Non-functional IgG transcripts in a case of hyper-IgM syndrome similar to type 4

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SUMMARY

86% of IgG heavy-chain gene transcripts were found to be non-functional in the peripheral blood B-cells of a patient initially diagnosed with common variable immunodeficiency, who later developed raised IgM, whereas no non-functionally rearranged transcripts were found in the cells of seven healthy control subjects. All the patient's IgM heavy-chain and κ light-chain transcripts were functional, suggesting that either non-functional rearrangements were being selectively class-switched to IgG, or that receptor editing was rendering genes non-functional after class-switching. The functional γ-chain sequences showed a normal rate of somatic hypermutation while non-functional sequences contained few somatic mutations, suggesting that most came from cells that had no functional gene and therefore were not receiving signals for hypermutation. However, apoptosis of peripheral blood lymphocytes was not impaired. No defects have been found in any of the genes currently known to be responsible for hyper-IgM syndrome but the phenotype fits best to type 4.

Abbreviations not listed by *Biochemical Journal*:

Ab	antibody
CSR	class-switch recombination (isotype-switching)
CVI	common variable immunodeficiency
C_γ	IgG constant region
C_μ	IgM constant region
dNTP	deoxyribonucleotide triphosphate
HCDR3	Ig heavy-chain complementarity-determining region 3
HBSS	Hanks' Balanced Salt Solution
HIGM	Hyper-IgM syndrome
mAb	monoclonal antibody
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PE	phycoerythrin
RSS	recombination signal sequence(s)
r.t.	room temperature
SHM	somatic hypermutation
V-genes	variable region gene elements
γ-chain	IgG heavy-chain
μ-chain	IgM heavy-chain

INTRODUCTION

Common variable immunodeficiency (CVI) and hyper-IgM syndrome (HIGM) both present with recurrent infections. In the former they are mainly bacterial whereas, in the latter, opportunistic parasitic and fungal infections are also common. CVI is the commonest symptomatic primary Ab-deficiency disorder.[1] By the standard criteria, the peripheral blood has IgG and IgA at least two standard deviations below the mean for age and sex (typically < 5 g/L and < 0·1 g/L respectively) and IgM may be low or within normal limits.[2-5] Its diagnosis is made by the exclusion of all secondary causes of immunodeficiency, and by lack of typical features of known single-gene disorders. Thus it is usually made on clinical and immunological grounds, rather than by genetic testing. The cause is unknown but it has been realised for a long time that it is heterogeneous.[1]

HIGM is less common. It exhibits low serum IgG, IgA and IgE along with a raised or normal IgM concentration and therefore may sometimes be confused with CVI. It is also heterogeneous.[6] Mutations in five genes have so far been identified as causing this syndrome. Of these the commonest cause is mutation of the CD154 (CD40 ligand) gene, *TNFSF5*, responsible for an X-linked form, HIGM1 (also previously known as X-HIGM).[7-12] CD154 is normally expressed transiently on the surface of activated T-cells, and the absence of CSR is due to failure of T-cell signalling to B-cells.[13] HIGM2 is autosomal recessive and caused by mutations in the activation-induced cytidine deaminase gene, *AICDA*, (*AID, CDA2*).[14] It is characterised by absence of CSR, lack of SHM, and lymph-node hyperplasia with giant germinal centres. Unlike types 1 and 3, opportunistic infections are not a feature of this type. HIGM3 is also autosomal recessive and is caused by mutations in the CD40 gene, *TNFRSF5*.[15] All cases reported so far

exhibit lack of expression of CD40[15,16] and also display lack of CSR, deficient SHM, and impaired generation of memory B-cells.

Recently, mutations in *UNG* (the uracil DNA glycosylase gene) have been found in three HIGM patients who lacked any of the above mutations.[17] These patients showed a profound impairment of CSR and a disturbance of the pattern of SHM; there was a deficit in transversion mutations of C·G base-pairs, compared with transitions, but no transition-transversion bias in mutations of A·T base-pairs. This condition is also autosomal recessive. Finally, a variety of mutations in the NF-κB essential modulator (NEMO) gene, *IKBKG* (aliases: *IP2, FIP3, NEMO, FIP-3, Fip3p, IKK-gamma*), cause an X-linked primary immunodeficiency characterized by hyper-IgM syndrome and hypohydrotic ectodermal dysplasia (XHM-ED, *alias* HEM-ID for hypohydrotic ectodermal dysplasia with immunodeficiency).[18-21] These patients' B cells are also unable to undergo CSR.

These latter two genetic entities may become known as HIGM5 and HIGM6 because the term HIGM4 has already been associated with yet another form of HIGM, for which the gene has not yet been identified.[22] This is a somewhat milder form symptomatically, clinically resembling HIGM2, including lymphoid organ hyperplasia but without giant germinal centres. CSR is impaired but there is some residual IgG production, and SHM is normal.

HIGM1 and HIGM3 are clinically indistinguishable[15] and are combined immunodeficiencies.[6,16] NEMO is involved in CD40-induced activation of the transcription factor NF-κB and hypomorphic mutations in it affect both T- and B-cells.[21] The other three types of HIGM described so far involve only B-cells. AICDA is necessary for both CSR and SHM.[14,23] Though CSR is absent in

HIGM1, a subset of B-cells do exhibit SHM.[24] It appears therefore that the CD40-ligand-CD40 interaction does not turn on AICDA expression but rather that it acts on a pathway downstream of AICDA that leads to CSR but not to SHM. UNG mutations clearly act downstream of AICDA in both pathways. The mechanism proposed is that AICDA deaminates cytosine to uracil in targeted DNA in Ig variable regions and switch regions and that UNG removes the uracil.[17] The defect in HIGM4 must also act downstream of AICDA (which is expressed normally) and appears to act in the CSR pathway only, as SHM is unaffected.[22]

Levy and colleagues[25,26] found that a subgroup of CVI patients have greatly reduced somatic mutation of their rearranged IgG heavy-chain genes, and that the mean mutation rate of these genes in the rest of their CVI patients was slightly below the mean rate in their healthy controls. From this one can infer that immunodeficiency in these patients may be due to poor affinity maturation resulting from reduced hypermutation. A study of hypermutation rates in CVI patients here revealed a patient whose rearranged IgG heavy-chain transcripts were nearly all non-functional, with the few functional ones having a normal hypermutation rate. All of the patient's IgM heavy-chain transcripts were functional. Thus, in this case, the immunodeficiency appears to be due to a very small IgG repertoire because there are very few functional rearrangements. The finding that a high proportion of IgG heavy-chain rearrangements are non-functional is extremely unusual. In seven healthy controls, no non-functionally rearranged γ-chain transcripts were found. Since diagnosis the patient's serum IgM has become raised. As SHM is normal, the pattern may best approximate to HIGM4. We present an analysis of the patient's rearranged genes and other investigations.

MATERIALS AND METHODS

Patient and healthy controls

As part of a survey of somatic hypermutation rates in CVI, peripheral blood was obtained from a patient diagnosed as having CVI and attending hospital for regular Ig infusions, and from seven healthy controls (working in our department). The patient is a 44-year-old man referred at age 39 with a history of upper and lower respiratory tract infections since adolescence, including otitis media and sinusitis, recurrent bronchitis and one episode of pneumonia. He also experienced recurrent conjunctivitis. In the four years prior to presentation, cervical lymphadenopathy had accompanied the upper respiratory tract infections, usually resolving completely with antibiotic therapy, but on one occasion precipitating lymph-node biopsy, which demonstrated reactive hyperplasia. There was no family history of immunodeficiency or recurrent infection. At the time of diagnosis he had daily purulent sputum production but no dyspnoea. No lymphadenopathy or other abnormality was present on physical examination. Haematological and radiological investigations excluded lymphoma. Immunoglobulin studies demonstrated IgG 0·08 g/L (normal range 5·0 – 16·0 g/L) IgA , 0·07 g/L (normal range 0·80 – 4·0 g/L), IgM 1·87 g/L (normal range 0·5 – 2·0 g/L) and no serum paraprotein. Routine peripheral blood lymphocyte phenotyping at the time revealed a total lymphocyte count of $2{\cdot}04 \times 10^9$/L, total T-cells ($CD3^+$) $1{\cdot}41 \times 10^9$/L (69%), total B-cells ($CD19^+$) $0{\cdot}102 \times 10^9$/L (5%) and total NK ($CD16^+CD56^+$) $0{\cdot}469 \times 10^9$/L (23%). B-cells were, and have remained, polyclonal with respect to κ/λ expression, and there has been no evidence of the presence of a clonal lymphocyte population in the peripheral blood. Lymphocyte

proliferative responses to mitogens, phytohaemaglutinin, concanavalin A and pokeweed mitogen were normal in comparison to a healthy control.

A diagnosis of CVI was made and he was commenced on intravenous replacement Ig infusions every three weeks. His subsequent course was characterised by decreased frequency of infections with the achievement of normal IgG levels. Eighteen months after diagnosis his IgM became raised, and since then, while it has varied between 1·94 and 6·02 g/L, the overall trend has been upward, though it remains polyclonal. However, testing for known genetic forms of hyper-IgM syndrome has been negative (see Results). Cervical lymphadenopathy has recurred intermittently and has prompted further biopsy but again only demonstrated reactive changes histologically. Unilateral parotid swelling has been present intermittently, biopsy has excluded malignant change, and the patient is under regular clinical and radiological review. There is a six-month history of xerophthalmia. The fluctuations in IgM levels have not correlated with recurrences of lymphadenopathy or parotid swelling, or any other clinical feature.

Cell preparation

PBMC were prepared from whole blood, anticoagulated with EDTA or heparin by centrifugation through Histopaque 1077 (Sigma Diagnostics).

Cytometry

For measurement of IgG^+ B-cells, 0·5 ml of whole blood, anticoagulated with EDTA, was washed twice 1/50 with HBSS at room temperature (r.t.), then incubated at 37°C 1/50 with FACSflow (Becton Dickinson) for 30 min, washed

once more with FACSflow at 37°C, and resuspended in the original volume. 100 µl portions of the washed blood were then incubated for 30 min at r.t. with 1 µl FITC-labelled mouse monoclonal anti-human CD19 (Sigma) and 4 µl of PE-labelled mouse monoclonal anti-human IgG (Pharmingen), or isotype control. The erythrocytes were then lysed by adding 2 ml of FACS Lysing Solution (Becton Dickinson) for 10 min, washed once with FACSflow at r.t. and analysed by flow-cytometry (FACScan, Becton Dickinson). For expression of IgM^+ the method was similar but the antibody was 5 µl PE-labelled polyclonal goat anti-human IgM (Sigma).

For CD40 ligand expression on T-cells, whole blood of the patient and a healthy control was diluted 1 in 10 with Iscove's medium and incubated without stimulants or with 0·5 µM PMA and 1·25 µM ionomycin for 4 hr at 37°C, 5% CO_2. Cells were then labelled with anti-human CD40L-PE and anti-human CD69-FITC (Becton Dickinson) followed by lysis of red cells, washing, and analysis by flow-cytometry as above. CD40 expression on B-cells was checked with 1 µl FITC-labelled mouse monoclonal anti-human CD19 (Sigma) and 10 µl PE-labelled mouse monoclonal anti-human-CD40 (Pharmingen) on 100 µl fresh blood.

For measurement of apoptosis, PBMC were prepared from ~12 ml of heparinised blood, washed 3 times with complete medium, and incubated in a culture flask in 10 ml of complete medium for 1-3 hr at 37°C in a 5% CO_2 atmosphere to enrich the suspension for lymphocytes by removing adherent cells (mainly monocytes). Non-adherent cells in the supernatant were adjusted to $1{\cdot}25 \times 10^6$ cells/ml and a sample (5×10^5 cells) removed (day 0). The remaining cells were cultured in 12-well flat-bottomed culture plates with 1 ml/well, 10^6 cells/ml,

in complete medium alone or with 1/250 pokeweed mitogen or dexamethasone 100 μM, and sampled at days 5 and 8. Sampled cells were permeablised by adding an equal volume of ice-cold 70% ethanol and incubating on ice for 15 min, then washed once with FACSflow and stained overnight with propidium iodide (50 μg/ml) in the dark at 4°C. Next day, cells were washed with FACSflow and data acquired by cytometry (FACScalibur, Becton Dickinson).

Immunoglobulin gene cloning and analysis

RNA was prepared from PBMC with RNAzol B (obtained through Biogenesis Ltd, Poole, U.K.) according to the manufacturer's instructions. cDNA was prepared by reverse-transcription with the SuperScript preamplification system (Life Technologies, Gibco BRL) according to the manufacturer's instructions using the following gene-specific Ig-constant-region primers: for IgG heavy chain, the 'CγA' primer;[25] for κ light chain, HuCKRT and for IgM heavy chain, HuCMRT (Table 1).

Table 1 about here

IgG and IgM heavy chain cDNA were amplified by PCR with the V_H3-30/3-33 leader primer HuVH3-30BACK2 (Table 1) and either the IgG heavy chain constant region primer 'CγB'[25] or the IgM constant region primer HuCMFOR (Table 1), and κ light chains with the Vκ family III leader primer HuVKIIIBACK and the kappa constant region primer HuCKFOR (both as in Table 1) with the Expand High Fidelity PCR System (Roche), 3·5 units/50 μl hot-start reaction, using AmpliWax Gem 50 wax beads (Perkin Elmer) and 1·25 × the enzyme buffer as recommended in the wax beads instructions, with final concentrations of 2 mM $MgCl_2$, 200 μM of each dNTP, 400 nM of each primer for heavy chains and 80 nM for light chains, and 1 - 3 μl of RT-reaction product

as cDNA template. Cycling conditions were 95°C, 2 min, followed by cycles of 94°C for 1 min, annealing at 70°C for IgG and IgM heavy-chain genes and 67°C for κ-chain DNA, all 1 min, then 72°C 2 min, 45 cycles for the heavy chains, 40 for κ chain, followed by 15 min at 72°C.

The products were separated by electrophoresis on agarose gels and stained with ethidium bromide. Appropriately sized bands were excised and the DNA extracted with a QIAquick gel extraction kit (QIAgen), ligated into the pCR2.1 vector ('Original TA cloning kit', Invitrogen), and cloned in *E. coli* INVαF′. DNA was recovered from single white colonies using QIAgen or Macherey-Nagel plasmid miniprep kits, sequenced on an ABI sequencer with fluorescent dye-terminators, and compared with the 'V Base' database of genomic human Ig DNA sequences (MRC Centre for Protein Engineering, Cambridge, U.K.) to identify the gene segments used and the mutations that have occurred. If any other sequence(s) had the same V, D and J the new sequence was then compared with them to (i) exclude contamination from PCR products of other subjects (ii) exclude any identical sequences from the same individual and (iii) identify related sequences with the same rearrangement but different mutations. All non-identical sequences with the same V segment from the same individual were aligned against the parent genomic sequence using BBEdit Lite and DNAPlot software for translation of all mutations and comparison of sequences.

TNFSF5 (CD40-ligand gene) genomic and cDNA inspection

Genomic DNA was prepared from blood using the QIAamp DNA Blood Minikit (QIAgen). Coding sequences of TNFSF5 exons with flanking intronic or untranslated sequence were amplified using the following primers: Exon 1, 40L1S

and 40L1A (Table 1); Exon 2, primers of Shimadzu *et al.*;[27] Exon 3, 40L3S (Table 1) and the antisense primer of pair 4 of Lin *et al.*;[28] Exon 4, 40L4S and 40L4A (Table 1); Exon 5, 40L5S and 40L5A (Table 1). PCR was carried out as for Ig genes with 80 nM primer concentrations, 45°C annealing for Exons 1 – 4 and 55°C for Exon 5, and 45 cycles for all. RNA was prepared as for Ig genes but from activated PBMC prepared as described[29] and cDNA was prepared by reverse transcription as for Ig genes but with an oligo-dT primer. The complete coding sequence of *TNFSF5* cDNA was then amplified with Primer P1 of Seyama *et al.*[29] and primer 40L5A (Table 1). All PCR products were gel-purified and sequenced as for Ig gene DNA and the results were compared with sequences in the NCBI database.

RESULTS

Immunoglobulin expression on B-cells

Fluorescence cytometry showed that similar percentages of the patient's and a healthy control's PBL were B-cells, 9 - 12 % on separate occasions (normal range 8 – 33 %[30]). 99·3 % of the patient's B-cells registered as IgM^+ compared with 89·0 % of the control's. In contrast, < 1% of the patient's B-cells were IgG^+ compared with about 5 % of the control's (Fig. 1).

Fig. 1 about here

Non-functionality in rearranged IgG genes

We amplified rearranged genes with the commonly used V_H segment, V_H3-30. The leader sequences of V_H3-30.3, V_H3-30.5 and V_H3-33 are the same as that of V_H3-30, so rearranged genes using these segments were also amplified and examined. V_H3-30 has 5 alleles and V_H3-33 has 2, the others having only one

known allele. V_H3-30.5 has a sequence identical to one of the alleles of V_H3-30, reducing the total possible number of different sequences to 8. All these genomic alleles have the same length and only a few base-differences.

The characteristics of the rearranged IgG heavy-chain sequences cloned from the patient are summarised in Table 2, and the details of the sequences shown in Fig. 2. Of 40 sequences cloned, there were 22 different sequences, of which only three were functional, and these all had exactly the same VDJ junction (Rearrangement 1 in Fig. 2) and were therefore derived from clonally-related B-cells. Among the non-functional sequences there were two pairs of clonally-related sequences and two groups of three clonally-related sequences, giving a total of 14 different rearrangements. Only Rearrangement 1 was both functional and completely normal. Rearrangement 2 was unusual in having no D segment but it was in frame and might have been functional. However, all three related sequences bearing this junction had a mutation producing a stop-codon in the V_H segment. Non-functional immunoglobulin genes can be generated either during V(D)J rearrangement or by mutation. All the other 12 of the patient's IgG rearrangements were non-functional due to the nature of the junction, either being out of frame, having D-segments in frames that generated stop-codons, having a stop-codon in the P/N bases, or a combination of these.

Table 2 and Fig. 2 about here

In 7 healthy controls a total of 72 different rearranged IgG cDNA sequences were examined and all junctions were functionally rearranged. Two of these sequences were non-functional due to frame-shifts within the V segments. Both of these may have been PCR artefacts as, in each case, the frame-shift occurred at a run of like bases and one of the sequences was identical to a functional sequence except for the frame-shift.

Functionality of other rearranged Ig genes

To determine whether the patient might have a general defect of Ig gene rearrangement, his rearranged kappa-chain genes were cloned. All of the 10 sequences examined were functional and none were related. Since class-switching to IgG changes the constant region expressed without changing the V_HDJ_H rearrangement, we examined the patient's IgM heavy-chain sequences. 18 were examined and again all were functional, and none related. The HCDR3 sequences of the μ-chain transcripts are shown in Fig. 3.

Fig. 3 about here

Length of junctions and HCDR3s in the patient's rearranged heavy-chain genes

Table 3 shows the lengths of the HCDR3s and all their components. The mean HCDR3 length of the IgG rearrangements is greater than that of the IgM rearrangements, but not in all components. The V_H and J_H components of the IgG HCDR3s are slightly shorter than those of the IgM rearrangements and the D segments slightly longer, and these approximately cancel out. In those rearrangements in which the D segments could be identified, the difference in mean HCDR3 length is approximately equal to the number of extra N base-pairs.

Table 3 about here

Somatic mutation rate

Somatic mutations are detected by comparing rearranged gene sequences with unrearranged genomic sequences. Somatic hypermutation of rearranged Ig genes occurs throughout the length of the V_H, D and J_H segments. However, as sequence changes also occur at the V_H-D and D-J_H junctions as part of the rearrangement

process, later somatic mutation at these places cannot be detected easily in peripheral blood B-cells. Therefore base-changes were only counted within the V_H segments of the rearranged genes for comparison within and between subjects. Further, they were only counted up to the end of 'codon 92' (Chothia nomenclature) because some bases may be deleted from the end of the V_H segment during rearrangement. This region comprises 96 codons, *i.e.* 288 bp, the full length of the V_H segments examined being 296 bp.

The average number of base changes per V_H segment in γ-chain transcripts for each control subject ranged between 10·5 changes (3·65%) and 28 changes (9·7%) and the overall average was 7·12% for the 7 control subjects.

In the patient, as shown in Table 2, the number of mutations in non-functional γ-chain genes was generally low but the three clonally related functional genes had 20 base-changes in common with two having a further 2 common and one of those having one more. With so few functional sequences, it is not possible to determine the patient's true average mutation rate in functionally rearranged genes, but these results – which give a figure of 7·5% - suggest that the mutation mechanism is not impaired. The two clonally related γ-chain sequences with additional base-changes were obtained from a blood sample taken 5 months after the first, showing long-term survival of cells with this functional rearrangement.

Of the 18 μ-chain transcripts, 16 had 0 – 2 mutations, one had 6, and one had 7. The overall mutation rate was 0·44%. Because cells expressing IgM were far more abundant than those expressing IgG, most if not all the κ transcripts are likely to have come from cells expressing IgM and hence would not be expected to be heavily mutated; they had 0 - 5 base-substitutions in the V_κ segment.

In view of the bias towards transitions at C·G base-pairs reported in patients

with mutations in UNG,[17] the different types of base-substitution were also counted. In their work on *UNG*[17] and HIGM4,[22] Imai *et al.* found a mean of 4% of bases substituted in the IgM heavy-chain transcripts of $CD19^{+}CD27^{+}$ memory B-cells. The rate of mutation in the IgM transcripts from our patient's unsorted cells was lower, as would be expected, so we investigated the transitions and transversions in the V segments of all transcripts: IgM heavy chains, functional and non-functional IgG heavy chains, and κ chains (Fig. 4). There was no transition bias in mutations at C·G base-pairs, though it is possible that there was some mild disturbance of mutation balance. Imai *et al.* gave the percentage of mutations at C·G base-pairs as 63·6% with a range of 62 – 66 in 7 control subjects while in our patient it was only 55%. Also, in comparison with their figures, our patient appears to have fewer G → C and more C → G mutations than expected, and also fewer C → T mutations, but this may just be due to small numbers.

Apoptosis of peripheral blood mononuclear cells

The finding that such a high proportion of γ-chain sequences were non-functional suggested that there must be surviving B-cells with no functional Ab. These cells would normally be eliminated by apoptosis. To test the hypothesis that this process is defective in our patient, apoptosis of PBL before and after culture in the presence of apoptosis inducers was assessed. No deficit in the ability of the patient's PBMC to enter apoptosis was demonstrated (Fig. 5).

Fig. 5 about here

Genetic testing for hyper-IgM syndromes

CD154 (CD40 ligand) gene, *TNFSF5*: The patient's activated T-cells showed normal CD154 expression by flow cytometry compared with healthy controls. In case the protein carried mutations affecting its function, the gene was also examined. Sequences of the five exons, amplified from genomic DNA, showed no mutations in coding sequences but two differences from the database sequence of intron 2 (accession no. D31795) were seen in four independent PCRs. There was replacement of C by A at the –57 position with respect to the beginning of Exon 3, and insertion of an A residue between positions –53 and –52. These differences do not affect RNA splicing as the cDNA showed only a single product and the sequence was normal.

CD40: Surface-expression of CD40 on the patient's B-cells was normal. All four patients so far reported with mutations of the CD40 gene, *TNFRSF5*, have lacked surface expression of CD40,[15,16] and since the patient's cells also exhibited CSR and SHM, the gene itself was not sequenced.

The *AICDA* (*AID*) and *UNG* genes were assessed for us by the laboratory of Anne Durandy (Hôpital Necker-Enfants Malades, Paris) and no abnormalities were found.

The (NEMO) gene, *IKBKG*, was not studied as the patient did not have hypohydrotic ectodermal dysplasia.

DISCUSSION

We present a novel case of an immunodeficient patient, originally diagnosed with common variable immunodeficiency who subsequently demonstrated raised IgM, in whom nearly all IgG heavy-chain transcripts from unsorted peripheral blood lymphocytes are non-functional. Since V(D)J rearrangement has an

approximately 2/3 chance of producing a non-functional result, and cells making such rearrangements may survive if they make a functional rearrangement on the paired chromosome, non-functional rearrangements are commonly found amongst V(D)J clones from chromosomal B-cell DNA. Our finding of non-functional transcripts, however, is highly unusual. We found no non-functional rearrangements amongst γ-chain transcripts from 7 healthy controls and, in our patient, all the μ- and κ-chain transcripts were functional. Chromosomal VDJ amplifications are carried out with V and J primers, because the constant-region exons are far downstream, so it is usually not known whether switching has occurred in the non-functional rearrangements, but our finding that all non-functional transcripts were γ-chains has not to our knowledge been reported previously. We conclude that either non-functional rearrangements were switched from C_μ to C_γ, or functional genes were switched and became non-functional afterwards.

Of the six hyper-IgM types described so far, this patient, with a history of recurrent bacterial but not opportunistic infections, lymphadenopathy without giant germinal centres, normal rate of hypermutation in his few functional IgG transcripts, and lack of transition bias, approximates best to the description of HIGM4.[22] Furthermore, genetic testing has ruled out HIGM1, 2 and 3, and UNG deficiency, and he does not exhibit hypohydrotic ectodermal dysplasia seen in patients with NEMO gene mutations. His age at diagnosis (39 years) was rather older than that of any of the 15 HIGM4 patients described (range 0·3 – 23 years) but he has a history of recurrent infections since adolescence. Like the HIGM4 patients described, he also had normal numbers of circulating lymphocytes, and normal percentages of T-cells and NK cells. His first B-cell percentage was below

the normal range but was normal on subsequent occasions.

In HIGM4, transcripts from excised circular DNA from Cμ to Iε were not detected, and 'functional' IgG and IgE transcripts (V_H-C) were both either in reduced quantities or undetected in different patients.[22] The method was RT-PCR. Sequencing was only performed on IgM transcripts from sorted IgM^+CD27^+ B-cells, so it is not known whether the IgG and IgE transcripts were functional in terms of being in frame and without stop-codons. The transcripts in memory cells would be expected to be functional or differentiation to memory cells would surely not have occurred. Our observation of non-functional IgG transcripts in unsorted B-cells therefore does not rule out HIGM4 in our patient.

Four possible explanations might be considered for our finding that a high proportion of γ-chain transcripts were non-functional while all μ-chain transcripts were functional:

1. There is no selection for B-cells with functional IgG, *i.e.* these cells are not given a survival signal.
2. There is no selection against B-cells that do not express any functional Ig, *i.e.* these cells are not deleted by apoptosis.
3. There is selection against cells bearing functional IgG.
4. Secondary rearrangement of V_HDJ_H segments (receptor revision[31,32]) is taking place soon after switching to IgG and much of this renders previously functional genes non-functional.

Most of our γ-chain transcripts were non-functional because of the nature of the rearrangements rather than mutation. This included the use of D-segments in

frames that read with stop codons, and J-segments out of frame with V segments. Therefore, the first three possibilities raise at least three problems: (a) they assume that non-functional VDJ rearrangements are class-switched from IgM to IgG, though we have been unable to find any evidence for this in the literature; (b) at least some cells with functional IgG are long-lived since the three functional IgG transcripts found were clonally related and two of them were derived from a blood sample taken five months after the one from which the first was amplified; (c) apoptosis of PBL is normal or even increased in the patient (Fig. 4).

On the other hand, evidence for secondary rearrangement after class-switching is also slim. To produce the observed non-functional rearrangements from previous functional ones would have required in some cases frame change at the V_H-D junction (achievable by V_H replacement or open-and-shut recombination), in other cases change at the D-J_H junction (possibly achievable by J_H replacement or open-and-shut recombination), and yet in other cases change at both junctions (to change the frame of the D segment whilst preserving the relationship of J_H to V_H). Hints that such processes could have occurred are restricted to: (i) the finding that the number of N base-pairs is greater at both V_H-D and D-J_H junctions in the IgG transcripts than in the IgM, significantly so in the former, while the lengths of other components of the HCDR3s are unchanged. Segment replacement tends to increase the number of N base-pairs[33] while the alternative possibility - of selection reducing HCDR3 length in the functional IgM – tends to reduce all components, not just the Ns.[34] (ii) the presence of bases in V_H-D junctions that could have belonged to previous in-frame V_H segments, although these are too short to provide strong evidence (data not shown) (iii) the observation of two possible cryptic RSS heptamers created by somatic hypermutation in the patient's

μ sequences (underlined in Fig. 3) suggesting that J_H replacement might be possible.

Imai *et al.*[22] concluded that in their HIGM4 cases there was a selective CSR defect downstream of AICDA activity, but suggested as an alternative that there might be a deficiency of a factor required for the survival of switched B-cells. The latter could also apply in our case but it would require the deficiency to occur only in cells with functional Ig genes. It will be interesting to know whether the switched gene transcripts in their cases also have a high proportion of non-functional ones.

In summary, we have found a case of hyper-IgM syndrome with no known genetic defect whose features correspond closely to the description of HIGM4. Rearranged IgM and κ transcripts are all functional but IgG transcripts are nearly all non-functional, suggesting that there is a cell-survival anomaly in switched B-cells (and possibly receptor revision after class-switching).

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