V(D)J recombination defects in lymphocytes due to *RAG* mutations: severe immunodeficiency with a spectrum of clinical presentations

Anna Villa, Cristina Sobacchi, Luigi D. Notarangelo, Fabio Bozzi, Mario Abinun, Tore G. Abrahamsen, Peter D. Arkwright, Michal Baniyash, Edward G. Brooks, Mary Ellen Conley, Patricia Cortes, Marzia Duse, Anders Fasth, Alexandra M. Filipovich, Anthony J. Infante, Alison Jones, Evelina Mazzolari, Susanna M. Muller, Srdjan Pasic, Gideon Rechavi, Maria Grazia Sacco, Sandro Santagata, Marlis L. Schroeder, Reinhard Seger, Dario Strina, Alberto Ugazio, Jouni Väliaho, Mauno Vihinen, Larry B. Vogler, Hans Ochs, Paolo Vezzoni, Wilhelm Friedrich, and Klaus Schwarz

Severe combined immunodeficiency (SCID) comprises a heterogeneous group of primary immunodeficiencies, a proportion of which are due to mutations in either of the 2 recombination activating genes (*RAG*)-1 and -2, which mediate the process of V(D)J recombination leading to the assembly of antigen receptor genes. It is reported here that the clinical and immunologic phenotypes of patients bearing mutations in *RAG*s are more diverse than previously thought and that this variability is related, in part, to the specific type of *RAG* mutation. By analyzing 44 such patients from 41 families, the following conclusions were reached: (1) null mutations on both alleles lead to the T-B-SCID phenotype; (2) patients manifesting classic Omenn syndrome (OS) have missense mutations on at least one allele and maintain partial V(D)J recombination activity, which accounts for the generation of residual, oligoclonal T-lymphocytes; (3) in a third group of patients, findings were only partially compatible with OS, and these patients, who also carried at least one missense mutation, may be considered to have atypical SCID/OS; (4) patients with engraftment of maternal T cells as a complication of a transplacental transfusion represented a fourth group, and these patients, who often presented with a clinical phenotype mimicking OS, may be observed regardless of the type of *RAG* gene mutation. Analysis of the *RAG* genes by direct sequencing is an effective way to provide accurate diagnosis of *RAG*-deficient as opposed to *RAG*-independent V(D)J recombination defects, a distinction that cannot be made based on clinical and immunologic phenotype alone. (Blood. 2001;97:81-88)

Introduction

The process of V(D)J recombination, leading to the assembly of genes coding for immunoglobulins and T-cell receptors (TCR), is central to the differentiation of B and T cells, to the establishment of a functional immune system, and to its ability to respond to antigenic challenge.[1]

All the immunoglobulin and TCR protein chains consist of 2 structural domains, the constant and the variable regions; the latter are responsible for the specific binding to antigen. V(D)J recombination is the process leading to the generation of variable domains through the assembly of one segment each from a set of variable (V), joining (J), and, in some cases, diversity (D) subgenic elements.[2] Rearrangement is directed by recombination signal sequences that flank each antigen receptor gene segment. These recombination signal sequences consist of a heptamer sequence (CACAGTG), directly adjacent to the coding element, and a nonamer element (ACAAAACC), separated from the heptamer by a spacer of either 12 or 23 base pairs.[3,4] Efficient recombination occurs between a pair of gene elements with recombination signal sequences that have different spacer lengths, the so-called 12/23 rule.

This process is carried out by several molecules that act in concert to create the diversity of the antigen receptor repertoire. Recombination activating genes (*RAG-1* and *RAG-2*) play a

From the Department of Human Genome and Multifactorial Disease, Istituto di Tecnologie, Biomediche Avanzate, Consiglio Nazionale delle Ricerche, Segrate (MI); Istituto di Medicina Molecolare "Angelo Nocivelli," and Clinica Pediatrica, Universita di Brescia, Italy; Children's Bone Marrow Transplant Unit, Newcastle General Hospital, Newcastle-upon-Tyne; University of Manchester, Academic Unit of Child Health, St Mary's Hospital, Manchester, United Kingdom; Department of Pediatrics, The National Hospital, Oslo, Norway; Lauterberg Center for General and Tumor Immunology, Hebrew University-Hadassah Medical School, Jerusalem; Pediatric Hematology-Oncology, Sheba Medical Center, Tel Hashomer and Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel; Queen Silvia Children's Hospital, Göteborg, Sweden; Department of Pediatrics and Ulm Germany Transfusion Medicine, University of Ulm, Ulm, Germany; Pediatric Immunology, Mother and Child Health Institute, Belgrade, Yugoslavia; University Childrens' Hospital, University of Zurich, Zurich, Switzerland; Institute of Medical Technology, University of Tampere, and Tampere University Hospital, Tampere, Finland; Department of Pediatrics, Child Health Research Center, University of Texas Medical Branch, Galveston, TX; Department of Immunology, St Jude Children's Research Hospital, Memphis, TN; Immunobiology Center and Ruttenberg Cancer Center, Mount Sinai School of Medicine of New York University, New York, NY; Children's Hospital Medical Center, Cincinnati, OH; Division of Hematology/Oncology/Immunology, Children's Cancer Research Center, University of Texas Health Science Center, San Antonio, TX; Pediatric Rheumatology Division, Emory University School of Medicine, Atlanta, GA; Department of Pediatrics, University of Washington School of Medicine, Seattle, WA; and Health Sciences Center, University of Manitoba, Canada.

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A.V. and C.S. contributed equally to this work.

Reprints: Anna Villa, ITBA, CNR, Via Fratelli Cervi 93, 20090 Segrate (MI), Italy.

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fundamental role by initiating the "cut-and-paste" process leading to the assembling of the V, (D), and J segments, which together form the variable portion of the receptors.[5,6] So far, the *RAG* genes are the only components of the gene rearrangement apparatus in which mutations leading to primary immunodeficiencies in humans have been described. This is at odds with investigations in the mouse, in which several spontaneously occurring or artificially created mutations in genes involved in DNA repair and V(D)J recombination, including those of the DNA-PK complex, *xrcc4* and *DNA ligase IV*, have been demonstrated to cause severe combined immunodeficiencies (SCID) with low numbers of both B- and T-lymphocytes (T-B-SCID).[7-14]

Gene targeting of the *rag-1* and *rag-2* in mice results in a complete absence of both B- and T-lymphocytes, whereas natural killer cells, which do not rearrange antigen receptor genes during their maturation, are not affected.[15,16] No murine model of partial inactivation of *RAG* function is available at present. In agreement with the mouse model, we have recently demonstrated that T-B-SCID in human is often due to *RAG* gene mutations.[17] Patients with SCID secondary to *RAG* mutations may have Omenn syndrome (OS),[18] a rare combined immune deficiency characterized by the presence of a substantial number of oligoclonal, activated T cells, and the lack of B lymphocytes, associated with particular clinical features (generalized erythroderma, lymphadenopathy, hepatosplenomegaly, increased occurrence of life-threatening infections).[19-21] On the basis of our findings, obtained in a limited number of *RAG*-deficient cases, we hypothesized that in humans the persistence of partial *RAG* activity could be responsible for OS, whereas mutations that completely abolish *RAG* function could give rise to T-B- SCID.[17,18,22] A role for nongenetic factors in the pathogenesis of OS cannot be ruled out. In addition, the extent to which a limited but detectable *RAG* activity can allow partial maturation of T or B cells has not yet been defined.

Here we report a large series of immunodeficient patients with *RAG* defects. Analysis of the type and site of the *RAG* mutations in relation to their clinical presentation and immunologic phenotype supports our previous hypothesis that partial *RAG* activity, allowing limited recombination events to occur, is the major determinant for the presence of a substantial number of oligoclonal T cells in OS. Furthermore, we have identified *RAG* defects in a cohort of patients with some, but not all, the clinical and immunologic features of OS, a condition referred to here as "atypical SCID/OS." Interestingly, missense mutations were overrepresented in patients with atypical SCID/OS, as they were in patients with OS. Finally, we have demonstrated that engraftment of maternal T cells into fetuses with *RAG* deficiency, regardless of the type and site of mutations, may result in a clinical and immunologic phenotype that mimics OS. Taken together, these findings suggest that *RAG*-dependent immunodeficiency covers a spectrum broader than previously thought and that the clinical picture is partly dependent on the specific mutation in the *RAG* genes.

Patients, materials, and methods

Patients

Patients were selected for analysis of *RAG* mutations if they had a B^- variant of SCID. If *RAG* mutations were discovered, for each patient a questionnaire was filled in at each participating center. Based on several criteria, patients were classified as follows: (1) complete absence of T and B cells and no clinical abnormalities to suggest Omenn syndrome; (2) complete absence of B cells, but presence of autologous T cells and typical clinical features compatible with OS; (3) patients with documented maternal T-cell engraftment (SCID with maternal T cells); (4) patients not fulfilling the above criteria. This group, labeled as "atypical SCID/OS," included a few patients in whom immunologic and genetic characterization was incomplete. Ten age-matched, healthy infants served as controls for the analysis of immunologic data. Approval for these studies was obtained from the institutional review board. Informed consent was provided according to the Declaration of Helsinki.

Mutation analysis at the *RAG-1* and *RAG-2* loci

Because of the restricted expression of *RAG-1* and *RAG-2* genes and the fact that the coding region of each gene is contained in a single exon, coding sequences were amplified from genomic DNA. For all patients undergoing bone marrow transplantation, DNA samples were obtained before this procedure. In some, but not all, patients, DNA was also obtained from the parents. Primers were designed for the amplification of the *RAG* genes based on the sequences reported in databases (*RAG-1*, M29474; *RAG-2*, M94633). *RAG* gene sequence information was obtained as previously specified.[17] Alternatively, the *RAG-1* gene was amplified in 2 segments (94-1852 and 1781-3262), and the *RAG-2* gene was amplified in one segment (1201-2922) with the following primers: *RAG-1*-90F, CTG AGC AAG GTA CCT CAG C; *RAG-1*-1852R, GCC TTC CAA GAT GTC TTC TTC; *RAG-1*-1781F, GCA AAG AGG TTC CGC TAT GA; *RAG-1*-3262R, CAT AAG TGG TTG CCC TAC TT; *RAG-2*-1201F, ATG TCT CTG CAG ATG GTA AC; *RAG-2*-2922R, CTG GCC CTT AAT TCA TGT AAC. Sequencing was performed directly on the PCR products purified from the gel with the Thermosequenase kit (Amersham Pharmacia Biotech UK, Buckinghamshire, United Kingdom). For patients who were compound heterozygotes, mutations were confirmed either by restriction analysis or by analysis of several clones from PCR amplification products cloned in TA vector (Invitrogen) and sequenced by the dideoxynucleotide chain termination method using the Sequenase kit (USB), as previously described.[23] To exclude polymorphisms, at least 100 normal chromosomes were investigated either by analysis of restriction enzyme sites eliminated or created by the mutations or by single-strand conformation polymorphism (SSCP) performed on small (fewer than 250 bp) PCR products amplified with pertinent primers and run according to standard methods. For patient 39, mutations were identified in his parents because no material was available from the affected child.

Results

More than 150 patients with SCID or OS, and negative for other genes known to be involved in SCID, were analyzed for *RAG* mutations. Forty-five patients from 41 unrelated families with *RAG* defects are reported in this paper (Table 1; Figure 1). Thirteen of them have been previously reported (patients 3, 9a with his brother 9b, 12 14, 20 in reference 17; patients 16b, 21, 31, 33-35, 37 in reference 18; patient 26 in reference 24). On the basis of the clinical presentation (Table 2) and the immunologic data (Table 3), we divided our patients into 4 subgroups. Nine patients (patients 1 to 9a) were classified as affected with T-B- SCID, 17 patients (27 to 41 and 16b) were diagnosed with classical OS (in 3 of these patients, maternal fetal engraftment [MFT] was not determined), 7 patients (patients 9b-15) were diagnosed with SCID with MFT, and 11 patients (patients 16a-26) were thought to have atypical SCID/OS (including 4 patients in whom MFT was not determined).

RAG mutations in T-B- SCID

Among 9 patients with T-B- SCID, 6 carried mutations in the *RAG-1* gene, and 3 carried mutations in the *RAG-2* gene. Of these 9 patients, 6 were homozygous for either nonsense (patients 1, 2, 5) or frameshift (patients 4, 6, 8) mutations and were therefore

Table 1. Summary of mutations in *RAG*-deficient patients

Patient	Gene	Nucleotide mutation	Effect	Diagnosis	Status
1	*RAG-2*	G2122A	W307X	T-B-SCID	Homoz
2	*RAG-1*	C1879G	Y589X	T-B-SCID	Homoz
3	*RAG-1*	G2276A	E722K	T-B-SCID	Heter
		G2432T	E774X		
4	*RAG-2*	ins 1665TGTTC	Frameshift	T-B-SCID	Homoz
5	*RAG-1*	T2727A	L872X	T-B-SCID	Homoz
6	*RAG-1*	del 2182-2189	Frameshift	T-B-SCID	Homoz
7	*RAG-1*	del T1173	Frameshift	T-B-SCID	Heter
		T2727A	L872X		
8	*RAG-1*	del 1521-1522	Frameshift	T-B-SCID	Homoz
9a	*RAG-2*	G2634A	C478Y	T-B-SCID	Homoz
10	*RAG-2*	A2622G	N474S	SCID with MFT	Homoz
11	*RAG-1*	A2676T	N8551	SCID with MFT	Homoz
12	*RAG-1*	C2801T	R897X	SCID with MFT	Heter
		G1983A	R624H		
13	*RAG-1*	G2988A	W959X	SCID with MFT	Homoz
9b	*RAG-2*	G2634A	C478Y	SCID with MFT	Homoz
14	*RAG-1*	T2926G	Y938X	SCID with MFT	Homoz
15	*RAG-1*	Del 1258	Frameshift	SCID with MFT	Homoz
16a	*RAG-1*	C1298T	R396C	Atypical SCID/OS	Homoz
17	*RAG-1*	G1533A	R474H	Atypical SCID/OS	Heter
		A2370T	H753L		
18	*RAG-1*	G1409A	V433M	Atypical SCID/OS	Heter
		C1443T	A444V		
19	*RAG-1*	C1631T	R507W	Atypical SCID/OS	Heter
		C1793T	R561C		
20	*RAG-2*	G1887A	R229Q	Atypical SCID/OS	Heter
		Locus deletion			
21	*RAG-1*	A1398G	D429G	Atypical SCID/OS	Heter
		del 368-369	Frameshift		
22	*RAG-1*	C2633T	R841W	Atypical SCID/OS	Heter
		G1341A	R410Q		
23	*RAG-1*	C1443T	A444V	Atypical SCID/OS	Homoz
24	*RAG-2*	C1886T	R229W	Atypical SCID/OS	Homoz
25	*RAG-1*	G1678T	W522C	Atypical SCID/OS	Heter
		G2276A	E722K		
26	*RAG-2*	G1887A	R229Q	Atypical SCID/OS	Homoz
27	*RAG-1*	T1313C	S401P	Omenn	Homoz
28	*RAG-1*	G1095A	C328Y	Omenn	Homoz
29	*RAG-2*	T1818G	F206C	Omenn	Heter
		C1643T	R148X		
30a	*RAG-2*	C1886T	R229W	Omenn	Homoz
30b	*RAG-2*	C1886T	R229W	Omenn	Homoz
31	*RAG-1*	G1299A	R396H	Omenn	Heter
		del 1723-1735	Frameshift		
32	*RAG-1*	A2118G	E669G	Omenn	Heter
		C2812A	C900X		
33	*RAG-1*	C1298T	R396C	Omenn	Heter
		A2847G	Y912C		
34	*RAG-2*	C1324G	C41W	Omenn	Heter
		T2055G	M285R		
35	*RAG-1*	G1794A	R561H	Omenn	Homoz
36	*RAG-1*	G1299T	R396L	Omenn	Heter
		G3036A	R975Q		
16b	*RAG-1*	C1298T	R396C	Omenn	Homoz
37	*RAG-1*	C1793T	R561C	Omenn	Heter
		G2322A	R737H		
38	*RAG-1*	del 368-369	Frameshift	Omenn	Heter
		G2276A	E722K		
39	*RAG-1*	del 368-369	Frameshift	Omenn	Heter
	RAG-1	C1982T	R624C		
40	*RAG-1*	G1789T	R559S	Omenn	Heter
		A1415G	M435V		
41	*RAG-1*	C1443T	A444V	Omenn	Homoz

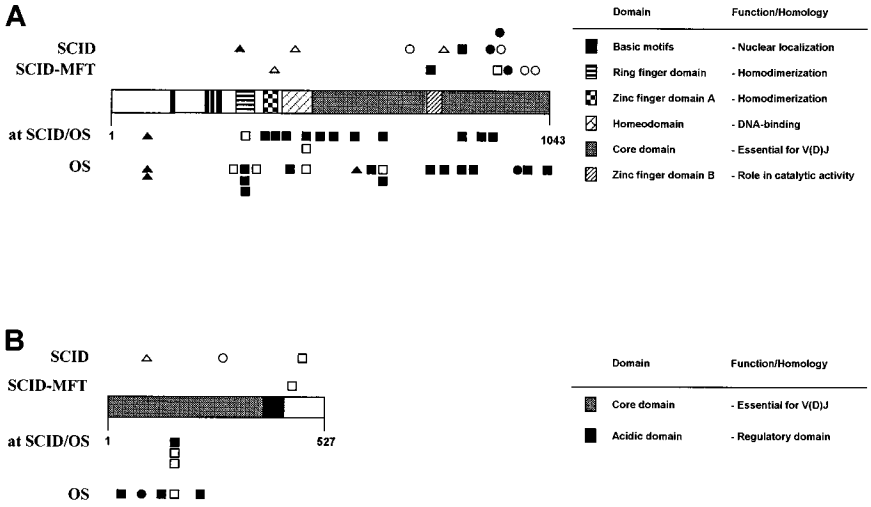

Figure 1. Summary of the position and nature of the mutations in *RAG*-dependent immunodeficiency. Frameshift mutations are indicated by a triangle, nonsense mutations by a circle, and missense mutations by a square. Open symbols refer to homozygous alleles, and filled symbols represent alleles found at the heterozygote status. (A) *RAG-1.* (B) *RAG-2.* In both panels, mutations are shown according to the 4 categories discussed in the text: T-B- SCID (SCID) and SCID with maternal fetal transfusion (SCID/MFT), which are shown above the bars; atypical SCID/OS (at SCID/OS) and Omenn syndrome (OS), which are shown below the bars. The various domains defined in *RAG-1* (A) and *RAG-2* (B) are shown at the right of the panels.

predicted to lack expression of functional protein. One additional patient (patient 7) was a compound heterozygote for 2 severe mutations; furthermore, patient 3 carried a nonsense mutation on one *RAG-1* allele and a missense mutation (E722K) on the other allele, previously shown to be completely inactive.[17] Only patient 9a in this series was homozygous for a *RAG-2* gene missense mutation (C478Y), the product of which was dead for function.[17]

RAG mutations in Omenn syndrome

Seventeen patients from 16 unrelated families (including patient 16b, whose brother was classified as affected with atypical SCID/OS) were defined as having classic OS. In 13 of these families, the mutation affected the *RAG-1* gene, and in 3 it affected the *RAG-2* gene. Seven patients from 6 families had homozygous mutations, whereas the remaining 10 unrelated patients were compound heterozygotes. All patients carried at least one missense mutation. Eleven patients (27-30b, 32, 36, 38-41) from 10 families in this series have not been previously reported and are described here in more detail. Of these, 4 were homozygous for a missense mutation (patients 27, 28, 30, 41). The other 6 patients were compound heterozygotes: 2 of them (patients 36 and 40) had missense mutations on both alleles; 2 (patients 29 and 32) had one missense and one nonsense mutation. The remaining 2 previously unreported patients (patients 38 and 39) had one novel missense mutation on one *RAG-1* allele and a 2-bp deletion on the other. Compound heterozygosity for a missense mutation and for the same 2-bp deletion was detected also in patient 21, who has been previously described.[18] Interestingly, all 3 patients with the 2-bp deletion were of Balkan (Serbian or Albanian) origin.

RAG mutations in SCID with MFT

Seven patients with SCID and MFT were identified through this study. Of these, as shown in Table 1, 5 had *RAG-1* and 2 had *RAG-2* gene mutations. Three patients were homozygous for severe mutations: nonsense mutations in the *RAG-1* gene were detected in patients 13 and 14, whereas a 1-bp deletion in the *RAG-1* gene was found in patient 15. Patient 12 was a compound heterozygote for a nonsense and a missense (R624H) mutation in the *RAG-1* gene; the remaining 3 patients were homozygous for missense mutations in either the *RAG-1* (patient 11) or the *RAG-2* (patients 10 and 9b) gene. The missense mutations of patients 9b and 12 lead to a nonfunctional protein, whereas the functional consequences of the remaining missense mutations are at present undetermined.

RAG mutations in patients with atypical SCID/OS

Altogether, 11 patients (including patient 16a, whose brother was diagnosed as affected with OS) were classified as having atypical SCID/OS, based on their clinical and immunologic phenotypes. Four of these patients (patients 16a, 20, 21, 26) have been previously reported.[17,18,24] Patient 16b had the same homozygous missense *RAG-1* mutation as did his brother. The remaining 7 were either compound heterozygotes for 2 missense mutations in the *RAG-1* gene (patients 17-19, 22, 25) or homozygous for missense mutations in the *RAG-1* (patient 23) or the *RAG-2* (patient 24) gene.

Analysis of the clinical and immunologic phenotypes

For each patient included in the study, clinical and immunologic data are shown in Tables 2 and 3, respectively. Cutaneous manifestations (documented in 31 patients), failure to thrive ($n = 30$), and protracted diarrhea ($n = 27$) were the most common clinical features in our series of 44 patients with *RAG* defects. However, though common manifestations of SCID (failure to thrive, pneumonia, and protracted diarrhea) were similarly documented in all subgroups, other signs were more common in specific cohorts. In particular, generalized erythroderma was documented in 15 of 17 patients with OS and in 7 of 11 patients with atypical SCID/OS SCID, but only in 2 of 7 patients with SCID and MFT and in none of the 7 patients with T-B- SCID. Lymphadenopathy was found only in patients with SCID and MFT (5 of 7 patients) and in patients with OS, the diagnosis of which required the presence of this clinical feature. Hepatomegaly was demonstrated in all 17 patients with OS and was less common in the other subgroups (1 of 9 patients with T-B- SCID; 2 of 7 patients with SCID with MFT; 4 of 11 patients with atypical SCID/OS).

Analysis of the clinical and immunologic phenotypes associated with SCID and MFT revealed a particularly high degree of heterogeneity. Although all 7 patients in this subgroup had failure to thrive, signs resembling OS (erythroderma, skin rash, lymphadenopathy, hepatosplenomegaly) were only present in 5 of them (patients 9b, 12-15). These patients also had higher proportions of circulating T-lymphocytes than patients 10 and 11. Analysis of total lymphocyte and eosinophil counts and of lymphocyte subset

Table 2. Clinical features of *RAG*-defective patients

Patient	Age at onset	Failure to thrive	Erythroderma	Skin rash	Pneumonia	Lymphoadenopathy	Hepatomegaly	Splenomegaly	Protracted diarrhea	Diagnosis
1	4 mo	+							+	SCID
2	2 mo	+			+				+	SCID
3	1.5 mo	+			+					SCID
4	2 mo	+								SCID
5	4 mo	+			+				+	SCID
6	1 mo						+		+	SCID
7	1 wk			+						SCID
8	4 mo	+		+	+				+	SCID
9a	Birth									SCID
10	1 wk	+							+	SCID with MFT
11	2 wk	+							+	SCID with MFT
12	NA	+			+	+				SCID with MFT
13	2 wk	+		+	+	+				SCID with MFT
9b	2 wk	+	+	+	+	+	+	+		SCID with MFT
14	1 mo	+	+	+		+	+	+	+	SCID with MFT
15	1 mo	+		+	+	+			+	SCID with MFT
16a	2 mo*	–	–	–	–	–	–	–	–	Atypical SCID/OS
17	3.5 mo	+							+	Atypical SCID/OS
18	2 mo				+		+		+	Atypical SCID/OS
19	3 mo			+						Atypical SCID/OS
20	2.5 mo		+				+		+	Atypical SCID/OS
21	1.5 mo	+	+	+					+	Atypical SCID/OS
22	1 mo	+	+	+	+				+	Atypical SCID/OS
23	1 mo		+	+	+					Atypical SCID/OS
24	Birth		+	+						Atypical SCID/OS
25	1.5 mo	+	+	+	+		+		+	Atypical SCID/OS
26	2 wk	+	+	+	+		+		+	Atypical SCID/OS
27	1.5 mo			+	+	+	+			OS
28	3 mo			+	+	+	+			OS
29	1 mo	+	+	+	+	+	+	+		OS
30a	4 mo	NA	+		NA	+	+	+		OS
30b	4 mo	NA	+		NA	+	+	+		OS
31	1 mo	+	+	+		+	+			OS
32	3 mo	+	+	+	+	+	+	+	+	OS
33	3 mo		+	+		+	+	+	+	OS
34	1 wk	+	+	+		+	+	+	+	OS
35	1 mo	+	+	+		+	+	+	+	OS
16b	4 mo	+	+	+	+	+	+	+	+	OS
36	4 mo	+	+	+	+	+	+	+	+	OS
37	1 mo	+	+	+	+	+	+	+	+	OS
38	1.5 mo	+	+	+	+	+	+	+	+	OS
39	1 mo	+	+	+	+	+	+	+	+	OS
40	1 mo	+	+	+	+	+	+	+	+	OS
41	3 mo	+	+	+	+	+	+	+	+	OS

*Diagnosed at 2 months of age because an older sibling died with typical OS. The patient immediately underwent allogeneic BMT when still asymptomatic.
NA, not applicable.

distribution and functions in the 4 subgroups of *RAG*-deficient patients is reported in Table 4.

Lymphopenia was documented in a particularly high proportion of patients with T-B- SCID and, to a lesser degree, in patients with atypical SCID/OS: 6 of 8 patients with T-B- SCID and 6 of 11 patients with atypical SCID/OS for which enough information was available had a total lymphocyte count of less than 1500 cells/μL. In contrast, only 2 of 7 patients with SCID and MFT and 4 of 14 patients with OS had such low counts. A high variability in the total lymphocyte count was documented in these latter 2 groups.

Patients with OS had a normal proportion of $CD3^+$ lymphocytes (65.4% ± 5.3%, mean ± SE) compared to normal controls (71% ± 6%). In all the other groups, a low proportion of $CD3^+$ cells was documented, with intermediate values in the atypical SCID/OS (32.3% ± 7.2%) and in the SCID with MFT (23.9% ± 9.3%) groups versus patients with T-B- SCID, who necessarily had very low T-cell counts (0.9% ± 0.5%). Data on the expression of activation markers (DR, CD45R0) were available for the subgroups of patients with OS and atypical SCID/OS. In particular, both groups showed increased percentages of DR^+ cells (OS, 52.3% ± 6%; atypical SCID/OS, 40.5% ± 8.4%) compared with controls (20% ± 3%). Furthermore, the proportion of $CD45R0^+$ cells within $CD4^+$ lymphocytes was markedly increased in patients with OS (94.8% ± 1.9%) and in patients with atypical SCID/OS (95.5% ± 2%) versus age-matched healthy controls (21% ± 9%).

All 4 groups of *RAG*-deficient patients had very low proportions of $CD19^+$ cells; however, residual (more than 3%) B cells were found in 5 of 11 patients with atypical SCID/OS and in 3 of 16 patients with OS. Although the proportion of $CD16^+$ natural killer

Table 3. Immunologic phenotype in 44 patients with *RAG* deficiency

Patient	WBC ($\times 10^3/\mu L$)	Lymph ($\times 10^3/\mu L$)	Eosin ($\times 10^3/\mu L$)	CD3 (%)	CD4 (%)	CD8 (%)	CD19/CD20 (%)	CD16 (%)	CD45RA (% CD4)	CD45R0 (% CD4)	DR (%)	IgG (mg/dL)	IgA (mg/dL)	IgM (mg/dL)	IgE (kU/L)	PHA (cpm $\times 10^3$)	MFT
1	7.2	0.72	0.14	<1	<1	<1	<1	74	ND	ND	ND	<100	<1	<1	ND	ND	ND
2	2.1	0.12	0	<1	<1	<1	<1	80	ND	ND	17	169	31	1	ND	ND	ND
3	3.5	0.35	0.17	2	<1	<1	<1	90	ND	ND	ND	subst.	<5	15	ND	5	−
4	11.0	0.66	0.44	<1	3	8	<1	70	ND	ND	ND	200	<10	<10	138	4	−
5	ND	ND	ND	<5	ND	ND	<5	ND	ND	ND	ND	ND	ND	ND	ND	1	−
6	9.0	0.04	<.05	<1	<1	<1	1	73	ND	ND	10	330	<30	<40	ND	1	ND
7	11.3	4.18	0.34	1	3	42	<1	64	ND	ND	ND	933*	69	56	ND	13	−
8	8.6	0.43	<.05	1	<1	1	<1	91	ND	ND	ND	105	<8	<7	ND	15	−
9a	5.0	2.0	0.25	4	<1	<1	<1	41	ND	ND	ND	460	<10	<10	<2	ND	−
10	2.8	1.12	ND	2	2	<1	<1	70	<1	2	<1	670*	7	8	87	ND	+
11	3.6	0.55	0.18	7	ND	31	1	75	ND	ND	25	110	<5	<5	<5	0.2	+
12	6.0	2.1	0.3	15	4	16	<1	67	ND	ND	ND	618*	32	51	ND	29	+
13	15	10.5	0.15	15	10	11	<1	19	<5	>95	61	4000	18	641	ND	7	+
9b	10	5.0	2.0	70	46	21	<1	41	ND	ND	ND	90	<5	<5	3	12	+
14	5.0	1.5	2.0	15	4	10	<1	67	ND	ND	ND	<250	<5	<5	2.7	28	+
15	20.6	1.6	ND	43.7	39.4	4	2	36.1	<1	93.8	ND	179	191	175	<2	17	+
16a	6.1	2.4	1.4	4	3	<1	<1	88	ND	ND	ND	580*	<7	7	ND	<1	−
17	3.2	0.32	.03	27	25	2	11	50	ND	ND	ND	ND	25	25	ND	49	−
18	1.0	0.19	0.14	27	20	7	4	56	5	95	31	378	5	32	ND	2	ND
19	7.5	3.15	0.6	4	5	56	3	83	ND	ND	18	858	<3	7	ND	1	ND
20	12.9	3.22	4.38	35	27	11	5	50	ND	ND	ND	208	11	<8	21	109	−
21	10.6	0.6	1.36	16	11	10	<1	57	<5	>90	25	350	<6	10	500	3	−
22	3.6	1.01	1.2	20	17	16	15	33	2	98	24	654	17	47	>1000	6.8	−
23	21.0	9.45	1.68	87	53	36	1	13	ND	ND	75	15	<8	<7	45	41	−
24	1.67	0.28	0.78	53	45	15	6	19	ND	ND	70	385	34	20	8500	4.5	ND
25	8.0	1.12	3.84	39	34	16	<1	39	ND	ND	22	120	16	17	5	12	ND
26	21.5	8.6	9.67	43	12	16	<1	46	1	99	59	205	8	87	9100	7.5	−
27	4.5	2.79	1.35	88	83	5	<1	6	<1	99	55	497	ND	ND	166	15	ND
28	1.0	0.64	0.01	58	26	27	<1	30	2.7	96.3	50	493	52	29	19.9	2.5	−
29	5.9	0.71	1.77	58	20	38	<1	35	ND	ND	ND	<100	<5	<5	5	6	−
30a	ND	ND	ND	77	74	4	4	2	ND	ND	72	ND	ND	ND	ND	2.5	−
30b	ND	ND	ND	61	59	8	2	19	8	96	37	ND	ND	ND	ND	ND	−
31	23.5	12.0	6.01	92	24	68	<1	7	8.3	91.7	70	37	<6	12	1	21.3	−
32	20.5	15.17	2.46	93	11	85	<1	11	ND	ND	ND	<130	<100	150	817	110	−
33	45.1	26.29	2.25	83	43	29	<1	7	3	97	72	776	54	74	>3000	1.6	−
34	16.0	5.88	3.37	45	12	35	<1	42	3	96	25	101	<6	6	316	7	−
35	12.0	0.72	4.54	34	32	24	2	43	4	97	27	228	<6	9	190	5.7	−
36	9.5	2.7	1.15	50	ND	ND	16	ND	ND	ND	ND	200	10	50	1690	24	−
16b	23.4	8.2	5.15	92	24	72	<1	ND	ND	ND	72	<33	<7	10	182	32	ND
37	26.4	9.5	5.2	54	48	17	2	ND	ND	ND	74	755*	62	33	45 000	2.5	−
38	10.5	2.94	1.47	49	44	12	3.9	26	<1	97	28	70	13	11	1938	<1	ND
39	24.2	15.0	3.0	80	50	18	0.9	5	21	79	35	60	10	13	2000	2.6	−
40	8.3	5.81	0.59	94	79	13	<1	6	<1	99	83	186	22	20	ND	<1	−
41	2.9	1.0	1.7	31	13	11	<1	68	ND	ND	ND	62	<7	13	ND	<1	−

*Maternally derived IgG.
§Maternal-fetal transfusion.
ND, not done.

cells was increased in all subgroups compared with controls, highest values were found in patients with T-B- SCID (72.9% ± 5.7%), SCID with MFT (53.6% ± 8.2%), and atypical SCID/OS (48.5% ± 7%) versus OS (21.6% ± 5.3%).

The proliferative response to PHA was markedly diminished in all subgroups; however, residual proliferation (greater than 10^4 cpm) was found in 2 of 6 patients with T-B- SCID, 4 of 6 patients with SCID and MFT, 4 of 11 patients with atypical SCID/OS, and 4

Table 4. Immunologic phenotype in 4 subgroups of patients with *RAG* deficiency

	T-B-SCID	SCID with MFT	Atypical SCID/OS	OS	Controls
Lymphocytes ($\times 10^3/\mu L$)	1.1 ± 0.5	3.2 ± 1.3	2.7 ± 1.0	7.2 ± 2.0	4.8 ± 0.35
Eosinophils ($\times 10^3/\mu L$)	0.18 ± 0.05	1.0 ± 0.4	2.28 ± 0.85	2.49 ± 0.47	0.25 ± 0.12
CD3 (%)	0.9 ± 0.5	23.9 ± 9.3	32.3 ± 7.2	65.4 ± 5.3	71.0 ± 6.0
CD16 (%)	72.9 ± 5.7	53.6 ± 8.2	48.5 ± 7.0	21.6 ± 5.3	11.0 ± 4.0
CD19 (%)	0.11 ± 0.11	0.43 ± 0.3	4.1 ± 1.5	1.9 ± 1.0	18.0 ± 5.0
Response to PHA (cpm $\times 10^3$)	6.5 ± 2.4	15.5 ± 4.7	21.4 ± 10.1	13.4 ± 7.1	103.0 ± 15.0

Values are expressed as mean ± SE.

Table 5. Distribution of mutations in patients with *RAG* deficiency

Mutation type	T-B-SCID (No. alleles = 18)	SCID with MFT (No. alleles = 14)	Atypical SCID/OS (No. alleles = 22)	OS (No. alleles = 34)
Missense (%)	3 (16.7)	7 (50)	20 (95.2)	29 (85.3)
Nonsense (%)	8 (44.4)	5 (35.7)	0 (0)	2 (5.9)
Frameshift (%)	7 (38.9)	2 (14.3)	1 (2.4)	3 (8.8)
Gross deletion (%)	0 (0)	0 (0)	1 (2.4)	0 (0)

of 16 patients with OS. Eosinophilia, a well-known feature of OS, was also observed in patients with atypical SCID/OS and, to a lesser degree, in patients with SCID and MFT, but not in patients with T-B- SCID.

Genotype-to-phenotype correlation

The different clinical picture seen in patients with defects in the same gene could be caused by the specific mutations, by other genetic factors, or by epigenetic mechanisms. Mutation analysis at the *RAG* loci failed to indicate a specific association of any of the 4 clinical and immunologic subgroups with the exclusive presence of mutations in either the *RAG-1* or the *RAG-2* gene. In contrast, type of mutation (missense vs nonsense or frameshift) has emerged as a major determinant of the clinical and immunologic phenotype. As shown in Figure 1 and in Table 5, 15 of 18 mutant alleles identified in patients with T-B- SCID were presumably null and were represented by nonsense (n = 8) or frameshift (n = 7) mutations, whereas the remaining 3 missense alleles are functional null mutants.[17] This contrasts the predominance of missense mutations in patients with OS (in whom they accounted for 29 of 34 alleles) and in patients with atypical SCID/OS (in which missense mutations accounted for 20 of 22 alleles). An even distribution of missense and severe (nonsense and frameshift) mutations was documented in patients with SCID and MFT.

To elucidate the role of specific *RAG* abnormalities, we analyzed homozygous or hemizygous patients with mutations affecting the same codon. These include 5 patients from 4 unrelated families in whom the *RAG-2* R229 codon is affected, one with a hemizygous R229Q (patient 20), one homozygous for R229Q (patient 26), one homozygous for R229W (patient 24), and 2 cousins homozygous for R229W (patients 30a and 30b). The 2 brothers had a complete picture of OS, whereas the other 3 were classified as having atypical SCID/OS. In addition, homozygosity for missense mutations at *RAG-1* residue A444 was found in 2 patients (23 and 41), one with a full picture of OS and the other with a diagnosis of atypical SCID/OS.

When only homozygous patients are examined, there is no overlap between SCID and OS. Heterozygosity for a biochemically inactive single amino acid substitution (E722K in *RAG-1*) was shared by one patient with T-B- SCID (patient 3) and another patient with OS (patient 38), but these patients differed for the second mutation. Two different missense mutations affecting codon 624 (R624H in patient 12 and R624C in patient 39) were found in a patient with SCID-MFT and in one patient with OS.

Although these data confirm that OS is caused by mutations that are permissive for *RAG* protein expression (and presumably function), they also indicate that a similar mechanism may apply to *RAG*-dependent atypical SCID/OS. It is unlikely that the distinction between these 2 forms is caused by specific differences in amino acid substitutions because patients 16a and 16b (2 siblings) had distinct phenotypes (atypical SCID/OS and OS, respectively) though sharing homozygosity for the R396C mutation in the *RAG-1* gene. Furthermore, homozygosity for the R229W mutation in the *RAG-2* gene also resulted in either atypical SCID/OS (as for patient 24) or in typical OS (as in siblings 30a and 30b), and the A444V mutation in the *RAG-1* gene, observed in 2 unrelated Turkish patients, also resulted in either atypical SCID/OS (patient 23) or OS (patient 41). This suggests that at least some patients with atypical SCID/OS in fact have Omenn Syndromes with partial expression or "incomplete" OS or OS variants.

RAG1$_{base}$ and *RAG2*$_{base}$ mutation databases

All the reported *RAG-1* and *RAG-2* mutations[17,18] were collected into databases called *RAG1*$_{base}$ and *RAG2*$_{base}$. The databases contain 4 main items: identification of the patient and mutation(s), reference either to published article(s) or a submitting physician, mutation information, and data related to disease and therapy. In addition to mutations, the registries contain information about, for example, lymphocyte counts, age at diagnosis, symptoms, and putative structural consequences of the mutations. The databases were constructed according to the concepts used in BTK$_{base}$, XLA mutation registry,[25] the MUT$_{base}$ system.[26] The *RAG*$_{bases}$ are freely accessible on the World Wide Web at http://www.uta.fi/imt/bioinfo/*RAG1*base and /*RAG2*base.

Discussion

T-B- SCID is a heterogeneous group of diseases, some of which are caused by abnormalities in *RAG* genes.[17,27] OS is also a combined immune deficiency with a peculiar immunologic and clinical phenotype that differs considerably from typical T-B- SCID but that, as previously shown, may be observed in patients with *RAG* mutations.[18] We have previously hypothesized that, as demonstrated in other diseases, the specific type of mutation found in individual patients could be responsible, at least in part, for these differences.[18,22] By investigating a larger number of patients with T-B- SCID and OS, we have shed further light on the molecular basis of *RAG*-dependent immunodeficiency, providing genetic evidence for the existence of 2 different classes of immune defects caused by complete or partial inactivation of *RAG* activity, respectively. In addition, we found an intermediate class of patients we refer to as having atypical SCID/OS, with some T (and occasionally B) cells but without the stigmata of OS.

This conclusion is supported by the following findings described in this paper. When homozygous mutations are considered, patients with severe truncations or premature terminations of *RAG-1* protein in both alleles (null mutations) are only found among those with classical T-B- SCID. Each patient with OS carries at least one allele with missense mutations, and most of them have missense substitutions on both alleles. In addition, though some patients with classical T-B- SCID have missense *RAG-1* mutations, the biochemical studies performed so far have shown that these amino acid changes completely abrogate recombination ability and, thus, represent null alleles, whereas those found

in OS maintain partial activity[17,18] (Gomez et al, manuscript submitted).

Through our study, we identified patients with an intermediate clinical picture, which we classified as atypical SCID/OS. With regard to their molecular basis, this category seems to be more like OS than classic SCID because all these patients carry at least one missense mutation, some of which are shared with OS patients. This conclusion is in agreement with the idea that in these patients, partial *RAG* activity is responsible for the development of a low number of T- (and possibly B-) lymphocytes. It can be speculated that the presence of partial *RAG* activity is a prerequisite for OS but that other epigenetic factors are needed to understand the disease fully. Alternatively, it is possible that individual differences result from early or delayed medical treatment. This contributes to clinical and immunologic heterogeneity, particularly because in some patients bone marrow transplantation performed very early in the course of the disease may eradicate the endogenous autoreactive T-cell clones and prevent the development of typical OS symptoms. One such example is represented in our series by patients 16a and 16b. Patient 16a underwent bone marrow transplantation at 2 months of age, in the absence of typical signs of OS, whereas his elder brother had clinical and laboratory findings of OS at 4 months of age. Finally, the possibility of the occurrence of stochastic events leading to the rearrangement of specific TCR molecules could modify the course of the disease.

It has been hypothesized that some recombinational events do occur in patients with OS and with atypical SCID/OS[18,21] and that they allow the differentiation of a few T- or B-cell clones to occur. Although the persistence of a limited *RAG* function is a prerequisite for this, the specific clinical picture shown by patients can, to some extent, be defined by further genetic or epigenetic (environmental) factors, including exposure to pathogens. Some mutations, such as the one found in patient 22, seem to be relatively mild; this patient showed a substantial number of lymphocytes in the peripheral blood. The possibility that even milder Rag mutations exist and are responsible for a less severe immunodeficiency, and possibly autoimmunity as the only manifestation, is worth investigating.

Additional analysis has revealed that truncated core proteins, encompassing amino acids 384 to 1008 for *RAG-1* and amino acids 1 to 387 for *RAG-2*, are necessary and sufficient to rearrange artificial V(D)J recombination substrates in vitro.[1,28] Our study has identified 3 homozygous missense mutations (Figure 1; Table 1) (*RAG-1*, patient 28; *RAG-2*, patients 9a, 9b, 10) that do not map to the *RAG* core regions.

We therefore conclude that a full description of the in vivo function of the *RAG* proteins cannot be obtained by the use of core proteins. This is supported by a recent study[29] showing that the removal of dispensable regions of *RAG-1* and *RAG-2* impairs proper processing of recombination substrates.

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