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B Cell Selection and Affinity Maturation During an Antibody Response in the Mouse with Limited B Cell Diversity

Naoki Kanayama,* Takafulmi Kimoto,* Kagefumi Todo,* Yumiko Nishikawa,† Masaki Hikida, Masaki Magari,* Marilia Cascalho,† and Hitoshi Ohmori

The quasi-monoclonal mouse has limited B cell diversity, whose major (∼80%) B cell Ag receptors are comprised of the knockin VH 17.2.25 (VH T)–encoded H chain and the A1 or A2 L chain, thereby being specific for 4-hydroxy-3-nitrophenylacetyl. The p-nitrophenylacetyl (pNP) was found to be a low affinity analog of nitrophenylacetyl. We examined affinity maturation of anti-pNP IgG by analyzing mAbs obtained from quasi-monoclonal mice that were immunized with this low affinity Ag. The results are: 1) Although VH T/A1 and VH T/A2 IgM were equally produced, VH T/A2 IgG almost exclusively underwent affinity maturation toward pNP. 2) A common mutation in complementarity-determining region 3 of VH T (T313A) mainly contributed to generating the specificity for pNP. 3) Because mutated VH T-encoded γ-chains could form A1-bearing IgG in Chinese hamster ovary cells, apparent absence of VH T/A1 anti-pNP IgG may not be due to the incompatibility between the γ-chains and the A1-chain, but may be explained by the fact that VH T/A1 B cells showed 50–100-fold lower affinity for pNP than VH T/A2 B cells. 4) Interestingly, a pNP-specific IgM mAb that shared common mutations including T313A with high affinity anti-pNP IgG was isolated, suggesting that a part of hypermutation coupled with positive selection can occur before isotype switching. Thus, even weak B cell receptor engagement can elicit an IgM response, whereas only B cells that received signals stronger than a threshold may be committed to an affinity maturation process. The Journal of Immunology, 2002, 169: 6865–6874.

When the peripheral lymphoid tissues are exposed to an Ag, several groups of B cells are clonally selected, and differentiate into plasma or memory cells. One characteristic feature of a T cell–dependent Ab response is that the isotype of the produced Abs is switched from IgM to other classes, including IgG with time after immunization. The other is a gradual increase of Ab affinity to an inducing Ag particularly in the switched isotypes, a process termed affinity maturation (1–6). It has been shown that isotype switching and affinity maturation are strongly dependent on germinat centers (GC) that are transiently formed from Ag-stimulated B and T cells in the follicular region of secondary lymphoid tissues (1, 2, 6). A series of previous reports have revealed that affinity maturation of an Ab is the result of somatic hypermutation of V region genes coupled with the positive selection of B cells (centroblasts) whose mutated cell Ag receptors (BCR) acquire higher affinity, while B cells that failed to improve their BCR may be deleted (1, 2, 7–9). It has been shown that mutated BCR are tested for their quality in terms of affinity to the inducing Ag that are trapped in the form of immune complexes onto follicular dendritic cells in GC (1, 10, 11). B cells with improved affinity are considered to receive survival signals via their BCR and the complement receptors (CD21/CD35) and from CD4+ T cells (1, 12, 13), thus leading to the retention of B cells that will subsequently secrete higher affinity Abs.

In wild-type mice, somatic hypermutation and affinity maturation have been systematically investigated in Ab responses to several haptons, including 2-phenyl-5-oxazolone (14, 15) and 4-hydroxy-3-nitrophenylacetyl (NP) (16–20), because limited Ig genes are preferentially used in these Ab responses, VH Ox1/VκOx1 in the former response, and VH 186.2 in the latter, respectively. However, it has not been fully elucidated how an Ag-stimulated B cell clone becomes a winner in the competition with others, and what criteria direct B cells to enter affinity maturation pathway. To examine these issues, we used the quasi-monoclonal (QM) mouse with limited BCR diversity, in which one of the JH loci is replaced with the 17.2.25 VH DJ segment (VH T) derived from an anti-NP mAb (21), with the other JH locus and both κ loci being disrupted (22). The advantage of this type of knockin strain is that the site-directed VH encoded (VH T+) H chain has been shown to undergo class switching and somatic mutation normally (23, 24). However, it has not been fully analyzed how a site-directed V gene is tuned during affinity maturation. Approximately 80% of QM B cells expressed VH T+ NP-specific BCR that bear A-chains (21, 22), of which A1- and A2-chains were major L chains used. By stimulating B cells with NP analogs of varying affinity, this mouse will enable us to examine how the B cell differentiation into plasma or memory cells is regulated by the intensity of BCR engagement. We tested various NP analogs, and found that p-nitrophenylacetyl (pNP) showed at least ∼20-fold lower affinity than NP for VH T+ QM B cells (25). In the present study, using QM mice that were immunized with pNP-conjugated chicken γ-globulin (CGG), we examined affinity maturation of anti-pNP IgG in the following two

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4 Abbreviations used in this paper: GC, germinal center; BCR, B cell Ag receptor; β-Gal, β-galactosidase; CDR, complementarity-determining region; CGG, chicken γ-globulin; CHO, Chinese hamster ovary; LN, lymph node; NIP, 4-hydroxy-5-iodo-3-nitrophenylacetyl; NP, 4-hydroxy-3-nitrophenylacetyl; pNP, p-nitrophenylacetyl; QM, quasi-monoclonal; sIgM, surface IgM.
respects: 1) How do originally NP-specific B cells acquire pNP specificity by somatic hypermutation during affinity maturation toward pNP? 2) How are two major B cell clones, V<sub>H</sub>T<sub>1</sub>A1 and V<sub>H</sub>T<sub>2</sub>A2, selected by pNP to be committed to affinity maturation process?

Materials and Methods

Hapten and Ags

NP, pNP, 4-hydroxy-5-ido-3-nitrophenylacetyl (NIP), 4-hydroxy-phenylacetyl, and m-nitrophenylacetyl were purchased as the free acids from Tokyo Kasei (Tokyo, Japan). NP, pNP, or pNP was conjugated to CGG (Sigma-Aldrich, St. Louis, MO) or BSA by reacting the N-hydroxysuccinimide ester of each acid, as described previously (25). Usually, CGG was conjugated with ~25 molecules of each hapten.

Estimation of affinity of NP-related haptons for V<sub>H</sub>T<sup>+</sup> anti-NP Ab

An appropriately diluted anti-pNP IgM Ab purified from a QM mouse serum was added at 50 μl to 96-well microplates (Nunc, Roskilde, Denmark) that were coated with rabbit anti-mouse IgM (Funakoshi Chemicals, Tokyo, Japan). After incubation for 2 h at 25°C, followed by washing, 50 μl of PBS containing 10 μg/ml NP-conjugated β-galactosidase (β-Gal) (~3 NP/enzyme molecule) and varying concentrations of a free hapten were added in each well. NP-β-Gal was prepared by reacting Escherichia coli β-Gal (Sigma-Aldrich) with N-hydroxysuccinimide ester of NP in the same fashion as described previously (26). The plate was incubated for 1 h at 25°C, followed by washing thoroughly. PBS containing 0.1% BSA was used for diluting reagents and washing. The bound NP-β-Gal activity was assayed by adding a fluorescent substrate, 4-methylumbelliferyl-β-D-galactoside (Sigma-Aldrich), as reported previously (27). The affinity of each hapten for the anti-NP IgM was estimated by measuring the free hapten concentration that is required for 50% inhibition of the binding of NP-β-Gal to the solid phase.

Flow cytometric analysis

B cells were stained with anti-mouse Ab in PBS containing 0.2% BSA, 0.1% sodium azide, and, when necessary, 50 μg/ml of normal rat IgG (ICN Pharmaceuticals, Costa Mesa, CA). FITC or CyChrome anti-B220 (RA3-6B2), PE anti-CD138 (281-2), biotinylated anti-αL L chain (R11-153), and biotinylated anti-αλ L chain (2B6) that was cross-reactive with αλ were purchased from BD Pharmingen (San Diego, CA). B cells bearing V<sub>H</sub>T<sub>1</sub>-encoded IgM were detected with biotinylated mAb to the Id of V<sub>H</sub>T, R2.438 (a gift from T. Imanishi-Kari, Tufts University, Boston, MA). Surface IgM (slgM) was detected using PE anti-IgM (Southern Biotechnology Associates, Birmingham, AL). Biotinylated Abs were visualized with FITC- or PE-labeled streptavidin. Stained cells were analyzed with FACSCalibur and CellQuest software (BD Biosciences, Mountain View, CA).

Mice and immunization

The quasi-monoclonal (QM) mouse is a gene-targeted strain whose genotype is V<sub>H</sub>T<sub>1</sub> αL<sup>+</sup> λ<sup>-+</sup> (22). QM mice were immunized in the footpads with 200/μl H9262 cells at 10<sup>6</sup> cells per mouse. The next day, the cells were washed to remove free Ags, and cultured for an additional 3 days. Culture supernatants were assayed for VH Ti Id<sup>+</sup> Abs by ELISA using anti-V<sub>H</sub>T<sub>1</sub> Id, anti-αL, and anti-α2 Abs, and further confirmed by RT-PCR using specific primers, as described previously (31, 32).

Hybridomas secreting nonmutated V<sub>H</sub>T<sub>1</sub>A1 IgM, V<sub>H</sub>T<sub>2</sub>A2 IgM, or V<sub>H</sub>T<sub>2</sub>A1 IgG1 mAbs were generated by the cell fusion with NSO<sup>–</sup>2 of QM B cells that were stimulated in vitro for 5 days with 20 μg/ml of LPS from E. coli (Sigma-Aldrich) in the presence or absence of 0.5 μg/ml of IL-4 (PeproTech EC, London, U.K.). The absence of somatic mutations in the V<sub>H</sub>T<sub>1</sub> mAbs was confirmed by sequencing V<sub>H</sub> and V<sub>L</sub> genes.

Sequence of V<sub>H</sub> and V<sub>L</sub> genes

cDNA was synthesized from total RNA of each anti-pNP mAb-secreting hybridoma, as described previously (25). V<sub>H</sub> and V<sub>L</sub> regions of mAbs were amplified with PCR using AmpliTaq Gold DNA polymerase (Applied Bio-systems, Foster City, CA). Primers for amplifying V<sub>H</sub> regions, CTGAATCTCAAAGGTCTTTACCT for V<sub>H</sub>T, and ARCTKWSGASGWCGWGRRSSW and ARCKTAWGSAWGWCGRGRSWG for endogenous VH as sense primers; GACAGGGMTCCKAGT TCCA for all IgG subclasses, and GTTCTGATACCTGGATGACCTC for IgM as antisense primers. For amplifying V<sub>L</sub> regions, CTGCTGCTGAC CAAATTTGAAAAGATAGACC and GCATGTTTCTCGTACG GCTTTG for murine IgM (Southern Biochemistry, Birmingham, AL) were used. Sequencing of V<sub>H</sub> and V<sub>L</sub> genes was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were compared to the Kabat database.

Expression of recombinant mAbs in Chinese hamster ovary (CHO) cells

Various combinations of germline or mutated V<sub>H</sub>T-encoded H chain with the αL<sup>-</sup>λ<sup>-+</sup> L chain derived from V<sub>H</sub>T<sub>1</sub>A1 or V<sub>H</sub>T<sub>2</sub>A2 mAbs were transfected into recombinant Abs in CHO cells. CHO cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 1×10<sup>-5</sup> M 2-ME. On day 3 of the culture, the cells were washed to remove free Abs, and cultured for an additional 3 days. Culture supernatants were assayed for V<sub>H</sub>T<sub>1</sub> αL<sup>+</sup> λ<sup>-+</sup> IgM Abs by ELISA using R2.438 anti-Id and anti-μ Abs. Data were presented as the mean values of triplicate experiments. SEs do not usually exceed 10% of the mean.

For observing down-regulation of slgM, QM B cells (3×10<sup>5</sup>/ml) were cultured with 1 μg/ml of pNP-CGG or pNP-CGG for 3 h. B220<sup>+</sup> λ<sup>-+</sup> B cells were assessed for the level of slgM by flow cytometry.

Assay of the level and the affinity of anti-pNP Abs

ELISA for anti-pNP Abs was performed using 96-well microplates coated with pNP<sub>20</sub>-BSA (a high hapten density) to detect both high and low affinity anti-pNP Abs. Each class of Abs bound to the plates was measured with peroxidase-conjugated goat IgG specific for mouse IgM (Southern Biotechnology Associates) or peroxidase-conjugated horse Abs to mouse IgG (Vector Laboratories, Burlingame, CA), respectively. IgG1 and IgG2b were major subclasses in the anti-pNP response. Usually, IgG1 was assayed without discriminating subclasses in the present experiments. Relative affinity of anti-pNP IgG in serum samples or mAbs was estimated by differential binding of the Ab to plates coated with pNP4-BSA (a low hapten density) and pNP20-BSA (a high hapten density), and expressed as the ratio of IgG bound to pNP4-IgG bound to pNP20 (sometimes abbreviated as pNP<sub>4</sub> pNP<sub>20</sub>), as reported previously (25, 26). As the positive control for the affinity assay, an anti-pNP IgG mAb (pNP<sub>4</sub> pNP<sub>20</sub> = 0.7) was used as a standard sample.

To compare the affinities of V<sub>H</sub>T<sub>1</sub>A1 and V<sub>H</sub>T<sub>2</sub>A2 mAbs for pNP and NP, a displacement ELISA was conducted (29). Briefly, 1 μg/ml of each mAb was incubated for 3 h at room temperature in microplates coated with pNP<sub>20</sub>-BSA or NP-BSA, and incubated at 25°C for 1 h. After washing, bound mAbs was assayed by reacting peroxidase-conjugated second Abs, as described above.

Generation and characterization of anti-pNP mAbs

On day 16 after immunization of QM mice with pNP-CGG/CFA in the footpads, popliteal lymph node (LN) cells were prepared and fused with a myeloma cell line, NSO<sup>–</sup>2 (30), which was given by B. Diamond (Albert Einstein College of Medicine, New York, NY). mAbs were screened on the basis of binding to pNP<sub>20</sub>-BSA. The retention of V<sub>H</sub>T and the usage of αL chains in the mAbs were examined by ELISA using anti-V<sub>H</sub>T<sub>1</sub> Id, anti-αL, and anti-α2 Abs, and further confirmed by RT-PCR using specific primers, as described previously (31, 32).

Hybridomas secreting nonmutated V<sub>H</sub>T<sub>1</sub>A1 IgM, V<sub>H</sub>T<sub>2</sub>A2 IgM, or V<sub>H</sub>T<sub>2</sub>A1 IgG1 mAbs were generated by the cell fusion with NSO<sup>–</sup>2 of QM B cells that were stimulated in vitro for 5 days with 20 μg/ml of LPS from E. coli (Sigma-Aldrich) in the presence or absence of 0.5 μg/ml of IL-4 (PeproTech EC, London, U.K.). The absence of somatic mutations in the V<sub>H</sub>T<sub>1</sub> mAbs was confirmed by sequencing V<sub>H</sub> and V<sub>L</sub> genes.

Using the IgM purification process to the bound mAbs to the plates, the mAbs were purified with QIAgex Gel Extraction Kit (Qiagen, Hilden, Germany) and directly sequenced using BigDye Terminator Cycle Sequencing FS Ready Kit equipped with ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The BLAST (http://www.ncbi.nlm.nih.gov/blast/) and IgBLAST (http://www.ncbi.nlm.nih.gov/igblast/) programs were used to find the closest matching sequences in the nonredundant database at the National Center for Biotechnology Information server, and the Seqhunt II (http://immuno.bme.nwu.edu/seqhunt.html) was used to search the Kabat database (33).
C region was amplified with CGGTCTCAGCCGCTAAAACAAACACCCCATCACGT and CTTTGGCCGCCGCTGTAGCTTATGACCC AGA, and the product was digested with EcoR1HI and Ncol. The digested V\textsubscript{H} fragments derived from V\textsubscript{H}1T2A IgM, G83, and G149 were each inserted in combination with the fragment of the V\textsubscript{D}2 C region between Ncol and NheI sites of a mammalian expression vector, pCI-neo (Promega, Madison, WI), which were designated as pCI-GV\textsubscript{H}1T2, pClG83, and pClG149, respectively. Another two constructs bearing the \( \gamma \)2\textsubscript{c} genes containing only one (T131A) or two (T131A/G163A) mutations in V\textsubscript{H} complementarity-determining region 3 (CDR3) were constructed by replacing a Kpn1/EcoR1HI fragment of pClGV\textsubscript{H}1T with the corresponding sequence from pClG83 or pClG149, respectively. A PvuII-BamHI fragment containing neomycin phosphotransferase gene of pCl-neo was exchanged with a Puromycin\textsubscript{acetyltransferase} gene of pBSpacDp (34). This expression vector was termed as pCl-puro.

Results

pNP as a low affinity analog of NP for QM B cells

The V\textsubscript{H}1D14 Ig gene segment, V\textsubscript{H}1T, is derived from 17.2.25 anti-NP mAb (\gamma1, \lambda) that binds to NP and its iodinated analog NIP. The affinity (\( \kappa \)) of 17.2.25 for NIP has been estimated to be \( 4 \times 10^5 \) M\textsuperscript{-1} (21). In QM mice bearing the site-directed V\textsubscript{H}1T with the genotype of V\textsubscript{H}1TID14\textsuperscript{+}, \( \kappa' /\kappa^*\Lambda'^{+}\lambda'^{+}\Lambda'^{-}\lambda'^{-}\), a majority (\( \sim 80\%\)) of B cells expressed V\textsubscript{H}1T-encoded BCR that were detected by an anti-Id mAb, R2.438 (35) (Fig. 1A). Because both \( \kappa \) loci are disrupted, QM B cells exclusively express \( \lambda \)-chains (22). Although an anti-\( \lambda \)-mAb used in the flow cytometric analysis has cross-reactivity with \( \lambda_3 \), of \( \sim 30 \) \( \lambda \)-chains randomly sequenced, \( \lambda_1 \) and \( \lambda_2 \), but not \( \lambda_3 \), were found (data not shown), thus suggesting that the \( \lambda_1 \)- and \( \lambda_2 \)-chains are major \( \lambda \) chains used. Flow cytometric analysis shows that each V\textsubscript{H}1T/A1 and V\textsubscript{H}1T/A2 B cell population has a comparable size (Fig. 1B). Both V\textsubscript{H}1T/A1 and V\textsubscript{H}1T/A2 Abs have been shown to bind to NP (21, 22). To examine various NP-related hapten for their binding activity to V\textsubscript{H}1T\textsuperscript{+} anti-NP IgM derived from QM mice, free hapten inhibition of the binding of NP-conjugated \( \beta \)-Gal to the immobilized anti-NP IgM was assessed. We found that pNP had \( \sim 20\text{-fold} \) lower affinity than NP (Fig. 2A). pNP has not been tested as a NP analog by other investigators. In contrast, m-nitrophenylacetyl showed a comparable affinity to NP, while NIP bound to the anti-NP Ab more strongly than NP, as observed in various anti-NP mAbs (21, 35). The 4-hydroxyphenylacetyl showed no significant binding. Thus, stimulation of QM B cells with haptons of varying affinity will provide useful means to analyze the correlation between the intensity of BCR engagement and subsequent B cell differentiation.

In the present work, QM mice were immunized with NP analogs that were conjugated to CGG. To confirm that pNP acts as a low affinity ligand to QM BCR, we compared pNP-CGG and NIP-CGG for the triggering activity of QM B cells by assessing down-regulation of slgM by these hapten carriers. slgM was strongly down-regulated after incubation of the splenic B cells with 1 \( \mu \)g/ml of NIP-CGG for 3 h, but to a much lesser extent with the same concentration of pNP-CGG (Fig. 2B). The down-regulating effects were dose dependent, and similar results were obtained when the incubation was prolonged up to 24 h (data not shown). In contrast, QM B cells were cultured in vitro with CGG-specific Th2 cells in the presence of varying concentrations of pNP-CGG or NIP-CGG. Consistent with the affinity of each Ag for QM BCR, NIP-CGG induced V\textsubscript{H}1T\textsuperscript{+} IgM at lower concentrations (0.01–0.1 \( \mu \)g/ml) than pNP-CGG, which elicited the Ab response significantly at greater than 1 \( \mu \)g/ml (Fig. 2C). CGG without haptons did not induce the Ab response at 0.01–10 \( \mu \)g/ml (data not shown).

These observations suggest that pNP behaves as a low affinity ligand for QM BCR in the form of CGG conjugate.

In addition, pNP was able to elicit the hapten-specific V\textsubscript{H}1T\textsuperscript{+} IgG response efficiently in QM mice, as shown in the following section. These observations led us to investigate how V\textsubscript{H}1T/A1 and V\textsubscript{H}1T/A2 B cells that are originally specific for NP or NIP are triggered by pNP and undergo affinity maturation toward this low affinity hapten, and how the site-directed V\textsubscript{H}1T is modified during the affinity maturation process.

Affinity maturation of anti-pNP IgG

When QM mice were immunized with pNP-CGG in the footpad, serum anti-pNP IgG Abs that were negligible on day 0 increased appreciably from day 8 to 16 after immunization (Fig. 3A). Anti-pNP IgM titer was apparently high in the preimmune serum because V\textsubscript{H}1T\textsuperscript{+} anti-NP IgM Abs that are spontaneously present at a high level showed cross-reactivity with pNP, but was further increased after immunization (data not shown). Concomitant with an increase in anti-pNP IgG titer, there was a significant increase in the affinity of IgG Abs to pNP that was assessed by the ratio of the Ab binding to the low density hapten (pNP\textsubscript{acetyl}-BSA) to the binding to the high density counterpa (pNP\textsubscript{acetyl}-BSA) (Fig. 3B). The ratio (pNP\textsubscript{pNP}\textsubscript{acetyl}) increased from \( \sim 0.1 \) on day 8 to \( \sim 0.6 \) on day 16, indicating that affinity maturation coupled with isotype switching occurred in this knockin strain.

Generation of anti-pNP mAbs and their Ag specificity

To investigate how V\textsubscript{H}1T\textsuperscript{+} Abs that were originally specific for NP underwent affinity maturation toward pNP by altering their Ag specificity, B cell hybridomas were generated from the draining LN cells on day 16 after immunization. Among 150 clones that secreted pNP-reactive IgG mAbs, 15 high affinity clones (pNP\textsubscript{pNP}>0.7) were selected, of which 8 clones were V\textsubscript{H}1T\textsuperscript{+}, and the rest were found to use endogenous V\textsubscript{H}1 genes that are considered to be generated by the replacement of the V\textsubscript{H}portion of V\textsubscript{H}1 with upstream endogenous V\textsubscript{H} or V\textsubscript{H}D, as reported previously (22,
36, 37) (Table I). V_H and V_L gene usage was examined by ELISA and/or RT-PCR. A characteristic feature is that all the V_H T/H11001 anti-pNP IgG mAbs examined used /H9261 2 as the L chain, while both /H9261 1 and /H9261 2 were used in V_H T/H11002 anti-pNP IgG mAbs (Table I). In contrast, all pNP-reactive IgM mAbs examined were V_H T/H11001, and used either /H9261 1 or /H9261 2 L chains (Table I), suggesting that both V_H T/H9261 1 and V_H T/H9261 2 B cells were activated in response to pNP to differentiate into IgM producers. More direct evidence is presented in the later section.

Ag specificity of these mAbs was analyzed by ELISA. The control unmutated V_H T/H11001 IgM and IgG1 mAbs were generated by the cell fusion of QM B cells that were stimulated in vitro with LPS in the presence or absence of IL-4. Nucleotide sequences of VH and VL genes in these mAbs were read to confirm the absence of somatic mutations (data not shown). Unmutated V_H T/H9261 2 IgM and

<table>
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FIGURE 2. A, Estimation of relative affinity of NP-related haptens for V_H T^- BCR of QM B cells. pNP is a low affinity analog of NP. NP-β-Gal was incubated in the microplates coated with anti-NP IgM purified from a QM mouse serum in the presence of varying concentrations of each hapten. Affinity of each hapten for V_H T^- BCR was estimated by measuring the free hapten inhibition of binding of NP-β-Gal to anti-NP IgM. IC_50 of each hapten is shown. Relative affinity of each hapten was calculated as the reciprocal of IC_50 and shown in the parentheses, in which the value of NP is designated as 1. B, Comparison of the potency to down-regulate sIgM on QM B cells between NIP-CGG and pNP-CGG. After incubation of spleen B cells with 1 μg/ml NIP25-CGG or pNP25-CGG for 3 h, B220^-gated cells were analyzed for the expression of sIgM by flow cytometry. Thick and thin diagrams represent NIP- or pNP-CGG-treated B cells and mock-treated B cells, respectively. Representative data from four repeated experiments are shown. C, Induction of IgM Ab response in QM B cells in vitro. The spleen B cells were cocultured in triplicate with CCG-specific Th2 cells in the presence of varying concentrations of pNP-CGG (○) or NIP-CGG (●). V_H T Id^- IgM levels in the culture supernatants were assayed. Data are the representative of five repeated experiments.

FIGURE 3. A, Anti-pNP IgG response in QM mice immunized with pNP-CGG. QM mice (n = 4–5) were immunized in the footpad with pNP-CGG/ CFA, and bled on indicated days. B, Affinity maturation of anti-pNP IgG. Sera collected on days 8 and 16 after immunization were assessed for the relative affinity of anti-pNP IgG Abs by measuring the ratio, the amount of IgG bound to pNP_4-BSA/the amount of IgG bound to pNP_20-BSA (pNP_4-pNP_20). In each panel, ○ and ● indicate two separate experiments.

Ag specificity of these mAbs was analyzed by ELISA. The control unmutated V_H T^- IgM and IgG1 mAbs were generated by the cell fusion of QM B cells that were stimulated in vitro with LPS in the presence or absence of IL-4. Nucleotide sequences of VH and VL genes in these mAbs were read to confirm the absence of somatic mutations (data not shown). Unmutated V_H T/A2 IgM and
Table 1. H and L chains used in anti-pNP mAbs obtained from QM mice immunized with pNP-CGG

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<sup>a</sup> Indicates that V<sub>H</sub>T-encoded IgH is used.

<sup>b</sup> The closest germline V<sub>H</sub> found in Kabat database (Ref. 33).

IgG1 mAbs thus obtained showed an Ag specificity (NIP > NP > pNP) that is similar to those reported previously (21, 35) (Fig. 4). In contrast, all of the high affinity V<sub>H</sub>T<sup>+</sup> anti-pNP IgG mAbs, six of which were shown as representatives (Fig. 4), acquired higher specificity for pNP (pNP > NP > pNP). Similar pNP specificity was observed in V<sub>H</sub>T<sup>+</sup> IgG mAbs listed in Table I (data not shown).

Most V<sub>H</sub>T/A1 or V<sub>H</sub>T/A2 IgM mAbs shown in Table I had similar Ag specificity (NIP > NP > pNP) to that of the unmutated V<sub>H</sub>T/A2 IgM (data not shown). One exception is a V<sub>H</sub>T/A2 clone, M53, that showed increased pNP specificity as V<sub>H</sub>T<sup>+</sup> anti-pNP IgG mAbs (Fig. 4). Taken together, it is shown that V<sub>H</sub>T/A2 B cells, but not V<sub>H</sub>T/A1 B cells, preferentially underwent affinity maturation toward pNP in response to pNP-CGG, while the IgM Ab response occurred comparably in V<sub>H</sub>T/A1 and V<sub>H</sub>T/A2 B cells. Following analyses were done to examine why the biased use of the A2-chain in anti-pNP IgG was brought about.

V<sub>H</sub> and V<sub>L</sub> sequences of V<sub>H</sub>T<sup>+</sup> anti-pNP mAbs

To identify somatic mutations that led to the change of Ag specificity from NP to pNP, nucleotide sequences of the V<sub>H</sub> and V<sub>L</sub> genes in V<sub>H</sub>T<sup>+</sup> anti-pNP IgG mAbs listed in Table I were analyzed. Data from six representative clones are shown (Fig. 5A).

There was a unanimously shared point mutation (T to A) at position 313 (T313A), which resulted in the replacement of Tyr 105 with Asn. An additional mutation (G to A) at position 316 (G316A) that caused the change of Ala<sup>106</sup> to Thr was found in four of six IgG clones (Fig. 5A). There were one to three point mutations in the A2-chain of each IgG clone, but no common replacement mutation was found (Fig. 5B).

All IgM mAbs that were obtained from pNP-CGG-immunized mice had V<sub>H</sub>T<sup>+</sup> μ-chains, which were associated with A1- or A2-chain (Table I). Consistent with their retention of the specificity for NP (Fig. 4), we confirmed that all the IgM mAbs except M53 had no point mutation in either chain (data not shown). V<sub>H</sub>T<sup>+</sup> μ-chain of M53 had a point mutation, T313A, that was shared by all V<sub>H</sub>T<sup>+</sup> anti-pNP IgG mAbs (Fig. 5A), while there was no mutation in its A2 gene (data not shown). Because M53 bound to pNP more efficiently than NP (Fig. 4), the T313A mutation is considered to be crucial for generating the pNP specificity. The occurrence of common mutations in IgM and IgG mAbs that are responsible for the affinity maturation is interesting because hypermutation and isotype switching generally have been reported to occur in a coordinated fashion (3, 7, 8).

A pivotal role of the T313A mutation played in the acquisition of pNP specificity was further confirmed by the following transfection experiments. When the V<sub>H</sub>T<sup>+</sup> γ2b chain possessing only T313A mutation was expressed in association with the A2-chain in CHO cells, the resultant IgG2b showed an increase in the binding to pNP compared with the native V<sub>H</sub>T<sup>+</sup>-encoded γ2b (Fig. 6A). When another mutation, G316A, was introduced in addition to T313A, the resultant IgG2b showed further increased to the level that was comparable to that observed in a high affinity anti-pNP IgG2b clone, G149, suggesting that these point mutations play a critical role in the affinity maturation toward pNP.

Although at least 40% of high affinity anti-pNP IgG Abs used endogenous V<sub>H</sub> genes, V<sub>H</sub>T<sup>+</sup> IgM clones may be rare. The 24 IgM mAbs not listed in Table I that were obtained on day 8 or 16 after immunization with pNP-CGG were also positive for the Id of V<sub>H</sub>T<sup>+</sup> anti-pNP mAbs not listed in Table I that were obtained on day 8 or 16 after immunization with pNP-CGG were also positive for the Id of V<sub>H</sub>T<sup>+</sup> anti-pNP mAbs (Fig. 4). When another mutation, G316A, was introduced in addition to T313A, the resultant IgG2b showed an increase in the binding to pNP compared with the native V<sub>H</sub>T<sup>+</sup>-encoded γ2b (Fig. 6A). When another mutation, G316A, was introduced in addition to T313A, the resultant IgG2b showed further increased to the level that was comparable to that observed in a high affinity anti-pNP IgG2b clone, G149, suggesting that these point mutations play a critical role in the affinity maturation toward pNP.

Analysis of the mechanism leading to predominant use of the A2-chains in high affinity V<sub>H</sub>T<sup>+</sup> anti-pNP IgG

Because V<sub>H</sub>T<sup>+</sup> QM B cells expressed A1 and A2 at comparable frequency (Fig. 1B), we investigated why A2 was predominantly used in high affinity V<sub>H</sub>T<sup>+</sup> anti-pNP IgG. This may be due to either that V<sub>H</sub>T<sup>+</sup> γ-chains harboring mutations that are responsible for the pNP specificity are structurally unfavorable in associating with the A1-chain, or that there is a difference in the affinity for pNP between V<sub>H</sub>T/A1 and V<sub>H</sub>T/A2 B cells. To examine whether the former possibility is correct, we transfected CHO cells with the mutated V<sub>H</sub>T<sup>+</sup> γ2b genes derived from the two representative anti-pNP IgG2b clones, G83 and G149, together with the A1 or A2 chain.
FIGURE 5. Nucleotide sequences of the V<sub>H</sub>T and VA2 genes in anti-pNP mAbs. A, V<sub>H</sub>T nucleotide sequences in anti-pNP mAbs. Results from six IgG clones (G51, 142, 166, 149, 83, and 14) and one IgM clone (M53) listed in Table I are shown as representatives. Dots indicate sequence identity with unmutated V<sub>H</sub>T (17.2.25). Capital and small letters indicate amino acid replacement mutations and silent mutations, respectively. Asterisks at position 313–315 show a unanimous replacement mutation (Tyr to Asn) in these anti-pNP mAbs. IgM clones other than M53 that are shown in Table I have no somatic mutation in VH<sub>T</sub> (data not shown). B, VA2 nucleotide sequences of the V<sub>H</sub>T<sup>+</sup> anti-pNP IgG mAbs shown in A.
FIGURE 6. Expression of recombinant IgG Abs in CHO cells. A, Contribution of mutations T313A and G316A in CDR3 of V_H T/2b to an increase in pNP specificity. V_H T/2b bearing none, T313A only, or G316A in addition to T313A (G316A/T313A) was each expressed together with L2 in CHO cells. As the control, γ2b from G149 anti-pNP IgG2b mAb (see Table I) and native V_H T/2b were expressed. Binding of secreted IgG to pNP was assayed by ELISA in appropriately diluted culture supernatants. B, Mutated V_H T/2b γ2b chains from G83 and G149 anti-pNP mAbs can associate with the L1-chain. The mutated V_H T/2b γ2b gene derived from the clones, G83 or G149, was expressed together with the native L1 or L2 gene in CHO cells. As the positive control, native V_H T-encoded γ2b was expressed. IgG secretion was measured by a sandwich ELISA using anti-IgG and anti-L1 or anti-L2. C, The L2-, but not the L1-chain forms pNP-specific IgG2b when expressed together with mutated V_H T/2b γ2b chains derived from G83 and G149 in CHO cells. Native V_H T/2b γ2b was used as a negative control.

FIGURE 7. A, Comparable binding of V_H T/1 and V_H T/2 IgM to NP. V_H T/1 and V_H T/2 IgM mAbs bound to a NP-BSA-coated plate were incubated with varying concentrations of free NP. NP-ε-aminocaproate was used as free NP. After washing, the level of residual IgM Abs on the plate was assayed. B, Comparison of the affinity for pNP between V_H T/1 and V_H T/2 IgM mAbs. V_H T/1 and V_H T/2 IgM mAbs bound to a pNP-BSA-coated plate were displaced by varying concentrations of free NP. In each panel, ○ and ▲ indicate V_H T/1 IgM and V_H T/2 IgM, respectively.

gene, respectively. Sandwich ELISA using anti-γ and anti-λ revealed that either γ2b chain was secreted in association with L1 or L2, respectively (Fig. 6B), thus ruling out that there is some incompatibility between the mutated γ2b and L1. In contrast, IgG2b comprised of these V_H T/2b γ2b and the L1-chain showed much lower binding to pNP than the original L2-associated counterpart, thus suggesting that V_H T/1 B cells cannot improve their affinity for pNP by using the same IgH mutations as those used in V_H T/2 anti-pNP IgG mAbs (Fig. 6C).

Next, we compared unmutated V_H T/1 and V_H T/2 IgM for their affinity for pNP to estimate the affinity of each QM BCR for the hapten. V_H T/1 and V_H T/2 IgM showed comparable affinity for NP, while the former is considered to bind pNP 50- to 100-fold less strongly than the latter, as assessed by the displacement of pNP-bound Abs with increasing concentrations of free NP (Fig. 7, A and B). Because V_H T/1 IgM has very low affinity for pNP, the relative affinity of pNP estimated in Fig. 2A may virtually represent that of V_H T/2. We compared the relative affinity of V_H T/2 IgM mAb for pNP and NP with displacement ELISA, and obtained a similar result to those shown in Fig. 2 (data not shown).

As a number of hybridomas secreting V_H T/1 IgM as well as those secreting V_H T/2 IgM were obtained (Table I), V_H T/1 B cells with very low affinity for pNP may have been activated in response to pNP-CGG in the initial phase of the immune response. To prove this more directly, we investigated whether V_H T/1 B cells acquire a plasma cell phenotype after immunization. On day 5 after immunization with pNP-CGG in the footpad, it was confirmed that there was a significant increase in the number of V_H T/1 Ab-secreting cells in the draining LN, as assessed by ELISPOT assay, 90–95% of which were IgM producers (350–500 ELISPOT/10^6 LN cells). The analysis of the same LN cells for the expression of CD138, a plasma cell marker, revealed that CD138+ V_H T/1 B cells increased significantly after immunization (Fig. 8A). V_H T/1- or CD138- B cells were further analyzed for the expression of the L1- or L2-chain. The results show that V_H T/1 as well as V_H T/2 B cells became CD138+ (Fig. 8B). The frequency of the latter appears to be higher than the former. This might reflect the differential affinity of these two populations for pNP. Although detailed data are not presented, we further confirmed the following two points: 1) There was a ~2-fold increase in the serum level of V_H T/1 IgM on day 8 after immunization with pNP-CGG, but the ratio of V_H T/1 IgM to V_H T/2 IgM in the preimmune sera (4:6) did not significantly change after immunization, thus suggesting that both V_H T/1 and V_H T/2 IgM were produced in response to
In consequence of affinity maturation of \( V_{H}^{+} \) anti-pNP IgG, the Ag specificity of the resultant Abs was changed to pNP > NP > NIP in contrast to that of the unmutated Abs (NIP > NP > pNP). A common point mutation, T313A in CDR3 of \( V_{H}^{+} \) is considered to be critical for generating the pNP specificity, although some other mutations, including G316A and those present in CDR2, may also be partly involved. The importance of the T313A mutation was confirmed by the observation that the \( V_{H}^{+} \)-encoded \( c \)-chain harboring only the T313A mutation constituted IgG2b with increased pNP specificity when it was expressed in CHO cells together with the unmutated A2-chain (Fig. 6A).

An IgM mAb, M53 is unique in that it showed higher specificity for pNP than NP. This is considered to be due to the presence of the same replacement mutations as those shared by \( V_{H}^{+} \) anti-pNP IgG mAbs (Fig. 5A). Somatic hypermutation and isotype switching are considered to occur in a coordinated fashion, with the latter initiating later than the onset of the former in GC (37). The mutation frequency is usually higher in the IgG classes than in the IgM class (7, 38). The link between somatic hypermutation and isotype switching has been recently explained at the molecular level by the discovery of activation-induced cytidine deaminase, a putative RNA-editing enzyme responsible for these two processes (3, 39, 40). The replacement mutations, including T313A and G316A, found in high affinity \( V_{H}^{+} \) anti-pNP IgG mAbs are considered to reflect the positive selection of high affinity B cells that occurred in GC (1, 2). Therefore, M53, whose Ag specificity is shifted to pNP with the same mutations, may have also been subjected to positive selection. It has been shown using lymphotixin-\( \alpha \)-deficient mice that affinity maturation occurred without GC, although with reduced efficiency (41). The occurrence of IgM Abs such as M53 implies that a part of hypermutation/positive selection can occur at the IgM level.

IgM mAbs that were isolated from pNP-CGG-immunized QM mice almost exclusively bore \( V_{H}^{+} \) IgH, whereas \( \sim 40\% \) of high affinity anti-pNP IgG mAbs used endogenous \( V_{H}^{+} \) genes. Because one \( I_{H} \) locus is disrupted, endogenous \( V_{H}^{+} \) genes found in the \( V_{H}^{+} \) IgG Abs may have been generated by the replacement of \( V_{H}^{+} \) in the \( V_{H}^{+} \) segment with upstream \( V_{H} \) or \( V_{H}^{+} \) segments, as reported previously (22, 36, 42). The apparent absence of \( V_{H}^{+} \) IgM clones in immunized mice implicates that these were very minor in an early stage of the immune response probably due to low frequency of the precursor cells, but expanded drastically during GC reactions through competition with an enormous number of \( V_{H}^{+} \) B cells. In a B cell line, LK35.2 expressing BCR specific for hen egg lysozyme, it has been shown that BCR-mediated triggering by mutant Ags with varying affinity, as assessed by Ag presentation to a T cell hybridoma, required a \( K_{D} \) value higher than \( \sim 10^{-6} \) M\(^{-1}\). The minimal concentration of an Ag that is required to trigger a response decreased as the affinity increased (43). The parallelism between the intensity of BCR engagement and B cell activation has also been reported in 3-83 anti-H-2K\(^{k}\) transgenic B cells stimulated with recombinant virus-displayed peptide Ags with low or high affinity for the BCR (44). These findings suggest the strict nature of the selection process depending on the affinity of BCR for an Ag.

Recently, dependence of a T cell-independent or a T cell-dependent anti-NP Ab response on BCR affinity for the hapten has been analyzed in two lines of mice that carry targeted VHBI-8 anti-NP IgH genes with low or high affinity for the hapten (45, 46). When these two populations compete with each other in the responses to NP-Ficoll and NP-keyhole limpet hemocyanin, only high affinity B cells were found to respond preferentially, while reasonable to analyze anti-pNP response in QM mice who exclusively express \( \lambda \)-chains (22).
low affinity B cells could respond significantly if there is no competitor. In the T cell-dependent anti-NP response, high affinity B cells predominately accumulated in GC (46). Similar observations have been made in mice transgenic for mutated Vμ 186.2-encoded μ genes that generate BCR with low or high affinity for NP when combined with the A1-chain (47). Consistent with these results, it has been reported that B cells with a wide variety of affinity for an inducing Ag populated early GC, but high affinity B cells gradually dominated during the course of an immune response (48).

Affinity maturation has not been fully analyzed in mice carrying a targeted Ab gene. QM mice are considered to be advantageous for analyzing selection and differentiation of B cells during an Ab response in that B cell repertoire is more limited due to the lack of κ genes, and that isotype switching and somatic mutation occur normally. In contrast, it may be argued that the situation in QM mice is unphysiological due to the abnormally high frequency of specific B cells. One of the experimental designs to avoid the unphysiological situation may be to use an adoptive transfer system. We immunized C57BL/6 mice (IgMb) that were injected with 2 × 10^6 QM B cells (IgM^α) with pNP-CGG or NP-CGG. NP-CGG with higher affinity for QM B cells than pNP-CGG induced IgM response in these mice. In contrast, while anti-pNP IgM^α Abs were induced by pNP-CGG, the production of IgM^α was negligible (H. Ohmori et al., manuscript in preparation). Thus, the transferred QM B cells with lower affinity for pNP are considered to be out-competed by high affinity B cells of the host, suggesting that the adoptive transfer system is not advantageous in this case.

In contrast, immunization of intact QM mice with pNP-CGG resulted in the induction of IgM production predominantly from VH T/ B cells. Besides the extremely high frequency of Vγ1 T/Ab cells (~80% of total B cells), this may be due to a lower level of competitor B cells in QM mice, because the VH T/ B cell diversity may be considerably limited due to disruption of the JH locus on one allele and both κ loci (22). As we reported previously, in (QM × C57BL/6)F1 mice (VH T/germline, κ^+/κ^−, λ^+/λ^−) that have 4–7% VH T/Ab cells and more diverse VH T/ B cell repertoire than QM mice, VH T/ B cells were preferentially activated by pNP-CGG (25). These are consistent with recent reports describing that even very low affinity B cells are activated by an Ag when relieved of competition from higher affinity B cells (46, 47). Despite some disadvantages relating to the unusual B cell repertoire, the anti-pNP Ab response in intact QM mice is likely to obey normal regulatory mechanisms, as follows: 1) The time course of the anti-pNP Ab response, IgG class switching, and GC formation (our unpublished observation) were normal in QM mice compared with wild-type mice. 2) The frequency of VH T/ IgM-secreting B cells after immunization (less than 0.1% in the draining LN cells) was comparable to that observed in wild-type mice. 3) pNP-CGG-induced IgM-secreting plasma cells were derived from VH T/Ab1 or VH T/Ab2 B cells, and a majority of VH T/ anti-pNP IgG Abs were the products of VH T/Ab2 B cells, which were found to bear a common point mutation in CDR3, contributing to affinity maturation toward pNP. Thus, it is suggested that VH T/ B cells were activated in response to pNP-CGG and recruited to GC, in which mutated B cells were normally selected on the basis of the affinity for pNP. In addition, when QM mice were immunized with viral Ags (49) or SRBC (50) that were irrelevant to NP, VH T/ B cells were exclusively involved in these Ag-specific Ab responses. We also confirmed that pNP-CGG, but not CGG without haptens, induced VH T/ Abs in QM B cells that were cultured with CGG-specific Th2 clone (Fig. 2C). These data suggest that VH T/ B cells in QM mice are normally subjected to affinity-dependent clonal selection.

Although pNP is a low affinity hapten for QM B cells, especially for VH T/Ab1 B cells (Figs. 2 and 7), pNP-CGG used is considered to have enough affinity for triggering QM B cells because both VH T/Ab1 and VH T/Ab2 IgM Abs were produced in the anti-pNP Ab response. The apparent absence of A1^+ anti-pNP IgG may be the result of competition between the two B cell populations with differential affinity for pNP that occurred in the early phase of the immune response. Although the detailed mechanism remains to be elucidated, weak signals via VH T/Ab1 BCR are sufficient for inducing IgM Ab responses, but may not satisfy the requirements for the recruitment to affinity maturation pathway. By using NP analogs of varying affinity, the QM system will provide an excellent means to investigate how BCR signal intensity regulates B cell selection and the subsequent differentiation.

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**References**
