

## Omenn's syndrome occurring in patients without mutations in recombination activating genes

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### Abstract

Omenn syndrome (OS) is characterised by hepatosplenomegaly, lymphadenopathy, erythema, eosinophilia, elevated IgE, oligoclonal T cell expansions and recombination activating gene (*RAG*) mutations. We investigated 9 cases of OS to correlate genotype with immunophenotype using a two-color flow cytometry with monoclonal antibodies against CD3 and TCRVB families to map TCRVB usage. T and B clonal cell populations were examined in peripheral blood lymphocytes by PCR and sequencing of *TCRB/TCRG* T cell and *IGH* FR2/FR3 B cell products. *RAG* and *Artemis* genes were sequenced from genomic DNA.

All patients demonstrated absent TCRVB families; six had predominant TCRVB families, six oligoclonal *TCR* gene rearrangements including *TCRGD* rearrangements. One demonstrated functional *IGH* rearrangement, an observation not previously reported. In this clinically homogeneous population, with similar immunological phenotype, *RAG* mutations were identified in only 2/9 patients.

OS is a genetically heterogeneous condition, and patients with similar immunophenotypes may have as yet unidentified gene defects.

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**Keywords:** Omenn syndrome; Recombination activating gene; *Artemis* gene; T cell receptor oligoclonality

### Introduction

A wide repertoire of T cell receptor or immunoglobulin diversity is achieved by somatic recombination of variable (V), diversity (D) and joining (J) gene segments. The recombination activating gene (*RAG*)-1 and *RAG*-2 endonuclease proteins initiate this recombination by cleaving DNA at recombination signal sequence sites. Cleaved DNA is subsequently repaired by the non-lymphoid-specific DNA double-strand break repair machinery [1]. Mutations in *RAG-1* or *RAG-2* genes result in a functional impairment of antigen receptor recombination, which causes T-negative B-

negative severe combined immunodeficiency (T-B-SCID) [2].

Omenn syndrome (OS), first described in an extended American-Irish family, is characterised by erythroderma, hepatosplenomegaly and lymphadenopathy with accompanying respiratory and gastrointestinal symptoms and failure to thrive [3]. Other features include raised serum IgE, eosinophilia and hypogammaglobulinemia as well as a T + B-peripheral lymphocyte profile dominated by activated, anergic T cells. Subsequent studies of small numbers of patients have found clonal T cell population expansions with restricted TCRVB family usage [4]. Skin biopsy shows an activated autologous T cell infiltrate. Lymph node architecture is abnormal, typically with an absence of germinal centres and paracortical expansion of S100 staining interdigitating reticulum cells [5].

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The description of a family with OS (T + B-SCID) in 1 sibling and a lymphocytosis-type severe combined immunodeficiency (T-B-SCID) in a subsequent sibling suggested that OS may be a “leaky” form of T-B-SCID [6]. Subsequently, missense mutations in *RAG-1* and *RAG-2* were identified in seven patients with OS [7], which severely restricted the function of the recombinase proteins but permitted the expansion of oligoclonal populations of host T cells. In one study of *RAG*-deficient patients, it was concluded that null mutations on both *RAG* alleles lead to the T-B-SCID phenotype whereas patients manifesting classic OS have missense mutations on at least one *RAG* allele and maintain partial V(D)J recombination activity, accounting for the generation of residual, oligoclonal T-lymphocytes [8]. However, both T-B-SCID and OS have been described in 3 different families with identical *RAG-1* or *RAG-2* missense mutations suggesting that the clinical phenotype is not explained by the underlying gene mutation alone [9]. The spectrum of *RAG* deficiency has expanded to include patients with atypical OS [8]. In this intermediate group of patients, erythroderma and hepatomegaly were present, but not lymphadenopathy. Some lymphocyte proliferation to PHA and presence of B cells were also described associated with *RAG* gene defects [8].

Recently 5 infants from 4 unrelated families with OS were reported, only 2 of whom had mutations in *RAG-1* [10]. Three other clinically similar patients had *RAG* gene polymorphisms, which do not affect endonuclease function, suggesting that mutations in other genes can give rise to a clinical phenotype similar to OS.

Mutations in the *Artemis* gene also cause T-B-SCID [11] and have been described in patients with features of OS but who had maternofetal engraftment (MFE) [12, 13]. The

demonstration of MFE precludes a diagnosis of OS. *Artemis* is involved in opening of the hairpin formed as an intermediate step during V(D)J recombination and thus forms part of the DNA double strand break repair mechanism required to complete V(D)J recombination [14]. More recently, a patient with a defect in *Artemis*, and with features of OS, but no evidence of MFE has been described [15].

In order to correlate gene defect with immunological phenotype, we have analysed a cohort of patients from a single centre, with a clinical diagnosis of OS, for clinical features and lymphocyte oligoclonal expansion, as well as *RAG* and *Artemis* gene defects.

## Patients

Nine consecutive patients with a presumptive diagnosis of OS were referred by clinical immunologists to the Northern Supra-Regional Bone Marrow Transplant Unit for SCID at Newcastle upon Tyne, UK, between 1996 and 2001 (Table 1). Clinical diagnostic criteria for OS were as previously described [7], namely the clinical triad of hepatosplenomegaly, lymphadenopathy, erythematous rash in the absence of maternofetal engraftment and presence of either a raised IgE or peripheral eosinophil count, together with immunodeficiency. Maternofetal engraftment was excluded in all cases by electrophoretic separation of radioactive PCR products following amplification of dinucleotide repeat polymorphisms in DNA from separated T cells using standard protocols, or where possible, by cytogenetic analysis. The median age of symptom onset was 3 weeks (range 0–7 weeks). The median age at referral was 10 weeks (range 0–16 weeks). Parental consanguinity was present in 4 families. All

Table 1  
Clinical details at presentation of 9 patients with Omenn syndrome

Clinical features	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5 <sup>a</sup>	Patient 6	Patient 7	Patient 8 <sup>a</sup>	Patient 9
Age at investigation (weeks)	10	10	16	3	10	11	12	birth	11
Sex	F	F	M	F	M	F	F	F	M
Ethnic origin	Caucasian	Caucasian	Caucasian	Asian	Asian	Asian	Caucasian	Asian	Asian
Consanguinity	no	no	no	cousin	cousin	cousin	no	cousin	cousin
Age at onset of rash (weeks)	1	3	6	birth	4	7	1	birth	7
Erythroderma	Generalised, scaly, scalp, face, limbs, trunk	Scalp, face, arms, trunk	Generalised, scaly, scalp, face, limbs, trunk	Generalised, scaly, scalp, face, limbs, trunk	Face, limbs, trunk	Generalised, exfoliative, face, trunk, limbs	Scalp, face, limbs, trunk	Face, limbs, trunk	Extensive thickened skin, face, limbs, trunk
Alopecia	complete	complete	complete	complete	partial	partial	complete	partial	complete
Hepatosplenomegaly (cm)	4/2	4/1	4/4	2/2	2/1	2/0	2/1	4/1	4/2
Features of CHARGE syndrome	no	no	no, 22q11 normal	no	no	no	no	no	no
Infection	nil	Av 41 enteritis	Aspergillus, PCP	nil	BCG	GAS, St aureus	VZV, PCP	nil	CMV

Av 41—adenovirus type 41; PCP—pneumocystis carinii pneumonia; GAS—group A streptococcus; VZV—varicella-zoster; CMV—cytomegalovirus.

<sup>a</sup> Siblings.

patients displayed extensive erythroderma, lymphadenopathy and hepatosplenomegaly. Six patients presented with invasive infection. Of the 8 patients who underwent lymph node biopsy, all had typical histological features previously described [5]. Eight patients had eosinophilia. Serum IgE levels were elevated in 6 of 7 patients in whom it was measured. Serum IgM levels were abnormally low in 8 patients (Table 2). No patient had features suggestive of DiGeorge anomaly or CHARGE syndrome. Specifically, none had hypocalcemia, cardiac defects, cleft palate or choanal atresia. One patient (patient 3) tested for 22q11 deletion was normal.

## Methods

Samples were collected prior to institution of immunomodulating therapy. Peripheral venous blood was analysed for TCRVB diversity at the Wessex Immunology laboratory, Southampton, United Kingdom. Control subjects were age matched, healthy children undergoing routine surgery.

### *Lymphocyte stimulation*

Mononuclear cells (MNC) were separated on a density gradient from whole blood taken in preservative-free heparin, washed and re-suspended in complete medium at a concentration of  $1 \times 10^6$  lymphocytes/ml. Two hundred microlitres of MNC was set up in triplicate on a microtitre plate with PHA at 10, 5 and 2.5  $\mu\text{g/ml}$  (final concentration). A background control with no stimulant was also set up and a normal sample was run in parallel on each plate, which was incubated at 37°C for 3 days, following which a pulse of tritiated thymidine was added to each well and the plate incubated for a further 8 h. The samples were then harvested onto filter paper, placed in scintillant and counted on a beta counter.

### *Maternofetal engraftment studies*

T cells were separated from whole blood using the Dynabeads method. Following extraction of DNA, short tandem repeat (STR) length polymorphism on 12 different STR markers was measured using multiplex PCR with fluorescent primers. Quantitation was determined by assessment of the area under the peak on a fragment analyser, with a lower limit of detection of 1–2% of the minor allele. In patients 3 and 9, cytogenetic analysis using fluorescent X/Y probes was undertaken, with lower limit of detection of 1%.

### *Immunofluorescence analysis of TCR repertoire*

Direct immunofluorescence was performed using FITC- or PE-conjugated antibodies. The following antibodies were used: anti-CD3: Leu4 (IgG2a) (Becton Dickinson, Mountain View,

CA), anti-TCRVB1, anti-TCRVB2, anti-TCRVB3, anti-TCRVB5S1, anti-TCRVB5S2/3, anti-TCRVB6S7, anti-TCRVB7S1, anti-TCRVB8.1/2, anti-TCRVB9, anti-TCRVB11, anti-TCRVB12, anti-TCRVB12S1, anti-TCRVB13S1, anti-TCRVB13.1/3, anti-TCRVB13.6, anti-TCRVB14, anti-TCRVB16, anti-TCRVB17, anti-TCRVB18, anti-TCRVB20, anti-TCRVB21S3, anti-TCRVB22 and anti-TCRVB23 (all from Serotec, Immunotech and A boyloston, Leeds, UK). The TCR framework antibodies used were TCRA and TCRGD (BD Biosciences). Whole blood analysis was performed with an anti-TCRBV antibody and anti-CD3 antibody. Staining was also performed with framework TCRA and TCRGD antibodies. Samples were analysed on a FACScan<sup>®</sup> analyser using the Lysis II programme (BD). Ten thousand events were collected within a lymphocyte gate. T lymphocytes were identified by CD3 staining and analysed for VB expression within the whole T cell population. TCRVB expression was represented as a percentage of CD3-positive cells for each family.

### *Genotypic analysis of TCR and IGH*

DNA was isolated from peripheral blood lymphocytes and paraffin-embedded material using the QIAGEN mini blood kit according to the manufacturer's protocol, with the following modification for paraffin-embedded material. Tissue was incubated in ATL buffer containing proteinase K (Qiagen) 400  $\mu\text{g/ml}$  at 42°C for 5 days, before purification through QIAamp spin columns. Samples were then amplified for *TCRB*, *TCRG* and *IGH* gene analysis. *TCRG* PCR analysis was performed using primer combinations V $\gamma$ J $\gamma$ , plus a V $\gamma$ 9 primer, and V $\gamma$ J $\gamma$ P [16]. *TCRB* PCR analysis was performed using primer pairs V $\beta$ J  $\beta$ 2 and D $\beta$ 1J $\beta$ 2 [17]. *TCRG* and *TCRB* reactions were subjected to 30 cycles of PCR (93°C for 1 min, 52°C (TCRB)/55°C (TCRG) for 1 min, 70°C for 1 min); PCR products were electrophoresed on a 10% polyacrylamide gel and stained with ethidium bromide.

*IGH* CDR3 PCR was performed as previously described using primers FR3 and JH [18]. Samples were subjected to 30 cycles of PCR (93°C for 1 min, 55°C for 1 min, 72°C for 1 min) and electrophoresed on a 10% polyacrylamide gel as above. *IGH* FR2 PCR analysis was performed in a semi-nested PCR amplification using primers FR2 LJH in the first round and FR2 VLJH in the second round [19]. Samples were subjected to 30 (first round)/20 (second round) cycles of PCR (93°C for 45 s, 50°C for 45 s, 72°C for 110 s) and electrophoresed on a 5% polyacrylamide gel. The PCR methods used in this study were validated with detection rates of >80%.

*TCRG*, *TCRB*, *TCRD* and *IGH* PCR analysis was also performed using the BIOMED-2-designed primers and protocols [20]. Briefly, VH family primers (VH1-VH7) designed to the FR1, FR2 and FR3 regions were used in conjunction with a single JH consensus primers (5' FAM labelled), in 3 multiplex reactions (FR1-JH, FR2-JH and

Table 2

Laboratory details at presentation of 9 patients with Omenn syndrome

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5 <sup>a</sup>	Patient 6	Patient 7	Patient 8 <sup>a</sup>	Patient 9
Eosinophils ( $\mu\text{l}$ ) [40–800]	30	7200	1100	12,100	5800	3900	1900	6200	6780
IgE (U/ml) [0–11]	95	1431	27	139	386	3	NA	175	NA
IgG (g/L) [3.7–12.6]	2.11	5.46	0.29	4.57	0.4	0.8	2.76	7.73	0.47
IgA (g/L) [0.02–0.15]	0.07	0.08	0.06	0.01	0.06	0.1	0.23	0	0.07
IgM (g/L) [0.05–0.29]	0.14	0.1	0.49	0.01	0.05	0.15	0.12	0.14	0.04
Calcium mmol/L [2.15–2.6]	2.48	2.35	2.4	2.36	2.26	2.49	2.32	2.49	2.16
Lymph node biopsy	Architectural effacement of node with S100 staining cell infiltrate, absence of germinal centres and abundant T cells	S100 staining cell infiltrate with absence of germinal centres and abundant T cells	S100 staining cell infiltrate with absence of germinal centres and abundant T cells	Architectural effacement of node with S100 staining cell infiltrate, absence of germinal centres and abundant T cells	S100 staining cell infiltrate with absence of germinal centres and abundant T cells	Abnormal architecture with S100 staining cell infiltrate, absence of germinal centres and abundant T cells	Architectural effacement of node with S100 staining cell infiltrate, absence of germinal centres and abundant T cells	S100 staining cell infiltrate with absence of germinal centres and abundant T cells	N/A
Skin biopsy	Epidermal necrosis consistent with severe GvHD	Diffuse dermal perivascular lymphocytic infiltrate resembling GvHD	Mild dermal perivascular lymphocytic infiltrate	N/A	Mild dermal perivascular lymphocytic infiltrate	Patchy dermal perivascular lymphocytic infiltrate equivalent to grade II GvHD	Diffuse dermal perivascular lymphocytic infiltrate	Diffuse dermal perivascular lymphocytic infiltrate	N/A
PHA (cpm $\times 10^3$ ) (control)	<1 (91.7)	<1 (171)	<sup>b</sup> 3.6 (12.1)	4.4 (87.8)	2.8 (114)	148 (153)	<1 (151)	1.1 (881)	N/A
Materno-fetal engraftment	Excluded by molecular genetic analysis	Excluded by molecular genetic analysis	Excluded by cytogenetic analysis	Excluded by molecular genetic analysis	Excluded by molecular genetic analysis	Excluded by molecular genetic analysis	Excluded by molecular genetic analysis	Excluded by molecular genetic analysis	Excluded by XY FISH analysis
Absolute leukocyte count ( $\mu\text{l}$ )	22,500	30,800	20,100	57,200	16,600	15,800	15,000	13,500	20,100
Absolute lymphocyte count ( $\mu\text{l}$ ) [4000–10,500]	11,475	16,940	11,600	35,870	9740	10,900	1500	4600	2920
Lymphocyte phenotype									
CD3 ( $\mu\text{l}$ ) [1700–3600]	10,755	16,160	7069	32,519	4362	10,219	1239	965	1387
CD4 ( $\mu\text{l}$ ) [1700–2800]	9675	9148	6126	19,888	3347	9180	1143	913	676
CD8 ( $\mu\text{l}$ ) [800–1200]	1238	7454	1021	14,655	1016	1880	83	35	109
CD19 ( $\mu\text{l}$ ) [500–1500] (%)	0	0	550 (4.7)	0	0	221 (4.4)	0	0	0
CD16/CD56 ( $\mu\text{l}$ ) [300–700]	675	1016	236	2442	1550	240	414	807	792
CD45RA (%CD3)	1	1	30	2	15	79	4	36	10
CD45RO (%CD3)	94	94	78	91	99	24	100	100	100
TCR $\alpha\beta$ (%CD3)	92	92	90	93	92	98	99	86	45
TCR $\gamma\delta$ (%CD3)	1	1	5	1	8	2	1	14	55

N/A—not available.

<sup>a</sup> Siblings.<sup>b</sup> Stimulation with anti-CD3 antibody.

FR3-JH). *TCRB* amplification was performed using 3 multiplex reactions ( $2 \times V\beta\text{-J}\beta$ ,  $1 \times D\beta\text{-J}\beta$ ). *TCRG* amplification was performed using 2 multiplex reactions: mix A and mix B have  $V\gamma\text{I}$  and  $V\gamma\text{10}$ , and  $V\gamma\text{9}$  and  $V\gamma\text{11}$  primers, respectively, in combination with a HEX-labelled J1.1/2.1 primer and a FAM-labelled J1.3/2.3 primer. *TCRD* amplification was performed using a single multiplex tube with  $6V\delta$  primers, 4 FAM-labelled  $J\delta$  primers, a HEX-labelled 5'  $D\delta$  primer and a NED-labelled 3'  $D\delta$  primer. The PCR reactions were set up using standard conditions and cycling parameters [20]. Appropriate clonal, polyclonal and negative controls were set up with each run. PCR products were visualised by heteroduplex analysis on PAGE and by GeneScan analysis on an ABI 3100 for the discrimination of monoclonal from polyclonal lymphocyte populations. Clonal populations were defined as those with 1 or 2 clonal PCR products present and oligoclonal populations as those with 3 or more distinct clonal PCR products present. A restricted profile demonstrated many more distinct clonal PCR products than an oligoclonal pattern but did not follow a normal Gaussian distribution. Polyclonal populations followed a normal Gaussian distribution.

Amplified *IGH* FR2 PCR products were gel purified using GeneClean kits (Anachem, Luton, Bedfordshire, UK), ligated into pGEM-T vector (Promega, Southampton, UK) and transformed into JM109 competent cells (Promega). Clones were randomly selected for each case and sequenced by the BigDye Terminator Ready Reaction cycle sequencing method (Perkin-Elmer, Warrington, Cheshire, UK). Sequence analysis was performed using MacVector 4.1.4 sequence analysis software with VBase (DNA plot) ([www.mrc-cpe.cam.ac.uk](http://www.mrc-cpe.cam.ac.uk)) and Igbblast ([www.ncbi.nlm.nih.gov/igblast](http://www.ncbi.nlm.nih.gov/igblast)) for determination of V, D, J gene usage and N region addition, as well as stop codons and frame shifts, which result in non-functional sequences.

#### Genotypic analysis of *RAG* and *Artemis*

Due to the restricted *RAG-1* and *RAG-2* gene expression, and because the coding region of both genes is contained in a single exon, coding sequences were amplified from genomic DNA. Primers were designed for the amplification of the *RAG* genes based on the sequences reported in databases (*RAG-1*: M29474; *RAG-2*: M94633). *RAG-1* gene was amplified in two segments (90–1852 and 1781–3309) and the *RAG-2* gene 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, UK). For compound heterozygote patients,

mutations were confirmed 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 [21]. To exclude polymorphisms, at least 100 normal chromosomes were investigated. Patients 5, 6 and 8 were also investigated by Southern blot analysis. Mutation screening of *Artemis* gene was performed by RT-PCR amplification of cDNA samples obtained from fibroblast cells of patients 5, 6, 7 and 8 using primers exon 1F/6R, exon 4F/10R and exon 9F/14R followed by sequencing the RT-PCR products. As no fibroblast cell line was available from patients 3, 4 and 9, genomic DNA was amplified using a series of oligonucleotide primer pairs as previously described [11]. In patients 5, 6 and 8, Southern blot analysis was also performed to investigate for deletions in *Artemis*.

#### Results

The clinical profiles of the patients are summarised in Table 1. Maternofetal engraftment was excluded in all cases by molecular or cytogenetic examination of peripheral blood. Additionally, HLA tissue typing of the patients did not support a diagnosis of maternofetal engraftment. All except patient 3 had subnormal serum IgM levels. All except patient 9 had a lymph node biopsy which showed disrupted lymph node architecture with absence of germinal centres and infiltration by S100 staining interdigitating reticulum cells (Fig. 1). Skin biopsies from the same 8 patients showed either changes similar to those seen in graft versus host disease, or dermatitis with T cell infiltrate (Fig. 2) (Table 2).

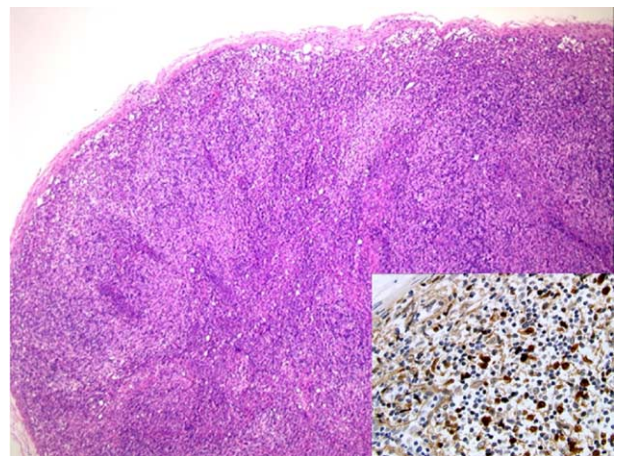


Fig. 1. The lymph node biopsy low power view (H&E  $\times 40$ ) shows absence of germinal centres and occupation of the node by sheets of large pale cells interspersed by T lymphocytes. The large cells are positive for S100 protein by immunohistochemistry (insert), consistent with interdigitating reticulum cells (Langerhans cells) (reproduced with permission of Department of Pathology, Newcastle upon Tyne Hospitals).

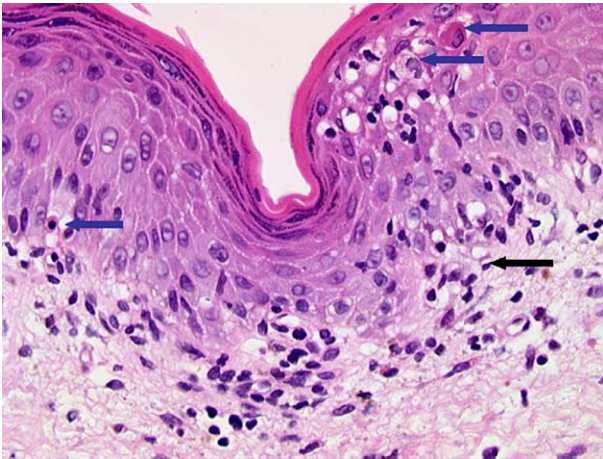


Fig. 2. The skin biopsy shows necrotic basal and suprabasal keratinocytes (blue arrows) and basal vacuolar degeneration in the epidermis (black arrow). A lymphocytic infiltrate is present both basally and in association with the intra-epidermal necrotic cells. These features are in keeping with graft versus host disease grade 2 (reproduced with permission of Department of Pathology, Newcastle upon Tyne Hospitals).

#### Analysis of T cell receptor oligoclonality

The absolute lymphocyte count at diagnosis ranged from 1500 to 35870 cells/ $\mu$ l (median 9740). CD3 counts ranged from 965 to 32519 cells/ $\mu$ l (median 7069) and ranged from 7 to 65 (median 35) percentage of the total leukocyte count. Eight patients demonstrated an excess of CD45 RO+ T cells consistent with an activated phenotype. All patients had detectable TCR $\alpha\beta$ + T cells; patient 9 had expansion of TCR $\gamma\delta$ + cells as identified with a framework *TCRGD* antibody. B cells were absent in all except patients 3 and 6 who had reduced B cell numbers (Table 2).

The immunophenotyping panel of TCRB family antibodies used in this study detects approximately 70% of all TCR $\alpha\beta$ + T cells. Previous studies have shown that all individual VB families are represented at a detectable level ( $>0.5\%$  of CD3 T cells) but rarely constitute greater than 10% of all CD3-positive cells in all healthy controls [22]. All 9 patients showed an absence of one or more TCRVB families (Table 3).

TCRVB family expansions were identified in 6 patients with 4 demonstrating this expansion in 2 TCRVB families and 1 demonstrating this expansion in 3 TCRVB families (Table 3). There was no single family that showed a shared expansion across these 6 individuals. Patients 1 and 4 had expansions of the TCRVB 6.7 and 5.1 families, respectively, at greater than 30%. Patients 4, 7 and 8 had expansions of 2 families, both greater than 10% of the total T cells. The distribution of these expansions across the CD4 and CD8 subsets was not assessed. Patient 9 had a TCRGD T cell expansion.

#### Lymphocyte receptor genotype

DNA from case 2 was not available for study. Clonal or oligoclonal *TCRG* and *TCRB* products were obtained in 6

Table 3

Flow cytometry analysis of the TCRVB repertoire in 9 patients with OS

TCRVB family	Case number								
	1	2	3	4	5	6	7	8	9
1	–	–	–	–	3.5	2.6	0.9	<b>11.9</b>	4.2
2	2.5	4.5	8.8	6.7	4.9	9.5	<b>14.3</b>	<b>15.4</b>	1.1
3	2.4	2.2	5.9	<i>0.1</i>	<i>0.2</i>	3.8	<b>14.1</b>	<i>0.4</i>	3.6
5.1	4.2	3.7	4.3	<b>30.1</b>	2.4	5.6	6.4	1.7	<b>20.2</b>
5.2/3	1.0	2.0	4.1	1.4	0.6	2.5	1.0	<i>0.3</i>	2.2
6.7	<b>38.3</b>	5.6	6.2	1.2	0.6	3.8	7.9	1.2	0.4
7.1	<i>0.3</i>	10.3	0	0	0	–	–	–	0.6
8.1/2	1.2	1.5	2.3	<i>0.3</i>	2.2	3.4	–	<i>0.4</i>	0
9	–	–	–	–	<i>0.3</i>	<b>10.7</b>	<b>6.1</b>	<i>0.3</i>	0
11	–	–	–	0	<i>0.2</i>	<i>0.4</i>	1.0	0	0.8
12	–	–	–	2.1	1.2	2.3	<i>0.4</i>	<i>0.4</i>	<i>0.2</i>
12.1	2.4	2.0	1.8	2.5	1.6	2.4	1.5	2.8	1.3
13.1	0.6	0.7	1.2	4.2	<i>0.2</i>	0.4	<i>0.2</i>	<i>0.2</i>	2.8
13.1/3	6.5	4.6	4.7	<b>12.5</b>	5.2	4.8	1.7	1.2	3.6
13.6	–	–	–	–	–	–	–	–	1.6
14	1.6	1.2	<i>0.4</i>	1.4	<i>0.2</i>	2.1	0.5	0.5	0
16	–	–	–	0	<i>0.2</i>	0.7	4.5	0.8	0
17	1.8	0	1.6	0.7	3.0	4.6	4.3	0.2	4.0
18	–	–	–	–	<i>0.3</i>	1.0	0.5	<i>0.2</i>	0
20	1.2	2.5	5.5	0.5	1.4	3.5	1.2	3.5	<b>9.1</b>
21.3	1.3	3.4	3.2	2.4	1.6	<b>6.7</b>	–	2.1	1.6
22	1.2	<i>0.2</i>	2.8	0	<i>0.1</i>	3.8	0.5	<i>0.2</i>	<i>0.4</i>
23	–	–	–	–	<i>0.4</i>	0.5	0	<i>0.2</i>	0
Total	66.5	44.4	52.8	66.1	30.3	75.1	67.0	43.9	57.7

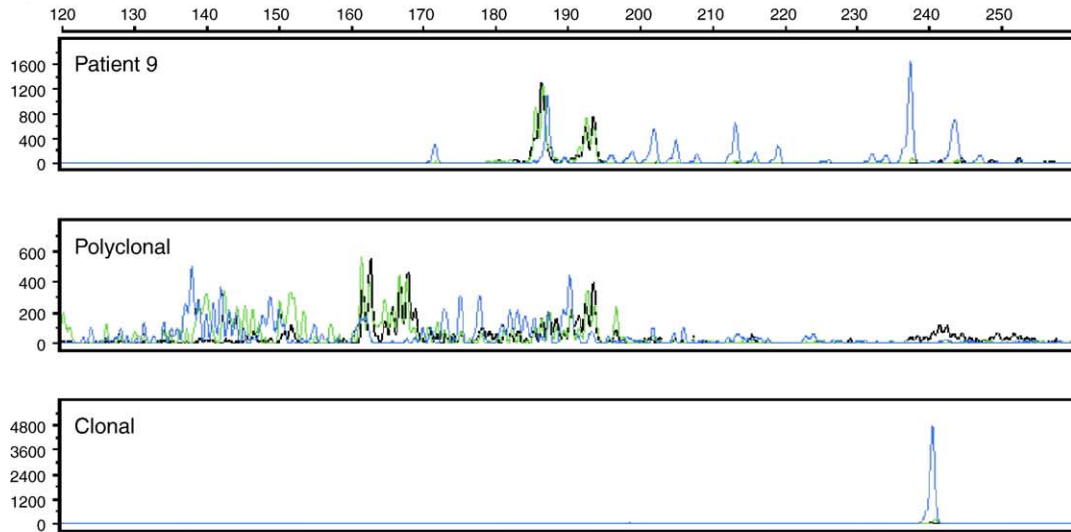
TCRVB expression as of percentage of CD3 TCR $\alpha\beta$ + positive T cells. The TCR immunophenotyping panel of antibodies usually detects 70% of all TCR $\alpha\beta$ + positive T cells in normal individuals. Figures in bold represent expanded TCRVB families ( $>p90$ , Ref. [21]). Figures in italics represent values  $<0.5\%$ . –: not done.

cases; in 2 cases all *TCR* products were polyclonal. In case 1 no *TCRB* products were found. In case 9 incomplete *TCRB* D-J clonal rearrangements were detected together with oligoclonal *TCRG* and *TCRD* rearrangements (Fig. 3, Table 4). In 3 of 6 patients with oligoclonal/clonal products identified there was no evidence of coexistent systemic infection. The other 3 patients had CMV, VZV and BCG [23] infection.

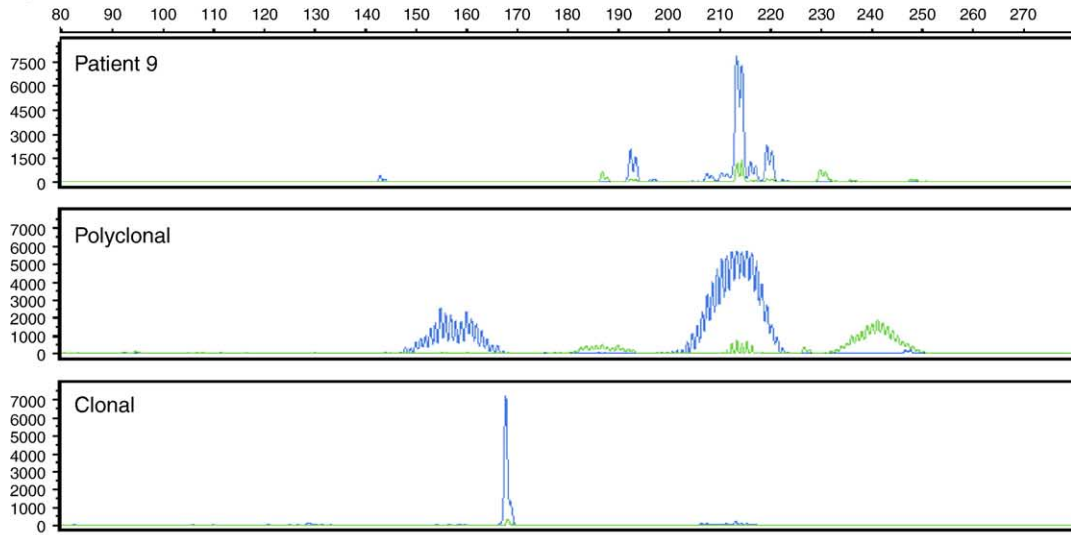
Previous studies of OS have not identified B cell oligoclonal expansion. In patients where no B cells were found, PCR for *IGH* products was not informative. In the 2 patients where peripheral B cells were detected, oligoclonal *IGH* products were found in one (case 3), and polyclonal products were found in the other (case 6). Sequence analysis of the *IGH* PCR products from patient 3 revealed 3 unique clonal in-frame IgH gene rearrangements consistent with functional *IGH* rearrangements. The gene usage of the 3 clones were VH4-59–JH6, VH3-23–JH3 and VH3.30–JH5. The first clonal sequence had 1 silent mutation and had 99.3% homology with the VH4-59 sequence, while the latter 2 sequences were devoid of somatic mutations.

Archived lymph node biopsy material was available from 6 patients (Table 4). Five showed clonal, oligoclonal or restricted TCR PCR products. In 2 (patients 4 and 5), this was a similar pattern to that seen in peripheral blood. These

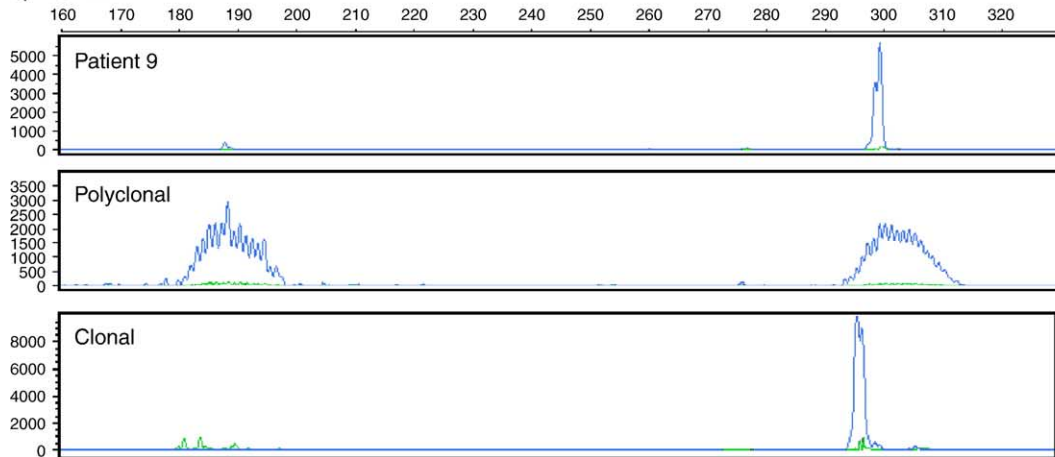
a) TCRD



b) TCRG



c) TCRB



patients had no BCR product, or showed a restricted PCR profile (Table 4). Patient 6, who demonstrated a polyclonal profile in peripheral blood, showed a similar profile in the lymph node. Patients 1, 4 and 8 showed the same TCRB VB family clonal expansion in the lymph node as in peripheral blood. Peripheral blood and lymph node lymphocyte receptor genotype analysis on control material taken from a patient with Nethertons syndrome showed a normal polyclonal profile (data not shown).

#### *RAG and Artemis mutations*

An analysis of the *RAG-1*, *RAG-2* and *Artemis* genes was undertaken in all patients. Patients 1 and 2 were the only individuals with disease-causing mutations in *RAG-1* (Table 4). Patient 1 was a compound heterozygote with a methionine to valine substitution at amino acid 435 and arginine to serine substitution at amino acid 449 in the core domain of the RAG1 protein. Patient 2 was homozygous for a deletion at nucleotide 887. All other patients, despite similar clinical and laboratory phenotypes, did not have a mutation in the *RAG-1*, *RAG-2* or *Artemis* coding regions. Radiosensitivity studies of fibroblasts from patients 5 to 8 were normal (P. Jeggo, personal communication), fibroblasts from other patients were unavailable for study. Southern blot analysis of *Artemis* in patients 5, 6 and 8 was normal, while a region of 1 kb at the 5' region of *Rag1* and *Rag2* genes was investigated by sequencing without finding any abnormalities.

#### Discussion

The patients studied had the characteristic clinical features of OS, including erythroderma, lymphadenopathy and hepatosplenomegaly, together with persistent infection, alopecia and an elevated leukocyte count due to eosinophilia and/or lymphocytosis. Lymph node and skin biopsies in 8 of the patients were consistent with the diagnosis of OS. The association of the above clinical features with low immunoglobulins, disseminated viral infection or pneumocystis carinii pneumonitis is strongly suggestive of underlying severe T cell immunodeficiency. While patients 3, 5, 6 and 8 had substantial numbers of CD45RA<sup>+</sup> cells, and patients 3 and 6 had low IgE levels with elevated CD19<sup>+</sup> counts, compared to classical OS, patients with defined RAG mutations and clinical OS have been described with

elevated CD45RA<sup>+</sup> cells, low IgE levels and even some B cells present [8]. Only 2 of 9 patients in our series had coding region *RAG-1* mutations. The two heterozygous missense mutations affecting the core domain of *RAG-1* identified in case 1 affect the binding of RAG1 to RSS in V(D)J gene rearrangements and have not been previously reported in OS or SCID. In case 2, in whom a homozygous deletion (del nt 887) was detected, no dominant TCRVB expansion was observed but <45% of the expected repertoire was detected by the antibody panel, which identifies 70–75% of the TCRV $\beta$  repertoire of normal individuals. This finding may indicate that an expanded TCRVB family was present amongst families not tested for. A lack of available DNA precluded clonality investigations in this case.

In 6 of the remaining 7 patients, non-disease-causing polymorphisms in *RAG-1/-2* were identified. No patients had mutations in the *Artemis* coding region, although a compound heterozygote intragenic deletion cannot be completely excluded in patients 3, 4 and 9 as no material was available for Southern blot analysis. While defects in the *RAG* or *Artemis* promoter regions have not been excluded, 4 patients (from 3 families) did not demonstrate fibroblast radiosensitivity, making a defect in *Artemis* unlikely. In all 7 of these patients predominance or absence of one or more TCRVB families was demonstrated by flow cytometry. Furthermore, oligoclonal or clonal rearrangements were apparent on molecular analysis in 6 of the 7 patients. In one (patient 6), no clonal populations were found, although *IgH* products were obtained. In 5 of 6 patients for whom archived lymph node biopsy material was available, clonal or oligoclonal TCR profiles were evident—in 3, the same TCRB VB family clonal expansion in the lymph node was found as in peripheral blood.

Three patients did not show the usual immunophenotype associated with OS. Two (patients 3 and 6) had significant numbers of B cells; functional clonal and oligoclonal *IGH* rearrangements were confirmed by sequence analysis in the absence of clonal or oligoclonal *TCR* rearrangements in one (patient 3), although no genetic mutation in *RAG* or *Artemis* was found. This finding is consistent with oligoclonal B cell populations, which do not represent cross lineage T cell gene rearrangements. This implies that the restricted OS T cell phenotype may extend to B cell oligoclonality where some partial B cell maturation can occur. Although functional antibodies have been described in a patient with atypical RAG deficiency, recipient B cells and maternal T

Fig. 3. GeneScan analysis of PCR products of *TCR* gene rearrangements using multiplex reactions. For all *TCR* loci (a, b and c) the GeneScan profiles illustrate the multiplex reactions of patient 9 in the upper panel, a polyclonal MNC control in the middle panel and a clonal *TCR* control in the lower panel. (a) GeneScan profiles of the TCRD multiplex reaction identify a wide distribution of PCR products size in a non-classical Gaussian distribution in the polyclonal control. A single dominant peak is demonstrated in the clonal control while several oligoclonal peaks are identified in patient 9. (b) GeneScan profiles of *TCRG* multiplex reaction (mix B-primers V $\beta$ 1, V10, J1.1/1.2 and J1.3/2.3) demonstrates four Gaussian distributions representing different recombinations in varying magnitudes in the polyclonal control. The clonal control demonstrates a clonal population with V10-J1.3/2.3 gene usage. Several peaks of variable intensities are identified in patient 9 and these populations are utilising V $\beta$ 1-J1.3/2.3. (c) GeneScan profiles of *TCRB* (DJ) multiplex reaction demonstrate two Gaussian distributions representing D2-J and D1-J gene recombinations in the polyclonal control. A single peak is identified in the clonal control and in patient 9 (D1-J).

Table 4  
Lymphocyte genotype in peripheral blood and lymph node biopsy

Patients	Sample	TCRG	TCRB	Gene mutation
1	PB	O	–	<sup>a</sup> <i>RAG1</i> compound heterozygous G1789T, A1415G
2	LN	C	O	<sup>a</sup> <i>RAG1</i> homozygous deletion nt887
		ND	ND	
3		P	P	Normal <i>RAG1</i> , <i>RAG2</i> , <i>Artemis</i>
@4	PB	O	O	<i>RAG1</i> heterozygous polymorphism A2571G, normal <i>RAG2</i> , <i>Artemis</i>
	LN	O	O	<i>RAG1</i> heterozygous polymorphism G858A, normal <i>RAG2</i> , <i>Artemis</i>
@5	PB	O	O	
	LN	O	O	<i>RAG1</i> homozygous polymorphism G858A, normal <i>RAG2</i> , <i>Artemis</i>
6	PB	P	P	
	LN	P	P	<i>RAG1</i> heterozygous G1457A and homozygous G858A normal <i>RAG2</i> , <i>Artemis</i>
7	PB	O	O	
	LN	O	O	<i>RAG1</i> heterozygous polymorphism G858A, normal <i>RAG2</i> , <i>Artemis</i>
@8	PB	C*	O	
	LN	O	C	<i>RAG1</i> heterozygous polymorphism G858A, normal <i>RAG2</i> , <i>Artemis</i>
@ + 9		O	C#	

The *TCR* genotyping assay records polyclonal profiles as 'P', oligoclonal as 'O', clonal as 'C', no products as '–' and 'ND' when samples were unavailable. C\* Clonal *TCRG* products with VJP primers, C# clonal product with *TCRB* DJ primers. @ DNA from these cases was also amplified with the BIOMED-2 primers. +Patient 9: oligoclonal *TCRD* gene rearrangements were also detected. In patients where no B cells were found, PCR for *IGH* products was not informative. In 1 patient where peripheral B cells were detected, oligoclonal *IGH* products were found (case 3). DNA extracted from lymph node biopsies were all analysed with the BIOMED-2 primers. Comparison of the peripheral blood and lymph node genotypic BIOMED-2 profiles demonstrated identical TCR peaks in both samples as well as additional TCR peaks in either the peripheral blood or lymph node DNA in patients 4, 5 and 6. In patients 1 and 7 no comparable BIOMED data were available for the peripheral blood; however, oligoclonal BIOMED-2 TCRB and TCRG profiles and oligoclonal TCRB and a clonal TCRG profile were demonstrated in the lymph node DNA from patient 1 and patient 7, respectively. In patient 6, good polyclonal TCR Gaussian distributions were demonstrated.

<sup>a</sup> Disease-causing mutation.

cells [24], *IGH* gene rearrangements have not previously been reported in OS [4,25–27]. It has been suggested that B cell differentiation relies more strictly on higher levels of *RAG* gene activity than *TCR* gene rearrangements [26–28]. Furthermore, a complete B cell differentiation block at the pre B cell stage has been described in the marrow of *RAG*-deficient SCID and OS patients where partial recombinase activity was associated with oligoclonal T cells [28]. Similar findings are described in *Artemis*-deficient SCID patients

[29], although B cell populations are found in combined immunodeficiency patients with hypomorphic *Artemis* mutations [30]. Our findings suggest that oligoclonal B cells as well as T cells may occur in clinically defined OS, albeit in patients with no *RAG1/2* mutations. As far as we are aware, this is the first description of B cell *IGH* clonal expansion in OS.

The ninth patient in whom no *RAG* or *Artemis* mutations were identified had an expanded TCRGD population of 55% of CD3 cells with restricted oligoclonal products for *TCRG* and *TCRD*. The preferential expression of VD2-JD1/VG9-JG1.2 in TCRGD T lymphocytes found in peripheral blood could not be excluded in this case due to the combination of antibodies and *TCRG* PCR primers utilised. However, oligoclonal VG1-J1.3/2.3 gene recombinations were identified and the functionality of these rearrangements would need to be confirmed by sequence analysis. Expanded TCRGD cell populations have been described in patients with clinically defined OS without characterisation of *RAG* status [6]. The aetiology of these populations in these patients is not clear.

The restricted TCRVB repertoires demonstrated in OS patients suggest that the defective recombination machinery can preferentially rearrange only a selected number of TCRVB families resulting in a 'leaky' phenotype [4]. Similar restricted TCRVB repertoires may be seen in severe infections due to expansion of immunologically functional T cell clones; importantly, absence of TCRVB families is not seen in this situation however. Restricted TCRVB family usage is found in other clinical conditions including malignancy and autoimmune disorders [31,32]. Superantigen infection may also cause clonal expansions or deletion of certain TCRVB families [33]. Thus, the clonal T cell populations seen in OS patients may represent an aberrant response to infection by non-antigen-specific T cell receptors, leading to a massive release of inflammatory cytokines. The discovery of identical CDR3 regions amongst TCRVB families from patients with OS, suggesting stimulation by few restricted antigenic determinants from recurrent infections supports this hypothesis [4,25]. However, in 3 of 4 of our patients with TCRVB predominance, no systemic infection was present. An alternative explanation for the clinical phenotype may be tissue damage by aberrant autoreactive autologous T cells [34]. Although it has been proposed that the OS phenotype is determined by missense *RAG* mutations, rather than nonsense mutations seen in T-B-SCID [8], both phenotypes have been identified in the same family [9]. As patients with *RAG*-deficient T-B-SCID, with no features of OS, also present with disseminated infection, factors other than the type of *RAG* mutation in combination with antigenic stimulation must drive the development of the clinical phenotype of OS rather than T-B-SCID.

Recently, a series of 5 patients with features of complete DiGeorge or CHARGE syndrome was described, who had some features of atypical OS, including spongiotic derma-

titis, oligoclonal T cell repertoire with PHA proliferative response and lymphadenopathy with B cell clustering, but absence of normal follicles [35], features similar to those described in OS. It is increasingly recognised that atypical manifestations of previously well-defined immunodeficiencies can occur, including some patients with adenosine deaminase deficiency [36] and JAK-3 deficiency [37]. Antigen-specific antibody production has been described in a patient with RAG1 deficiency [24].

The definition of OS was originally based on a clinical description of patients from a consanguineous Irish-American kindred with early onset erythematous rash, infections, hypogammaglobulinemia, leukocytosis with eosinophilia, generalised lymphadenopathy and hepatosplenomegaly [3]. Since then, other distinguishing features have been described, including a restricted T cell receptor Vbeta repertoire [4], and disordered lymph node architecture with absence of B cell follicles and germinal centres [5]. It is possible that patients with features suggestive of OS or atypical OS may not have defects in the RAG genes, as demonstrated in those with DiGeorge or CHARGE syndromes [35]. It is plausible that defects in other genes associated with SCID may lead to a leaky phenotype in which OS features are manifest.

We have described a number of clinically defined OS patients both with disease-associated *RAG* gene mutations and those in whom no mutation could be demonstrated. Those without demonstrable *RAG* mutations demonstrated abnormal lymph node appearances with oligoclonal expansions of T cell families in peripheral blood and in 1 case, oligoclonal expansions of B cell families, a phenomenon not previously reported in OS. Other gene defects are postulated in T-B-SCID, although the genes have yet to be identified [38]. While splicing or promoter mutation defects cannot be entirely excluded, in patients 5, 6 and 8 normal Southern blot analysis of *Rag* and *Artemis* and normal radiation studies would suggest that defects in these genes are unlikely. These data from our study suggest that defects in genes other than *RAG* and *Artemis* may cause OS with a phenotype indistinguishable from that due to *RAG* mutations.

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