

# Novel RAG1 Mutation in a Case of Severe Combined Immunodeficiency

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**ABSTRACT.** *Objective.* The recombination activating enzymes RAG1 and RAG2 are essential to the process of V(D)J rearrangement in B and T cells and thus to the development of normal immune function. Mutations in RAG1 or RAG2 can lead to a spectrum of disorders, ranging from typical B<sup>-</sup>T<sup>-</sup> severe combined immunodeficiency to Omenn's syndrome. We present a unique presentation of RAG1 deficiency.

*Patient.* We report on a 6-month-old girl who presented with severe respiratory distress, which continued to progress despite antibiotic therapy but seemed to respond to treatment with corticosteroids. The patient exhibited no erythroderma or eosinophilia, and her lymphoid organs were not enlarged.

*Results.* Investigation of the immune system showed normal numbers of CD3<sup>+</sup> T cells, which expressed either CD4 or CD8. Subsequent analysis of the T-cell receptor demonstrated that nearly all CD3<sup>+</sup> T cells were clonal; one clone expressed CD4, whereas the other expressed CD8. The extremely restricted T-cell repertoire and the lack of circulating B cells prompted analysis of the RAG1 gene, which revealed a novel homozygous thymine to cytosine substitution at nucleotide position 2686.

*Conclusions.* This case underscores the importance of more extensive evaluation of the immune system even when widely available, standard, flow cytometric analysis shows normal numbers of T cells that express CD4 or CD8, especially in the absence of circulating B cells. *Pediatrics* 2005;116:e445–e449. URL: [www.pediatrics.org/cgi/doi/10.1542/peds.2005-0369](http://www.pediatrics.org/cgi/doi/10.1542/peds.2005-0369); *immunodeficiency, gene mutation.*

ABBREVIATIONS. PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; TCR, T-cell receptor.

Severe combined immunodeficiency (SCID) comprises a genetically diverse group of immunologic disorders, which present invariably at infancy with profound T-cell abnormalities that cause extreme susceptibility to infections. Most patients have lymphopenia, usually attributable to reduced numbers of circulating T cells, and they present at 6 to 8 months of age with protracted

diarrhea, pneumonitis, and failure to thrive. Some patients also lack circulating B cells at presentation, and they are found most frequently to have mutations in the recombination activating genes RAG1 and RAG2.<sup>1</sup> These enzymes play a critical role in the complex process of assembling the V(D) and J segments, which together form the variable parts of immunoglobulin and T-cell receptor (TCR) protein chains.<sup>2–4</sup> In the absence of effective recombination, the development of a full repertoire of T or B cells is greatly limited,<sup>5,6</sup> leading to the reduced numbers of circulating T and B cells among patients with abnormal RAG1 or RAG2 activity.

Not all patients with mutations in RAG1 or RAG2 present with typical features.<sup>6,7</sup> Patients may have all or some of the features associated with Omenn's syndrome.<sup>7</sup> This syndrome, which may or may not be caused by a mutation in a RAG gene, consists of SCID associated with severe erythroderma, lymphadenopathy, hepatosplenomegaly, and eosinophilia.<sup>8,9</sup> Many of these features are thought to be triggered by the abnormal expansion of oligoclonal T cells.<sup>9</sup> We report an unusual and potentially misleading presentation of an infant with SCID caused by a novel mutation in RAG1.

## METHODS

### Peripheral Lymphocyte Function Assays

Cell surface markers of peripheral blood cells were determined with immunofluorescence staining and flow cytometry (Epics V; Coulter Electronics, Hialeah, FL), with antibodies purchased from Coulter Diagnostics. To assay lymphocyte proliferation, peripheral blood mononuclear cells (isolated through Ficoll-Hypaque gradient centrifugation) were incubated at 37°C (5% CO<sub>2</sub>) in complete RPMI 1640 culture medium supplemented with 10% (vol/vol) fetal calf serum (Gibco/BRL, Gaithersburg, MD). Cells were incubated in round-bottomed tissue culture plates, with or without phytohemagglutinin (Difco Laboratories, Detroit, MI) or formalin-treated *Staphylococcus aureus* Cowan A (Calbiochem, La Jolla, CA). Four hours before termination of the culture, 1 μCi of [<sup>3</sup>H]thymidine was added to each well. The cells were then harvested, and samples were counted in a liquid scintillation counter.<sup>10</sup>

### Lymphocyte Cell Surface Markers

Peripheral leukocyte markers were determined with immunofluorescence staining and flow cytometry, with labeled antibodies for CD2, CD3, CD4 and CD8 (T cells), CD16 and CD56 (natural killer cells), and CD19 (B cells), as described previously.<sup>11</sup>

### TCR V<sub>β</sub> Repertoire

TCR V<sub>β</sub> repertoire quantitative analysis was performed with flow cytometry with the IOTest β mark kit (product number IM3497; Beckman, Fullerton, CA). Cells were stained for CD3, CD4, or CD8, followed by staining for expression of 24 different TCR V<sub>β</sub> chains.

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## Assessment of Maternal-Fetal T-Cell Engraftment

The patient's lymphocytes were typed for HLA, to ascertain whether there was maternal-fetal engraftment of T cells.

## DNA Preparation and RAG1 Gene Sequencing

Genomic DNA was isolated from peripheral blood mononuclear cells after Ficoll-Hypaque gradient centrifugation and was extracted with a Promega Wizard genomic purification kit (Promega, Madison, WI) (detailed procedure as listed in the manufacturer's instructions). Genomic DNA was extracted from peripheral blood leukocytes of the patient and her parents as described previously.<sup>11</sup> Because RAG1 is a single-exon gene, genomic DNA was used for polymerase chain reaction (PCR) amplification of the entire coding region of RAG1.

The following primers, which flanked the 2 sides of the RAG1 single coding exon and its intron-exon junctions, were used for PCR: 5'-AGCAAGGTACCTCAGCCAGCATG (forward) and 3'-ACCAACTCATAAGTGGTTGCCCTAC (reverse). PCR conditions included 1 cycle of 2 minutes at 94°C, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 68°C for 3 minutes. PCR assays were performed with elongase (Gibco/BRL), with a Perkin-Elmer GeneAmp 9600 PCR system (Perkin-Elmer, Boston, MA).

Sequencing was performed directly on the PCR products, which were subjected to electrophoresis on agarose gels and purified with a QIAquick gel extraction kit (Qiagen, Chatsworth, CA). The PCR products were sequenced with a ThermoSequenase kit (Amersham, Piscataway, NJ), as specified by the manufacturer. Primers used in sequencing were as follows: 5'-AGCAAGGTACCTCAGCCAGCATG (forward), 5'-CAGCCAAACTTG-CAGCTCAGC, 5'-AAGCTGCAAGTCAAAGCCTTT, 5'-GAT-GATTACCCAGTGGACACC, 5'-CTGGAACGTTATGAGGCTC, and 3'-ACCAACTCATAAGTGGTTGCCCTAC (reverse).

## RESULTS

### Case Report

This female patient of Pakistani descent was born after a normal pregnancy and delivery, to parents who were first cousins. At 4.5 months of age, she developed fever, cough, and vomiting and was treated as an outpatient with antibiotics,  $\beta$ -receptor agonist nebulizers, and prednisolone. Her fever resolved, but her respiratory symptoms persisted, necessitating admission to the hospital for 3 days. Subsequently the patient developed thrush, which responded to nystatin, but she did not have lymphadenopathy, splenomegaly, erythroderma, or failure to thrive. Chest radiographs showed pneumonitis. There were no acid-fast bacilli seen in the sputum, and PCR analyses for *Bordetella pertussis* and *Bordetella parapertussis* also yielded negative results. Despite treatment with multiple antibiotic agents, the patient developed a progressive oxygen requirement and was transferred to the Hospital for Sick Children intensive care unit at the age of 8 months. Sweat chloride test, urinalysis, serum  $\alpha$ -antitrypsin assay, and upper gastrointestinal imaging results were normal. Stool assays for ova, parasites, and viruses yielded negative results, but the urine tested positive for cytomegalovirus.

Repeat chest radiographs showed bilateral interstitial shadowing and, because of her respiratory distress, the patient began nasal continuous positive airway pressure treatment. Because the patient was suspected to have immunodeficiency, she was placed in reverse isolation and treated with gancyclovir, intravenously administered immunoglobulins, and trimethoprim. However, only after corticosteroids (1 mg/kg prednisone) were added did her

TABLE 1. Studies of Humoral and Cellular Immunity

	Patient	Normal Range
Immunoglobulins, g/L		
IgG	0.1	3.2–12.7
IgM	0.4	0.5–3.1
IgA	<0.1	0.4–3.7
IgE	<3.0	<90
Specific antibodies		
Tetanus, IU/mL	<0.01	>0.05
Polio, titer	<1:8	>1:16
Mitogenic responses* (patient/control)		
PHA	14/173	
SAC	1/19	
Lymphocyte markers, cells per $\mu$ L		
CD3	1660	1600–6700†
CD4	493	1000–4600
CD8	1164	400–2100
CD19	0	600–2700

PHA indicates phytohemagglutinin; SAC, *Staphylococcus aureus* Cowan A.

\* Stimulation index.

† Normal range for 9 to 15 months of age.<sup>12</sup>

pulmonary condition begin to improve gradually. After 10 days of steroid treatment, the patient no longer needed oxygen or ventilatory assistance, and she was transferred to the bone marrow transplantation unit. She died shortly after bone marrow transplantation, as a result of overwhelming cytomegalovirus infection.

### Clinical Features

The patient presented with pneumonitis, which led to progressively deteriorating respiratory distress that improved gradually with corticosteroid treatment. Other than the episode of pneumonitis, the patient had no other features typical of SCID. Small lymph nodes of normal size were palpated in the neck, but there was no enlargement of lymph nodes, spleen, or liver. The thymus shadow was reported to be within normal limits. There was no significant skin rash except for irritation on the face that was thought to be related to the mask and nebulized medications. There was no diarrhea and no positive microbial isolate in multiple samples obtained from nasal, pharyngeal, or bronchial secretions. There was no family history of early infant death or profound immunodeficiency.

### Immunologic Evaluation

The patient had normal numbers of circulating lymphocytes (2200 cells per  $\mu$ L) at the time of admission to the hospital; numbers were reduced to 958 cells per  $\mu$ L during corticosteroid treatment, as expected. With the cessation of treatment, the lymphocyte count returned to the normal range (2970 cells per  $\mu$ L). Eosinophils were not detected and other hemopoietic components were all normal. Flow cytometric analysis at 12 months of age showed a normal number of CD3<sup>+</sup> cells (1660 cells per  $\mu$ L) and a reversed CD4/CD8 ratio, with 493 CD4<sup>+</sup> cells per  $\mu$ L and 1164 CD8<sup>+</sup> cells per  $\mu$ L. There were 1087 natural

killer cells per  $\mu\text{L}$  but no detectable  $\text{CD19}^+$  or  $\text{CD20}^+$  B cells.

Despite the normal numbers, the patient's T cells responded poorly to mitogens (Table 1). To define more completely why the T cells failed to respond to mitogen stimulation, we studied the repertoire of lymphocytes. To our surprise, almost the entire population of  $\text{CD3}^+$  T cells was composed of only 1  $\text{CD4}^+$   $\text{V}\beta 17$  T-cell clone and 1  $\text{CD8}^+$   $\text{V}\beta 12$  T-cell clones (Fig 1). Careful molecular analysis showed that these T cells were not of maternal origin.

Humoral immunity was also abnormal, with low serum IgG levels and undetectable IgA and IgM (Table 1). The patient had no detectable serum antibodies to tetanus and polio, despite 3 previous vaccinations. The clonal nature of the T cells and the remarkably small number of circulating B cells raised the possibility of a defect in immunoglobulin gene recombination.

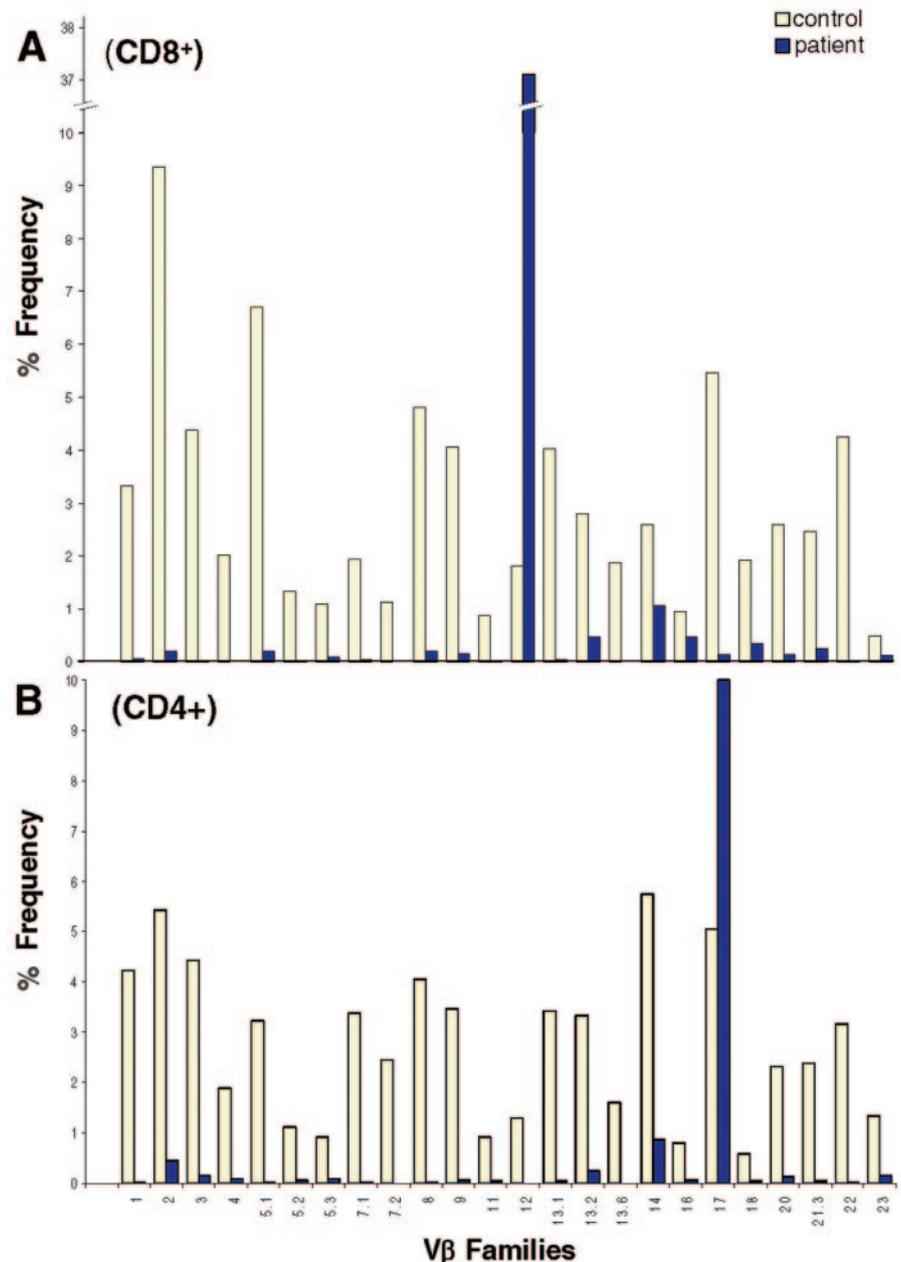
### Molecular Defect

Mutations in RAG1 or RAG2 were shown previously to restrict the production of mature B and T cells, leading to a  $\text{T}^- \text{B}^-$  SCID phenotype or alternatively to SCID with features of Omenn's syndrome, including erythroderma, eosinophilia, lymphadenopathy, and hepatosplenomegaly.<sup>6,7</sup> Despite observing no such features, we reasoned that the lack of B cells for this female patient might indicate an atypical variant of RAG deficiency.

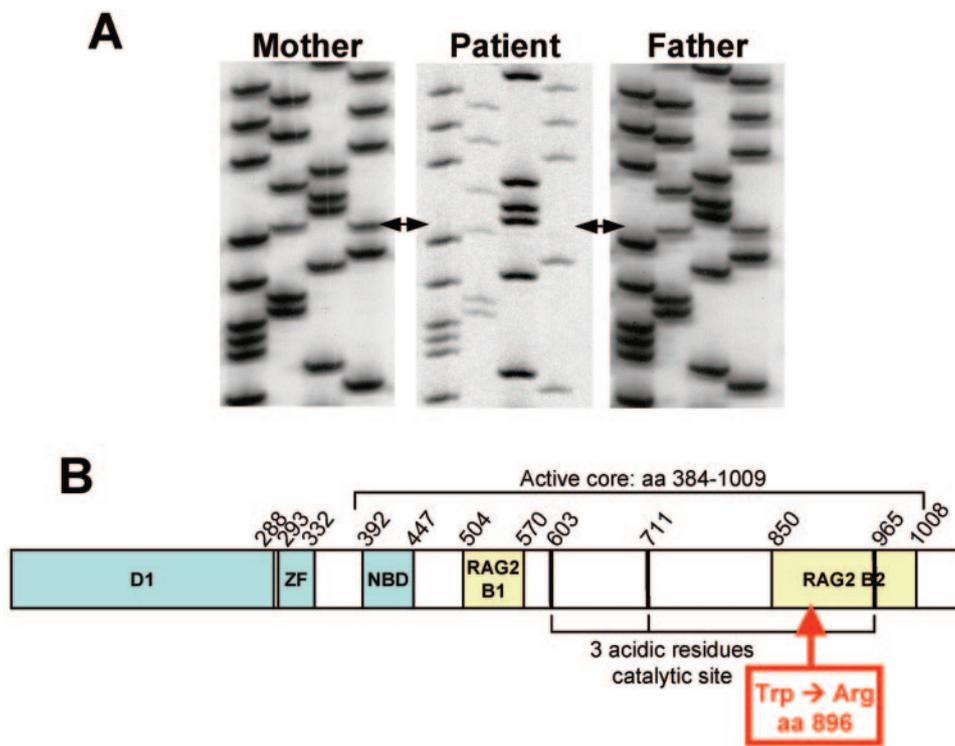
Sequence analysis of the patient's RAG1 gene revealed a homozygous mutation, ie, T2686C, which predicted a putative tryptophan to arginine substitution at position 896 (Fig 2A). The parents were both heterozygous for this mutation and were asymptomatic.

### DISCUSSION

The recombination activating enzymes RAG1 and RAG2 are essential in the assembly of V(D) and J



**Fig 1.** TCR repertoire. Relative representation of various  $\text{V}\beta$  families in a patient sample, compared with a control sample. A represents  $\text{CD8}^+$  T cells; B,  $\text{CD4}^+$  T cells.



**Fig 2.** Molecular analysis of the RAG1 gene. (A) Patient DNA shows a homozygous T2686C point mutation, whereas the parents are heterozygotes (arrows) (B). The mutation is located within the RAG2 binding domain<sup>15</sup> (arrow). aa indicates amino acids; ZF, zinc finger; NBD, nonamer binding domain.

segments, which form the variable portions of immunoglobulin and TCR proteins.<sup>2-5</sup> Initially, mutations in either the RAG1 or RAG2 genes were identified to cause SCID, with markedly reduced numbers of T and B cells,<sup>1</sup> and were also implicated as a cause of Omenn's syndrome, which is characterized by erythroderma, lymphadenopathy, hepatosplenomegaly, eosinophilia, and high IgE levels.<sup>6,7</sup> Some patients with Omenn's syndrome were shown to have mainly missense mutations, leading to partial activity of RAG1 or RAG2.<sup>6</sup> Such patients usually have variable but detectable numbers of activated, anergic, oligoclonal T cells. Analysis of the T-cell repertoire for some of these patients showed recombination of most TCR  $V_{\beta}$  segments, but there was restricted heterogeneity of several TCR  $V_{\beta}$  subfamilies. It was therefore proposed that a complete lack of RAG function leads to the  $B^{-}T^{-}$  SCID phenotype but partial RAG function may lead to SCID with clinical features of Omenn's syndrome.

Our patient presented with progressive respiratory distress, which did not respond to antibiotic therapy but improved after treatment with corticosteroid. This suggested a major inflammatory component contributing to the pathogenesis of the lung disease. In similar circumstances, corticosteroids with or without cyclosporine were shown previously to suppress autologous lymphocyte numbers and activity in SCID with an interleukin-2 receptor  $\alpha$  deficiency<sup>13</sup> or Omenn's syndrome,<sup>9,14</sup> leading to marked clinical improvement.

This patient had no other features typical of Omenn's syndrome. She had no persistent candidiasis, failure to thrive, or chronic diarrhea. She also

did not have features reminiscent of Omenn's syndrome, such as erythroderma, lymphadenopathy, hepatosplenomegaly, or eosinophilia. Even atypical Omenn's syndrome cases have some of these features. Of 10 atypical cases reported recently in a large cohort, only 3 presented without erythroderma in addition to pneumonitis. Unlike the patient presented here, those 3 patients demonstrated failure to thrive, protracted diarrhea, or splenomegaly.<sup>7</sup>

Evaluation of the patient's immune system revealed normal numbers of circulating lymphocytes, neutrophils, and eosinophils. Flow cytometric analysis showed markedly reduced numbers of circulating B cells but normal numbers of CD3<sup>+</sup> T cells expressing either CD4 or CD8.

Functionally, the patient's T cells failed to respond to mitogens or antigens, raising the possibility of primary immunodeficiency (Table 1). This discrepancy between the normal number and lack of function could be explained on the basis of oligoclonal expansion of T cells. Indeed, all T cells comprised predominantly 2 clones, one CD4<sup>+</sup> and the other CD8<sup>+</sup>.

The lack of circulating B cells, together with oligoclonal expansion of T cells, raised the possibility of RAG deficiency despite the unusual clinical presentation. Sequence analysis of RAG1 demonstrated a homozygous thymine to cytosine transition at position 2686, which seems to be a novel missense mutation. The location of this mutation is within the RAG2 binding domain, which was defined with coprecipitation of murine RAG1 subdomains with RAG2 (Fig 2B).<sup>15</sup> This mutation for our patient is in close proximity to several other previously described

mutations. It is interesting to note that these neighboring mutations have various clinical presentations, ranging from T<sup>-</sup>B<sup>-</sup> SCID to Omenn's syndrome.<sup>7</sup>

### CONCLUSIONS

This patient had a unique phenotype, on the basis of clinical and immunologic features. This case underscores the need for thorough immunologic evaluation of infants who present with persistent respiratory problems, even in the presence of normal numbers of circulating lymphocytes and the absence of obvious clinical features such as Omenn's syndrome.

### ACKNOWLEDGMENTS

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