

Affinity maturation of secreted IgM pentamers on B cells

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Abstract

We prepared a series of hapten-BSA conjugates with varying ratios of biotin to measure ligand–receptor interactions on B cells by flow cytometry using avidin for detection. Surface plasmon resonance measurements of the interaction with a monoclonal anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibody suggested that NP₅-BSA or NP₇-BSA harboring 29 or 23 biotin molecules (NP₅-BSA-bio₂₉ or NP₇-BSA-bio₂₃) would be suitably sensitive for flow cytometric analysis. By using NP-BSA-bio, we analyzed NP-binding cells in immunized mice. Unexpectedly, 30–40% of spleen cells expressing IgM could bind to NP₅-BSA or NP₇-BSA after immunization of mice with NP₄₀-chicken γ -globulin. The proteins binding to NP₇-BSA-bio₂₃ on the cell surface were analyzed by immunoprecipitation and western blotting. Surprisingly, most of the proteins binding NP-BSA-bio on the cell surface were not the membrane form of IgM monomer, but a secreted IgM pentamer. It is likely that the IgM pentamer bound through Fc receptors for polymeric IgA or IgM and contributed to antigen binding. Comparison of the binding ratio of NP_{0,9}-BSA:NP₅-BSA between B cells of primary and secondary immunization suggested that the affinity of IgM matured during immunization.

Introduction

Hapten-conjugated proteins have long been used as T cell-dependent antigens for studies on the immune response, especially in the investigation of antibody affinity maturation (1–7). Although these studies have employed various conjugates which differed in the number of haptens or in the size of carrier proteins, such as ovalbumin, chicken serum albumin, chicken γ -globulin (CGG) or keyhole limpet hemocyanin, little is known about the effects of these factors on the magnitude of antibody production or memory cell formation during the immune response. Recently, we have examined these issues by means of surface plasmon resonance (SPR) measurements and found that the apparent affinity (avidity) largely depended on both the valence of the haptens and the size of the carrier proteins (8). The valence of haptens in particular would be expected to affect the magnitude of

signals through BCR, since the retention time or occupancy of the bound antigens as well as the degree of BCR cross-linking depend largely on hapten valence (9,10). Differences in the signal strength of antigens with different hapten valences would alter the quality as well as the intensity of the immune response.

It has been well established that after stimulation with T cell-dependent antigens, B cells within the periarteriolar lymphoid sheaths enter the follicles, and acquire a centroblast and centrocyte phenotype in the germinal center (11–16). Somatic hypermutation (SHM) takes place through these B cell stages and B cells with mutated BCR are continuously selected on the basis of antigen specificity as well as affinity. It was shown that some of these selected B cells move into the bone marrow (BM) and remain there as long-lived plasma cells (17,18).

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Memory B cells were characterized as having higher affinity than naive B cells due to SHM and selection by antigens (19,20). BCR of these cells were sometimes believed to have undergone class switching to isotypes other than IgM (21,22). Therefore, in previous studies, memory B cells were characterized as hapten-binding, IgM⁻IgG1⁺CD38⁻ cells. However, isotype switching was not necessarily required for establishing memory B cells (23,24). Therefore, increased affinity as a result of SHM and antigen selection would provide the most reliable condition for memory B cell generation.

Although the affinity maturation of secreted IgG has been shown by ELISA (25), it is rather difficult to measure the affinity of BCR on the cell surface. We attempted to develop a method for measuring the relative affinity/avidity of BCR by flow cytometry by employing a series of (4-hydroxy-3-nitrophenyl)-acetyl (NP)-BSA-biotin conjugates (NP-BSA-bio) with various valences of NP and biotin. The specificity of the binding of these conjugates to anti-NP mAb and the sensitivity of detection of NP-BSA-bio bound to BCR by streptavidin (SA) were tested with the BIAcore system, which utilizes SPR as a means of detection, as well as with flow cytometry using K46 μ , a B cell transfectant with a BCR of anti-NP binding specificity (26).

Through the use of NP-BSA-bio in combination with SA-phycoerythrin (PE), we studied the binding of NP-BSA to B cells obtained from mice immunized with NP-CGG of various NP valences. Unexpectedly, we observed that a massive number of B cells capable of binding with NP₅-BSA appeared transiently with a peak at around day 7 following primary immunization with NP₄₀-CGG or NP₁₃-CGG. By analyzing the proteins responsible for this phenomenon, it became evident that a secreted form of IgM pentamer and not the membrane form of IgM, i.e. BCR, was involved in the NP binding. By comparing the binding activity to NP_{0.9}-BSA-bio and NP₅-BSA-bio, the affinity maturation of IgM on B cells was investigated.

Methods

Reagents

NP was prepared from 4-hydroxyphenylacetic acid as described previously (27). The hydroxysuccinimide ester of NP was prepared as described (28). 2,4,6-Trinitrobenzenesulfonic acid (Wako, Tokyo, Japan) and ethoxymethylene-2-phenyl-2-oxazolin-5-one (Sigma-Aldrich, St Louis, MO) were purchased for use in preparing trinitrophenyl (TNP)- or phenyloxazolone (phOx)-conjugated proteins respectively. For preparing NP-BSA-bio and phOx-BSA-bio, biotin was first conjugated to BSA with NHS-biotin and this was followed by haptenylation with the respective reagents. The number of conjugated biotin molecules was estimated using HABA reagents (Pierce, Rockville, MD) after treatment with pronase for 16 h according to the manufacturer's instructions. The numbers of hapten moieties introduced were determined spectrophotometrically using the molar extinction coefficients of NP (4.23×10^3 at 430 nm) and phOx (3.2×10^4 at 348 nm) (29). Protein concentrations were estimated using the BCA assay (Pierce).

Cell lines

The K46 μ lymphoma line (26) was kindly provided by Dr J. C. Cambier (Denver, CO), and cultured in Iscove's modified Dulbecco's medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM glutamine and 50 μ M β -mercaptoethanol. A hybridoma secreting the anti-NP mAb, N1G9, was donated by Dr K. Rajewsky (30,31).

SPR measurements

The BIAcore biosensor system, BIAcore 2000 (BIAcore, Uppsala, Sweden), was used to measure real-time interactions between NP-CGG or NP-BSA-bio and the anti-NP mAb, N1G9, that was bound to 6000 RU of rabbit anti-mouse IgG Fc (Pierce) coupled to a CM5 sensor chip. An isotype-matched mAb was used to discount non-specific binding.

Mice and immunization

Mice were purchased from KWL (Wakayama, Japan) or SLC (Shizuoka, Japan) and housed in our institutional animal facility. The experiments were carried out under the Guidelines for Animal Protocols of our institution. Groups of C57BL/6 mice were immunized with a single i.p. injection of 100 μ g of NP₄, NP₁₃, NP₄₀ or phOX₃₅-TNP₄₀-CGG with alum. A booster immunization was carried out using 100 μ g NP₄₀-CGG in PBS. Splenocytes were isolated as previously described (32).

Flow cytometric analysis

FITC-anti-IgM (R6-60.2 or II-41), -Ig κ (187.1) and -IgM^a (DS-1), biotin-anti-Ig λ (R26-46) and -IgM^b (AF6-78), and SA-PE were purchased from BD Bioscience Pharmingen (San Diego, CA). FITC-goat anti-mouse IgM was purchased from Southern Biotechnology Associates (Birmingham, AL). Splenocytes were stained with 1 μ g/ml of NP-BSA-bio or phOx-BSA-bio in the combination with FITC-anti-IgM, followed by SA-PE staining. To minimize non-specific staining, anti-Fc γ RII-specific mAb, 2.4G2, were added. For pre-incubation with sera, spleen cells from non-immunized mice were incubated with serum for 30 min on ice followed by washing and staining with NP-BSA-bio and FITC-anti-IgM. Data were acquired by a FACSCalibur or a FACSVantage and analyzed with CellQuest software (BD Bioscience, Mountain View, CA). Lymphocytes were gated for each figure.

ELISA

Seven days after immunization with NP₄-CGG, NP₁₃-CGG, or NP₄₀-CGG with alum, antisera were collected from the immunized mice. Mice were then sacrificed and tissue samples were obtained. For measuring anti-NP antibody production, plastic plates coated with NP₉-BSA were used. Peroxidase-conjugated goat anti-mouse IgM or IgG (Southern Biotechnology Associates) was used for detection of bound antibodies. Antisera from hyper-immunized mice were used as standard.

Single-cell sorting and RT-PCR

Spleen cells of normal or immunized mice were stained with FITC-anti-Ig κ plus biotin-anti-Ig λ followed by SA-PE. Single cells were sorted on a FACSVantage into a 96-well plate containing RT buffer. cDNA was synthesized by SuperScript II reverse transcriptase with oligo-dT primer (Invitrogen,

Carlsbad, CA). Ig λ 1 cDNA was amplified with primers specific to V λ (5'-ATGGC CTGGA TTCA CTTAT ACTCT CTC) and C λ 1 (5'-CTTTC CCATG CTCTT GCTGT CAGG). A small aliquot of amplified cDNA was re-amplified with internal primers specific to J λ 1 (5'-GTGTT CGGTG GAGGA ACCAA ACTG) and C λ 1 (5'-GTTGC TCTGT TTGGA AGGCT GGGT). Ig λ 2 and Ig λ 3 messages were amplified using the same sets of primers, which were V λ and C λ 2 and 3 (5'-TGAGA TCTCC ACTGG TCCGA TGTC) for the first round of PCR, and J λ 2 and 3 (5'-RTTTT CGGCR GTGGA ACCAA GGTC) and internal C λ 2 and 3 (5'-GTTGY CCTCT TTGGT GGGAT TTGA) for the second round. V κ cDNA was amplified using the universal V κ primer (5'-GGCTG CAGST TCAGT GGCAG TGGRT CWGGR AC) (33) in combination with a C κ primer (5'-GTCGT TCATA CTCGT CCTTG GTCAA C). The second round of PCR was performed using a mixture of J κ primers (J κ 1: 5'-GTTTCG GTGGA GGCAC CAAGC TGGA; J κ 2: 5'-GTTTCG GAGGG GGGAC CAAGC TGA; J κ 4: 5'-GTTTCG GCTCG GGGAC AAAGT TGGA; J κ 5: 5'-GTTTCG GTGCT GGGAC CAAGC TGGA) with an internal C κ primer (5'-GTTCA GGACG CCATT TTGTC GTTCA C). The PCR products were analyzed by agarose-gel electrophoresis and stained with ethidium bromide. In order to determine the V–J junctional sequences, amplified cDNA was re-amplified with V L and internal C L primers, and amplified fragments were sequenced by Beckman Coulter (Fullerton, CA) ACE2000-XLE DNA sequencer.

Western blotting

One week after immunization with NP₄₀-CGG/alum or 5 days after booster immunization, cell suspensions were prepared from spleen cells of normal or immunized mice. Cells were incubated with 1 μ g/ml of NP₇-BSA-bio₂₃ in PBS containing 1% BSA. After washing the unbound NP-BSA-bio, cells were suspended in lysis buffer for 30 min on ice. The lysis buffer contained 1% NP-40, 50mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM PMSF, aprotinin, leupeptin and pepstatin A. The detergent-soluble fraction was collected after centrifugation and proteins bound to NP₇-BSA-bio₂₃ were precipitated with SA–agarose beads (Pierce). The serum was prepared and reacted with NP₇-BSA-bio₂₃ in the lysis buffer and antibodies bound to NP-BSA-bio were precipitated with SA–agarose. The pellet was suspended in sample loading buffer containing 20 mM Tris–HCl (pH 7.0), 30% glycerol, 1% SDS and bromophenol blue. In order to prepare the total-cell lysate, cells were incubated in the lysis buffer, and the detergent soluble fraction was collected and diluted in the sample loading buffer. Samples were subjected to 3% polyacrylamide–0.5% agarose gel electrophoresis (34) and the protein was transferred to a PVDF membrane (Millipore, Bedford, MA). The blot was probed with peroxidase-labeled goat anti-mouse IgM or IgG, followed by detection with ECL reagent (Pharmacia, Piscataway, NJ).

Results

Characterization of antigens for immunization and binding assays

In this study, CGG and BSA were used as carrier proteins to which hapten and/or biotin were conjugated. Average

numbers of hapten or biotin molecules per carrier protein are expressed with subscripts (e.g. NP₅-BSA-bio₂₉ or NP₄₀-CGG).

NP₄-CGG, NP₁₃-CGG, and NP₄₀-CGG were used as immunogens, and binding of these to a primary anti-NP mAb, N1G9, was examined by SPR measurement. Since this mAb was shown to have an affinity to NP-Cap of 5×10^5 M⁻¹, and to lack SHM in V_H and V_L regions (30,31), it was considered to be useful as a model of BCR on naive B cells. N1G9 was immobilized on BIACore sensor chips through rabbit anti-mouse IgG Fc antibodies and the interactions of NP-CGG with this chip-coupled mAb were used to mimic antigen–BCR interactions on the cell surface. The results were analyzed using Scatchard plots (Fig. 1A). Since the binding of NP-CGG to N1G9 was characterized in terms of multivalent–divalent interactions, the plots would not be expected to fall in a linear fashion. However, within a limited range of NP-CGG concentrations, the plots generate straight lines and the apparent association constant (K_a) estimated from the slopes was 1.4×10^7 M⁻¹ for NP₄-CGG, 9.3×10^7 M⁻¹ for NP₁₃-CGG and 2.4×10^8 M⁻¹ for NP₄₀-CGG. It is clear that the binding of NP-CGG increased with an increase in the NP valence from 5 to 40.

We used NP-BSA for the binding analyses of antigen–BCR interactions on the cell surface. For these analyses, we needed to employ the biotin–SA system for the detection by flow cytometry. First, we analyzed the interaction of N1G9 with biotinylated NP-BSA (NP-BSA-bio) by SPR measurement in order to examine the effects of NP valence as well as biotinylation on the interaction (Fig. 1B). NP_{0.9}-BSA-bio₂₉, NP_{1.6}-BSA-bio₁₄ and NP_{2.3}-BSA-bio_{1.8} showed marginal binding ($K_a < 1 \times 10^6$ M⁻¹), while NP-BSA-bio with an NP valence >5, i.e. NP₅-BSA-bio₂₉, NP_{8.4}-BSA-bio₁₄ and NP₁₁-BSA-bio_{1.8}, bound to N1G9 efficiently with K_a values of 1.6×10^7 , 4.2×10^7 and 6.3×10^7 M⁻¹ respectively. Since similar NP valence-dependent binding was observed using NP-BSA with no biotinylation (8), it was concluded that NP₅-BSA bound to BCR with high avidity and an affinity as low as 10^5 M⁻¹, whereas NP_{0.9}-BSA did not, and that biotinylation provided little inhibition of the antigen–antibody interactions. Next, we examined the interaction between SA and NP-BSA-bio in the chip-coupled antigen–antibody complex (Fig. 1C). Considering the high affinity between biotin and SA, it was rather surprising that the sensitivity depended largely on the number of biotin moieties. The binding of SA to NP_{2.3}-BSA-bio_{1.8}, NP₁₁-BSA-bio_{1.8}, NP_{1.6}-BSA-bio₁₄ or NP_{8.4}-BSA-bio₁₄ was inefficient compared with the binding to NP_{0.9}-BSA-bio₂₉ or NP₅-BSA-bio₂₉.

The binding of NP-BSA-bio to BCR was examined using a B cell transfectant, K46 μ , which expresses B1-8 μ chains and λ 1 chains, and possesses anti-NP binding activity (35). Since the amino acid sequences of V_H and V_L regions of N1G9 and B1-8 are identical (30,36), binding data on N1G9 obtained from SPR measurements can be applied to those of K46 μ . As expected from the SPR results, essentially no binding was detected with either NP_{2.3}-BSA-bio_{1.8} or NP₁₁-BSA-bio_{1.8} (Fig. 2). An increase in the number of biotin molecules to 14 (NP_{8.4}-BSA-bio₁₄) improved the binding significantly. Finally, we found that NP-BSA with 23–29 biotin molecules had maximum sensitivity for K46 μ , but showed little non-specific binding to the parent K46 cells (data not shown) and,

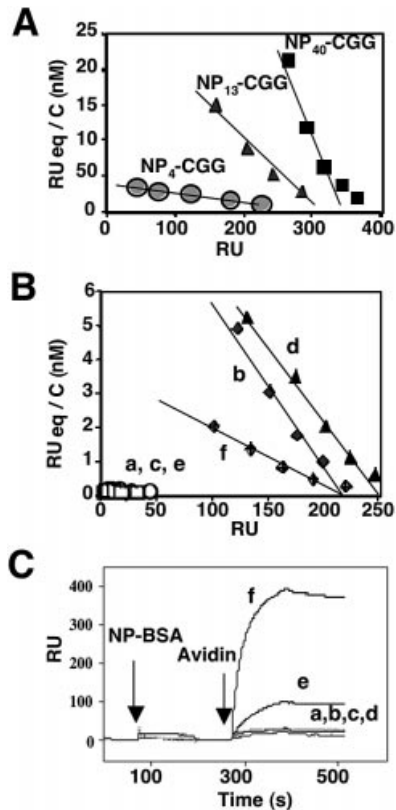


Fig. 1. Binding of NP-CGG or NP-BSA-bio to the NP-specific mAb, N1G9 (A and B), and binding of SA to NP-BSA-bio in the antigen-antibody complex (C). Scatchard plots for interactions with NP-CGG (A) and NP-BSA-bio (B) to N1G9 immobilized on the chips through rabbit anti-mouse IgG Fc. The apparent association constants were estimated from the slope of the plots. For analysis of the interaction between SA and NP-BSA-bio in the antigen-antibody complexes in (C), the initiation point of the reaction of 500 nM NP-BSA-bio with N1G9 on the chip is shown by an arrow. After washing with buffer, 500 nM SA was added to react with NP-BSA-bio in the pre-formed antigen-antibody complex at the point indicated by the arrow. Non-specific binding of SA has been removed from the sensorgrams. The binding of NP₅-CGG, NP₁₃-CGG, and NP₄₀-CGG are analyzed in (A), and binding of NP_{2.3}-BSA-bio_{1.8} (a), NP₁₁-BSA-bio_{1.8} (b), NP_{1.6}-BSA-bio₁₄ (c), NP_{8.4}-BSA-bio₁₄ (d), NP_{0.9}-BSA-bio₂₉ (e) and NP₅-BSA-bio₂₉ (f) are shown in (B) and (C).

therefore, used these higher-valence conjugates for further flow cytometry analysis of antigen-BCR interactions on B cells.

It has been shown previously that the binding ratio of antibodies to low and high hapten number conjugates, as determined by ELISA, is a measure of antibody affinity (25). Results of SPR measurement also provided information on the relative affinity of sensor chip-immobilized antibodies. As shown in Fig. 1(C), N1G9 exhibited an RU of ~60 in the binding of NP_{0.9}-BSA-bio₂₉ and an RU of ~350 in binding to NP₅-BSA-bio₂₉. In this case, the ratio of binding of NP_{0.9}-BSA-bio₂₉ and NP₅-BSA-bio₂₉ was ~0.2. Therefore, the primary immature antibodies are likely to have a ratio of ~0.2. The mature mAb, C6, however, had a value of close to 1 (data not shown). The relative affinity of BCR on the cell surface was examined by flow cytometry (Fig. 2). In the case of BCR on K46 μ , the ratio of binding to NP_{0.9}-BSA-bio₂₉ and NP₅-BSA-bio₂₉ was 0.4–0.5.

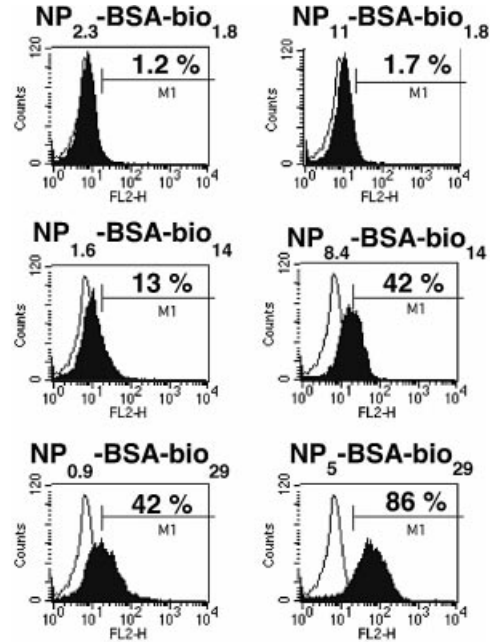


Fig. 2. Flow cytometric profiles for the binding of NP-BSA-bio to BCR on K46 μ . The percentages of NP-BSA-bio binding cells in the total cell population were compared. NP-BSA-bio used for staining is indicated for each figure. Negative control was stained with BSA-bio₂₉.

Although this value was higher than that for N1G9 from results obtained from SPR measurements, we considered that the binding ratio to NP_{0.9}-BSA-bio and NP₅-BSA-bio corresponded to BCR affinity.

Immunization of NP-CGG and appearance of IgM⁺NP⁺ B cells

In order to examine the relationship between the hapten valence of CGG and the immune response, C57BL/6 mice were immunized with NP-CGG and the spleen cells collected on day 7 were analyzed by flow cytometry using FITC-anti-IgM antibodies and NP₇-BSA-bio₂₁ (Fig. 3A). FITC-anti-IgM antibodies stained ~50% of unimmunized spleen cells in which NP-binding cells were under the detection level. Fewer NP₇-BSA-bio₂₁-binding cells were detectable after immunization with NP₄-CGG. Immunization with NP₁₃-CGG caused 76% of IgM⁺ B cells to acquire the ability to bind to NP₇-BSA-bio₂₁. A further increase in the valence of NP (NP₄₀-CGG) resulted in no increase in the number of NP₇-BSA-bio₂₁-binding IgM⁺ cells (65%). CD4⁺ or CD8⁺ T cells did not show any NP binding (data not shown). We referred to these cells as IgM⁺NP⁺ B cells.

The titers of anti-NP IgM and IgG in serum were measured by ELISA (Fig. 3B). After immunization with NP₄-CGG, a slight increase in anti-NP antibodies was observed. On the other hand, NP₁₃-CGG and NP₄₀-CGG caused a clear immune response of IgM and IgG1 production, and titers were indistinguishable between sera prepared from mice immunized with these two immunogens. We analyzed the number of antibody-forming cells in spleen by the ELISPOT assay and found a smaller number of anti-NP IgM- or IgG1-producing

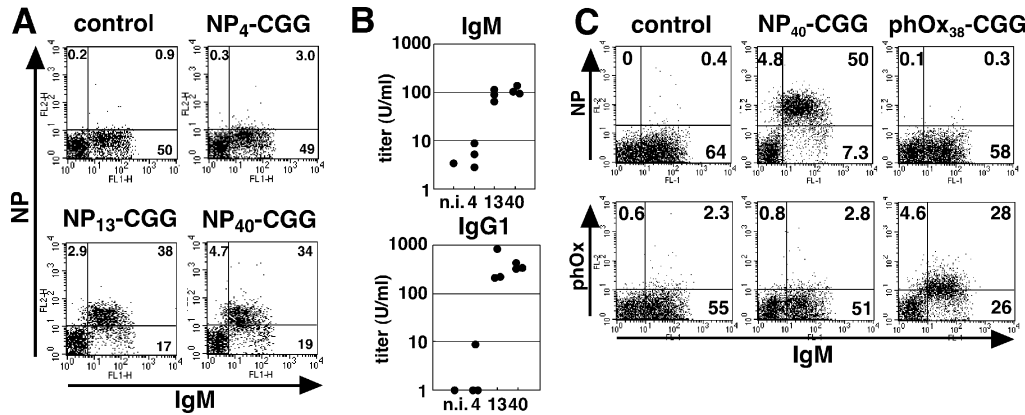


Fig. 3. Appearance of hapten-binding B cells in spleens of mice immunized with hapten-conjugated CGG. (A) Binding of NP₇-BSA-bio₂₁ cells was analyzed using a single-cell suspension of spleen cells obtained from mice on day 7 after immunization with NP-CGG having different NP valences or from non-immunized control mice. The amounts of bound NP-BSA-bio and of IgM BCR expressed on the cell surface were measured by flow cytometry and the percentages of each in the total lymphocytes are shown. (B) Mice were immunized with NP-CGG and sera were prepared on day 7. Titers of anti-NP IgM (top) and IgG1 (bottom) in sera were measured by ELISA, and plotted against the number of NP haptens on NP-CGG. Serum from non-immunized mice was used as a control (n.i.). (C) Mice were immunized with antigens indicated at the top, and spleen cells were stained with anti-IgM and NP₇-BSA-bio₂₃ or phOx₁₀-BSA-bio₂₃ in combination with FITC-anti-IgM.

cells in mice immunized with NP₄-CGG, but indistinguishable numbers in NP₁₃-CGG or NP₄₀-CGG immunized mice (data not shown). These results suggested that the appearance of IgM⁺NP⁺ B cells correlated with the increase in anti-NP antibodies in serum.

We examined whether the large number of hapten-binding IgM⁺ B cells which appeared on day 7 after the primary immunization was also observed with other hapten systems. After immunization of C57BL/6 mice with phOx₃₈-CGG, spleen cells were stained with phOx₁₀-BSA-bio₂₃. We found IgM⁺NP⁺ and IgM⁺phOx⁺ B cells in NP-CGG- and phOx-CGG-immunized mice respectively (Fig. 3C). The hapten-binding activity depended on the antigens used for immunization, indicating that the binding was specific. We also found TNP binding to B cells after immunization with TNP₄₀-CGG (data not shown). Therefore, the appearance of a large number of hapten-bound cells seems to be a phenomenon common to hapten systems.

Characterization of proteins responsible for NP binding

The spleen cells from NP₄₀-CGG-immunized mice obtained on day 7 were analyzed by flow cytometry using anti-Ig λ and anti-Ig κ , since anti-NP antibodies of the primary response were known to bear λ 1 chains. A large fraction of B cells was sorted as $\kappa^+\lambda^{\text{dull}}$ with minor single λ^+ and single κ^+ fractions (Fig. 4). This $\kappa^+\lambda^{\text{dull}}$ fraction was absent in B cells from unimmunized mice and was considered to be responsible for NP binding. In order to determine the type of L chains synthesized by these cells, single-cell analysis was performed by RT-PCR using primers specific to respective L chains (Table 1). Although expression of κ chains was identified, no λ 1 chains were detected in the $\kappa^+\lambda^{\text{dull}}$ fraction. On the other hand, λ chains were detected in all B cells of the single λ^+ fraction. These results suggested that the B cells in the $\kappa^+\lambda^{\text{dull}}$ fraction synthesized only κ chains, but not λ chains, and that the λ chains detected were derived from Ig bound to the B cell surface.

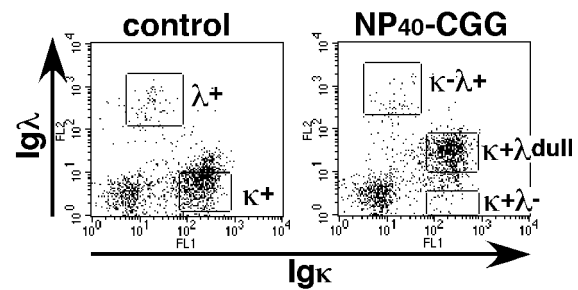


Fig. 4. Flow cytometric analysis using anti- κ or anti- λ antibodies. A single-cell suspension of spleen cells obtained from mice on day 7 post-immunization or from non-immunized control mice was analyzed using a FACSVantage. The fractions indicated were sorted and analyzed by single-cell RT-PCR (Table 1).

Next, we analyzed the materials responsible for NP binding. Spleen B cells from NP₄₀-CGG-immunized mice were isolated and reacted with NP₇-BSA-bio₂₃ followed by solubilization with a buffer containing NP-40. Materials forming a complex with NP₇-BSA-bio₂₃ in detergent lysate were precipitated with SA-agarose and analyzed by non-reducing SDS-PAGE followed by western blotting (Fig. 5A). The NP-binding proteins of cell lysates from NP₄₀-CGG-immunized mice showed a broad band corresponding to IgM pentamers, which was almost absent in those from non-immunized mice (Fig. 5A, NP-binding cell lysate). These results suggested that a secreted form of the IgM pentamer with anti-NP specificity existed on the B cell surface. On the other hand, we did not recover any NP-binding BCR, i.e. monomer IgM or secreted IgG, in spite of the existence of a large amount of NP-specific IgG in the sera (Fig. 5A, NP-binding sera). These results indicated that the BCR from most of the IgM⁺NP⁺ B cells was not specific to NP and that the binding of secreted IgM enabled B cells to bind NP. When B cells from non-immunized mice were solubilized with lysis buffer and analyzed by SDS-PAGE without reacting

with NP₇-BSA-bio₂₃ and SA-agarose, bands corresponding to both pentamer and monomer IgM were observed (Fig. 5A, total cell lysate), indicating that B cells possess two types of IgM irrespective of immunization; a monomer type that functions as a BCR and a pentamer type that might bind to the cell surface through an unidentified mechanism. Comparison of the band intensity provided an approximate ratio of pentamer relative to monomer that was <0.1.

In order to determine that the anti-NP antibodies in serum can provide B cells with NP-binding ability, spleen cells

from non-immunized mice were incubated with serum from immunized mice and analyzed by flow cytometry (Fig. 5B). When the spleen cells were pre-incubated with non-immunized serum, no NP-binding activity was observed, while after treatment with serum of NP₄₀-CGG immunized mice, IgM⁺NP⁺ B cells appeared. These results suggested that spleen B cells acquired NP-binding activity by absorbing the secreted anti-NP IgM on the cell surface.

The detection of IgM on B cells was rather surprising because IgM pentamers absorbed by these cells would result in more than one IgM on the cell surface. In order to examine whether this phenomenon occurs in normal situations, we analyzed the IgM allotype on B cells of C57BL/6 (IgM^b allotype), BALB/c (IgM^a allotype) and CBF1 mice. Splenic B cells expressed either IgM^b or IgM^a on the cell surface in C57BL/6 or BALB/c mice respectively (data not shown). By mixing an equal number of cells from C57BL/6 and BALB/c mice and staining with anti-IgM^a and -IgM^b antibodies, B cells were found to express only one of two allotypes and not both at once on the cell surface. Although B cells of CBF1 mice were also expected to express either IgM^a or IgM^b because of allelic exclusion, they provided populations of IgM^a and IgM^b cells which were weakly, but significantly, positive for IgM^b and IgM^a (referred as IgM^aIgM^b/dull and IgM^a/dull/IgM^b cells respectively) (Fig. 5C). These results suggested that B cells expressed two forms of IgM, one which existed as a BCR and the other which was present as an adsorbed form, although the amount of the latter was less than that of the former.

Table 1. Ig L gene expression in different populations of spleen B cells

Mice	Populations ^a	Ig L expressed ^b		
		Ig κ	Ig λ1	Ig λ2 or Ig λ3
Non-immunized	Ig κ ⁻ Ig λ ⁺	0	5	5
	Ig κ ⁺ Ig λ ⁻	13	0	0
Immunized (day 7)	Ig κ ⁻ Ig λ ⁺	0	16	8
	Ig κ ⁺ Ig λ ^{dull}	22	0	0
	Ig κ ⁺ Ig λ ⁻	18	0	0

^aThe cells of different populations were isolated by flow cytometry.

^bThe expression of Ig L was analyzed by PCR (24 cells were analyzed for each fraction). Numbers of detected cells expressing a functional message are shown.

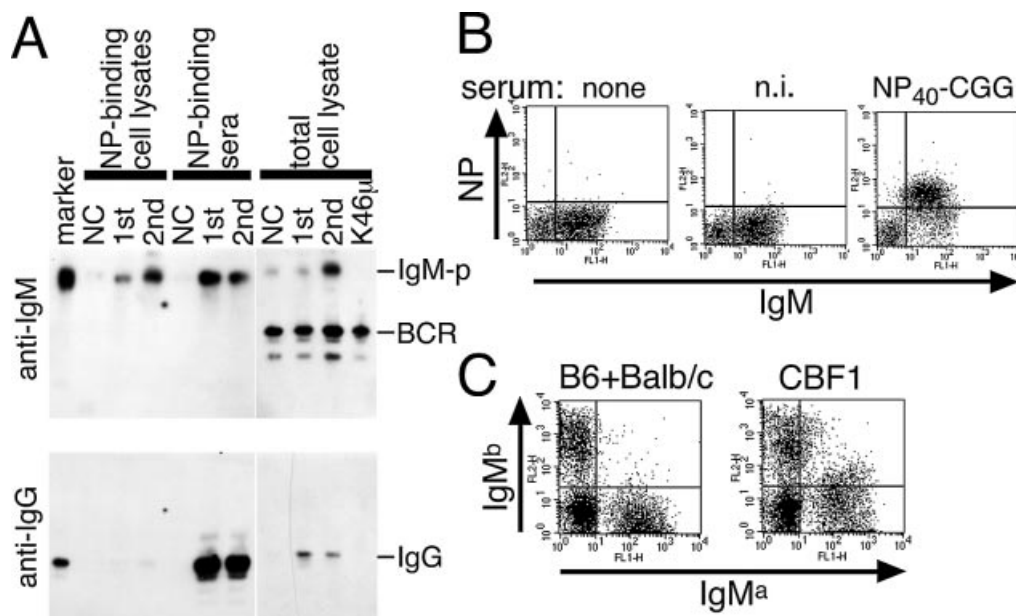


Fig. 5. Existence of IgM pentamer on spleen B cells. (A) Detergent lysates (NP-binding cell lysates) were prepared after reacting with NP₇-BSA-bio₂₃. Sera (NP-binding sera) were reacted with NP₇-BSA-bio₂₃. NP-binding proteins were precipitated with SA-agarose and analyzed on western blots using peroxidase-labeled anti-IgM (top) or anti-IgG (bottom). Lysates not reacting with NP₇-BSA-bio₂₃ and SA-agarose (total lysates) were also analyzed to show the BCR position. The marker lane contained a mixture of purified IgM pentamer and IgG. The total lysate of K46μ cells shows the position of IgM BCR on the cell surface. The positions of IgM pentamer (IgM-p), IgM BCR (BCR) and IgG (IgG) are indicated on the right. NC, serum or lysates from a non-immunized mouse; 1st, from an NP₄₀-CGG immunized mouse after primary immunization; 2nd, from a mouse 5 days after booster immunization. (B) Spleen cells of immunized mice were pre-incubated without (left), with non-immunized serum (middle) or with NP₄₀-CGG-immunized serum (right), followed by staining with NP-BSA-bio and FITC-anti-IgM. (C) Mixtures of spleen cells of C57BL/6 and BALB/c mice or a CBF1 mouse were stained with anti-IgM^a and -IgM^b antibodies.

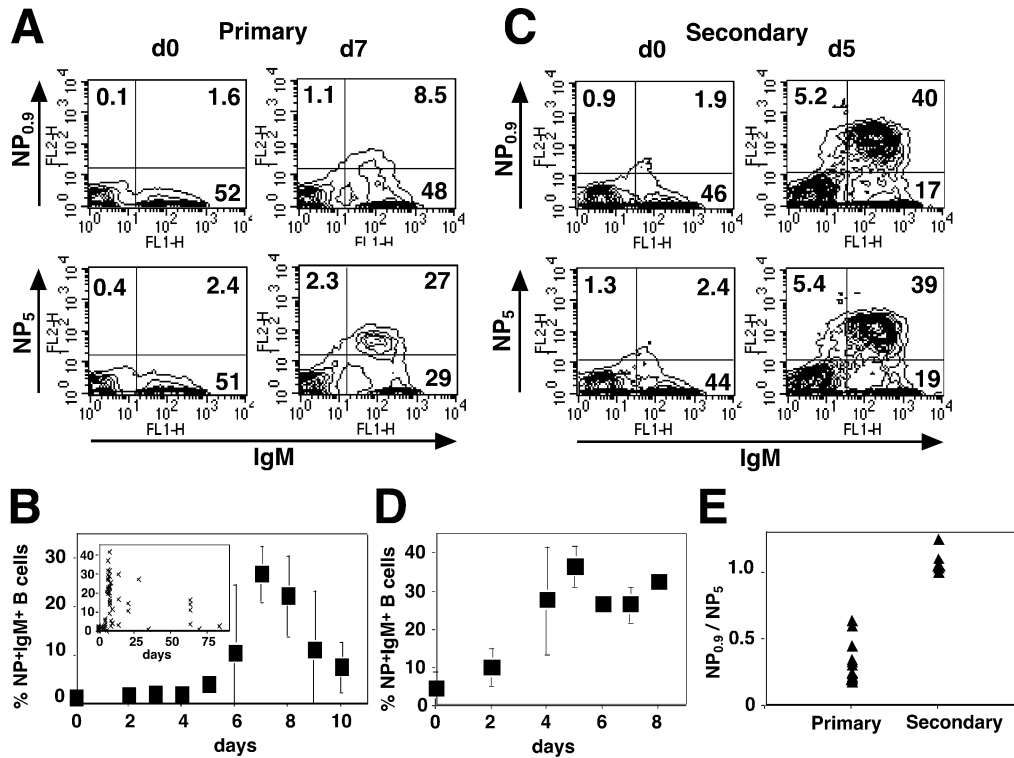


Fig. 6. Affinity maturation of IgM+NP⁺ B cells during the immune response. (A) Spleen cells were obtained 7 days after immunization with NP₄₀-CGG and stained with either NP₅-BSA-bio₂₉ + SA-PE or NP_{0.9}-BSA-bio₂₉ + SA-PE, followed by staining with FITC-anti-IgM antibodies. Percentages of each fraction are indicated. (B) The kinetics of the appearance of IgM+NP⁺ B cells in primary immunization is shown. The numbers represent average percentages obtained from a total of 60 mice used. No IgM+NP⁺ B cells existed in mice on day 70 post-immunization with NP₄₀-CGG. (C) On day 70 after primary immunization (d0), mice were immunized with NP₄₀-CGG and 5 days later, spleen cells were isolated and analyzed. (D) The kinetics of the appearance of IgM+NP⁺ B cells in secondary immunization of NP₄₀-CGG. The numbers represent average percentages obtained from a total of 30 mice used for secondary immunization. (E) The binding ratio of NP_{0.9}-BSA-bio₂₉ relative to NP₅-BSA-bio₂₉ is shown. The ratio was calculated based on the percentage of NP_{0.9}-BSA-bio₂₉ binding cells and that of NP₅-BSA-bio₂₉ binding cells on days 6–8 after primary immunization and days 5–8 after secondary immunization.

Affinity maturation of IgM+NP⁺ B cells

The binding of NP_{0.9}-BSA or NP₅-BSA to spleen cells and changes in the number of IgM+NP⁺ B cells were examined after immunization with NP₄₀-CGG. The primary IgM+NP⁺ B cells were characterized by weak binding of NP_{0.9}-BSA (Fig. 6A). These cells were absent on day 4, but started to appear on day 5. On day 7, their number reached a maximum followed by a gradual decrease with time to day 70 (Fig. 6B). Following a booster immunization with NP₄₀-CGG, IgM+NP⁺ B cells reappeared as early as day 4 and reached a maximum on day 5 (Fig. 6C and D). The binding ratios of NP_{0.9}-BSA:NP₅-BSA were 0.2–0.6 for primary B cells, whereas this ratio of NP_{0.9}-BSA:NP₅-BSA for the secondary B cells was close to 1 (Fig. 6E). These results indicated that the IgM+NP⁺ B cells of the primary immunization possessed an affinity similar to that of B1-8 and that those which reappeared following the secondary immunization had an elevated affinity to NP.

Discussion

The avidity of N1G9 to NP-CGG increased with an increase in the NP valence (Fig. 1A). We examined this effect on the

immune response at the cellular level. The biotin–SA system has been widely used for flow cytometric detection of antigens or antibodies bound to the cell surface. Since the level of detection would depend on the number of SA molecules bound to the biotin conjugates, an increase in the number of biotin molecules would raise this level. In the case of antibody biotinylation, relatively low numbers of biotin molecules were conjugated to antibodies in order to avoid inhibition by biotin introduced into the antibody combining sites. In interactions between NP-BSA-bio and N1G9, however, essentially no inhibition by biotin was observed (Fig. 1B). Thus, an increase in the number of biotin molecules resulted in an increase in the number of SA molecules bound to NP-BSA-bio (Fig. 1C). This brought about an enhanced sensitivity in detection of NP-BSA-bio bound to BCR on K46 μ and enabled us to estimate the number of NP binding cells by flow cytometry using SA-PE. Recently, we have shown that the relative affinity of BCR on the cell surface could also be estimated by the binding ratio of NP₁-BSA-bio and NP₅-BSA-bio (37). In addition, due to the high specificity of the biotin–SA interaction, no non-specific binding was observed either with SPR measurement or flow cytometry, in spite of the high numbers of biotin molecules in the conjugates (Fig. 2).

We detected a large fraction of IgM⁺NP⁺ B cells in spleens after immunization with NP-CGG, but none in pre-immune spleens (Fig. 3A) or in mice immunized with unrelated hapten conjugates or CGG alone (Fig. 3C and data not shown). The IgM⁺NP⁺ B cells were evident after immunization with NP₁₃-CGG or NP₄₀-CGG, comprising 30–40% of the total number of spleen cells, although they were hardly detected after immunization with NP₄-CGG. We examined whether the appearance of IgM⁺NP⁺ B cells was related to the amount of anti-NP IgM in the sera and found that NP₁₃-CGG or NP₄₀-CGG induced a similar amount of IgM production, while that of NP₄-CGG was rather weak (Fig. 3B). Such a parallel relationship in the ability of NP-CGG between induction of anti-NP IgM secretion and of IgM⁺NP⁺ B cells suggested that the appearance of IgM⁺NP⁺ B cells was closely related with the amount of secreted anti-NP IgM in the blood. Immunization with NP₄₀-CGG also resulted in a large fraction of $\kappa^+\lambda^{\text{dull}}$ B cells (Fig. 4). RT-PCR analysis revealed that these B cells synthesized only κ chains and not λ chains (Table 1). It has been shown previously that immunization of C57BL/6 mice with NP-CGG induced a predominant secretion of antibodies bearing $\lambda 1$ chains (38). Therefore, it is suggested that predominant production of anti-NP IgM bearing $\lambda 1$ chains resulted in the appearance of $\kappa^+\lambda^{\text{dull}}$ B cells possessing NP-binding activity.

We analyzed the molecules responsible for the NP binding. The NP-binding proteins recovered from the detergent-soluble fraction of immunized B cells were a secreted form of IgM pentamer (Fig. 5A). The monomer IgM of BCR was not recovered in the NP-binding material. In addition, although the immune sera contained both anti-NP IgM and anti-NP IgG, only secreted IgM could be detected. This indicated that IgM molecules on the B cell surface were not in the form of immune complexes with NP-CGG, since these complexes should contain both anti-NP IgM and anti-NP IgG. It was also suggested that IgG, which could be bound to Fc γ RIII on B cells, did not contribute to the NP-binding capacity of B cells. B cells from non-immunized mice had no NP-binding activity, but did acquire it after treatment with immune sera from NP₄₀-CGG-immunized mice (Fig. 5B). All these results suggested that secreted IgM bound to B cells. Recently, Shibuya *et al.* (39) reported the presence of Fc α / μ R on B220⁺ cells and it is possible that the IgM pentamers bound to the B cells through Fc α / μ R. However, a question was raised as to the binding of the secreted IgM, since not all B cells were capable of NP binding. This may be related to the broad expression of Fc α / μ R on B220⁺ cells (39). Among splenic B cells, NP binding was detected in IgM^{dull}IgD⁺ mature B cells, but not in IgM^{hi}IgD⁻ immature B cells (data not shown), which corresponded with the Fc α / μ R expression level (40). It is likely that B cells with weak expression of Fc α / μ R could be sorted as NP⁻ since they would fall below the detection level of flow cytometry analysis. We are currently examining the relationship between Fc α / μ R and appearance of IgM⁺NP⁺ cells.

If the binding of secreted IgM to B cells really occurred, it was predicted that F₁ mice from parents of different IgM allotypes should have B cells that are double positive in terms of IgM allotype, although such a fraction had not been reported previously. However, careful analysis of the flow cytometry pattern showed that the expression of IgM allotypes

of CBF1 B cells was different from that of a mixture of BALB/c and C57BL/6 B cells. It was evident that a significant amount of IgM^aIgM^{b/dull} or IgM^{a/dull}IgM^b B cells existed (Fig. 5C). Since the sera from CBF1 mice contain both secreted IgM^a and IgM^b, these IgM would compete for B cell binding sites. In addition, the band intensity in the western blots suggested that IgM pentamers represent <10% of IgM BCR (Fig. 5A). Therefore, the IgM pentamers bound to the B cells induced only a slight shift in allotype expression, which was observed as IgM^aIgM^{b/dull} or IgM^{a/dull}IgM^b. Our sensitive assay using NP-BSA-bio enabled us to detect such a low amount of NP-specific IgM pentamer on B cells.

In primary immunization, after a rapid appearance, which peaked on day 7, the percentage of IgM⁺NP⁺ B cells decreased gradually and disappeared by day 70 (Fig. 6B). These B cells reappeared after booster immunization with NP₄₀-CGG and reached a maximum number on day 5 (Fig. 6D). The binding ratio of NP_{0.9}-BSA-bio₂₉:NP₅-BSA-bio₂₉ was in the range of 0.2–0.6 for primary IgM⁺NP⁺B cells on day 7, but it increased to 1.0–1.1 with secondary immunization (Fig. 6E), suggesting that the secondary IgM possessed enhanced affinity, i.e. that affinity maturation proceeded in IgM antibodies as it does in IgG antibodies. Although amino acid replacements of IgM by SHM, typical for high-affinity anti-NP antibody, were observed (41), affinity maturation of IgM antibodies has not been shown during immunization by an ELISA method similar to that used for IgG (25). This was explained in terms of high avidity of IgM. In fact, the binding ratio of NP₁-BSA:NP₁₀-BSA was ~1, even in the case of low-affinity IgM (Shimizu, unpublished results). Recently, Tobita *et al.* (42) reported that IgM immobilized on a biosensor chip behaved similarly to IgG in the interaction of NP-BSA with different hapten valence, suggesting that the present method using NP-BSA-bio was applicable to both IgM and IgG, when these antibodies were immobilized on a biosensor chip through anti-Fc antibodies. This situation may be similar to IgM pentamer bound on B cells through Fc α / μ R, suggesting that affinity of IgM could be estimated by binding of NP-BSA-bio with different NP valency. In the present experiment, we were able to show affinity maturation of B cell-bound IgM. Since matured IgM antibodies can only be secreted after secondary stimulation with NP-CGG, maturation proceeded to the stage of generation of memory B cells in possession of IgM BCR. Recently, the presence of IgM⁺ memory cells was demonstrated in response to pHox, although direct binding to pHox was not shown (43). These IgM⁺ memory B cells would later convert to IgM-producing plasma cells as a result of booster immunization.

It is likely that the immune system responds to antigens more dynamically than was previously thought. The high sensitivity of the reagents used for flow cytometry analysis allows for the detection of virus-specific T cells and massive expansion of virus-specific CD8⁺ T cells has been reported (44–47). In the present study, we showed that B cells were coated with IgM pentamers, most likely through Fc α / μ R, which caused a massive increase in antigen-binding B cells. The immunological significance of the appearance of these IgM-coated B cells remains unresolved and is currently under investigation.

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Abbreviations

CGG	chicken γ -globulin
NP	(4-hydroxy-3-nitrophenyl)acetyl
NP-BSA bio	NP-BSA-biotin conjugates
PE	phycoerythrin
phOx	phenyloxazolone
SA	streptavidin
SHM	somatic hypermutation
SPR	surface plasmon resonance
TNP	trinitrophenyl

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