An asymmetric antibody repertoire is shaped between plasmablasts and plasma cells after secondary immunization with (4-hydroxy-3-nitrophenyl)acetyl chicken γ-globulin

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Abstract

Studies on the structural basis of antibody affinity maturation have been carried out by measuring the affinity of secreted antibodies, and information on structures has often been obtained from nucleotide sequences of BCRs of memory B cells. We considered it important to establish whether the repertoire of secreted antibodies from plasma cells is really in accord with that of BCRs on memory B cells at the same time points post-immunization. We isolated plasma cells secreting antibodies specific to (4-hydroxy-3-nitrophenyl)acetyl (NP) hapten by affinity matrix technology using biotin–anti-CD138 and streptavidin–NP–allophycocyanin, to which anti-NP antibodies secreted by autologous plasma cells bound preferentially. We found that plasmablasts occupied >90% of the antibody-secreting cell compartment in the primary response and that they secreted antibodies whose V_H regions were encoded by V186.2+Tyr95+ sequences, which provided an increase in the medium level of affinity by somatic hypermutation (SHM) of heavy chains at position 33. After secondary immunization, a further increase in antibody affinity was observed, which was explained by the appearance of a number of plasma cells secreting V186.2+Gly95+ antibodies that acquired high affinity by multiple SHMs as well as plasmablasts secreting V186.2+Tyr95+ antibodies. However, we did not detect any plasmablasts secreting V186.2+Gly95+ antibodies, showing that plasmablasts and plasma cells have a different antibody repertoire, i.e. their respective repertoires are asymmetric. On the basis of these findings, we discussed the relationship between the BCR affinity of memory B cells and plasmablasts as well as plasma cells as pertaining to their ontogeny.

Keywords: affinity maturation, antibody, memory B cells, somatic hypermutation

Introduction

The immune response of C57BL/6 mice to a hapten, (4-hydroxy-3-nitrophenyl)acetyl (NP), has been widely employed in the analysis of affinity maturation since anti-NP antibodies have unique structural properties; they are encoded by the canonical gene segments, V186.2, DFL16.2 and JH2 for the heavy chain and V_K1 and J_K1 for the light chain (1) although V_H genes analogous to V186.2 have also been employed (2). Therefore, anti-NP antibodies are rather homogeneous in terms of usage of their gene segments. However, even though they are encoded by canonical genes, these antibodies are heterogeneous in sequences of their complementarity-determining regions (CDRs), especially in the third CDR of heavy chains (CDRH3), due to the addition of an N-region by terminal deoxyribonucleotidyl transferase, which adds non-germline-encoded nucleotides during immunoglobulin gene rearrangement (3). By preparing hybridomas, we previously demonstrated two distinct anti-NP antibody populations that were characterized by the amino acid residue at position 95...
while V186.2 were dominant at the early phase of the immune response studied extensively by focusing on V186.2 (NP–APC (NP–APC cytometry) to which anti-NP antibodies secreted from autologous cells, and they are then captured by second antibody reagents so that the secreting cells can be detected by flow cytometry, and the average molar ratio of NP per carrier protein was calculated by measuring OD at 280 and 430 nm except in the case of 0.15 M sodium bicarbonate buffer (pH 8.5) containing 0.15 M NaCl for 1 h at room temperature. Any excess reagents or byproducts of the reaction were removed by passing the mixture through a Sephadex G-25 column (Amersham Biosciences) or performing dialysis against PBS. The average molar ratio of NP per carrier protein was calculated by measuring OD at 280 and 430 nm except in the case of NP–APC or NP–APC<sub>av</sub> The number of NPs per APC or APC<sub>av</sub> was approximated based on the molar ratio of NP–OSu and APC or APC<sub>av</sub> in the reaction mixture and expressed as NP<sub>av</sub>, NP<sub>med</sub> and NP<sub>max</sub>, respectively. Hybridomas producing the anti-NP antibodies, B2 and E11 were prepared as described previously (16). N1G9 was provided by Prof. K. Rajewsky. The equilibrium association constants (K<sub>a</sub>) of N1G9, B2 and E11 to NP–e-aminocaproic acid, as determined by isothermal titration calorimetry, are 2.9 × 10<sup>5</sup> M<sup>-1</sup>, 3.4 × 10<sup>6</sup> M<sup>-1</sup> and 3.3 × 10<sup>5</sup> M<sup>-1</sup>, respectively (6, 7).

**Methods**

**NP–protein conjugates and anti-NP antibodies**

NP–protein conjugates were prepared as described previously (16). Briefly, the hydroxysuccinimide ester of NP (NP–OSu) was coupled to carrier proteins such as chicken γ-globulin (CGG), BSA, APC and streptavidin–APC (APC<sub>av</sub>) by incubation in 0.1 M sodium bicarbonate buffer (pH 8.5) containing 0.15 M NaCl for 1 h at room temperature. Any excess reagents or byproducts of the reaction were removed by passing the mixture through a Sephadex G-25 column (Amersham Biosciences) or performing dialysis against PBS. The average molar ratio of NP per carrier protein was calculated by measuring OD at 280 and 430 nm except in the case of NP–APC or NP–APC<sub>av</sub>. The number of NPs per APC or APC<sub>av</sub> was approximated based on the molar ratio of NP–OSu and APC or APC<sub>av</sub> in the reaction mixture and expressed as NP<sub>av</sub>, NP<sub>med</sub> and NP<sub>max</sub>, respectively. Hybridomas producing the anti-NP antibodies, B2 and E11 were prepared as described previously (16). N1G9 was provided by Prof. K. Rajewsky. The equilibrium association constants (K<sub>a</sub>) of N1G9, B2 and E11 to NP–e-aminocaproic acid, as determined by isothermal titration calorimetry, are 2.9 × 10<sup>5</sup> M<sup>-1</sup>, 3.4 × 10<sup>6</sup> M<sup>-1</sup> and 3.3 × 10<sup>5</sup> M<sup>-1</sup>, respectively (6, 7).

**Immunization**

The experiments using mice were carried out in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of Tokyo University of Science. The protocol was approved by the Committee on the Ethics of Animal Experiments of Tokyo University of Science (Permit Number: S13017). All efforts were made to minimize suffering. Female C57BL/6 mice (Crea) at 6–10 weeks of age were immunized i.p. with 100 μg NP<sub>av</sub>–CGG adsorbed onto 100 μl of alum gel. For analysis of the memory response, mice received a secondary i.p. immunization with 100 μg NP<sub>av</sub>–CGG in PBS at 7 weeks after the primary immunization.

In the present experiments, the process of antibody affinity maturation was tracked by measuring the binding ratio of NP<sub>av</sub>–BSA to NP<sub>26</sub>–BSA (NP<sub>av</sub>/NP<sub>26</sub> ratio) of secreted antibodies by ELISA; by measuring the number of memory B cells, plasmablasts and plasma cells at the same immunization time points by flow cytometry and by analysis of nucleotide sequences of V186.2 genes of isolated cells. We found here that the affinity maturation of anti-NP antibodies proceeds in two steps. The first step begins with a GC reaction through which memory B cells as well as plasmablasts are generated from GC B cells accompanied by a gradual increase in antibody affinity. Plasmablasts are dominant components of ASCs and secrete V186.2–Tyr95–Leu33<sup>+</sup> antibodies having an NP<sub>av</sub>/NP<sub>26</sub> ratio of ~0.7 during primary immunization. The second step begins with secondary immunization, and the appearance of a large number of plasma cells secreting V186.2–Gly95<sup>+</sup> antibodies with multiple SHMs (SHM<sup>+</sup>), which was thought to raise the NP<sub>av</sub>/NP<sub>26</sub> ratio to ~1.0. Surprisingly, V186.2–Gly95–SHM<sup>+</sup> plasma blasts were absent even in the secondary response, suggesting that the repertoire of plasma cells is different from that of plasmablasts. On the basis of these findings together with those reported by Murakami et al. (15), we discuss here the relationship between plasmablasts and plasma cells that appeared after secondary immunization with respect to plasma cell ontogeny. We also suggest that secondary immunization is necessary for inducing differentiation of silent memory B cells that have gained higher affinity as plasma cells over the course of the vaccination schedule.
Spleens were excised at various time points and single-cell suspensions were prepared. In some experiments, bone marrow was aspirated from both femurs.

**Transfectants**

A mouse B-cell lymphoma, K46 and a plasmacytoma, SP2/0, were used for preparing B-cell transfectants expressing NP-specific BCRs and secreting anti-NP antibodies, respectively. The membrane-form immunoglobulin (mlg) μ chain gene was amplified by PCR from cDNA of BALB/c mouse spleen cells using the primers: CH-Xhol-F1, 5′-agtctcgagt-gagagtctcctc-3′ and IgMm-R1, 5′-taagcggccagcggc-gctctgacttacacgaacaggg-3′. V(D)J genes of N1G9, B2 and E11 were obtained by PCR using cDNA from the respective hybridomas and primers: Bud Hs, 5′-aggccgaggccagcagc-gcacgccagcag-3′ and VH-Xhol, 5′-taactcagctgtgagagttggtc-cct-3′. The μc gene was cloned into pCR2.1-TOPO vector, followed by ligation with V(D)J genes. The complete anti-NP mlg μ gene was cloned into pBudCE4.1 vector (Invitrogen). The x1 chain gene was amplified from cDNA of N1G9, B2 and E11, using primers: BudEF-Notl-F, 5′-aggccgaggccagc-gcacgccagcag-3′ and EFLa, 5′-aggccgaggccagcag-3′, and cloned into pBudCE 4.1 vector. These transfectants were selected in the presence of 0.4 mg ml⁻¹ Zeocin. The SP2/0 plasmacytoma secreting anti-NP antibodies, N1G9, B2 and E11 were prepared by transfecting the respective H-chain genes of secreted-form antibodies used for flow cytometry were anti-mouse IgM (sIg) and anti-NP antibodies, N1G9, B2 and E11 were obtained by transfecting the respective H-chain genes of secreted-form antibodies, N1G9, B2 and E11 were obtained by PCR using cDNA from the respective hybridomas and primers: Bud Hs, 5′-aggccgaggccagcagc-gcacgccagcag-3′ and VH-Xhol, 5′-taactcagctgtgagagttggtc-cct-3′. The μc gene was cloned into pCR2.1-TOPO vector, followed by ligation with V(D)J genes. The complete anti-NP mlg μ gene was cloned into pBudCE4.1 vector (Invitrogen). The x1 chain gene was amplified from cDNA of N1G9, B2 and E11, using primers: BudEF-Notl-F, 5′-aggccgaggccagc-gcacgccagcag-3′ and EFLa, 5′-aggccgaggccagcag-3′, and cloned into pBudCE 4.1 vector. These transfectants were selected in the presence of 0.4 mg ml⁻¹ Zeocin. The SP2/0 plasmacytoma secreting anti-NP antibodies, N1G9, B2 and E11 were prepared by transfecting the respective H-chain genes of secreted-form antibodies used for flow cytometry were anti-mouse IgM (sIg) and μ-chain genes. In some experiments, SP2/0 cells transfected with cyan fluorescence protein (CFP) were used.

**Flow cytometry**

Cells were stained on ice for 15 min in a FACS washing solution (PBS + 1% BSA) at 5 × 10⁶ ml⁻¹ with biotin- or fluorophore-conjugated antibodies. The mean fluorescence intensity of the cells was recorded using a FACSCanto II (BD Biosciences). Antibodies used for flow cytometry were anti-mouse IgM (PerCP-Cy5.5-II/41), anti-mouse IgG1 (PE-A85-1), FITC/PE-labeled rat anti-mouse λ chain (Igλ), anti-CD45R/B220 (PE-Cy7-RA3-6B2), biotin-anti-CD138/synedecan (281.12) and PE–streptavidin. The data were analyzed using FlowJo software (Tree Star).

**cDNA synthesis and sequencing**

Cells were sorted using a FACSaria II (BD Biosciences). Individual cells were sorted into 5 μl of an oligo(dT)-primed cDNA reaction mixture containing murine leukemia virus reverse transcriptase with the recommended reverse transcriptase buffer, 0.5mM spermidine, 100 μg ml⁻¹ BSA, 10ng μl⁻¹ oligo(dT), 200 μM each dNTP, 1mM dithiothreitol, 200U ml⁻¹ RNAsin and 100 μg ml⁻¹ Escherichia coli tRNA, in 96-well microtiter plates. Reaction mixtures were incubated at 37°C for 90 min and stored at −80°C until analysis was performed. Twenty-five microliters of reaction mixtures containing 2 μl of cDNA from individual single-cell cDNA preparations were set up using the following reagents: 2U ml⁻¹ Taq polymerase with the recommended 1x reaction buffer, 0.1mM of each dNTP and varying concentrations of primers and MgCl₂. The first PCR was performed for 35 cycles.

**Results**

**Affinity maturation of anti-NP IgG antibodies**

Antigens harboring a different number of hapten groups per carrier protein, such as NP–BSA, are widely employed in estimating the relative affinity. Figure 1(A) shows the binding of control antibodies to NP₂⁻ and NP₂⁻ BSA. Our antibodies bound to NP₂⁻ BSA irrespective of affinity, while the binding to NP₂⁻ BSA was affinity dependent. Therefore, the ratio of binding to NP₂⁻ BSA versus NP₂⁻ BSA (NP/NP₂⁻) provided a useful measure of antibody affinity; N1G9 (V186.2’Tyr95’Trp33’, Kₐ = 2.9 × 10⁹ M⁻¹) had an NP/NP₂⁻ ratio close to 0, B2 (V186.2’Tyr95’Leu33’, Kₐ = 3.4 × 10⁶ M⁻¹) had a ratio of ~0.7 and E11 (V186.2’Gly95’SHM⁶, Kₐ = 9 × 10⁶ M⁻¹) exhibited the highest ratio, ~1.2 (Fig. 1A).

The changes in the amounts of IgG1 antibodies bound to NP₂⁻ and NP₂⁻ BSA as well as the NP/NP₂⁻ ratios are shown in Fig. 1(B). Day 7 antibodies were characterized by little binding to NP₂⁻ BSA and provided an NP/NP₂⁻ ratio <0.1. Antibodies capable of binding to NP₂ appeared on day 14 and their amounts increased with time. The NP/NP₂⁻ ratio reached a plateau value of ~0.7 on day 42. After secondary immunization, antibodies with higher affinities were produced, having NP/NP₂⁻ ratios of ~1. We divided the process of antibody production into three periods based on the magnitude of the NP/NP₂⁻ ratio of the secreted antibodies as indicated in Fig. 1(B); Phase I corresponds to early primary antibody production on day 7, Phase II, to the subsequent period of antibody production accompanied by a gradual increase in affinity between day 14 and 49 and Phase III, to the period during which antibody production is maintained after secondary immunization.

**Establishment of a method for isolating plasma cells secreting anti-NP antibodies**

CD138 molecules on SP2/0 cells were reacted with biotin–anti-CD138, allowing for NP–streptavidin–APC (NP–APCₙ) with different NP valences (NP₂⁻, NP₀⁻) to be conjugated through the biotin–avidin reaction. Biotin–anti-CD138/NP–APCₙ labels CD138⁺ cells with fluorescent chromophore and, simultaneously, provides NP groups to which anti-NP antibodies secreted from autologous CD138⁺ cells can bind. Since the bound antibodies were detected by FITC–anti-Igλ, anti-NP ASCs were detected by flow cytometry as APC⁺/FITC⁺ double-positive cells. We first examined whether the anti-NP antibodies added to the medium could bind to NP–APCₙ via CD138 on SP2/0 cells. All anti-NP antibodies bound to NP₂⁻APCₙ irrespective of their affinities (Fig. 2A), while antibody binding to NP₀⁻APCₙ and NP₂⁻APCₙ was affinity dependent; N1G9 failed to bind to both, while B2 bound to NP₀⁻APCₙ but not to NP₂⁻APCₙ. Only E11 bound to NP₂⁻APCₙ (Fig. 2A).

Next, we examined the binding of antibodies secreted from autologous plasma cells. In order to distinguish between BCRs (mlg) expressed on the surface and antibodies (sig) bound to the anti-CD138/NP–APCₙ complex, we
used anti-Igλ conjugated with different chromophores, FITC or PE. PE–anti-Igλ was added before cultivation to ensure that BCR+ cells were labeled with PE. We used the following cells in our mixture; K46 (IgG2a, κ) as B cells of unknown specificity, K46 transfected with membrane-form anti-NP IgG or IgM as memory B cells with anti-NP specificity, hybrids secreting anti-NP antibodies as plasmablasts, SP2/0-CFP as plasma cells secreting antibodies with unknown specificity (CFP genes were transfected for labeling SP2/0) and SP2/0 transfectants secreting B2 (SP2/0-B2γ) as plasma cells secreting anti-NP antibodies. After independent cultivation of these cells, antibodies secreted from SP2/0-B2γ or B2 hybridoma, which bound to biotin–anti-CD138/NP, were detected with FITC–anti-Igλ (Fig. 2B). The antibodies secreted were trapped by the biotin–anti-CD138/NP complex but not by biotin–anti-CD138/NP–APC, that lacked NP.

Although only cells secreting anti-NP antibodies, B2 hybridoma and SP2/0-B2γ were detected by FITC–anti-Igλ when experiments were carried out using independent cultures (Fig. 2B), we observed antibody binding to all CD138+ cells when a mixture of cells was cultured, suggesting that antibodies secreted from plasma cells diffused during cultivation and bound to biotin–anti-CD138/NP–APC complexes on neighboring plasma cells (data not shown). To prevent this diffusion, we cultured cells in a medium that contained both 1% agarose and 50 ng ml−1 NP–BSA. The former was expected to decrease the diffusion rate of the secreted antibodies (14) and the latter, to absorb the diffused antibodies. When the same numbers of SP2/0-CFP and SP2/0-B2γ were cultured

Fig. 1. Changes in the amount of antibodies bound to NP–BSA or NP0–BSA and in the NP/NP0 ratio after immunization of C57BL/6 mice with NP40–CGG. (A) Binding of control IgG antibodies having different $K_a$ values to NP, N1G9 ($K_a = 2.9 \times 10^5$ M$^{-1}$), B2 ($K_a = 3.4 \times 10^6$ M$^{-1}$) and E11 ($K_a = 9 \times 10^8$ M$^{-1}$) (NP–BSA, open bars and NP0–BSA, closed bars). NP/NP0 ratios are represented by hatched circles. (B) Changes in the binding of IgG1 antibodies in the immune sera. Serum from an individual immunized mouse ($n = 5$) was diluted (20000-fold) and its binding measured by ELISA. Open circles (individual mice) and open bars (averaged values) represent binding to NP–BSA. Closed circles (individual mice) and closed bars (averaged values) represent binding to NP0–BSA. The time course of antibody production was divided into three periods: Phase I, Phase II and Phase III.
Fig. 2. Development of a modified cellular affinity matrix system for isolation of plasma cells. (A) Binding of control antibodies to the biotin–anti-CD138/NP–APC complex on an SP2/0 surface. The complex was pre-formed by adding biotin–anti-CD138 to an SP2/0 cell suspension followed by a biotin–streptavidin reaction with NP–APC having different NP valences, NP$_{\text{lo}}$, NP$_{\text{med}}$ and NP$_{\text{hi}}$. The control antibodies were added to the SP2/0 cell suspension and bound antibodies were titrated by flow cytometry using FITC–anti-Ig$.\lambda$. (B) Binding of secreted antibodies from SP2/0 transfectants to the biotin–anti-CD138/NP–APC complex on an autologous cell surface. Cells were cultured for 30 min and bound antibodies were measured using FITC–anti-Ig$.\lambda$. (C) Specificity of detection of ASCs, B2 and SP2/0-B2γ, with NP$_{\text{hi}}$–APC. Secreted antibodies were detected on the cell surface of ASCs using NP$_{\text{hi}}$–APC but not NP$_{\text{lo}}$–APC, which lacked NP. No difference in binding between NP$_{\text{hi}}$–APC and NP$_{\text{lo}}$–APC was observed in the B-cell lymphoma, K46 and its transfectant, K46B2μ. Discrimination between mIg and sIg was carried out using PE–anti-Ig$\lambda$ and FITC–anti-Ig$\lambda$, respectively. (D) The sIg$\lambda^+$ fraction showed no CFP fluorescence, and conversely, the CFP$^+$ fraction showed no sIg$\lambda^+$ cells (E), indicating that there was little contamination by non-anti-NP antibody-secreting plasma cells in those of the secreting (sIg$\lambda^+$) fraction.
with K46, K46B2μ and B2 hybridomas, ~50% of CD138+ cells were detected as an slgγ− population, indicating that aggrose and NP_{40}–BSA synergistically prevented antibodies from binding to neighboring cells (Fig. 2C). In addition, secreted antibodies were detected only on CFP− cells (SP2/0-B2γ) and not on CFP+ cells (Fig. 2D).

Isolation of ASCs from mice immunized with NP_{40}–CGG

We isolated plasma cells from day 7 mice immunized with NP_{40}–CGG, using biotin–anti-CD138/NP–APC_{av}. We then carried out detection of the bound antibodies with FITC–anti-Igλ, as described above. B cells and plasmablasts were sorted into NP_{av}B220−CD138−Igλ (PE)+ cells and NP_{av}B220−CD138−Igλ (PE)+ cells, respectively (Fig. 3A). Plasmablasts were also characterized as NP_{av}−APC-binding (NP+) CD138+ cells (Fig. 3B). On the other hand, plasma cells were separated as CD138−Igλ (FITC)+ cells and were characterized by their large size, dull expression of B220 and the absence of BCR expression (Fig. 3B), phenotypes which were consistent with those reported previously (17–20). RT–PCR analysis of cDNA revealed that mRNA encoding mlg was not expressed in plasma cells, in contrast to the significant expression of mRNA encoding slg (Fig. 3C). Although both plasmablasts and plasma cells contained mRNA encoding slg, mRNA encoding mlg was detected only in the former. On the other hand, B cells (represented by NP_{av}B220−CD138− cells) synthesized only a small amount of mRNA encoding slg (21).

Variation in the numbers of ASCs and B cells during immunization

Figure 4 shows the variation in the numbers of plasmablasts, plasma cells and B cells with time after immunization with NP_{40}–CGG. A large number of NP_{av}–APC_{av}-binding IgG1+ plasmablasts were present on day 7 in spleen (~5 × 10^5, Fig. 4A) and bone marrow (~1 × 10^6, Fig. 4C). Plasma cells were observed in spleen on day 7 (~1 × 10^4, Fig. 4B) but not in bone marrow (Fig. 4D). Therefore, the ratio of plasma cells to total ASCs at these time points was <0.02. Judging from the small number of plasma cells compared to plasmablasts, the former seemed to provide a rather small contribution to

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**Fig. 3.** Isolation of NP-specific B cells as well as plasmablasts (PBs) or plasma cells (PCs) secreting anti-NP antibodies from mice immunized with NP_{av}–CGG. (A) NP-specific B cells and PBs were isolated using NP_{av}−APC, which bound to their BCRs on the cell surface. B cells were then separated as B220−CD138− cells and PBs, as B220−CD138+ cells. (B) PCs secreting anti-NP antibodies were isolated as CD138+ mIgλ− cells, while PBs were distinguished as NP-binding mIgλ+ cells. (C) RT–PCR analysis of mRNAs encoding the membrane-form (mIgγ) or secreted-form (slgγ) of γ-chains obtained from memory B cells, PBs and PCs, respectively. β-Actin was used as a control.
Fig. 4. Appearance of plasmablasts, plasma cells and memory B cells in spleen and bone marrow (BM) with time post-immunization with NP$_{40}$-CGG. (A) Changes in the numbers of plasmablasts secreting anti-NP IgG antibodies at the early stage of immunization. (B) Variation in the number of IgG$^+$ plasma cells in spleen. (C) Changes in the number of IgG$^+$ plasmablasts in BM. (D) Changes in the number of IgG$^+$ plasma cells in bone marrow. (E) NP$_{med}$/NP$_{hi}$ ratio of plasmablasts and memory B cells. (F) Changes in the number of IgG$^+$ memory B cells in spleen.
primary IgG1 antibody production. The absence of plasma cells in bone marrow on day 7 suggested that they were generated in spleen but did not migrate into bone marrow immediately.

The number of plasmablasts and plasma cells in spleen had decreased to ~1/10 by day 14, suggesting that these cells were short-lived (Fig. 4A and B). They were further decreased by day 28 and their numbers remained the same thereafter. The decrease in these cells on day 14 was not seen in bone marrow (Fig. 4C and D) (22). We also examined the number of plasmablasts capable of binding to NP<sup>med</sup>–APC<sub>avi</sub> (Fig. 4A and C). A certain fraction of NP<sup>med</sup>–APC<sub>avi</sub>-binding cells were present in spleen and bone marrow. The ratio of NP<sup>med</sup>–APC<sub>avi</sub>-binding cells relative to NP<sup>hi</sup>–APC<sub>avi</sub> binding cells (NP<sup>med</sup>/NP<sup>hi</sup> ratio) also increased with time, indicating that affinity maturation had proceeded in plasmablasts (Fig. 4E), although the number of plasma cells was too small to allow for an accurate estimation of the NP<sup>med</sup>/NP<sup>hi</sup> ratio (data not shown). On the other hand, memory B cells exhibited a rather small change in their number compared with ASCs (Fig. 4F). On day 42, at a time point when primary immunization had reached a steady state, there were ~4.5 x 10<sup>3</sup> memory B cells but only ~1.2 x 10<sup>3</sup> plasmablasts and ~0.1 x 10<sup>3</sup> plasma cells, suggesting that more memory B cells were generated compared with ASCs during Phase II. Plasma cells occupied only 6–7% of the ASC compartment, and it is not yet clear whether the antibodies have a similar repertoire to that of plasmablasts. It is also not clear whether such small numbers of plasma cells are derived from plasmablasts.

Secondary immunization on day 49 caused a remarkable increase in the cell number on day 56, especially in the number of plasma cells (Table 1). The ratio of cell numbers on day 56 versus day 42 was 20 in spleen and 19 in bone marrow for plasmablasts, while that of plasma cells was 91 in spleen and 19 in bone marrow. Since plasma cells were unable to proliferate in response to antigen stimulation, they may have differentiated either from memory B cells or plasmablasts, although no information was available regarding whether plasmablasts are capable of responding to antigen stimulation (23, 24). Regardless of their origin, this selective expansion of plasma cells brought their percentage in total ASCs to 26%, much higher than the 7% seen with primary immunization on day 42, suggesting that plasma cells contributed significantly to antibody production after secondary immunization. In contrast to the rather large increase in the number of ASCs, B cells showed a small change in their number following secondary immunization (Fig. 4F).

**Frequency of SHM in antibody V<sub>H</sub> regions secreted from plasmablasts and BCRs of memory B cells with time post-immunization**

We examined the frequency of SHM in antibodies secreted from plasmablasts and in BCRs of memory B cells. SHM was not observed in antibodies on day 7 but became apparent on day 14, and the average frequency increased with time (Fig. 5). These profiles of SHM induction were similar to those of BCRs of memory B cells and GC B cells (10). In fact, SHMs had accumulated to a similar extent in the plasmablast and memory B cell compartment on day 42. V<sub>H</sub> gene sequence analysis showed that V<sub>H</sub> regions of both memory B cells and plasmablasts were predominantly encoded by V186.2-Tyr95-Leu33 sequences and those encoded by V186.2-Gly95 sequences were observed only in memory B cells and not in plasmablasts. Although we attempted to analyze V<sub>H</sub> gene sequences of the plasma cells, only preliminary sequence data were obtained and were insufficient for statistical analysis because of the low numbers of cells recovered (data not shown).

**Characterization of ASCs responsible for recall antibody production**

V<sub>H</sub> gene sequence analysis revealed that there was no significant difference in the frequency of SHM between ASCs on day 42 and 56, or among memory B cells, plasmablasts and plasma cells. These results suggested that additional SHMs would not be induced by secondary immunization. Analysis focusing on the amino acid residue at position 95 also showed that little change in the repertoire of memory B cells occurred with secondary immunization, which consisted largely of components encoded by V186.2-Tyr95-Leu33 sequences in addition to those encoded by V186.2-Gly95 sequences, suggesting that the immunological memory was maintained without drastic changes in the repertoire brought on by boost immunization. Alteration in the repertoire was also not observed in antibodies secreted from plasmablasts; these antibodies were encoded by V186.2-Tyr95-Leu33 sequences and the components encoded by V186.2-Gly95-SHM<sup>+</sup> sequences were absent. On the other hand, the repertoire of plasma cells was different, i.e. components secreting V186.2-Gly95-SHM<sup>+</sup>

**Table 1.** Cell number of each subset at indicated time points

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<td>Plasmablasts</td>
<td>337 (±93.1)</td>
<td>4.64 (±4.2)</td>
<td>1.12 (±1.05)</td>
<td>0.64 (±0.83)</td>
<td>12.9 (±10.7)</td>
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<td>Plasma cells</td>
<td>8.92 (±4.2)</td>
<td>0.69 (±4.2)</td>
<td>0.07 (±0.06)</td>
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<td>4.55 (±3.56)</td>
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<td>Memory B cells</td>
<td>23.3 (±10.8)</td>
<td>5.91 (±3.02)</td>
<td>5.01 (±4.37)</td>
<td>4.5 (±1.35)</td>
<td>18.8 (±3.32)</td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmablasts</td>
<td>1.7 (±0.46)</td>
<td>1.13 (±0.52)</td>
<td>0.8 (±0.3)</td>
<td>0.66 (±0.41)</td>
<td>12.3 (±4.11)</td>
<td></td>
</tr>
<tr>
<td>Plasma cells</td>
<td>n/d</td>
<td>0.13 (±0.11)</td>
<td>0.11 (±0.1)</td>
<td>0.21 (±0.14)</td>
<td>3.97 (±3.08)</td>
<td></td>
</tr>
</tbody>
</table>

Cell number (x10<sup>5</sup>) (±SD). BM, bone marrow; n/d, not detected.
antibodies became predominant. These results suggested that V186.2$^+$Gly95$^+$SHM$^+$memory B cells that had gained high affinity differentiated into plasma cells that were responsible for affinity maturation at the late stage of immunization.

**Discussion**

Comparison of the $V_{\text{H}}$ repertoires of plasma cells and plasmablasts or memory B cells at the same time points of immunization deepened our knowledge of the relationships between these cells in the developmental pathway as well as of the mechanism of antibody affinity maturation at the cellular level. To achieve this understanding, we first established experimental conditions for isolating plasma cells using a method based on affinity matrix technology (18). We employed CD138 as an absolute marker of ASCs and labeled these cells using biotin–anti-CD138 and NP–APC$_{avi}$. With 30-min cultivation in the presence of 50 ng ml$^{-1}$ NP$_{13}$–BSA and 1% agarose, anti-NP antibodies secreted from autologous plasma cells bound preferentially to the biotin–anti-CD138/NP–APC$_{avi}$ complex. Plasma cells were discriminated from plasmablasts on the basis of the expression of BCRs on the cell surface. The number of ASCs thus obtained in the present experiments was in good agreement with those reported previously (24).

We tracked antibody affinity changes using the NP$_2$/NP$_{26}$ ratio as a measure of affinity, which was found to increase in a stepwise manner (Fig. 1B). Accordingly, we divided the time course of antibody production into three periods, Phases I, II and III. Day 7 IgG antibodies had an NP$_2$/NP$_{26}$ ratio of <0.1 (Fig. 1B) and were known to be encoded largely by the germline V186.2$^+$Tyr95$^+$Trp33$^+$ sequences (17, 19). Since antibodies having an NP$_2$/NP$_{26}$ ratio of <0.1 appeared only on day 7 but not later, the ASCs responsible for their production would be short-lived. In fact, a large number of plasmablasts appeared on day 7 in spleen but their incidence declined to 1/20–1/50 by day 14. A similar decline in the number of ASCs in bone

![Figure 5](image_url)
marrow was not evident, suggesting that they had achieved longevity (22). The ratio of plasma cells versus plasmablasts was ~0.07, which is in agreement with results reported by Smith et al. (17) who showed that 90% of ASCs were capable of binding to antigens. Therefore, at Phase I, only a small fraction of the ASC compartment was occupied by plasma cells. If we assume that plasmablasts are intermediates of plasma cell ontogeny, most of them seem to remain undifferentiated into the final stage of development.

The antibodies secreted from short-lived plasmablasts were substituted by those capable of binding to NP–BSA after day 14. Phase II corresponds to the duration of GC activity and induction of SHM in GC B cells which then differentiate into either memory B cells or plasmablasts. Since GC B cells at this time point consisted largely of V186.2-Tyr95* cells, they were able to raise affinity promptly by a single amino acid substitution of Trp33 (Trp33* to Leu (Leu33*) (5, 6). Simultaneous to the increase in the NP/NPNP ratio, SHMs began to accumulate in plasmablasts (Fig. 5A and B). The NP/NPNP ratio reached a plateau value of ~0.7 on day 42, which was similar to the value of V186.2-Tyr95*Leu33* antibodies such as B2 (Fig. 1A). On day 42 when the primary immune response had reached a steady state, plasmablasts occupied 93% of the ASC compartment (Table 1) and 65% of them secreted V186.2-Tyr95*Leu33* antibodies (Fig. 5D).

From these results, we concluded that the gradual increase in the NP/NPNP ratio up to ~0.7 during Phase II reflected the increase in the fraction of V186.2-Tyr95*Leu33* plasmablasts. In addition, the fraction of plasma cells in the ASC compartment on days 7 and 42 was as low as ~8%, suggesting plasma cells are not generated in the primary response.

Antibody affinity maturation at Phase II was demonstrated by the appearance of V186.2-Tyr95*Leu33* plasmablasts and the essential lack of contribution from V186.2-Gly95* cells, although V186.2-Gly95* memory B cells were observed at this time point. Since Nishimura et al. (10) showed that V186.2-Gly95* B cells were rarely observed during the primary response, we speculated that V186.2-Gly95* GC B cells differentiated into plasmablasts but were too rare to be detected. It should be noted that the GC reaction generated 3-fold more memory B cells, consisting of both V186.2-Tyr95* and V186.2-Gly95* cells, than ASCs (Table 1), suggesting that Phase II represents the period in which immunological memory is established.

Although the NP/NPNP ratio reached a plateau on day 42, secondary immunization induced a further increase in the ratio to ~1.0 (Fig. 1B). Since a mutation that brings a further increase in the affinity of V186.2-Tyr95*Leu33* antibodies has not yet been found (25), V186.2-Gly95*SHM* antibodies were considered to be candidates responsible for this increase in the NP/NPNP ratio. In fact, secondary immunization induced a large number of plasma cells of which 65% secreted V186.2-Gly95*SHM* antibodies (Fig. 6D). These plasma cells are expected to have differentiated from memory B cells or plasmablasts. However, Murakami et al. (15) showed that hardly any V186.2-Tyr95* antibodies were converted to V186.2-Gly95* antibodies by SHM; not only replacement of Tyr95 to Gly but also shortening of the CDR3H region were necessary for the conversion of Tyr95 antibody to Gly95 antibody. They also showed that V186.2-Tyr95* and V186.2-Gly95* antibodies employed different strategies for affinity maturation; the mutation of Trp33Leu contributed to an increase in the affinity of the former while it had a negative effect on that of the latter. Therefore, we considered that the V186.2-Gly95*SHM* plasma cells that appeared after secondary immunization were not derived from V186.2-Tyr95*Leu33* plasmablasts but from V186.2-Gly95*SHM* memory B cells via V186.2-Gly95*SHM* plasmablasts, although the latter plasmablasts were rarely observed.

Plasma cell ontogeny is considered to consist of the processes of plasmablast and plasma cell differentiation. We suppose that each process depends on the BCR affinity of memory B cells. The plasma cell compartment generated after secondary immunization consisted of ~30% V186.2-Tyr95*Leu33* and ~65% V186.2-Gly95*SHM* cells (Fig. 6D), and therefore contained only affinity-maturated cells. This is contrast to the plasmablast compartment that contained V186.2-Tyr95*Trp33* cells that had immature affinity (Fig. 6D). These results suggest that the affinity threshold required for differentiation of plasmablasts into plasma cells is higher than that of memory B-cell differentiation into plasmablasts. After secondary immunization, memory B cells differentiate into plasmablasts and only a portion of V186.2-Tyr95*Leu33* plasmablasts would be converted into plasma cells because their affinities would hardly reach the threshold and the majority would continue to reside in the plasmablast compartment. On the other hand, V186.2-Gly95*SHM* plasmablasts would differentiate promptly into plasma cells because of their high affinities and did not remain in the plasmablast stage. Therefore, the absence of V186.2-Gly95*SHM* cells in the plasmablast compartment and the abundance of V186.2-Gly95*SHM* cells in the plasma cell compartment can be explained in terms of the high affinity of V186.2-Gly95*SHM* memory B cells that tend to differentiate into plasma cells (25).

Memory B cells that have completed affinity maturation during primary immunization either remain memory B cells or become plasmablasts or plasma cells after secondary immunization. Since no significant difference was observed between the memory B-cell repertoire before and after secondary immunization, selective expansion of particular components did not occur on antigen stimulation, and ‘memory’ was preserved after proliferation. In view of the longevity of memory B cells and their limited variation in number during immunization, the role of these cells would appear to be to preserve and transmit their complete ‘memory’ to ASCs and daughter cells. On the other hand, the quantity and quality of antibodies in blood were regulated by a change in the numbers of plasmablasts and plasma cells which were promptly generated from the appropriate memory B cells when necessary; V186.2-Tyr95*Leu33* memory B cells differentiated into plasmablasts and V186.2-Gly95*SHM* cells, into plasma cells. Plasmablasts and plasma cells appear to have different roles in antibody production; the former are responsible for the prompt secretion of antibodies, while the latter act to secrete antibodies with maturated affinity after secondary immunization.

In conclusion, we showed here the functional roles of memory B cells, plasmablasts and plasma cells in the immune response to NP–CGG. In particular, we showed that secondary immunization is essential for memory B cells that require
a long period for affinity maturation in order to differentiate into plasma cells. Together with the dynamic changes in antibody affinities reported in other immune systems (26–29), the observations obtained from the NP hapten system used here are expected to help in understanding the immune response and to contribute to the establishment of effective vaccination schedules.

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Fig. 6. Comparison of the frequency of SHM (A) and the V, repertoire (B) among memory B cells, plasmablasts and plasma cells 7 days after secondary immunization, corresponding to 56 days after primary immunization. Data are taken from three independent experiments using two mice per experiment.


