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

Naoki Kanayama,* Takafumi 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 VH17.2.25 (VH T)-encoded H chain and the λ1 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 λ1-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 λ1-chain, but may be explained by the fact that VH T/A1 B cells showed 50- to 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 germinal 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 B 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, V H 1-Ox1/V k-Ox1 in the former response, and V H 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 V H 1-DJ H segment (V H T) derived from an anti-NP mAb (21), with the other J H locus and both κ loci being disrupted (22). The advantage of this type of knockin strain is that the site-directed V H 1-encoded (V H 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 V H T+ NP-specific BCR that bear a-chains (21, 22), of which λ1- 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 V H 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_{H} T/1 and V_{H} T/2, selected by pNP to be committed to affinity maturation process?

Materials and Methods

Hapten and Abs

NP, pNP, 4-hydroxy-5-iodo-3-nitrophenylacetamid (NIP), 4-hydroxy-phenylacetyl, and n-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-hydroxysuccimide ester of each acid, as described previously (25). Usually, CGG was conjugated with 25–250 molecules of each hapten.

Estimation of affinity of NP-related hapten for V_{H} T/1 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 mouse Abs 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-α-L chain (R11-153), and biotinylated anti-α-L chain (2B6) that was cross-reactive with B220 were purchased from BD Pharmingen (San Diego, CA). B cells bearing V_{H} T-encoded IgH were detected with biotinylated mAb to the Id of V_{H} T, R2.438 (a gift from T. Imanishi-Kari, Tufts University, Boston, MA). Surface IgM (sgIM) 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_{H} T/1−, Jκ/Jκ, β/λ/λ (22). QM mice were immunized in the hind footpad with 20 μg of pNP-CGG emulsified in CFA, and bled on indicated days after immunization. All mice were treated in accordance with the guidelines approved by the Committee of Laboratory Animal Care, Okayama University.

In vitro culture of QM B cells

In the induction of an Ab response in vitro, QM spleen B cells (3 × 10^6), prepared as described (27), were cultured with 3 × 10^4 CGG-specific Th2 clone, CTH, which was established in our laboratory, in the presence of varying concentrations of pNP-CGG or pNP-CGG in 1 ml of RPMI 1640 medium supplemented with 10% FCS and 1 × 10^−5 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_{H} T/1 IgH Abs by a sandwich 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 sgIM, QM B cells (3 × 10^6/ml) were cultured with 1 μg/ml of pNP-CGG or NIP-CGG for 3 h. B220−/Id−B cells were assessed for the level of sgIM 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_Alg-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, IgG3 was not detected with the present assay, but occasionally appeared when mAbs were studied. The displacement of the bound Ab by the free hapten was estimated by determining the residual Ab on the plates.

Estimation of Ag specificity of mAbs

An appropriately diluted mAb was added to microplates that were coated with pNP-BSA, NP-BSA, or NIP-BSA (6–7 each hapten/BSA molecule), and incubated at 25°C for 1 h. After washing, bound mAb 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/CFCA in the footpads, popliteal lymph node (LN) cells were prepared and fused with a myeloma cell line, NSO-5 (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, NP, NIP, and pNP, as described previously (31, 32). Recombinant mAbs secreting nonmutated V_{H} T/1 IgM, V_{H} T/2 IgM, or V_{H} T/2 IgG1 were generated by the cell fusion with NSO-5 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 10 ng/ml of IL-4 (PeproTech EC, London, U.K.). The absence of somatic mutations in the V_{H} T/1 mAbs was confirmed by sequencing V_{H} and V_{L} genes.

Sequence of V_{H} and V_{L} genes

cDNA was synthesized from total RNA of each anti-pNP mAb-secreting hybridoma, as described previously (25). V_{H} and V_{L} regions of mAbs were amplified with PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). Primers used were as follows: For amplifying V_{H} regions, CTGAATCTCAAGGTCCTTAC specified for V_{H} T, and ARCTKWSGAGWGCGWGGRSSWG and ARCTKAWSGAGWGCGGRSG for endogenous VH as sense primers; GACAGGGTTTCAAKGTTCCA for all IgG subclasses, and GTTCTGATACCCTGGATGACTTC for IgM as antisense primers. For amplifying VH regions, CTGCTGCTGAC CAATTATTGAAAAGATAGAACCC and GCACATGTTCTCAGGCCTCGT for endogenous VH as sense primers; GACAGGGTTTCAAKGTTCCA for all IgG subclasses, and GTTCTGATACCCTGGATGACTTC for IgM as antisense primers. For amplifying VH regions, CTGCTGCTGAC CAATTATTGAAAAGATAGAACCC and GCACATGTTCTCAGGCCTCGT for endogenous VH as sense primers; GACAGGGTTTCAAKGTTCCA for all IgG subclasses, and GTTCTGATACCCTGGATGACTTC for IgM as antisense primers. For amplifying VH regions, CTGCTGCTGAC CAATTATTGAAAAGATAGAACCC and GCACATGTTCTCAGGCCTCGT for endogenous VH as sense primers; GACAGGGTTTCAAKGTTCCA for all IgG subclasses, and GTTCTGATACCCTGGATGACTTC for IgM as antisense primers.
C region was amplified with CCCTCTCGAGGCTAAAAACAAACAAC CCCCATCAGTC and CTTTGGCCGCGCTGACTTCTGACCCCG AGA, and the product was digested with EcoRI and NotI. The digested VH fragments derived from VH×2 IgM, G83, and G149 were each inserted in combination with the fragment of the y2B region C between Nhel and NdeI sites of a mammalian expression vector, pCI-neo (Promega, Madison, WI), which were designated as pCIGVH×T, pCIG83, and pCIG149, respectively. Another two constructs bearing the y2B genes containing only one (T313A) or two (T313A/G316A) mutations in VH complementarity-determining region 3 (CDR3) were constructed by replacing a PspBl-EcoRIII fragment of pCIGVH×T with the corresponding sequence from pCIG83 or pCIG149, respectively. A PspBl-BamHI fragment containing puromycin phosphotransferase gene of pC neo was exchanged with a PspBl-BamHI fragment containing puromycin N-acetyltransferase gene of pBluescript (34). This expression vector was termed as pC1-pto. The λ1- and λ2-chains were amplified from VH×1A1 IgM and VH×1A2 IgM hybridomas using ACCCTGGCG CAAGCGGACCAGGTGGTATTGACTATAC as a sense primer for both λ1 and λ2, and GGAGGCGGCGGTCCCTTGTAGAGCTTCTGCGAGG as an anti-sense primer for λ1 and λ2, respectively. The λ1 and λ2 fragments were digested with Sall and NotI, and cloned into pC1-pto. Sequence analysis of all the gene constructs confirmed that no mutation was introduced during PCR amplification. The IgG2b C region and the λ1- and λ2-chains had germinal sequences. All constructs were linearized by digesting with BamHI or BglII before transfection. CHO cells were transfected with an appropriate combination of a y2B and a λ gene using Lipofectamine (Life Technologies, Rockville, MD). Stable transfectants were selected and maintained in the presence of 500 μg/ml G418 and 5 μg/ml puromycin (Sigma-Aldrich).

**Results**

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

The VH×D1JH gene segment, VH×T, is derived from 17.2.25 anti-NP mAb (γ1, λ) that binds to NP and its iodinated analog NIP. The affinity (Kd) of 17.2.25 for NP has been estimated to be 4 × 10^5 M⁻¹ (21). In QM mice bearing the site-directed VH×T with the genotype of VH×1JH*×, κ*/κ*, λ*λ*, a majority (~80%) of B cells expressed VH×T-encoded BCR that were detected by an anti-Id mAb, R2.438 (35) (Fig. 1A). Because both κ loci are disrupted, QM B cells exclusively express λ-chains (22). Although an α-λ2 mAb used in the flow cytometric analysis has cross-reactivity with λ3, of ~30 λ-chains randomly sequenced, λ1 and λ2, but not λ3, were found (data not shown), thus suggesting that the λ1- and λ2-chains are major L chains used. Flow cytometric analysis shows that each VH×1JH/A1 and VH×1JH/A2 B cell population has a comparable size (Fig. 1B). Both VH×1JH/A1 and VH×1JH/A2 Abs have been shown to bind to NP (21, 22). To examine various NP-related haptons for their binding activity to VH×1T⁺ anti-NP IgM derived from QM mice, free hapten inhibition of the binding of NP-conjugated β-Gal to the immobilized anti-NP IgM was assessed. We found that pNP had ~20-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 μg/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 VH×1T⁺ IgM at lower concentrations (0.01–0.1 μg/ml) than pNP-CGG, which elicited the Ab response significantly at greater than 1 μg/ml (Fig. 2C). CGG without haptons did not induce the Ab response at 0.01–10 μg/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 VH×1T⁺ IgG response efficiently in QM mice, as shown in the following section. These observations led us to investigate how VH×1JH/A1 and VH×1JH/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 VH×T 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 VH×1T⁺ 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×-BSA) to the binding to the high density counterpart (pNP×-BSA) (Fig. 3B). The ratio (pNP×-pNP×) increased from ~0.1 on day 8 to ~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 VH×1T⁺λ⁺ 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 (pNP4-pNP30 > 0.7) were selected, of which 8 clones were VH×1T⁺, and the rest were found to use endogenous VH× genes that are considered to be generated by the replacement of the VH× portion of VH× with upstream endogenous VH× or VH×D, as reported previously (22, 23).
36, 37) (Table I). V<sub>H</sub> and V<sub>L</sub> gene usage was examined by ELISA and/or RT-PCR. A characteristic feature is that all the V<sub>H</sub> T<sub>H9261</sub> anti-pNP IgG mAbs examined used /H9261<sub>2</sub> as the L chain, while both /H9261<sub>1</sub> and /H9261<sub>2</sub> were used in V<sub>H</sub> T<sub>H11002</sub> anti-pNP IgG mAbs (Table I). In contrast, all pNP-reactive IgM mAbs examined were V<sub>H</sub> T<sub>H11001</sub>, and used either /H9261<sub>1</sub> or /H9261<sub>2</sub> L chains (Table I), suggesting that both V<sub>H</sub> T<sub>/H9261<sub>1</sub></sub> and V<sub>H</sub> T<sub>/H9261<sub>2</sub></sub> 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<sub>H</sub> T<sub>H11001</sub> 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<sub>H</sub> T<sub>/H9261<sub>2</sub></sub> IgM and

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**FIGURE 2.** A, Estimation of relative affinity of NP-related haptens for V<sub>H</sub> T<sup>+</sup> 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<sub>H</sub> T<sup>+</sup> BCR was estimated by measuring the free hapten inhibition of binding of NP-β-Gal to anti-NP IgM. IC<sub>50</sub> of each hapten is shown. Relative affinity of each hapten was calculated as the reciprocal of IC<sub>50</sub> and shown in the parentheses, in which the value of NP is designated as 1.

B, Comparison of the potency to down-regulate slgM on QM B cells between NIP-CGG and pNP-CGG. After incubation of spleen B cells with 1 µg/ml NIP<sub>25</sub>-CGG or pNP<sub>25</sub>-CGG for 3 h, B220<sup>+</sup>-gated cells were analyzed for the expression of slgM 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 CGG-specific Th2 cells in the presence of varying concentrations of pNP-CGG (•) or NIP-CGG (○). V<sub>H</sub> T<sup>+</sup> IgM levels in the culture supernatants were assayed. Data are the representative of five repeated experiments.
IgG1 mAbs thus obtained showed an Ag specificity (NIP > NP > pNP) that is similar to that reported previously (21, 35) (Fig. 4). In contrast, all of the high affinity V_{H}T^{+} anti-pNP IgG mAbs, six of which were shown as representatives (Fig. 4), acquired higher specificity for pNP (pNP > NP > NIP). Similar pNP specificity was observed in V_{H}T^{+} IgG mAbs listed in Table I (data not shown).

Most V_{H}T/A1 or V_{H}T/A2 IgM mAbs shown in Table I had similar Ag specificity (NIP > NP > pNP) that to that of the unmutated V_{H}T/A2 IgM (data not shown). One exception is a V_{H}T/A2 clone, M53, that showed increased pNP specificity as V_{H}T^{+} anti-pNP IgG mAbs (Fig. 4). Taken together, it is shown that V_{H}T/A2 B cells, but not V_{H}T/A1 B cells, preferentially underwent affinity maturation toward pNP in response to pNP-CGG, while the IgM Ab response occurred comparably in V_{H}T/A1 and V_{H}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.

**FIGURE 4.** Ag specificity of anti-pNP IgG or IgM mAbs obtained from pNP-CGG-immunized QM mice. The Ag specificity was assessed by measuring the binding of an appropriately diluted mAb to microplates coated with pNP-, NP-, or NIP-BSA. As the control, the Ag specificity of unmutated V_{H}T/A2 IgM or V_{H}T/A2 IgG1 mAb is shown. Data are presented as relative binding to each hapten, in which the highest value in each mAb is expressed as 100. Anti-pNP IgG mAbs shown here exhibit high affinity for pNP (pNP_{pNP} > 0.7). All mAbs listed in Table I were examined, and results from representative clones are presented.
FIGURE 5. Nucleotide sequences of the \( V_{\text{H}T} \) and \( V_{\text{A2}} \) genes in anti-pNP mAbs. A. \( V_{\text{H}T} \) 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 \( V_{\text{H}T} \) (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 \( V_{\text{H}T} \) (data not shown). B. \( V_{\text{A2}} \) nucleotide sequences of the \( V_{\text{H}T}^\text{m} \) anti-pNP IgG mAbs shown in A.
gene, respectively. Sandwich ELISA using anti-\( \gamma \) and anti-\( \lambda \) revealed that either \( \gamma 2b \) chain was secreted in association with \( \lambda 1 \) or \( \lambda 2 \), respectively (Fig. 6B), thus ruling out that there is some incompatibility between the mutated \( \gamma 2b \) and \( \lambda 1 \). In contrast, IgG2b comprised of these \( \gamma 1T^\gamma 2b \) chains and the \( \lambda 1 \)-chain showed much lower binding to pNP than the original \( \lambda 2 \)-associated counterpart, thus suggesting that \( \gamma 1T^\gamma 2b \) B cells cannot improve their affinity for pNP by using the same IgH mutations as those used in \( \gamma 1T^\lambda 2b \) anti-pNP IgG mAbs (Fig. 6C).

Next, we compared unmutilated \( \gamma 1T^\gamma 1T^\lambda 2 \) and \( \gamma 1T^\lambda 2 \) IgM for their affinity for pNP to estimate the affinity of each QM BCR for the hapten. \( \gamma 1T^\gamma 1T^\lambda 2 \) and \( \gamma 1T^\lambda 2 \) IgM showed comparable affinity for pNP, 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 \( \gamma 1T^\gamma 1T^\lambda 2 \) IgM has very low affinity for pNP, the relative affinity of pNP estimated in Fig. 2A may virtually represent that of \( \gamma 1T^\gamma 1T^\lambda 2 \). We compared the relative affinity of \( \gamma 1T^\gamma 1T^\lambda 2 \) 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 \( \gamma 1T^\gamma 1T^\lambda 2 \) IgM as well as those secreting \( \gamma 1T^\gamma 1T^\lambda 2 \) IgM were obtained (Table I), \( \gamma 1T^\gamma 1T^\lambda 2 \) 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 \( \gamma 1T^\gamma 1T^\lambda 2 \) 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 VH T\( ^\gamma 1T^\lambda 2 \) B 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– VH T\( ^\gamma 1T^\lambda 2 \) B cells increased significantly after immunization (Fig. 8A). VH T\( ^\gamma 1T^\lambda 2 \)-gated CD138– B cells were further analyzed for the expression of the \( \lambda 1 \)- or \( \lambda 2 \)-chain. The results show that \( \gamma 1T^\gamma 1T^\lambda 2 \) as well as \( \gamma 1T^\lambda 2 \) 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 VH T\( ^\gamma 1T^\lambda 2 \) IgM on day 8 after immunization with pNP-CGG, but the ratio of VH T\( ^\gamma 1T^\lambda 2 \) IgM to VH T\( ^\gamma 1T^\lambda 2 \) IgM in the preimmune sera (4:6) did not significantly change after immunization, thus suggesting that both VH T\( ^\gamma 1T^\lambda 2 \) and VH T\( ^\gamma 1T^\lambda 2 \) IgM were produced in response to
FIGURE 8. A, Detection of CD138+ B cells in the draining LN of QM mice after immunization. On day 5 after immunization with pNP-CGG in the footpad, the popliteal LN cells were analyzed for the expression of the Id of V\textsubscript{H}1 and CD138. B, Id\textsuperscript{-}gated CD138 + cells from the day 5 LN cells were analyzed for the expression of the \( \lambda 1 \) (left panel) or the \( \lambda 2 \) (right panel) L chain. The number in each diagram represents percentage of the indicated population in the total gated cells.

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\textsubscript{H}1T+ 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 frequency pNP-immunized QM mice provide a unique system in which to study these processes (11, 22, 36, 42). The apparent absence of V\textsubscript{H}2 T+ 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\textsubscript{H}1T+ IgH, whereas \(~40\%\) of high affinity anti-pNP IgG mAbs used endogenous V\textsubscript{H}1 genes. Because one J\textsubscript{G} locus is disrupted, endogenous V\textsubscript{H}1 genes found in the V\textsubscript{H}1T+ IgG Abs may have been generated by the replacement of V\textsubscript{H}1 in the V\textsubscript{H}1T segment with upstream V\textsubscript{H}1 or V\textsubscript{H}1-D segments, as reported previously (22, 36, 42). The apparent absence of V\textsubscript{H}1T+ 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\textsubscript{H}1T+ 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 \(~10^6 \text{ M}^{-1}\). 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\textsuperscript{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).

In consequence of affinity maturation of V\textsubscript{H}2T 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\textsubscript{H}2T 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\textsubscript{H}2T-encoded \( \gamma 2b \) 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).

Discussion

In QM mice with limited B cell diversity, we monitored how the two major B cell populations bearing NP-specific BCR, V\textsubscript{H}1T/A1 and V\textsubscript{H}1T/A2, were clonally selected and committed to affinity maturation in the immune response to a hapten pNP, a low affinity analog of NP. NP shows comparable affinity for V\textsubscript{H}1T/A1 and V\textsubscript{H}1T/A2 BCR, while pNP binds to V\textsubscript{H}1T/A2 BCR \(~20\)-fold less strongly than NP. In contrast, V\textsubscript{H}1T/A1 B cells have very low affinity for NP and pNP, which bind pNP 50- to 100-fold less efficiently than V\textsubscript{H}1T/A2 B cells. The QM system is considered to have the following two characteristic features. One is that, by stimulating QM B cells with pNP, it is possible to investigate how originally NP-specific B cells somatically mutate their V region genes and change the Ag specificity toward pNP during affinity maturation. The other is that one is able to analyze how differentially V\textsubscript{H}1T/A1 and V\textsubscript{H}1T/A2 B cells respond to pNP depending on the intensity of BCR signals. Because pNP preferentially induces IgG Abs bearing the \( \lambda \) L chain, as we reported previously (25), it is considered to re...
low affinity B cells could respond significantly if there is no competitor. In the T cell-dependent anti-NP response, high affinity B cells predominantly accumulated in GC (46). Similar observations have been made in mice transgenic for mutated V_{H} 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 (IgM) that were injected with 2 × 10^{9} QM B cells (IgM^{a}) with pNP-CGG or NP-CGG. NP-CGG with higher affinity for QM B cells than pNP-CGG induced IgM^{a} response in these mice. In contrast, while anti-pNP IgM^{a} Abs were induced by pNP-CGG, the production of IgM^{a} 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 V_{H}^{T} B cells. Besides the extremely high frequency of V_{H}^{T} B cells (∼80% of total B cells), this may be due to a lower level of competitor B cells in QM mice, because the V_{H}^{T} B cell diversity may be considerably limited due to disruption of the J_{H} locus on one allele and both κ loci (22). As we reported previously, in (QM × C57BL/6)F_{1} mice (V_{H}^{T}/germline, κ⁺/κ⁻, λ⁺/λ⁻) that have 4–7% V_{H}^{T} B cells and more diverse V_{H}^{T} B cell repertoire than QM mice, V_{H}^{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 V_{H}^{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 V_{H}^{T}TA1 or V_{H}^{T}TA2 B cells, and a majority of V_{H}^{T} anti-pNP IgG Abs were the products of V_{H}^{T}TA2 B cells, which were found to bear a common point mutation in CDR3, contributing to affinity maturation toward pNP. Thus, it is suggested that V_{H}^{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, V_{H}^{T} B cells were exclusively involved in these Ag-specific Ab responses. We also confirmed that pNP-CGG, but not CGG without hapten, induced V_{H}^{T} Abs in QM B cells that were cultured with CGG-specific Th2 clone (Fig. 2C). These data suggest that V_{H}^{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 V_{H}^{T}/A1 B cells (Figs. 2 and 7), pNP-CGG used is considered to have enough affinity for triggering QM B cells because both V_{H}^{T}TA1 and V_{H}^{T}TA2 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 V_{H}^{T}/A1 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**

AFFINITY MATURATION IN THE QM MOUSE