Becton Dickinson, San Jose, CA) on his $CD3^+$ T lymphocytes, whereas CD40L (LL48; Schering-Plough, Dardilly, France) expression was absent (Figure 1). Fluorescent sequencing of the CD40L gene showed a 782C→T mutation (expected protein alteration T254M). The family was screened, and in his cousin patient no. 2, who is now 2 years old, XHIM was identified shortly after birth (Figure 2). At 9 months of age, the serum IgG level had decreased to less than 0.5 g/L. Although there were no clinical symptoms, Ig replacement therapy was started because of the increased risk of serious infections.

The second index patient, patient no. 3 (of family B), is a British boy, now 11 years of age, who was diagnosed as having XHIM at 6 months of age when he presented with PCP. After successful treatment of this infection, he has remained asymptomatic on Ig replacement therapy and Co-trimoxazole prophylaxis except for 1 episode of a diarrheal illness at 7 years of age that was of unknown etiology, lasted for 2 months, and was associated with weight loss. Recent studies show no evidence of cholangiopathy on liver function tests, liver his-

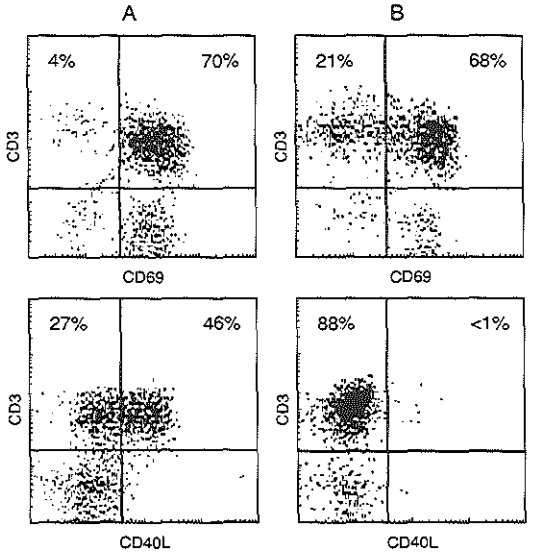

Figure 1. CD69 and CD40L expression on CD3+ T lymphocytes after stimulation of PBMC with PMA and ionomycin.
(A) Normal control. (B) Patient no. 1.

tology, and endoscopic retrograde cholangio-pancreatogram. His younger brother (patient no. 4), who is now 8 years old, was diagnosed soon after birth based on the family history. He has remained completely well on Ig replacement therapy and Co-trimoxazole prophylaxis. His liver is also normal. The mutation in these boys has been previously reported (case 13 in Katz *et al.*).[2]

In our opinion, the issue of a phenotype-genotype relation in XHIM remains debatable. Not all patients with a staining pattern type 1 or 2 described by Seyama *et al.* showed a mild clinical phenotype.[1] Comparison of our 2 index patients with the 782C→T mutation with the 2 mild patients described by Seyama *et al.* does not support a tight phenotype-genotype relation in XHIM patients, although our 4 patients all showed a favorable course after Ig replacement therapy.[1] We agree that symptomatic PCP may occur in non-immunocompromised infants, although this is very unusual. Nevertheless, we do not believe that the life-threatening episode of PCP in our patient no. 3 is compatible with a label of mild phenotype. Furthermore, our XHIM cases also show that a favorable clinical course on Ig replacement therapy is compatible with a diagnosis of XHIM. This implies that testing of CD40L expression on activated PBMC should be considered in all patients with unexplained hypogammaglobulinemia.

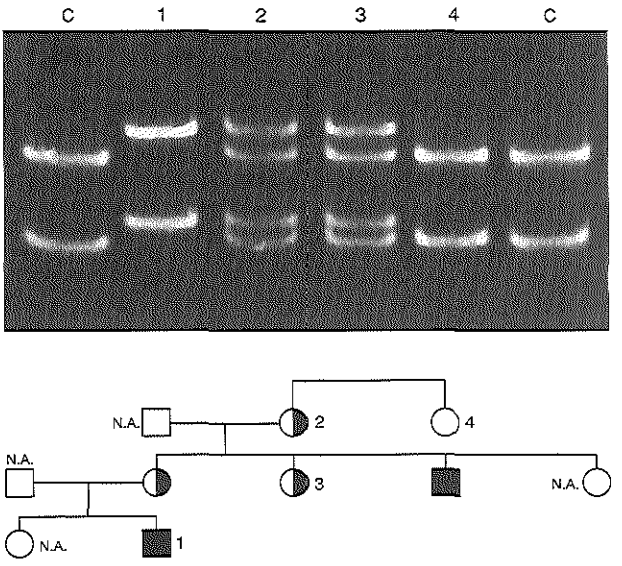

Figure 2. Single-strand conformation polymorphism (SSCP) analysis of XHIM family A with T254M mutation.
PCR products (198 bp) of exon 5 of the *CD40L* gene were denatured and run on a 12.5% polyacrylamide gel at 150 V for 14 hours. The 2 control (C) lanes show the positions of the DNA strands of the unaffected X-chromosome. The C→T mutation leads to a shift in the position of these bands, as is shown in lane 1 (XHIM patient no. 2). Carriers (lanes 2 and 3) show both mutated and unmutated DNA strands. Noncarriers (lane 4) only show the unmutated DNA strands.

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Chapter 6

COMPOSITION OF THE PRECURSOR B CELL COMPARTMENT IN PATIENTS WITH X-LINKED AGAMMAGLOBULINEMIA COMPARED TO HEALTHY CHILDREN

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SUMMARY

X-linked agammaglobulinemia (XLA) is characterized by a severe B-cell deficiency, resulting from a differentiation arrest in the bone marrow (BM). Since XLA is clinically and immunologically heterogeneous, we investigated whether the B-cell differentiation arrest in BM of XLA patients is heterogeneous as well. First, we analyzed BM samples from 19 healthy children by flow cytometry. This resulted in a normal B-cell differentiation model with eight consecutive stages.

Subsequently, we analyzed BM samples from nine XLA patients. Eight patients had amino acid substitutions in the Bruton's tyrosine kinase (BTK) kinase domain or premature stop codons, resulting in the absence of functional BTK proteins. In seven of these eight patients a major differentiation arrest was observed at the transition between cytoplasmic Igμ (CyIgμ) negative pre-B-I cells and CyIgμ$^+$ pre-B-II cells, consistent with a role for BTK in pre-B-cell receptor (pre-BCR) signaling. However, one patient exhibited a very early arrest at the transition between pro-B cells and pre-B-I cells, which could not be explained by a different nature of the *BTK* mutation. We conclude that the absence of functional BTK proteins generally leads to an almost complete arrest of B-cell development at the pre-B-I to pre-B-II transition.

The ninth XLA patient had a splice site mutation associated with the presence of low levels of wild type *BTK* mRNA. His BM showed an almost normal composition of the precursor B-cell compartment, suggesting that low levels of BTK can rescue the pre-BCR signaling defect, but do not lead to sufficient numbers of mature B-lymphocytes in the peripheral blood.

INTRODUCTION

X-linked agammaglobulinemia (XLA) is a primary immunodeficiency, which affects approximately 1 in 100,000 boys and is characterized by agamma- or hypogammaglobulinemia and strongly reduced numbers of B-lymphocytes in the peripheral blood (PB).[1] The clinical picture of XLA generally develops during the first year of life, after maternal antibodies (Ab) have disappeared from the serum, with frequent infections of different organ systems, particularly upper and lower respiratory tract and gastrointestinal infections, but also bacterial skin infections, meningitis, septicemia or osteomyelitis.[1,2]

XLA is caused by mutations in the gene encoding Bruton's tyrosine kinase (BTK), located on Xq21.3.[3,4] BTK is a cytoplasmic protein of 659 amino acids (aa), composed of five different domains, the pleckstrin homology (PH), Tec homology (TH), Src homology 3 (SH3), SH2 and kinase domain, respectively.[1] So far, 341 unique mutations have been reported which are scattered throughout the gene.[5] No correlation has been described between the type and position of the mutations and phenotypic parameters, such as age or serum Ig lev-

els at diagnosis or severity of infections.[2,6] XLA is a heterogeneous disease,[7,8] which could be the result of differences in mutations in the BTK gene, in combination with other genetic or environmental factors, infections or age of the patients.

Early studies have shown that in XLA a B-cell differentiation arrest occurs in bone marrow (BM).[9-11] However, controversy exists regarding the stage in B-cell development that is primarily affected by mutations in the BTK gene. Differentiation arrests have been described between cytoplasmic Igμ (CyIgμ) positive precursor B cells and surface membrane IgM (SmIgM) positive immature B cells as well as at the transition of CyIgμ- into CyIgμ+ precursor B cells.[10,11] These findings could reflect heterogeneity in the stages at which B-cell development is arrested, in parallel with the clinical and immunological heterogeneity of XLA.[12-14] In a recent study, monoclonal Ab (MAb) against the surrogate light chains (SL) were used to characterize the differentiation arrest.[15] It was shown that the major blockade in XLA patients was located at the transition from large to small $CyIg\mu^{low}SL^{bright}$ pre-B cells.[15] However, the authors did not discuss the severity of the blockade. The severity of this arrest might be variable, because XLA patients differ in the absolute numbers of mature B-lymphocytes in their PB, which suggests that variable degrees of "leakiness" exist.[16] The residual B cells can proliferate, undergo IgH isotype switching, and differentiate into specific antibody producing cells.[17] We hypothesized that an accurate characterization of the B-cell differentiation arrest in BM of XLA patients would help to explain the level of immunological variation and provide information on the function of BTK in early B-cell development in man.

Several recent flow cytometric studies have shown that in human BM five to six B-cell differentiation stages can be distinguished.[18-22] According to Ghia *et al.*, pro-B, pre-B-I, large pre-B-II, small pre-B-II, immature B, and mature B cells can be recognized in man on the basis of Ig rearrangement and cytoplasmic or surface Ig expression.[19,21] BTK functions as a signal-transducing element downstream of the B-cell receptor (BCR), thereby directing B-cell development.[23]

To unravel the precise B-cell differentiation arrest in XLA patients and to obtain information on the degree of "leakiness", we performed detailed flow cytometric studies of nine BM samples from XLA patients together with 19 BM samples from healthy children under the age of 16 years as controls.

MATERIALS AND METHODS

Cell samples

We received BM samples from 19 healthy children (youngest age 1y7m, oldest 16y1m, median 8y2m; 10 males, 9 females), who were donors for BM transplantation of their siblings (Department of Pediatrics, Leiden University Medical Center; Drs. P.M. Hoogerbrugge and J.M.J.J. Vossen). These control BM samples were used for detailed flow cytometric studies, via the lysed whole BM (LWBM) method[24] as well as after Ficoll-Paque

density centrifugation (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden).

We received BM and PB samples from nine XLA patients over the last 15 years. BM samples from four recent XLA patients were used for flow cytometric analysis according to the LWBM method. The BM samples from the other five XLA patients were subjected to Ficoll density centrifugation. The recovered mononuclear cells (MC) were frozen and stored in liquid nitrogen and thawed later for flow cytometric immunophenotyping studies.

Granulocytes and MC were isolated from PB by Ficoll density centrifugation and used for DNA and/or RNA extraction.

All cell samples were obtained according to the informed consent guidelines of the Medical Ethics Committees of the Leiden University Medical Center and the University Hospital Rotterdam.

Comparison of cell samples

In four healthy children, we compared the results of flow cytometric analysis of fresh BM via the LWBM method with the results obtained from thawed bone marrow mononuclear cells (BMMC). Furthermore, in five other healthy children, we compared the results of flow cytometric analysis of fresh BM via the LWBM method with the results obtained from freshly isolated BMMC. All flow cytometric data concerning the precursor B-cell compartment were analyzed independently by two experienced technicians, and did not show significant changes before and after Ficoll density centrifugation as well as before and after freezing and thawing of MC, implying that no major selective loss of particular precursor B-cell subsets occurred. Anyway, using BMMC from our cell bank (storage in liquid nitrogen) was the only way we could obtain BM samples from enough XLA patients to perform this study.

Flow cytometric analysis of BM from healthy children and XLA patients

BM samples were diluted with phosphate buffered saline (PBS, pH7.8) to a concentration of 15 x 10^6 cells/ml. Twenty-five µl aliquots of whole BM and/or BMMC were incubated for 10 min. at room temperature with combinations of optimally titrated MAb: 25 µl fluorescein isothiocyanate (FITC) conjugated MAb, and/or 25 µl phyco-erythrin (PE) conjugated MAb, and/or 25 µl PE-Cyanine5 (PE/Cy5) conjugated MAb were used to detect membrane bound antigens. After incubation, the cells were washed and further processed depending on the type of triple labeling. The 14 applied triple labelings are summarized in Table 1.

Triple labelings for three membrane bound antigens (labelings 1-7 in Table 1) were directly analyzed by flow cytometry using FACScan (Becton Dickinson, San Jose, CA, USA) in case of BMMC, whereas whole BM samples were first subjected to lysis of the erythrocytes using FACS Lysing Solution (Becton Dickinson).

Permeabilization of the BM cells using FACS Lysing Solution (Becton Dickinson) was performed prior to intranuclear staining of terminal deoxynucleotidyl transferase (TdT) both on whole BM samples and BMMC (labelings 8-10 in Table 1).[24,25]

Intracellular staining of Igµ (CyIgµ) was performed after permeabilization of the BM cells using IntraPrep Permeabilization Reagent (Immunotech, Marseille, France) both on whole BM samples and BMMC (labelings 11-14 in Table 1).[24, 25]

Table 1. Triple labelings for analysis of B-cell differentiation.

	FITC-conjugates	PE-conjugates	PE-Cy5-conjugates
MAb directed against membrane antigens			
1	CD34 (HPCA-2)[a]	CD22 (Leu-14)[a]	CD19 (HIB19)[d]
2	CD34 (HPCA-2)	CD10 (J5)	CD19 (HIB19)
3	CD34 (HPCA-2)	CD38 (Leu-17)[a]	CD19 (HIB19)
4	CD34 (HPCA-2)	CD20 (Leu-16)[a]	CD19 (HIB19)
5	CD10 (J5)[b]	CD20 (Leu-16)	CD19 (HIB19)
6	SmIgM[c]	CD10 (J5)	CD19 (HIB19)
7	SmIgD[c]	SmIgM (MB-11)[c]	CD19 (HIB19)
MAb directed against membrane antigens and TdT via FACS Lysing Solution			
8	TdT (hTdT-6)[f]	CD22 (Leu-14)	CD19 (HIB19)
9	TdT (hTdT-6)	CD34 (HPCA-2)	CD19 (HIB19)
10	TdT (hTdT-6)	CD10 (J5)	CD19 (HIB19)
MAb directed against membrane antigens and CyIgμ via IntraPrep			
11	CyIgμ[e]	CD34 (HPCA-2)	CD19 (HIB19)
12	CyIgμ	CD10 (J5)	CD19 (HIB19)
13	CyIgμ	CD38 (Leu-17)	CD19 (HIB19)
14	CyIgμ	SmIgM (MB-11)	CD19 (HIB19)

MAb were derived from: [a] Becton Dickinson (San Jose, CA, USA); [b] Beckman Coulter (Brea, CA, USA); [c] Sigma Aldrich (St. Louis, MO, USA); [d] Serotec (Raleigh, NC, USA); [e] Kallestad / Sanofi-Synthelabo (Paris, France); [f] Supertechs (Bethesda, MD, USA).

DNA and RNA extraction and reverse transcriptase (RT) reaction

DNA was extracted from PBMC and/or granulocytes using the QIAamp Blood kit (Qiagen, Chatsworth, CA, USA).[26] Total RNA was isolated from PB granulocytes according to the method of Chomczynski using RNAzol B (Tel-Test, Friendswood, TX, USA).[27] cDNA was prepared from 1μg mRNA as described before, using oligo(dT) and AMV reverse transcriptase.[28]

PCR amplification of (c)DNA

PCR was performed as described previously.[29] Exons 1 to 13 and exon 19 of the *BTK* gene were amplified separately, while exons 14 to 18 were amplified in one long-range (LR)-PCR reaction. In each 100 μl PCR reaction 0.1μg (c)DNA, 20 pmol of 5' and 3' oligonucleotides and 1 U Ampli*Taq* gold polymerase (PE Biosystems, Foster City, CA, USA) were used. PCR conditions were 2-10 min at 94°C, followed by 60 sec at 92°C, 60 sec at 57-60°C, 2 min at 72°C for 40 cycles, followed by a final extension step (7 min at 72°C).

In each 100 μl LR-PCR reaction 0.1μg DNA sample, 5.25 U Expand enzyme mix (Boehringer Mannheim, Mannheim, Germany), and 30 pmol of 5' and 3' oligonucleotides were used. LR-PCR conditions were 2 min at 94°C, followed by 15 sec at 94°C, 30 sec at

57°C, and 4 min at 68°C for 40 cycles using 10 sec auto-extension from cycle 21 onward. After the last cycle an additional step of 10 min at 72°C was performed for final extension.

The sequences of the oligonucleotides used for PCR amplification of *BTK* were based on Oeltjen *et al.*[30] (Genbank accession number U78027) and were designed with the OLIGO 6 program (Dr. W. Rychlik, Molecular Biology Insights, Cascade, CO).[31] Primer sequence data will be made available on request to interested readers.

Fluorescent sequencing reaction and analysis

LR-PCR products of *BTK* exons 14 to 18 were first purified with the QIAquick PCR purification kit (Qiagen), while short PCR products (exons 1 to 13 and 19) were used directly for sequencing with 5 µl dRhodamine dye terminator mix (PE Biosystems), using 3.3 pmol sequencing primers. The sequencing primers were positioned in the *BTK* introns just upstream and downstream of the exon-intron borders, so that the splice site sequences could be evaluated as well. All sequencing was performed as described before[32] and run on an ABI Prism 377 fluorescent sequencer (PE Biosystems).

Isolation of PCR products from agarose gels

RT-PCR products from XLA patient 9 were separated in a 1% agarose gel stained with ethidium bromide. Under UV illumination, two bands of different sizes were cut out of the gel and frozen for 15 min. at -20°C. After thawing, the PCR products were isolated from the gel fragments using ultrafree-mc Millipore columns (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's instructions and used directly for fluorescent sequencing.

RESULTS

Composition of the BM lymphogate

To characterize B-cell development in young children, BM samples from 19 healthy children and nine XLA patients were analyzed by flow cytometry, using 14 triple labelings (Table 1). The obtained percentages of $CD22^+$ B cells in the BM lymphogate are shown in Table 2. This table illustrates the variation in composition of the lymphogate. This variation is caused by 'contamination' of the lymphogate with T-lymphocytes, NK cells, myeloid precursors and normoblasts (Table 2). Therefore, reliable comparison of B-cell subpopulations in BM samples from healthy children and XLA patients requires analysis within a B-cell gate.

The use of CD22 as a pan-B-cell marker has been described previously.[33,34] To confirm the reliability of this marker we performed quadruple labelings using a mixture of PerCP-Cy5.5 labeled MAb (CD3, CD33 and CD16) together with CD22. Analyses of BM samples from two healthy children showed that CD22 is rarely expressed on $CD3^+$ T cells, $CD33^+$ myeloid cells or $CD16^+$ NK-cells (Figure 1), thereby excluding the possibility that the

Table 2. Composition of the BM lymphogate

	CD22+ B cells (%)		CD3+	CD16+/	CD13+/	CD71+/GpA+/
	Precursor B cells[a]	Mature B cells[b]	T-lympho-cytes (%)	CD56+ NK cells (%)	CD33+ myeloid cells (%)	CD45- normoblasts (%)
Healthy children <5y (n=6)	36±7	12±9	22±13	3±1	6±3	25±16
Healthy children 5-10y (n=7)	27±9	7±3	28±8	3±2	5±2	31±7
Healthy children 10-16y (n=6)	26±11	7±4	25±5	3±1	5±2	35±13
XLA1 (LWBM)	15	0.2	55	6	5	20
XLA2 (thawed BMMC)	8	0.2	39	15	11	26
XLA3 (thawed BMMC)	40	<0.01	22	11	3	13
XLA4 (thawed BMMC)	21	0.04	38	6	6	22
XLA5 (thawed BMMC)	39	0.2	23	12	5	17
XLA6 (LWBM)	12	0.03	68	5	7	15
XLA7 (thawed BMMC)	20	0.03	52	16	2	6
XLA8 (LWBM)	9	0.1	56	6	3	25
XLA9 (LWBM)	26	2.2	29	7	5	34

[a] The percentage of precursor B cells was calculated by subtracting the percentage of mature B cells from the total percentage of CD22+ B cells.

[b] The percentage of mature B cells was based on the CD10-/SmIgM+ and/or SmIgM+/SmIgD+ populations (labelings 6 and 7 from Table 1).

CD22+ gate would include substantial numbers of non-B lineage cells. Furthermore, labelings containing both CD22 and CD79a showed a small CD22+/CyCD79a- pro B-cell population (data not shown). Therefore, we choose CD22 as a pan B-cell marker instead of CyCD79a.

B-cell subsets in bone marrow from healthy donors

The markers CD22 and CD19 were used to identify the total B-cell population in BM, because these two markers are regarded as pan-B-cell markers, which are expressed by virtually all precursor B cells. In each triple labeling, the expression pattern of the two other

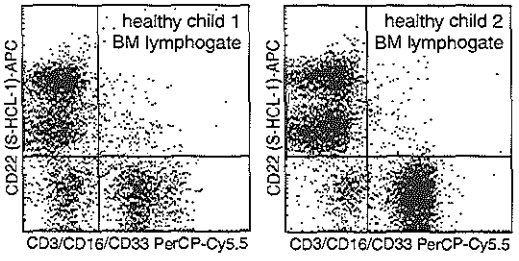

Figure 1. Flow cytometric analysis of BM samples from 2 healthy children using a mix of PerCP-Cy5.5 labeled MAb to confirm the reliability of CD22 as a pan-B-cell marker.
Flow cytometric analyses within a BM lymphogate showed that CD22 is rarely expressed on CD3+ T cells, CD16+ NK cells, or CD33+ myeloid cells, thereby confirming the reliability of CD22 as a pan-B-cell marker.

MAb was analyzed within the CD22 or CD19 gate, resulting in normal differentiation pathways (Figure 2). The apparent heterogeneity of the $CD22^+$ gate in Figure 2A was caused by different expression levels ($CD22^+$ and $CD22^{bright}$) on precursor B-cells, which was also clear from Figure 1.

Virtually all events within the B-cell gates were grouped using different regions, bringing the total percentage of identified B-cell subpopulations in each gate to approximately 100%. For example, the CyIgμ-SmIgM-CD19 labeling was analyzed within the CD19 gate (Figure 2B). Three subpopulations could be distinguished (from immature to mature): $CyIg\mu^-/SmIgM^-$, $CyIg\mu^+/SmIgM^-$, and $CyIg\mu^+/SmIgM^+$. An identical approach was followed

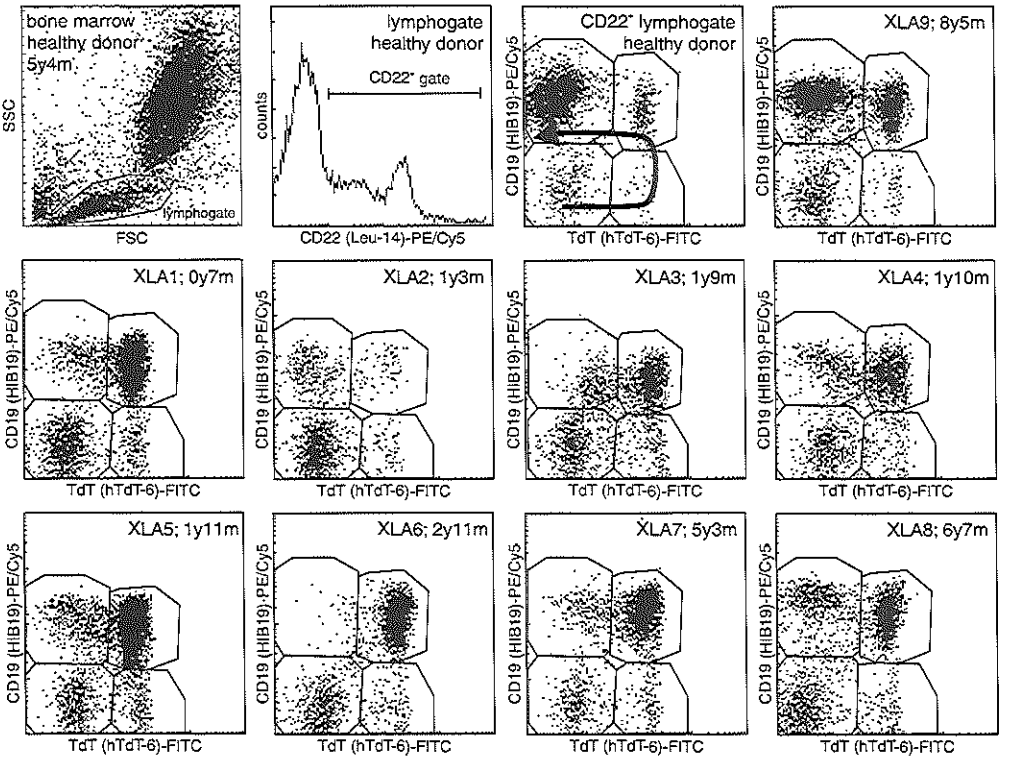

Figure 2. Flow cytometric analysis of BM samples from a healthy child and nine XLA patients, showing the normal composition of the precursor B-cell compartment and the differentiation arrests in XLA patients, respectively.

The composition of the precursor B-cell compartment in the healthy child was representative of that in all other healthy children (19). The composition of the precursor B-cell compartment was analyzed within a lymphogate and a $CD22^+$ (A) or $CD19^+$ histogram gate (B and C). The order of the B-cell differentiation stages in the healthy child is indicated with arrows. In general, XLA patients lacked or showed a severe reduction in the more mature B-cell differentiation stages with a relative increase in the immature B-cell differentiation stages.

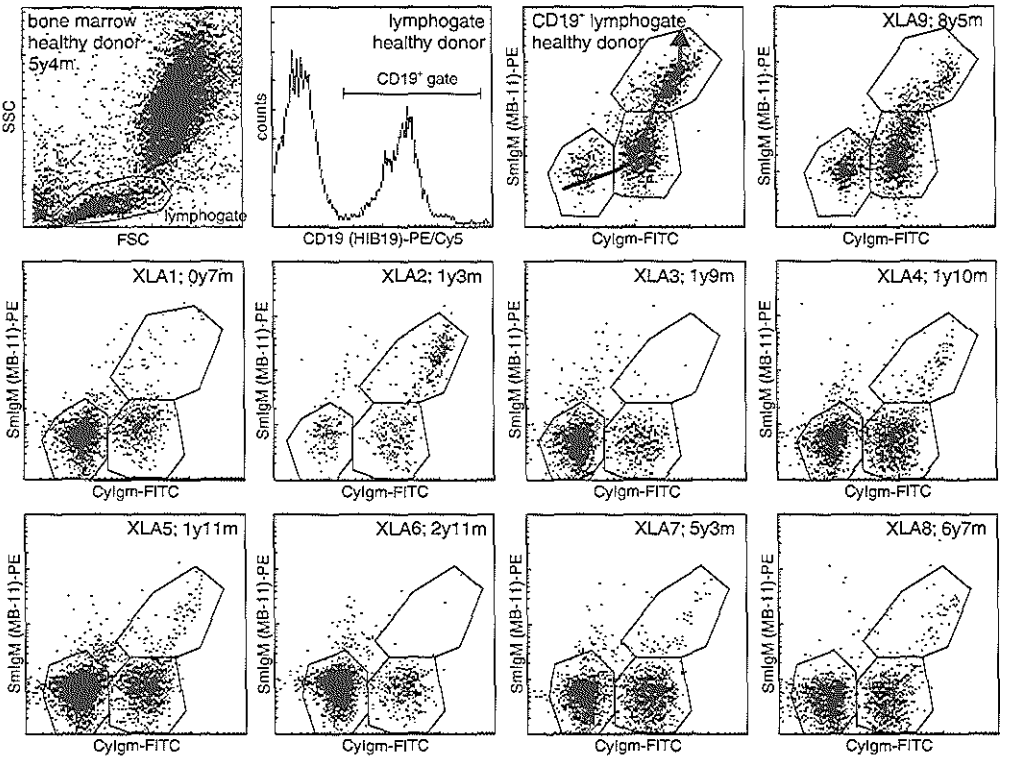

Figure 2. Continued

for other markers within the CD22 or CD19 gates of all 14 triple labelings, resulting in the differentiation scheme in Table 3.

Several human B-cell differentiation schemes with five to six precursor B-cell subsets have been published.[18-22] Pro-B, pre-B, immature B and mature B cells were designated according to Ghia *et al.*[21] The $CD19^-$ fraction was termed pro-B cells, and was further subdivided on the basis of TdT expression. Pre-B-I cells were defined as $CD19^+$, $CD10^+$, TdT^+, $CD34^+$, and $CyIg\mu^-$ and were further subdivided on the basis of the level of CD10 expression. Pre-B-II cells were $CD19^+$, $CD10^+$, TdT^-, $CD34^-$, and $CyIg\mu^+$, and were further subdivided on the basis of the presence or absence of CD20 expression. Immature B cells were $CD19^+$, $CD10^+$, $SmIgM^+$, and $SmIgD^-$, whereas mature B cells were $CD19^+$, $CD10^-$, $SmIgM^+$, and $SmIgD^+$.

All percentages of precursor-B cell subpopulations in healthy children within a $CD22^+$ or $CD19^+$ gate are listed in the lower part of Table 3. It has been published that the relative composition of the precursor B-cell compartment is age related with several differences between children and adults.[22] We subdivided the healthy children into three age groups in order to check for differences between children of different age groups. Our results showed

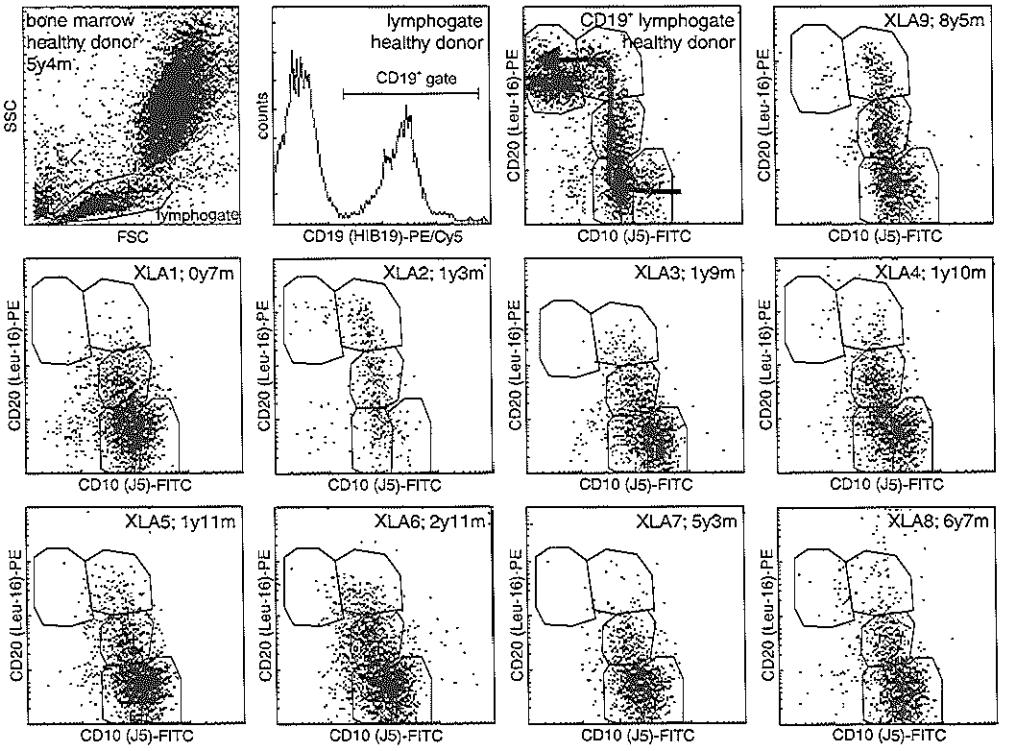

Figure 2. Continued

only minor non-significant differences in the composition of the precursor B-cell compartment in BM from children of different age groups.

Definition of CD22+ and CD19+ B-cell compartments

We identified the entire B-cell compartment as being CD22+. In healthy children below five years of age, between five and ten years of age, and between ten and 16 years of age, the CD19+ fraction within this CD22+ B-cell compartment was very large (labelings 1 and 8), i.e. 91.4%, 88.7%, and 90.9%, respectively, implying that the CD19-/CD22+ pro-B-cell population was small in all three age groups (Table 3). Consequently, the percentages of the different subpopulations within the CD19 gate could be recalculated into percentages of the CD22+ population, by multiplying with 0.914, 0.887, and 0.909, respectively. The same approach was followed for XLA patients.

Correction of the B-cell compartment in BM for blood contamination

The mature B-cell population (stage 8) varied between healthy children mainly because of blood contamination with CD10-/SmIgM+/SmIgD+ B-lymphocytes: 1% to 30%

Table 3. B-cell differentiation stages in BM from healthy children (< 16 years).

		1	2	3	4	5	6	7	8
		Pro-B		Pre-B-I		Pre-B-II		Immature B	Mature B
Immunological markers	CD19	-	-	+	+	+	+	+	+
	CD22	+	+	+	+	+	+	+	++
	CD34	+	+	+	+	-	-	-	-
	TdT	-	+	+	+	-	-	-	-
	CD10	-	-	++	+	+	+	+	-
	CD20	-	-	-	-	-	+	++	++
	CD38	NA	NA	++	++	++	++	++	+
	CyIgM	-	-	-	-	+	+	+	+
	SmIgM	-	-	-	-	-	-	+	+
	SmIgD	-	-	-	-	-	-	-	+
Mean % within CD22 gate (SD)	< 5 years (n=6)	6.0 (1.7)	1.2 (1.4)	7.6 (4.0)			83.8 (7.0)		
	5-10 years (n=7)	8.2 (4.8)	1.0 (0.6)	9.1 (1.5)			79.7 (6.2)		
	10-16 years (n=6)	6.8 (2.2)	0.8 (0.3)	8.3 (3.1)			82.6 (4.6)		
Mean % within CD19 gate (SD)	<5 years (n=6)	-	-	3.7 (2.4)	3.2 (2.3)	23.2 (16.3)	22.4 (5.0)	19.6 (8.0)	25.6 (14.0)
	5-10 years (n=7)	-	-	4.6 (1.4)	4.9 (2.1)	34.8 (12.7)	18.2 (4.7)	15.2 (3.2)	24.4 (11.5)
	10-16 years (n=6)	-	-	5.7 (2.3)	4.9 (3.1)	25.8 (8.0)	20.4 (6.7)	18.1 (3.8)	28.8 (13.4)

NA = not analyzed. Since CD38 was not used in combination with CD22 (Table 1), no precise information about CD38 expression on pro-B cells was available. However, analysis of CD34 and CD38 expression in the lymphogate showed that more than 99% of $CD34^+$ cells were also $CD38^+$. Hao *et al.* showed that the $CD38^-$ population comprised 3.53% ± 0.68% of the $CD34^+$ cells.[53]

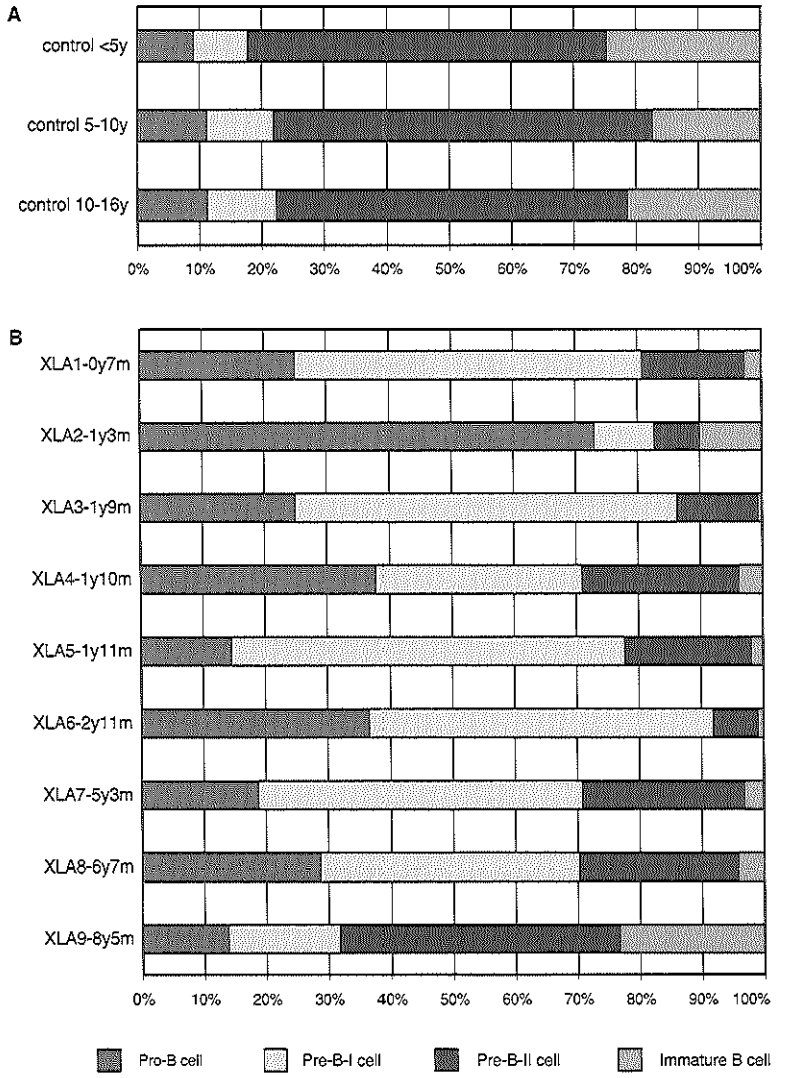

Figure 3. Composition of the precursor B-cell compartment in healthy children (A) compared to XLA patients (B), corrected for the composition of the lymphogate.
The precursor B-cell compartment (Table 2) was set to 100%. To this aim, $CD10^-/SmIgM^+/SmIgD^+$ mature B cells were excluded (see text for details). Seven out of nine XLA patients showed a similar differentiation arrest with approximately 80% of the precursor B cells in the pro-B and pre-B-I stages, thereby revealing an arrest at the transition from $CyIg\mu^-$ to $CyIg\mu^+$ pre-B cells, with a variable degree of "leakiness". XLA patients 2 and 9 showed different compositions of their precursor B-cell compartments.

Table 4. Characteristics of XLA patients.

Patient	Age at diagnosis[a]	Serum Ig levels at diagnosis[b]			Age at BM puncture	Genomic mutation[c]		Predicted effect at protein level	
		IgM (g/l)	IgG (g/l)	IgA (g/l)		Exon	Nucleotide	Amino Acid	Affected domain
XLA1	0 y 7 m	0.11	0.3	0.01	0 y 7 m	Exon 17	C 53 T	R 562 W	Kinase
XLA2	1 y 2 m	0.26	0.27	0.17	1 y 3 m	Exon 7	26 del G	L 198 stop	TH
XLA3	1 y 9 m	<0.1	0.36	<0.1	1 y 9 m	Exon 2	142 del TC	Y 40 stop	PH
XLA4	0 y 7 m	0.13	0.41	0.04	1 y 10 m	Exon 15	G 78 A	M 509 I	Kinase
XLA5	1 y 10 m	0.13	0.01	0	1 y 11 m	Exon 15	C 106 A	Y 485 stop	Kinase
XLA6	2 y 11 m	<0.05	<0.1	<0.06	2 y 11 m	Exon 3	75 ins A	E 84 stop	PH
XLA7	1 y 8 m	NA	<0.3	NA	5 y 3 m	Exon 14	C 98 A	Y 425 stop	Kinase
XLA8	6 y 7m	<0.1	3.0	<0.05	6 y 7m	Exon 18	T 10 C	M 587 T	Kinase
XLA9[d]	8 y 4 m	0.05	4.7	0.4	8 y 5 m	Exon 3	G 99 A	L 119 stop	PH

[a] XLA diagnosis was based upon strongly reduced or absent numbers of B-lymphocytes in the PB and hypogammaglobulinemia.

[b] Lower level normal serum Ig values according to reference 35: 6-12m: IgM 0.47; IgG 4.34; IgA 0.15. 1-2y: IgM 0.39; IgG 3.69; IgA 0.15. 2-3y: IgM 0.53; IgG 5.10; IgA 0.25. 6-9y: IgM 0.54; IgG 6.83; IgA 0.32.

[c] The beginning of each exon is numbered as 1.

[d] Mutation in donor splice site of exon 3 with mutant and wt *BTK* mRNA detectable.

NA = not analyzed.

within the lymphogate (Table 2) and 8% to 42% within the $CD19^+$ gate. This variable degree of blood contamination was also clear from the comparable variation of $CD3^+$ T-lymphocytes within the lymphogate, ranging from 9% to 35% (Table 2). To correct for this variance in our comparative studies between precursor B cells in normal BM and in BM from XLA patients, we excluded the mature $CD10^-$/$SmIgM^+$/$SmIgD^+$ B-cell population from our calculations (Table 2). Consequently, the percentages of stages 1 to 7 were recalculated to 100% (Figure 3). Via this recalculation the percentages of precursor B cells in Table 2 were set to 100%.

Patient characteristics and mutations in the BTK gene

The nine boys were clinically diagnosed as XLA based upon hypo- or agammaglobulinemia[35] and strongly reduced or undetectable numbers of mature B-lymphocytes in the PB. Analysis of PB mature B-lymphocytes for SmIgM/SmIgD expression showed that XLA patients 1, 4, 5, and 7 had very low percentages (<0.1% of lymphocytes) of $SmIgM^{++}$/$SmIgD^+$ B-lymphocytes. In XLA patients 3 and 6, no $SmIg^+$ B-lymphocytes could be detected. XLA patient 2 had 0.6% $SmIgM^{+/++}$/$SmIgD^+$ B-lymphocytes at the age of 6y5m, while XLA patient 8 had 0.3% $SmIgM^{++}$/$SmIgD^+$ B-lymphocytes at the age of 6y7m. XLA patient 9 was diagnosed at eight years of age and had 1.0% of $SmIgM^{+/++}$/$SmIgD^+$ B-lymphocytes in the PB and almost normal levels of IgG and IgA, but decreased levels of IgM (Table 4).

Mutation analysis of the *BTK* gene was performed at the DNA level. We found five point mutations, one splice site mutation, two small deletions and one insertion. Five mutations (XLA patients 1, 4, 5, 6, and 7) had already been observed in other patients,[5,36-41] while the other three mutations (XLA patients 2, 3, and 9) were previously unknown. The entire

BTK gene of each patient was sequenced to exclude additional mutations. The predicted effects of these mutations on the protein level are summarized in Table 4. Except for XLA patient 9, all *BTK* mutations would lead to absence of functional BTK proteins, either because of aa substitutions in the BTK kinase domain or because of premature stop codons.

In XLA patient 9, the last nucleotide of exon 3 was mutated. This mutation did not give rise to an aa substitution. However, RT-PCR analysis on mRNA from PB granulocytes generated two bands of different sizes, which were isolated and sequenced. We identified a dominant mutated form of *BTK* mRNA with 106 nucleotides inserted from intron 3, but also low levels of wt *BTK* mRNA (Figure 4). Quantitative analysis by real-time quantitative PCR with TaqMan technology of wt *BTK* mRNA expression in PB granulocytes of this patient indicated that less than 5% of normal levels were present (data not shown).

Flow cytometric analysis of BM precursor B cells from XLA patients

Because of lack of BM cells, a few triple labelings could not be performed in some XLA patients. Particularly labeling 7 (SmIgD/SmIgM/CD19) could not be performed on BM samples from XLA patients 2, 3, 4, and 7. In these four XLA patients we used the $CD10^-/SmIgM^+$ population to identify mature B cells. In five XLA patients we used thawed BMMC because no fresh BM samples were available (see Materials and Methods).

In BM from healthy children, approximately 20% of the B-cell compartment consisted of $CyIg\mu^-$ precursor B cells, equally distributed over pro-B cells and pre-B-I cells (pro-B/pre-B-I ratio of 1.2±0.8), whereas in BM from eight out of nine XLA patients approximately 80% (71% to 92%) of the B-cell compartment was located in these stages (Figure 3). Therefore, our results indicate that the differentiation arrest in the eight XLA patients result-

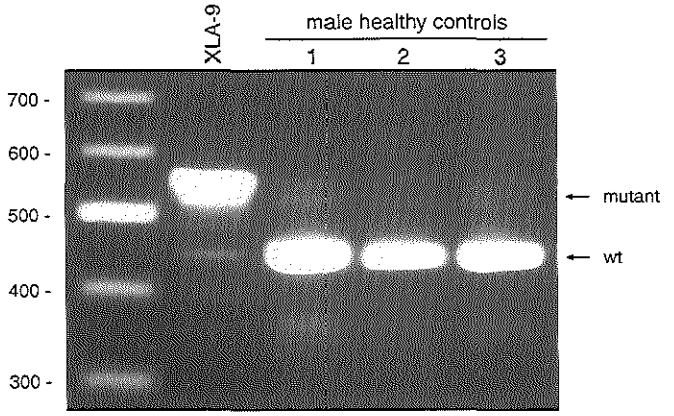

Figure 4. RT-PCR analysis of BTK mRNA from PB granulocytes of XLA patient 9 and three healthy male controls.
XLA patient 9 predominantly expressed the mutated form (upper band) with 106 nucleotides inserted from intron 3. Small amounts of wt *BTK* mRNA (lower band) could be detected.

ed in a four-fold relative accumulation of B-cell subpopulations located before the transition from CyIgμ$^-$ to CyIgμ$^+$ pre-B cells. The relative accumulation of CyIgμ$^-$ precursor B cells was somewhat lower for pro-B cells (18% to 42%) as compared to pre-B-I cells (34% to 63%) (Figure 3B), resulting in a mean pro-B/pre-B-I ratio of 0.6±0.3 in seven of the eight XLA patients. Although this differentiation arrest was virtually identical in seven of the eight XLA patients, the percentage of B-cell subpopulations located behind the arrest (CyIgμ$^+$ pre-B-II cells and immature B cells) was variable, indicating "leakiness" (Figure 3B).

Of the eight XLA patients with absence of functional BTK proteins, only XLA patient 2 displayed an atypical differentiation pattern with 73% CD19$^-$/CD22$^+$ pro-B cells and 10% pre-B-I cells (pro-B/pre-B-I ratio of 7.4). Apparently, in this patient a major differentiation arrest was located at a very early stage, i.e. at the transition from pro-B cells to CyIgμ$^-$ pre-B-I cells (Figure 3B). However, the SmIgM$^+$ B-cell population was extensive compared to other XLA patients, suggesting a second arrest at the transition from immature SmIgM$^+$ to mature SmIgM$^+$/SmIgD$^+$ B cells.

XLA patient 9 showed an apparently normal composition of the precursor B-cell compartment. Nevertheless, this patient had diminished percentages of mature B cells in his BM (Table 2) and in his PB.

In general, seven of the eight XLA patients with absence of functional BTK proteins showed a similar differentiation arrest at the transition from CyIgμ$^-$ to CyIgμ$^+$ pre-B cells, with a variable degree of "leakiness" (Figure 3B).

Atypical B-cell differentiation patterns in BM from XLA patients

In BM samples from healthy children, co-expression of the markers CD34 and CD20 was virtually absent (Table 3), since <4.4% CD34$^+$/CD20$^+$ cells were detectable (Figure 5). However, in BM from most XLA patients a substantial CD34$^+$/CD20$^+$/CD19$^+$ population could be identified. This atypical B-cell population varied between XLA patients from 11% to 20%, but was smaller (6%) in XLA patient 9. The atypical marker expression was predominantly caused by prolonged expression of CD34, since in BM samples from XLA patients 9% to 16% of CyIgμ$^+$ pre-B-II cells was CD34$^+$ (4% in XLA patient 9), compared to <2.4% in healthy children (Figure 5).

DISCUSSION

We have analyzed BM samples from 19 healthy children by flow cytometry, using 14 triple labelings, revealing the normal composition of the precursor B-cell compartment (Figure 2). This resulted in an accurate human B-cell differentiation scheme, which resembles the classification according to Ghia *et al.*[21] Our scheme, in which Ig expression patterns are dominant parameters, consists of eight consecutive stages and is summarized in Table 3. Similar analysis of the composition of the precursor B-cell compartment in BM from nine XLA patients showed that approximately 80% (71% to 92%) of the B-cell compartment in

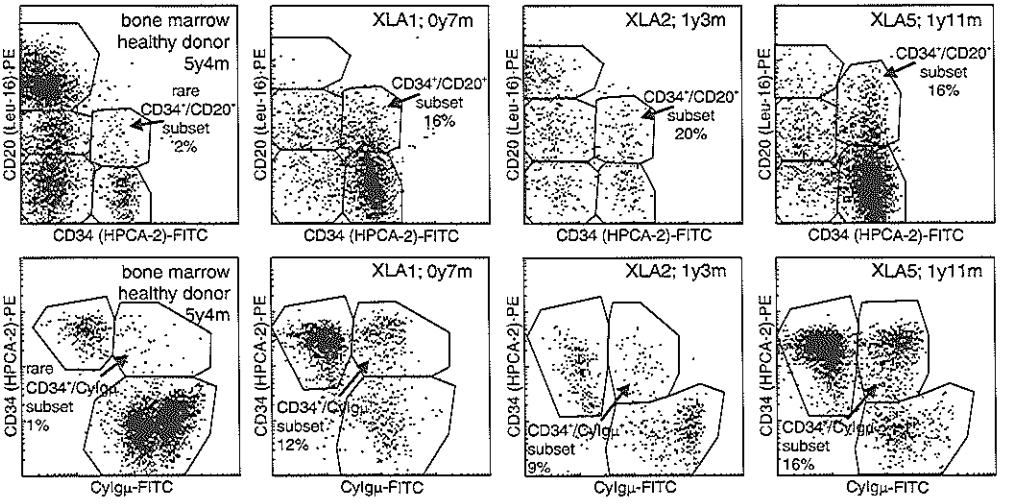

Figure 5. Flow cytometric analysis of CD34/CD20 co-expression within the CD19+ lymphogate of BM from a healthy child and three XLA patients.
In all healthy children the markers CD34 and CD20 were virtually exclusive ($CD34^-/CD20^-$ population, stage 5). However, most XLA patients showed a dominant $CD34^+/CD20^+$ population (upper panel). This $CD34^+/CD20^+$ population originated from prolonged CD34 expression, since in XLA patients a large proportion of $CyIg\mu^+$ pre-B-II cells was also $CD34^+$ (lower panel). XLA patients 2 and 5 showed the smallest and largest $CD34^+/CyIg\mu^+$ populations, respectively. XLA patient 1 was selected as a representative example of all other XLA patients (except for XLA patient 9).

eight of these patients consisted of CyIgμ- precursor B cells (pro-B cells and pre-B-I cells), in contrast to approximately 20% in healthy children. This is in line with the results of Campana *et al.*,[11] who described a maturation arrest at the transition between CyIgμ- and CyIgμ+ pre-B cells in BM of XLA patients. The localization of this arrest at the transition between CyIgμ- and CyIgμ+ pre-B cells in seven of the eight XLA patients with absence of functional BTK proteins suggests that BTK proteins are required for the expansion of the CyIgμ+ pre-B-II population by signaling via the pre-BCR, which is expressed on CyIgμ+ pre-B cells.[21,42] This is supported by the finding that BTK is constitutively phosphorylated in a pre-B cell-line, which implicates a functional role for BTK in pre-BCR signaling during B-cell development.[43]

B-cell differentiation arrests have been analyzed in BM samples from patients with non X-linked causes of agammaglobulinemia. These patients have mutations in genes that encode other components of the pre-BCR, such as the μ heavy chain or λ5/14.1,[44,45] or in genes that encode proteins involved in signaling via the pre-BCR, such as CD79a or B-cell linker protein (BLNK).[46,47] BLNK is an adapter protein, which plays a role in the same signal-transduction pathway as BTK.[48] The B-cell differentiation arrest in BM from these non X-linked agammaglobulinemia patients seems to be comparable to the arrest in XLA patients

and is also located at the transition from CyIgμ- pre-B-I cells to CyIgμ+ pre-B-II cells.[49] These results strongly support a role for BTK at the pre-BCR checkpoint in human B-cell development.

The flow cytometric data of the XLA patients clearly show a relative accumulation of CyIgμ- precursor B cells, particularly of pre-B-I cells (Figure 3B). This is in contrast with the studies by Campana *et al.*, who described normal frequencies of CyIgμ- precursor B cells, and Pearl *et al.*, who described normal frequencies of CyIgμ+ precursor B cells in their XLA patients.[10,11] These discrepancies are probably due to differences in techniques and in age of the studied patients. Our results are in line with the study by Nomura *et al.*, who described significantly increased percentages of precursor B cells located before the blockade.[15]

We hypothesized that accurate characterization of the B-cell differentiation arrest in BM of XLA patients would help to explain the level of immunological variation previously reported.[10,11] In seven out of the eight XLA patients with absence of functional BTK proteins, this differentiation arrest appeared to be very similar with a large CyIgμ- precursor B-cell compartment and a more prominent accumulation of pre-B-I cells as compared to pro-B-cells, resulting in a pro-B/pre-B-I ratio of 0.6±0.3, which is significantly lower (Mann-Whitney U test for unrelated groups with unequal standard deviations; $p<0.03$) than in normal BM (1.2±0.8). Although the arrest was similar, the degree of "leakiness", indicated by the percentage of B-cell subpopulations located behind the arrest, varied between these seven XLA patients (Figure 3B). The age of the XLA patients was comparable and the percentage of myeloid cells in the lymphogate did not differ significantly between patients, indicating that none of the patients suffered from infections at the time of BM donation. It seems therefore most likely that other genetic factors influence the degree of "leakiness", as has also been suggested with respect to the heterogeneity of the clinical picture of XLA.[7,8]

In the eighth XLA patient (XLA-2) the CyIgμ- precursor B cell compartment mainly consisted of pro-B cells (pro-B/pre-B-I ratio of 7.4). This particularly concerned the most immature CD22+/CD19-/TdT- pro-B cells (stage 1), which represented 66% of the total precursor B-cell compartment in this patient (Figures 2A and 3B). Curiously, also the percentage of SmIgM+ B cells was increased in this patient compared to other XLA patients, indicating a second arrest at the transition from immature to mature B cells. We do not have an explanation for this unique composition of the precursor B-cell compartment.

It is interesting to notice that the precursor B cells of XLA patient 9, which can generate low levels of wt BTK mRNA (Figure 4), apparently can overcome the differentiation arrest at the pre-BCR level, since CyIgμ+ pre-B-II cells were present in near normal frequencies in the BM of this patient as shown in Figure 3B. However, the reduced numbers of mature B-lymphocytes in the PB of this patient indicate that the differentiation arrest at the BCR level is more severe. This means that, if BTK proteins are indeed involved in signaling via the pre-BCR, lower amounts of BTK are needed for signal-transduction from the pre-BCR compared to the BCR. The phenotype of this patient resembles that of X-linked immunodeficiency mice, which have a point mutation in the PH domain or a targeted deletion of BTK. These mice show normal precursor B-cell compartments in BM, but reduced numbers

of mature B-lymphocytes in PB and predominantly lack IgM and IgG3 in the serum.[50-52]

The marker expression on precursor B cells in BM samples from the seven similar XLA patients seemed to be normal until the pre-B-II stage was reached in which CyIgμ is expressed. The markers CD34 and CD20 showed virtually no co-expression in healthy children, but were co-expressed in these XLA patients, resulting in a clearly detectable CD34+/CD20+/CD19+ population (Figure 5). The co-expression appeared to be caused by prolonged CD34 expression, since a large proportion of CyIgμ+ pre-B-II cells was CD34+. This suggests that normally CD34 downregulation is mediated via pre-BCR signaling.

In conclusion, the B-cell differentiation arrest in the majority of XLA patients appeared to be homogeneous, with approximately 80% of the precursor B-cell compartment being negative for CyIgμ expression. The size and nature of the residual more mature B-cell population ("leakiness") varied between patients, independent of the type of BTK mutation. Furthermore, it seems that the composition of the precursor B-cell compartment in BM of some XLA patients can be influenced by low levels of wt BTK mRNA as was particularly illustrated by XLA patient 9. It would be interesting to analyze the presence of wt BTK mRNA levels in other XLA patients with splice site mutations in the BTK gene. Since low levels of wt BTK mRNA appear to be sufficient for a seemingly normal composition of the precursor B-cell compartment, one might expect more mature B-lymphocytes in the PB and higher serum Ig levels in these patients.

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Chapter 7

XLA PATIENTS WITH *BTK* SPLICE SITE MUTATIONS PRODUCE LOW LEVELS OF WILD TYPE *BTK* TRANSCRIPTS

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Submitted

SUMMARY

X-linked agammaglobulinaemia (XLA) is caused by mutations in the Bruton's tyrosine kinase (*BTK*) gene, which result in a precursor-B-cell differentiation arrest at the transition from cytoplasmic (Cy) Igμ$^-$ pre-B-I cells to CyIgμ$^+$ pre-B-II cells in bone marrow (BM). In some XLA patients, this differentiation arrest is incomplete, leading to low but detectable numbers of functionally intact B-lymphocytes with an immature phenotype in the peripheral blood (PB).

We recently identified an XLA patient with a remarkably mild clinical phenotype, low numbers of PB B-lymphocytes, and a splice site mutation in the *BTK* gene. The precursor-B-cell compartment in the BM of this patient did not show the usual differentiation arrest as found in the BM of other XLA patients, but was almost identical to that in healthy children. Using real-time quantitative (RQ)-PCR, we were able to show low levels of wild type (wt) *BTK*-mRNA in the PB granulocytes of this patient. Based on these observations, we speculated that wt *BTK*-mRNA transcripts might be responsible for a milder clinical and immunophenotype, as has been shown in several other diseases.

Consequently, we studied wt *BTK*-mRNA expression using RQ-PCR in PB granulocytes of eight additional XLA patients with splice site mutations in the *BTK* gene and compared their phenotypes with 17 BTK-deficient XLA patients with other types of mutations. However, the presence of low levels of wt *BTK*-mRNA did not correlate with the percentage, absolute numbers, or immunophenotype of the PB B-lymphocytes, nor with the age or serum immunoglobulin levels at diagnosis.

INTRODUCTION

X-linked agammaglobulinaemia (XLA) is a primary antibody deficiency disease, which affects approximately 1 in 100,000 boys and is characterised by agamma- or hypogammaglobulinaemia and strongly reduced numbers of B-lymphocytes in the peripheral blood (PB).[1] The clinical phenotype of XLA generally develops during the first year of life, after maternal antibodies (Ab) have disappeared from the serum, with frequent infections of different organ systems, particularly upper and lower respiratory tract and gastrointestinal infections, but also bacterial skin infections, meningitis, septicaemia or osteomyelitis.[1,2]

The strongly reduced number of B-lymphocytes in the PB of XLA patients is caused by a precursor-B-cell differentiation arrest in the bone marrow (BM) at the transition from cytoplasmic (Cy) Igμ– pre-B-I cells to CyIgμ+ pre-B-II cells, i.e. at the pre-B-cell receptor checkpoint.[3-6] This differentiation arrest can be incomplete, resulting in low but detectable numbers of B-lymphocytes in the PB of some XLA patients.[7] These residual B-lymphocytes have an immature immunophenotype with significantly brighter expression of surface membrane (Sm) IgM than B-lymphocytes from healthy controls,[7] can proliferate, undergo

immunoglobulin (Ig) heavy chain isotype switching, and differentiate into specific Ab producing plasma cells.[8]

XLA is caused by mutations in the gene encoding Bruton's tyrosine kinase (BTK), located on Xq21.3.[9,10] BTK is a cytoplasmic protein of 659 amino acids (aa), composed of five different domains, the pleckstrin homology (PH), Tec homology (TH), Src homology 3 (SH3), SH2 and kinase domain, respectively.[1] So far, more than 400 unique mutations have been reported which are scattered throughout the gene.[11,12] No correlation has been described between the type and position of the mutations and phenotypic parameters, such as age or serum Ig levels at diagnosis or severity of infections.[2,13] On the contrary, XLA is a clinically heterogeneous disease, in which null mutations in the BTK gene can be associated with mild disease.[14,15] Furthermore, the clinical presentation of XLA patients can be reminiscent of common variable immunodeficiency, resulting in aberrant classification of patients or the first occurrence of symptoms during adulthood.[16-18]

However, in some diseases it has been shown that genomic mutations, which affect mRNA splicing, can result in milder clinical phenotypes.[19-22] We recently described an XLA patient whose composition of the precursor-B-cell compartment in the BM differed significantly from the composition of the precursor-B-cell compartment in other XLA patients, and was almost identical to the composition of the precursor-B-cell compartment in healthy children (Figure 1).[6] We detected a splice site mutation in exon 3 of the BTK gene, leading to the insertion of 106 nucleotides (nt) from intron 3 and subsequently a frame shift and premature stop codon. Using real-time quantitative (RQ)-PCR technology, we demonstrated low levels of wt BTK-mRNA in this patient (XLA-9 in this paper). This patient was diagnosed at a late age (8y4m) with 1% of mature B-lymphocytes in his PB and normal serum IgA, slightly reduced IgG, and almost absent IgM levels.[6] Consequently, we hypothesised that XLA patients with splice site mutations but low levels of wt BTK transcripts might have low frequencies of PB B-lymphocytes and a mild clinical phenotype.

We studied the expression of wt BTK-mRNA in PB granulocytes of nine XLA patients from six different families with splice site mutations, and compared the percentage, absolute numbers, and immunophenotype of PB B-lymphocytes with the same parameters of 17 unrelated XLA patients with other BTK mutations.

MATERIALS AND METHODS

Cell samples

We received fresh PB samples from 26 boys, who were clinically diagnosed as XLA based upon hypo- or agammaglobulinaemia[23] and reduced numbers of PB B-lymphocytes. Immunophenotyping, DNA and RNA isolation, and freezing of the mononuclear cell (MC) fraction in liquid nitrogen were performed within 24 hours after venous puncture. Frozen MC were thawed at a later stage for protein isolation from monocytes. Fresh PB samples of five male healthy controls were used as source of normal granulocytes. All cell samples were

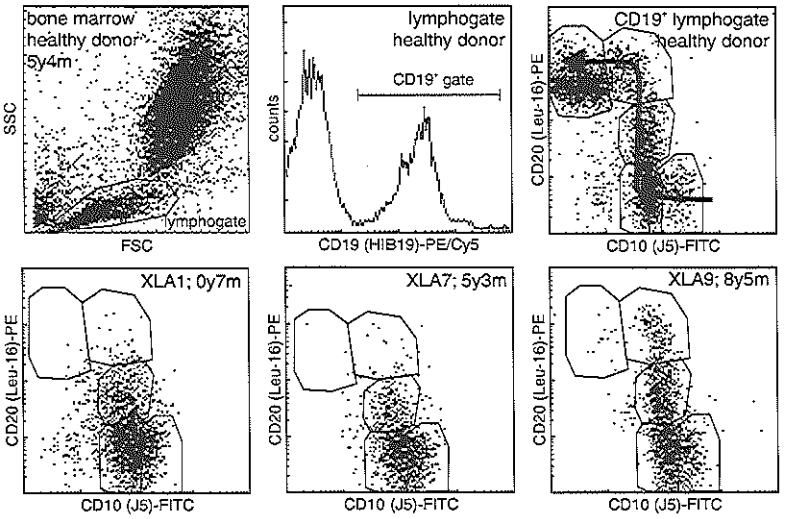

Figure 1. Flow cytometric analysis of BM samples from a healthy child and three XLA patients, showing the normal composition of the precursor B-cell compartment and the differentiation arrests in XLA patients, respectively.

The composition of the precursor-B-cell compartment was analysed within a lymphocyte gate and a $CD19^+$ histogram gate. The order of the B-cell differentiation stages in the healthy child is indicated with arrows. In general, XLA patients lacked or showed a severe reduction in the more mature B-cell differentiation stages with a relative increase in the immature B-cell differentiation stages. However, XLA patient 9 showed an almost normal composition of his precursor-B-cell compartment. The $CD10^-/CD20^{bright}$ mature B-cell population is the result of blood contamination and was not included in the analysis of the precursor-B-cell compartment. The age at bone marrow puncture is indicated.

obtained according to the informed consent guidelines of the Medical Ethics Committee of the University Hospital Rotterdam.

DNA and RNA extraction and reverse transcriptase reaction

BTK-mRNA is expressed in PB B-lymphocytes, monocytes and granulocytes. Since XLA patients have virtually no B-lymphocytes in their PB and only a small percentage of the MC consists of monocytes, we used PB granulocytes of XLA patients and healthy controls to study the expression of wt *BTK*-mRNA. Granulocytes and MC were isolated from PB by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. Granulocytes were separated from erythrocytes by NH_4Cl lysis. DNA was extracted from granulocytes using the QIAamp Blood kit (Qiagen, Chatsworth, CA, USA).[24] Total RNA was isolated from granulocytes according to the method of Chomczynski using RNAzol B (Tel-Test, Friendswood, TX, USA).[25] cDNA was prepared from mRNA as described before, using random hexamers and Superscript reverse transcriptase.[26] The cDNA quality and purity was checked by PCR amplification of *ABL*, showing only small products derived from mRNA

and virtually no large products derived from genomic DNA. The RQ-PCR primers for detection of wt *BTK*-mRNA were positioned in such a way that only wt *BTK*-mRNA could be amplified and no mutated *BTK*-mRNA or genomic *BTK* DNA (see below).

PCR amplification of DNA

PCR was performed as described previously.[26] Exons 1 to 13 and exon 19 of the *BTK* gene were amplified separately, while exons 14 to 18 were amplified in one long-range (LR)-PCR reaction. In each 100 µl PCR reaction 0.1µg DNA, 20 pmol of 5' and 3' oligonucleotides and 1 U Ampli*Taq* gold polymerase (Applied Biosystems, Foster City, CA, USA) were used. PCR conditions were 2-10 min at 94°C, followed by 60 sec at 92°C, 60 sec at 57-60°C, 2 min at 72°C for 40 cycles, followed by a final extension step (7 min at 72°C).

In each 100 µl LR-PCR reaction 0.1µg DNA sample, 5.25 U Expand enzyme mix (Boehringer Mannheim, Mannheim, Germany), and 30 pmol of 5' and 3' oligonucleotides were used. LR-PCR conditions were 2 min at 94°C, followed by 15 sec at 94°C, 30 sec at 57°C, and 4 min at 68°C for 40 cycles using 10 sec auto-extension from cycle 21 onward. After the last cycle an additional step of 10 min at 72°C was performed for final extension.

The sequences of the oligonucleotides used for PCR amplification of *BTK* were based on Oeltjen *et al.* [27] (Genbank accession number U78027), and were designed with the OLIGO 6 program (Dr. W. Rychlik, Molecular Biology Insights, Cascade, CO).[28] Primer sequences will be made available on request to interested readers.

Fluorescent sequencing reaction and analysis

LR-PCR products of *BTK* exons 14 to 18 were first purified with the QIAquick PCR purification kit (Qiagen), while short PCR products (exons 1 to 13 and 19) were used directly for sequencing with 5 µl dRhodamine dye terminator mix (Applied Biosystems), using 3.3 pmol sequencing primers. The sequencing primers were positioned in the *BTK* introns sufficiently upstream and downstream of the exon-intron border to evaluate the splice site sequences as well. All 19 *BTK* exons of each patient were sequenced to ensure the presence of only one mutation per patient. All sequencing was performed as described before[29] and run on an ABI Prism 377 fluorescent sequencer (Applied Biosystems).

Real-time quantitative (RQ) PCR analysis of wt BTK-mRNA expression

After identification of the mutations in the *BTK* gene and subsequent analysis of the effect of splice site mutations at the *BTK*-mRNA level, specific primers and TaqMan probes were designed for each individual XLA family, allowing amplification of wt *BTK*-mRNA only. Amplification from genomic DNA was ruled out by using genomic DNA as a template in the RQ-PCR reaction and the subsequent absence of RQ-PCR product. Primers and probes were designed as described before using the Primer Express 1.0 program (Applied Biosystems).[28] cDNA of patients or healthy controls was added to TaqMan universal PCR master mix (Applied Biosystems) and divided over four tubes to ensure an equal amount of cDNA per reaction. Two tubes were used for wt *BTK*-mRNA amplification and two for

GAPDH-mRNA amplification. RQ-PCR was performed as described before and run on an ABI Prism 7700 (Applied Biosystems).[28] RQ-PCR products were size-checked on a 2% agarose gel. The PCR cycle at which a fluorescent signal became detectable (threshold cycle or C_T) was comparable in the duplicate tubes ($\Delta C_T < 1.0$).

Relative quantification of wt *BTK*-mRNA was performed by correction for the expression of *GAPDH*-mRNA (Applied Biosystems) and related to the expression of wt *BTK*-mRNA and *GAPDH*-mRNA in a human EBV transformed B-cell line, derived from an X-linked hyper IgM syndrome (XHIM) patient with a mutation in the *CD40 ligand* gene, since XHIM patients have no intrinsic B-cell defects.[30] The cDNA obtained from this B-cell line was used to compare the wt *BTK*-mRNA expression levels in granulocytes of XLA patients and healthy controls (relative quantification). Using the relative standard curve method with separate tubes (Applied Biosystems, user bulletin #2, relative quantitation of gene expression), the expression of wt *BTK*-mRNA in the B-cell line was arbitrarily set to 1.0.

The slope of the standard curves should be between -3.3 and -3.7, which ensures optimal amplification, while the correlation coefficient should be close to 1.0. The amplification curves should reach a fluorescence signal intensity (ΔRn) >1.0. Standard curves and amplification curves of XLA patients using identical patient specific primer-probe sets, were generated in the same experiment to exclude inter-experiment variation. All RQ-PCR experiments were performed twice and in duplicate, showing identical results.

Flow cytometric detection of low numbers of B-lymphocytes in PB of XLA patients

Immunophenotyping with triple labelings of membrane and intracellular markers was performed as described before.[31,32] Fifty µl aliquots of whole PB or thawed PBMC were incubated for 10 min. at room temperature with combinations of optimally titrated MAb: 50 µl fluorescein isothiocyanate (FITC) conjugated MAb, phyco-erythrin (PE) conjugated MAb, and PE-Cyanine5 (PE/Cy5) conjugated MAb were used to detect membrane bound antigens. After incubation, the cells were washed and further processed depending on the type of triple labeling and the origin of the blood sample (whole PB or thawed PBMC).

Triple labelings for membrane bound antigens were directly analysed by flow cytometry using FACScan (Becton Dickinson, San Jose, CA, USA) in case of PBMC, whereas whole PB samples were first subjected to lysis of the erythrocytes using FACS Lysing Solution (Becton Dickinson).

Permeabilization of the cells using FACS Lysing Solution (Becton Dickinson) was performed prior to intranuclear staining of terminal deoxynucleotidyl transferase (TdT) both on whole PB samples and PBMC.[33,34]

Highly sensitive B-cell detection could be achieved via two consecutive gating steps, consisting of a light-scatter based lymphocyte gate and a subsequent exclusion gate using a mixture of PE conjugated MAb (CD3, CD14, CD15, CD16, and CD56) to exclude T cells, NK cells, monocytes and granulocytes from the lymphocyte gate (Figure 2).[35]

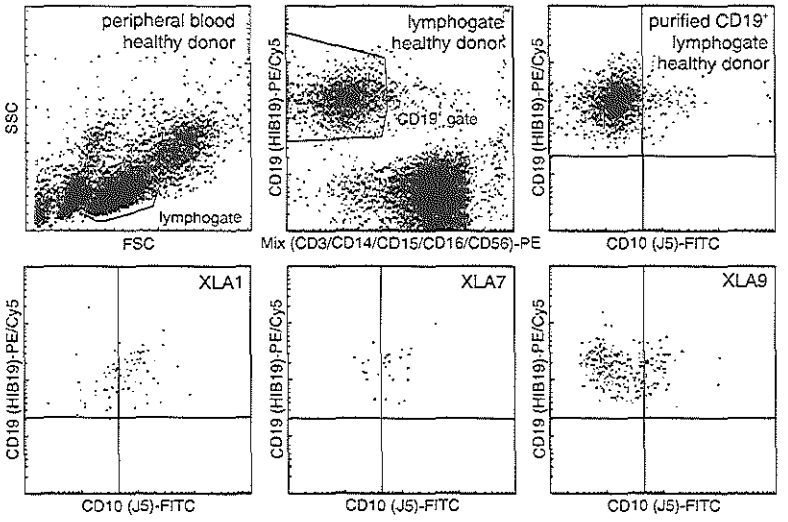

Figure 2. Flow cytometric analysis of fresh or thawed PB samples from a healthy child and three XLA patients.

The PB sample was analysed within a lymphocyte gate and a purified CD19+ gate. Due to the very low percentage of CD19+ B-lymphocytes in the PB of some XLA patients, this purification step appeared to be essential to obtain reliable results. CD19+ B-lymphocytes in the PB of XLA patients were analysed for the expression of a variety of antigens. For percentages see Table 3.

Isolation of monocytes using magnetic cell sorting (MACS), protein isolation and Western blot analysis

Monocytes were isolated from fresh or thawed PBMC by incubation of CD14 MAb (Miltenyi Biotec, Bergisch Gladbach, Germany) labelled with magnetic beads and subsequent MACS analysis (Miltenyi Biotec). After MACS analysis, the percentage CD14+ monocytes was determined by flow cytometry (purity>95%).

Cytoplasmic proteins were isolated from CD14+ monocytes by incubation with a lysis buffer (1% Triton X-100, 0.1 mM Na_3VO_4, 50 mM Tris/HCl pH 8.0, 100 mM NaCl) as described before.[36] Denatured cytoplasmic protein fractions were separated on a 12.5% polyacrylamide gel and run for 5 min at 100 V and 60 min at 200V. Gels were blotted overnight at 4°C on a nylon membrane (Schleicher & Schuell, Dassel, Germany) and blocked overnight at 4°C in 0.6% bovine serum albumine. Two different anti-BTK Abs ("Vienna", gift from Dr. M. von Lindern, and 48-2H, gift from Dr. H. Kanegane.[16]) were used to detect BTK protein expression. Visualisation was performed using peroxidase-labeled anti-isotype Ab, followed by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

RESULTS

Mutation detection in the *BTK* gene

Mutation analysis of the BTK gene was performed at the DNA level. All 19 BTK exons of each patient were sequenced to exclude more than one mutation.

We detected novel splice site mutations in six out of 23 XLA families (26%), compared to 15% in the international BTKbase.[11,12] The effects of the splice site mutations in the nine patients of the six families were evaluated at the mRNA level and are summarised in Table 1.

For comparison, we studied 17 XLA patients from unrelated families, showing 15 different mutations in the BTK gene (2 patients from unrelated families had identical mutations) and one large deletion. The effects of these mutations are summarised in Table 2. We identified eight aa substitutions (35%). Four of these aa substitutions were published before.[37-42] Three point mutations resulted in the formation of a direct stop codon (nonsense mutation = 13%). Two of these three direct stop codons were already reported by other groups.[43-45] Four small deletions (three novel ones) resulted in frame-shifts and premature stop codons (17%).[46,47] We found one previously unknown insertion leading to a frame-shift and premature stop codon (4%). Finally, we detected an unusually large deletion spanning exon 8 up to and including exon 18, resulting in an in-frame deletion of 439 aa. There was no significant difference between these percentages and the percentages in BTKbase.

As it has been described for other diseases that non-splice site mutations can also lead to aberrant mRNA splicing,[48] we wished to analyse the effect of the genomic BTK mutations at the mRNA level in the 17 XLA patients without splice site mutations. RNA was available from 11 of these 17 XLA patients (not from XLA-6, 17, 19, 20, 21, and 22). We did not detect alternatively spliced BTK-mRNA in PB granulocytes of these 11 XLA patients.

Splice site mutations in the *BTK* gene and their effects at the mRNA level

Splice sites can be divided in 5' or donor splice sites (between exon and intron) and 3' or acceptor splice sites (between intron and exon). Consensus splice site sequences of vertebrates have been identified.[49] In 100% of sequences analysed, the first two nucleotides of the intron consisted of GT, while the last two nucleotides of the same intron consisted of AG (Figure 3). Furthermore, in 77% of sequences analysed, the last nucleotide of the exon (donor splice site) was a G nucleotide (Figure 3). Fifteen percent of all point mutations in human genetic diseases cause an mRNA splicing defect.[50] The effects of mutations in a splice site include exon skipping, intron inclusion, or activation of a proximal or distal cryptic splice site.[51]

We detected splice site mutations in six XLA families, consisting of four donor and two acceptor splice site mutations (Table 1 and Figure 3). In three out of six XLA families (XLA-11, 13, and 18), we found that the first G nucleotide in the donor splice site of the intron was mutated (Figure 3), resulting in skipping of exon 5 in XLA-13, activation of a

Table 1. Characteristics of XLA patients with splice site mutations.

Patient	Genomic mutation[a]		Effect on	Predicted effect at protein level		Position of primers/	Amount of wt	Healthy	% of
	Position	Nucleotide	BTK-mRNA	Amino Acid	Domain	TaqMan probe[b]	BTK-mRNA[c]	controls[c]	healthy control
XLA-9	Exon 3	G 99 A[d]	Ins 106 nt intron 3	FS; codon 119 stop	PH	Forward primer over border between exons 3 and 4	5.2×10^{-2}	1.7 ± 0.6	3
XLA-10.1	Intron 15	A 1356 C	Del exon 16	FS; codon 527 stop	Kinase	Forward primer in exon 16	1.4×10^{-1}	1.1 ± 0.4	13
XLA-10.2							3.0×10^{-1}		27
XLA-11	Intron 18	G 1 A	Del last 33 nt exon 18	In frame del aa 626-636	Kinase	Forward primer in deleted 33 nt	4.7×10^{-4}	1.7 ± 1.1	0.03
XLA-13.1	Intron 5	G 1 T	Del exon 5	FS; codon 104 stop	PH	Probe and reverse primer in exon 5	1.4×10^{-3}	2.2 ± 1.1	0.06
XLA-13.2							6.2×10^{-3}		0.3
XLA-18.1	Intron 3	G 1 C	Ins 106 nt intron 3	FS; codon 119 stop	PH	Forward primer over border between exons 3 and 4	0[f]	3.7 ± 1.3	0
XLA-18.2							0		
XLA-23	Intron 4	A 1552 G	Ins 21 nt intron 4	In frame ins 7 aa	PH	Forward primer over border between exons 4 and 5	1.8×10^{-1}	2.7 ± 0.6	7

[a] The beginning of each exon or intron is numbered as 1.

[b] Primers and TaqMan probes were positioned in such a way that only wt BTK-mRNA and no genomic DNA or mutated BTK-mRNA could be amplified.

[c] Amount of wt BTK-mRNA expressed in PB granulocytes and corrected for the amount of GAPDH-mRNA. The amount of BTK-mRNA in a human EBV transformed B-cell line, derived from an XHIM patient, was used as positive control and arbitrarily set to 1.0.

[d] This mutation did not result in an aa substitution (silent), but did affect the splice site.

[e] Amount of wt BTK-mRNA in PB granulocytes of male healthy controls (n=5), using the patient specific TaqMan primer-probe set.[3]

[f] As wt BTK-mRNA was not detectable in PB granulocytes, these patients were classified as having a frame-shift and premature stop codon upon statistical analysis (Table 3).

Abbreviations: Ins = insertion; Del = deletion; FS = frame-shift.

Table 2. Characteristics of XLA patients with other than splice site mutations.

Patient	Genomic mutation[a]		Predicted effect at protein level	
	Exon	Nucleotide	Amino Acid	Affected domain
XLA-1[b]	Exon 17	C 53 T	R 562 W	Kinase
XLA-4	Exon 15	G 178 A	M 509 I	Kinase
XLA-8	Exon 18	T 10 C	M 587 T	Kinase
XLA-15	Exon 15	T 77 G	Y 476 D	Kinase
XLA-17	Exon 2	G 33 T	M 1 I	PH
XLA-20[b]	Exon 17	C 53 T	R 562 W	Kinase
XLA-21	Exon 15	T 177 C	M 509 T	Kinase
XLA-22	Exon 18	C 105 T	P 619 S	Kinase
XLA-2	Exon 7	26 del G	FS; codon 198 stop	TH
XLA-3	Exon 2	142 del TC	FS; codon 40 stop	PH
XLA-6	Exon 3	75 ins A	FS; codon 84 stop	PH
XLA-14[c]	Exon 3	91 del 15 nt	FS; codon 114 stop	PH
XLA-19	Exon 8	54 del TG	FS; codon 223 stop	SH3
XLA-5	Exon 15	C 106 A	Y 485 stop	Kinase
XLA-7	Exon 14	C 98 A	Y 425 stop	Kinase
XLA-16	Exon 8	C 175 T	R 255 stop	SH3
XLA-12	Exon 8-18	Large deletion	In frame del aa 197-636	TH, SH3, SH2, Kinase

[a] The beginning of each exon is numbered as 1.

[b] Unrelated patients with identical mutation.

[c] Splice-site deletion resulting in the insertion of 100 nucleotides from intron 3.

Ins = insertion; Del = deletion; FS = frame-shift.

	Donor splice site							Acceptor splice site			
Exon	G	G	T	Intron				A	G		Exon
Frequency	77%	100%	100%					100%	100%	Effect of mutation	wt mRNA
XLA-9	A	.	.	.	.	.	.	.	.	Downstream SS	+
XLA-10	.	.	.	.	.	.	.	C	.	Exon skipping	+
XLA-11	.	A	.	.	.	.	.	.	.	Upstream SS	+
XLA-13	.	T	.	.	.	.	.	.	.	Exon skipping	+
XLA-18	.	C	.	.	.	.	.	.	.	Downstream SS	-
XLA-23	.	.	.	.	.	.	.	G	.	Upstream SS	+

Figure 3. Consensus donor and acceptor splice sites with mutations identified in the six XLA families. Mutations in the splice sites resulted in activation of a proximal (XLA-11 and 23) or distal (XLA-9 and 18) cryptic splice site, or in exon skipping (XLA-10 and 13). The absence of wt *BTK*-mRNA in XLA family 18 suggests that a C nucleotide at position 1 of intron 3 results in a fully non-functional donor splice site.

proximal donor splice site in exon 18 with consequent deletion of a part of exon 18 in XLA-11, and activation of a distal cryptic splice site with inclusion of a part of intron 3 in XLA-18. In XLA-11 and XLA-18, the cryptic donor splice sites contained a GT sequence, comparable to normal donor splice sites. In XLA-11, other GT sequences were present in closer approximation to the original donor splice site of intron 18, but usage of these other

cryptic donor splice sites would generate out-of-frame mRNA.

We identified mutations in the acceptor splice site of intron 15 (XLA-10), resulting in the complete deletion of exon 16, and in the acceptor splice site of intron 4 (XLA-23), leading to activation of a more proximal intronic cryptic splice site (Table 1).

The donor splice site of intron 3 was mutated in three families (XLA-9, 14, and 18) (Tables 1 and 2). In XLA-14 a deletion of 15 nt (9 nt from exon 3 and 6 nt from intron 3) of the donor splice site occurred, resulting in the insertion of 100 nt from intron 3, leading to a frame-shift and premature stop codon.[46] Since the donor splice site was deleted in XLA-14, this patient was predicted to be unable to generate wt *BTK*-mRNA and was therefore classified as having a frame-shift and premature stop codon (Table 2).

Splice site mutations and the ability to produce wt BTK-mRNA

The splice site mutation in XLA-9 caused an out-of-frame insertion of 106 nt from intron 3 (Table 1), but also gave rise to low levels of wt *BTK*-mRNA.[6] The wt *BTK*-mRNA expression in XLA-9 was proven by direct fluorescent sequencing of the isolated PCR product.[6] Therefore, we investigated the expression of wt *BTK*-mRNA in PB granulocytes of the eight other XLA patients with splice site mutations from five different families. The primers and TaqMan probes were positioned in such a way, that only wt *BTK*-mRNA could be amplified (Table 1) and the RQ-PCR products were size-checked on a 2% agarose gel.

The amplification curves are shown in Figure 4. C_T values of XLA patients were higher than C_T values of the B-cell line, indicating that the expression level of wt *BTK*-mRNA in PB granulocytes of XLA patients with splice site mutations was lower than the expression level in the human B-cell line. We corrected the expression levels of wt *BTK*-mRNA by determining the expression levels of *GAPDH*–mRNA in the same sample. The standard curves for calculation of the wt *BTK* mRNA levels all had slopes between –3.4 and –3.6 and correlation coefficients > 0.99, except for XLA-18.1 and 18.2 (correlation coefficient of 0.93).

As can be seen in Table 1, different splice site mutations had different effects on the ability to produce wt *BTK*-mRNA. For example, PB granulocytes of patients XLA-10.1 and 10.2 with a mutation in the acceptor splice site of intron 15 expressed 1.4×10^{-1} and 3.0×10^{-1} wt *BTK*-mRNA, respectively (compared to 1.0 in a human EBV^+ B-cell line), while wt *BTK*-mRNA could not be detected in PB granulocytes of patients XLA-18.1 and 18.2 with a mutation in the donor splice site of intron 3. Since we hypothesised that the presence of wt *BTK*-mRNA would result in low frequencies of PB B-lymphocytes and a mild clinical phenotype, XLA-18.1 and 18.2 were reclassified as having a frame shift and premature stop codon in Table 3.

As mRNA was isolated from PB granulocytes separated by Ficoll-Paque density centrifugation, the differences in wt *BTK*-mRNA expression cannot be attributed to heterogeneity of the cell population. The expression level of wt *BTK*-mRNA in PB granulocytes of healthy controls varied between 1.1 and 3.7 (compared to 1.0 in a human EBV^+ B-cell line), depending on the primer-probe set used (Table 1). This variation is minor in the context of PCR studies, as illustrated by the robustness of the RQ-PCR analyses with comparable con-

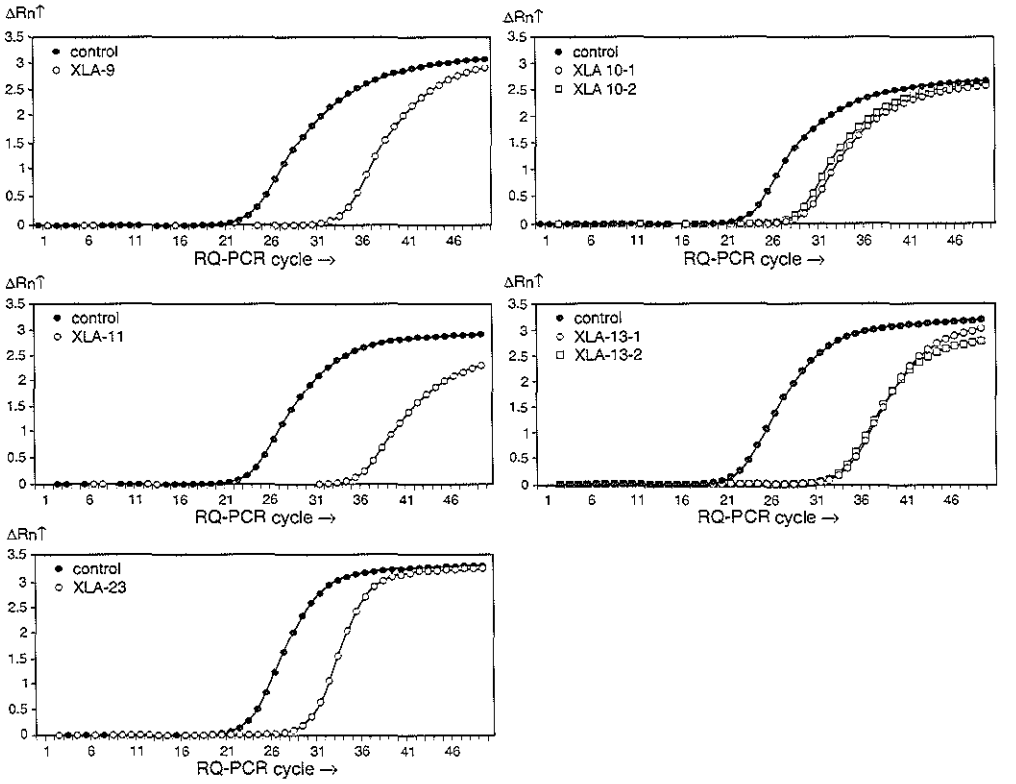

Figure 4. Expression of wt *BTK*-mRNA in PB granulocytes of seven XLA patients from five families with splice site mutations in the *BTK* gene compared to the expression level in a human EBV transformed B-cell line.

The increase in fluorescence (ΔRn) is plotted on the y axis against the number of RQ-PCR cycles on the x axis. A robust RQ-PCR should reach a ΔRn>1.0. Curves with closed symbols represent the amplification of wt *BTK*-cDNA from a human EBV-transformed B-cell line (undiluted) using different wt *BTK* primer-probe sets. C_T-values derived from a dilution series of this cDNA were used to generate standard curves (not shown). Open symbols represent amplification curves of wt *BTK*-cDNA from PB granulocytes of XLA patients with splice site mutations. In PB granulocytes of XLA patients 18-1 and 18-2, wt *BTK*-mRNA expression was not detectable.

trol C_T values and high ΔRn values for all primer-probe sets used (Figure 4). Based on the minor variation of wt *BTK* transcript levels in healthy controls, we suggest that more than 5 to 10 fold differences in these levels might have a biological significance. In this respect, wt *BTK*-mRNA levels were comparable in patients of the same family (Table 1).

Western blotting for BTK protein detection

We investigated the presence of BTK proteins in PB monocytes of XLA patients, which were purified using magnetic cell sorting. However, using two different Ab against BTK on Western blot, we were not able to show BTK protein expression in PB monocytes

Table 3. Absolute counts and phenotype of B-lymphocytes in PB of XLA patients.

Patient	Mutation	% CD19+ B-cells (within lymphogate)	Absolute numbers CD19+ B-cells (x10⁹/l)	Age at diagnosis (months)	% CD10+	Igκ/Igλ ratio	Phenotype (within CD19+ lymphogate) %SmIgM^{hi}/ SmIgD^{lo}	%SmIgM^{hi}/ SmIgD^{hi}	%SmIgM^{lo}/ SmIgD^{hi}	Serum Ig levels at diagnosis IgA (g/l)	IgG (g/l)	IgM (g/l)
XLA-9	SS	1.04	0.015	100	44	1.1	16	40	43	0.4	4.7	0.05
XLA-10.1	SS	0.04	0.0021	41	79	1.8	77	23	0	<0.05	<0.4	<0.04
XLA-10.2	SS	0.28	0.011	23	66	1.6	62	20	15	<0.05	0.76	0.12
XLA-11	SS	0.011	<0.001	22	NE	NE	NE	NE	NE	<0.05	<1.0	<0.04
XLA-13.1	SS	<0.01	<0.001	36	NE	NE	NE	NE	NE	<0.05	<0.1	0.06
XLA-13.2	SS	0.079	0.0026	31	91	1.6	54	32	9	<0.1	0.18	<0.1
XLA-23	SS	0.057	0.0044	18	43	1.0	46	50	4	<0.05	0.33	<0.04
XLA-1	AA	0.096	0.010	7	84	0.9	37	35	18	0.01	0.3	0.11
XLA-4	AA	0.062	0.0022	7	80	2.6	82	18	0	0.04	0.41	0.13
XLA-8	AA	0.26	0.0047	79	78	0.8	51	41	7	<0.05	3.0	<0.1
XLA-15	AA	0.26	0.0054	7	74	1.2	24	56	19	0.26	1.8	0.24
XLA-17	AA	<0.01	<0.001	24	NE	NE	NE	NE	NE	<0.05	2.5	0.1
XLA-20	AA	0.029	<0.001	NA	NE	NE	NE	NE	NE	NA	NA	NA
XLA-21	AA	0.018	<0.001	26	NE	NE	NE	NE	NE	0.05	0.19	0.03
XLA-22	AA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
XLA-2	FS	0.56	0.010	14	63	1.3	50	21	27	0.17	0.27	0.26
XLA-3	FS	<0.01	<0.001	21	NE	NE	NE	NE	NE	<0.1	0.36	<0.1
XLA-6	FS	<0.01	<0.001	35	NE	NE	NE	NE	NE	<0.06	<0.1	<0.05
XLA-14	FS	0.053	<0.001	12	NE	NE	NE	NE	NE	0.15	0.21	0.91
XLA-18.1	FS	0.016	<0.001	NA	NE	NE	NE	NE	NE	0.02	NA	<0.29
XLA-18.2	FS	0.66	0.019	43	71	1.1	24	48	26	0.12	0.26	0.33
XLA-19	FS	0.32	0.005	36	88	1.3	67	1	25	NA	NA	NA
XLA-5	Stop	0.08	0.0020	22	59	1.1	51	18	24	0	0.01	0.13
XLA-7	Stop	0.042	0.0011	20	86	2.0	73	27	0	0.18	<0.3	0.28
XLA-16	Stop	0.77	0.051	15	85	1.2	57	26	14	<0.05	<0.4	<0.04
XLA-12	LD	<0.01	<0.001	11	NE	NE	NE	NE	NE	0.19	1.29	0.29

Sm = surface membrane; NE = not evaluable, because of low numbers of CD19+ B cells (<0.001 x 10⁹/l); hi = high expression; lo = low expression; SS = splice site mutation; AA = amino acid substitution; FS = frame shift; stop = direct change into a stop codon; LD = large deletion; NA = not analysed.

from any of the XLA patients analysed (data not shown).

Sensitive flow cytometric detection of mature PB B-lymphocytes

Patient XLA-9 expressed low levels of wt *BTK*-mRNA (Table 1), showed an almost normal composition of the precursor B-cell compartment in his BM, had 1% of PB B-lymphocytes and normal serum IgA, slightly reduced IgG, and almost absent IgM levels, and was diagnosed at a late age (8y4m).[6] Based on these observations, we hypothesised that XLA patients with splice site mutations in the *BTK* gene, who retained the ability to produce low levels of wt *BTK*-mRNA, would have higher absolute numbers of PB B-lymphocytes. Therefore, we performed sensitive flow cytometric detection (with a detection limit of 0.01% or 0.001 x 10^9/l) of PB B-lymphocytes in nine XLA patients with splice site mutations and 17 XLA patients with other mutations in the *BTK* gene, as described before (Figure 2).[35]

Absolute numbers of $CD19^+$ B-lymphocytes were compared with age-matched reference values as reported by Comans-Bitter *et al.*[52] When the absolute number of $CD19^+$ B-lymphocytes was less than 0.001 x 10^9/l, the immunophenotype of the B-lymphocytes could not be interpreted properly. The percentage and absolute number of PB B-lymphocytes did not differ significantly between the three groups of XLA patients (one way analysis of variance (ANOVA); p=0.65 and p=0.652, respectively).

$CD19^+$ B-lymphocytes were analysed for the expression of CD34, TdT, CD10, SmIgM, SmIgD, SmIgκ and SmIgλ. $CD19^+$ B-lymphocytes in PB of healthy controls are $CD10^-$, $CD34^-$, and TdT^-, mature from $SmIgM^{high(hi)}/SmIgD^{low(lo)}$, via $SmIgM^{hi}/SmIgD^{hi}$ to $SmIgM^{lo}/SmIgD^{hi}$ [53] and show a mean κ/λ ratio of 1.4. $CD19^+$ B-lymphocytes in the PB of XLA patients did not express CD34 or TdT, but 43-91% (mean: 73%) of $CD19^+$ B-lymphocytes in the PB of XLA patients expressed CD10 (Table 3). The percentage of $CD10^+/CD19^+$ B-lymphocytes did not differ significantly between the three groups of XLA patients (ANOVA; p=0.336). Furthermore, we could not detect the most mature $SmIgM^{lo}/SmIgD^{hi}$ B-lymphocytes in the PB of three out of 15 XLA patients analysed, indicating that also a peripheral maturation arrest occurred, in addition to the incomplete ("leaky") differentiation arrest in the BM of XLA patients.[4,6,7]

DISCUSSION

Until now, it has been impossible to establish a genotype-phenotype relationship in XLA patients.[14, 15] Also in this study, we did not observe a significant difference in age and serum IgA, IgG, and IgM levels at diagnosis between the three groups (ANOVA; p=0.34, p=0.876, p=0.23, and p=0.158, respectively). Moreover, variation in clinical phenotype has been shown in families with XLA patients that carry identical *BTK* mutations.[14,15] This phenotypic variation within families is generally attributed to environmental factors and/or additional genetic differences, such as polymorphisms.

We identified an 8-year-old boy (XLA-9) with a mild clinical phenotype and 1%

B-lymphocytes in his PB, which we attributed to a splice site mutation in the *BTK* gene.[6] Using RQ-PCR, we showed low levels of wt *BTK*-mRNA in the PB granulocytes of this XLA patient, leading to our hypothesis of a milder clinical phenotype in XLA patients with splice site mutations in the *BTK* gene, which allow generation of low levels of wt *BTK*-mRNA.

We used sensitive flow cytometric detection of PB B-lymphocytes as a phenotypic read-out system and compared nine XLA patients from six different families with splice site mutations in the *BTK* gene with 17 XLA patients with mutations in the coding exons of the *BTK* gene. The levels of wt *BTK*-mRNA in the XLA patients with splice site mutations were highly variable and did not correlate with percentages or absolute numbers of $CD19^+$ B-lymphocytes in PB. In contrast, XLA-16 with a premature stop codon in the *BTK* gene showed the highest absolute numbers of PB B-lymphocytes. This result could not be explained by an in-frame deletion of the sequence with the premature stop codon in XLA-16, although it has been described for several other diseases that mutations in exons could also result in aberrant mRNA splicing.[48]

The base pair substitution of XLA-9 was not at an invariant site within the splice site consensus sequence, whereas all of the other patients with splice site mutations had alterations at invariant sites (Figure 3). Therefore, it was surprising to find that the relative expression level of wt *BTK* transcripts was not highest in PB granulocytes from XLA-9 (Table 1). This suggests that it might be more important to recognise whether XLA patients with splice site mutations are able to generate wt *BTK* transcripts at all, rather than to precisely quantify the transcript levels.

A substantial fraction of PB B-lymphocytes (mean: 73%) in XLA patients showed an immature phenotype ($CD10^+/CD19^+$ B-lymphocytes), resembling regenerating B-lymphocytes present in the PB of children after completion of treatment for acute lymphoblastic leukaemia.[54] As the precursor-B-cell compartment in the BM of XLA patients shows a differentiation arrest at the transition from $CyIg\mu^-$ pre-B-I cells to $CyIg\mu^+$ pre-B-II cells,[4,6] most precursor-B cells present in the BM of XLA patients are $CD34^+$ and TdT^+.[6] B-lymphocytes present in the PB of XLA patients were $CD34^-$ and TdT^-, indicating that these B-lymphocytes were different from the pre-B-I cells found in the BM. The B-lymphocytes in the PB of XLA patients have slipped through the differentiation arrest, which has therefore been termed 'leaky'. In addition to the B-cell differentiation arrest in the BM, it has been described that B-lymphocytes present in the PB of XLA patients show an immature phenotype with a significantly brighter expression of SmIgM than B-lymphocytes from healthy controls, indicating a peripheral maturation arrest.[7] Accordingly, we could not detect the most mature $SmIgM^{lo}/SmIgD^{hi}$ B-lymphocytes in the PB of three out of 15 XLA patients analysed (Table 3). However, the percentages of $SmIgM^{hi}/SmIgD^{lo}$, $SmIgM^{hi}/SmIgD^{hi}$, and $SmIgM^{lo}/SmIgD^{hi}$ on $CD19^+$ B-lymphocytes were independent of the type of mutation in the *BTK* gene (ANOVA; $p=0.615$, $p=0.34$, and $p=0.938$, respectively).

Recently, Dingjan *et al.* found that *BTK* deficient mice showed diminished usage of Igλ (2.5 compared to 5.0 in wt mice), resulting in an increased Igκ/Igλ ratio.[55] A similar finding was published by Conley, who reported that PB B-lymphocytes in XLA patients had a

higher Igκ/Igλ ratio than in healthy controls (2.9 versus 1.1).[7] However, as shown in Table 3, we did not detect abnormalities in the Igκ/Igλ ratio in XLA patients (mean of 1.4 ± 0.5). Furthermore, the Igκ/Igλ ratio did not differ significantly between the three groups of XLA patients (ANOVA; p=0.962).

The presence of wt *BTK*-mRNA normally results in BTK protein expression. Therefore, we used Western blot to analyse $CD14^+$ monocytes from XLA patients for BTK protein expression, which was not detected in any of the XLA patients analysed (data not shown). However, Western blot is insensitive and the low *BTK*-mRNA expression levels in XLA patients with splice site mutations (Table 1) do probably not allow the detection of BTK proteins on Western blot. Flow cytometric BTK detection as reported by a Japanese group might be more sensitive,[16,44] but this approach was not available at the time of our study.

In conclusion, the finding that a splice site mutation in the *BTK* gene, resulting in low levels of wt *BTK*-mRNA, occurred in an XLA patient with an almost normal composition of his precursor-B-cell compartment in the BM and a mild clinical phenotype (XLA-9),[6] suggested that XLA patients with splice site mutations in the *BTK* gene in general would show a milder clinical and/or immunological phenotype. Our detailed analyses show that low levels of wt *BTK*-mRNA are indeed present in virtually all XLA patients with splice site mutations, but this appeared not to be associated with a significantly milder immunological or clinical phenotype. We recently performed flow cytometric analysis of the precursor-B-cell compartment in the BM of a second patient with a splice site mutation (XLA-23) and found a differentiation arrest at the transition from $CyIg\mu^-$ pre-B-I cells to $CyIg\mu^+$ pre-B-II cells with more than 70% of the precursor-B-cell compartment negative for CyIgμ expression (data not shown), which is identical to the precursor-B-cell differentiation arrest found in the majority of XLA patients.[6] These findings underline that the heterogeneity of the clinical and immunological XLA phenotype is influenced by other (genetic) factors, such as polymorphisms.

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Chapter 8

MAPPING OF A HOMOZYGOUS *IGH-C$_\mu$* DELETION IN A FEMALE AGAMMAGLOBULINEMIA PATIENT USING DNA FIBER FISH

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Submitted

SUMMARY

Most patients suffering form inherited forms of agammaglobulinemia have mutations in one of five candidate genes encoding Bruton's tyrosine kinase (BTK), immunoglobulin heavy chain constant mu (IGH-C_μ), λ14.1, CD79a, or B-cell linker protein. Mutations in the *BTK* gene result in X-linked agammaglobulinemia, while defects in the other four genes show autosomal recessive inheritance patterns.

We present a female agammaglobulinemia patient with complete absence of PB B-lymphocytes. Flow cytometric analysis of bone marrow showed complete absence of cytoplasmic (Cy) Igμ, surface membrane (Sm) IgM, and SmIgD expression, while CyCD79a and Cyλ14.1 expression levels were normal. A bi-allelic *IGH-*C_μ deletion was identified by PCR and Southern blot analysis. Subsequent DNA fiber fluorescent *in situ* hybridization allowed us to precisely map a D_H-J_H-C_μ-C_δ deletion on both *IGH* alleles. This is the third report describing a defect in *IGH-*C_μ as cause of agammaglobulinemia, indicating the low prevalence of *IGH* gene defects.

INTRODUCTION

Children suffering from inherited forms of agammaglobulinemia generally present with bacterial infections of the upper and lower respiratory tract after maternal antibodies have disappeared from the serum. Until now, five different genetic defects resulting in agammaglobulinemia have been described. Approximately 85% of agammaglobulinemia patients are males, suffering from X-linked agammaglobulinemia, caused by mutations in the Bruton's tyrosine kinase (*BTK*) gene.[1,2] The remaining 15% of agammaglobulinemia patients suffer from autosomal-recessively inherited genetic defects, such as mutations in the immunoglobulin heavy chain constant mu (*IGH-*C_μ),[3] *λ14.1*,[4] *CD79a*,[5] B-cell linker protein (*BLNK*),[6] or yet unknown genes.[7]

Agammaglobulinemia results from the absence or strongly reduced numbers of B-lymphocytes in the peripheral blood (PB), as a consequence of an arrest in precursor-B-cell differentiation in bone marrow (BM). This arrest is comparable for all five genetic defects and is positioned at the pre-B-cell receptor (pre-BCR) checkpoint[7,8] However, the severity of the arrest varies between the genetic defects, resulting in low numbers of PB B-lymphocytes in some BTK and λ14.1 deficient patients, while Igμ, CD79a, and BLNK deficient patients present with complete absence of PB B-lymphocytes.[7]

We studied a female agammaglobulinemia patient and identified a homozygous *IGH-*C_μ deletion, which was precisely mapped using DNA fiber fluorescent *in situ* hybridization (FISH).

PATIENT, MATERIAL AND METHODS

Case report

The patient was diagnosed as having agammaglobulinemia at the age of 16 months. Serum IgG and IgA were not detectable and only a minor amount of IgM was present. Despite substitution with gammaglobulin and prophylactic antibiotics, she suffered from severe infections and impaired growth. At the age of 2 years a devastating panophthalmitis caused blindness of both eyes. Recurrent and persisting pneumonias led to bronchiectasias for which a lobotomy of the left lung was performed at the age of 4 years. The remaining part of the left lung had to be removed when she was 12 years old. Chronic middle ear infections resulted in impaired hearing.

The parents are consanguineous. The patient has one healthy older sister and a one-year-old baby sister who also suffers from agammaglobulinemia. Three brothers of the patient's father died in Turkey at 1, 2 and 3 years of age, respectively. The causes of their death are unknown.

Flow cytometric analysis of PB and BM samples

Heparinized PB (25 or 50 µl; undiluted) and BM (50µl; 15 x 10^6 nucleated cells/ml) were incubated for 10 min. at room temperature with quadruple combinations of monoclonal antibodies as described before.[9] Lysis of erythrocytes was performed using BD Lysing Solution (Becton Dickinson, San Jose, CA, USA) or IntraPrep Permeabilization Reagent (Immunotech, Marseille, France). Labeled cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

DNA extraction and (long range (LR))-PCR amplification

DNA was extracted from PB granulocytes with the QIAamp Blood kit (Qiagen, Chatsworth, CA, USA).[10] The 6 exons of the *IGH-*C_μ gene were amplified individually in 50 µl PCR reactions, while the entire *IGH-*C_μ gene was amplified in a single 100 µl LR-PCR reaction, using 50 or 100 ng DNA, 10 or 30 pmol of 5' and 3' oligonucleotides, and 0.5 U Ampli*Taq* gold polymerase (Applied Biosystems, Foster City, CA, USA) or 5.25 U Expand DNA polymerase (Boehringer Mannheim, Mannheim, Germany), respectively. PCR and LR-PCR conditions were 7 or 2 min at 95 or 94°C, followed by 1 min or 15 sec at 94°C, 1 min or 30 sec at 57 or 60°C, 2 or 7 min at 72 or 68°C for 40 cycles (using 10 sec auto-extension from cycle 21 onward in case of LR-PCR), followed by a final extension step (10 min at 72°C). The sequences of the oligonucleotides will be made available upon request.

Southern blot (SB) analysis of the *IGH* locus

*Eco*RI or *Bam*HI digested genomic DNA from PB granulocytes of the patient and healthy controls was hybridized with the following probes: IGHJ6, *Sau*3A-J_H, IGHMU, Cγ, and TCRBJ1 as described before.[11-13]

DNA fiber FISH analysis of the *IGH* locus

DNA fiber FISH was performed on PB mononuclear cells (MC) as described before,[14] using the following probes: 4394 (U2-2) recognizing *IGH-D_H* and *IGH-J_H* (red); 4395 (Cos3/64) recognizing *IGH-C_μ* and *IGH-C_δ* (green); 4237 (CosIg6) recognizing *IGH-C_γ* and *IGH-C_α* gene segments (green); 4555 recognizing *IGH-C_γ* gene segments (red); and 4551 recognizing *IGH-C_α* gene segments (red).

RESULTS AND DISCUSSION

Flow cytometric analysis of PB showed complete absence of B-lymphocytes ($<0.001 \times 10^9$/l) in our patient. Subsequent flow cytometric analysis of BM showed normal expression levels of cytoplasmic (Cy) CD79a and Cyλ14.1, but no expression of CyIgμ (Figure 1), surface membrane (Sm) IgM, and SmIgD. Based on the flow cytometric results, we performed PCR analysis of the *IGH-C_μ* gene. However, both LR-PCR amplification of the entire *IGH-C_μ* gene and PCR amplification of the six separate *IGH-C_μ* exons did not result in detectable PCR products.

As these results suggested a bi-allelic deletion of the *IGH-C_μ* gene, we performed SB analysis of the *IGH* locus for confirmation. Indeed no hybridization signals were obtained with the IGHJ6, *Sau*3A-J_H, or IGHMU probes,[11] while hybridization with a control probe (TCRBJ1) showed bands of the expected size. Hybridization with a Cγ specific probe showed a banding pattern that corresponded to the presence of all five *IGH-C_γ* gene exons.[12] These results indicated a bi-allelic deletion of the entire *IGH-J_H* and *IGH-C_μ* region with presence of the *IGH-$C_\gamma 3$* gene, which is the most upstream member of the *IGH-C_γ* gene cluster.

To assess this bi-allelic deletion more precisely, DNA fiber FISH analysis was performed. As expected in a consanguineous family, a homozygous deletion was observed, affecting *IGH-D_H*, *IGH-J_H*, *IGH-C_μ*, and *IGH-C_δ* gene segments (Figure 2).

Until now, only two reports described patients with defects in the *IGH-C_μ* gene.[3,15] Yel *et al.* described four mutations in three families, including two large deletions and two point mutations in exon 4.[3] Schiff *et al.* described a homozygous insertion of one nucleotide in exon 1, resulting in a frame shift and premature stop codon.[15] BM samples were analyzed from one of the patients with an amino acid substitution[3] and from the patient with the insertion in exon 1.[15,16] The first patient contained normal percentages of CyIgμ$^-$/CD19$^+$ pre-B-I cells, but a marked decrease was observed in the number of CyIgμ$^+$/CD19$^+$ pre-B-II cells, which was probably the result of unstable Igμ proteins.[3] In the other patient diminished amounts of CyIgμ$^-$/CD19$^+$ pre-B-I cells were reported, whereas CyIgμ$^+$/CD19$^+$ pre-B-II cells were completely missing.[15] Our patient showed 6% precursor-B-cells within the lymphogate (29±10% in fresh BM samples of healthy children).[9] However, no CyIgμ could be detected, revealing a complete differentiation arrest at the pre-BCR checkpoint, i.e. at the transition from CyIgμ$^-$ pre-B-I cells to CyIgμ$^+$ pre-B-II cells (Figure 1). This arrest is comparable to the arrests described in the two other Igμ deficient patients.[3,15] Expression of Igμ is apparently essential

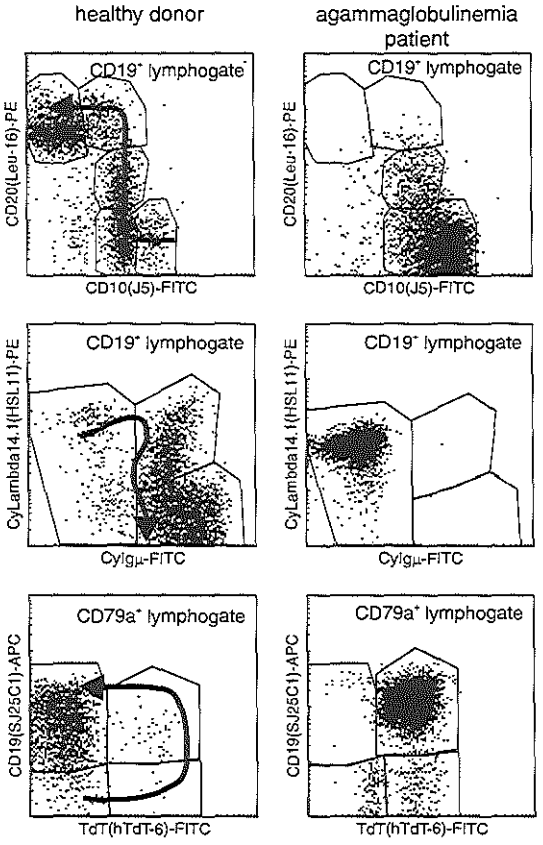

Figure 1. Flow cytometric analysis of fresh BM from a healthy control and the Igμ deficient female agammaglobulinemia patient.
The expression levels of CyCD79a and Cyλ14.1 in the patient were normal, but no CyIgμ expression could be detected, revealing a complete differentiation arrest at the pre-BCR checkpoint. Consequently, the vast majority of precursor-B-cells consisted of CyCD79a$^+$/CD19$^+$/CD10$^+$/TdT$^+$/Cyλ14.1$^+$ cells.

for normal human precursor-B-cell differentiation as shown by the complete absence of PB B-lymphocytes in Igμ deficient agammaglobulinemia patients. This is in contrast to the incomplete precursor-B-cell differentiation arrest in BM of some BTK and λ14.1 deficient patients.[7,9]

The detailed flow cytometric studies of PB and BM in our female agammaglobulinemia patient directed the mutation analysis towards the *IGH-*C_μ gene. PCR and SB analysis indeed showed a homozygous deletion, which was precisely mapped using DNA fiber FISH analysis.

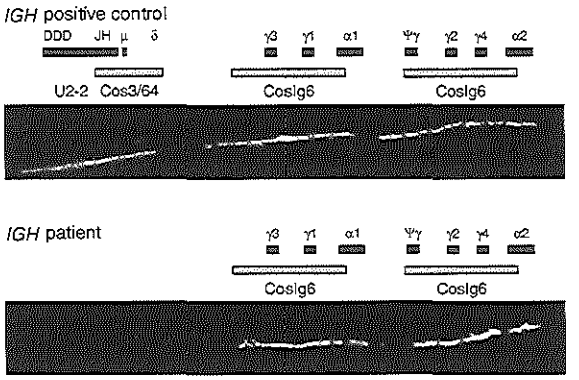

Figure 2. DNA fiber FISH analysis on PBMC from the Igμ deficient female agammaglobulinemia patient.
A homozygous deletion was identified in the patient, encompassing the *IGH-*D_H, *IGH-*J_H, *IGH-*C_μ, and *IGH-*C_δ gene segments. The human embryonic lung-fibroblast cell line MRC-5 (ATCC number CCL-171) was used as positive control.

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Chapter 9

INTRODUCTION SEVERE COMBINED IMMUNODEFICIENCY DISEASES

INTRODUCTION SEVERE COMBINED IMMUNODEFICIENCY DISEASES

Patients suffering from severe combined immunodeficiency diseases (SCID) in general suffer from failure to thrive, protracted diarrhea, and opportunistic infections.[1] The clinical phenotype of SCID is caused by the absence or dysfunction of T lymphocytes in the peripheral blood (PB).[1] The immunological classification of SCID is based on the absence or presence of T, B, and NK cells in PB. Consequently, five forms of SCID can be recognized: $T^-/B^+/NK^+$ SCID,[2-16] $T^-/B^+/NK^-$ SCID,[17,18] $T^-/B^-/NK^+$ SCID,[19-21] $T^-/B^-/NK^-$ SCID, and $T^{low}/B^+/NK^-$ SCID.[22] In SCID patients with absence or decreased numbers of T cells but normal numbers of B and NK cells in PB, B-cell function is often severely hampered due to a lack of T-cell help.

Each subgroup of SCID is associated with several genetic defects (Table 4 in Chapter 3). Careful immunophenotyping of PB samples from SCID patients can therefore result in an enhanced molecular diagnosis. However, transplacental T-cell engraftment from mother to fetus (MFT) has been described and this phenomenon can result in confusing immunophenotyping results. Clinical signs of graft-versus-host-disease (GVHD) may alert the pediatrician to analyze MFT. A recent publication showed that MFT occurred in approximately 40% of SCID patients.[23] However, 60% of these SCID patients with MFT did not show clinical signs of GVHD.[23]

The authors of this paper showed that MFT occurred only in reticular dysgenesis, T^-/B^- SCID, and T^-/B^+ SCID patients, and not in SCID patients with ADA/PNP deficiency, MHC class II deficiency, Omenn syndrome, $CD8^+$ T-cell deficiency, or other forms of T^+ SCID.[23] Furthermore, the simultaneous presence of both maternal and autologous T cells was never observed. Maternally derived B cells and monocytes were not detected, while maternal NK cells were detected in only one out of 30 patients with MFT.[23]

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Chapter 10

REVIEWING OMENN SYNDROME

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SUMMARY

Omenn syndrome is a form of SCID associated with high mortality. Early recognition is required in order to initiate life-saving therapy. This review provides information on the clinical symptoms, laboratory parameters and pathology of the disease, supporting early diagnosis in suspect patients. A literature search was performed using Medline, encompassing the period 1965-1999. Sixty-seven cases were identified. With the addition of a recently diagnosed patient at our hospital, 68 children were included. Median age at onset of symptoms was 4 weeks. Key symptoms were erythematous rash (98%), hepatosplenomegaly (88%), lymphadenopathy (80%), often accompanied by recurrent infections (72%) and alopecia (57%). An elevated white blood cell count (WBC) was frequently observed (55%), due to eosinophilia and/or lymphocytosis. B-cell counts were significant decreased, whereas T-cell counts were elevated. A high serum IgE was another frequent finding (91%). Therapeutic options included bone marrow transplantation or cord blood stem cell transplantation. However, the mortality still was 46%.

Conclusion: Omenn syndrome is a fatal disease if untreated. The mortality may be reduced when diagnosis is established early and treatment is initiated rapidly by using early compatible BMT or cord blood stem cell transplantation.

INTRODUCTION

Omenn syndrome (OS) is an autosomal recessive form of severe combined immunodeficiency (SCID) with autoreactive manifestations.[13,33] Although most patients have normal or even elevated numbers of peripheral blood (PB) T lymphocytes, they suffer from a profound immunodeficiency, which usually leads to death due to recurrent life-threatening infections before 6 months of age, unless treated by bone marrow transplantation (BMT) or cord blood stem cell transplantation (CBT). In 1965 the disease was first described by Gilbert Omenn in a child of consanguineous parents.[33] Since then at least 67 cases of patients with this syndrome have been published.[2-10,12-15,19-29,32,33,35,36,38,40,42-46]

Villa *et al.* recently elucidated the genetic defect underlying OS.[44] They reported mutations in the recombination activating genes (*RAG1* and *RAG2*) in 7 children with a clinical picture of OS. These mutations lead to partial V(D)J recombination activity in precursor B and T cells which was concluded to be the basic defect in OS. This finding in principle enables a molecular approach to the diagnosis of this syndrome by screening for the presence of mutations in *RAG1* or *RAG2*.[31]

We recently diagnosed a new patient with OS that has been published elsewhere.[30] Subsequently, we studied the previous publications on this syndrome. However, the descriptions of the clinical features or the laboratory findings were not always consistent. Because most patients die before BMT can be undertaken, rapid recognition of OS is important in

order to initiate treatment at an early stage.[25] The aim of this paper is to provide an overview of the published cases of OS, and to delineate the clinical picture, the most important laboratory results and the pathological findings, thus supporting early recognition of OS patients.

MATERIALS AND METHODS

In order to review published cases of OS a search in Medline (1966-1999) was performed using the keywords "Omenn syndrome", "reticuloendotheliosis", "eosinophilia", and "severe combined immunodeficiency". Also a search was performed on the names of a number of authors, who have been involved in research on OS. We additionally searched the references from the articles obtained this way. The following criteria for inclusion in this review were used:

1. articles that were written and published in English or French
2. articles published between 1965 and 1999
3. studies containing case report(s) in which the diagnosis OS was made only relevant new information was used from cases that were reported twice

In some cases more than one laboratory result was published. In such circumstances we used the earliest obtained value. We removed all cases from the study in which maternal chimerism was found. Thus, 67 cases were identified and listed in Figure 1. With the addition of our own case, 68 cases could be evaluated.

RESULTS

Clinical data

Sex was mentioned in 60 of 68 cases (88%). The male-to-female ratio was 0.9. Birth weight was noted in 16 cases with an average of 3.2 kg (range: 2.7-4.1). Onset of the disease at birth or in the first two weeks of life occurred in 29 of 66 cases (44%). In 60 of 66 cases (91%) the disease presented during the first two months of life. The median age at clinical presentation was 2.5 weeks. For all cases the onset of disease occurred before the age of 20 weeks. Consanguinity was present in 22 cases and reported to be absent in 26 cases (Table 1).

A total of 58 case reports (85%) described an erythematous skin disorder present at the onset of disease. All children developed cutaneous symptoms during the course of the disease. In addition alopecia was also a frequent finding (39 cases, 57%). Absence of alopecia

Figure 1 (see page 131). Summary of characteristics of patients reported in the literature.
Data in the first column reflect first 3 letters of the author followed by the patient number in that original report. The numbers between brackets refers to the reference list. M= male, F = female, + = present, - = absent, n = normal, * = absolute number calculated from percentage, empty box = information not mentioned in original report.

Figure 1	Patient Data			Symptoms										Course			Analysis											Outcome		
Reference	Sex	Consanguinity	Birth Weight (kg)	Age at onset (w/s)	Presentation with erythrodermia	Erythrodermia	Alopecia	Hepatosplenomegaly	Lymphadenopathy	Diarrhea	Failure to thrive	Pneumonia	Recurrent infections	P. Carinii Pneumonia (PCP)	Candida infection	White blood cell count (5-21E9/L)	Lymphocytes (2-13E9/L)	Eosinofilia (0-0.5 E9/L)	IgE (0.0-6.1 IU/ml)	B-cell deficiency	Hypogammaglobulinaemia	Skin biopsy: reticuloendotheliosis	Lymphnode biopsy: histiocytosis	Thymus on X-Thorax	Maternal chimerism	BMT or CBT* (months)	Time to death (months)	Cause of death
OME-1 (33)	M	+	4.0	4	+	+	+	+	+	+	+	+	+	+	+	14.0	4.5*	7.6*			+	+	+			-	5	septic shock
BAR-1 (2)	M			4	+	+		+	+				+			10.0		2.1*			+	+	+				4	
BAR-2 (2)	F			3	+	+		+	+				+			68.0		17*									4	sepsis
CED-1 (6)	F	-	2.8	3	+	+	+	+	-			+	+	+		60.0	30.0*	10.8*			-	+	+				4.5	
CED-2 (8)	F	+	3.0	12	+	+	+	+	+				+	+		50.0	25.0*	7.0*			-	-	+				0.5	
CED-3 (6)	F	-	4.0	0	+	+	+	+	-	+		+	+	+		14.6	1.3*	1.9*			+	+	+				7	
CED-4 (6)	F		n	0	+	+	+	+	-	+		+	+			22.0	8.4*	7.0*			-	+	+	-			1.5	pneumonia / meningitis
OCH-1 (32)	F	-	3.6	2	+	+	+	+	+	+	+	+	+		+		14.5	3.1	2000	+	-	+	+	-	-	-	5.5	
COH-1 (10)	M	-	2.7	2	+	+	+	+		+					+	22.0	2.2*	11.9*	+	-	+	+		-			<12	
WYS-1 (46)	M	+	n	0	+	+			+	+	+	+	+		+	64.0	21.9	20.8	0.16	+	+	+	+	-		-	18	pseudomonas pneumonia
KAR-1 (24)	M	+	2.8	0	+	+			+	+		+	+		+	10.0	4.3*	2.2*		+						3	4	pneumonia
KAR-2 (24)	F	+		2	+	+			+	+	+		+		+	28.0	7.7*	14.9*		+				-		4		
VEL-1 (43)	M	-		6	+	+			-	+	+		+			20.0		6.0*	n	+	+		+	+			20	toxic shock
GEL-2 (15)		-		<20	+	+			+	+								6.5		-	-					-	<12	
GEL-3 (15)		+		<20			+		+	+								1.5		-	+					-	<12	
HON-1 (21)	M			0	+	+	+	+	+				+			24.6	10.5*	3.4*					+	+		-	2.5	
HON-2 (21)	M			0	+	+	+	+	+				+		+		0.2	+					+	+		-	+	
LED-1 (13)	F	-		0	+	+	+	+	+	+	+	+	+	+		25.0	15.0	11.0	40	+	+	+	+	+			+	9	respiratory distress
LED-2 (13)	M	-		5	+	+	+	+	+		+	+	+			20.0	10.0	3.0	692	+	+	+	+				-	12	
LED-3 (13)	M	+		1	+	+	+	+	+	+	+	+	+	+		100.0	30.0	15.0		+	+	+	+					15	pericarditis
LED-5 (13)	F	-		2	+	+	+	+	+	+	+		+			20.0	8.0	10.0	160	+	+	+	+			-	+		
RUC-1 (35)	F	+	3.4	2	+	+	+	+	+	+	+	+	+		+	25.0		10.0	4600	+	+						3	interstitial pneumonia
RUC-2 (35)	F	+	3.2	6	+	+	+	+	+	+	+	+	+	+	+	20.7	6.0	11.1	1845	+	-	+	+			-	7	interstitial pneumonia
BUS-3 (7)	F	+	3.3	6	+	+		+	+	+	+	+	+		+	+	1.3	1.3		+	+						5	resp. and cardiovascular failure
BUS-4 (7)		+	3.3	2	+	+	+			+			+			+	4.3	0.4	390	+	-					-	+	7	GVHD
JOU-2 (22)	F			5		+	+	-	+	+			+			14.3	3.5	0.7	2280		+			+	+			+	
JOU-4 (22)	M			2	+	+	+	+	+	+			+			22.0	8.0	4.4										+	
JOU-7 (22)	F					+				+			+					0.8										+	
JOU-8 (22)	F			6		+		+	+				+			16.0	5.0	4.0										+	
JOU-9 (22)	M			1		+	+	+	+	+						20.0		3.0										+	
WIR-1 (45)	M	-		8	+	+	+	+	+	+	+	+	+		+		110.0	20.0			+	+		+	-	-		n.a.	
JUN-1 (23)	F	+		2	+	+	+	+	+		+		+		+	13.0	10.4	3.1	516	+	+	+	+	-	-	+		
BRU-1 (5)	M	+	3.1	1	+	+		+	+	+	+	+	+	+	+		1.3	13.4		+	+	+	+		-	8		
DYK-1 (14)	F	-	2.6	0	+	+	+	+	+	+	+	+				8.2	4.9	1.1		-	+	+	+	-			2	cardiac failure + sepsis
HEY-1 (20)	F	+		18	+	+		+	+		+		+			42.0	24.0	8.0		+	+				-	+		
SAI-1 (12)		+		<8	+	+	+	+	+	+	+	+	+					+			+				-		1	pneumoniae
SAI-2 (12)		+		<8	+	+	+	+	+	+	+		+					+	+		+	+			-	+		
SAI-3 (12)	F	-		<8	+	+	+	+	+	+	+		+					+	+		+	+			-	+	+	
SAI-4 (12)	M	-		<8	+	+	+	+	+	+	+		+					+	+		+	+			-	+		
SAI-5 (12)		-		<8	+	+	+	+	+	+	+	+	+					+	+		+				-		12	pneumonitis
SCH-1 (37)	F	-	2.9	3	+	+	+	+	+	+			+			13.8	4.1				+		+			7	8	gram-negative septicemia
MOR-1 (26)	M	-		6	+	+	+	+	+				+			10.0	7.0	3.5	150		+	+	+	-	-	-		
SCH-1 (38)	F	+		<8	+	+		+	+	+	+	+	+		+		1.6	2.3	164		-					-	n.a.	
MEL-1 (27)	M	-		12	+	+	+	+	+		+	+	+	+	+		10.0	22.0	8		+			+	-	11		
MAR-1 (26)	F			2	+	+		+	+							79.6	68.6*	7.2*	3844				+	-		+		
LOE-1 (25)	M	-		2	-	+	+	+	+	+	+		+	+	+		5.0	+	10000		-					-	+	+	Pseudomonas sepsis
LOE-2 (25)	F	-		2	+	+	+	+	+	+			+	+	-			+	+							-	+	+	aspergillosis
LOE-3 (25)	F	-		2	+	+	+	+	+	+	+		+	+	+	4.2	=	+	+		+	+				-	+	+	Echo vir. meningoencephalitis
LOE-4 (25)	M	+		16	+	+	+	+	+	+	+		+	+	+		2.4	9.1	0140		+					-	+	+	GVHD + sepsis
LOE-6 (25)	M	-		6	+	+	+	+	+	+	+		+	+	+		6.2	+	+		+					-	+	+	EBV B-cell lymphoma
CHI-1 (9)	M	-		0	+	+		+	+	+	+		+				5.9	3.4	316	+	+	+	+			-	8		
CHI-2 (9)	F	-		4	-	+		+	+	+	+	+	+		+		12.0	2.2	42	-		+	+			-	6	8	pneumoniae / pulmonary embolism
CHI-3 (9)	F	-		6		+		+	+	+	+		+				0.7	4.6	190	+		+	+			-	6		
HAR-1 (19)	M			2	+	+		+	+		+		+		+	89.1	58.1	12.5	183	+	+	+	+			-	4		
RYB-1 (36)	M	+		0	+	+	+	+										+										1	GE haemorrhage
RYB-2 (36)	M	+	2.8	3	-	+	+	+			+	+				60.0		42.0*			+	+	+					2	sepsis
BRU-3 (6)	F			2	+	+		+			+	+						6.2	160				+			-		3	cardiac failure (myocarditis)
BRU-4 (6)	M			4	+	+					+							5.3	3000							-	+		
BRU-5 (6)	M			4	+	+			+		+					23.5		6.0				+				-	+		
NAZ-1 (29)	M	-		4	+	+	+	+	+	+	+	+	+			18.0	7.6*	2.2*	n	+	+	+	+			-	-	9	toxic shock
VIL-5 (44)	F	-		8	+	+					+	+						2.0	500		+					-	+	+	sepsis
VIL-6 (44)																	2.7	1.4			+					-	+		
VIL-7 (44)				3	+	+		+			+	+					13	5.2	1800	-		+				-	+	+	sepsis
BEN-1 (3)	M			4	+	+	+	+	+	+	+		+		+	7.0	3.1*	1.4*	3528	+	+	+	+			-	19*		
BRO-1 (4)	F			8	+	+		+	+	+	+	+						+	+			-			-	-		n.a.	
BRO-2 (4)	F			4	+	+		+	+	+								1.5	2617			+						n.a.	
SIG-1 (45)	F	+		2	+	+				+	+	+						8.6	9100	+	+					-	-	3	heart failure/resp distress
NOO-1 (30)	F	+	4.1	0	+	+		+	+	-	+	+	+	-	-	32.4	10.7	5.8			+			+	+	-	-	-	1	respiratory insufficiency

Table 1. Summary of clinical data of 68 patients with OS.

	Evaluable (n)	Median	Average	Range	
Birth weight (kg)	16	3.2	3.2	2.7-4.1	
Age at onset (weeks)	58 (+8)	2.5	3.8	0-20	
		Positive (n)	Negative (n)	Percentage of evaluable positive (%)	Percentage of total positive (%)
Consanguinity	48	22	26	45.8%	32.4%
Erythrodermia at onset	61	58	3	95.1%	85.3%
Erythrodermia in course	67	67	0	100.0%	98.5%
Alopecia	39	39	0	100.0%	57.4%
Hepatosplenomegaly	62	60	2	96.8%	88.2%
Lymphadenopathy	57	54	3	94.7%	79.4%
Diarrhoea	45	44	1	97.8%	64.7%
Failure to thrive	42	42	0	100.0%	61.8%
Pneumonia	26	26	0	100.0%	38.2%
Recurrent infections	49	49	0	100.0%	72.1%
P. carinii infection	14	13	1	92.9%	19.1%
Candida infection	24	22	2	91.7%	32.4%

Number between brackets in the second column refers to number of patients from whom only an indication of age of onset is provided.

was not reported. Most authors have reported the presence of hepatosplenomegaly (60 cases, 88%) and lymphadenopathy (54 cases, 79%). Other frequent signs and symptoms included diarrhoea (44 cases, 65%), failure to thrive (42 cases, 62%) and pneumonia (26 cases, 38%).

Patients with OS generally suffered from recurrent infections (49 cases, 72%). These infections were often caused by opportunistic pathogens like Candida (22 cases, 32%) and *Pneumocystis carinii* (13 cases, 19%).

Laboratory results

The white blood cell count (WBC) was available in 36 cases. The median WBC of these cases was $22.0x10^9/l$ with a range of $4.2\text{-}100x10^9/l$. In 21 cases (55%) the WBC was higher than the upper limit of normal reference values ($21x10^9/l$). The lymphocyte count (LC) was available in 48 cases, either as a percentage of the white blood cell differentiation, or as an absolute number. The median LC was $6.6x10^9/l$ with a range of $0.2\text{-}110.0x10^9/l$. Thirteen cases (26%) had lymphocytosis above the upper limit of normal age-matched reference values ($13.1x10^9/l$).[11] The median eosinophil count (EC), based on 51 cases, was $5.3x10^9/l$ with a range of $0.4\text{-}42.0x10^9/l$. All these cases, except one, had eosinophilia with an EC higher than $0.5x10^9/l$. In 12 additional cases eosinophilia was mentioned without documenting the EC. In 6 cases eosinophilia or EC was not mentioned (Table 2).

In 27 cases (38%) the value of IgE levels was documented. The median IgE level was 516 IU/ml with a range of 0.18-10,000 IU/ml. In another 8 cases IgE levels were reported to be elevated, while in 2 cases a normal IgE level was found. Thus in 34 of 37 cases (92%) elevated IgE levels were found. In 30 cases PB lymphocytes were studied with B-cell markers. In 24 of these cases (81%) B cells were absent or strongly decreased. In 6 cases (19%) nor-

Table 2. Summary of laboratory data in 68 patients with OS.

	Evaluable (n)	Median	Range	Reference of range	Elevated (n)	Percentage of evaluable (n)	Percentage of total (%)
WBC (x10e9/l)	36 (+2)	22.0	4.2-100	5.0 - 21.0	21	55.3%	30.9%
Lymphocytes (x10e9/l)	48 (+2)	6.6	0.2-110	3.5 - 13.1	13	26.0%	19.1%
Eosinophils (x10e9/l)	51 (+12)	5.3	0.4-42	0.0 - 0.5	62	98.4%	91.2%
IgE (IU/ml)	27 (+10)	516.0	0.18-1x104	0.0 - 6.1	34	91.9%	50.0%

		Positive (n)	Negative (n)	Percentage positive (%)
Hypogammaglobulinemia	50	40	10	80.0%
Thymus absent on chest X-ray	13	10	3	76.9%
Reticuloendotheliosis	34	33	1	97.1%
Histiocytosis	31	31	0	100.0%

Number in brackets of second column represents number of patients from whom only semi-quantitative results were available.

mal numbers of B lymphocytes were found. In 50 cases information on serum immunoglobulines (Ig) levels was presented. Hypogammaglobulinemia was frequently encountered (40 cases, 80%), especially in older children. In 10 cases there was no thymus shadow on chest X-ray. In 3 cases a positive thymic shadow was observed.

In 34 cases a skin biopsy was performed. All specimens, except one (CED-2), showed a picture consistent with histiocytic infiltrations. In 31 children a lymph node biopsy was performed. The histologic findings were always compatible with histiocytic infiltration.

Outcome

In 28 of the 68 published cases a BMT was performed and 15 of these patients recovered completely. Two patients recovered without BMT. One patient (MOR-1) only received supportive care. The other patient (BEN-1) received CBT and survived. BMT or CBT was attempted at a median age of 7.5 months. Out of 64 patients, 47 died (73%). The most frequent causes of death were respiratory distress (11 cases, 23%) and septicaemia (11 cases, 23%). The median age at death was 5 months (Table 3).

Table 3. Summary of outcome in 68 patients with OS.

	Evaluable (n)	Deaths (n)	Survival (n)	Not available (n)	Percentage deaths of evaluable
Without therapy	12	11	1		92%
BMT	28	13	15		46%
CBT	1	0	1		0%
no information on therapy	27	23		4	85%
Total	41	24	17		59%

DISCUSSION

Clinical symptoms

Incomplete description and emphasis on the presence and not the absence of symptoms complicate the interpretation of data and symptoms from previous described case reports. However, an adequate description of frequently available symptoms is of great value to help the clinician in an accurate diagnosis. We defined classical or typical symptoms as those symptoms that could be evaluated in more than 80% of the cases and were positive in >80% of the evaluable cases. Using these criteria, OS is best described as a clinical entity presenting during the first 8 weeks of life with a combination of the following three major symptoms: erythrodermia, hepatosplenomegaly and lymphadenopathy. Since the differential diagnosis of erythrodermia at a very young age is limited, this symptom is a key-sign in the diagnosis of OS. In addition, alopecia, chronic diarrhea, failure to thrive, and recurrent infections are frequently encountered. The triad of erythrodermia, hepatosplenomegaly and lymphadenopathy requires further immunological studies comprising analysis of eosinophils, lymphocyte subsets and serum Ig levels, including IgE. It is now possible to perform genetic analysis of the *RAG1* and *RAG2* genes in patients suspected of OS, thus confirming the clinical diagnosis.[31,44] Furthermore, genetic analysis may be helpful in prenatal diagnosis.

The differential diagnosis of OS includes severe atopic dermatitis, GVHD, and histiocytosis X. Occasionally, the clinical picture of OS is seen in other immunodeficiency syndromes.[1,17] The clinical features of OS are similar to those one would expect to find in GVHD. In fact, engraftment of maternal T cells in a child with a T$^-$SCID can be so reminiscent of OS that it has often been called Omenn-like syndrome,[9] or that GVHD has been proposed to be the pathogenetic mechanism in OS.[22] In our opinion cases with maternal engraftment of T cells do not fit with OS, and were excluded from this review.

Another differential diagnostic entity is histiocytosis X, also known as Letterer-Siwe syndrome.[26] This syndrome is also characterised by a skin eruption, hepatosplenomegaly and lymphadenopathy. However, the eruption is mostly of a patchy type, preferentially located around orifices, e.g. mouth, nose and anus. Within the skin lesions purpura are discerned frequently. This diagnosis can be confirmed by the demonstration of CD1a molecules on the surface of involved histiocytes. Cederbaum *et al.*[8] reported 4 cases which were diagnosed as Letterer-Siwe syndrome, but were clinically indiscernible from OS. Unfortunately, immunological studies could not confirm the exact diagnosis. Based on the generalised erythematous rash and alopecia, together with eosinophilia and characteristic pathology, all four patients were included in this review.

Immunology

The number of circulating PB T-lymphocytes may vary greatly in OS, but in general these T cells are of oligoclonal origin with highly restricted T-cell receptor (TCR) heterogeneity.[12,34] Furthermore, the T cells have very poor proliferative responses *in vitro*.[4,5,7,27]

Frequently, they express the CD45RO isoform which is characteristic of primed/memory cells, and show an activated phenotype ($CD25^+/HLA\text{-}DR^+$).[6,37] *in vitro* activation leads to an increased production of interleukin (IL) 4 and 5, while IL-2 and interferon-γ are hardly synthesised, thereby showing a T-helper 2 (Th2) phenotype.[9,37] This preferential Th2 phenotype is thought to be responsible for high levels of IgE and eosinophilia,[37] two characteristic laboratory features in OS. The expansion of T cells is thought to be the consequence of continuous antigenic stimulation in combination with a profound defect in the development of T cells, leading to autoreactive T lymphocytes.[34,45]

Recently the basis for oligoclonal expansion was elucidated by Villa *et al.*[44] They showed a genetic defect in activity of RAG1 and RAG2 enzymes. Both enzymes have a crucial role in initiating rearrangement of TCR as well as Ig genes. Complete absence of recombination activity of RAG1 and RAG2 proteins will result in a B^-/T^- SCID.[31,41] However, partial activity of mutated RAG1 and RAG2 proteins leads to the generation of only a small number of T cell clones, as observed in OS. Intrathymic restriction and peripheral expansion will subsequently lead to an oligoclonal TCR repertoire in the peripheral blood.[42] Villa *et al.*[44] described 7 cases with mutations in *RAG1* or *RAG2* genes. This partially functioning of the RAG1 and RAG2 enzymes now seems to explain the oligoclonal TCR repertoire. However, it remains to be elucidated why partial V(D)J recombination activity in OS leads to strongly reduced numbers of B lymphocytes, since B lymphocytes are frequently quantitatively and functionally deficient with minimal specific antibody response to immunisations and reduced production of allohemagglutinins.

Serum Ig levels may be normal but are usually low, whereas IgE levels are very high.[7,24] Hypogammaglobulinemia is generally found in older infants suffering from OS, thereby suggesting that normal levels of IgG in the younger ones are acquired transplacental from their mother. RAG1 and RAG2 are also essential in Ig gene rearrangements.[31,41] Depending on the degree of enzym restactivity to rearrange Ig genes, some "leaky" clones of B cells might be expected. These few B cells might subsequently be forced by the Th2 cells to produce IgE preferentially. In our case it has been shown that the 631delT mutation leads to N-terminal truncated RAG1 proteins that were not able to rearrange Ig genes,[30] thereby blocking maturation of B cells completely. This implies that under such circumstances B cells are not found in the peripheral blood and production of Ig isotypes is abolished. In our patient PB B cells were indeed absent and levels of IgA and IgM were not detected. Unfortunately, IgE levels were not measured.

Pathology

The pathological observations in patients with OS are very similar to those found in GVHD.[22,45] Thymus, lymph nodes and spleen, as well as the lamina propria of the intestinal mucosa, which also displays absence of solitary lymphoid follicles and Peyer's patches, are profoundly depleted of T cells. Post mortem analysis of the thymus shows severe atrophy and absence of Hassall's corpuscles. Furthermore, the internal architecture consists almost entirely of epitheloid-appearing cells.[2,6-8,14,16,21,29,33,35,46]

Skin biopsies show psoriatiform hyperplasia of the epidermis with parakeratosis and cellular dyskeratosis and necrosis. The basement membrane is destructed, allowing the pen etration of some inflammatory cells into the epidermis. Dermal infiltration is abundant and consists of histiocytes, eosinophils and T-lymphocytes.[2,9,22,28,29,46]

Lymph node biopsy reveals a total effacement of the normal microscopic architecture. Lymph nodes are increased in size and show a picture consistent with dermatopathic lymphadenopathy with an increase of cells with histiocytic appearance and a dense infiltrate of eosinophils.[9,22,26]

In addition to abnormalities of the skin and superficial lymph nodes, histiocytic infiltrations of other organs has frequently been observed. Most often it concerns aseptic organitis, containing infiltration of T cells, histiocytes and eosinophils. The organitis contributes to life-threatening complications.[2,5,12,21,35,46] This condition is difficult to discern from opportunistic infections and may react on immunosuppressive medication.[18,25] In several cases the aseptic organitis recovers completely after BMT.[20]

Treatment

OS is fatal when untreated.[18] Despite intensive supportive care, 69% of the patients died within the first year of life. Corticosteroids, cyclosporin, and interferon-γ showed transient symptomatic efficacy.[13,33,37,45]

HLA-identical and haploidentical BMT can cure OS, although fewer patients have survived than in other types of SCID. In patients who lack a histocompatible sibling, the use of unrelated donors did not have equal success compared to other forms of SCID, due to more severe and more frequent complications, contributing significantly to transplantation-related mortality.[25] The more aggressive conditioning required to eliminate the autoreactive cells found in OS may explane the high frequency of graft failure.[2,5,18,20,23,25] Nutritional support and control of T cell activation and proliferation (ATG and cyclosporin) before BMT apparently reduces the risk of fatal complications of aggressive conditioning in these fragile patients.[18] Interferon-γ is an interesting option to attenuate Th2-like immune responses.[18,37]

Remarkable are the 3 children in whom other therapies were used. One child described by Morren *et al.* (MOR 1)[28] was cured by supportive care only. The clinical picture was indeed in conformity with OS, and GVHD was excluded. Based on the proposed genetic defect, one could predict that cases of OS cannot be cured by supportive care alone. The authors noticed that the clinical course of their patient was peculiar and they proposed a diagnosis of Omenn-like syndrome. The other child, described by Benito *et al.* (BEN-1),[3] was successfully treated with stem cells from umbilical cord blood of an unrelated donor. This type of treatment may serve as an alternative option for BMT.[3] Le Deist *et al.*[13] described another case of OS. This child was cured with corticosteroids and epipodophyllotoxin. However, maternal chimerism was not excluded. Furthermore, the patient had an intestinal disease suggestive for severe cow-milk protein allergy. In addition to the aforementioned medication, a cow-milk protein free diet was prescribed. It would be interesting to know whether immune-reconstitution has been observed during long term follow-up.

CONCLUSION

Early recognition of OS is of great importance in order to initiate appropriate treatment, since OS is lethal when BMT or cord blood stem cell transplantation is delayed. In this review we provide information on clinical aspects, immunology and pathology that may help the clinician to establish the diagnosis at an early stage, thereby improving the prognosis for a child with OS. Clinical hallmarks are erythrodermia, hepatosplenomegaly and lymphadenopathy. Characteristic laboratory findings include eosinophilia and high IgE levels. Molecular diagnostic procedures are now available to determine exact diagnosis or to serve as a tool in carriership evaluation and prenatal diagnosis.

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Chapter 11

N-TERMINAL TRUNCATED HUMAN RAG1 PROTEINS CAN DIRECT TCR BUT NOT IG GENE REARRANGEMENTS

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SUMMARY

The proteins encoded by RAG1 and RAG2 can initiate gene recombination by site-specific cleavage of DNA in Ig and TCR loci. We identified a new homozygous *RAG1* gene mutation (631delT), leading to a premature stop codon in the 5' part of the *RAG1* gene. The patient carrying this 631delT *RAG1* gene mutation, died at the age of 5 weeks from an Omenn syndrome-like T^+/B^- SCID. The high number of blood T-lymphocytes (55 x 10^6/ml) showed an almost polyclonal TCR gene rearrangement repertoire and were not of maternal origin. In contrast, B-lymphocytes and Ig gene rearrangements were hardly detectable. We showed that the 631delT *RAG1* gene can give rise to an N-terminal truncated RAG1 protein, using an internal AUG codon as translation start site. Consistent with V(D)J recombination in T-cells, this N-terminal truncated RAG1 protein was active in a plasmid V(D)J recombination assay. Apparently, the N-terminal truncated RAG1 protein can only recombine TCR but not Ig genes. We conclude that the N-terminus of the RAG1 protein is specifically involved in Ig gene rearrangements.

INTRODUCTION

Severe combined immunodeficiency (SCID) is clinically characterized by failure to thrive and opportunistic infections, usually starting within the second month of life.[1] SCID consists of a heterogeneous group of diseases, including an X-linked form,[2] and multiple autosomal recessive forms.[3] SCID can be immunologically classified by the absence or presence of T-, B-, and NK-cells, a phenomenon that is associated with different disease categories defined on a molecular basis. For instance the non-B, non-T form of SCID is frequently caused by mutations in the recombination activating genes (*RAG1* or *RAG2*).[4] During recombination of an immunoglobulin (Ig) or T-cell receptor (TCR) gene, a combination of the available variable (V), diversity (D), and joining (J) gene segments is made, resulting in a V-D-J exon. The RAG1-RAG2 protein complex first cleaves the DNA at specific sites, called recombination signal sequences (RSS), which are characterized by a heptamer-nonamer sequence separated by a spacer of 12 or 23 basepairs (bp). The cleaved DNA is finally linked together by factors involved in double strand break repair, such as Ku70, Ku80 and $DNA\text{-}PK_{cs}$.[5-7]

Absence of functionally rearranged Ig and TCR genes blocks the B- and T-cell differentiation in an early stage, resulting in the absence of mature B- and T-lymphocytes in the peripheral blood (PB).[8,9] However, some SCID patients have T-lymphocytes due to intrauterine transfer from mother to child.[10,11] These maternal T-lymphocytes can hamper the diagnosis of a non-B, non-T SCID. Careful proof or exclusion of the maternal origin of T-lymphocytes is needed for correct diagnosis, because in another form of SCID, the Omenn syndrome (OS), patients have oligoclonal, (non-maternal) activated T-cells in their PB, and very low

numbers of B-cells accompanied by hypogammaglobulinemia and high levels of IgE.[1] T-cells from OS patients have a T-helper (Th)2-phenotype, which can account for the high IgE levels.[12,13] OS is generally characterized by failure to thrive, erythrodermia, eosinophilia, hepatosplenomegaly, lymphadenopathy and a graft-versus-host (GVH)-like disease.[1] Recently, Villa *et al* described OS patients with oligoclonal T-cells who had mutations in their *RAG* genes, implying that these mutations do not necessarily completely abolish the function of the RAG proteins.[14] However, it remained unclear why the B-cell lineage seems to be more affected than the T-cell lineage by these partially functional RAG proteins.[15]

The essential parts of the murine *RAG1* and *RAG2* genes have been characterized by studies in cell lines on the residual function of deletion constructs.[16-22] The RAG1 core domain consists of amino acid (aa) 384 to 1008 of the 1040 aa long murine RAG1 protein. Most deletions in this core domain abolish recombination activity. The N-terminal part of the RAG1 protein is not essential, although its presence may enhance the recombination activity.[20,21] The portion of the N-terminus responsible for this enhancement is localized in a small region between aa 216 and 238 (basic aa motif BIIa).[20] Although the murine and human RAG1 proteins have an overall 90% amino acid sequence identity,[23] the N-terminal first 350 aa have a homology of only 77%, while the core domain has a high homology of 95%.

Here we describe a patient with OS-like T^+/B^- SCID showing a homozygous T nucleotide deletion in her *RAG1* gene (631delT). We showed that the 631delT *RAG1* gene can give rise to an N-terminal truncated RAG1 protein, which was active in a plasmid V(D)J recombination assay. The patient had large numbers of (non-maternal) T-cells in her PB, but no B-cells could be detected. The N-terminal truncated RAG1 protein is apparently able to direct TCR, but not Ig gene rearrangements.

CASE REPORT

The patient was the first born girl from consanguineous healthy Moroccan parents. Six hours after birth she was admitted to a local hospital with an erythematous skin rash and a tachypnea of 60 to 80/min. (O_2 saturation: 92%). First laboratory results showed a leukocytosis of 32.4 x 10^9/l with the following differential blood count: 11% eosinophils, 20% metamyelocytes, 5% band forms, 12% polymorphonuclear leukocytes, 51% lymphocytes, and 1% monocytes. Neonatal sepsis was considered and antibiotic treatment was started, but no improvement was observed. Gradually a hepatosplenomegaly and a generalized lymphadenopathy developed. On the ninth day of life, she experienced a generalized convulsion. After this event she was admitted to the neonatal intensive care unit of the university hospital for further diagnosis and treatment.

The differential diagnosis included metabolic disorder, sepsis or toxic shock-like syndrome, histiocytosis, neonatal leukemia, autoimmune disease or GVHD. Laboratory results showed that the leukocytosis had increased to 68.5 x 10^9/l. The differential blood count showed 1% eosinophils, 1% metamyelocytes, 1% band forms, 8% polymorphonuclear leuko-

cytes, 87% lymphocytes, and 2% monocytes. Serum Ig levels were: IgG 2.73 g/l, IgA <0.10 g/l, IgM 0.11 g/l. IgE levels were not determined.

Immunophenotyping with triple labelings of membrane and intracellular markers was performed as described before[24,25] and showed complete absence of B-lymphocytes in the PB (< 0.01 % of lymphocytes). Sensitive B-cell detection could be achieved via a double lymphocyte and exclusion gate, using CD3, CD14, CD15, CD16, and CD56 to exclude T-cells, NK-cells, monocytes and granulocytes from the lymphogate (Figure 1). In the bone marrow (BM) virtually no precursor-B-cells could be detected (<1% $CD34^+$, <0.5% $CD117^+$, <0.5%

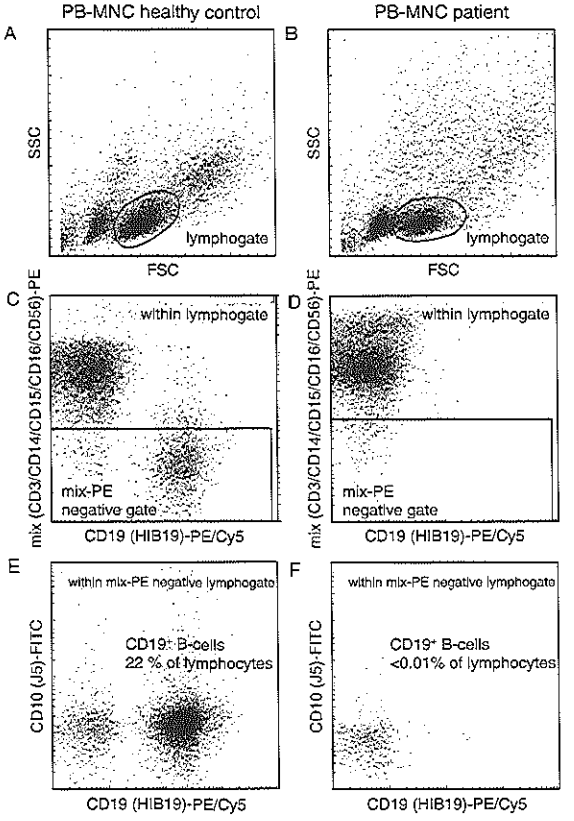

Figure 1. Sensitive flow cytometric analysis of PB-MNC of a healthy control and the patient with the 631delT *RAG1* gene.

Based on scatter characteristics, gating was performed on lymphocytes (A and B). To further reduce background staining in our attempts to detect rare events ($CD19^+$ B-cells), we used a so-called exclusion gate with negativity for labeling with the PE-conjugated CD3, CD14, CD15, CD16, and CD56 antibodies (C and D). This exclusion of T-lymphocytes, monocytes, granulocytes, and NK-cells resulted in sensitive detection of $CD19^+$ B-lymphocytes. The patient had less than 0.01% $CD19^+$ B-lymphocytes (F), compared to 22% in the healthy control (E).

TdT+, 3% CD10+, <1% CD19+). On the other hand, 66% of PB leukocytes consisted of CD3+ T-lymphocytes with the following immunophenotype: 35% CD4+, 59% CD8+, 89% TCRαβ+, 4% TCRγδ+, and 66% CD45RO+, thereby showing substantial immunophenotypic heterogeneity within the expanded T-cell population. The origin of PB T-lymphocytes was determined by HLA typing, which showed that the T-lymphocytes were not of maternal origin.

Despite all supportive care the child gradually deteriorated. Respiratory insufficiency required artificial ventilation. Due to an interstitial pulmonary inflammatory reaction, high ventilation pressures were needed to obtain sufficient pO_2 and SaO_2 values. Treatment with methylprednisolon and cyclosporin on the tentative diagnosis of autoimmune disease or GVHD could not reverse the inflammatory reaction. She died at the age of 32 days due to severe hypoxemia despite maximal respiratory support. The clinical picture together with the immunophenotyping results suggested an OS-like form of SCID.[14]

OS is characterized by a dominance of Th2-cells, which are characterized by high levels of interleukin (IL)-4 and IL-5, as compared to Th1-cells, which produce interferon (IFN)-γ. IL-4, IL-5 and IFN-γ levels were not detectable in plasma or in the supernatant of unstimulated PB mononuclear cells (MNC). PB-MNC stimulated with Ca-ionophore and phorbol myristate acetate (PMA) produced 4430 ng/ml IFN-γ, which was comparable to the amount of IFN-γ produced by polyclonally stimulated PB-MNC isolated from neonatal cord blood (NCB) of control patients (235-9900 ng/ml). Also the IL-4 levels were normal (32 pg/ml compared to 1-72 pg/ml in NCB), while the IL-5 levels were raised slightly (145 pg/ml compared to 2-82 pg/ml in NCB).[26,27] Thus, we did not find a typical Th2-profile in this OS-like T^+/B^- SCID patient.

MATERIALS AND METHODS

DNA and RNA extraction and reverse transcriptase (RT) reaction

Granulocytes and/or MNC were isolated from PB or BM by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. DNA was extracted from PB-MNC, PB granulocytes and BM-MNC using the QIAamp Blood kit (Qiagen, Chatsworth, CA, USA).[28] Total RNA was isolated from PB-MNC according to the method of Chomczynski using RNAzol B (Tel-Test, Friendswood, TX, USA).[29] cDNA was prepared from mRNA as described before, using oligo(dT) and AMV reverse transcriptase.[30]

(RT)-PCR amplification and analysis of Ig and TCR gene rearrangements

PCR was performed as described previously.[31] In each 100 µl PCR reaction 0.1-1 µg DNA sample, 12.5 pmol of 5' and 3' oligonucleotides and 1 U Ampli*Taq* gold polymerase (PE Biosystems, Foster City, CA, USA) were used. The *TCRB* RT-PCR amplification used multiple Vβ family primers in combination with a single Cβ primer as was described before.[32] Most oligonucleotides for amplification of the *IGH, IGK, IGL, TCRB, TCRG,* and *TCRD*

genes were published before[33-35] (RT)-PCR conditions were 2-10 min 94°C, followed by 45 sec 92°C, 90 sec 57-65°C, 2 min 72°C for 40 cycles, followed by a final extension step (7 min 72°C). Heteroduplex analysis of PCR products was used to analyze the mono-, oligo-, or polyclonal nature of the amplified rearrangements as described before[32] The PCR products were cloned in pGEM-T easy vector and subsequently sequenced. The minimal number of nucleotides used for identification of a D gene segment was 3 in case of Dδ1 and Dδ2, 4 in case of Dδ3 and Dβ1, and 5 in case of Dβ2.[36,37] Ig gene rearrangements were identified using IMGT, the international ImMunoGeneTics database http://imgt.cnusc.fr:8104 (Initiator and coordinator: Marie-Paule Lefranc, Montpellier, France, lefranc@ligm.igh.cnrs.fr).[38]

Long range (LR)-PCR for amplification of *RAG* genes

The entire *RAG1* or *RAG2* gene was amplified in one LR-PCR reaction (100 µl). When the LR-PCR product was generated for sequencing 4 U r*Tth* DNA polymerase XL (PE Biosystems) were used. While in case of cloning the LR-PCR product 5.25 U Expand enzyme mix (Boehringer Mannheim, Mannheim, Germany) were used. We used 30 pmol of 5' and 3' oligonucleotides. The sequences of the oligonucleotides used for the LR-PCR of *RAG1* and *RAG2* will be made available on request. LR-PCR conditions were 2 min 94°C, followed by 15 sec 94°C, 30 sec 60°C, and 3 min 68°C for 25 cycles using 15 sec auto-extension from cycle 11 onward. After the last cycle an additional step of 10 min at 72°C was performed for final extension.

Fluorescent sequencing reaction and analysis

LR-PCR products of *RAG1* and *RAG2* were purified using QIAquick PCR purification kit (Qiagen). 5-9 µl purified PCR product was sequenced with 5 µl dRhodamine dye terminator mix (PE Biosystems), using 3.3 pmol internal sequencing primers. All sequencing was performed as described before[33] and run on an ABI Prism 377 fluorescent sequencer (PE Biosystems).

Cloning of the mutated and wild-type *RAG1* genes

LR-PCR products of the *RAG1* gene were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). The DNA fragment containing the entire *RAG1* open reading frame was isolated after digestion with *Mlu*I and partial cleavage with *Xho*I, and cloned into the *Xho*I-*Mlu*I cut vector pMSE1 (a pCDM8-based vector[22]). Protein expression will result in a C-terminal fusion of a myc epitope tag to the RAG1 protein, which can be used for easy detection. The cloned wt and 631delT *RAG1* genes were sequenced to exclude the presence of any additional mutations.

In vitro transcription and translation

0.5 µg of pGEM-T easy construct was added in a 25 µl reaction volume of TNT® Coupled Reticulocyte Lysate System (Promega), using 0.5 µl of T7 RNA polymerase and 1 µl of ^{35}S labeled Methionine. *In vitro* transcription and translation took place at 30°C for 90

min. Protein products were separated on a 7.5% polyacrylamide gel and visualized by autoradiography.

Western blotting

5 x 10^5 COS cells (40% confluent) were transfected with 2 μg expression construct for 631delT or wt *RAG1* using SuperFect Transfection Reagent (Qiagen), and cultured for 2 days at 37°C. Proteins were separated on a 6% polyacrylamide gel and blotted onto a nylon membrane (Schleicher & Schuell, Dassel, Germany). The RAG1 protein was detected by the anti-c-myc (9E10) murine monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

V(D)J recombination assay

2 μg of pMSE1-*RAG1* (631delT or wt) together with 2 μg pMSE1-*RAG2* wt and 1 μg of the recombination substrate (pDVG93) containing two RSS elements was transfected into CHO9 cells using SuperFect Transfection Reagent (Qiagen). Transfected cells were cultured for 2 days at 37°C and 5% CO_2 before harvesting. Upon V(D)J recombination, the sequence in between the RSS elements is inverted, which can be detected by PCR (Figure 5A). The level of recombination activity of the 631delT *RAG1* was compared to the wt *RAG1*. DNA recovered from these transfection experiments was diluted as indicated and used as template for PCR. The PCR products were detected by blotting onto a nylon membrane (Schleicher & Schuell) and hybridization with the ^{32}P labeled oligonucleotide FM23 and visualized by phosphor imaging.

RESULTS

Mutation detection in the *RAG1* gene

Based on the clinical presentation and immunodiagnostic results, this patient was classified as SCID. Since it is known that both T^-/B^- SCID and OS with oligoclonal T-cells can be caused by mutations in the *RAG* genes,[4,14] we analyzed the *RAG1* and *RAG2* genes. Fluorescent sequencing of LR-PCR products of *RAG1* and *RAG2* revealed a homozygous deletion of one T-nucleotide in *RAG1* at position 631 (631delT) (numbering according to Schatz *et al.* Genbank accession number M29474). This point mutation leads to a frameshift at codon 173, giving rise to a polypeptide of 199 aa. The consanguineous parents of the patient were both heterozygous for this mutation. We did not detect any mutations in the *RAG2* gene of the patient.

Analysis of TCR gene rearrangements

The maternal origin of the PB T-lymphocytes was excluded by HLA typing. Furthermore, after Ficoll-Paque density centrifugation, the PB granulocyte fraction was immunophenotyped and appeared to consist of >80% T-cells, probably due to the unusually

high T-cell counts. DNA isolated from these cells was used for mutation detection and revealed a homozygous *RAG1* mutation, whereas the mother of the child was heterozygous for this mutation, again excluding the maternal origin of the T-cells.

Because of the extremely elevated PB T-lymphocyte counts (55.5 x 10^6/ml), a T-cell leukemia was suspected. We therefore investigated the clonality of the PB T-lymphocytes by PCR amplification followed by heteroduplex analysis of *TCRB*, *TCRG*, and *TCRD* gene rearrangements. This technique depends on denaturation and renaturation of PCR products, which results in formation of homo- and heteroduplexes, showing single homoduplex bands in case of monoclonality, whereas heteroduplex 'smears' or 'staircase' patterns indicate polyclonality or oligoclonality, respectively.[32] Figure 2 shows the result of the heteroduplex analysis, indicating full usage of the TCR repertoire with some oligoclonal patterns. Since the leukocyte count in the PB was extremely elevated with 81% $CD3^+$ T-lymphocytes, we assume that the oligoclonal pattern is largely caused by expansion of several T-lymphocyte clones in an otherwise polyclonal background. This assumption was supported by flow cytometric analysis of TCRVβ protein expression using a panel of 22 different Vβ antibodies[39] which showed elevated percentages of Vβ1 (within $CD8^+$ T-lymphocytes), and Vβ14 and Vβ5.1 (within $CD4^+$ T-lymphocytes), while percentages of Vβ3, Vβ5.2/5.3, Vβ7, Vβ8.1/8.2, Vβ9.1, Vβ13.1/13.3, Vβ13.6, Vβ16, Vβ17, Vβ22, and Vβ23 were decreased (data not shown) as compared to healthy neonates and children.[46]

To evaluate deletion and insertion of nucleotides and the usage of D gene segments, cloned *TCRB* and *TCRD* gene rearrangements were sequenced (Table 1). The sequenced rearrangements consisted of V, D, and J gene segments. Also insertion and deletion of nucleotides had taken place in most rearrangements.

Overall, the combinatorial TCR repertoire in this patient was comparable to that of healthy individuals[36] as deduced from the finding that almost all tested V, D, and J gene segments were used (Figure 2). The junctional region repertoire seemed to be somewhat reduced because some primer combinations resulted in PCR products with oligoclonal heteroduplex patterns (Figure 2), but this is probably due to the increased T-cell counts.

Analysis of Ig gene rearrangements

To study the effect of the 631delT *RAG1* mutation on Ig gene rearrangements we could only use DNA from BM-MNC (with <1% $CD19^+$ precursor-B-cells), because no B-lymphocytes were detected in the PB (<0.01% of lymphocytes). Due to the very limited amount of available BM cells, we could only study a limited number of potential Ig gene rearrangements. PCR amplification of *IGH* rearrangements in BM-MNC were negative (D_H3-J_H, D_H6-J_H, D_H7-J_H, and V_H3-J_H) except for a D_H2-J_H rearrangement (Table 1), which appeared monoclonal on heteroduplex analysis. The same D_H2-J_H rearrangement was amplified from PB-DNA. As no B-cells were detectable in PB, we conclude that the incomplete D_H2-J_H rearrangement was probably derived from T-cells, occuring as a crosslineage Ig gene rearrangement.[33]

PCR amplification of *IGK* and *IGL* rearrangements in BM-MNC showed faint bands

Table 1. Analysis of *TCRB, TCRD, IGH* and *IGL* gene rearrangements.

Analyzed gene	V gene segment	Del	Junctional region	Del	D gene segment	Del	Junctional region	Del	D gene segment	Del	Junctional region	Del	J gene segment	Frame
TCRB	Vβ13.4	?	TATCTTG	-3	Dβ1	-4	CC	-	-	-	-	-2	Jβ2.7	+
	Vβ17 (S1A1T)	-3	CGGTG	-4	Dβ1	-4	ACGGG	-	-	-	-	-6	Jβ1.1	+
	Vβ17 (S1A1T)	-3	CCT	-	-	-	-	-	-	-	-	-3	Jβ2.1	+
	Vβ12 (S1A1N2)	0	**G**	0	Dβ1	-6	AAAA	-	-	-	-	-2	Jβ2.1	+
TCRD	Vδ2	-11	-	-5	Dδ1	0	GGGGGGGG	-2	Dδ3	-	-	-	-	NA
	Vδ2	-6	ATGGGA	-	-	-	-	-3	Dδ3	-	-	-	-	NA
	-	-	-	-	Dδ2	-1	GCGTTAGATC	-4	Dδ3	0	CGT	0	Jδ1	NA
	-	-	-	-	Dδ2 [a]	-14	GGG [b]	-	-	-	-	-4	Jδ1	NA
	-	-	-	-	Dδ2	-3	CGAGGAG	-2	Dδ3	-5	CTAT	-4	Jδ1	NA
	Vδ1	0	**AC**	-	-	-	-	-5	Dδ3	-4	**CT**	0	Jδ1	+
	Vδ1	-5	CCACCCGTACTGG	-2	Dδ2	-3	CCTGGG	-8	Dδ3	0	**CGGGTTTGTC**	-7	Jδ1	+
	Vδ3	0	**A**	-3	Dδ2	-2	**GGT**	0	Dδ3	-1	CGGGCGG	-1	Jδ1	+
	Vδ3	-2	**GT**	-	-	-	-	0	Dδ3	-5	-	0	Jδ1	+
	Vδ3	-4	CG	-3	Dδ2	-3	-	-1	Dδ3	-5	TTATCG	0	Jδ1	+
	Vδ2	0	**GGAAC**	0	Dδ2	-4	AGC	-3	Dδ3	-5	TTTGG	-4	Jδ1	+
	Vδ2	-1	-	-	-	-	-	-3	Dδ3	-6	-	-2	Jδ1	+
	Vδ2	-2	TG	-	-	-	-	-3	Dδ3	-2	ATGT	-3	Jδ1	-
	Vδ2	0	**GGTTGG**	0	Dδ1	-5	CCCG	-2	Dδ3	-6	-	-2	Jδ1	+
IGH	-	-	-	-	-	-	-	-	D_H2-15	-5	T	-2	J_H4	NA
IGL	Vλ2	-4	CCT	-	-	-	-	-	-	-	-	0	Jλ3	+

[a] Only 5' sequence of Dδ2 present

[b] Four nucleotides required to identify Dδ3

Del = deletion of germline gene segment nucleotides; P-nucleotides are in bold face; NA=not applicable

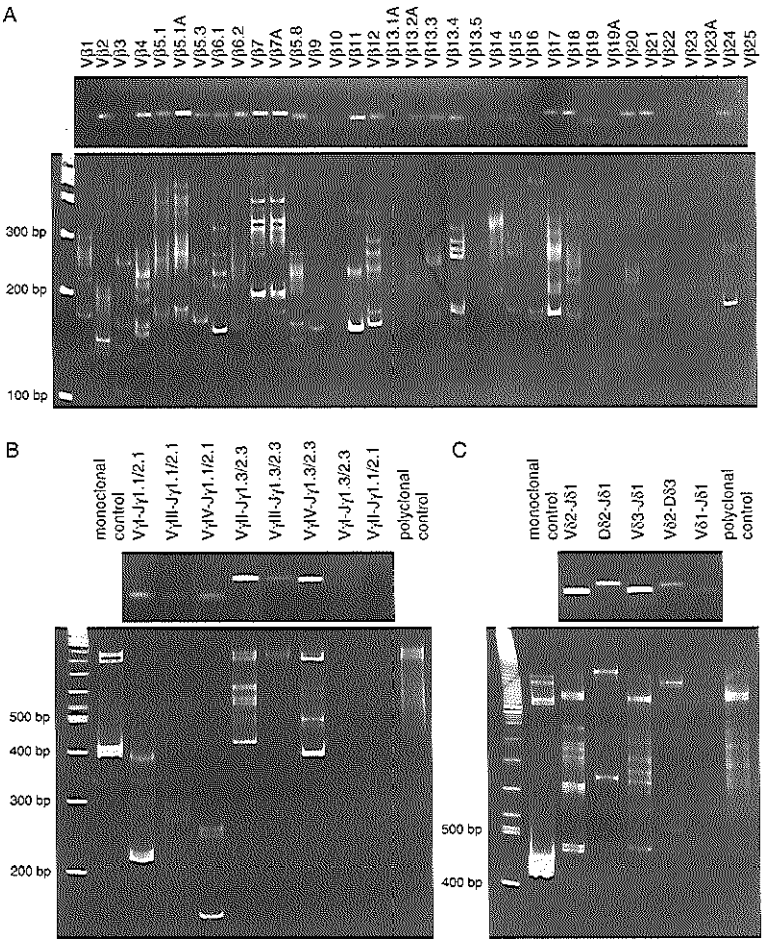

Figure 2. Agarose gel and heteroduplex PCR analysis of *TCRB* gene rearrangements (RT-PCR with Vβ-Cβ primer) (A), *TCRG* gene rearrangements (B) and *TCRD* gene rearrangements (C), showing oligoclonal rearrangement patterns, in an otherwise polyclonal background.
The upper part of each panel shows the presence of PCR products in agarose gels for virtually each primer combination, whereas the lower part of each panel shows heteroduplex analysis of the obtained PCR products. The oligoclonal patterns are probably related to the high T-cell counts with expansion of several T-lymphocyte clones.

on agarose gels. These amounts of PCR products were not sufficient for heteroduplex analysis. Also cloning and fluorescent sequencing was hardly possible and only a single VλII-Jλ3 rearrangement was identified (Table 1).

Expression of N-terminal truncated RAG1 protein

The human wt RAG1 protein has a molecular weight of 119 kilodalton (kD).[23] Usage of a second (codon position 183) or third (codon position 202) AUG codon as an alternative translation start site would theoretically lead to an N-terminal truncated RAG1 protein of approximately 100 kD; the third AUG codon is in a Kozak consensus context. We did not have sufficient BM cells to perform Western blot analysis for the detection of RAG1 proteins. Therefore, we decided to clone the 631delT and wt *RAG1* gene in two different expression vectors, pGEM-T easy and pMSE1, which were used for in vitro transcription and translation and for transfection of COS cells followed by Western blot analysis, respectively. The pGEM-T easy expression vector carries the T7 promotor for initiation of transcription. The in vitro transcription and translation experiment with the wt *RAG1* construct generated a protein of the expected size, while the 631delT *RAG1* construct generated a smaller protein (Figure 3). The same smaller protein band was also visible in the wt lane, suggesting that the alternative translation start site can also be used in the wt *RAG1* gene.

RAG1 protein expression from the pMSE1 vector will result in a C-terminal fusion of a myc epitope tag to the RAG1 protein, which can be used for easy detection. RAG1 protein expression was analyzed after transfection of the expression constructs into COS cells. Western blot analysis showed a number of protein products (Figure 4). In the lane of the N-terminal truncated RAG1 protein, the same pattern is observed as in the wt lane, except for absence of the 119 kD wt product. The 100 kD polypeptide, which is present in both lanes, represents the N-terminal truncated RAG1 protein, thereby suggesting once more that also the wt *RAG1* gene can use this alternative translation start site. Furthermore, some smaller bands were present in both lanes at the same positions, probably representing degradation products, which is consistent with the short half-life of the RAG1 protein.[22]

Analysis of V(D)J recombination activity

V(D)J recombination activity of the wt and N-terminal truncated RAG1 proteins was tested using the recombination substrate pDVG93 (Figure 5A). Upon recombination, the sequence in between the RSS elements is inverted, which can be detected by PCR. As shown in Figure 5B, both the wt and the N-terminal truncated RAG1 protein were able to recombine this substrate, although the activity of the truncated RAG1 protein may be slightly reduced.

DISCUSSION

We identified a novel mutation in the *RAG1* gene (631delT) of a newborn from consanguineous parents, who showed the clinical picture of SCID. In line with the presence of high blood T-cell counts with an almost complete polyclonal TCR repertoire, we observed that this 631delT *RAG1* gene can direct V(D)J recombination on plasmid recombination substrates. Western blotting and in vitro transcription and translation showed the usage of a second translation start site in the 631delT *RAG1* gene, leading to an N-terminal truncation of

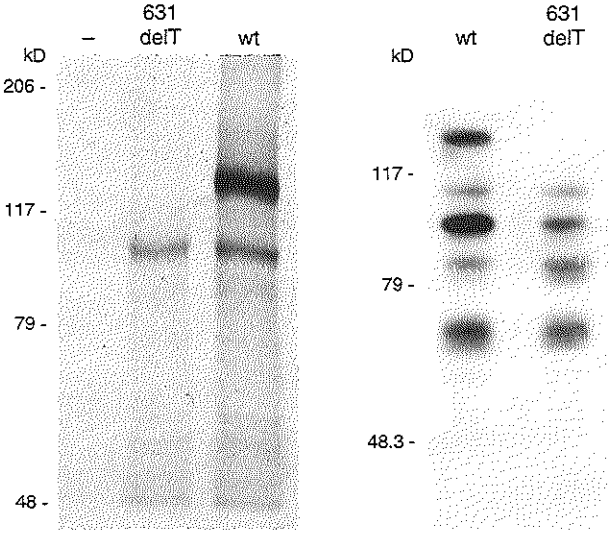

Figure 3. In vitro transcription and translation assay.
The 631delT *RAG1* gene and the wt *RAG1* gene were both cloned in a pGEM-T easy expression vector using the T7 promotor. Transcription and translation of the 631delT *RAG1* gene showed absence of the 119 kD wt protein band, which was present in the lane of the wt *RAG1* gene. The 631delT only showed the smaller 100 kD N-terminal truncated protein band, which was also present in the wt *RAG1* gene lane.

Figure 4. Western blotting after transfection of COS cells with wt *RAG1* and 631delT *RAG1* constructs.
RAG1 proteins with c-myc tag were detected using a c-myc Ab and visualized by enhanced chemiluminescence. The upper band in the wt lane represents the 119 kD wt RAG1 protein, which is absent in the 631delT *RAG1* lane. Both the wt *RAG1* and the 631delT *RAG1* gene express the 100 kD N-terminal truncated protein. In both lanes additional protein products are seen, representing degradation products of the RAG1 protein, which is in line with the short half-life of this protein.

the RAG1 protein. This N-terminal truncation apparently has major effect on B-cell differentiation, since sensitive flow cytometric analysis showed virtually no B-cells in the PB (<0.01% of lymphocytes) and <1% $CD19^+$ precursor-B-cells in the BM of the patient.

In their original manuscript, Schwarz *et al.* found that a number of B^- SCID patients had *RAG* mutations resulting in severely decreased RAG activity (recombination frequency of *RAG1* mutants <0.7%).[4] Villa *et al.* observed that OS patients with oligoclonal T-cells and diminished numbers of B-cells had *RAG* gene defects with residual RAG activity (recombination frequency *RAG1* mutants 5-23%).[14] Our SCID patient with a homozygous 631delT *RAG1* mutation appeared to have high blood T-cell counts with an almost complete TCR repertoire, but undetectable numbers of B-cells, while the RAG activity was only slightly diminished. Our patient showed some clinical symptoms characteristic of OS, such as GVH-like disease, erythrodermia, hepatosplenomegaly, lymphadenopathy, and agammaglobulinemia. However, the clinical picture was unusually severe, regarding the early onset and her

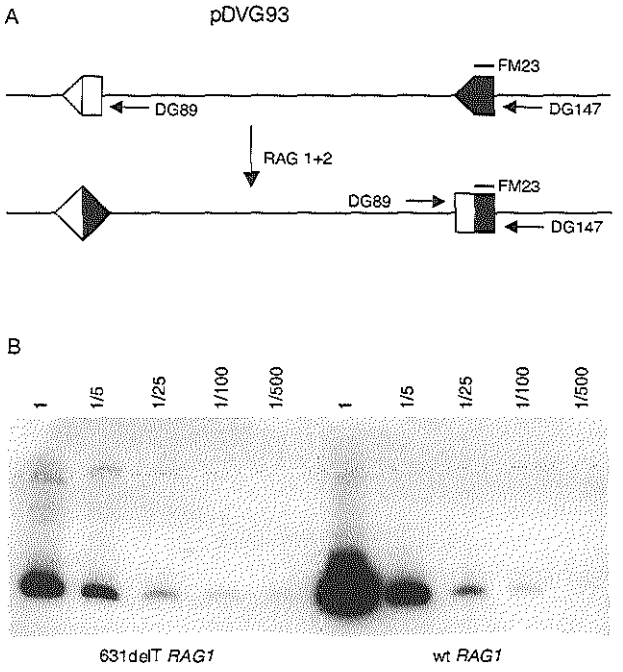

Figure 5. Plasmid recombination assay using construct pDVG93.
Transfection of pDVG93, *RAG1*, and *RAG2* in CHO cells will lead to an inversional rearrangement of pDVG93, which can be detected by primers DG89 and DG147 (A). PCR reactions with primers DG89 and DG147 on serial dilutions of recombination products as indicated. PCR products were visualized by hybridization with ^{32}P-labeled oligonucleotide FM23 followed by phosphor imaging. The N-terminal truncated RAG1 protein is still able to perform inversional rearrangement of pDVG93, although the activity may be slightly reduced as compared to wt RAG1 protein (B).

death before the fifth week of life. Other OS characteristics, such as a predominance of Th2-cells, were not present. IgE levels were not determined, but the complete absence of B-lymphocytes in the PB makes high IgE levels unlikely. Eosinophilia was present at first admission, but was absent upon referral to the university hospital. The strongly increased T-lymphocyte counts (55.5 x 10^6/ml) suggested the presence of a T-cell leukemia, but only limited TCR oligoclonality was observed in an otherwise polyclonal background as demonstrated by Vβ antibody studies, heteroduplex PCR, and sequencing of TCR gene rearrangements. This is not in line with studies on OS patients, which show that the peripheral T-cell repertoire in OS is highly restricted.[40,41] The combined clinical and laboratory data are not in line with a genuine OS, but with an OS-like T^+/B^- SCID.

Despite the polyclonal TCR repertoire, B-lymphocytes and Ig gene rearrangements were hardly detectable in our patient. As reported before, deletion of part of the N-terminus of the murine RAG1 protein did not have a major effect on recombination activity in a V(D)J

recombination assay.[16,22] Furthermore, the basic aa motif BIIa, responsible for enhancement of the recombination activity, was still present in the N-terminal truncated RAG1 protein.[20] These combined data indicate that the N-terminus of the RAG1 protein is probably particularly important for Ig gene rearrangements. Similarly, the deletion constructs of Kirch *et al.* suggested that the C-terminus of the RAG2 protein is essential for efficient V_H to DJ_H rearrangement.[17] Thus the N-terminal part of RAG1 and the C-terminus of RAG2 are dispensable for basic recombination activity, but they may have a role in targeting of Ig loci.

We considered the following explanations for the absence of Ig gene rearrangements in our patient: 1) The RSS might differ in sequence between Ig and TCR genes, and the RAG1 N-terminus would only be required for Ig RSS recognition and/or cleavage. However, published data on RSS do not support this suggestion.[42,43] 2) The N-terminal truncated RAG1 protein might be able to mediate 'one-step' rearrangements (V to J joining), but not 'two-step' rearrangements (V to D-J joining). The positioning of the 12 and 23 bp spacer length in the RSS of the V, D, and J segments of the *IGH, TCRB,* and *TCRD* genes differ in such a way, that the *TCRB* and *TCRD* genes can skip D gene segments and thereby produce 'one-step' V-J joinings. *IGH* gene rearrangements in principle always include D-segments and thus have to be 'two-step' rearrangements. Sequencing of *TCRB* and *TCRD* gene rearrangements showed usage of D gene segments, proving the occurrence of 'two-step' rearrangements in our patient (Table 1). 3) Recruitment of the RAG1 protein to the Ig genes might be established via its N-terminus, implying that the N-terminal truncated RAG1 protein is hampered in reaching Ig loci. 4) The N-terminus of the RAG1 protein might be involved in opening of the chromatin structure of the Ig loci prior to recombination, implying that an N-terminal truncation of the RAG1 protein would prevent a complete rearrangement of Ig genes. 5) The N-terminus of the RAG1 protein might interact with a B-cell specific factor, which would form a complex necessary for rearrangement of Ig genes. 6) Alternatively, Ig gene rearrangements might require higher levels of RAG activity than TCR gene rearrangements. This hypothesis is supported by the observation that cross-lineage *TCRB, TCRG* and *TCRD* gene rearrangements occur at high frequency in precursor-B-ALL (>90%), while cross-lineage *IGH, IGK* and *IGL* gene rearrangements are rare in T-ALL or do not occur at all.[33,44,45] Explanations 3 to 6 are not mutually exclusive, and development of model systems will be required to clarify this issue.

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Chapter 12

THE IMMUNOPHENOTYPIC AND IMMUNOGENOTYPIC B-CELL DIFFERENTIATION ARREST IN BONE MARROW OF RAG DEFICIENT SCID PATIENTS CORRESPONDS TO RESIDUAL RECOMBINATION ACTIVITIES OF MUTATED RAG PROTEINS

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Submitted

SUMMARY

The protein products of the recombination activating genes (*RAG1* and *RAG2*) initiate the formation of immunoglobulin (Ig) and T-cell receptors, which are essential for B- and T-cell development, respectively. Mutations in the *RAG* genes result in severe combined immunodeficiency disease (SCID), which can be subdivided into several forms, based on clinical characteristics and immunophenotype and counts of blood lymphocytes. Biochemically, mutations in the *RAG* genes result either in non-functional proteins or in proteins with partial recombination activity.

The mutated *RAG* genes of nine patients from seven families were analyzed for their recombination activity using inversional recombination substrates, rearrangement of endogenous Ig loci in *RAG* gene transfected non-lymphoid cells, or the presence of Ig gene rearrangements in bone marrow (BM). Recombination activity was virtually absent in all six patients with mutations in the RAG core domains, but partial activity was present in the other three RAG deficient patients, two of them having Omenn syndrome with oligoclonal T-lymphocytes.

Using four-color flow cytometry, we could define the exact stage at which B-cell differentiation was arrested in the BM of five RAG deficient SCID patients. In four out of five patients, the absence of recombination activity was associated with a complete B-cell differentiation arrest at the transition from cytoplasmic (Cy) Igμ- pre-B-I-cells to CyIgμ+ pre-B-II-cells. However, the fifth patient showed low frequencies of precursor-B-cells with CyIgμ and surface membrane IgM, in line with the partial recombination activity of the patient's mutated *RAG* gene and the detection of in-frame Ig gene rearrangements in BM.

INTRODUCTION

Human severe combined immunodeficiency disease (SCID) refers to a group of disorders, which can be classified based on the pattern of inheritance (e.g. X-linked versus autosomal recessive) and the immunological phenotype, e.g. the presence or absence of T, B, and NK-cells in the peripheral blood (PB).[1] Characteristic for all types of SCID is the absence or dysfunction of T-lymphocytes. Consequently, affected children present soon after birth with opportunistic infections, failure to thrive and protracted diarrhea. The available treatment options for SCID are bone marrow transplantation (BMT), polyethylene glycol-conjugated adenosine deaminase (ADA) substitution for ADA deficient SCID, and somatic gene therapy for the X-linked form of SCID with common gamma chain deficiency.[2]

One type of SCID is characterized by autosomal recessive inheritance, absence of T- and B-lymphocytes in PB, but presence of NK-cells (non-T non-B SCID). A number of patients suffering from non-T non-B SCID have been shown to carry mutations in the recombination activating genes (*RAG*) 1 or 2.[3] The two human *RAG* genes are located in a tail to

tail orientation on chromosome 11p13 and are expressed during T- and B-cell development in the thymus and BM, respectively.[4-8] The RAG proteins are essential to initiate the recombination of the immunoglobulin (Ig) and T-cell receptor (TCR) loci.[9-11] During this recombination, the RAG proteins introduce double strand breaks at the recombination signal sequences (RSS) flanking the variable (V), diversity (D), and joining (J) gene segments, which are then joined to form a VDJ exon encoding the antigen binding part of the Ig and TCR molecules.[12,13] At the junction of the gene segments, nucleotides can be inserted by terminal deoxynucleotidyl transferase (TdT) or deleted, which contributes enormously to the diversity of the Ig and TCR repertoire.[14] Non-functional RAG1 or RAG2 proteins result in the absence of mature B- and T-lymphocytes in the PB.[15,16]

The RAG1 and RAG2 proteins both contain a core domain, which is essential for the recombination activity.[17-20] Although the N-terminus of the RAG1 protein and the C-terminus of the RAG2 protein seem to be dispensable for recombination activity, it has been shown that these presumed dispensable portions might both harbor Ig specificity.[21-25] Most mutations in the *RAG* genes causing non-T non-B SCID affect the core domain and encode non-functional proteins ("null" alleles).[3] On the other hand, some *RAG* mutations in the core domain appear to be less severe, because they are associated with partial VDJ recombination activity, causing a variant of non-T non-B SCID, called Omenn syndrome (OS).[26] OS is characterized by oligoclonal, auto-reactive T-cells with a T-helper 2 phenotype, absence of B-lymphocytes, but high serum IgE levels. The clinical phenotype of OS resembles the clinical phenotype resulting from maternal-fetal T-cell engraftment (MFT) in a newborn with non-T non-B SCID. Altogether, the RAG deficient SCID phenotype can be subdivided into four groups, i.e. non-T non-B SCID, OS, non-T non-B SCID with MFT, and an atypical form.[27] For reasons of clarity, we will use the terms B-cell negative or RAG deficient SCID here.

The absence of T- and/or B-lymphocytes in the PB of patients suffering from RAG deficient SCID results from a differentiation arrest during T- and B-cell development in thymus and BM, respectively. The localization of this differentiation arrest has been precisely defined in murine thymus and BM.[15,16] However, no such data are available in man. From a theoretical point of view, one would expect that RAG deficient SCID patients have a complete B-cell differentiation arrest at the transition from CyIgμ- to CyIgμ+ precursor-B-cells, but this has not been shown yet.[28] Here we present nine RAG deficient SCID patients from seven families. The recombination activity of the mutated *RAG* genes was analyzed using inversional recombination substrates, rearrangement of endogenous Ig loci in (mutated) *RAG* gene transfected human non-lymphoid cells, or the occurrence of Ig gene rearrangements (immunogenotype) in BM of the SCID patients. In addition, from five RAG deficient SCID patients, the composition of the precursor-B-cell compartment (immunophenotype) in BM could be analyzed with four-color flow cytometry. The immunophenotypic and immunogenotypic B-cell differentiation arrest in BM corresponded to residual recombination activities of the mutated RAG proteins.

MATERIALS AND METHODS

Cell samples

Over the last 15 years, we received BM samples from seven B-cell negative SCID patients, and Epstein-Barr virus (EBV) transformed B-cell lines derived from BM precursor-B-cells of two siblings with B-cell negative SCID.[29]

The BM samples from the seven SCID patients were subjected to Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. The recovered mononuclear cells (MC) were frozen and stored in liquid nitrogen and thawed later for flow cytometric immunophenotyping studies. Granulocytes were used for DNA extraction.[30]

We have previously reported on an extensive study concerning the composition of the precursor-B-cell compartment in the BM of 18 healthy children using triple labelings.[31] We repeated our flow cytometric analyses using quadruple labelings in thawed BM samples from six of these healthy children (age range: 1y7m to 5y11m; mean age 3y10m; 4 males, 2 females), who were BM donors for their siblings (Department of Pediatrics, Leiden University Medical Center; Drs. P.M. Hoogerbrugge and J.M.J.J. Vossen).

All cell samples were obtained according to the informed consent guidelines of the Medical Ethics Committees of the Leiden University Medical Center and the University Hospital Rotterdam.

DNA extraction, PCR amplification, and analysis of Ig gene rearrangements

DNA was extracted from BMMC and transfected φNX-WTA cells (a human epithelial kidney cell line) with the QIAamp Blood kit (Qiagen, Chatsworth, CA, USA).[32] PCR was performed as described previously.[33] In each 100 µl PCR reaction 0.1-1 µg DNA, 10-12.5 pmol of 5' and 3' oligonucleotides and 1 U Ampli*Taq* gold polymerase (Applied Biosystems, Foster City, CA, USA) were used. Most oligonucleotides for amplification of the *IGH*, *IGK*, and *IGL* genes were published before.[34-36] PCR conditions were 7 min at 95°C, followed by 45 sec at 94°C, 90 sec at 57-65°C, 2 min at 72°C for 40 cycles, followed by a final extension step (7 min at 72°C). DNA from BMMC was analyzed for rearrangements of D_H1, D_H2, D_H3, D_H4, D_H5, D_H6, D_H7, $V_H1/7$, V_H2, V_H3, V_H4, V_H5, and V_H6 to J_H. The PCR products were cloned in the pGEM-T easy vector and subsequently sequenced. When *IGH* rearrangements were identified, DNA from BMMC was also analyzed for Vκ-Jκ and Vλ-Jλ rearrangements. Involved Ig gene segments were identified using IMGT, the international ImMunoGeneTics database http://imgt.cnusc.fr:8104 (Initiator and coordinator: Marie-Paule Lefranc, Montpellier, France, lefranc@ligm.igh.cnrs.fr).[37]

Long range (LR)-PCR for amplification of *RAG* genes

The entire *RAG1* or *RAG2* gene was amplified in a single LR-PCR reaction (100 µl), using 4 U r*Tth* DNA polymerase XL (Applied Biosystems) for subsequent sequencing analysis of the LR-PCR product or 5 U *Pfu* enzyme mix (Stratagene, La Jolla, CA) for cloning the

LR-PCR product; we used 30 or 14 pmol of oligonucleotides, respectively. The sequences of the oligonucleotides used for the LR-PCR of *RAG1* and *RAG2* will be made available on request. Restriction-sites for *Bam*HI and *Not*I were incorporated into the 5' and 3' oligonucleotides used for cloning, respectively. LR-PCR conditions were 2 min at 94°C, followed by 15 sec at 94°C, 30 sec at 57°C, and 4 min at 68°C for 40 cycles using 10 sec auto-extension from cycle 21 onward. In case of cloning, the LR-PCR conditions were 45 sec at 94°C, followed by 45 sec at 94°C, 45 sec at 62°C, and 3.5-6.5 min at 72°C for 30-35 cycles using 10 sec auto-extension from cycle 21 onward. After the last cycle an additional step of 10 min at 72°C was performed for final extension.

Fluorescent sequencing reaction and analysis

LR-PCR products of *RAG1* and *RAG2* were purified using QIAquick PCR purification kit (Qiagen). 5-9 µl purified PCR product was sequenced with 5 µl dRhodamine dye terminator mix (Applied Biosystems), using 3.3 pmol internal sequencing primers. All sequencing was performed as described before,[35] and run on an ABI Prism 377 fluorescent sequencer (Applied Biosystems).

Cloning of mutated and wild type *RAG* genes

LR-PCR products containing the entire *RAG1* or *RAG2* open reading frame were isolated after digestion with *Bam*HI and *Not*I, and cloned into the *Bam*HI-*Not*I cut vector pEBB under control of the EF-1 promoter.[22] The cloned wt and mutated *RAG* genes were sequenced to exclude the presence of PCR-induced mutations.

V(D)J recombination assay using an inversional recombination substrate

2 µg of pEBB-*RAG1* (mutated or wt) together with 2 µg pEBB-*RAG2* (mutated or wt) and 1 µg of the recombination substrate (pDVG93) containing two RSS elements were transfected into Chinese hamster ovary (CHO9) cells using SuperFect Transfection Reagent (Qiagen). Transfected cells were cultured for 2 days before harvesting. Upon V(D)J recombination, the sequence in between the RSS elements is inverted, which can be detected by PCR.[24] The level of recombination activity of the mutated *RAG* genes was compared to that of the wt *RAG* genes. DNA recovered from these transfection experiments was diluted and used as template for PCR. The PCR products were detected by blotting onto a nylon membrane (Schleicher & Schuell) and hybridization with the ^{32}P labeled oligonucleotide FM23 and visualized by phosphor imaging.[24]

Analysis of V(D)J recombination of endogenous Ig loci following transfection of cloned *RAG* genes in combination with E47 in φNX-WTA cells

Transfection was performed as described before.[38] In short, 6 µg of pEBB-*RAG1* (mutated or wt) together with 6 µg pEBB-*RAG2* (mutated or wt) and 6 µg βhAP-E47 (under control of the human β actin promoter) were transfected into confluent φNX-WTA human kidney epithelial cells using 2M $CaCl_2$ and 2x HBS (4.1 gram NaCl, 2.975 gram HEPES,

0.0525 gram Na_2HPO_4, in 250 ml H_2O, pH 7.05). Transfected cells were cultured for 3 days before harvesting. DNA was isolated and analyzed for rearrangement of endogenous loci, such as D_H4-J_H, Vκ1-Jκ, and Vλ3-Jλ1/2/3. These rearrangements were earlier shown to be specifically induced upon transfection of E47 and RAG1 and RAG2.[38]

Real-time quantitative (RQ)-PCR assay for analysis of relative recombination activity of mutated *RAG* genes

Two RQ-PCR assays were developed to quantify the recombination activity. The first RQ-PCR assay focussed on the inversional recombination substrate pDVG93, using the original primers (DG89 and DG147) and a TaqMan probe modified from probe FM23.[24] The second RQ-PCR assay focussed on Vκ1/6-Jκ rearrangements for quantification of recombination activity following transfection of φNX-WTA cells. This assay used a consensus forward primer recognizing Vκ1 and 6 family gene segments and a consensus reverse primer recognizing the Jκ1, 2, 3, and 4 gene segments. The consensus TaqMan probe was positioned on the reverse strand in the Vκ gene segment. Primers and probes were designed as described before using the Primer Express 1.0 program (Applied Biosystems).[39]

In both RQ-PCR assays, the amount of DNA added was standardized using a control primer-probe set. First, non-replicated pDVG93 plasmid DNA was removed by *Dpn*I digestion.[40] Subsequently, the amount of replicated pDVG93 added to the RQ-PCR reaction was determined using two primers and a TaqMan probe with several *Dpn*I restriction sites between the primer annealing sites (forward primer 5'-CACAATTCCCCTATAGTGAGTCGTATT-3'; reverse primer 5'-AAGTGGCGAGCCCGATCT-3'). For RQ-PCR analysis of Vκ1/6-Jκ rearrangements in φNX-WTA cells, the *albumin* gene was used as a control set.[39]

Plasmids or DNA were added to the TaqMan universal PCR master mix (Applied Biosystems) and divided over four tubes to ensure an equal amount of DNA per reaction. Two tubes were used for specific amplification and two for control amplification. RQ-PCR was performed as described before and run on an ABI Prism 7700 (Applied Biosystems).[39] The PCR cycle at which a fluorescent signal became detectable (threshold cycle or C_T) was comparable in the duplicate tubes (ΔC_T<1.0). All RQ-PCR experiments were performed multiple times, showing virtually identical results.

Transfection of wt *RAG* genes was used as a positive control for recombination activity in both assays. Using these RQ-PCR results, standard curves for the two specific amplifications and the two control amplifications were generated. All four standard curves met the following requirements: slopes between -3.3 and -3.7 to ensure optimal amplification, correlation coefficient close to 1.0, and a fluorescent signal intensity (ΔRn)>1.0. Using the relative standard curve method with separate tubes (Applied Biosystems, user bulletin #2, relative quantitation of gene expression), the relative recombination activity of the wt *RAG* genes was arbitrarily set to 100%. The relative recombination activity of mutated *RAG* genes was calculated from the specific standard curve and corrected using the control standard curve.

Flow cytometric analysis of BM samples from healthy children and SCID patients

Fifty µl aliquots of thawed BMMC (10 x 10^6 cells/ml) were incubated for 10 min. at room temperature with combinations of optimally titrated MAb: 50 µl fluorescein isothiocyanate (FITC) conjugated MAb, 50 µl phyco-erythrin (PE) conjugated MAb, 50 µl peridinin chlorophyll protein (PerCP) cyanin (CY) 5.5 conjugated MAb, and 50 µl allophycocyanin (APC) conjugated MAb were used to detect membrane bound antigens. After incubation, the cells were washed and further processed depending on the type of quadruple labeling. The 14 applied quadruple labelings are summarized in Table 1.

Quadruple labelings for membrane bound antigens (labelings 1-7 in Table 1) were directly analyzed by flow cytometry using FACSCalibur (Becton Dickinson, San Jose, CA, USA). For quadruple labelings involving intracellular staining of cytoplasmic (Cy) CD79a, CyIgµ, and CyVpreB,[41] and intranuclear staining of TdT (labelings 8-14 in Table 1), we first performed the membrane labelings, followed by permeabilization of the BM cells using IntraPrep Permeabilization Reagent (Immunotech, Marseille, France), and subsequent intracellular staining.[42,43]

Table 1. Quadruple labelings for analysis of the precursor B-cell compartment.

	FITC-conjugates	PE-conjugates	PerCP-Cy5.5-conjugates	APC-conjugates
MAb directed against membrane antigens				
1	IgG	IgG		IgG
2	CD10 (J5)[a]	CD20 (Leu-16)[b]	CD3 (SK7)[b] / CD16[b, h] / CD33[b, h]	CD19 (SJ25C1)[b]
3	CD34 (HPCA-2)[b]	CD20 (Leu-16)	CD3 (SK7) / CD16 / CD33	CD19 (SJ25C1)
4	SmIgD[c]	SmIgM (MB-11)[f]	CD3 (SK7) / CD16 / CD33	CD19 (SJ25C1)
5	CD36 (CLB-IVC7)[d]	CD19 (Leu-12)[b]	CD3 (SK7) / CD16 / CD33	CD22 (S-HCL-1)[b]
6	CD71 (T9)[b]	CD16 (Leu-11c) [b] / CD56 (Leu-19)[b]	CD45 (2D1)[b, i]	CD3 (SK7)[b]
7	CD34 (HPCA-2)	CD13 (Leu-M7) [b] / CD33 (hP676)[b]	CD19 (SJ25C1)[b]	CD22 (S-HCL-1)
MAb directed against membrane antigens, TdT, CyCD79a, CyIgµ, and CyVpreB following IntraPrep permeabilization				
8	CyIgG	CyIgG		IgG
9	CyIgµ[e]	SmIgM (MB-11)	CD3 (SK7) / CD16 / CD33	CD19 (SJ25C1)
10	CD36 (CLB-IVC7)	CyVpreB (HSL96)	CD3 (SK7) / CD16 / CD33	CD22 (S-HCL-1)
11	CyIgµ	CyVpreB (HSL96)	CD3 (SK7) / CD16 / CD33	CD19 (SJ25C1)
12	CD19 (Leu-12)[b]	CyCD79a (HM47)[a, g]	CD3 (SK7) / CD16 / CD33	CD22 (S-HCL-1)
13	TdT (hTdT-6)[e]	CyCD79a (HM47)	CD3 (SK7) / CD16 / CD33	CD19 (SJ25C1)
14	CD36 (CLB-IVC7)	CyCD79a (HM47)	CD3 (SK7) / CD16 / CD33	CD22 (S-HCL-1)

MAb were derived from: [a] Beckman Coulter (Brea, CA); [b] Becton Dickinson (San Jose, CA); [c] Kallestad / Sanofi-Synthelabo (Paris, France); [d] CLB (Amsterdam, The Netherlands); [e] Supertechs (Bethesda, MD); [f] Sigma Aldrich (St. Louis, MO).

[g] Staining of CyCD79a as well as the cytoplasmic tail of SmCD79a.

[h] Custom-made MAb.

[i] PerCP labeled MAb.

RESULTS

Patient characteristics and mutations in the *RAG* genes

Based on clinical characteristics and immunophenotyping results of PB, nine patients from seven families were diagnosed as having B-cell negative SCID (Table 2). SCID-1 was studied extensively and published before.[24] MFT in patients diagnosed with OS (SCID-1 and SCID-5) was ruled out by HLA typing, the detection of a homozygous *RAG* gene mutation in DNA isolated from PBMC, and the detection of oligoclonal Vβ TCR gene rearrangements.[24,44]

We detected mutations in the *RAG1* or *RAG2* genes in all nine B-cell negative SCID patients (Table 2). The *RAG1* genes contained four different mutations, including one novel mutation. Two of the four mutations (R249H and K820R) represented polymorphisms. The R249H mutation has been shown to be a functionally intact polymorphism by recombination activity studies,[3] while the K820R mutation was interpreted as a polymorphism based on its allelic frequency.[45] In this study, we show that the K820R mutation indeed retained intact recombination activity (see below). The other two RAG1 mutations (codon 199 stop in SCID-1 and R404Q in SCID-7), were shown to have disease-causing effect by recombination activity studies (see below).

We detected four different mutations in the *RAG2* genes, including three novel mutations (Table 2). The disease-causing effect of the three novel mutations (codon 247 stop in

Table 2. Mutation analysis in patients presenting with B-cell negative SCID.

Patients	Diagnosis	Mutation		Family members		Consanguinity
		DNA level[a]	Protein level	Carriers	Non-carriers	of parents
RAG-SCID 1	OS	*RAG1* 631delT[d]	Codon 199 stop	3	0	Yes
		RAG1 G858A[d]	R249H			
RAG-SCID 2.1[c]	T-B- SCID	*RAG1* A2571G[d]	K820R	2	0	Yes
RAG-SCID 2.2		*RAG2* 1913delG[d]	Codon 247 stop			
RAG-SCID 3	T-B- SCID	*RAG2* A2643C[e]	H481P	NA	NA	Yes
RAG-SCID 4[c]	T-B- SCID	*RAG1* A2571G[e]	K820R	NA	NA	Unknown
		RAG2 1913delG[e]	Codon 247 stop			
RAG-SCID 5	OS	*RAG1* G858A[d]	R249H	2	0	No
		RAG2 T2558A[d]	W453R			
RAG-SCID 6	T-B- SCID	*RAG2* C1247T[e]	Q16 stop	NA	NA	No
RAG-SCID 7.1	T-B- SCID	RAG1 G858A[e]	R249H	1[b]	NA	No
RAG-SCID 7.2		RAG1 G1323A[e]	R404Q			

[a] Numbering of RAG1 gene according to reference 4, RAG2 gene according to reference 6; [b] Carriership in the mother was based on family history instead of DNA analysis; [c] Patients from different families with identical mutations; [d] Homozygous mutation, both parents were carrier; [e] Seemingly homozygous mutation, but deletion of the other allele was not ruled out; NA = not analyzed.

SCID-2 and SCID-4, H481P in SCID-3, and Q16 stop in SCID-6) was shown by recombination activity studies (see below). It has been shown before that the W453R mutation in SCID-5 retained partial RSS nicking and hairpin formation function, as well as a reduced capacity to form signal and coding joints, compatible with the clinical diagnosis of OS in SCID-5 (Table 2).[46] For this reason, we did not clone the W453R mutation for recombination studies.

In total, we found two different homozygous disease-causing *RAG1* mutations and four different homozygous disease-causing *RAG2* mutations in nine patients from seven families. According to the information obtained by the clinicians, families SCID-2 and SCID-4 were unrelated. Nevertheless, we identified the same disease-causing mutation in RAG2 (codon 247 stop) and the same polymorphism in RAG1 (K820R) in both families.

V(D)J recombination activity

The "gold standard" for analysis of V(D)J recombination activity is the transformation assay, in which additional antibiotic resistance is acquired upon V(D)J recombination of a plasmid. After transformation, the ratio between single and double resistant colonies gives a reliable estimate of the remaining recombination activity of mutated *RAG* genes. Villa *et al.* compared different assays for analysis of V(D)J recombination activity and showed that the transformation assay and the inversional recombination assay, which we used in this study, gave comparable results.[26] Furthermore, the *RAG1* 631delT mutation in SCID-1 was found recently in other patients. The recombination activity of this mutation was shown to be approximately 10% of wt recombination activity, using an adapted version of the transformation assay.[47] This is similar to the result of our inversional recombination assay (see below).

After transfection, plasmid pDVG93 was used as template for PCR and subsequent hybridization with a radioactive probe (results not shown), as well as for RQ-PCR reaction after digestion with the restriction enzyme *Dpn*I. The results of the RQ-PCR reaction are shown in Table 3. Transfection of the wt *RAG1* gene or the K820R mutation resulted in identical amplification curves. After correction for transfection efficiency and the amount of pDVG93 added to the RQ-PCR reaction, the relative recombination activities were comparable, indicating that the K820R mutation represents a functional polymorphism (Table 3).

After correction for transfection efficiency and the amount of pDVG93 added to the RQ-PCR reaction, the mutated *RAG* genes of SCID-1 and SCID-3 showed the highest relative recombination activities (Table 3). SCID-1 suffered from an OS-like T+/B- SCID.[24] The *RAG1* 631delT mutation in this patient was shown to result in an N-terminal truncation of the RAG1 protein, leaving the core domain unaffected.[24] SCID-3 suffered from a genuine non-T, non-B SCID. The aa substitution (H481P) in this patient was located outside the core domain in the C-terminus of the RAG2 protein. The *RAG* core domain mutations in SCID-2, SCID-4, SCID-6, and SCID-7 resulted in very low relative recombination activities (Table 3). It has been published recently that a RAG1 R404W mutation (comparable to the R404Q mutation in SCID-7) resulted in complete absence of recombination activity as well.[47]

Table 3. Relative recombination activity of mutated *RAG* genes.

Patient	RAG1 protein	RAG2 protein	Relative recombination activity using pDVG93 (%)[b]	E47 induced rearrangements of endogenous loci in φNX-WTA cells			
				Vκ1-Jκ	RQ-PCR Vκ1/6-Jκ (%)[c]	D_H4-J_H	Vλ3-Jλ
Healthy control	wt	wt	100	+	100	+	+
RAG-SCID 1	N-terminal truncation	wt	9.3 ± 2.9	+	0.6 ± 1.1	-	+
RAG-SCID 2 and 4	K820R	Codon 247 stop	1.4 ± 1.6	-	0	-	-
	K820R	wt	115.1 ± 54.4	+	91.0 ± 88.1	+	+
	wt	Codon 247 stop	1.6 ± 2.4	-	0.1 ± 0.2	-	-
RAG-SCID 3	wt	H481P	3.2 ± 2.0	+	21.2 ± 22.4	-	+
RAG-SCID 6	wt	Q16 stop	0	-	0	-	-
RAG-SCID 7	R404Q	wt	1.3 ± 1.5	-	0	-	-
Mock transfection[a]	-	-	0	-	0	-	-

wt = wild type

[a] Mock transfection consisted of H_2O in case of transfection of CHO9 cells and of E47 without wt *RAG* genes in case of transfection of φNX-WTA cells.

[b] Mean of four RQ-PCR reactions using plasmids from four different transfections.

[c] Mean of four RQ-PCR reactions using DNA from one transfection.

Rearrangement of endogenous Ig loci after transfection of φNX-WTA human kidney epithelial cells

To study the ability of mutated *RAG* genes to rearrange endogenous Ig loci, we transfected wt and/or mutated *RAG* genes together with E47 in φNX-WTA cells. φNX-WTA cells are human kidney epithelial cells that do not express RAG proteins and for this reason provide a good model system to study the effect of cloned *RAG* mutations. Transfection of E47 together with wt *RAG* genes was shown to specifically induce D_H4-J_H, Vκ1-Jκ, and Vλ3-Jλ rearrangements.[38] Therefore, we analyzed the occurrence of these rearrangements in our transfection assay (Table 3). Furthermore, we quantified the recombination activity by using DNA from transfected φNX-WTA cells as template for a Vκ1/6-Jκ RQ-PCR reaction (Table 3).

The TaqMan primer-probe set for quantification of Vκ1/6-Jκ rearrangements, showed a ΔRn>1.0 when DNA from PBMC was used as template. However, when DNA from transfected φNX-WTA cells was used as template, the ΔRn after 50 cycles was lower, probably due to the low frequencies of rearrangements. Transfection of E47 without wt *RAG* genes did not result in rearrangements (Table 3).

The mutated *RAG* genes of SCID-1 and SCID-3 with the highest relative recombination activity in the inversional recombination assay were also able to perform rearrangement of Vκ1-Jκ and Vλ3-Jλ when co-transfected with E47 (Table 3). Except for the K820R polymorphism in RAG1, we could not detect D_H4-J_H rearrangements after transfection of mutated *RAG* genes (Table 3).

Ig gene rearrangements in BMMC

In order to study the capacity of mutated *RAG* genes to rearrange Ig loci during human B-cell differentiation *in vivo*, we isolated DNA from BMMC and investigated the occurrence of incomplete D_H-J_H and complete V_H-J_H rearrangements. *IGH* rearrangements were detected in two out of seven RAG deficient SCID patients studied (Table 4), the same patients (SCID-1 and SCID-3) that were shown to harbor partial recombination activity (Table 3). The two patients with *IGH* rearrangements were also analyzed for the presence of *IGK* and *IGL* rearrangements. As the presence of complete and in-frame heavy and light chain Ig gene rearrangements (as in SCID-3, Table 4) would theoretically allow the B-cell to surpass the (pre)-BCR checkpoints and to enter the periphery, the V to J rearrangements were also analyzed for the presence of somatic mutations. As expected in patients suffering from RAG deficient SCID, no somatic mutations could be detected (Table 4).

Patients SCID-7.1 and 7.2 were two sisters suffering from the same *RAG1* mutation. This R404Q mutation was shown to cause a complete loss of function (Table 3). The EBV-transformed B-cell lines of both patients were reported to have germline *IGH*, *IGK*, and *IGL* genes, as assessed by Southern blotting.[29]

Composition of the BM lymphocyte gate and design of an optimal B-cell gate

As shown previously, the composition of the BM lymphocyte gate in healthy children is highly variable, because of 'contamination' with T-lymphocytes, NK-cells, myeloid precursors and normoblasts.[31] Therefore, reliable comparison of B-cell subpopulations in BM samples from healthy children and RAG deficient SCID patients requires analysis within a well-defined and "purified" B-cell gate (Figures 1 and 2).

We have previously shown that CD22 is a reliable pan-B-cell marker, which is rarely expressed on $CD3^+$ T-cells, $CD33^+$ myeloid cells or $CD16^+$ NK-cells.[31] Labelings containing both CD22 and CyCD79a (labelings 12 and 14 from Table 1), showed a consistently present but very small $CD22^+$/$CyCD79a^-$ pro-B-cell population (Figure 1), indicating that expression of CD22 precedes CyCD79a expression. This was illustrated by the clear presence of this population in BM of RAG deficient SCID patients (see below).

During our initial flow cytometric analyses of SCID BM samples, we detected variable percentages of non-B-lineage cells in the B-cell gate. To ensure that the B-cell gate included B-lineage cells only, we added a mix of PerCP-Cy5.5 labeled CD3, CD16, and CD33 MAb to all B-cell labelings to exclude T-cells, NK-cells, granulocytes and other myeloid cells (Table 1 and Figure 1, purification gate 1). Furthermore, we detected co-expression of CD36 and the pan-B-cell markers CD22, CyCD79a, and CD19 in SCID patients, not in healthy controls. CD36 is expressed on platelets, mature monocytes and macrophages, during stages of erythroid cell development and on some macrophage-derived dendritic cells. Therefore, we added CD36 to some essential labelings (labelings 5, 10, and 14 in Table 1, purification gate 2 in Figure 1), resulting in increased purity of the B-cell gates.

We identified the entire B-cell compartment as being $CD22^+$. In healthy children the $CyCD79a^+$ and the $CD19^+$ fractions within the $CD22^+$ B-cell compartment were very large

Table 4. Analysis of *IGH*, *IGK*, and *IGL* gene rearrangements in BMMC of RAG deficient SCID patients.

Patient	Origin of DNA	Analyzed gene	PCR products	V gene segment	Del	Junctional Region	Del	D gene segment	Del	Junctional region	Del	J gene segment	Frame	Somatic mutations	Number identical clones
RAG-SCID 1	BMMC	*IGH*	Identified					D_H2-15	-5	T	-2	J_H4	NA	NA	1
		IGK	Not identified												
		IGL	Identified	Vλ2	-4	CCT	-	-	-	-	0	Jλ3	+	Absent	1
RAG-SCID 2.1	BMMC	*IGH*	Not identified												
RAG-SCID 2.2	BMMC	*IGH*	Not identified												
RAG-SCID 3	BMMC	*IGH*	Identified	V_H3-7	0	TTGTA	-2	D_H3-22	0	-	-1	J_H3b	+	Absent	4
				V_H5-51	-1	GC	-1	D_H2-15/INV	-15	GGGCTACGGGG	-6	J_H4b	+	Absent	6
								D_H3-10	-7	G	-11	J_H2	NA	NA	4
								D_H2-21	-7	AAGAC	-4	J_H3b	NA	NA	1
								D_H4-23	-3	GACTGCGA	-11	J_H4	NA	NA	1
								D_H4-17	-1	ACTAG	-4	J_H5	NA	NA	3
		IGK	Not identified												
		IGL	Identified	Vλ3-10*01	0	-	-	-	-	-	0	Jλ3*01	+	Absent	3
RAG-SCID 4	BMMC	*IGH*	Not identified												
RAG-SCID 5	BMMC	*IGH*	Not identified												
RAG-SCID 6	BMMC	*IGH*	Not identified												

Del = deletion of germline gene segment nucleotides; NA=not applicable; INV=inverted.

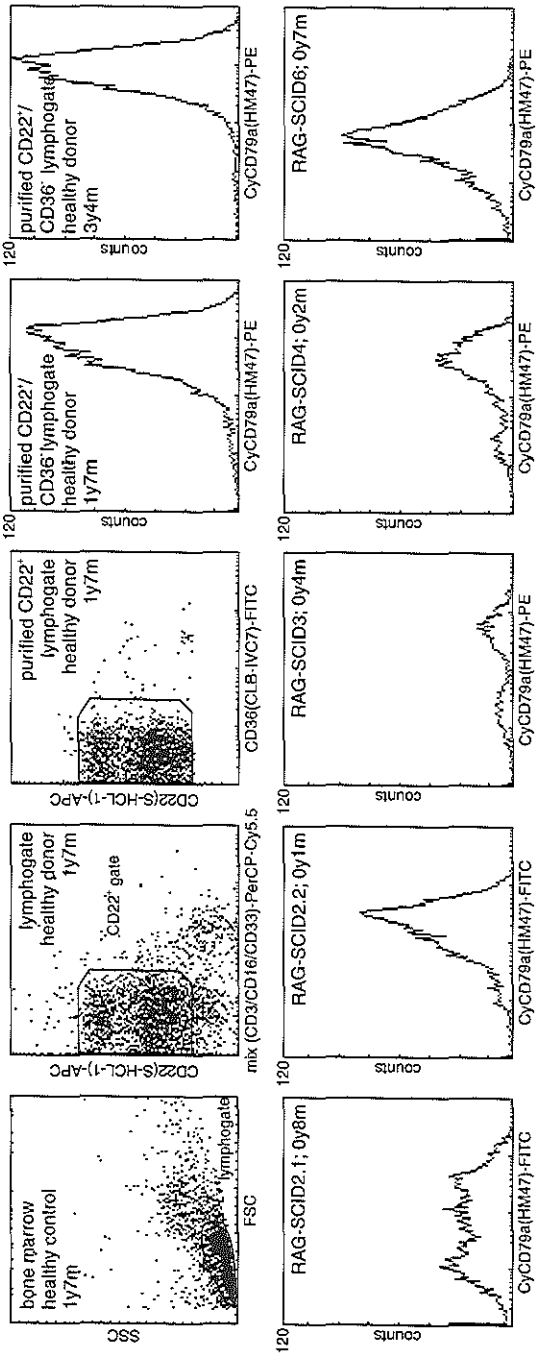

Figure 1. BM samples of RAG deficient SCID patients contain increased numbers of the most immature CD22⁺/CyCD79a⁻ pro-B-cells.
The composition of the precursor-B-cell compartment in the two healthy children was representative of that in other healthy children (n=6). The composition of the precursor-B-cell compartment was analyzed within a lymphocyte gate and a purified CD22⁺ gate. Patient SCID-6 showed an overall weak expression of the pan-B-cell markers CD22, CyCD79a, and CD19. The age at BM sampling is indicated.

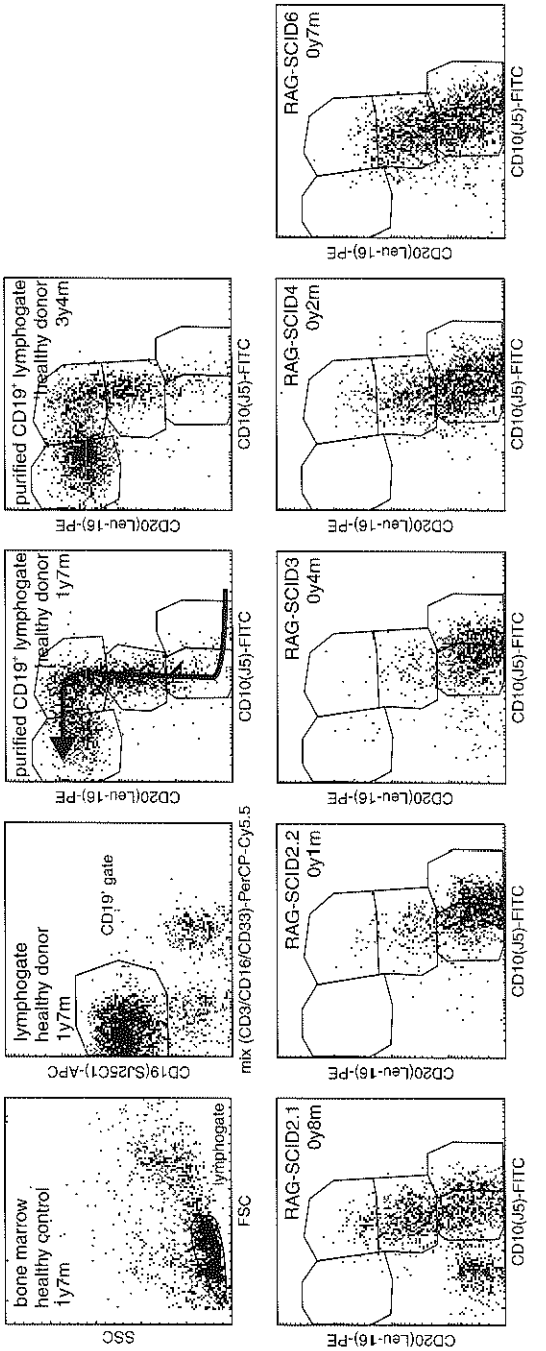

Figure 2. Flow cytometric analysis of thawed BM samples from five RAG deficient SCID patients compared to healthy children.
The composition of the precursor-B-cell compartment in the two healthy children was representative of that in other healthy children (n=6). The composition of the precursor-B-cell compartment was analyzed within a lymphocyte gate and a purified CD19+ gate. The sequential order of the B-cell differentiation stages in the healthy children is indicated with arrows. In general, RAG deficient SCID patients lacked the more mature B-cell differentiation stages with a strong relative increase in the immature B-cell differentiation stages. According to Nomura *et al.*, expression of CyVpreB preceded expression of CD19.[51] Within a CD19+ lymphocyte gate, all cells should thus be CyVpreB+ and/or CyIgμ+ (C). Therefore, we suggest that the double negative events represent apoptotic cells. The age at BM sampling is indicated.

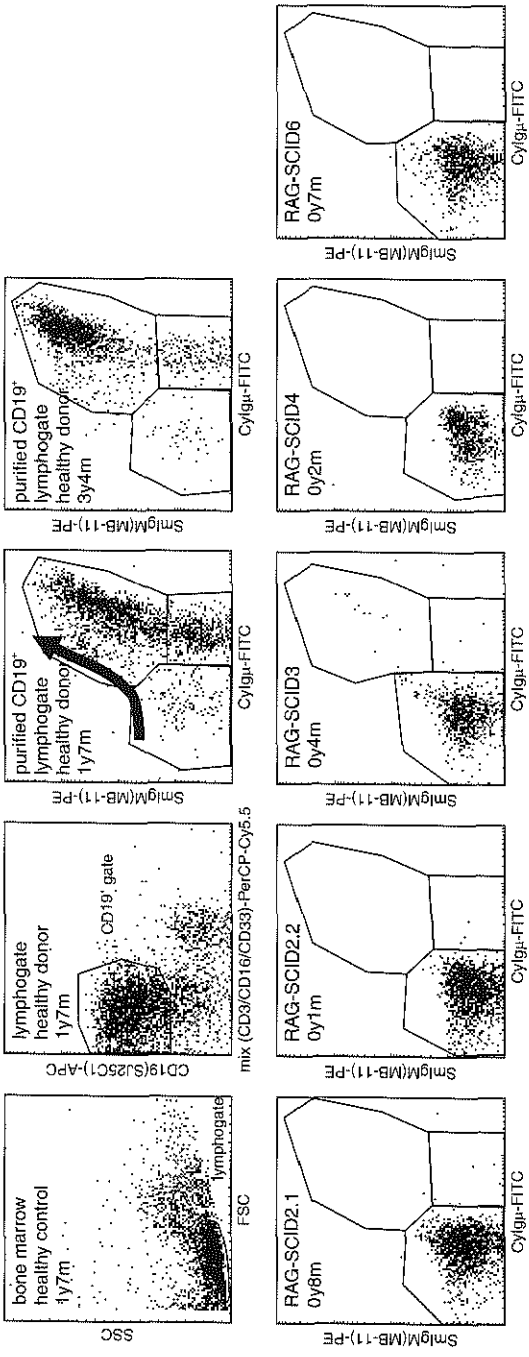

Figure 2. Continued

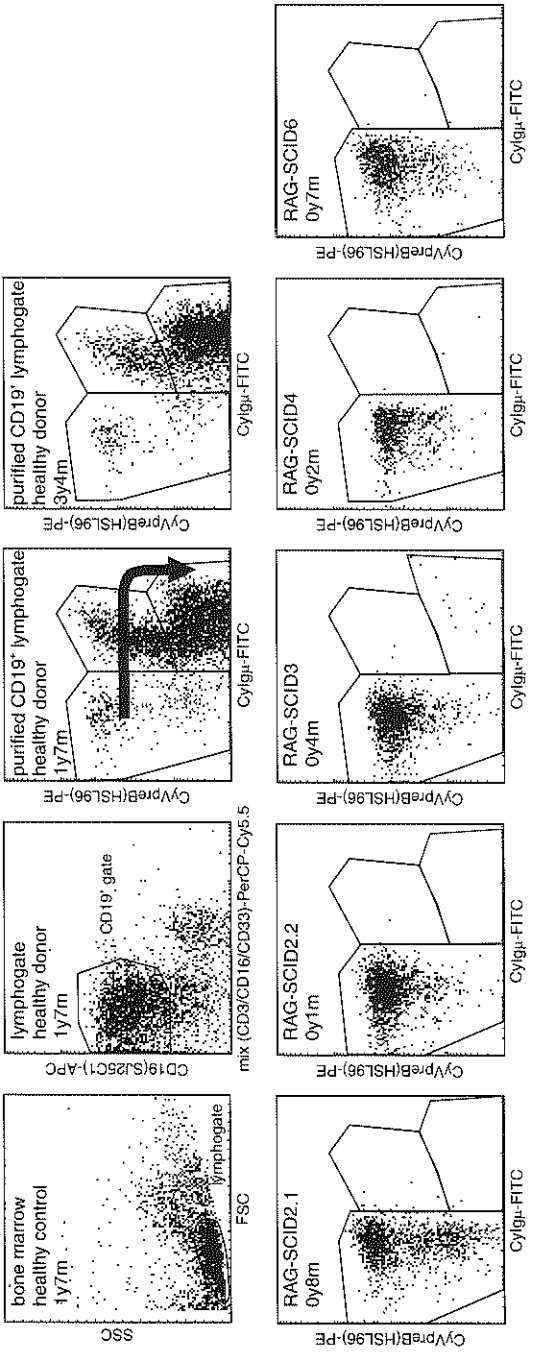

Figure 2. Continued

(labelings 5 and 14 from Table 1), i.e. 98.7% and 97.6%, respectively, implying that the $CD19^-/CD22^+$ pro-B-cell population was very small (2.4%). The percentages of the different subpopulations within the $CyCD79a^+$ or $CD19^+$ gates were recalculated into percentages of the $CD22^+$ population, by multiplying with 0.987 and 0.976, respectively. The same approach was followed for SCID patients.

B-cell subsets in BM from healthy donors

Based on detailed triple flow cytometric labelings, we previously presented the composition of the precursor-B-cell compartment in healthy children and found that its composition is stable during childhood.[31,48] As we now extended our flow cytometric protocol to 14 quadruple labelings, we have repeated our flow cytometric analyses on thawed BM samples from six of the previously studied healthy children, resulting in the recognition of nine consecutive stages. We have previously compared the composition of the precursor-B-cell compartment in fresh BM and thawed BMMC from healthy children and we could not detect major selective loss of particular precursor-B-cell subsets.[31] This information was essential for our study, because the BMMC samples in our cell bank (storage in liquid nitrogen) were the only source for studying the precursor-B-cell compartment in RAG deficient SCID patients.

Pro-B, pre-B, immature B and mature B-cells were designated according to Ghia *et al.*[49] The $CD19^-$ fraction was termed pro-B-cells, and was further subdivided on the basis of CyCD79a and TdT expression (stages 1-3). Pre-B-I cells were defined as $CD19^+$, $CD10^+$, TdT^+, $CD34^+$, $CyVpreB^+$ and $CyIg\mu^-$ and were further subdivided on the basis of the level of CD10 expression (stages 4-5). Pre-B-II cells were recognized as $CD19^+$, $CD10^+$, TdT^-, $CD34^-$, and $CyIg\mu^+$, and were further subdivided on the basis of the presence or absence of CyVpreB expression, respectively (stages 6-7). Pre-B-II-cells in stage 7, which are $CyIg\mu^+$, but $CyVpreB^-$, were also recognized by Schiff *et al.*, and probably represent non-cycling small pre-B-cells which have upregulated RAG expression, allowing rearrangement of the Ig light chain genes.[50] However, TdT expression was beyond detection during this stage.[50] Immature B-cells were defined as $CD19^+$, $CD10^+$, $SmIgM^+$, and $SmIgD^-$ (stage 8), and mature B-cells as $CD19^+$, $CD10^-$, $SmIgM^+$, and $SmIgD^+$ (stage 9).

Correction of the precursor-B-cell compartment in BM for blood contamination

The mature B-cell population in BM varied between healthy children mainly because of blood contamination with $CD10^-/SmIgM^+/SmIgD^+$ B-lymphocytes: 15% to 27% within the lymphocyte gate with a mean of 19%, and 29% to 60% within the $CD19^+$ gate with a mean of 40%. This variable degree of blood contamination was also clear from the comparable variation of $CD3^+$ T-lymphocytes within the lymphocyte gate, ranging from 13% to 35% with a mean of 20%. To correct for this variance in our comparative studies between precursor-B-cells in normal BM and in BM from SCID patients, we excluded the mature $CD10^-/SmIgM^+/SmIgD^+$ B-cell population (stage 9) from our calculations. Consequently, the percentages of stages 1 to 8 were recalculated and set at 100% (Figure 3).

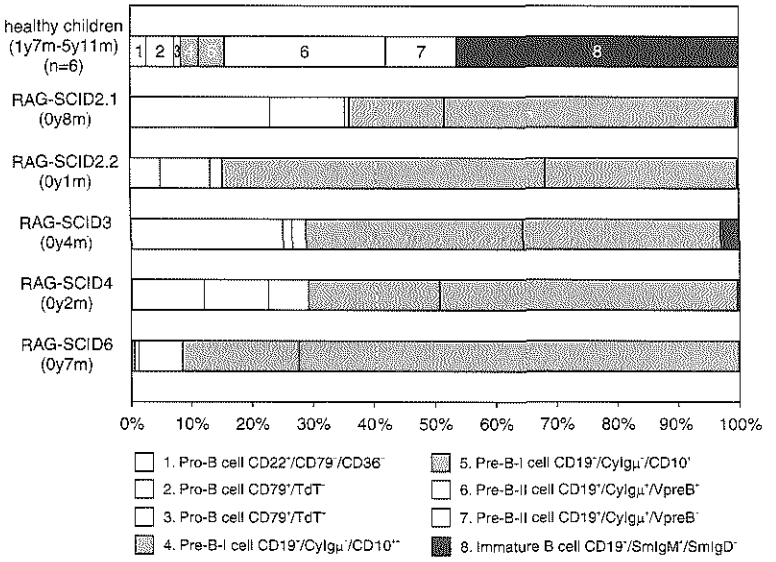

Figure 3. Composition of the precursor-B-cell compartment in RAG deficient SCID patients compared to healthy children.
The precursor-B-cell compartment was set to 100%, after exclusion of CD10$^-$/SmIgM$^+$/SmIgD$^+$ mature B-cells (see text for details). All RAG deficient SCID patients showed a similar differentiation arrest with approximately 99% of the precursor-B-cells in the pro-B and pre-B-I stages, thereby revealing an arrest at the transition from CyIgμ$^-$ pre-B-I to CyIgμ$^+$ pre-B-II-cells. Only in SCID-3 leakiness was observed with >3% Ig$^+$ B-cells.

Flow cytometric analysis of precursor-B-cells in BM of SCID patients

BM samples from seven RAG deficient SCID patients were available, but the BM samples from OS patients SCID-1 and SCID-5 could not be analyzed using flow cytometry due to the very low percentage of precursor-B-cells (<2% CD22$^+$/CD36$^-$ precursor-B-cells within the lymphocyte gate).

In BM from healthy children, approximately 15% of the precursor-B-cell compartment consisted of CyIgμ$^-$ precursor-B-cells, distributed over pro-B-cells and pre-B-I-cells with a pro-B/pre-B-I ratio of 1.5±1.0, whereas in BM from all five analyzed RAG deficient SCID patients approximately 99% (97% to 100%) of the precursor-B-cell compartment was located in these stages (Figure 3). Therefore, our results indicate that the differentiation arrest in the five SCID patients resulted in a more than six-fold relative accumulation of precursor-B-cell subpopulations located before the transition from CyIgμ$^-$ to CyIgμ$^+$ pre-B-cells. Although this differentiation arrest was virtually identical in the five evaluated SCID patients, some CyIgμ$^+$ pre-B-II-cells and SmIgM$^+$ immature B-cells could be detected in SCID-3 (Figures 2 and 3). Furthermore, the composition of the remaining precursor-B-cell compart-

ment in the five SCID patients was highly variable, even in the three patients with the same homozygous *RAG2* mutation (SCID-2.1, 2.2, and 4). The relative accumulation of CyIgμ- precursor-B-cells was lower for pro-B-cells (8% to 36%) as compared to pre-B-I-cells (63% to 91%) (Figure 3), resulting in an inverted pro-B/pre-B-I ratio of 0.3±0.2 in the SCID patients.

DISCUSSION

We have identified nine patients from seven families with B-cell deficient SCID due to RAG mutations. The disease-causing effect of these RAG mutations was shown by transfection into CHO9 cells together with the inversional recombination substrate pDVG93 in eight out of nine patients; the effect of the mutation of the ninth patient (SCID-5) was reported before (Table 5).[46] In the eight patients, we also studied the recombination activity of the mutated RAG genes by transfection into non-lymphoid φNX-WTA cells together with the basic helix-loop-helix protein E47, followed by analysis of endogenous Ig loci recombination. From five out of nine patients, BM samples could be analyzed for the immunophenotypic composition of the precursor-B-cell compartment and for their *in vivo* immunogenotype, i.e. the occurrence of Ig gene rearrangements.

The mutations in the RAG genes of SCID-2, SCID-4, SCID-6, and SCID-7 affected the core domains of the RAG proteins and were shown to abrogate recombination activity (almost) completely (Table 3). The other three RAG mutations were located outside the core domain (SCID-1, SCID-3, and SCID-5) and were all associated with partial recombination activity. The partial recombination activity was reported previously for the W453R RAG2 mutation of OS patient SCID-5, whereas the mutated RAG genes of SCID-1 and SCID-3 were shown to have partial recombination activity in our inversional recombination assay (Table 3). The N-terminal truncated RAG1 protein of SCID-1 had strongly decreased potential to direct Ig gene rearrangements (Table 3), but this OS patient contained many oligoclonal TCR gene rearrangements.[24] In line with some remaining recombination activity of the C-terminal mutated RAG2 protein in SCID-3, some SmIgM+ immature B-cells were detectable in the BM of this patient (Figures 2 and 3). Consequently, complete and in-frame *IGH* and *IGL* rearrangements were detectable in the BM precursor B-cells (Table 4). The aa substitution in the C-terminus of the RAG2 protein in SCID-3 was located outside the functionally important core domain, but this mutation might result in diminished protein stability, thereby explaining the strongly reduced recombination activity. Kirch *et al.* have shown that C-terminal truncated RAG2 proteins can initiate incomplete D_H-J_H rearrangements, but not complete V_H-J_H rearrangements.[23] We indeed detected more incomplete D_H-J_H rearrangements than complete V_H-J_H rearrangements in SCID-3 (Table 4), but this might also be caused by the relative increase of pro-B-cells and pre-B-I-cells in the BM of RAG deficient SCID patients (Figure 3).

The composition of the normal precursor-B-cell compartment in BM was assessed by flow cytometric analysis of BM samples from six healthy children using 14 quadruple label-

Table 5. Summary of *RAG* gene mutations, their recombination activity, and effects on the *in vivo* lymphoid compartment.

Gene	Mutation	Recombination activity	Diagnosis	Lymphoid compartment		
				Precursor B cells in BM		Immunogenotype
				Relative size	Differentiation arrest	
RAG1	N-terminal truncation	Strongly decreased	OS in SCID-1	<2%	Not evaluable	Oligoclonal T cells
	R249H	Normal (polymorphism)				
	R404Q	Absent	T^-B^- in SCID-7	No BM available		No Ig rearrangements
	K820R	Normal (polymorphism)				
RAG2	Q16 stop	Absent	T^-B^- in SCID-6	57%	Absolute arrest	No Ig rearrangements
	Codon 247 stop	Absent	T^-B^- in SCID-2 and 4	5-57% [1]	Absolute arrest	No Ig rearrangements
	W435R	Strongly decreased	OS in SCID-5	<2%	Not evaluable	Oligoclonal T cells
	H481P	Strongly decreased	T^-B^- in SCID-3	4%	Leakiness	*IGH/IGL* rearrangements

[1]5% within lymphocyte gate in SCID-2.1, 57% in SCID-2.2, and 47% in SCID-4

ings (Figures 1, 2 and 3). This resulted in an accurate human B-cell differentiation scheme, which resembles the classification according to Ghia *et al.*[49] Our scheme consists of nine consecutive stages, in which CD79, TdT, CD10, and Ig expression patterns (CyIgμ, CyVpreB, and SmIg) are dominant parameters. Similar analyses of the composition of the precursor-B-cell compartment in BM from five RAG deficient SCID patients showed that approximately 99% (97% to 100%) of the precursor-B-cell compartment in the SCID patients consisted of CyIgμ– precursor-B-cells (pro-B-cells and pre-B-I-cells), in contrast to approximately 15% in healthy children. The localization of the virtually complete arrest at the transition between CyIgμ– and CyIgμ+ pre-B-cells in all five RAG deficient SCID patients is in line with the essential role of the RAG proteins in the initiation of the VDJ recombination process. Absence of functional RAG proteins results in the absence of functional IGH rearrangements and thus to absence of CyIgμ and pre-BCR expression.

In contrast to the leakiness of the B-cell differentiation arrest in XLA patients,[31,51] the RAG deficient SCID patients show a rather strict B-cell differentiation arrest with respect to both immunophenotype and immunogenotype. Only in SCID-3 some minor leakiness was seen (3% Ig+ B-cells and detectable Ig gene rearrangements), which was clearly related to the low levels of recombination activity (Table 3). These data show that in RAG deficient SCID patients the immunophenotypic and immunogenotypic B-cell differentiation arrest are closely linked.

As was observed earlier, a small subpopulation of CD34+/CD20+ precursor-B-cells (mean of 1.7±1.0%) was detectable within the CD19+ gate in BM from healthy children (labeling 3 from Table 1).[31] However, in BM from most SCID patients a substantial CD34+/CD20+/CD19+ population could be identified. This atypical precursor-B-cell population varied from 9% (SCID-2.2) to 29% (SCID-6) between SCID patients, but was smaller (3%) in SCID-3 (data not shown). We observed that the CD34+/CD20+ population is also relatively large in the precursor-B-cell compartment of XLA patients (11% to 20%), suggesting that downregulation of CD34 is normally mediated via pre-BCR signaling.[31]

Although it has been shown that OS is caused by mutated RAG genes with partial recombination activity,[26] it is not understood why these patients present with oligoclonal T-lymphocytes, but absence of B-lymphocytes. The presence of high serum IgE levels suggests that Ig producing plasma cells might be present in patients with OS. However, in BM samples from two patients with OS (SCID-1 and SCID-5) and oligoclonal T-cells, in whom MFT was excluded, flow cytometric analysis was impossible as the percentage CD22+/CD36– precursor-B-cells was <2% of lymphocytes, revealing a severe suppression of the B-cell compartment.

In conclusion, we observed a complete B-cell differentiation arrest at the pre-BCR checkpoint in the BM of RAG deficient SCID patients with absence of recombination activity. One patient (SCID-3) showed some SmIgM+ immature B-cells (minor leakiness of the differentiation arrest), which corresponded with partial recombination activity of the mutated RAG2 protein and the presence of in-frame Ig gene rearrangements. The partial recombination activity in two other patients was associated with OS and oligoclonal T-cells.

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Chapter 13

RADIOSENSITIVE SCID PATIENTS WITH ARTEMIS GENE MUTATIONS SHOW A COMPLETE ARREST AT THE PRE-BCR CHECKPOINT IN BONE MARROW

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Submitted

SUMMARY

Severe combined immunodeficiency disease (SCID) can be immunologically classified by the absence or presence of T, B, and NK-cells. About 30% of patients with $T^-B^-NK^+$ SCID carry mutations in the recombination activating genes (*RAG*). Some $T^-B^-NK^+$ SCID patients without mutations in the *RAG* genes are sensitive to ionizing radiation and several of these radiosensitive (RS)-SCID patients were recently shown to have mutations in the *Artemis* gene, implying a role for Artemis in DNA double strand break (dsb) repair.

We identified five RS-SCID patients without *RAG* gene mutations and with normal expression of the DNA dsb repair proteins Ku70, DNA-PK_{cs}, Ligase IV, Mre11, and/or Ku80, XRCC4, NBS1, and ATM. Four of these patients appeared to have *Artemis* gene mutations. One patient had a large genomic deletion, but the other three patients carried simple missense mutations in the SNM1 homology domain of the Artemis protein, resulting in amino acid substitutions in regions which are highly conserved between yeast, mouse and man. Extrachromosomal V(D)J recombination assays showed normal and precise signal joint formation, but inefficient coding joint formation in fibroblasts of all four patients, confirming a role for Artemis in V(D)J recombination. Immunogenotyping and immunophenotyping of bone marrow samples of two RS-SCID patients showed the absence of complete V_H-J_H gene rearrangements and consequently a complete B-cell differentiation arrest at the pre-BCR checkpoint, i.e. at the transition from $CyIg\mu^-$ pre-B-I-cells to $CyIg\mu^+$ pre-B-II-cells. The completeness of this arrest illustrates the importance of Artemis at this stage of lymphoid differentiation.

INTRODUCTION

Severe combined immunodeficiency disease (SCID) is clinically characterized by opportunistic infections, protracted diarrhea, and failure to thrive.[1] Patients generally die within the first year of life, unless treated by bone marrow (BM) transplantation. Other treatment options include enzyme substitution in case of adenosine deaminase deficient SCID, and gene therapy in case of common gamma chain deficient X-linked SCID.[2]

Although SCID consists of a heterogeneous group of diseases, it is immunologically characterized by the absence or dysfunctioning of T lymphocytes. SCID can be subdivided based on the additional presence or absence of B lymphocytes and NK cells in peripheral blood (PB). A number of T–B–NK+ SCID patients were shown to have mutations in the recombination activating genes (RAG1 and RAG2).[3] However, not all patients with T–B–NK+ SCID have mutations in the RAG genes. Subsequently, it was shown that fibroblasts of several T–B–NK+ SCID patients without RAG gene mutations were sensitive to ionizing radiation and thus likely to have a defect in DNA double strand break (dsb) repair.[4]

During the process of immunoglobulin (Ig) and T-cell receptor (TCR) gene recombi-

nation in differentiating lymphocytes, the RAG proteins introduce DNA dsb's, which are repaired via non homologous end joining (NHEJ).[5] NHEJ requires active DNA dependent protein kinase (DNA-PK), which consists of Ku70, Ku80, and the catalytic subunit (DNA-PKcs), and it requires DNA ligase IV, and XRCC4. The DNA-PK protein complex functions as a DNA damage sensor, with Ku70 and Ku80 forming a heterodimer that binds to DNA ends, while DNA-PKcs has Serine and Threonine protein kinase activity.[6] Although the exact function of the DNA-PK complex in DNA end joining is not yet clear, the Ku heterodimer binds to the broken DNA ends and probably brings them together. In the final phase, the two broken DNA ends are ligated by the DNA ligase IV / XRCC4 complex.[7,8] However, it was shown that a number of radiosensitive (RS) T–B–NK+ SCID patients without mutations in the RAG genes did not suffer from mutations in any of these five DNA dsb repair components.[4]

Recently, a new gene involved in the pathogenesis of RS-SCID was identified and named Artemis.[9,10] Two observations were reported that might point towards the presently unknown function of the Artemis protein: (1) some of the patients carried two complete null alleles of Artemis caused by a homozygous deletion of the 5' coding part of the gene, but this condition was apparently not embryonically lethal, as seen in XRCC4 and DNA ligase IV gene knockout mice; (2) patients with mutations in Artemis showed a defect in coding joint formation, but not in signal joint formation as seen in DNA-PKcs gene knockout mice. Based on these observations, the authors suggested that the Artemis protein might not be involved in ligation of broken DNA ends, but in opening of the hairpin at the coding ends through its possible hydrolase activity.[10]

We studied five RS-SCID patients without RAG gene mutations and found that four of them (from three families) had mutations in the Artemis gene. Immunophenotyping and immunogenotyping of BM samples from two RS-SCID patients with Artemis gene mutations provided additional information about the role of Artemis during precursor B-cell differentiation.

MATERIALS AND METHODS

Cell samples

We received PB and/or BM samples and/or fibroblasts from 32 $T^-B^-NK^+$ SCID patients. These patients were analyzed for the presence of mutations in the *RAG* genes.[11] Fibroblasts from 13 $T^-B^-NK^+$ SCID patients without mutations in the *RAG* genes were analyzed for sensitivity to ionizing radiation.

BM samples from $T^-B^-NK^+$ SCID patients were subjected to Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. The recovered mononuclear cells (MC) were frozen and stored in liquid nitrogen and thawed later for flow cytometric immunophenotyping studies. Granulocytes were used for DNA extraction.

All cell samples were obtained according to the informed consent guidelines of the

Medical Ethics Committees of the Erasmus University Rotterdam / University Hospital Rotterdam and the Leiden University Medical Center.

Sensitivity of fibroblasts to ionizing radiation

Primary skin fibroblasts in exponential growth were trypsinized and 500-2,000 cells (5,000-80,000 cells for the highest doses) were seeded into 10 cm plastic dishes (two dishes per dose, three for non-irradiated control) and irradiated at room temperature at a dose of approximately 2.7 Gy/min (200 kV, 4.0 mA, 0.78 mm Al). After 12-14 days, the cells were rinsed with 0.9% NaCl and stained with 0.25% methylene blue for survival assessment. At least three independent survival experiments were performed for each sample.

mRNA and DNA isolation, and cDNA reaction

DNA was extracted from granulocytes or fibroblasts using the QIAamp Blood kit (Qiagen, Chatsworth, CA, USA).[12] Total RNA was isolated from BMMC or fibroblasts using the GenElute Mammalian RNA kit (Sigma-Aldrich, St. Louis, MO). cDNA was prepared from mRNA as described before, using random hexamers and Superscript reverse transcriptase (Life Technologies, Paisley, UK).[13]

PCR amplification and analysis of Ig gene rearrangements

PCR was performed as described previously.[13] In each 100 µl PCR reaction 0.1-1 µg (c)DNA, 10-12.5 pmol of 5' and 3' oligonucleotides and 1 U Ampli*Taq* gold polymerase (Applied Biosystems, Foster City, CA, USA) were used. Oligonucleotides for amplification of the *Artemis* and Ig heavy chain (*IGH*) gene rearrangements (V_H-J_H and D_H-J_H) were published before.[10,14-16] PCR conditions were 7 min at 95°C, followed by 45 sec at 94°C, 90 sec at 55-60°C, 2 min at 72°C for 40 cycles, followed by a final extension step (7 min at 72°C). DNA from BMMC was analyzed for rearrangements of D_H1, D_H2, D_H3, D_H4, D_H5, D_H6, D_H7, $V_H1/7$, V_H2, V_H3, V_H4, V_H5, and V_H6 to J_H. The *IGH* PCR products were cloned in the pGEM-T easy vector (Promega, Madison, WI) as described before and subsequently sequenced.[11]

Fluorescent sequencing reaction and analysis

PCR products of *Artemis* were purified using QIAquick PCR purification kit (Qiagen). Cloned *IGH* gene rearrangements were isolated via GenElute Plasmid MiniPrep Kit (Sigma-Aldrich). 2-9 µl template was sequenced with 5 µl BigDye terminator mix (Applied Biosystems), using 3.3-6.6 pmol sequencing primers. All sequencing was performed as described before,[15] and run on an ABI Prism 377 fluorescent sequencer (Applied Biosystems).

Western blot analysis of proteins involved in DNA dsb repair

Protein samples from fibroblasts were separated on a 11% polyacrylamide gel and analyzed by Western blotting. The expression of Mre11 (rabbit polyclonal Ab nr. 2244),[17]

XRCC4 (rabbit polyclonal Ab NIH14), Ku70 (goat polyclonal Ab C19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Ku80/86 (goat polyclonal Ab C20, Santa Cruz Biotechnology), DNA-PK_{cs} (rabbit polyclonal Ab nr. 2129), Ligase IV (goat polyclonal Ab T20, Santa Cruz Biotechnology), ATM (gift of Dr. S. Jackson), and NBS1 (p95NBS1, Calbiochem, San Diego, CA, USA) proteins was analyzed.

Analysis of NHEJ via transfection of linearized DNA constructs

Linearized DNA constructs were transfected into fibroblasts of RS-SCID patients and fibroblasts of a patient with a mutation in *DNA ligase IV* (180BR) as described before.[18] Newly formed junctions were PCR amplified and the relative use of a particular microhomology was assayed by digestion with the restriction enzyme *Bst*XI.[18]

Extrachromosomal VDJ recombination assay

2 μg of *RAG1* and 2 μg of *RAG2* expression vectors together with 1 μg of the recombination substrate (pDVG93) containing two recombination signal sequence (RSS) elements were transfected into fibroblasts using SuperFect Transfection Reagent (Qiagen) as described before.[11] Transfected cells were cultured for 2 days at 37°C and 5% CO_2 before harvesting. Upon V(D)J recombination, the sequence between the RSS elements is inverted, after which coding and signal joint formation can be detected by PCR. DNA recovered from these transfection experiments was diluted and used as template for PCR. The PCR products were detected by blotting onto a nylon membrane (Schleicher & Schuell) and hybridization with the ^{32}P labeled oligonucleotide FM23 and visualized by phosphor imaging.[11]

Flow cytometric analysis of BM samples from RS-SCID patients with *Artemis* gene mutations

Fifty μl aliquots of thawed BMMC (10 x 10^6 cells/ml) were incubated for 10 min. at room temperature with combinations of optimally titrated MAb: 50 μl fluorescein isothiocyanate (FITC) conjugated MAb, 50 μl phyco-erythrin (PE) conjugated MAb, 50 μl peridinin chlorophyll protein (PerCP) cyanin (CY) 5.5 conjugated MAb, and 50 μl allophycocyanin (APC) conjugated MAb were used to detect membrane bound antigens. After incubation, the cells were washed and further processed depending on the type of quadruple labeling.[19]

Quadruple labelings for membrane bound antigens were directly analyzed by flow cytometry using FACSCalibur (Becton Dickinson, San Jose, CA, USA). For quadruple labelings involving intracellular staining of cytoplasmic (Cy) CD79a, CyIgμ, CyVpreB,[20] and intranuclear staining of TdT, we first performed the membrane labelings, followed by permeabilization of the BM cells using IntraPrep Permeabilization Reagent (Immunotech, Marseille, France), and subsequent intracellular staining.[21,22]

RESULTS

Patient characteristics, Western blot analysis, and disease-causing *Artemis* gene mutations

We analyzed material from 32 T⁻B⁻NK⁺ SCID patients for the presence of *RAG* gene mutations, both at the genomic and at the transcriptional level. We could not detect mutations in the *RAG* genes in 23 patients (72%). From 13 T⁻B⁻NK⁺ SCID patients without *RAG* mutations, fibroblasts were available and analyzed for sensitivity to ionizing radiation in a clonogenic survival assay.

Fibroblasts from five T⁻B⁻NK⁺ SCID patients from four families showed a 2-3 fold increased sensitivity towards X-rays, in comparison to wild type primary fibroblasts (FN1). The degree of the observed X-ray sensitivity was similar to fibroblasts derived from a patient with Nijmegen breakage syndrome (NBS1-LBI) (Figure 1). Western blot analysis of proteins Ku70, DNA-PK_{cs}, Ligase IV, Mre11, and/or Ku80, XRCC4, NBS1, or ATM derived from fibroblasts from the five RS-SCID patients showed normal protein expression. Finally, *Artemis* gene mutations were detected in four RS-SCID patients from three families. The clinical characteristics of the four patients are summarized in Table 1.

Patient Artemis-1 showed a homozygous deletion of exons 10, 11, and 12 of the *Artemis* gene. At the mRNA level, exon 9 was coupled to exon 13, resulting in a frameshift and premature stop at codon 269 in the SNM1 homology domain (numbering according to Moshous *et al.*).[10]

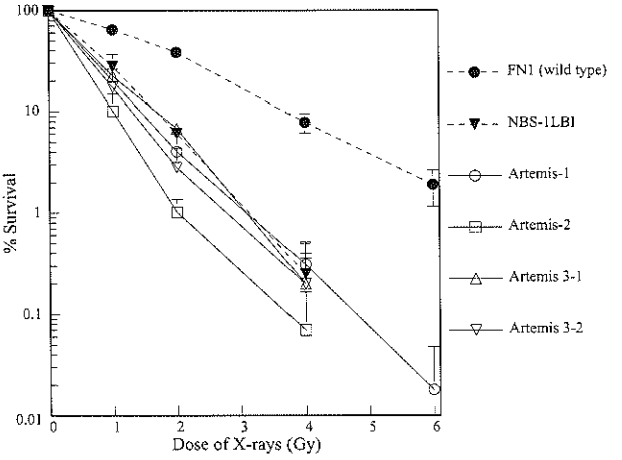

Figure 1. Clonogenic survival assay of fibroblasts after ionizing radiation.
Fibroblasts from five T⁻B⁻NK⁺ SCID patients from four families without *RAG* mutations were radiosensitive. Data from the four patients with *Artemis* gene mutations are shown. NBS = Nijmegen breakage syndrome. Each survival curve represents the mean of at least three independent experiments. Error bars represent standard errors of the mean.

Table 1. Clinical characteristics of the four RS-SCID patients with *Artemis* gene mutations.

Patient	Sex	Age at diagnosis	Diagnosis	Consanguinity of parents	Infections at diagnosis	Treatment	Outcome
Artemis-1	F	5 months	$T^-B^-NK^+$	Yes	PCP	BMT	Alive
Artemis-2	F	5 months	$T^{low}B^-NK^+$ [a]	Yes	PCP, CMV	BMT	No B-cell take, receives IVIG
Artemis 3-1	M	4 months	$T^-B^-NK^+$	No	Persistent VZV infection	BMT	Died from disseminated infections
Artemis 3-2	M	1 day	$T^-B^-NK^+$	No	None	BMT	Died from multi-organ failure

[a] Maternal T lymphocytes were shown to be present in her PB, but did not cause graft-versus-host-disease due to identical HLA. PCP=*Pneumocystis carinii* pneumonia; CMV=Cytomegalo virus; VZV=Varicella zoster virus; BMT=BM transplantation; IVIG=intravenous Ig substitution.

Patient Artemis-2 showed a homozygous G to T mutation at position 47 in exon 5 of the *Artemis* gene. This resulted in mutation of a Glycine to Valine residue at position 111 in the SNM1 homology domain of the Artemis protein. We did not identify this point mutation in 18 healthy controls. Furthermore, Glycine at position 111 is highly conserved between yeast PSO2, mouse SNM1, human SNM1A, and human SNM1B proteins and therefore likely to represent an essential amino acid (aa).[23]

Patients Artemis 3-1 and 3-2 showed a homozygous G to A mutation at position 42 in exon 6 of the *Artemis* gene. This resulted in mutation of a Glycine to Glutamic Acid residue at position 128 in the SNM1 homology domain of the Artemis protein. We did not identify this point mutation in 14 healthy controls. Furthermore, Glycine at position 128 is highly conserved between yeast PSO2, mouse SNM1, human SNM1A, and human SNM1B proteins and therefore likely to represent an essential aa.[23]

Additional non disease-causing mutations in the *Artemis* gene

The entire *Artemis* gene was sequenced in all five RS-SCID patients to exclude the presence of additional mutations. We identified a total of four additional alterations compared to the published sequence. Two base changes were present in all five RS-SCID patients analyzed, and were therefore likely to represent polymorphisms or sequencing errors in the published sequence. The first alteration concerned a G to T change at position 522 in exon 14 of the *Artemis* gene. This alteration resulted in a change from a Valine to Leucine residue at position 553 outside the SNM1 homology domain of the Artemis protein. The second alteration concerned a C to T change at position 653 in exon 14 of the *Artemis* gene, which did not result in an aa substitution.

We identified another silent alteration at position 106 in exon 8 of the *Artemis* gene (a T to C change) in patients Artemis 3-1 and 3-2. Finally, patient Artemis-1 carried a homozygous A to G change at position 50 in exon 9 of the *Artemis* gene. This alteration resulted in a change from the basic aa Histidine to the basic aa Arginine at position 236 in the SNM1 homology domain. We do not know whether this alteration represents a polymorphism or an additional disease-causing mutation.

DSB repair

All published NHEJ yeast mutants showed a shift from direct joining to microhomology.[24,25] Therefore, we transfected linearized DNA constructs in fibroblasts of all four patients with *Artemis* gene mutations to analyze the use of direct joining and microhomology pathways. However, all patients showed equal usage of direct joining and microhomology pathways (Figure 2).

V(D)J recombination also involves DSB repair by NHEJ. We analyzed coding and signal joint formation in fibroblasts of all four patients with *Artemis* gene mutations. In all cases we found normal levels of precise signal joint formation, but inefficient coding joint formation (data not shown).

Ig gene rearrangements in BMMC

In order to study the capacity of mutated Artemis proteins to perform DNA dsb repair during human precursor B-cell differentiation *in vivo*, we isolated DNA from BMMC and investigated the occurrence of incomplete D_H-J_H and complete V_H-J_H gene rearrangements. BM samples were available from patients Artemis-1 and Artemis-2. In both patients, incom-

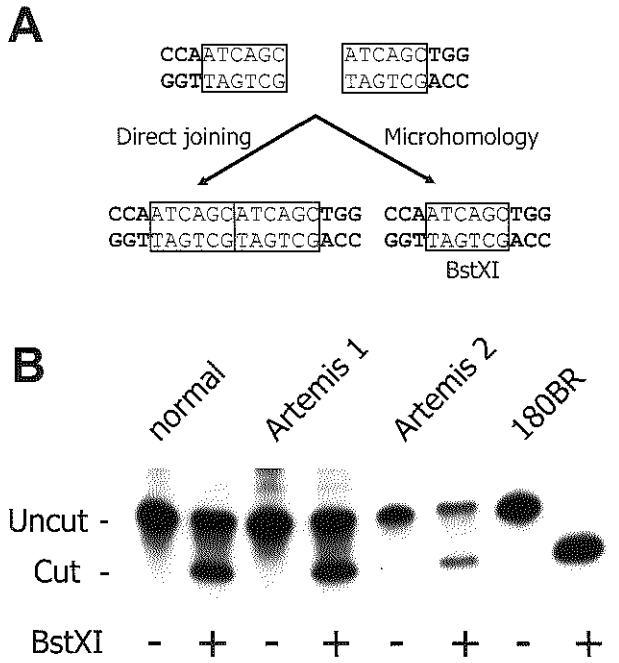

Figure 2. Fibroblasts of RS-SCID patients with mutations in the *Artemis* gene show equal usage of direct joining and microhomology when rejoining linearized DNA constructs.
Linearized DNA constructs can be rejoined via direct joining or via microhomology. Joining via microhomology results in the generation of a *Bst*XI restriction site. Human fibroblasts with a mutation in *DNA ligase IV* (180BR) show complete absence of direct joining and full usage of the microhomology pathway.

plete D_H-J_H gene rearrangements could be amplified by PCR. However, after cloning these PCR products, we could only identify a single aberrant D_H-J_H gene rearrangement in patient Artemis-1, involving the J_H4b gene segment coupled to the intron between the D_H6-6 and D_H1-7 gene segments. Complete V_H-J_H gene rearrangements could not be detected in both patients.

Flow cytometric analysis of precursor-B-cells in BM of RS-SCID patients with Artemis gene mutations

BM samples from two RS-SCID patients with *Artemis* gene mutations (Artemis-1 and -2) were available and analyzed as described before.[19] The percentage of B cells within the lymphocyte gate differed between the two RS-SCID patients with *Artemis* gene mutations (Table 2).

In BM from healthy children, approximately 15% of the precursor B-cell compartment consisted of CyIgμ$^-$ precursor B-cells, distributed over pro-B cells and pre-B-I cells with a pro-B/pre-B-I ratio of 1.3±0.8, whereas in BM from both RS-SCID patients with *Artemis* gene mutations 100% of the precursor B-cell compartment was located in the pro-B and pre-B-I cell stages (Figures 3 and 4). Therefore, our results indicate that the differentiation arrest in the two RS-SCID patients with *Artemis* gene mutations resulted in a more than six-fold relative accumulation of precursor B-cell subpopulations located before the transition from CyIgμ$^-$ pre-B-I cells to CyIgμ$^+$ pre-B-II cells, with an inverted pro-B/pre-B-I ratio of 0.6±0.7 in the RS-SCID patients with *Artemis* gene mutations.

DISCUSSION

T$^-$B$^-$NK$^+$ SCID can be caused by mutations in the *RAG* genes.[3] Fibroblasts from T$^-$B$^-$NK$^+$ SCID patients without mutations in the *RAG* genes were shown to be sensitive to ionizing radiation, but no mutations in DNA-PK, Ligase IV, or XRCC4 were detected.[4] Subsequently, it was shown that these RS-SCID patients suffered from mutations in the *Artemis* gene.[10] In accordance with the suggestion of Moshous *et al.*, that the *Artemis* gene might represent a hot spot for gene deletion, we identified a large genomic deletion in patient Artemis-1. Although none of the *Artemis* mutations reported so far were simple missense mutations, the RS-SCID phenotype in patients Artemis-2, 3-1, and 3-2 was most probably

Table 2. Percentage B cells within the BM lymphocyte gate.

	CD22$^+$ B cells (%)		CD79$^+$ B cells (%)	CD19$^+$ B cells (%)
	Precursor B cells[a]	Mature B cells		
Healthy children <6y (n=6)	31±10	19±5	53±10	50±10
Artemis-1	3	0.004	1	1
Artemis-2	23	0.006	17	16

[a] The percentage of precursor B cells was calculated by subtracting the percentage of mature SmIgM$^+$/SmIgD$^+$ B cells from the total percentage of CD22$^+$ B cells.

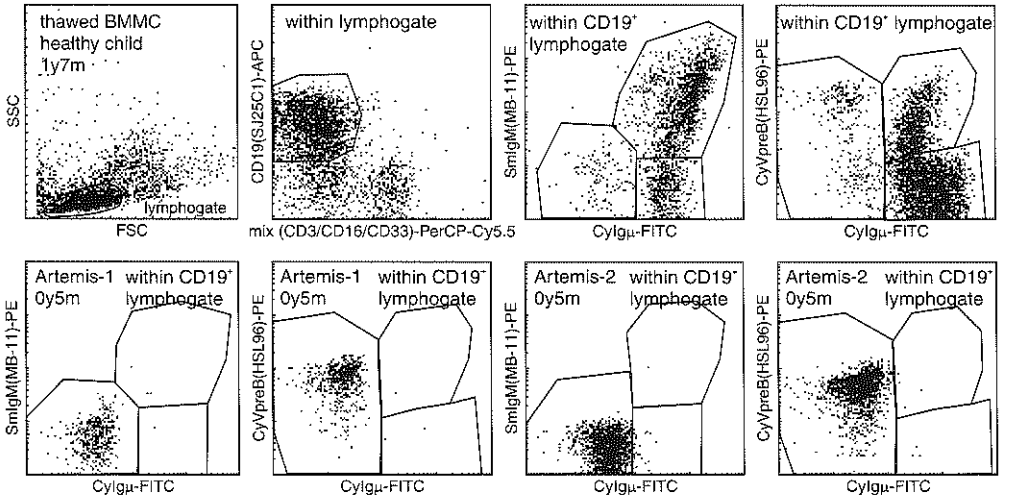

Figure 3. Flow cytometric analysis of BMMC from two RS-SCID patients with *Artemis* gene mutations. Both patients showed a complete arrest at the transition from CyIgμ⁻ pre-B-I cells to CyIgμ⁺ pre-B-II cells. Although no CyIgμ expression was detected, virtually all CD19⁺ precursor B cells were positive for CyVpreB. This is fully in line with the expression of CyVpreB in early precursor B cells.

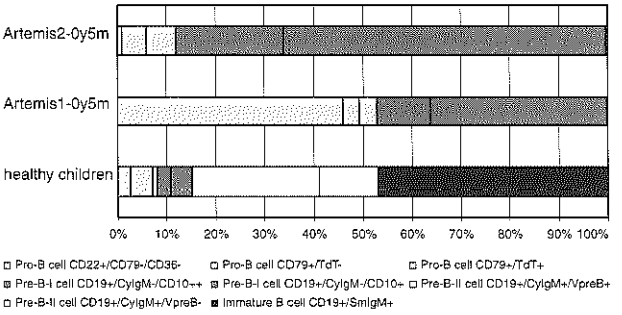

Figure 4. Composition of the precursor B-cell compartment in the two studied RS-SCID patients with *Artemis* gene mutations.
Although the composition of the precursor B-cell compartment was different in the two RS-SCID patients with *Artemis* gene mutations, a complete arrest was observed at the transition from pre-B-I cells to pre-B-II cells.

caused by aa substitutions in the SNM1 homology domain of the Artemis protein, because the involved aa sequences appeared to be highly conserved between yeast, mouse, and man. Furthermore, signal joint formation was preserved in these patients, while coding joint formation was very inefficient.

Defective direct joining of linearized DNA constructs is highly diagnostic of abnormal NHEJ in yeast.[24,25] However, our four RS-SCID patients with mutations in the *Artemis*

gene showed normal direct joining (Figure 2). This suggests that the Artemis protein functions in a different step in the end joining process. The fifth patient had no mutations in the Artemis gene, suggesting that mutations in other genes can also account for the RS-SCID phenotype. Until now, five RS patients with mutations in *DNA ligase IV* have been described. One patient suffered from acute lymphoblastic leukemia, but showed largely normal V(D)J recombination and normal development of the immune system.[26,27] The clinical phenotype of the other four RS patients closely resembled the DNA damage response disorder NBS.[28]

We previously described detection of *IGH* gene rearrangements in human BMMC as a parameter for *in vivo* RAG activity.[11] We now used detection of *IGH* gene rearrangements in BMMC of patients Artemis-1 and -2 as a parameter for residual activity of the Artemis protein. In patient Artemis-1, one aberrant incomplete D_H-J_H gene rearrangement was detectable, in which the J_H gene segment was coupled to the intron between two D_H gene segments, but we could not detect complete V_H-J_H gene rearrangements. The large genomic deletion in patient Artemis-1 resulted in a truncated Artemis protein with absence of part of the SNM1 homology domain, which makes the presence of residual Artemis protein activity highly unlikely. Although patient Artemis-2 contained relatively high frequencies of precursor-B-cells (23% of BM lymphocytes, Table 2), we could not detect *IGH* gene rearrangements.

Flow cytometric evaluation of the BM precursor B-cell compartment in patients Artemis-1 and -2 showed a complete arrest at the transition from CyIgμ$^-$ pre-B-I cells to CyIgμ$^+$ pre-B-II cells. We have previously shown that in primary immunodeficiency diseases with a comparable arrest in precursor B-cell differentiation, the pro-B/pre-B-I ratio is inverted.[19] This pro-B/pre-B-I ratio differs significantly between healthy controls and XLA patients,[19] and between healthy controls and RAG deficient SCID patients (Table 3). Also in the two RS-SCID patients with *Artemis* gene mutations, the pro-B/pre-B-I ratio was decreased, caused by the relative accumulation of pre-B-I cells before the differentiation arrest. The completeness of this B-cell differentiation arrest illustrates the importance of Artemis at this stage of lymphoid differentiation.

Table 3. Pro-B / Pre-B-I ratio's in healthy children and patients suffering from an arrest in precursor B-cell differentiation.

BM samples	Pro-B / Pre-B-I ratio
Healthy children (n=22) [a]	1.27 ± 0.82]
XLA patients (n=12) [a]	0.51 ± 0.33]
Thawed BMMC RAG deficient SCID patients (n=6)	0.29 ± 0.21
Thawed BMMC Artemis deficient SCID patients (n=2)	0.63 ± 0.70

Mann-Whitney U test for unrelated groups with unequal standard deviations

[a] no significant difference between lysed whole (LW) BM and thawed BMMC.

] significant difference between healthy and immunodeficient children ($p<0.01$).

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Chapter 14

INTRODUCTION PHAGOCYTE DEFECTS

INTRODUCTION PHAGOCYTE DEFECTS

Enhanced susceptibility to mycobacterial infections can be caused by mutations in the genes encoding the interferon (IFN)-γ receptor (R) 1 or 2 chains,[1-3] the β1 chain of the interleukin (IL)-12R,[4,5] IL-12p40,[6] or signal transducer and activator of transcription (STAT)-1.[7] In Chapter 15, we discuss two patients suffering from mycobacterial infections with mutations in the *IFNGR1* gene.

Mutations in the *IFNGR1* gene can cause either complete or partial signaling defects, resulting in two distinct phenotypes.[8-10] *Complete signaling defects* can be subdivided into two groups, distinguishable by the absence or the presence of IFN-γR1 protein expression on the surface membrane (Sm). In cases with SmIFN-γR1 expression, the causative defect is in the IFN-γ binding capacity of the IFN-γR.[11] Both subgroups show an autosomal recessive (AR) inheritance.

Partial signaling defects caused by mutations in the *IFNGR1* gene may inherit either as an AR trait, in which the mutations generally lead to partially perturbed IFN-γR signaling,[12] or as an autosomal dominant (AD) trait, in which the mutations lead to an intracellularly truncated form of the IFN-γR1 chain, which still can bind IFN-γ, but lacks intracellular signal-transducing and recycling domains, and fails to be internalized from the Sm.[13] Thus, the AD form displays a dominant negative, inhibitory phenotype.

In general, patients with a complete signaling defect have a poor prognosis and often die without bone marrow transplantation, while patients with a partial signaling defect generally have a better prognosis.

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Chapter 15

COMPLETE DEFECTS IN INTERFERON GAMMA RECEPTOR DEPENDENT SIGNALING ARE ASSOCIATED WITH DIFFERENT CLINICAL PHENOTYPES

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Submitted

SUMMARY

Unusual susceptibility to mycobacterial infections can be caused by deleterious mutations in genes that encode the interferon-γ receptor (IFN-γR) 1 or 2 chains, the interleukin (IL)-12Rβ1 chain, the IL-12p40 chain, or the signal transducer and activator of transcription (STAT)-1. Such mutations hamper the activation of macrophages by a type 1 immune response and result in enhanced survival of intracellular pathogens.

We here report two patients with unusual mycobacterial infections, whom were both diagnosed with homozygous deleterious *IFNGR1* mutations. Patient 1 became ill after BCG vaccination at the age of nine months and died at the age of 18 months. She carried a homozygous C71Y mutation in the extracellular part of the IFN-γR1 protein, resulting in the lack of surface membrane (Sm) IFN-γR1 protein expression and absence of IFN-γ dependent signaling.

Patient 2 became ill at the age of three years, is still alive at 19 years of age, and has suffered from five subsequent infection episodes with *Mycobacterium gordonae*, *M. peregrinum*, *M. mageritense*, *M. szulgai*, and *M. mageritense*. A homozygous splice site mutation in intron 3 was identified, resulting in the deletion of exon 3 at the mRNA level and consequently a truncated IFN-γR1 protein with absence of the transmembrane domain. SmIFN-γR1 protein expression and IFN-γ dependent signaling were completely absent. Despite the complete signaling defect, patient 2 showed a relatively mild clinical phenotype. These results indicate that the outcome of mycobacterial infections in patients with complete IFN-γR1 deficiency is variable, ranging from rapid fatality to survival in adulthood.

INTRODUCTION

The interferon-γ receptor (IFN-γR) consists of two chains, the ligand-binding chain (IFN-γR1), and an accessory chain (IFN-γR2), which is required for signal transduction. The IFN-γR is expressed on most nucleated cells. Upon binding of IFN-γ, two IFN-γR1 chains will dimerize and subsequently associate with two IFN-γR2 chains to form a tetrameric complex. The intracellular domains of IFN-γR1 and 2 are constitutively bound to Janus kinases (JAK)-1 and -2, respectively, that upon activation will phosphorylate a Tyrosine at amino acid (aa) position 440 (Y440) in the intracellular domain of IFN-γR1. This phosphorylation generates a docking site for signal transducer and activator of transcription (STAT) 1α, which in turn becomes phosphorylated. Phosphorylated STAT1α homodimers will translocate to the nucleus and bind to specific sequences in the promoter region of early IFN-γ inducible genes.[1-4] Furthermore, IFN-γ can augment the IL-12 production by macrophages, which will stimulate T-helper 1 (Th1) and NK cells to produce IFN-γ.[4]

Intracellular signaling from the IFN-γR is required for killing of intracellular pathogens, such as mycobacteria, by macrophages.[5, 6] Mutations in the genes encoding the

IFN-γR1 or 2 chains,[7-9] the β1 chain of the IL-12R,[10,11] IL-12p40,[12] or STAT-1[13] have been shown to result in enhanced susceptibility to mycobacterial infections.

Mutations in the *IFNGR1* gene can cause either complete or partial signaling defects, resulting in two distinct phenotypes.[14-16] First, patients suffering from a complete signaling defect in general present with diffuse and poorly differentiated, lepromatoid-like granulomas with many bacilli, have a poor prognosis, and can often only be treated by bone marrow transplantation (BMT). Complete signaling defects can be divided into two subgroups, distinguishable by the absence or the presence of IFN-γR1 protein expression on the surface membrane (SmIFN-γR1). In cases with complete signaling defects but presence of SmIFN-γR1, the causative defect is in the IFN-γ binding capacity of the IFN-γR.[17] Both subgroups show an autosomal recessive (AR) inheritance.

In contrast to patients with complete IFN-γR1 signaling defects, patients suffering from a partial IFN-γR1 signaling defect generally present with well-circumscribed and well-differentiated tuberculoid granulomas with few visible acid-fast rods, have a better prognosis, and may respond to treatment with IFN-γ or anti-mycobacterial chemotherapy. Partial signaling defects caused by mutations in the *IFNGR1* gene may inherit either as an AR trait, in which the mutations generally lead to partially perturbed IFN-γR signaling,[18] or as an autosomal dominant (AD) trait, in which the mutations lead to an intracellularly truncated form of the IFN-γR1 chain, which still can bind IFN-γ, but lacks intracellular signal-transducing and recycling domains, and fails to be internalized from the surface membrane.[19] Thus, the AD form displays a dominant negative, inhibitory phenotype.

Here we present the clinical status and the functional and genetic analysis of two patients who suffered from mycobacterial infections due to complete IFN-γR1 signaling defects.

MATERIALS AND METHODS

Patients

Patient 1 (female) had consanguineous parents. In this family, four out of eight children died, two after BCG vaccination. The disease course of this patient was described before.[20] In short, she became sick after BCG vaccination at the age of nine months. Although a systemic BCG-itis was suspected, *M. avium* was cultured from peripheral blood (PB), stomach, liver, lymph node and bone. Monocytes showed defective up-regulation of HLA-DR as detected by monoclonal antibodies (MAb) L243 and OKIA after one day of maturation with metrizamide, T3 + insulin, TP1, or TP5. She was treated with anti-tuberculous drugs and short-term IFN-γ, but died at the age of 18 months.

Patient 2 (male) is the only child of Dutch, non-consanguineous parents and became sick at the age of three years. *M. gordonae*, *M. peregrinum*, *M. mageritense* and *M. szulgai* have been cultured from lymph node biopsies during four separate consecutive disease episodes. He has been treated repetitively with anti-tuberculous drugs and has shown a vari-

able clinical phenotype. He is now alive at the age of 19 years. Recently, he suffered from his fifth infection (*M. mageritense*). Serum IgG titers against cytomegalo virus, Epstein-Barr virus, herpes simplex virus, hepatitis B virus, *Q fever*, *Toxoplasma*, *Toxocara*, *Yersinea*, and *Brucella* were all negative.

All cell samples were obtained according to the informed consent guidelines of the Medical Ethics Committee of the University Hospital Rotterdam.

IFN-γR1 protein expression

SmIFN-γR1 protein (CDw119) expression was measured by flow cytometry on patient or control PHA blasts using specific, non-blocking MAb's (clones from Genzyme, Cambridge, MA or Pharmingen, San Jose, CA). Either PB mononuclear cells (MC) stimulated for 3 days with PHA at a final concentration of 2 μg/ml, or PHA-stimulated T-cell lines were stained for 30 minutes at 4°C with specific MAb or the respective isotype controls, washed, fixed and analyzed by FACSCalibur (Becton Dickinson, San Jose, CA).

IFN-γ responsiveness of cells from patient 2

The response of cells from patient 2 to IFN-γ was monitored by the IFN-γ mediated upregulation of CD64 on the PBMC-derived monocytic cell fraction as determined by flow cytometry using a CD64-specific MAb (Pharmingen).

The response of patient 2 and control cells to IFN-γ was also analyzed by measuring the enhancement of IL-12 and TNF-α secretion and by the inhibition of IL-10 secretion after stimulation of whole blood cells with LPS (from E.coli, Sigma) at a final concentration of 100 ng/ml. Briefly, 200 μl of a whole blood cell suspension, that was collected in endotoxin-free tubes and five times diluted in Iscove's Modified Dulbecco's Medium (Bio-Whittaker, Verviers, Belgium), was incubated with LPS in the presence or absence of up to $1x10^3$ U IFN-γ (Boehringer Ingelheim). After overnight incubation, IL-12p40 and IL-10 were measured in the collected supernatant of three wells by ELISA (R&D Systems).

Furthermore, polymorphonuclear cells (PMN) from patient 2 were stimulated *in vitro* with IFN-γ and analyzed for phosphorylation of STAT-1, binding of STAT-1 to DNA, and intracellular killing of *Toxoplasma gondii* and *Salmonella typhimurium*.

STAT-1 DNA binding assay

After stimulation of PMN with IFN-γ (10, 100, 1,000, and 10,000 U) for 20 minutes, cellular extracts were prepared. Protein-DNA complexes were detected by electrophoretic mobility shift assay (EMSA). 10 μg of extract was incubated for 30 minutes at 4°C in a 10mM Hepes buffer containing 60mM KCl, 1mM EDTA, 1mM DTT, 10mM Na_3PO_4, 10% glycerol, 1 μg poly(dI-dC), 0.5 μg sonicated herring sperm ssDNA, and 1 ng of ^{32}P radio-labeled dsDNA probe corresponding to the IFN-γ response region. Supershift experiments were performed by incubating formed complexes with STAT-1 specific antibody E23 (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes on ice. Samples were separated by electrophoresis on a 6% non-denaturing polyacrylamide gel. Gels were fixed with 10% methanol

and 10% acetic acid, dried onto Whatmann 3M paper and exposed to an X-ray film.

DNA and RNA extraction and reverse transcriptase reaction

Granulocytes and MC were isolated from PB by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. DNA was extracted from granulocytes using the QIAamp Blood kit (Qiagen, Chatsworth, CA, USA).[21] Total RNA was isolated from PBMC according to the method of Chomczynski using RNAzol B (Tel-Test, Friendswood, TX, USA).[22] cDNA was prepared from mRNA as described before, using random hexamers and Superscript reverse transcriptase.[23]

PCR amplification of (c)DNA

PCR was performed as described previously.[23] In each 100 µl PCR reaction 0.1 µg (c)DNA, 20 pmol of 5' and 3' oligonucleotides and 1 U Ampli*Taq* gold polymerase (Applied Biosystems, Foster City, CA, USA) were used. PCR conditions were 7 min at 95°C, followed by 30 sec at 94°C, 90 sec at 57-60°C, 2 min at 72°C for 40 cycles, followed by a final extension step (10 min at 72°C).

The sequences of the oligonucleotides used for PCR amplification of the *IFNGR1* gene and mRNA were based on human sequences with Genbank accession numbers AL050337 and J03143,[24] respectively, and were designed with the OLIGO 6 program (Dr. W. Rychlik, Molecular Biology Insights, Cascade, CO).[25] Primer sequences will be made available on request to interested readers.

Fluorescent sequencing reaction and analysis

PCR products were first purified with the QIAquick PCR purification kit (Qiagen), and were subsequently used for sequencing with 5 µl big dye terminator mix (Applied Biosystems), using 3.3 pmol sequencing primers. The sequencing primers were positioned in the *IFNGR1* introns sufficiently upstream and downstream of the exon-intron borders to evaluate the splice site sequences. All sequencing was performed as described before,[26] and run on an ABI Prism 377 fluorescent sequencer (Applied Biosystems).

RESULTS

Flow cytometric analysis of IFN-γR1 expression

Relatively frequently, the syndrome of high susceptibility to unusual mycobacterial infections has been associated with causative, deleterious genetic mutations in the *IFNGR1* gene.[15] Therefore, we screened our two patients suffering from unusual *M. avium* infection or consecutive infections with *M. gordonae*, *M. peregrinum*, *M. mageritense*, *M. szulgai*, and *M. mageritense*, respectively, for the expression of IFNγR1 proteins (CDw119), using two different MAb. As shown in Figure 1, we could not find any detectable levels of SmIFN-γR1 protein expression on PHA blasts at day 3 after stimulation, neither on cells from patient 1,

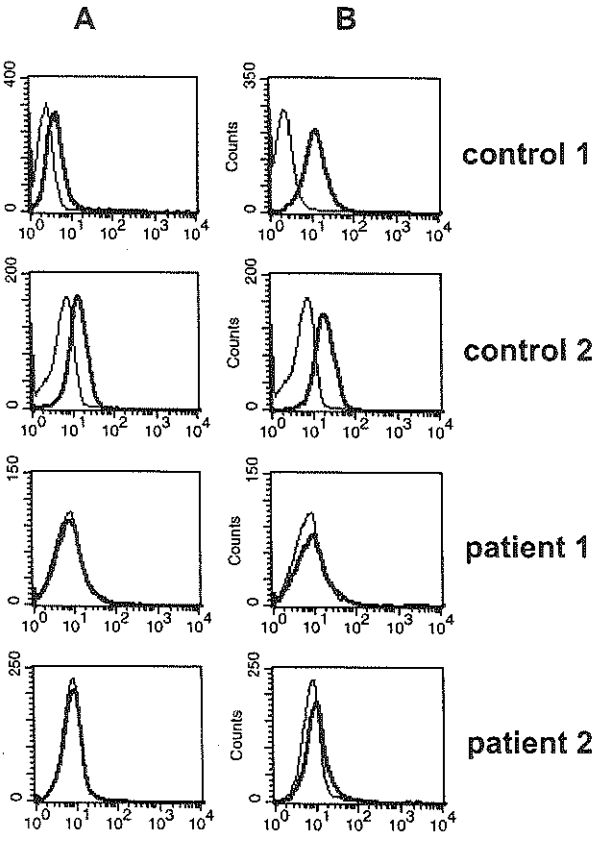

Figure 1. Flow cytometric analysis of IFN-γR1 (CDw119) protein expression on PHA blasts.
Patient or control cells were stained with two different IFN-γR1 specific, non-blocking MAb's (clones from Pharmingen (A) and Genzyme (B)), and analyzed by flow cytometry. Both controls, but neither of the two patients, showed detectable levels of IFN-γR1 proteins at their cell surface. Control 2 was the mother of patient 2. Shown are histograms of the fluorescent signal with isotype controls depicted by thin lines and specific staining depicted by bold lines.

nor on cells from patient 2.

Functional analysis of IFN-γ mediated responses

In order to assess the capacity of cells from patient 2 to respond to IFN-γ, we monitored by flow cytometry the upregulation of CD64 (FcγR1) on monocytic cells from patient 2 and controls in response to increasing doses of IFN-γ. No CD64 upregulation was found (data not shown).

Additionally, for patient 2 we performed an *in vitro* stimulation of whole blood cells with LPS in the presence or absence of recombinant human IFN-γ (Figure 2). As read-out we

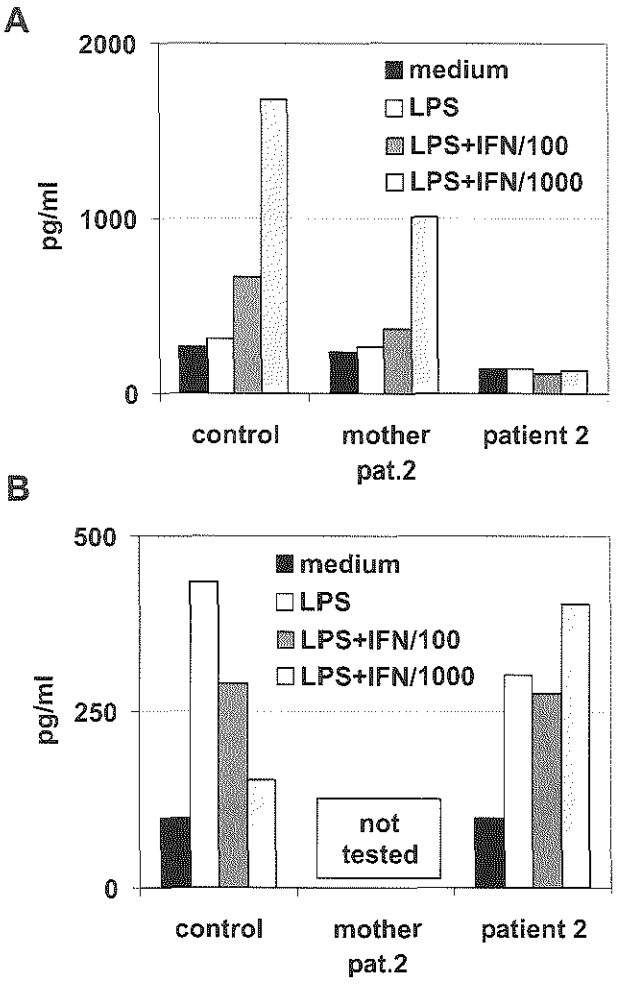

Figure 2. Production of IL-12p40 and IL-10 after stimulation with IFN-γ.
Whole blood cells from controls or patient 2 were tested for IFN-γ mediated enhancement of IL-12p40 secretion or IFN-γ mediated inhibition of IL-10 secretion upon stimulation by LPS. **A.** Both a healthy control and the mother of patient 2, but not patient 2 himself, showed a concentration-dependent increase in the level of secreted IL-12p40 upon LPS stimulation in the presence of IFN-γ. **B.** In contrast to a healthy control, patient 2 showed no concentration-dependent decrease in the amount of secreted IL-10 in the presence of IFN-γ.

measured the secreted levels of IL-12p40 or IL-10 by ELISA, which should be increased and decreased, respectively, if the IFN-γR signaling cascade would still be intact. In accordance with the absence of SmIFN-γR1 protein expression on patient cells and supporting the lack of CD64 upregulating-capacity, cells from patient 2 did not show an augmented IL-12p40 secretion nor an inhibited IL-10 secretion in response to IFN-γ (Figure 2). We also failed to

detect an IFN-γ mediated upregulation of TNF-α production in cells from patient 2 (data not shown).

Finally, after stimulation of PMN from patient 2 with IFN-γ, we were not able to detect STAT-1 phosphorylation, binding of STAT-1 to DNA, or intracellular killing of *Toxoplasma gondii* or *Salmonella typhimurium* (data not shown).

Characterization of IFNGR1 gene mutations

Since the aforementioned phenotypic and functional analyses indicated that both patient 1 and patient 2 suffered from a complete IFN-γR1 signaling defect due to the absence of detectable SmIFN-γR1 protein expression, we aimed at the identification of mutations in the *IFNGR1* gene. Direct fluorescent sequencing of PCR products from patient 1 showed a homozygous G to A substitution at nucleotide position 54 in exon 3 of the *IFNGR1* gene (numbering according to Genbank accession number AL050337). This mutation was confirmed in *IFNGR1*-mRNA and results in a C71Y aa substitution. The father and mother of this patient as well as her sister were heterozygous carriers without clinical manifestations. Her brother was homozygous for the wild type *IFNGR1* gene.

Sequencing of PCR products from patient 2 showed a homozygous G to T substitution at nucleotide position 1 in intron 3 of the *IFNGR1* gene. This splice site mutation lead to the deletion of 173 nucleotides starting at position 249 of the *IFNGR1*-mRNA,[24] which corresponds to the complete absence of exon 3. This leads to a frame shift and a premature stop at codon 73, with only part of the extracellular domain of the IFN-γR1 protein translated. Both non-consanguineous parents were heterozygous for this mutation, as shown at the mRNA (Figure 3), and DNA level. This same mutation has been described before in a compound heterozygous child.[27]

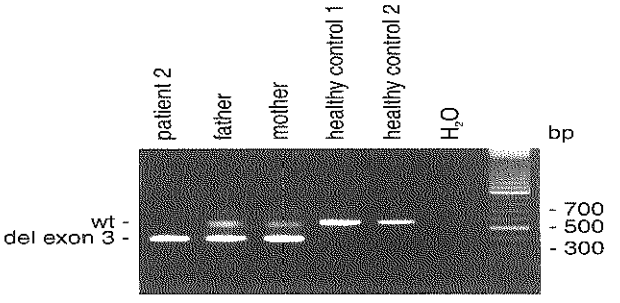

Figure 3. RT-PCR products of *IFNGR1*-mRNA on agarose gel.
The homozygous splice site mutation in patient 2 leads to a complete deletion of exon 3 at the mRNA level. PBMC of his parents expressed both the wt and the mutated mRNA. Their carrier status was confirmed at the genomic DNA level.

Table 1. Summary of *IFNGR1* mutations published.

No.	Inheri-tance	Surface membrane expression	IFN-γ binding	Signaling defect	Group[a] (17)	Onset	Type of mutation	Ref.
1	AR	Absent	Absent	Complete	D	Childhood	C395A, premature stop in EC domain	(7)
2	AR	Absent	Absent	Complete	D	2.5 mo	131delC, premature stop in EC domain	(8)
3	AR	Present	Reduced	Partial	B	Childhood	T260C, I87T in EC domain	(18)
4	AR	Absent	Absent	Complete	D	3y	107ins4, premature stop in EC domain	(30)
							200+1G→A, premature stop in EC domain	(30)
5	AR	Absent	Absent	Complete	D	8 mo	201-2A→G, in frame deletion of 34 aa in EC domain	(31)
6	AR	Absent	Absent	Complete	D	18 mo	22 del C, premature stop in EC domain	(31)
7	AD	Increased	Normal	Partial	A	Childhood	818del4, premature stop after TM domain (lack IC domain)	(19)
8	AD	Increased	Normal	Partial	A	Childhood	818delT, premature stop after TM domain (lack IC domain)	(19)
9	AR	Absent	Absent	Complete	D	4 mo	561del4, premature stop in EC domain	(27)
							200+1G→T, premature stop in EC domain	(27)
10	AR	Present	Absent	Complete	C	1y	295del12, In frame deletion of 4 aa in EC domain	(17)
11	AR	Present	Absent	Complete	C	Childhood	G230A, C63Y in EC domain	(17)
12	AR	Present	Absent	Complete	C	Childhood	T182A, V47Q in EC domain	(17)
							652del3, in frame deletion of 1 aa in EC domain	(17)
13	AR	Absent	Absent	Complete	D	8 weeks	165delG, premature stop in EC domain	(32)
14	AR	Present	Absent	Complete	C	5y	T188G, V63G in EC domain	(33)
15	AD	Increased	Normal	Partial	A	7y	G832T, premature stop after TM domain (lack IC domain)	(34)
P1	AR	Absent	Absent	Complete	D	9 mo	G254A, C71Y in EC domain	This
P2	AR	Absent	Absent	Complete	D	3y	200+1G→T, premature stop in EC domain	report

[a] Group A: AD inheritance, Sm expression increased, normal IFN-γ binding, partial signaling defect.
Group B: AR inheritance, Sm expression present, reduced IFN-γ binding, partial signaling defect.
Group C: AR inheritance, Sm expression present, absent IFN-γ binding, complete signaling defect.
Group D: AR inheritance, Sm expression absent, absent IFN-γ binding, complete signaling defect.

AR = autosomal recessive; AD = autosomal dominant; y = years; mo = months; EC = extracellular; TM = transmembrane; IC = intracellular.

DISCUSSION

Signaling defects resulting from deleterious mutations in the *IFNGR1* gene can be subdivided into four groups, based on the severity of the signaling defect, the mode of inheritance, absence or presence of remaining SmIFN-γR1 proteins, and IFN-γ binding capacity (Table 1).[17,28] Patients with a complete signaling defect generally have a poor prognosis, while patients with a partial signaling defect generally have a better prognosis. In this study two patients with complete IFN-γR1 signaling defects were carefully analyzed, both at the molecular, biochemical and cellular level. However, despite the diagnosis of complete IFN-γR1 deficiency in both cases, there was an unexpected difference in clinical outcome.

Patient 1 became ill after BCG vaccination at the age of nine months and died at the age of 18 months. She had a homozygous C71Y mutation. We could not detect any IFN-γR1 protein expression on PHA blasts (Figure 1). The Cysteine at aa position 71 forms an intrachain disulfide bridge with the Cysteine at aa position 63.[29] Site-directed mutagenesis of Cysteine at position 71 and/or position 63 to Serine completely abolished IFN-γ binding.[29] Furthermore, it has been reported that an IFN-γR1 C63Y mutation in a patient with mycobacterial infections, also completely abolished IFN-γ binding.[17] Consequently, the severe clinical phenotype and the laboratory data of patient 1 fit with a complete signaling defect without expression of IFN-γR1 proteins on the cell membrane (Table 1).

The disease course in patient 2 is relatively mild for a patient with a complete signaling defect, since such patients, like patient 1, generally die at early age without BMT (Table 1). Although patient 2 showed a relatively mild clinical phenotype compared to patient 1, we could neither detect IFN-γR1 protein expression nor IFN-γ mediated responses in this patient, indicative for a complete IFN-γR1 signaling defect (Figures 1 and 2). Indeed, molecular analysis of the *IFNGR1* gene revealed the presence of a homozygous splice site mutation in intron 3, resulting in the absence of exon 3 at the mRNA level and the absence of the transmembrane domain at the protein level. This splice site mutation has been described before in a compound heterozygous patient.[27] However, splice site mutations may allow low levels of normal splicing, resulting in the presence of wild type (wt) mRNA. Such a mechanism could putatively explain the milder clinical phenotype in patient 2. We detected, albeit only after 36 cycles of amplification in real-time quantitative (RQ)-PCR, very low levels of wt *IFNGR1*-mRNA, which were $1x10^4$ times lower than the levels detected in a myeloid cell line (data not shown). Yet, patient 2 did not respond clinically to exogenous administration of IFN-γ. The latter is in contrast to findings of Newport *et al.*, who observed clinical improvement after IFN-γ administration to patients with a homozygous mutation in the *IFNGR1* gene resulting in truncated proteins that lacked the transmembrane domain.[7]

In conclusion, our report confirms that deleterious *IFNGR1* mutations result in increased susceptibility to mycobacterial infections. However, although both patients suffered from complete signaling defects from the IFN-γR, they presented with different clinical phenotypes. It might be that other factors, such as genetic background or access to spe-

cialized pediatric treatment, explain the difference in clinical phenotypes between these two patients.

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Chapter 16

GENERAL DISCUSSION

During our studies in patients suffering from primary immunodeficiency diseases (PID) over the last five years, we have focused on correlations between the genetic defect and immunological characteristics, such as the type of precursor B-cell differentiation arrest in bone marrow (BM) and the residual activity of mutated enzymes (genotype-phenotype relationships). Via these studies and our molecular diagnostic service, we have identified several patients with well-characterized antibody (Ab) or T-cell deficiencies but without mutations in known candidate genes, indicating that yet unidentified genetic defects play a role in the pathogenesis of Ab and T-cell deficiencies.

Genotype-phenotype relationship in PID of the lymphoid system

In the last decade, many genetic defects have been identified that are involved in the pathogenesis of PID of the lymphoid system (Table 1 in Chapter 3). The elucidation of a causative genetic defect was frequently followed by studies focussing on a possible genotype-phenotype relationship. In this context genotype refers to the position of the mutation in the gene (upstream versus downstream) and the type of mutation (deletions, nonsense, missense, or splice site mutations), whereas phenotype refers to clinical presentation, general laboratory parameters, and/or immunological characteristics. The occurrence of genotype-phenotype relationships has indeed been reported, for example in the X-linked hyper IgM syndrome (HIGM1), caused by mutations in the CD40 ligand (*CD40L*) gene,[1] and in patients suffering from B-cell negative SCID, caused by mutations in the recombination activating genes (*RAG*) 1 and 2.[2,3] However, these genotype-phenotype relationships became less clear by subsequent publications describing patients that did not follow the earlier established genotype-phenotype relationships.[4-6] Such discrepancies are generally explained by the presumed influence of additional genetic factors, mostly polymorphisms, or environmental factors, such as antigenic exposure or access to specialized treatment.

The role of splice site mutations in genotype-phenotype relationships is intriguing, because these mutations may allow the production of normally spliced transcripts. These wild type (wt) transcripts encode normal proteins, which might subsequently account for a milder disease phenotype.[7,8]

Phenotypic heterogeneity of lymphoid PID

The clinical and molecular diagnosis of lymphoid PID is hampered by the phenotypic heterogeneity of lymphoid PID. This phenotypic heterogeneity also complicates the selection of patients who are likely to carry the same genetic defect and who can be studied to identify new genetic defects. This phenotypic heterogeneity results in misclassification of patients suffering from lymphoid PID. This is best illustrated by the common variable immunodeficiency (CVID). CVID patients (both males and females) are characterized by hypogammaglobulinemia and generally also by the presence of low numbers of circulating B lymphocytes. CVID is regarded as a separate disease-entity in which the causative genetic defect is still unknown. However, recent studies have shown that the group of CVID patients is 'contaminated' with patients suffering from other lymphoid PID with atypical presentation.

Kanegane *et al.* used flow cytometric analysis of Bruton's tyrosine kinase (BTK) protein expression and showed that in as many as seven out of nine male Japanese CVID patients with decreased numbers of peripheral blood (PB) B lymphocytes, BTK protein expression in monocytes was absent.[9] The XLA diagnosis in these CVID patients was confirmed by mutation analysis of the *BTK* gene. On the other hand, Weston *et al.* showed a *BTK* mutation (using the less sensitive single strand conformation polymorphism (SSCP) technique) in only 1 out of 24 male CVID patients, from whom 12 had normal absolute numbers of B lymphocytes and 12 had decreased or no detectable numbers of B lymphocytes.[10] Furthermore, it appeared that a subset of CVID patients had defects in the generation of antigen-specific antibodies (Ab) via somatic hypermutation (SHM).[11,12]

Consequently, various genetic defects causing hypogammaglobulinemia should be excluded, including "leaky" severe combined immunodeficiency (SCID), XLA, HIGM, and X-linked lymphoproliferative disease (XLP), before the diagnosis CVID can be made.[13]

Unknown gene defects causing Ab deficiencies

Although many genetic defects causing lymphoid PID have been identified, there are still some subgroups that remain genetically unidentified (Table 1 in Chapter 3). As discussed before, approximately 5% of agammaglobulinemia patients with decreased numbers of PB B lymphocytes have no mutations in one of the five currently identified candidate genes (*BTK*, C_{μ}, *CD79a*, *λ14.1*, or B-cell linker protein (*BLNK*)).[14] Cell material of those patients is a valuable source to search for new genetic defects. However, the molecular identification of such patients is time-consuming, as more and more possible genetic causes need to be ruled out before an agammaglobulinemia patient with decreased numbers of PB B lymphocytes can be categorized into the group of patients with unknown genetic cause.

Another group concerns patients suffering from Ab deficiencies with normal numbers of PB B lymphocytes, which are unable to perform immunoglobulin (Ig) class switching or to generate antigen-specific Ab, due to a defect in SHM.[11,12] Recently, a new gene was identified, which encodes the activation-induced cytidine deaminase (*AICDA*) protein, required for class switch recombination (CSR) and SHM, but not for the formation of germinal centers.[15] This gene was shown to be mutated in several patients with the autosomal recessive (AR) form of HIGM (HIGM2),[16] but not in patients with defects in SHM. Furthermore, the group of Anne Durandy states that up to 50% of patients with HIGM2 do not carry mutations in the *AICDA* gene. Some of these patients were recently shown to carry mutations in the *CD40* gene.[52] We also identified several patients with the clinical phenotype of HIGM, without mutations in the *CD40L* and *AICDA* genes, and normal expression of CD28, CD40, CD80, and CD86 proteins as evaluated by flow cytometry. This group of HIGM patients and the group of patients with defects in SHM provide a valuable source to study the mechanisms of CSR and SHM, respectively.

Unknown gene defects causing SCID

We have studied patients with SCID who were phenotypically classified as $T^-B^-NK^+$

SCID or Omenn syndrome (OS) patients. Some of these patients have mutations in the *RAG* genes.[2,3] $T^-B^-NK^+$ SCID or OS patients without mutations in their *RAG* genes could theoretically suffer from a transcriptional defect. Therefore, we studied the *RAG*-mRNA expression in BM of these patients using real-time quantitative (RQ)-PCR. However, until now we could not detect decreased *RAG*-mRNA expression levels after correcting for the relative size of the precursor B-cell compartment. Nicolas *et al.* tested fibroblasts from $T^-B^-NK^+$ SCID patients without mutations in their *RAG* genes for sensitivity to X-rays and found that some of these patients suffered from a radiosensitive form of SCID (RS-SCID).[17] This phenotype was accompanied by a profound defect in coding joint formation, whereas signal joints were normal,[17] thereby resembling the phenotype of the SCID mouse, which suffers from a mutation in its DNA-dependent protein kinase catalytic subunit ($DNA\text{-}PK_{cs}$).[18,19] However, the radiosensitivity in these SCID patients was not caused by defects in one of the known components of the DNA double strand break (dsb) repair pathway involving non-homologous end-joining (NHEJ), such as Ku70, Ku80, $DNA\text{-}PK_{cs}$, DNA ligase IV, or XRCC4.[17] Recently, a new gene named *Artemis* was cloned and shown to be mutated in RS-SCID patients.[20] However, our preliminary results indicate that not all RS-SCID patients suffer from *Artemis* mutations (Noordzij *et al.*, unpublished results).

Therefore, in contrast to published data,[17] it might be that some RS-SCID patients do suffer from mutations in one of the known NHEJ components, or in another yet unknown component. Mice carrying a defective gene of either one of the known NHEJ components, exhibit a profound arrest in B- and T-cell differentiation.[21] In addition to the SCID phenotype, the *Ku70* and *Ku80* gene knockout mice are also growth retarded [22,23] and the *XRCC4* and *DNA ligase IV* gene knockout mice die during embryogenesis,[24,25] but the mechanism underlying these phenotypes is not yet clear. Until now, five RS patients with mutations in *DNA ligase IV* have been described. One patient suffered from acute lymphoblastic leukemia, but showed largely normal V(D)J recombination and normal development of the immune system.[26,27] The clinical phenotype of the other four RS patients closely resembled the DNA damage response disorder Nijmegen breakage syndrome.[53] No human SCID patients with mutations in one of the above mentioned genes have been reported so far.

DNA-microarray technology in the search for new genetic defects

DNA-microarrays now make it possible to analyze the mRNA expression profile of more than 10,000 genes at one time. This provides a powerful tool to compare the mRNA expression profiles between different cell types, or between cells of the same lymphoid lineage in subsequent differentiation stages.

Upon stimulation with CD40L, relevant cytokines, and other co-stimulatory molecules, a human surface membrane (Sm) IgM^+/IgD^+ B-cell line derived from a Burkitt's lymphoma, could be induced to perform CSR and SHM in culture.[28,29] It would be interesting to study whether these processes can be induced in normal or Epstein Barr virus (EBV) transformed human PB $SmIgM^+/SmIgD^+$ B lymphocytes or tonsillar B cells in culture as well. If it is possible to induce CSR and/or SHM in non-malignant human B lymphocytes, compari-

son of mRNA expression profiles in stimulated B lymphocytes from healthy individuals and patients suffering from HIGM or defects in SHM, might provide insight in the possible genetic defects resulting in these Ab deficiencies.

Composition of the precursor B-cell compartment in human bone marrow

The composition of the precursor B-cell compartment in human BM is stable during childhood.[30-32] Using CD22, CyCD79a, CD19, terminal deoxynucleotidyl transferase (TdT), CD10, and Ig expression patterns (CyIgμ, CyVpreB, and SmIg) as dominant parameters, we were able to recognize nine different precursor B-cell differentiation stages.[33] Overall, our scheme was in line with the schemes proposed by Paulo Ghia and Claudia Schiff.[34,35] Validation by cell sorting into separate stages and subsequent analysis of sterile Ig transcripts, incomplete D_H-J_H and complete V_H-J_H, Vκ-Jκ, and Vλ-Jλ rearrangement patterns,[36] and expression of transcription factors, which are known to be essential for precursor B-cell differentiation, such as E2A, PAX5, and early B-cell factor (EBF),[37] can further confirm our results.

When comparing different precursor B-cell differentiation schemes that have been published, there is controversy about the large, cycling cells in the pre-B-II stage (stage 6 in Figure 1 from the General Introduction). Ghia *et al.* recognized three separate CyIgμ$^+$ pre-B-II cell stages. The pre-B-II cells were subdivided in large, cycling cells and small, resting cells, and the large, cycling pre-B-II cells were further subdivided on the basis of pre-BCR expression: (1) pre-B-cell receptor (BCR)$^+$/RAG$^-$ cycling pre-B-II cells; (2) pre-BCR$^-$ cycling pre-B-II cells; and (3) pre-BCR$^-$/RAG$^+$ resting pre-B-II cells.[34,36] However, Nomura *et al.* recognized three different CyIgμ$^+$ pre-B-II cell stages. All large, cycling pre-B-II cells expressed the pre-BCR, while the small, resting pre-B-II cells were subdivided on the basis of pre-BCR expression: (1) large CyVpreB^{bright} pre-B-II cells; (2) small CyVpreB$^+$ pre-B-II cells; and (3) small CyVpreB$^-$ pre-B-II cells.[31] Our results showed that the CyIgμ$^+$/CyVpreB$^+$ pre-B-II cells in stage 6 had a small forward scatter and were therefore unlikely to represent cycling cells (Noordzij *et al.*, unpublished results).

More detailed insight into the precursor B-cell differentiation stages can be obtained by sorting and pooling the precursor B-cell subsets of BM samples from different donors to minimize inter-individual variation, followed by mRNA expression profile analysis using DNA-microarrays. Such analyses will probably show differences in mRNA expression levels of genes known to be involved in cell cycle regulation, Ig gene rearrangement, and/or transcription. These differences can be validated by means of RQ-PCR analysis. The DNA-microarray analysis might also show differences in mRNA expression levels of genes, which are yet unknown to be involved in precursor B-cell differentiation. Identification of such genes might help to further unravel the regulation of precursor B-cell differentiation.

Precursor B-cell differentiation arrest in BM of patients suffering from B-cell deficient PID

The localization of the precursor B-cell differentiation arrest has been determined in

BM from patients suffering from lymphoid PID, characterized by the complete absence or decreased numbers of B lymphocytes in the PB. This included patients suffering from X-linked agammaglobulinemia (XLA),[31,32] several causes of autosomal recessive agammaglobulinemia (C_μ,[38] λ14.1,[39] CD79a,[40] and BLNK deficiency[41]), and RAG deficient SCID patients.[33] All patients showed a B-cell differentiation arrest at the transition from CyIgμ$^-$ to CyIgμ$^+$ pre-B cells, i.e. at the pre-BCR checkpoint.[14,42] However, the severity of this arrest appears to be variable ("leakiness"), as XLA patients and λ14.1 deficient agammaglobulinemia patients can present with decreased but clearly detectable numbers of B lymphocytes in the PB, while C_μ, CD79a, BLNK, and RAG-deficient SCID patients in general present with complete absence of PB B lymphocytes.

It has been shown in wt mice that some precursor B cells can rearrange Ig light chain (*IGK* and *IGL*) genes before Ig heavy chain gene (*IGH*) rearrangement is completed.[43] Moreover, B-cell development in λ5 (the murine form of λ14.1) deficient mice can be rescued by premature expression of Ig light chains.[37] Although rearrangement of *IGH*, *IGK*, and *IGL* genes during human precursor B-cell differentiation occurs via an ordered model,[44] one might hypothesize that in λ14.1 deficient patients, *IGK* and/or *IGL* gene rearrangement occurs in an earlier phase, thereby circumventing the requirement for λ14.1 expression, and explaining the leaky phenotype.[14,39] Recently it was shown that, when expressed in *Drosophila melanogaster* Schneider cells, the VpreB proteins alone were capable of forming complexes with Igμ, while the λ5 protein alone was unable to bind properly to Igμ, thereby providing another explanation for the leaky phenotype of λ14.1 deficient patients.[45]

BTK, CD79a, and BLNK proteins are all three involved in pre-BCR signaling, but it remains unclear why some BTK deficient XLA patients can produce mature B lymphocytes, while the reported CD79a and BLNK deficient agammaglobulinemia patients showed a complete arrest in precursor B-cell differentiation. However, one should bear in mind that until now, only one CD79a deficient and one BLNK deficient patient have been reported and that the diagnosis of additional patients might show leakiness in these diseases as well.

Specific functions of dispensable portions of RAG proteins

Both RAG proteins contain core domains, which are essential for recombination activity. The N-terminal part of the RAG1 protein and the C-terminal part of the RAG2 protein are both dispensable for recombination activity *in vitro*, although the presence of the RAG1 N-terminus enhances recombination activity.[46] However, Kirch *et al.* have shown that C-terminal truncated RAG2 proteins can initiate incomplete D_H-J_H rearrangements, but not complete V_H-J_H rearrangements.[47] This suggested that the C-terminus of the RAG2 protein harbors Ig specificity.

We have shown that a frameshift mutation within the 5' coding region of the *RAG1* gene can be encompassed by the translation machinery, using an alternative UTG translation initiation codon downstream of the deletion. This resulted in an N-terminal truncation of the RAG1 protein, leaving the core domain unaffected, with a recombination activity of approximately 10% of wt function.[33,48] As this mutation was identified in a patient suffering from an

OS-like T^+/B^- SCID with an almost polyclonal T-cell receptor (TCR) gene rearrangement repertoire, but hardly detectable Ig gene rearrangements, our results suggested that the N-terminus of the RAG1 protein might harbor Ig specificity as well.[48,49]

Santagata *et al.* also identified several OS patients suffering from N-terminal truncations of the RAG1 proteins, resulting in decreased recombination activities.[50] They suggested that the presence of partial V(D)J recombination activity resulted in OS and might be explained as follows: (1) Translation from an alternative translation initiation codon is less efficient. (2) Deletion of part of the N-terminus results in the absence of nuclear localization signals and subsequent aberrant localization of RAG1 proteins in the cytoplasm instead of in the nucleus. (3) Deletion of part of the N-terminus results in absence of regions involved in the enhancement of recombination efficiency. For example, initiation of translation at codon 183 (i.e. the deletion of the first 183 N-terminal amino acids) resulted in a RAG1 protein with wt levels of recombination activity, while initiation of translation at codon 263 generated a RAG1 protein with 10% of wt recombination activity.[50]

Until now, it remains unknown why mutated RAG proteins with partial VDJ recombination activity can produce oligoclonal TCR gene rearrangements, but no Ig gene rearrangements, although the presence of high serum IgE levels in OS patients suggests the presence of Ig producing plasma cells. Villa *et al.* indeed reported decreased absolute numbers of B lymphocytes in the PB of some OS patients.[51] However, we have identified two OS patients with proven partial recombination activity and oligoclonal TCR gene rearrangements, but severe suppression of precursor B-cells in the BM (<2% of the lymphocytes) and complete absence of B lymphocytes in PB.[33]

CONCLUSION

Many genetic defects resulting in PID of the lymphoid system have already been identified, but still several defects have to be unraveled. We feel that careful selection of patients (with exclusion of known candidate genes) and careful comparative studies between cells of patients suffering from PID and healthy children will contribute to the identification of new genetic defects and subsequently the molecular diagnosis of these patients.

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LIST OF ABBREVIATIONS

aa	:	amino acid
Ab	:	antibody
AD	:	autosomal dominant
ADA	:	adenosine deaminase
Ag	:	antigen
AICDA	:	activation-induced cytidine deaminase
AMV	:	avian myeloblastosis virus
ANOVA	:	one way analysis of variance
ALPS	:	auto-immune lymphoproliferative syndrome
APC	:	antigen-presenting cell / allophycocyanin
AR	:	autosomal recessive
ATG	:	anti thymocyte globulin
BCR	:	B-cell receptor
BLNK	:	B-cell linker protein
BM	:	bone marrow
BMT	:	bone marrow transplantation
bp	:	base pair
BTK	:	Bruton's tyrosine kinase
c	:	complementary
CBT	:	cord blood stem cell transplantation
CD	:	cluster of differentiation
CD40L	:	CD40 ligand
CHO	:	Chinese hamster ovary
CSR	:	class-switch recombination
CVID	:	common variable immunodeficiency
Cy	:	cytoplasmic
Cy5	:	Cyanine5
D	:	diversity
DISC	:	death-inducing signaling complex
DNA-PK_{cs}	:	DNA-dependent protein kinase catalytic subunit
dsb	:	double strand break
EBF	:	early B-cell factor
EBV	:	Epstein-Barr virus
EC	:	eosinophil count / extracellular
EMSA	:	electrophoretic mobility shift assay
ENT	:	ear-nose and throat
ESE	:	exonic splicing enhancer
FACS	:	fluorescence activated cell sorter
FADD	:	Fas-associated via death domain

FISH	:	fluorescent *in situ* hybridization
FITC	:	fluorescein isothiocyanate
FSC	:	forward scatter
GVHD	:	graft-versus-host-disease
HDA	:	Heteroduplex analysis
hi	:	high
HIGM	:	hyper IgM syndrome
HIV	:	human immunodeficiency virus
IC	:	intracellular
ICF	:	immunodeficiency-centromeric instability-facial dysmorphism
IFN	:	interferon
Ig	:	immunoglobulin
IGH-C_μ	:	Ig heavy chain constant mu
IL	:	interleukin
J	:	joining
JAK	:	Janus associated kinase
LC	:	lymphocyte count
lo	:	low
LR	:	long range
LW	:	lysed whole
MACS	:	magnetic cell sorting
MC	:	mononuclear cell
MFT	:	maternal-fetal T-cell engraftment
mo	:	month
NBS	:	Nijmegen breakage syndrome
NHEJ	:	non-homologous end joining
NK	:	natural killer
nt	:	nucleotide
OS	:	Omenn syndrome
PB	:	peripheral blood
PCP	:	*Pneumocystis carinii* pneumonia
PE	:	phyco-erythrin
PerCP	:	peridinin chlorophyl protein
PH	:	pleckstrin homology
PID	:	primary immunodeficiency disease
PMA	:	phorbol myristate acetate
PMN	:	polymorphonuclear cell
PNP	:	purine nucleoside phosphorylase
R	:	receptor
RAG	:	recombination activating gene
Rn	:	normalized reporter signal

RQ	:	real time quantitative
RS	:	radiosensitive
RSS	:	recombination signal sequence
RT	:	reverse transcriptase
SB	:	Southern blot
SCID	:	severe combined immunodeficiency disease
SH	:	Src homology
SHM	:	somatic hypermutation
Sm	:	surface membrane
SSC	:	side scatter
SSCP	:	single strand conformation polymorphism
STAT	:	signal transducer and activator of transcription
TCR	:	T-cell receptor
TdT	:	terminal deoxynucleotidyl transferase
TH	:	Tec homology
Th	:	T-helper cell
TM	:	transmembrane
UTR	:	untranslated region
V	:	variable
WASP	:	Wiskott-Aldrich syndrome protein
WBC	:	white blood cell count
wt	:	wild type
XLA	:	X-linked agammaglobulinemia
XLP	:	X-linked lymphoproliferative disease
XLT	:	X-linked thrombocytopenia
XRCC	:	X-ray cross complementation group
y	:	year
ZAP	:	zeta-chain-associated protein kinase
ψL	:	pseudo-light chain
γ_c	:	common gamma chain

SUMMARY

Primary immunodeficiency diseases (PID) are hereditary diseases, characterized by dysfunctioning of the immune system. They can be divided into five categories, based on the component of the immune system that is affected. Two of these categories, antibody and T-cell deficiencies, comprise more than 90% of all PID. These PID of the lymphoid system are the topic of interest in this thesis.

PID are clinically heterogeneous diseases. Therefore, it can be difficult to establish the correct diagnosis based on clinical presentation alone. We feel that the diagnosis of PID should be based on mutation analysis of the affected gene. The clinical symptoms and immunophenotyping results of blood and bone marrow (BM) are important tools to select candidate genes for mutation analysis. **Part 2** of this thesis focuses on immunophenotyping (**Chapter 2**) and immunogenotyping (**Chapter 3**) techniques.

Many attempts have been made to relate the clinical heterogeneity of PID to the type of mutation (genotype-phenotype relationship). For a few PID, among which the severe combined immunodeficiency disease (SCID) caused by mutations in the recombination activating genes (RAG), a (partial) genotype-phenotype relationship has been documented. **Chapter 5** comments on a report in which a genotype-phenotype relationship for X-linked hyper IgM syndrome caused by mutations in the CD40 ligand gene was described. However, phenotype can also be defined as the localization of the precursor B-cell differentiation arrest in BM (immunophenotype), the presence of wild type mRNA transcripts, the absence of immunoglobulin gene rearrangements, or the residual recombination activity of mutated RAG proteins. In **Chapters 6, 7, 11, and 12**, we have analyzed the possible relationship between the genotype and these kinds of phenotypes in patients with X-linked agammaglobulinemia (XLA) or RAG deficient SCID.

Performing mutation analysis as a diagnostic service results in patients, in whom no initial molecular diagnosis could be established. These patients can be used to search for other, more rare genetic defects. **Chapters 8 and 13** describe patients with antibody deficiencies or SCID, in whom eventually rare genetic defects were discovered.

Although phagocyte defects do not belong to PID of the lymphoid system, we studied two patients suffering from mycobacterial infections. **Chapter 15** discusses how two patients with absence of the interferon-γ receptor 1 chain can present with different clinical phenotypes.

In conclusion, we studied possible genotype-(immuno)phenotype relationships in patients with PID of the lymphoid system, using a broader definition of the term phenotype. In XLA patients, we found that a patient with a *BTK* splice site mutation had an almost normal composition of his precursor B-cell compartment in BM. In RAG deficient SCID patients, the residual recombination activity of the mutated RAG proteins appeared to correlate with the "leakiness" of the precursor B-cell differentiation arrest in BM.

SAMENVATTING VOOR NIET-INGEWIJDEN

Primaire immunodeficiënties (PID) zijn aangeboren stoornissen in het immuunsysteem. PID leiden over het algemeen tot ziekte op de kinderleeftijd. PID worden onderverdeeld in vijf groepen, afhankelijk van welk onderdeel van het immuunsysteem is aangedaan.

De grootste groep (±70%) van de PID wordt gevormd door stoornissen in de antistofvorming. Deze antistof deficiënties kunnen worden veroorzaakt doordat de antistofproducerende B cellen afwezig zijn (bijvoorbeeld bij X-gebonden agammaglobulinemie of XLA), doordat de helper T cellen een eiwit missen dat belangrijk is voor de vorming van verschillende soorten antistoffen (X-gebonden hyper IgM syndroom of HIGM1), of doordat de antistoffen niet verder kunnen "uitrijpen". Ongeveer 85% van de patiënten met een antistof deficiëntie lijdt aan XLA. XLA wordt veroorzaakt door een fout (mutatie) in het X-chromosoom. De ziekte komt alleen bij mannen (XY) voor, vrouwen (XX) kunnen draagster zijn. De mutatie die XLA veroorzaakt zit in een stuk DNA (gen) dat informatie bevat voor het Bruton's tyrosine kinase (BTK) eiwit. Dit BTK eiwit is belangrijk om voorloper B cellen in het beenmerg te laten uitrijpen tot antistofproducerende B cellen. Kinderen met XLA missen het BTK eiwit en hebben geen antistofproducerende B cellen. Deze kinderen krijgen maandelijks via een infuus antistoffen van donoren toegediend.

Ongeveer 20% van de PID wordt gevormd door stoornissen in de T cellen. De cytotoxische T cellen zijn belangrijk voor de afweer tegen virussen. De helper T cellen zijn de "dirigenten van het immunologisch orkest". Afwezigheid van helper T cellen kan ertoe leiden dat ook andere afweercellen niet goed functioneren. Stoornissen in de T cellen leiden tot ernstige, gecombineerde immunodeficiënties ("severe combined immunodeficiencies" of SCID). Kinderen met SCID worden over het algemeen binnen een paar maanden na de geboorte ernstig ziek van virussen of bacteriën waar gezonde kinderen niet ziek van worden (opportunistische infecties). Tot voor kort konden deze kinderen alleen worden behandeld met behulp van een beenmergtransplantatie. Recent is gepubliceerd dat jongens met de X-gebonden vorm van SCID kunnen worden behandeld met behulp van gentherapie.

In dit proefschrift worden vooral de antistof deficiënties en de T cel deficiënties beschreven. De overige drie categorieën (fagocyten deficiënties, complement deficiënties, en overige PID) vormen ongeveer 10% van de PID en blijven in dit proefschrift buiten beschouwing. Naast PID bestaan er ook secundaire immunodeficiënties. Deze niet-aangeboren afweerstoornissen kunnen in de loop van het leven ontstaan, bijvoorbeeld door infectie met het humaan immunodeficiëntie virus (HIV), leidend tot het "acquired immunodeficiency syndrome" (AIDS). Ook de secundaire immunodeficiënties komen in dit proefschrift niet aan de orde.

In dit proefschrift wordt de genetische diagnostiek van kinderen met een PID beschreven (Hoofdstukken 2 en 3). Daarnaast hebben we geprobeerd een relatie te leggen tussen genotype en fenotype bij deze kinderen. Onder genotype wordt verstaan de plaats van

de mutatie in een gen en het soort mutatie. Van fenotype is een brede definitie te geven. Hieronder kan worden verstaan de leeftijd waarop kinderen ziek worden en de ernst van de infecties (klinisch fenotype), maar ook het aantal B cellen in bloed of beenmerg en de restfunctie van aangedane eiwitten (immunologisch fenotype).

Van een aantal PID (zoals SCID veroorzaakt door mutaties in de recombinatie activerende genen en HIGM1) werd in de afgelopen jaren ontdekt dat er een relatie bestond tussen genotype en fenotype. We hebben deze relaties verder kunnen uitdiepen of nuanceren (Hoofdstukken 5 en 12). Verder was bekend dat er bij XLA geen relatie bestond tussen genotype en fenotype. Door het fenotype van XLA patiënten te definiëren als de ernst van de uitrijpingsblokkade van voorloper B cellen in het beenmerg konden we aantonen dat een milde mutatie in het *BTK* gen gepaard ging met een milde uitrijpingsblokkade (Hoofdstuk 6). Helaas hebben we dit niet bij andere XLA patiënten met een milde *BTK* mutatie kunnen bevestigen (Hoofdstuk 7).

Concluderend blijkt het vaak moeilijk te zijn om de ernst van de PID enkel te verklaren op grond van de ernst van de mutatie. Dit suggereert dat andere factoren, zoals polymorfismen (natuurlijk voorkomende genvariaties), mede bepalend zijn voor de ernst van het ziektebeeld.

DANKWOORD

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