

RAG MUTATIONS IN SEVERE COMBINED IMMUNODEFICIENCY AND OMENN'S SYNDROME

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In vertebrates, the adoptive immune system recognizes and responds to many foreign antigens by way of the antigen-specific immunoglobulin/B-cell receptor (Ig/BCR) or T-cell receptor (TCR) molecules expressed on the cell surface of B and T lymphocytes, respectively. The Ig receptor of B cells binds to soluble antigens, whereas the TCR recognizes peptide antigens presented by human leukocyte antigen (HLA) molecules.

BCR and TCR genes are generated through the lymphocyte-specific V(D)J recombination process. Lymphocyte-specific and ubiquitous DNA repair proteins involved in this process have been highly conserved throughout evolution. Naturally occurring mutations in the two lymphocyte-specific V(D)J recombination machinery components, recombinase-activating gene 1 and 2 (*RAG1* and *RAG2*), lead to primary immunodeficiencies, indicating the crucial need for the integrity of V(D)J recombination for lymphoid development. A complete *RAG1* or *RAG2* deficiency causes severe combined immunodeficiency without B and T cells ($B^{-}T^{-}$ severe combined immunodeficiencies [SCID]). In contrast, partial *RAG* defects result in Omenn's syndrome, an immune deficiency with autoreactive manifestations caused by a residual, oligoclonal lymphocyte repertoire.

This work was supported by Grant IZKF CO5 from the Bundesministerium für Bildung und Forschung (KS), and the Telethon Grant E.0917 (AV).

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V(D)J RECOMBINATION

B and T lymphocytes share a common precursor that is subsequently committed to either of these lineages. During the many differentiation steps leading to the production of mature lymphocytes, lymphoid cells acquire specific features that finally distinguish every single cell from the other. Some of the components of these differentiation programs are shared by B and T lymphocytes.^{10, 29}

Central to the differentiation of B and T lymphocytes is the ability, so far described only in these cells, to rearrange germ-line subgenic DNA elements to produce an unlimited variety of somatic genes for membrane receptors that can recognize their cognate antigens.⁴⁷ B cells control humoral immunity through the production of (soluble) antigen-specific immunoglobulins, whereas T cells are responsible for cell-mediated immunity through their T-cell receptors.

The process of antigen receptor gene assembly by the rearrangement of gene segments during lymphocyte development is termed V(D)J recombination.

Each Ig and TCR protein chain consists of two structural parts, the constant and variable regions, the latter being specifically responsible for antigen binding. V(D)J recombination leads to the generation of the variable regions by the assembly of a unique coding frame from a set of Variable (V), Joining (J), and, in some cases, Diversity (D) subgenic elements that are dispersed on the chromosome, coding for a respective antigen receptor chain. During this process, intervening sequences are usually lost from the genome, allowing the formation of fused V(D)J segments, each different from cell to cell:

Recombination is directed specifically to coding elements by recombination signal sequences (RSSs), flanking each receptor gene segment. These RSSs consist of a conserved heptamer that flank the coding region and an AT-rich nonamer. Heptamers and nonamers are separated by a nonconserved spacer of either 12 or 23 nucleotides.^{35, 37, 62} According to the 12/23 rule, efficient recombination occurs only between a pair of subgenic V(D)J segments with RSSs that have different spacer lengths.

Two stages can be recognized during the recombination process. During the first stage, the initiation of recombination is mediated by the heterodimeric endonucleolytic recombination activating genes, RAG1 and RAG2, which bind to the RSSs and introduce a double-strand break (dsb) at the border between a coding element and the heptamer of a RSS. This break generates covalently sealed coding-end hairpins and blunt DNA ends at the RSSs. RAG proteins remain associated with coding ends and signal ends after cleavage.²

The second stage involves the resolution and processing of the coding-end hairpins, which ultimately leads to the formation of modified coding joints. Signal ends are ligated without alteration to produce signal joints.

In vitro evidence has demonstrated that the RAG1/RAG2 complex is involved in the DNA hairpin opening.^{7, 71} The coding ends are processed further by the deletion or addition of nucleotides (N or P), a process which adds more diversity to the variable region of the receptor. Terminal deoxynucleotidyltransferase (TdT), a tightly regulated enzyme, whose expression overlaps that of RAG proteins, is responsible for the adding of N nucleotides, whereas another source of nucleotide insertion (P nucleotides) is caused by the possible asymmetric opening of the coding-end hairpin, which leads to the addition of palindromic nucleotides.

The joining reaction is characterized by the action of a number of ubiquitously expressed proteins involved in DNA dsb repair. These factors include

XRCC4, DNA ligase IV, KU70 and KU80, and the catalytic subunit of the DNA-dependent protein kinase (DNA-PK_{cs}).^{31, 40, 41}

RAG STRUCTURE

RAG1 and RAG2 are essential components of the V(D)J recombination process, as pointed out by the phenotype of murine knock-out models, which show a complete block of T- and B-cell development.^{53, 70} These genes are highly conserved throughout evolution from shark to humans and were originally cloned by their ability to rearrange an integrated artificial V(D)J recombination substrate in a fibroblast cell line.^{58, 67} The two human genes are tightly linked on chromosome 11p13 in a tail-to-tail configuration, and are convergently transcribed.⁵⁸ Their linked chromosomal localization and ability to activate the rearrangement of exogenous substrates have suggested that both genes were possibly acquired because of a transpositional insertion. This suggestion has been supported recently by two independent groups demonstrating that RAG1 and RAG2 possess a transposase-like ability capable of excising *in vitro* an RSS-containing DNA segment and inserting it into unrelated target DNA.^{3, 38} The RAG1 gene codes for 1043 amino acids, and the RAG2 gene codes for 527 amino acids. RAG1 and RAG2 proteins do not share sequence homology.

Mutational analysis has revealed that truncated proteins of approximately 30% and 25% of RAG1 and RAG2, respectively, are still able to carry out the recombination of plasmid substrates in transfected fibroblasts.^{19, 20, 43, 52} These studies have allowed the definition of biologically active core regions encompassing the codons 384 to 1008 for RAG1, and 1 to 387 for RAG2; these deleted mutants have been used widely in *in vitro* tests because they are more soluble.

Despite the ability of the RAG1/RAG2 core molecules to recombine episomal plasmid substrates *in vitro*, it seems likely that the N-terminal portion of RAG1 is not dispensable *in vivo*.⁶⁶ Computer analysis of the sequence of the N-terminal domain of RAG1 reveals amino acid residues highly conserved throughout evolution.^{51, 73} In particular, three basic motifs (BI, BII, and BIII) have been identified as being responsible for the interaction with a nuclear transport protein, Srp-1.^{17, 73} This region could be involved in the correct transport of RAG1 to the nucleus. In addition, a recent study⁷⁵ has shown that the removal of the dispensable regions of RAG1 and RAG2 impairs proper processing of recombination intermediates.

In the RAG1 core segment, the region spanning amino acids 376–477 is crucial for its DNA-binding activity. The RSS nonamer recognition is mediated by a RAG1 domain with homology to the helix-turn-helix structure of homeodomains.^{23, 74} This region of RAG1 is highly conserved during evolution and has sequence similarity to the Hin family of bacterial invertases, whose members also recognize a nonamer-like sequence. Another well-conserved region of RAG1 (amino acids 265–380) containing cysteine-rich domains, including a zinc C3HC3 ring finger flanked by a classic C2H2 zinc finger, has been described as a homodimerization element.^{6, 64} The core region also contributes to the heterodimerization domain that allows interaction with RAG2; it has been mapped in the large region between amino acids 504 and 1008.⁵²

RAG2 alone is unable to bind double-stranded DNA.^{23, 36, 74} The active core of RAG2 located in the first three quarters of the sequence shows a sixfold repeated motif of approximately 50 amino acids related to the kelch/mipp motif.¹³ Despite being dispensable for *in vitro* assays, the last quarter of the protein seems to play an essential role for efficient recombination of Ig heavy-

chain segments⁴⁴ and shows highly conserved amino acids in all species examined so far. It contains a plant homeodomain finger-like motif, believed to mediate association with chromatin proteins. A conserved degradation signal, including an essential CDK phosphorylation site at threonine 490, is responsible for the rapid degradation of RAG2 during the cell cycle.²²

RAG EXPRESSION

RAG proteins are expressed at the earliest stages of lymphocyte development and form a complex localized at the periphery of the nucleus.⁷³

In immature lymphocytes, *RAG1* and *RAG2* are tightly regulated, and their expression is not only stage-specific but also depends on the cell cycle.²² The isolation and characterization of promoter and enhancer elements helped to better define the tight temporal and spatial regulation of the *RAG* genes. Basal promoters for the murine and human *RAG1* and human *RAG2* gene have been described,^{11, 25, 79} but none shows tissue specificity. Highly conserved regions in these promoters support the idea that the mechanism of their regulation is conserved. A minimal basal promoter of *RAG1* (nucleotide [nt] -111 to +97 relative to the position of the major transcriptional start site) has been isolated and shows several potential recognition sites for the E2A, Ikaros, and Ets family transcription factors. A reduction of the *RAG1* promoter activity is caused by the mutagenesis of the *RAG1* Y-promoter box motif that is responsible for the binding of the nuclear factor Y (NF-Y). The mouse and human *RAG1*-promoter region lacks a TATA sequence, but several transcription initiation sites closely match the consensus sequence for the initiator element. Recently, the murine *rag2* promoter has been cloned and sequenced.⁴⁵ Its analysis has revealed that the promoter is regulated differently in B and T cells. In particular, the B-cell specific transcription factor BSAP (Pax-5)—a transcription factor expressed only in B cells, the central nervous system, and the testes—binds to a critical element in the *rag2* promoter, whereas a more distal region of the *rag2* promoter is essential for its activity in T cells.

Recently, the re-expression of *rag1* and *rag2* genes has been demonstrated in mouse germinal center B cells, and the possibility of a secondary V(D)J recombination reaction leading to the modification of the antibody repertoire of peripheral B cells has been implied.^{33, 56} Murine peritoneal B-1B cells (a self-renewing population of B cells involved in autoantibody production) also can transcribe *rag1* and *rag2* and undergo secondary immunoglobulin-gene rearrangements.⁶¹

B-T⁻ SCID: A COMPLETE RAG DEFICIENCY

Clinical and Laboratory Findings

Glanzmann and Riniker²⁸ first described siblings with diarrhea, candidiasis, and lymphopenia. Thymic dysplasia and hypogammaglobulinemia subsequently were described in immunodeficiency patients.^{18, 39} The knowledge of the antigen-receptor gene structures and their recombination allowed the identification of infants with SCID who were defective in V(D)J recombination.^{1, 68} Based on these findings, the authors identified mutations of the *RAG* genes in patients with B-T⁻ severe combined immunodeficiencies.⁶⁹

Severe combined immunodeficiency has an estimated incidence of about

1:100,000 live births.⁷⁶ The clinical presentation is relatively uniform. During pregnancy, birth, and within the first weeks of life, complete RAG-deficient patients with SCID generally exhibit no overt symptoms. Infectious complications start within the second or third month after birth. These children may present with opportunistic infections; chronic, persistent disease of the airways; therapy-resistant candidiasis; and local or systemic bacterial infections (e.g., otitis, mastoiditis, purulent rhinitis, conjunctivitis, sepsis, meningitis, arthritis, and abscesses). The recurrent infections and a chronic enteritis lead to a failure to thrive. Viruses (e.g., Epstein-Barr virus, cytomegalovirus) may cause lethal complications.

Noninfectious clinical symptoms may result from graft-versus-host disease (GVHD), caused by allogeneic cell transfusions with nonirradiated blood components or a maternal-fetal transfusion. Vaccination with live viral or bacterial organisms may have a fatal outcome.

A physical examination of patients with complete RAG deficiency reveals infections and characteristically the absence of lymph nodes and tonsils.

The patients with RAG defects exhibit no mature B and T cells in the peripheral blood (B⁻T⁻ SCID). Maternal T cells are detectable in more than 50% of the cases. The reactivity of these maternal cells in *in vitro* and *in vivo* lymphocyte-function analysis has to be accounted for. Natural killer (NK) cell numbers and function are not affected by the RAG mutations. After the decline of the initial maternal Ig, no antibodies circulate in the peripheral blood.

No simple tests exist that reveal V(D)J recombination defects in general or a specific RAG defect. Protein analysis is not a practical alternative, because the relevant cells are a minority in the bone marrow cell population or are amenable to analyses only after thymic biopsy. Only a direct or indirect (e.g., linkage analysis) genetic analysis of the RAG genes reveals their mutational status. Carriers of the mutant RAG genes are healthy without any immunologic disturbances; therefore, they are detected only through a molecular identification of the mutation in question. Genetic prenatal diagnosis of a known RAG defect has been performed (K. Schwarz, MD, unpublished data); alternatively a B⁻T⁻ immunophenotype in cord blood may suggest a RAG mutation.

The treatment of children with RAG-defects combines supportive, anti-infectious measures and the curative therapy of peripheral stem-cell or bone marrow transplantation. The survival of the transplantation procedure in the small RAG-deficient group presently equals 60% to 70%.

MOLECULAR ANALYSIS OF PATIENTS WITH COMPLETE RAG DEFICIENCY

Mutation analysis in 14 B⁻ SCID cases detected 6 patients with RAG defects (Table 1), encompassing missense, nonsense, and deletional mutations.⁶⁹

All missense mutations in the RAG1 gene map to phylogenetically conserved residues in the active core; the RAG2-R229Q substitution locates to the active core, whereas the C478Y exchange maps to the C-terminal region of RAG2. All of the mutations were inherited, with consanguinity contributing to the Y938X-RAG1 and C478Y-RAG2 mutations.

The RAG mutations did not affect steady-state RAG RNA or protein levels nor did they influence subcellular RAG1 or RAG2 localization. In transient transfection experiments with artificial extrachromosomal rearrangement substrates, all these mutants were practically devoid of V(D)J recombination activity ($\leq 1\%$ wild-type, Fig. 1). The few signal and coding joints recovered seemed

Table 1. RAG GENE MUTATIONS WITH THEIR CONSEQUENCES IN PATIENTS WITH B⁻ T⁻ SCID OR OMENN'S SYNDROME

Disease	Gene	Mutation	Region Affected
B ⁻ T ⁻ SCID	RAG1	E722K	Active core
		E774X	Active core, truncated protein
B ⁻ T ⁻ SCID	RAG1	Y938X	Active core, truncated protein
B ⁻ T ⁻ SCID	RAG1	R897X	Active core, truncated protein
		R624H	Active core
B ⁻ T ⁻ SCID*	RAG2	R229Q	Active core
		Deletion encompassing RAG1 and RAG2	
B ⁻ T ⁻ SCID	RAG2	C478Y	Active core
Omenn's	RAG1	R561H	RAG2 interaction
Omenn's	RAG1	R396C	Homeodomain (DNA binding)
Omenn's	RAG1	R396H	Homeodomain (DNA binding)
		Deletion 1723-1735nt	Out of frame, predicted truncated protein
Omenn's	RAG1	R396C	Homeodomain (DNA binding)
		Y912C	Active core
Omenn's	RAG1	D429G	Homeodomain
		Deletion 368-369nts	Loss of N-terminal domain, caused by possible usage of downstream ATG codon
Omenn's	RAG1	R561H	RAG2 interaction
		R737H	Active core
Omenn's	RAG2	C41W	Active core
		M285R	Active core

*This patient also bears an A156V change in RAG1 considered as a polymorphism.

qualitatively normal, indicating that the mechanisms of dsb repair are not affected in individuals with complete RAG-deficiency.

In the cases of complete RAG deficiencies, V(D)J recombination cannot be initiated; thus, IgM and TCR- β chains are not synthesized and pre-B-/pre-T-cell receptors are not assembled. Pre-B and pre-T cells do not survive during lymphocyte development if they do not receive a survival-proliferation signal from their pre-B-/pre-T-cell receptors. The lymphocytosis of complete RAG deficiency is caused by the premature death of these lymphocyte precursors.

OMENN'S SYNDROME: A PARTIAL RAG DEFICIENCY

Clinical and Laboratory Findings

The first report of this peculiar combined immunodeficiency with hyper-eosinophilia was described by Gilbert Omenn in 1965 in relation to a large inbred Irish Catholic family.⁵⁹

Omenn's syndrome (OS), in contrast to other combined immunodeficiencies, presents with a number of unusual clinical and laboratory features. The early onset of a severe and generalized exudative erythrodermia, resembling acute graft-versus-host disease, is the hallmark. Lymphadenopathy and hepatosplenomegaly, generalized edema caused by hypoprotidemia (protein loss through the skin and chronic diarrhea), are usually present. At variance with other forms of

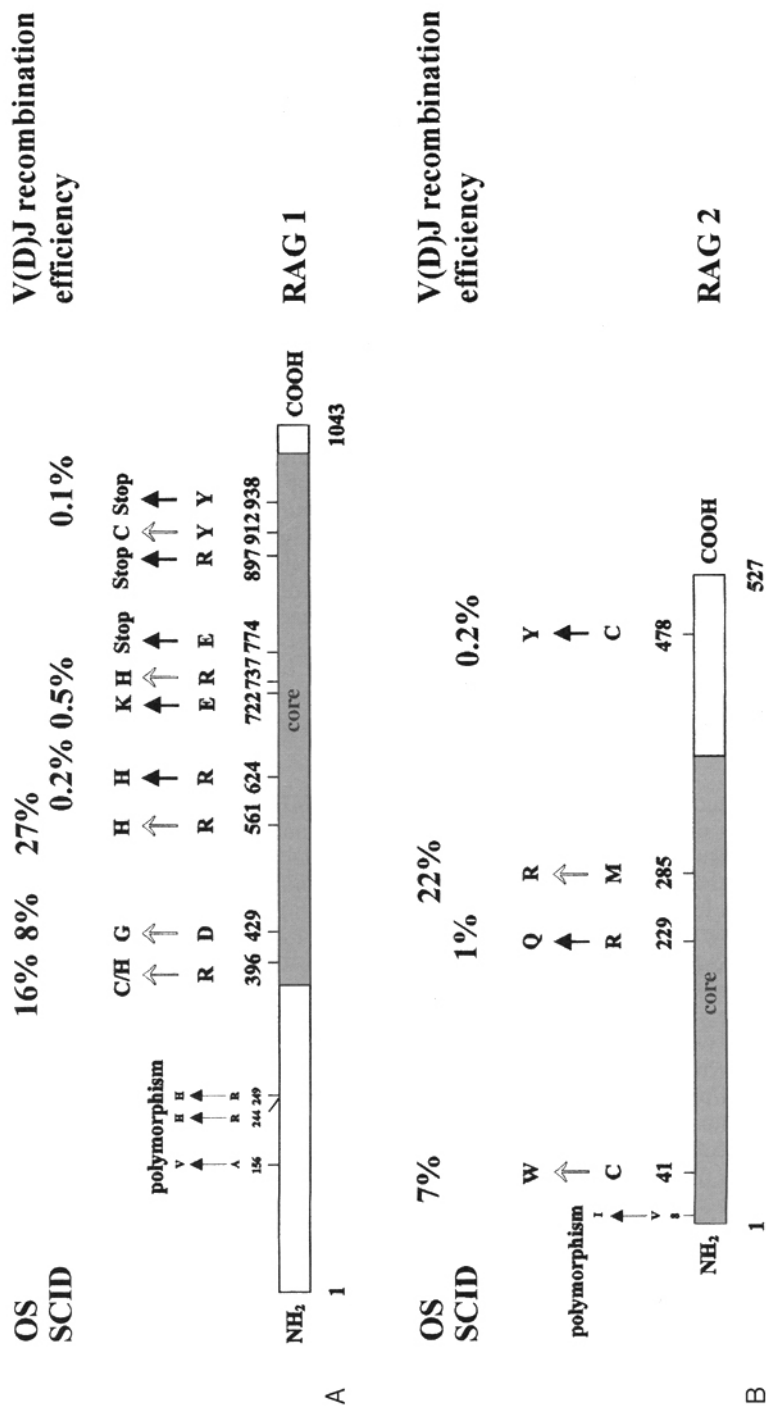


Figure 1. V(D)J recombination efficiency of B-T⁻ SCID and Omenn's syndrome RAG defects. Mutational and functional analysis of RAG1 (A) and RAG2 (B) and representation of the full-length human RAG1 and RAG2 protein. Mutations identified in patients with B-T⁻ SCID (solid arrows) and in children with Omenn's syndrome (OS) (open arrows) and polymorphic amino acids are indicated above the diagram of the genes. The residual V(D)J recombination capability of some mutants, as measured in a transient transfection assay with extrachromosomal artificial V(D)J recombination substrates, is related to 100% wild-type activity in the respective assays.

SCID, patients with OS have a variable number of circulating T cells, coexpressing activation markers, such as CD45R0, HLA-DR, CD25, CD95, CD30, and CD70.^{12, 15, 50} Despite this activated phenotype, T cells are poorly responsive to in vitro stimulation by mitogens and antigens. B cells are strongly reduced in number, with consequent low levels of immunoglobulins, but high levels of IgE, whose origin is poorly understood. The in vivo antibody response to T-dependent antigens is impaired, but not completely abrogated and restricted to IgM.^{46, 57}

The remarkable hypereosinophilia and the increased IgE serum levels suggest skewing towards a Th2 phenotype, as supported by the predominant IL-4 and IL-5 production and a strong reduction of the Th1 cytokines IL-2 and IFN- γ .^{12, 15, 16}

An analysis of the T-cell repertoire in patients with OS reveals the presence of a restricted oligoclonality.^{34, 63, 72} The limited usage of distinct variable gene segments could reflect the preferential recruitment of TCR segments caused by the basic defect in the V(D)J recombination process or it could be the result of antigen selection. Imberti et al have recently shown that in the only patient with OS analyzed so far at the peripheral tissue and the thymus level, oligoclonal T cells were already present at the thymic level,⁷² although different percentages of the same clones were detected in different peripheral tissues. Based on these finding, it has been suggested that the restricted repertoire is caused by a defect in the recombination process, but that antigen encounter allows the expansion of selected clones.

MOLECULAR ANALYSIS OF PATIENTS WITH OMENN'S SYNDROME

The restricted T-cell repertoire of patients with OS suggested a possible defect in one of the proteins involved in the recombination process. Initially, a RAG involvement in OS seemed unlikely, but the description of a family in which one member was affected by B⁻ T⁻ SCID and another by OS²¹ prompted an investigation for RAG gene mutations.

The analysis of RAG genes in seven patients fulfilling the criteria for Omenn's syndrome has revealed mutations in the RAG1 (six cases) or RAG2 (one case) genes.⁷⁸ Patients with OS had mutations in both alleles (see Table 1), according to the autosomal recessive inheritance of the disease.

The patient with a RAG2 defect carried two missense mutations, C41W and M285R, located in the active core at the end of the putative 2-3 loop of the β propeller model proposed by Callebaut and Mornon.¹³

Among patients with RAG1 gene defects, two subjects were homozygous for the amino acid substitutions R561C and R396C. A third case bore a missense mutation on the first allele, leading to a D429G exchange and a deletion of 2 nts (368-369) at the 5' portion of the second allele. All these mutations involve amino acids highly conserved during evolution; in particular, R396 and D429 located in the homeodomain region of RAG1 are responsible for the recognition and binding to the nonamer motif of the RSS. R561 maps to the region mediating the interaction with RAG2. A fourth case showed a deletion of 13 nts resulting in a frame shift and premature stop codon in one allele, whereas the second allele carried a missense mutation at the codon 396 and lead to a different amino acid change R396H. RAG1 missense mutations, R561C, R737H and Y912C, and R396C, were present in the other two examined patients, respectively.

This analysis demonstrated the presence of at least one missense mutation in

every patient, suggesting the possibility of a residual RAG activity. In addition, a comparison with the mutations present in patients with raised B⁻T⁻ SCID raised the possibility that OS mutations involve specific domains. These hypotheses were tested at the biochemical level by performing a series of assays that investigated different aspects of RAG function.

The analysis of the mutants for DNA binding to RSS, cleavage activity, homodimerization ability, RAG1-RAG2 interaction and for the recombination activity on extrachromosomal substrates⁷⁸ has confirmed our assumptions and allowed us to better understand the mechanisms leading to the particular defects observed in OS. The mutations affecting the RAG1 homeodomain region (R396C, R396H, D429G) lead to a decreased but not absent DNA-binding activity and cleavage, whereas R561H determines a reduced level of interaction with RAG2. All these mutants, together with the RAG2 mutants (C41W and M285R), showed a reduced but not abolished recombination activity with extrachromosomal substrates in reconstituted cells (see Fig. 1).

Genetic and biochemical data suggest that OS is a B-T⁺ SCID caused by mutations that impair but do not abolish the V(D)J recombination activity of RAG1 or RAG2. The mutations seem to affect the correct formation of the stable complex between RSSs, RAG proteins and other potential molecules involved in the cleavage step of V(D)J recombination. According to this hypothesis, a few productive recombination events still occur in thymic lymphocytes. These T lymphocytes exit from the thymus and are selected and expanded in the periphery, where, for reasons which are poorly understood, they give rise to a CD4⁺ cell distribution skewed toward the Th2 subset. This alteration in the balance between T lymphoid subsets is probably responsible for some aspects of OS that for a long time have contributed to the enigmatic features of this disease.

DEFECTS IN V(D)J COMPONENTS OTHER THAN RAG

After the initial generation of the DNA dsbs at RSSs through the RAGs, the final step of the recombination process is completed by ubiquitously expressed factors (Table 2). Several recent reports extensively reviewed the biochemical pathways involved in the end joining processes of V(D)J recombination and dsb repair^{30, 40, 41}; therefore, the concentration is on animal models with site-specific recombination defects and their possible relationship to human immunodeficiencies.

Genetic studies defined the central role of the heterotrimer DNA-dependent protein kinase (DNA-PK) in the DNA nonhomologous end-joining (NHEJ) process. DNA-PK components include the heterodimeric KU70 and KU80 proteins and the catalytic subunit of DNA-PK (DNA-PK_{cs}).^{30, 40, 41} DNA-bound KU recruits and activates DNA-PK_{cs}, which phosphorylates, among others, a series of factors that are involved in DNA-dsb repair.

KU70 and KU80 null-mutant mice (KU70^{-/-}, KU80^{-/-}) were generated through homologous recombination.^{32, 55, 60, 80} Both strains are proportional dwarfs with about 50% wild-type weight from neonates through adults. Primary fibroblasts of KU70^{-/-} and KU80^{-/-} mice are irradiation sensitive, and display signs of premature senescence.

KU70^{-/-} and KU80^{-/-} mice are severely immunodeficient, with B cells lacking and T cells reduced in the periphery. The lymphocyte depletion is caused by reduced signal and coding joint formation in V(D)J recombination.^{8, 32, 55, 60, 80}

KU70 and KU80 are required in Ig heavy chain class switch recombination, as previously has been suggested for DNA-PK_{cs}.^{14, 49, 65}

Table 2. COMPONENTS OF THE V(D)J RECOMBINASE

	Human Gene Locus	Human Disease	Animal Model
<i>Lymphocyte-Specific Components</i>			
RAG1	11p13	B ⁻ T ⁻ SCID/ Omenn's syndrome	KO mouse
RAG2	11p13	B ⁻ T ⁻ SCID/ Omenn's syndrome	KO mouse
TdT	10p23-24	—	KO mouse
<i>DNA Double-Strand Break Repair Components</i>			
KU70	22q13	—	KO mouse
KU80	2q33-35	—	KO mouse
DNA-PK _{cs}	8q11	—	<i>scid</i> mouse KO mouse Equine SCID
XRCC4	5q13-14	—	KO mouse
Ligase IV	13q33-34	—	KO mouse

KO = knockout.

In the *scid* mouse, the 460-kd PI3-like DNA-PK_{cs}, the product of the *prkdc* gene, is inactivated through a premature stop codon resulting in an 83 amino acid C terminally truncated protein. DNA-PK_{cs} protein levels in *scid* mice are approximately 10% of wild-type, with barely any DNA-PK activity detectable. Mice with DNA-PK_{cs} defects are not growth-retarded, but are sensitive to ionizing radiation. Because of severe defects in V(D)J coding joint formation (up to a 1000-fold reduction), the mice with DNA-PK_{cs} deficiency are profoundly T- and B-cell deficient. Signal joint formation is reduced only about tenfold, but many of these joints exhibit deletional anomalies compared with the wild-type. These features of the *scid* mouse were independently confirmed by intentional *prkdc* inactivation.^{9, 26, 42, 77}

An additional component of the DNA-dsb repair pathway, the x-ray cross-complementing group 4 (XRCC4) gene product was identified on the basis of its ability to complement the x-ray-sensitive XR-1 cell line for V(D)J recombination and DNA-dsb repair.⁴⁸ In mice, XRCC4 deficiency causes late embryonic lethality accompanied by a defective lymphogenesis and neurogenesis.²⁷ Thymic T-cell development is arrested at the CD4⁻CD8⁻ (DN) CD44⁺ progenitor stage, slightly different than in RAG^{-/-} mice, who proceed to the CD44⁻CD25⁺DN stage. B cells are arrested at the B220⁺IgM⁻ stage, reminiscent of the block in RAG^{-/-} B-cell development. Severe cell death throughout the developing central nervous system and peripheral sensory ganglia, including the dorsal root ganglia is observed in postmitotic neuronal populations, whereas proliferative progenitors are spared. Primary fibroblasts of XRCC4^{-/-} mice exhibit irradiation sensitivity and premature senescence.

XRCC4 associates with DNA ligase IV and stimulates its activity in vitro. A targeted disruption of both human ligase IV alleles in a pre-B-cell line rendered the cells irradiation sensitive and ablated V(D)J recombination.³¹ In vivo ligase IV^{-/-} mice revealed a phenotype identical to XRCC4^{-/-}-defective animals^{4, 24}; thus, the XRCC4^{-/-} phenotype may be explained in the context of the functional

association with ligase IV. A common feature of these animal models is the increased sensitivity to ionizing radiation. In irradiation-sensitive primary fibroblast lines, established from patients with B⁻ SCID⁵⁴ (K. Schwarz, MD, and A. Villa, MD, unpublished data), all candidate genes of the NHEJ process have proved so far to be negative for mutations of DNA-dsb repair components. In contrast, these studies even suggested possible novel factors involved in human V(D)J recombination and DNA-dsb repair. This implication is supported by fractionation studies of human cell extracts for DNA end-joining components.⁵

SUMMARY

RAG gene mutations determine different forms of primary immunodeficiencies. A complete lack of RAG function leads to a block of B- and T-lymphocyte development resulting in a B⁻T⁻ SCID. On the other hand, hypomorphic RAG alleles allow T-cell but not B-cell differentiation, which is observed in patients with Omenn's syndrome. These observations confirm that an undisturbed generation of T- and B-cell repertoires is required for effective immune responses, and that B-cell development tends to rely more strictly on an intact V(D)J recombination machinery. The observation that several of the OS clinical features resemble autoimmune manifestations raises the question whether RAG mutations may contribute to human autoimmune diseases. Presently, RAG defects can be confirmed reliably at the genetic level. The knowledge of RAG mutations in diseased children helps to facilitate prenatal and family diagnoses. RAG deficiencies are treated by provision of allogeneic hematopoietic stem cells. At present, gene therapy is not a therapeutic option, but it might become an attractive alternative in the future.

ACKNOWLEDGMENT

The authors are grateful to all contributors in the human primary immunodeficiency field and in the antigen receptor rearrangement field. They apologize to those whose work is not cited because of space limitation of the review, and thank A. Jacobs for excellent secretarial assistance.

References

1. Abe T, Tsuge J, Kamachi Y, et al: Evidence for defects in V(D)J rearrangements in patients with severe combined immunodeficiency. *J Immunol* 152:5504, 1994
2. Agrawal A, Schatz DG: RAG1 and RAG2 form a stable postcleavage synaptic complex with DNA containing signal ends in V(D)J recombination. *Cell* 89:43, 1997
3. Agrawal A, Eastman Q, Schatz D: Transposition mediated by Rag1 and Rag2 and its implications for the evolution of the immune system. *Nature* 394:744, 1998
4. Barnes DE, Stamp G, Roswell I, et al: Targeted disruption of the gene encoding DNA ligase IV leads to lethality in embryonic mice. *Curr Biol* 8:1395, 1998
5. Baumann P, West SC: DNA end-joining catalyzed by human cell-free extracts. *Proc Natl Acad Sci U S A* 95:14066, 1998
6. Bellon SF, Rodgers KK, Schatz DG, et al: Crystal structure of the RAG1 dimerization domain reveals multiple zinc-binding motifs, including a novel zinc binuclear cluster. *Nat Struct Biol* 7:586, 1997
7. Besmer E, Mansilla-Soto J, Cassard S, et al: Hairpin coding end-opening is mediated by Rag1 and Rag2 proteins. *Mol Cell* 2:817, 1998

8. Bogue MA, Wang C, Zhu C, et al: V(D)J recombination in KU-deficient mice: Distinct effects on coding, signal, and hybrid joint formation. *Immunity* 7:37, 1997
9. Bogue MA, Jhappan C, Roth DB: Analysis of variable (diversity) joining recombination in DNA-dependent protein kinase (DNA-PK)-deficient mice reveals DNA-PK-independent pathways for both signal and coding joint formation. *Proc Natl Acad Sci U S A* 95:15559, 1998
10. Boyd RL, Hugo P: Towards an integrated view of thymopoiesis. *Immunol Today* 12:72, 1991
11. Brown S, Miranda C, Galic Z, et al: Regulation of the Rag1 promoter by the NF-Y transcription factor. *J Immunol* 158:5071, 1997
12. Brugnani D, Airò P, Facchetti F, et al: In vitro cell death of activated lymphocytes in Omenn's syndrome. *Eur J Immunol* 27:2765, 1997
13. Callebaut, Moron JP: The V(D)J recombination activating Rag2 consists of a six-bladed propeller and a PHD finger-like domain, as revealed by sequence analysis. *Cell Mol Life Sci* 54:880, 1998
14. Casellas R, Nussenzweig A, Waerffel R, et al: KU80 is required for immunoglobulin isotype switching. *EMBO J* 17:2404, 1998
15. Chilosi M, Facchetti F, Notarangelo LD, et al: CD30 cell expression and abnormal soluble CD30 serum accumulation in Omenn's syndrome: Evidence for a T helper 2-mediated condition. *Eur J Immunol* 26:329, 1996
16. Chilosi M, Pizzolo G, Facchetti F, et al: The pathology of Omenn's syndrome. *Am J Surg Pathol* 20:773, 1996
17. Cortes P, Ye ZS, Baltimore D: Rag-1 interacts with the repeated amino acid motif of the human homologue of the yeast protein SRP1. *Proc Natl Acad Sci U S A* 91:7633, 1994
18. Cottier H: Zur Histopathologie des Antikörpermangel-Syndroms. In *Transaction of 6th Congress of the European Society for Haematology*, Copenhagen, 1957. Basel, S Karger, 1958
19. Cuomo CA, Oettinger MA: Analysis of regions of Rag2 important for V(D)J recombination. *Nucleic Acids Res* 22:1810, 1994
20. Cuomo CA, Mundy CL, Oettinger MA: DNA sequence and structure requirements for cleavage of V(D)J recombination signal sequences. *Mol Cell Biol* 16:5683, 1996
21. De Saint Basile G, Le Deist F, De Villartay JP, et al: Restricted heterogeneity of T lymphocytes in combined immunodeficiency with hypereosinophilia (Omenn's syndrome). *J Clin Invest* 87:1352, 1991
22. Desiderio S, Lin WC, Li Z, et al: The cell cycle and V(D)J recombination. *Curr Top Microbiol Immunol* 217:45, 1996
23. Difilippantonio M, McMahan CJ, Eastman QM, et al: Rag1 mediates signal sequence recognition and recruitment of Rag2 in V(D)J recombination. *Cell* 87:253, 1996
24. Frank KM, Sekiguchi JM, Seidl KJ, et al: Late embryonic lethality and impaired V(D)J recombination in mice lacking DNA ligase IV. *Nature* 396:173, 1998
25. Fuller K, Storb U: Identification and characterization of the murine Rag1 promoter. *Mol Immunol* 34:939, 1997
26. Gao Y, Chaudhuri J, Zhu C, et al: A targeted DNA-PK ϵ null-mutation reveals DNA-PK-independent functions for KU in V(D)J recombination. *Immunity* 9:367, 1998
27. Gao Y, Sun Y, Frank KM, et al: A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. *Cell* 95:891, 1998
28. Glanzmann E, Riniker P: Essentielle Lymphocytophtisie: Ein neues Krankheitsbild aus der Säuglingspathologie. *Ann Pediatr* 175:1, 1950
29. Glimcher LH, Singh H: Transcription factors in lymphocyte development: T and B cells get together. *Cell* 96:13, 1999
30. Grawunder U, West RB, Lieber MR: Antigen receptor gene rearrangement. *Curr Opin Immunol* 10:172, 1998
31. Grawunder U, Zimmer D, Fugmann S, et al: DNA ligase IV is essential for V(D)J recombination and DNA double-strand break repair in human precursor lymphocytes. *Mol Cell* 2:477, 1998
32. Gu Y, Seidl KJ, Rathbun GA, et al: Growth retardation and leaky SCID phenotype of KU70-deficient mice. *Immunity* 7:653, 1997

33. Han S, Zheng B, Schatz DG, et al: Neoteny in lymphocytes: Rag1 and Rag2 expression in germinal center B cells. *Science* 274:2094, 1996
34. Harville TO, Adams DM, Howard TA, et al: Oligoclonal expansion of CD45RO+ T lymphocytes in Omenn's syndrome. *J Clin Immunol* 17:322, 1997
35. Hesse JE, Lieber MR, Mizuuchi K, et al: V(D)J recombination: A functional definition of the joining signals. *Genes Dev* 3:1053, 1989
36. Hiom K, Gellert M: A stable Rag1-Rag2 DNA complex that is active in V(D)J cleavage. *Cell* 88:65, 1997
37. Hiom K, Gellert M: Assembly of a 12/23 paired signal complex: A critical control point in V(D)J recombination. *Mol Cell* 1:1011, 1998
38. Hiom K, Melek M, Gellert M: DNA transposition by the Rag1 and Rag2 proteins: A possible source of oncogenic translocations. *Cell* 94:463, 1998
39. Hitzig WH, Biro Z, Bosch H, et al: Agammaglobulinämie und Alymphozytose mit Schwund des lymphatischen Gewebes. *Helv Pädiatr Acta* 13:55, 1958
40. Jeggo PA: DNA breakage and repair. *Adv Genet* 38:185, 1998
41. Jeggo PA: Identification of genes involved in repair of DNA double-strand breaks in mammalian cells. *Radiat Res* 15(suppl 5):80, 1998
42. Jhappan C, Morse III HC, Fleischmann RD et al: DNA PK ϵ : T-cell tumor suppressor encoded at the mouse scid locus. *Nat Genet* 17:483, 1997
43. Kirch SA, Sudarsanam P, Oettinger MA: Regions of Rag1 protein critical for V(D)J recombination. *Eur J Immunol* 26:886, 1996
44. Kirch SA, Rathbun G, Oettinger MA, et al: Dual role of Rag2 in V(D)J recombination: Catalysis and regulation of ordered Ig gene assembly. *EMBO J* 17:4881, 1998
45. Lauring J, Schlissel MS: Distinct factors regulate the murine RAG-2 promoter in B- and T-cell lines. *Mol Cell Biol* 19:2601, 1999
46. Le Deist F, Fischer A, Durandy A, et al: Déficit immunitaire mixte et grave avec hyperéosinophilie. *Arch Fr Pédiatr* 42:11, 1985
47. Lewis SM: The mechanisms of V(D)J joining: Lessons from molecular, immunological, and comparative analyses. *Adv Immunol* 56:27, 1994
48. Li Z, Otevrel T, Gao Y, et al: The XRCC4 gene encodes a novel protein involved in DNA double-strand break repair and V(D)J recombination. *Cell* 83:1079, 1995
49. Manis JP, Gu Y, Lansford R, et al: Ku70 is required for late B-cell development and immunoglobulin heavy chain class switching. *J Exp Med* 187:2081, 1998
50. Martin JV, Willoughby PB, Giusti V, et al: The lymph node pathology of Omenn's syndrome. *Am J Surg Pathol* 19:1082, 1995
51. McMahan CJ, Difilippantonio J, Rao N, et al: A basic motif in the N-terminal region of RAG1 enhances V(D)J recombination activity. *Mol Cell Biol* 17:4544, 1997
52. McMahan CJ, Sadofsky MJ, Schatz DG: Definition of a large region of Rag1 that is important for coimmunoprecipitation of Rag2. *J Immunol* 158:2202, 1997
53. Mombaerts P, Iacomini J, Johnson RS, et al: RAG-1 deficient mice have no mature B and T lymphocytes. *Cell* 68:869, 1992
54. Nicolas N, Moshous D, Cavazzana Calvo M, et al: A human severe combined immunodeficiency (SCID) condition with increased sensitivity to ionizing radiations and impaired V(D)J rearrangements defines a new DNA recombination/repair deficiency. *J Exp Med* 188:627, 1998
55. Nussenzweig A, Chen C, Da Costa Seares V, et al: Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. *Nature* 382:551, 1996
56. Nussenzweig MC: Immune receptor editing: Revise and select. *Cell* 95:875, 1998
57. Ochs HD, Davis SD, Mickelson E, et al: Combined immunodeficiency and reticuloendotheliosis with eosinophilia. *J Pediatr* 85:463, 1974
58. Oettinger MA, Schatz DG, Gorka C, et al: Rag1 and Rag2: Adjacent genes that synergistically activate V(D)J recombination. *Science* 248:1517, 1990
59. Omenn GS: Familial reticuloendotheliosis with eosinophilia. *N Engl J Med* 273:427, 1965
60. Ouyang H, Nussenzweig A, Kurimasa A, et al: Ku70 is required for DNA repair but not for T-cell antigen receptor gene recombination in vivo. *J Exp Med* 186:921, 1997
61. Qin X-F, Schwerts S, Yu W, et al: Secondary V(D)J recombination in B1 cells. *Nature* 397:355, 1999

62. Ramsden DA, Baetz K, Wu GE: Conservation of sequence in recombination signal sequence spacers. *Nucleic Acids Res* 22:1785, 1994
63. Rieux-Laucat F, Bahadoran P, Brousse N, et al: Highly restricted human T-cell repertoire in peripheral blood and tissue infiltrating lymphocytes in Omenn's syndrome. *J Clin Invest* 102:312, 1998
64. Rodgers KK, Bu Z, Fleming KG, et al: A zinc-binding domain involved in the dimerization of RAG1. *J Mol Biol* 260:70, 1996
65. Rolink A, Melchers F, Andersson J: The SCID but not the RAG2 gene product is required for Smu-Sepsilon heavy chain class switching. *Immunity* 5:319, 1996
66. Roman CAJ, Cherry SR, Baltimore D, et al: Complementation of V(D)J recombination deficiency in RAG1^{-/-} B cells reveals a requirement for novel elements in the N-terminus of Rag1. *Immunity* 7:13, 1997
67. Schatz DG, Oettinger MA, Baltimore D: The V(D)J recombination activating gene Rag1. *Cell* 59:1035, 1989
68. Schwarz K, Hansen-Hagge TE, Knobloch C, et al: Severe combined immunodeficiency (SCID) in man: B-cell-negative (B-) SCID patients exhibit an irregular recombination pattern at the JH locus. *J Exp Med* 174:1039, 1991
69. Schwarz K, Gauss GH, Ludwig L, et al: RAG mutations in human B cell-negative SCID. *Science* 274:97, 1996
70. Shinkai Y, Rathbun G, Lam K-P, et al: RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68:855, 1992
71. Shockett PE, Schatz DG: DNA hairpin opening mediated by the Rag1 and Rag2 proteins. *Mol Cell Biol* 19:4159, 1999
72. Signorini S, Villa A, Imberti L, et al: Intrathymic restriction of the T-cell repertoire in Omenn's syndrome. *Blood* 94:3468, 1999
73. Spanopoulou E, Cortes P, Huang E, et al: Localization, interaction, and RNA-binding properties of the V(D)J recombination activating proteins Rag1 and Rag2. *Immunity* 3:715, 1995
74. Spanopoulou E, Zatseva F, Wang FH, et al: The homeodomain region of Rag1 reveals the parallel mechanisms of bacterial and V(D)J recombination. *Cell* 87:263, 1996
75. Steen SB, Han JO, Mundy C, et al: Roles of the dispensable portions of Rag1 and Rag2 in VDJ recombination. *Mol Cell Biology* 19:3010, 1999
76. Stephan JL, Vlekova V, Le Deist F, et al: Severe combined immunodeficiency: A retrospective single center study of clinical presentation and outcome in 117 cases. *J Pediatr* 123:564, 1993
77. Taccioli GE, Amatiucci AG, Beamish HJ, et al: Targeted disruption of the catalytic subunit of the DNA-PK gene in mice confers severe combined immunodeficiency and radiosensitivity. *Immunity* 9:355, 1998
78. Villa A, Santagata S, Bozzi F, et al: Partial V(D)J recombination activity leads to Omenn's syndrome. *Cell* 93:885, 1998
79. Zarrin A, Fong I, Malkin L, et al: Cloning and characterization of the human recombination activating gene 1, RAG1, and RAG2 promoter regions. *J Immunol* 159:4382, 1997
80. Zhu C, Bogue MA, Lim DS, et al: KU 86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. *Cell* 86:379, 1996

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