Low-affinity IgM antibodies lacking somatic hypermutations are produced in the secondary response of C57BL/6 mice to (4-hydroxy-3-nitrophenyl)acetyl hapten

Akikazu Murakami, Hayato Moriyama, Mina Osako-Kabasawa, Kanako Endo, Miyuki Nishimura, Keiko Udaka, Masamichi Muramatsu, Tasuku Honjo, Takachika Azuma and Takeyuki Shimizu

1Laboratory of Structural Immunology, Division of Bioinformatics, Research Institute for Biomedical Sciences (RIBS), Tokyo University of Science, Chiba 278-0022, Japan
2Department of Immunology, Kochi Medical School, Kochi University, Nankoku-shi, Kochi 783-8505, Japan
3Department of Molecular Genetics, Graduate School of Medical Science, Kanazawa University, Kanazawa 920-8640, Japan
4Department of Immunology and Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan

Correspondence to: T. Shimizu; E-mail: tshimizu@kochi-u.ac.jp

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Abstract

Class-switched memory B cells, which are generated through the processes of somatic hypermutation (SHM) and affinity-based selection in germinal centers, contribute to the production of affinity-matured IgG antibodies in the secondary immune response. However, changes in the affinity of IgM antibodies during the immune response have not yet been studied, although IgM memory B cells have been shown to be generated. In order to understand the relationship between IgM affinity and the recall immune response, we prepared hybridomas producing anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) IgM antibodies from C57BL/6 mice and from activation-induced cytidine deaminase (AID)-deficient mice. Binding analysis by ELISA showed that mAbs obtained from the secondary immune response contained IgM mAbs with affinity lower than the affinity of mAbs obtained from the primary response. By analyzing sequences of the IgM genes of hybridomas and plasma cells, we found many unmutated \(V_H\) genes. \(V_H\) genes that had neither tyrosine nor glycine at position 95 were frequent. The repertoire change may correlate with the lower affinity of IgM antibodies in the secondary response. The sequence and affinity changes in IgM antibodies were shown to be independent of SHM by analyzing hybridomas from AID-deficient mice. A functional assay revealed a reciprocal relationship between affinity and complement-dependent hemolytic activity toward NP-conjugated sheep RBCs; IgM antibodies with lower affinities had higher hemolytic activity. These findings indicate that lower affinity IgM antibodies with enhanced complement activation function are produced in the secondary immune response.

Keywords: affinity maturation, antibody, memory B cell, plasma cell, somatic hypermutation

Introduction

When naive B cells recognize T cell-dependent antigens via the BCR and receive help from \(T_H\) cells, they become activated and undergo proliferation and differentiation. Some cells migrate to the extrafollicular region and differentiate into short-lived plasma cells (PCs) (1). These cells secrete IgM or IgG antibodies, which are in germline configuration and have relatively low affinity to antigens. Other cells migrate into follicles and form germinal centers (GCs) (2–4) where somatic hypermutation (SHM) and class-switch recombination (CSR) are considered to take place. Both processes are dependent on activation-induced cytidine deaminase (AID) (5).

In GCs, the affinity of BCRs is changed through SHM and B and B cells are screened by antigens on follicular dendritic cells; B cells that obtain BCRs of higher affinity to antigens are selected and leave GCs, while B cells that acquire
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self-reactivity or lose affinity to antigens will disappear (6, 7). After leaving GCs, the antigen-selected B cells differentiate into long-lived PCs, which reside in the bone marrow (8, 9), or become memory B cells. Upon encountering the same antigen, memory B cells predominantly respond and differentiate into PCs. Therefore, affinity-matured and class-switched antibodies are abundant in the secondary immune response. The affinity maturation and repertoire changes of anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) IgG antibodies have been reported (10–12). The canonical anti-NP antibodies produced in C57BL/6 mice are composed of heavy chains encoded by the V_{H}186.2 gene segment and light chains (13, 14), most of which have tyrosine or glycine at position 95 (according to the amino acid residue numbering system of Kabat et al. (15)) of the heavy chain. Tyr95-type antibodies have tyrosine at this position and are found frequently in the primary response. Gly95(H)-type antibodies, which have glycine at position 95 and histidine at position 100, are found in the secondary response (11, 16). Tyr95-type antibodies can increase affinity quickly because a single amino acid substitution at position 33 (referred to as the W33L mutation) can raise affinity to NP ~10-fold (17–19), with added mutations causing a modest increase in affinity. Gly95(H)-type antibodies require multiple mutations in both heavy and light chains for an increase in affinity (20, 21). Therefore, affinity maturation progresses slowly, but after accumulating multiple mutations, their affinity reaches ~1000-fold higher than those of germline antibodies. The rapid maturation enables the rise of Tyr95-type antibodies in the early phase of the immune response, while Gly95(H)-type antibodies can overcome competition later by higher evolvability. As described, the affinity maturation of anti-NP IgG antibodies is closely related to generation of memory B cells capable of acquiring higher affinity by SHM through a GC-dependent pathway. The induction of SHM and antibody evolvability determined by the amino acid residue at position 95 are critical factors for establishment of IgG+ memory B cells.

Memory B cells were formerly defined as those possessing class-switched and somatically mutated Ig genes since they were thought to be generated only through a GC-dependent pathway. Recently, however, heterogeneity of memory B cells, in terms of BCR isotypes and SHM expression, has been reported (22–24) and IgM+ memory B cells have been shown to exist (25–28) and to be generated through a GC-independent pathway (29, 30). Although IgM antibodies are considered to be secreted at the early stage of primary immunization and have lower affinity than IgG antibodies, it is interesting to examine how these IgM+ memory B cells are involved in antibody production and to determine whether IgM antibodies of the primary and secondary responses are different from each other in terms of structure and function. In addition, IgM antibodies in the secondary immune response may be different from IgG antibodies since the numbers of mutations possessed by IgM+ and IgG+ memory B cells were reported to be different (23, 31).

In this study, we established hybridomas producing anti-NP antibodies and analyzed sequences of Ig genes and their affinities to NP. We report here that the IgM repertoire of the secondary response is more heterogeneous than the IgG repertoire in terms of V_{H} gene usage. There are many unmutated V_{H} genes. V_{H} genes that have codons corresponding neither to tyrosine nor to glycine at position 95 (designated as X95 type) are frequently found. These genes encode antibodies with a very low affinity to NP but grant them a higher capacity to activate the complement pathway. V_{H} genes with these features are frequently found in IgM+ PCs in the secondary response. These results suggested that B cells with different types of BCRs are selected as IgM+ memory B cells and differentiate into PCs in the secondary immune response.

Methods

Mice and immunization

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). AID-deficient mice (5) were crossed with C57BL/6 mice for more than 10 generations. Mice more than 8 weeks old were used for immunization. NP-CGG (chicken γ-globulin) (Rockland, Gilbertsville, PA, USA) whose NP valence was 30–40 per CGG molecule was prepared as previously described (32). NP-CGG was precipitated with alum, and 100 μg of antigen was intra-peritoneally injected into mice. The boost immunization consisted of an intra-peritoneal injection of 100 μg of NP-CGG in PBS. The experiments were carried out following the Guidelines for Animal Protocols of Tokyo University of Science or those of Kochi Medical School.

Establishment of hybridomas secreting anti-NP antibodies and sequence analysis of Ig genes

To establish primary-response hybridomas, mice were immunized with NP-CGG/alum, and on day 7 or 14, mice were sacrificed and spleens were taken. For secondary-response hybridomas, mice were challenged with NP-CGG in PBS on day 53, and sacrificed 3 days later (day 56). Single-cell suspensions were prepared and the cells were fused with SP2/0-Ag14 myeloma cells using PEG1500 (Roche Diagnostic, Mannheim, Germany). Selection was started 2 days after cell fusion using culture media containing HT media supplements and aminopterin (Sigma, St Louis, MO, USA). Two weeks later, growing cells were screened for anti-NP antibody production by ELISA in which NP-BSA was coated onto microtiter plates and bound antibodies were detected by peroxidase-conjugated goat anti-mouse IgG or IgM antibodies. IgG subclasses and types of light chains (κ or λ chain) were determined using antibodies specific to these molecules (Southern Biotech, Birmingham, AL, USA). Clones secreting antibodies reacting to NP-BSA but not to BSA were subcloned by limiting dilution. mAbs were purified from culture supernatants on an NP-BSA column as previously described (11). In some experiments, unpurified culture supernatants were used after antibody concentrations were determined by ELISA.

Total RNA samples were prepared from hybridoma cells with Trizol (Invitrogen, Carlsbad, CA, USA) and cDNAs were synthesized with Reverscript reverse transcriptase (Wako Chemicals, Osaka, Japan) using an oligo dT primer (Invitrogen). IgH genes were amplified by PCR using the sense V_{H}186.2-specific primer V_{H}S1, and one of the C region-specific antisense primers, Cμ-A1, Cγ1-A1 or Cγ2b-A1 (the primer sequences used in this report are summarized in...
Supplementary Table 1, available at International Immunology Online. IgL genes were amplified using the \( \lambda \)-specific primer \( \lambda \)-S1 and the \( \mu \)-specific primer \( \mu \)-A1 or the \( \lambda \), \( \mu \)-specific primer \( \lambda \)-S1 and \( \mu \)-A1. Amplified fragments were purified by agarose gel electrophoresis and sequences were determined using an ACE2000XLE DNA sequencer (Beckman Coulter, Brea, CA, USA). The primers used for sequencing reactions were \( \mu \)-A2, \( \lambda \)-A2, \( \lambda \)-A1, and \( \mu \)-B2. Amplified fragments were digested with \( Kpnl \) and \( XbaI \), purified by agarose gel electrophoresis and ligated into pBluescript II KS+ vector (Stratagene, La Jolla, CA, USA) to be introduced into Escherichia coli. Isolated plasmids were subjected to sequence analysis using a Genetic Analyzer 3130 (Applied Biosystems, Foster City, CA, USA).

DNA containing exons 1–4 of the \( \mu \) gene was amplified from genomic DNA of C57BL/6 mice by PCR using \( \mu \)-A and \( \mu \)-S and \( \mu \)-A3 primers and cloned into pEF1/My-HisA vector (Invitrogen). \( \mu \) genes from PCs were then excised and cloned into the 5′ end of \( \mu \) exons, and the resulting expression vectors were transfected into J558L cells. After selecting with G418 (Wako Chemicals), stable transfectants were cloned by limiting dilutions. Culture supernatants were collected and \( \mu \) concentrations were determined by ELISA using goat anti-mouse antibodies.

Preparation of control IgM and affinity measurement by ELISA

The genes encoding \( \lambda \)-D and part of \( \lambda \)-J were amplified from cDNA of the anti-NP IgG1-producing hybridomas, N1G9 (18) and B2 (11), by PCR using the 5′ \( \lambda \) primer \( \lambda \)-S2 and the \( \lambda \)-J antisense primer \( \lambda \)-J-A2. The gene encoding \( \lambda \)-J and \( \mu \)-C was amplified from cDNA prepared from spleen cells of C57BL/6 mice using the \( \lambda \)-J sense primer \( \lambda \)-J-A-S and the 3′ \( \mu \)-C primer \( \mu \)-C-A3. In order to obtain the hybrid cDNA of the anti-NP \( \lambda \) region fused with the \( \mu \) domain, the amplified products were mixed and reamplified with primers \( \lambda \)-S2 and \( \mu \)-C-A3. The hybrid cDNA was cloned into the cloning site downstream of the EF-1α promoter in pBudCE4.1 vector (Invitrogen). These constructs were transfected into the J558L myeloma cell line (33) by electroporation as previously described (34). After selection with Zeocin (Invitrogen), stable transfectants clones were isolated by dilution limit. IgM in the culture supernatant was then purified on an NP-BSA column.

Anti-NP mAbs were diluted to 1 μg ml\(^{-1}\) in PBS containing 0.1% (v/v) Tween 20. When culture supernatants were used without purification, IgM was diluted with medium. NP-BSA with various NP valences was prepared as described previously (32). The molecular ratios of NP per BSA are expressed as subscripts. MaxiSorp ELISA plates (Nunc, Roskilde, Denmark) were coated with 50 μl of NP-BSA of varying NP valence (10 μg ml\(^{-1}\)) in PBS overnight at 4°C. After washing and blocking (3% skimmed milk in PBS), the wells were seeded with 50 μl of the diluted samples and incubated for 1 h at room temperature. After washing, the amount of antibody bound to the plates was detected by reacting with either peroxidase- or alkaline phosphatase-conjugated goat anti-mouse IgG or anti-mouse IgM antibodies (Southern Biotech). For estimation of the relative affinity, the ratios of absorbance values of antibody binding to NP \( \lambda \)-BSA (NP \( \lambda \)-BSA) and NP \( \mu \)-BSA (NP \( \mu \)-BSA or NP \( \mu \)-BSA) were calculated.

\( \lambda \)-gene analysis of PCs and expression of recombinant IgMs

PCs were enriched by means of a CD138+ PC isolation kit using VarioMACS equipment (Miltenyi Biotec, Bergisch Gladbach, Germany). PCs were fixed and permeabilized with buffers purchased from BioLegend (San Diego, CA, USA) and stained with FITC-goat anti-mouse IgG, PE-goat anti-mouse IgM antibodies (Southern Biotech). IgM+IgG+ cells and IgM-IgG- cells were sorted using a FACSM Vantage cell sorter (BD Bioscience, Franklin Lakes, NJ, USA). Sorted PCs were digested with proteinase K (Sigma), and rearranged \( \lambda \)-186.2 genes were amplified by PCR. First-round PCR was carried out using \( \lambda \)-S3 or \( \lambda \)-S4 with a mixture of four \( \lambda \)-primers (3′J\(_{\lambda 1} \)-1-A, 3′J\(_{\lambda 2} \)-2-A, 3′J\(_{\lambda 3} \)-3-A and 3′J\(_{\lambda 4} \)-4-A). The second-round PCR was performed with nested \( \lambda \)-S4 or \( \lambda \)-S5 primer with one of the \( \lambda \) primers. Amplified fragments were digested with \( Kpnl \) and \( XbaI \), purified by agarose gel electrophoresis and ligated into pBluescript II KS+ vector to be introduced into Escherichia coli. Isolated plasmids were subjected to sequence analysis using a Genetic Analyzer 3130 (Applied Biosystems, Foster City, CA, USA).

Dynamics of anti-NP IgM antibody production

An NP-RBC suspension was prepared by reacting sheep RBCs (1 x 10\(^6\) cells ml\(^{-1}\)) with 1 mM succinimide ester of NP for 30 min. After the reaction, the cells were washed with isotonic veronal buffer with Ca\(^2+\), Mg\(^2+\) and gelatin. Culture supernatants of hybridomas at various dilutions in medium were added to NP-RBC suspensions and incubated for 90 min on ice. In order to normalize the amount of IgM mAbs bound to the NP-RBCs, the bound IgM was detected using an FITC-labeled goat anti-mouse \( \lambda \) chain antibody (Southern Biotech) and levels were measured by flow cytometry. NP-RBCs to which IgM had bound were washed and reacted with 32-fold dilute rabbit sera for 60 min at 37°C. After the reaction, the remaining cells were centrifuged and the absorbance of the supernatant was measured at 415 nm. After subtracting the amount of non-specific hemolysis, specific hemolysis was calculated as the percentage of total hemolysis in distilled water.

Results

The IgM mAb repertoire was different from the IgG repertoire in the secondary immune response

We prepared hybridomas by fusing splenocytes of NP-CGG-immunized mice with myeloma cells, and screened anti-NP mAb production by ELISA. Numbers of anti-NP mAb-producing clones are shown in Table 1. We obtained 30 and 10 clones on days 7 and 14 after primary immunization of C57BL/6 mice, respectively. Three clones produced mAbs bearing \( \lambda \) light chains, while the others secreted mAbs with \( \mu \) light chains. The nucleotide sequences of V-region genes of \( \lambda \)-bearing mAbs were determined after amplification by RT-PCR. It was found that the heavy chains of 24 mAbs were encoded by the canonical \( \lambda \)-186.2 gene and those of six mAbs by \( \mu \)-186.2-related genes. The cDNA of other mAbs could not be amplified by the primers, and they are considered to be encoded by genes unrelated to the \( \lambda \)-186.2 gene. Among \( \mu \)-186.2A-producing clones, 7 produced IgM and 17 produced IgG. In order to analyze the effect of SHM and CSR...
on the antibody repertoire, hybridomas were also prepared from immunized AID-deficient mice at the same time points. Nineteen anti-NP IgM-producing clones were isolated on day 7 and five on day 14. By sequence analysis, we found eight clones bearing V_{H}186.2 heavy chains and \lambda light chains. The preferential usage of these genes was consistent with previous studies (13, 14) and was not affected by AID deficiency. In order to compare the antibody repertoire between the primary and secondary immune response, we prepared hybridomas from boost-immunized mice. Splenocytes were taken 3 days after boost immunization with NP-CGG in PBS. At this time point, anti-NP antibodies in sera were clearly detectable and the absorbance values of anti-NP IgMs and IgGs were hardly detectable and the absorbance values of anti-NP IgMs were essentially the same as those of day 14 mAbs except that the former generated IgM mAbs only. Six out of eight IgM mAbs were of the Tyr95 type and the other two had either glycine or arginine at position 95. Because SHM does not occur in AID-deficient mice, the variation in the amino acid at position 95 was not a result of SHM.

Although the frequency was low, we found mutations in day 7 IgM mAbs. 1B366 showed three amino acid replacements in V_{H}3 and V_{\lambda}186.2, while 1B269 also had a replacement in V_{H}3. In contrast, no amino acid replacement was observed in V genes of day 7 IgG mAbs, which was in agreement with previous observations (10, 14, 35, 36). The day 14 IgG mAbs had tyrosine at position 95. All had acquired amino acid replacements by SHM. Two IgG clones and one IgM clone were of the Tyr95 type and one had a W33L mutation, suggesting that affinity-based selection in the GC has occurred by this time point. As expected, no SHM was observed in hybridomas obtained from AID-deficient mice.

The secondary-response IgG mAbs from C57BL/6 mice were characterized by the frequent appearance of the Gly95(H) type (4 out of 7, 57%). This type of mAb showed multiple replacements of amino acids in both V_{H} and \lambda regions, which were caused by SHM, and all had a lysine to arginine mutation in the heavy chain at position 58 (16). Similarly, Tyr95-type mAbs also showed a number of amino acid replacements, including the W33L mutation, although no Tyr95 mAb, 8B550, harbored no mutations at all. Therefore, as described previously (11), the preferential appearance of the highly mutated Gly95(H) type was characteristic of the IgG produced in the secondary immune response. In contrast, IgM mAbs from boost-immunized mice lacked amino acid replacements caused by SHM. We detected X95-type mAbs, which were rarely found in the primary repertoire or in IgG mAbs of the secondary response. These results suggested that, in C57BL/6 mice, B-cell populations activated for the production of IgG and IgM in the secondary immune response are different. Four out of nine clones from AID-deficient mice had glycine at position 95, but they did not have histidine at position 100. In contrast, in AID-deficient mice, all mAbs were IgM and had not undergone SHM.

### Table 1. Numbers of anti-NP hybridoma clones

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>AID&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 7</td>
<td>Day 14</td>
</tr>
<tr>
<td>IgG</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>IgM</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>V_{H}186.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6</td>
<td>12</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers of clones expressing the V_{H}186.2 gene among \lambda light chain-bearing clones are indicated in the parentheses.
light chains without heavy chains (33). The recombinant IgMs with N1G9 or B2 \( V_{\lambda} \) region genes were termed N1G9-IgM and B2-IgM, respectively. We first measured the interaction between NP-\( \epsilon \)-aminocaproic acid and the recombinant antibodies, as well as between other mAbs, using isothermal titration calorimetry (11, 12). However, the measurement of IgM mAbs from NP-CGG-immunized mice was not possible because their affinities were too low to be determined by this method, although levels of recombinant N1G9-IgM and B2-IgM were determined (data not shown). Therefore, we analyzed affinity by ELISA. Calculation of ratios of antibody binding to NP-BSA with different NP valences using ELISA has been widely used as a conventional method for estimating the affinity of IgG antibodies (37, 38). We examined
Table 3. Structural phenotypes of Ig genes of anti-NP mAbs with V_{\mu}186.2 heavy chains and \lambda light chains from AID-deficient mice

<table>
<thead>
<tr>
<th>Clone</th>
<th>Heavy chain</th>
<th>Light chain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V_{\mu}</td>
<td>D_{\mu}</td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A8</td>
<td>186.2</td>
<td>SP2</td>
</tr>
<tr>
<td>1A86</td>
<td>186.2</td>
<td>FL16.1</td>
</tr>
<tr>
<td>1A72</td>
<td>186.2</td>
<td>FL16.1</td>
</tr>
<tr>
<td>1A440</td>
<td>186.2</td>
<td>FL16.1</td>
</tr>
<tr>
<td>1A499</td>
<td>186.2</td>
<td>FL16.1</td>
</tr>
<tr>
<td>1A350</td>
<td>186.2</td>
<td>SP2</td>
</tr>
<tr>
<td>1A376</td>
<td>186.2</td>
<td>SP2</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2A122</td>
<td>186.2</td>
<td>FL16.1</td>
</tr>
</tbody>
</table>

*\text{D}_{\mu} genes of some clones could not be identified and are indicated as x. SP2 represents a D_{\mu} family member.*

*\text{Amino acid residues at position 95. G(H) indicates glycine at position 95 with histidine at position 100.}

*\text{Numbers of amino acid replacements by SHM in the V_{\mu} region.}

*\text{Numbers of amino acid replacements by SHM in the V_{\lambda} region.}

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NP_{1\mu}/NP_{2\mu} binding ratios using absorbance values measured in NP-BSA- and NP_{3\mu}-BSA-coated wells. In the case of IgG mAbs, N1G9 had a binding ratio close to zero, while that of B2 was 0.6 (Fig. 2A). This result indicated that B2 has a relatively higher affinity than N1G9, as expected. In contrast to IgG, N1G9-IgM and B2-IgM had a ratio of ~1, suggesting that an IgM with an affinity higher than that of N1G9-IgM would provide the same ratio due to its high avidity involving 10 antigen-binding sites of IgM (39). Because our IgM mAbs showed lower affinity than that of N1G9-IgM in isothermal titration calorimetry experiments, we searched for experimental conditions for comparing the relative affinities of these IgM mAbs. We chose five IgM antibodies including N1G9-IgM and compared profiles of the binding of NP-BSA of different NP valences (Fig. 2B). N1G9-IgM bound to NP-BSA with little dependence on the NP valence. 1A236, 1A8 and 8A507 bound NP_{1\mu} and NP_{2\mu}-BSA less efficiently, although they were able to bind to antigen with an NP valency of more than 6. 1A32, which is an anti-NP IgM with \lambda light chains, showed inefficient binding to NP_{2\mu}-BSA. All IgM mAbs bound to NP_{3\mu}-BSA to the same degree. These results suggested that binding to NP_{1\mu}-BSA (NP_{1\mu}) depended on affinity, while binding to NP_{3\mu}-BSA with an NP valence of more than 20 (NP_{2\mu}) was independent of affinity. Next, we examined the effect of the IgM concentration. The amount of N1G9-IgM that bound to NP_{2\mu}-BSA rose with increasing concentrations, but that of
1A32 remained marginal, even at 10 μg ml⁻¹ (Fig. 2C). On the other hand, in binding to NP₂₆-BSA, little difference was observed among IgM mAbs (Fig. 2D). Therefore, the relative affinity of IgM could be compared using the NP Lo/NP Hi binding ratio at an antibody concentration of 1 μg ml⁻¹, when the IgM mAb in question has an affinity lower than that of N1G9-IgM.

Very low-affinity anti-NP IgM antibodies were frequently found in secondary responses

The day 7 IgG mAbs from C57BL/6 mice had a ratio of 0–0.3 (Fig. 3A). An increase in the binding ratio was observed in a day 14 IgG mAb with the W33L mutation. With two exceptions, most mAbs obtained from boost-immunized mice showed higher ratios (> 0.7) than day 7 IgG mAbs, suggesting that affinity-matured IgG antibodies were selected during the immune response.

Next, we analyzed the relative affinities of IgM mAbs by ELISA. We tentatively classified IgM mAbs on the basis of their NP Lo/NP Hi binding ratios. IgM mAbs with a ratio lower than 0.2 were termed NP Lo⁻, while other mAbs with a ratio higher than 0.2 were termed NP Hi⁺. Their affinities were relatively high, but perhaps not higher than that of N1G9-IgM. Primary-response IgM mAbs of both C57BL/6 (Fig. 3B) and AID-deficient mice (Fig. 3C) belonged to the NP Lo⁻ group except for 1A376, with arginine at position 95, which had a ratio of 0.03. Therefore, the frequency of NP Lo⁻ IgMs among these IgM mAbs was 12/13 (92%). On the other hand, NP Hi⁺ IgM mAbs were abundant among IgMs from boost-immunized C57BL/6 mice, comprising 50% of the total. These results suggest that, in contrast to IgG mAbs, the relative affinities of IgM mAbs do not increase but actually decrease during the immune response in C57BL/6 mice. Two X95-type IgMs were found in day 56 mAbs and both were NP Lo⁻. The frequency of NP Lo⁻ IgM mAbs of AID-deficient mice was 4/7 (57%). In spite of glycine at position 95, 8A38 and 8A303 were NP Lo⁻, while the affinity of the Gly95(H) type without mutation (1A350) was the same as that of the Tyr95 type, as described previously (21). Therefore, they are considered to be the X95 type. NP Lo⁻ IgM mAbs were abundant among Ty95-type IgMs, while X95 types tended to be NP Hi⁺ in the absence of SHM. However, some Ty95-type mAbs (8B668, 8A507 and 8A307) were NP Lo⁻, structural differences between NP Lo⁻ and NP Lo⁺ IgM may not be explained simply by the amino acid residue at position 95.

Unmutated and X95-type V₄ genes were frequently found in secondary-response IgM PCs

The frequent appearance of the X95-type and very low-affinity IgM mAbs in the secondary response was unexpected. Therefore, we examined whether such IgM antibodies are produced in vivo. ELISA showed that the relative affinities

Fig. 2. The binding of IgM mAbs to NP-BSA of varying NP valence. (A) Binding of IgG mAbs and recombinant IgM to solid-phase NP₂⁻ and NP₂₆-BSA was measured and NP Lo/NP Hi binding ratios were calculated. (B) Plates were coated with NP₂⁻, NP₄⁻, NP₆⁻, NP₈⁻, NP₁₀⁻, NP₂₆⁻ and NP₂₆⁺-BSA and the binding of IgM mAbs was detected by anti-IgM antibody. Absorbance values are shown. (C and D) The binding (absorbance values) of IgM to NP₂⁻-BSA and NP₂₆⁺-BSA, respectively, is plotted as a function of the IgM concentration.
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of anti-NP IgM in secondary immune response sera were lower than those of anti-NP IgM in the primary immune response (Supplementary Figure 1, available at International Immunology Online), suggesting the production of very low-affinity IgM antibodies in the secondary response. In order to demonstrate the presence of the X95-type IgM, we analyzed the \( V_H \) gene repertoire in PCs. We enriched PCs from spleens of immunized mice by magnetic cell sorting. IgM\(^+\)Ig\(\lambda\)^+ PCs and class-switched IgM\(^-\)Ig\(\lambda\)^+ PCs were then sorted (Supplementary Figure 4, available at International Immunology Online). \( V_H \) genes of Ig\(\lambda\)^+ cells were considered to encode anti-NP antibodies and were amplified by PCR. Amplified products were cloned into plasmid vector and the sequences were determined. Critical amino acid residues and the number of amino acid replacements by SHM are listed in Supplementary Tables 2–4, available at International Immunology Online and summarized in Fig. 4.

In the primary immune response (day 7), all \( V_H \) genes of IgM\(^+\) PCs and 17 out of 19 class-switched PCs were of the Tyr95 type without amino acid replacements. This was consistent with the results of hybridoma analysis. In the \( V_H \) repertoire of class-switched PCs from boost-immunized mice (day 3 and day 5), most sequences had amino acid replacements, and 35 of 65 (54%) were of the Tyr95 type with the W33L mutation. From the repertoire of day 5 after boost immunization, we also found the Gly95(H) type with multiple mutations. The appearance of affinity-matured sequences was similar to findings in the repertoire of IgG-producing hybridomas. When we analyzed the \( V_H \) repertoire of IgM\(^+\)Ig\(\lambda\)^+ PCs on day 3 after boost immunization, which was the same time point as when hybridomas were prepared, we found that most of the sequences did not contain amino acid replacements and 32% (6 out of 19) were of the X95 type. In the \( V_H \) repertoire of IgM\(^+\)Ig\(\lambda\)^+ PCs on day 5 after boost immunization, we found more clones with mutations including the W33L mutation. This suggested that unmutated X95-type IgM antibodies and affinity-matured IgM antibodies are produced in the secondary response.

Because we did not analyze sequences from single cells, we could not gain sequence information on pairing light chains. Amino acid replacements in light chains contribute to affinity maturation (20), so that we could not speculate on the affinities of mutated \( V_H \) genes from PCs. We chose \( V_H \) genes without mutations, assuming that their partner light chains were in germline configuration. \( V_H \) genes were recloned into the expression vector containing C\(\mu\) exons and transfected into J558L cells. Culture supernatants containing recombinant

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**Fig. 3.** The relative affinity of mAbs. NP\(_{Lo}\)/NP\(_{Hi}\) binding ratios using IgG mAbs of C57BL/6 mice are shown in (A) and ratios for IgM mAbs of C57BL/6 and AID-deficient mice are shown in (B) and (C), respectively. Data are expressed as means ± SD.
IgM were subjected to ELISA. Nine out of ten (90%) recombinant Tyr95-type IgMs from primary immunization were NP<sub>10</sub><sup>+</sup> (Fig. 5A). On the other hand, 7 out of 14 (50%) of the IgMs constructed with sequences of the secondary immune response were NP<sub>10</sub><sup>−</sup> (Fig. 5B). X95-type IgMs showed lower affinity, and two of them were NP<sub>10</sub><sup>−</sup> but the ratios were relatively low. Therefore, we concluded that unmutated very low-affinity IgM antibodies, which were found in hybridomas, were produced in vivo in the secondary immune response.

Low-affinity IgM mAbs showed higher complement-dependent hemolysis activity

It was rather surprising that, in contrast to affinity maturation of IgG, very low-affinity IgM antibodies are produced in the secondary immune response. In order to address the question of whether such very low-affinity IgM antibodies have any role in biological defense, we analyzed the relationship between affinity and antibody function. Because one of the most important functions of IgM is to activate the classical pathway of the complement cascade, we compared hemolytic activity toward NP-RBCs using rabbit sera as the source of complement factors. Flow cytometry analysis showed that all of IgM mAbs bound to NP-RBCs when the NP valence on the surface was sufficiently high. Because the amount of bound IgM on RBCs is a critical factor in initiating hemolysis, it was analyzed by flow cytometry and the concentration of each IgM mAb was adjusted to give similar geometric means of fluorescence intensity (Fig. 6A). Interestingly, IgM mAbs having higher affinity...
activated hemolysis less efficiently than mAbs with lower affinity (Fig. 6B). It is evident that hemolytic activity was inversely correlated with the NP\textsubscript{Lo}/NP\textsubscript{Hi} binding ratio, i.e. affinity (Fig. 6C). This cannot be explained by the lack of complement activation by IgM mAbs with higher affinity since when NP-RBCs with a low number of NPs were used, hemolysis was effectively induced (Supplementary Figure 5, available at International Immunology Online). These results suggested that low-affinity IgM antibodies secreted during the secondary response were superior to higher affinity IgM antibodies in their ability to activate complement when the epitope density was high.

**Discussion**

Class-switched memory B cells are important for affinity maturation and the repertoire shift of IgG antibodies in the secondary immune response. Recent studies of B-cell memory revealed the presence of IgM\textsuperscript{+} or unmutated memory B cells. Antigen-dependent selection in GCs establishes affinity-matured memory B cells, while memory B cells can also be derived via a GC-independent pathway (30). Although some IgM\textsuperscript{+} memory B cells have mutated V\textsubscript{H} genes, a significant portion of IgM\textsuperscript{+} memory B cells are unmutated (40). It is reported that memory B cells can be generated even by T cell-independent antigens (41). Marginal-zone B cells (42) or B1b cells (43) contribute to IgM\textsuperscript{+} memory. We have analyzed cDNA sequences of NP-binding memory B cells and found not only affinity-matured but also unmutated low-affinity IgM\textsuperscript{+} memory B cells (submitted). However, it was not clear whether these IgM\textsuperscript{+} memory B cells contribute to IgM antibody production in the secondary immune response.
In order to compare antibody repertoires during the immune response, we determined sequences of Ig genes of hybridomas and PCs at different time points after immunization. Since both sequence analysis and functional analysis are important for elucidating the biological significance of antibodies, we analyzed affinities and complement activation capacities. On day 7 after immunization, most IgG hybridomas secreted Tyr95-type mAbs. The absence of mutations in their V\(_g\) regions suggests that they developed directly from naive B cells without GC involvement. Similarly, most of the V\(_g\) genes of class-switched PCs were of the Tyr95 type without mutation, indicating that the hybridoma repertoire represented the repertoire of antibodies produced in vivo. Since two of the IgG mAbs prepared from mice on day 14 after immunization were of the Tyr95W33L type and showed increased affinity to NP, the GC reaction and affinity-based selection had already occurred at this time point. In IgG mAbs prepared from boost-immunized mice, we found an increased frequency of affinity-matured types, i.e. Tyr95W33L and Gly95(H) mAbs with multiple mutations. They presumably originated from GC-dependent memory B cells. As expected, ELISA showed that most of them had increased affinity to NP. There were two exceptional IgG mAbs with low affinity. 8B529 had the W33L mutation but had also additional mutations that decreased affinity, as described previously (21). 8B550 had the germline sequence, so that it may have originated from a GC-independent memory B cell. V\(_g\) sequences of class-switched PCs frequently contained the Tyr95W33L type, and the Gly95(H) type appeared on day 5 after boost immunization. Therefore, the affinity-matured IgG antibodies that were found in the hybridoma repertoire were produced in vivo. In contrast to the hybridoma repertoire, the class-switched PC repertoire contained more of the Tyr95 type without the W33L mutation. We could not measure the affinities of these mutated antibodies because paired light chains were not identified.

Similar to the IgG repertoire, most IgM mAbs of the primary response on day 7 were of the Tyr95 type without mutations, and all of the V\(_g\) genes isolated from IgM\(^+\) PCs were also of the Tyr95 type without mutations. These results suggested that, at this time point, almost the same populations of naive B cells are selected to differentiate into IgM PCs and IgG PCs without passing through GCS. The fact that IgM mAbs harbor mutations suggested that these cells had undergone SHM without CSR in GCS. The IgM repertoire of boost-immunized mice was different. We found not only Tyr95-type but also X95-type IgM mAbs, and all of them were in the germline configuration. Most IgM mAbs of the Tyr95 type had a higher NP\(_{X95}\)/NP\(_{Tyr95}\) binding ratio (NP\(_{Tyr95}\)) even without mutations, while X95-type IgM mAbs had very low-affinity (NP\(_{X95}\)). V\(_g\) genes of PCs from mice 3 days after boost immunization contained many unmutated genes including the X95 type. On generating recombinant antibodies with V\(_g\) genes from PCs, we confirmed that these antibodies bind NP with relatively lower affinity than Tyr95-type V\(_g\) genes of the primary PC repertoire. One possible explanation for why the X95 type was not found in the IgG hybridoma repertoire is that its affinity was too low to be detected by ELISA in the form of IgG. The formation of the IgM pentamer increases avidity so that it was able to be detected by hybridoma screening. However, PCs were sorted as Ig\(\gamma^+\) cells and the repertoire was not biased by the NP-binding capacity. The presence of the X95 type in the IgM\(^+\) PC repertoire and not in the class-switched PC repertoire suggests that these types of B cells are only selected if class switching has not occurred. On day 5 after boost immunization, we found the affinity-matured W33L mutation in V\(_g\) genes of IgM\(^+\) PCs. These cells may be derived from GC-dependent IgM\(^+\) memory B cells. Our sequence analysis and affinity measurements revealed that there are three types of IgM antibodies that are produced by antigen recall. The first type has the V\(_g\) gene of the germline configuration and is typically of the Tyr95 type, and its affinity is not matured but is relatively high (NP\(_{X95}\)). These mAbs may have derived from memory B cells, which develop in a GC-independent manner. V\(_g\) genes of the second type show such affinity-matured sequences as Tyr95W33L or Gly95(H), and these B cells are selected in the GC as are IgG\(^+\) memory B cells. The third type, which has very low-affinity (NP\(_{X95}\)) BCRs without mutations, is unique in the IgM repertoire of secondary responses. Since this later type appeared in AID-deficient mice, SHM is not necessary for these B cells and they may develop independently of GCS.

Since memory B cells can respond more rapidly and vigorously than naive B cells, most secondary-response antibodies can be considered to be derived from memory B cells. It is still possible that naive B cells can be stimulated with help from memory T\(_m\) cells to produce IgM after secondary immunization, as shown in rat chimeras (44). However, when mice were immunized with CGG to induce T\(_m\) cells and boost immunized with NP-CGG, we found a slight increase in anti-NP IgM production (Supplementary Figure 6, available at International Immunology Online). This result suggests that naive B cells can contribute to IgM production in the secondary immune response. In addition to the IgM repertoire of hybridomas and GCSs of the secondary response, we have also found X95-type V\(_g\) sequences in low-affinity BCR-expressing memory B cells (submitted). Taken together, the IgM antibody repertoire produced in the secondary response is thought to reflect the repertoire of IgM\(^+\) memory B cells.

Our results suggest that the fate of naive B cells is determined by BCR affinity. Before encountering antigen, naive B cells of the Tyr95, Gly95(H) or X95 type without mutations can be found. Tyr95- and Gly95(H)-type BCRs have an affinity of 10\(^{2}\) M\(^{-1}\) even without mutations, while X95-type BCRs have lower affinity (21). Higher affinity B cells can compete with lower affinity B cells in GC formation (45–47). Preferential differentiation of high-affinity B cells into short-lived PCs was shown in studies using transgenic mice (48, 49). Relatively high-affinity Tyr95- or Gly95(H)-type B cells are, therefore, preferentially activated to proliferate, to differentiate into either PCs or GC B cells, and to induce CSR. A GC reaction generates affinity-matured memory B cells with either IgM or IgG BCRs. Some Tyr95-type B cells may differentiate into memory B cells without passing through GCSs. On the other hand, B cells that have extremely low-affinity BCRs like the X95 type may not efficiently differentiate into PC or GC B cells in the primary response. However, after antigen recognition, they survive as memory B cells without inducing SHM and CSR. Consequently, in addition to GC-dependent affinity-matured and GC-independent memory B cells, very low-affinity IgM\(^+\) memory B cells without mutations are generated.
Upon antigen recall, these memory B cells are activated and differentiate into PCs. Therefore, in the secondary response, affinity-matured IgG antibodies, unmutated antibodies and very low-affinity IgM antibodies are produced. Although IgM+ memory B cells preferentially differentiate into GC B cells but not into PCs on restimulation (40), some are thought to differentiate into IgM-producing PCs (27, 30, 50). Our results suggested that they do differentiate into IgM+ PCs, although we did not determine the relative incidence of these two IgM+ memory B-cell fates.

The $V_{\gamma}$ repertoire of IgM+ PCs on day 3 after boost immunization contained mainly unmutated genes, while the day 5 repertoire had many sequences with mutations, which included those with mutations such as W33L, which result in higher affinity. These results suggest that IgM+ memory B cells without mutations respond to boost immunization more quickly than do mutated memory B cells. This may be because of the difference between GC-independent and GC-dependent IgM+ memory B cells, with the former being more susceptible to activation than the latter. Alternatively, B cells with low-affinity BCRs may be activated more efficiently in the presence of memory T cells. It was shown that antigens that are bound to lower affinity BCRs are more efficiently presented on class II MHC molecules (51) and that these cells may receive more help from T cells. In addition, after boost immunization, high-affinity IgG antibodies are produced in vivo, which may inhibit B-cell activation especially that of cells with low-affinity BCRs (31) on day 5 after immunization. These possibilities need to be elucidated by further experiments.

We showed dynamic changes in antibody production following immunization with NP-CGG. A boost immunization induced the production of IgM antibodies, in addition to the affinity-matured IgG antibodies, which play an important neutralizing role. The high avidity of IgM antibodies is known to be due to their harboring of 10 antigen-combining sites per molecule. In this study, we used NP-CGG with a relatively high density of hapten so that IgM could bind antigens efficiently even if their affinity was low. This type of polyvalent structure is widely observed in the lectin family and is considered to be useful for recognizing repeated molecular patterns on bacteria or viruses (52). IgM antibodies are known to have a strong capacity for aggregation and to efficiently activate the classical complement pathway. Because of these functional advantages, IgM antibodies are able to play an important protective role in influenza virus infection (53, 54). IgM produced from B1b cells can eliminate a Borelia hermsii infection and confer T cell-independent long-lasting immunity (43). Before affinity-matured IgG antibodies have made their appearance, primary IgM antibodies provide the only antibody defense against invasion by microorganisms. However, even after matured IgG antibodies appear, IgM antibodies would likely have a continuing role in secondary immunization. The production of low-affinity IgM in the secondary response was observed in transgenic mice (46), although its biological significance was unclear. In the present study, we showed that IgM mAbs with very lower affinity were superior to those with higher affinity in a hemolytic assay using NP-RBC. Moreover, Fossati-Jimack et al. (55) demonstrated the strong hemolytic activity of low-affinity autoantibodies toward RBCs, and Swanson et al. (56) reported that anti-FITC IgM mAbs with high affinity ($\sim 10^{12} M^{-1}$) bind C1 less efficiently than IgG does. IgM consists of five subunits, each of which has two antigen-binding sites. Without antigen binding, this large structure has a planar conformation, which does not induce activation of the complement cascade because C1 can bind to it with low affinity. The interaction with antigen can induce conformational changes, which depend on many factors such as affinity, epitope density and the flexibility of IgM pentamer. The formation of a staple-like structure enables C1 to bind to IgM efficiently (57, 58). When a high-affinity IgM pentamer binds to a high-epitope-density ligand such as NP-RBC (Fig. 6), it is assumed that the interaction of one or two antigen-binding sites of one IgM subunit is sufficient for binding. Therefore, there is no need to change the conformation so that the IgM pentamer binds to NP-RBC in the planar form. If the same IgM binds to antigen when the span of each epitope is too long for two antigen-binding sites of one IgM subunit to bind simultaneously, other subunit(s) in the pentamer will be involved in antigen binding and the IgM will assume a staple-like conformation, which enhances C1 binding and complement activation. On the other hand, low-affinity IgM cannot bind to NP-RBCs with one IgM subunit and participation of the other subunits of the IgM pentamer is required for sufficient binding. When several antigen-binding sites are occupied, the IgM conformation will assume a staple-like form. Therefore, during interactions with NP-RBCs of high NP density, high-affinity and low-affinity IgM could preferentially form planar and staple-like conformations, respectively. Although further study is required to verify this hypothesis, our results suggest that very low-affinity IgM antibodies have unique immunological functions. IgM likely competes with IgG in binding to epitopes on pathogens through the high avidity provided by its ten antigen-combining sites. Since an increase in binding by multiple interactions is more pronounced when antibody-combining sites have a lower intrinsic affinity (32), it is speculated that IgM with very low affinity can recognize pathogens with multiple epitopes through its high avidity, even during secondary immunization with T cell-dependent antigens.

Supplementary data
Supplementary data are available at International Immunology Online.

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