Thirteen Japanese patients with hyper-IgM syndrome but normal CD40 ligand were characterized. All patients had mutations in AID (activation-induced cytidine deaminase) gene. Five of them had a missense mutation of Arg112His. In all patients, serum IgG, IgA and IgE levels were undetectable, B cells failed to produce detectable amounts of IgE even if cultured them with anti-CD40 and IL-4. Somatic hypermutation (SHM) was also impaired in their peripheral blood B cells. These results suggest that Arg112 is the hot spot of AID mutation and demonstrate that AID plays indispensable roles in class switch recombination (CSR) and somatic hypermutation (SHM) in human B cells. In addition, serum IgM levels in the patients have been continuously high even after proper intravenous immunoglobulin infusion (IVIG) and without infection, indicate that AID has the function to induce spontaneous IgM production in B cells.

Key words: Immunodeficiency, Hyper-IgM Syndrome, AID, Class Switch Recombination, Somatic Hypermutation.
APOBEC-1, an mRNA editing enzyme that deaminates a cytidine to uridine within the transcript for apoB mRNA. Physiological role of AID is still unknown; however, AID may function, like APOBEC-1, as an enzymatic component of an RNA-editing complex. Together with an ACF (APOBEC-1 complementation factor)-like RNA-binding protein, AID may edit the RNA of hypermutation and/or CSR-control factor(s). AID may also function as a nick-inducing deaminase by deamination of C to U in the hypermutation domain or CSR of Ig genes.

Revy et al. identified ten different mutations in AID in eighteen patients with autosomal recessive form of the hyper-IgM syndrome. In those cases, family consanguinities were frequent. Minegishi et al. identified three different mutations in AID in eighteen patients in a closed society. Mutation analysis of AID has not yet been reported in Japan. In order to know whether there is any unique AID mutation in Japanese patients, we sequenced AID gene of thirteen patients with hyper-IgM syndrome without defect in CD40 ligand. In this paper, we showed ten independent mutations observed in thirteen patients. The mutation of Arg112His was found in five patients. This implies that Arg112His is the hot spot of AID mutation in Japanese. We also demonstrated that B cells from the patients possessed defective CSR and defective generation of SHM in Ig variable region genes. Possible role of AID in excessive IgM production will be discussed.

Subjects and Methods

This study was designed in accordance with the declaration of Helsinki and approved by institutional ethical committee. Written informed consents were obtained from all patients or their parents before the participation of this study.

Patients

Thirteen patients from eleven unrelated families were diagnosed of having hyper-IgM syndrome because of markedly diminished serum IgG, IgA and IgE levels with strikingly elevated serum IgM. CD40 ligand expression on activated T cells of these patients was normal and no mutation was found in their CD40 ligand gene. Three patients (P1, P2 and P13) were from two cousin marriage families. Two patients, P3 and P4, were elder brother and younger sister, but they did not come from consanguineous family. There was no family history or consanguineous marriage in other cases. All the patients had experienced recurrent bacterial pneumonia and otitis media, however, did not suffer from opportunistic infections, such as Pneumocystis carinii or Cryptosporidium. All the patients had lymphoid hyperplasia that was seldom observed in HIGM1 patients (Table 1). Once diagnosis was made, nine patients were treated with intravenous Ig substitution (IVIG).

Cell preparation and ELISA

Peripheral blood mononuclear cells (PBMCs) were isolated as described previously. B cells were obtained from the PBMCs with Dynabeads M-450 CD19 (Dynal), and the beads were detached from the B cells using DETACHaBEAD CD19 (Dynal). Purified B (5 × 10⁶/well) cells were incubated with anti-CD40 mAb (1μg /ml) and IL-4 (20 ng/ml) for 12 days, and the production of IgE in supernatants was measured by specific IgE ELISA. All values were determined in triplicate.

Detection of CD40L expression with anti-CD40L antibody by flow cytometry

PBMCs were stimulated with PMA (20ng/ml) plus ionomycin (1μg/ml) for 6 hours, and then PBMCs

Table 1. Clinical and laboratory findings of 13 non-X-linked hyper-IgM syndrome patients.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex</th>
<th>Age</th>
<th>IgG (mg/dl)</th>
<th>IgA (mg/dl)</th>
<th>IgM (mg/dl)</th>
<th>IgD (mg/dl)</th>
<th>IgE (mg/dl)</th>
<th>Lymphoid hyperplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>F</td>
<td>32</td>
<td>3160</td>
<td>2660</td>
<td>1380</td>
<td>5400</td>
<td>6440</td>
<td>+</td>
</tr>
<tr>
<td>P2</td>
<td>F</td>
<td>37</td>
<td>3160</td>
<td>1380</td>
<td>6440</td>
<td>5400</td>
<td>3160</td>
<td>+</td>
</tr>
<tr>
<td>P3</td>
<td>F</td>
<td>25</td>
<td>1520</td>
<td>1380</td>
<td>6440</td>
<td>5400</td>
<td>5400</td>
<td>+</td>
</tr>
<tr>
<td>P4</td>
<td>M</td>
<td>31</td>
<td>5400</td>
<td>3160</td>
<td>5400</td>
<td>6440</td>
<td>5400</td>
<td>+</td>
</tr>
<tr>
<td>P5</td>
<td>F</td>
<td>48</td>
<td>6440</td>
<td>5400</td>
<td>6440</td>
<td>5400</td>
<td>5400</td>
<td>+</td>
</tr>
<tr>
<td>P6</td>
<td>F</td>
<td>27</td>
<td>5400</td>
<td>5400</td>
<td>6440</td>
<td>5400</td>
<td>5400</td>
<td>+</td>
</tr>
<tr>
<td>P7</td>
<td>M</td>
<td>35</td>
<td>6440</td>
<td>6440</td>
<td>6440</td>
<td>6440</td>
<td>6440</td>
<td>+</td>
</tr>
<tr>
<td>P8</td>
<td>F</td>
<td>21</td>
<td>5400</td>
<td>5400</td>
<td>6440</td>
<td>5400</td>
<td>5400</td>
<td>+</td>
</tr>
<tr>
<td>P9</td>
<td>F</td>
<td>1</td>
<td>3160</td>
<td>3160</td>
<td>3160</td>
<td>3160</td>
<td>3160</td>
<td>+</td>
</tr>
<tr>
<td>P10</td>
<td>F</td>
<td>60</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>+</td>
</tr>
<tr>
<td>P11</td>
<td>F</td>
<td>31</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>+</td>
</tr>
<tr>
<td>P12</td>
<td>M</td>
<td>40</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>+</td>
</tr>
<tr>
<td>P13</td>
<td>M</td>
<td>35</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>5380</td>
<td>+</td>
</tr>
</tbody>
</table>

Normal values: 870-1700 110-410 10-190 0-11.5 0-170

*; these patients are siblings. nd: not done. IVIG: intravenous Ig substitution.
were stained with FITC anti-CD40L mAb (PharMingen) or 5c8 for 30 min. Expression of CD40 ligand was analyzed by a FACScan flow cytometry.

The DNA sequence of CD40 ligand was also analyzed in genomic DNA obtained from granulocytes of patients as described previously.

**AID Gene Sequencing**

Genomic DNA was extracted from granulocytes of patients using Sepa Gene (Sanko Junyaku, Tokyo, Japan). The five exons of AID were amplified by polymerase chain reaction (PCR) using Taq High Fidelity (GibcoBRL). The PCR products were sequenced with a BigDye Terminator Cycle Sequencing Kit (ABI PRISM) and analyzed with a 310 Genetic Analyzer (ABI PRISM) according to manufacturer’s instructions.

Total RNA was extracted from EB cell lines established from patients’ PBMCs by ToTALLY RNA Kit (Ambion). First-strand cDNA was produced from 2 µg total RNA with ThermoScript™ RT-PCR System (GibcoBRL) using oligo dT as a primer. AID RT-PCR was performed with a forward primer: 5'-ATGGACAGCCTCCTTGATGAA-3' and a reverse primer: 5'-TCAAAGTCCCAAAGTACGAA-3' (1 cycle at 94°C 5 min, then 30 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min, with a final extension for 7 min at 72°C). PCR products obtained from cDNA were subsequently subcloned into pGEM-T Easy Vector System (Promage), and at least five clones were sequenced and analyzed.

**Cloning and Sequencing of Ig VH5 genes**

Total RNA extraction and cDNA synthesis from unstimulated PBMCs were performed as previously described. Ig VH5 genes were amplified using the primers corresponding to the 5' region of VH5 leader sequences 5'-ATGGGCTCAACCCGCATCTC-3' and to the 3' constant region 5'-GTTCCTGTGC-GAGGCAGCCCA-3' (14,15). The PCR condition was 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, with a final 7 min-extension at 72°C. PCR products obtained from cDNA were subsequently subcloned into pGEM-T Easy Vector System (Promage), and at least eight clones were sequenced and compared with germ-line sequence.

### Results

**Mutation Analysis of the HIGM2 Patients**

Thirteen patients with hyper-IgM syndrome but normal CD40 ligand were monitored for mutations in AID gene. Ten independent mutations (six missence mutations, one insertion and two deletion mutations, one splice donor site mutation) were found through our analysis (Table 2).

Although we found eleven homozygous mutations in thirteen patients, only two families were consanguineous. A missence mutation, Arginine112Histidine, was observed in five patients (P3, P4, P5, P6, and P7) from four unrelated families. Parents of P6 and P7 were heterozygous for the R112H mutation. Other homozygous missense mutations were found in P10, P12 and P1, P2. Two mutations, Isoleucine136Lysine in P10 and Serine43Proline in P12 were not reported before. Arginine24Tryptophan mutation found in P1, P2 consanguineous family was published previously.

Nucleotides 428-543 corresponding to the exon 4 were deleted in the cDNA of P11. By analyzing her genomic DNA, one base pair mutation was found at intron 4, +1g→c, which is important for the splicing of

<table>
<thead>
<tr>
<th>Patients</th>
<th>Nucleotide change</th>
<th>Predicted effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1, P2</td>
<td>▲▲C70T</td>
<td>Homo R24W</td>
</tr>
<tr>
<td>P3, P4</td>
<td>▲▲G335A</td>
<td>Homo R112H</td>
</tr>
<tr>
<td>P5</td>
<td>▲▲G335A</td>
<td>Homo R112H</td>
</tr>
<tr>
<td>P6</td>
<td>▲▲G335A</td>
<td>Homo R112H</td>
</tr>
<tr>
<td>P7</td>
<td>▲▲G335A</td>
<td>Homo R112H</td>
</tr>
<tr>
<td>P8</td>
<td>▲C334T</td>
<td>Hetero R112C</td>
</tr>
<tr>
<td>P9</td>
<td>▲T31G,</td>
<td>Hetero P11V</td>
</tr>
<tr>
<td></td>
<td>▲226T ins</td>
<td>Hetero aa 116 stop (p1)</td>
</tr>
<tr>
<td>P10</td>
<td>▲▲T407A</td>
<td>Homo I136K</td>
</tr>
<tr>
<td>P11</td>
<td>▲▲intron4 +1g→c</td>
<td>Homo Exon4 del</td>
</tr>
<tr>
<td>P12</td>
<td>▲▲T127C</td>
<td>Homo S43P</td>
</tr>
<tr>
<td>P13</td>
<td>▲▲235 del 9bp</td>
<td>Homo in-frame 3aa del</td>
</tr>
</tbody>
</table>

Abbreviation: homo, homozygous; hetero, heterozygous; ins, insertion; dele, deletion; aa, amino acid; pt, premature termination. R, Arginine; W, Tryptophan; H, Histidine; C, Cysteine; F, Phenylalanine; V, Valine; I, Isoleucine; K, Lysine; S, Serine; P, Proline.

In 11 out of 13 patients, the mutations were homozygous. Only P8 and P9 had compound heterozygote mutations. 226T ins and 235 del 9bp were located in the cytidine deaminase catalytic region.
Defect of Class Switch Recombination in B cells from HIGM2 patients stimulated with anti-CD40 mAb and IL-4

AID is indispensable for CSR. Serum IgG is undetectable in HIGM2 patients, but can be detected in most of HIGM1 patients. In order to know whether detected AID mutations truly affect Ig class switch in B cells from patients, we tested the capacity of their B cells to mount Ig response in vitro. IgE production was induced in B cells from controls and HIGM1 patients after activation of B cells by anti-CD40 mAb+ IL-4, whereas IgE production was undetectable when the B cells from AID-deficient patients were incubated in the same condition (Table 3).

Ig VH5 Genes Somatic Mutations in HIGM2 Patients

The somatic hypermutation process is initiated in germinal center in lymph nodes and spleen when B cells encounter antigens that can react with their specific Ig molecules. After extensive proliferation of B cells, B cells produce mutated Igs with a high affinity for the immunizing antigens and are selected for survival and become long-lived memory cells. To investigate consequences of AID defect in B cell differentiation, we analyzed the frequency of somatic mutation in the Ig variable region genes belonging to the small VH5 family of the patients. Frequency of SHM in four normal adults ranged from 2.17% to 2.61%, whereas the VH5 genes sequences of P3, P6 and P9 ampliﬁed from unstimulated PBMCs were 100% identical to published VH5 germ-line genes sequences (VH251 or VH32). In P5, P7, P12 and P13, low rates of SHM were detected, while signiﬁcant frequency of SHM was found in P8 (Table 4).

Table 3. In vitro IgE production by B cells obtained from patients with hyper-IgM syndrome type 2.

<table>
<thead>
<tr>
<th>Pts</th>
<th>IgE production (pg/ml)</th>
<th>anti-CD40 + IL-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)</td>
<td>(&lt;50)</td>
<td>(&lt;50)</td>
</tr>
<tr>
<td>P1</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>P2</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>P3</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>P4</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>P5</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>P6</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>P7</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>P8</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Normal</td>
<td>18377±6624</td>
<td>16107±5096</td>
</tr>
<tr>
<td>HIGM</td>
<td>(&lt;50)</td>
<td>(&lt;50)</td>
</tr>
</tbody>
</table>

Purified B cells were incubated with anti-CD40 mAb (1ug/ml) and IL-4 (20ng/ml) for 12 days, and the production of IgE in supernatants was measured by IgE speciﬁc ELISA. All values were determined in triplicate.

Table 4. Somatic Hypermutations in VH5-Ig genes.

<table>
<thead>
<tr>
<th>clones</th>
<th>mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>Unmutated</td>
</tr>
<tr>
<td>Control 1</td>
<td>10</td>
</tr>
<tr>
<td>Control 2</td>
<td>9</td>
</tr>
<tr>
<td>Control 4</td>
<td>8</td>
</tr>
<tr>
<td>Control 5</td>
<td>10</td>
</tr>
<tr>
<td>P3</td>
<td>10</td>
</tr>
<tr>
<td>P5</td>
<td>14</td>
</tr>
<tr>
<td>P6</td>
<td>10</td>
</tr>
<tr>
<td>P7</td>
<td>10</td>
</tr>
<tr>
<td>P8</td>
<td>10</td>
</tr>
<tr>
<td>P9</td>
<td>10</td>
</tr>
<tr>
<td>P12</td>
<td>10</td>
</tr>
<tr>
<td>P13</td>
<td>10</td>
</tr>
</tbody>
</table>

PCR error: 0.05 per bp % | bp %: 100 base pairs

* nucleotide exchange frequency in the VH5 segments of Cμ transcripts. The total number of nucleotides of VH5 segments is 294 base pairs.
Discussion

AID is a responsible gene for hyper-IgM syndrome type 2. In this study, we analyzed the sequence of AID gene from thirteen patients with non-X-linked hyper-IgM syndrome. Ten independent mutations were found in thirteen patients. Only two families were consanguineous. The mutation of R112H was found in five patients who were from four unrelated families. The parents of two patients were heterozygous for this mutation. These data, including two Japanese patients who also had R112H in AID gene reported by Minegishi et al., imply that R112H may be the hot spot of AID mutation in Japanese and the number of carriers with R112H mutation may be higher in Japan than anywhere in the world. In case of P13, the expected protein alteration was in-frame 3 aa deletion. Because the mutation is located in the cytidine deaminase catalytic region, it can severely interfere with the function of AID. B cells from the same patient showed defective SHM as well as CSR. These data imply that catalytic domain is indispensable for SHM and CSR. Except R24W, R112H and R112C, other mutations have never been reported before.

AID is shown to be required for CSR. Consistently, all the patients with AID mutations show undetectable amounts of serum IgG, IgA and IgE. This finding is in contrast to the observation that most of the HIGM1 patients have traceable amounts of serum IgG. IgE production was not detected even if B cells from the HIGM2 patients were incubated with anti-CD40 + IL4. IgD^CD27^B cells could be detected in PBMCs of one HIGM2 patient (P1) and when stimulated B cells with anti-CD40 + IL4, CD23 was also increasingly induced in three patients with AID mutations (data not shown). These results were in concordant with data by Revy and Minegishi. There were normal proportions of memory B cells, but these B cells could not undergo CSR. All of these results indicate that AID play indispensable role in CSR.

AID is the first gene that was found to be required for SHM. In consistent with this, all of our HIGM2 patients except one patient showed defective SHM in their B cells (Figure 1). However, in one patient (P8), the rate of SHM was within normal range of age-matched controls (6 years old control: 0.81%–0.96%). SHM increases the affinity of the antibody so that it can neutralize viruses and toxins and inactivate pathogenic organisms. Truly, P8 did not receive intravenous Ig infusion even if she also had recurrent severe infections. Interestingly, the class switch was completely impaired in P8. This result suggests that AID may function differently in CSR and SHM using the diverse mechanisms. Alternatively, this would be explained by residual AID activity in some missence mutations or environmental factors. Clinical characteristics of patient, for example, intensity of infections that occurred in patient could also affect the AID functions.

Most of HIGM2 patients display lymphoid hyperpla-
The reason for this phenomenon is currently unknown. Germinal center proliferating B cells of HIGM2 patients express IgM, IgD, and CD38. Lymphoid hyperplasia did not disappear after the intravenous Ig infusion as well. These results suggest that in absence of functional AID, B cells are continuously triggered to proliferate by antigens, but no successful CSR and Ig variable region gene somatic mutations have occurred. As shown in Table 1, the amounts of serum IgG were undetectable in HIGM2 patients, and IgM levels were highly increased in all patients. It is of note that even in pediatric patients (P8, P9), serum IgM levels were more than 1,000mg/dl. In addition, these high amounts of IgM are less likely to be caused by recurrent infections, since patients (P1, 3, 5, 6, 7, 9, 10, 11, 12) under appropriate immunoglobulin replacement therapy (IVIG) show the high amounts of serum IgM levels (Figure 2). These findings indicate that defective AID results in spontaneous production of high amounts of IgM and serum IgG does not suppress the production of IgM. Thus, AID might play an essential role in regulating IgM production and in lymphoid hyperplasia.

Acknowledgment

We thank the following physicians for referring patients: Kawano S, Moriguchi N, Yosida M, Endo M, Kashimura M, Tanabe T, Kodaka Y, Osawa M, Ogura T, Takada M, Kawahara K, Ito T, Ishimoto K, Dan K, and Sako M.

References