Molecular Immunology 70 (2016) 8–12

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Affinity maturation of anti-(4-hydroxy-3-nitrophenyl)acetyl antibodies accompanies a modulation of antigen specificity

Masayuki Oda[a,*], Takachika Azuma[b]

[a] Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, 1-5 Hangi-cho, Shimogamo, Sakyo-ku, Kyoto, Kyoto 606-8522, Japan
[b] Research Institute for Biological Sciences (RIBS), Tokyo University of Science, 2669 Yamazaki, Noda, Chiba 278-0022, Japan

ARTICLE INFO

Article history:
Received 17 September 2015
Received in revised form
20 November 2015
Accepted 1 December 2015

Keywords:
Antigen-antibody interaction
Antigen-binding specificity
Heterocliticity
pH dependency
Surface plasmon resonance

ABSTRACT

Anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibodies bearing λ1 chains are known to possess fine specificity, referred to as heterocliticity, which causes these antibodies to bind to hapten analogues such as (4-hydroxy-3-iodo-5-nitrophenyl)acetyl (NIP) and (4-hydroxy-3,5-dinitrophenyl)acetyl (NNP) with higher affinity than to the autologous hapten, NP. They also show preferential binding to the phenolate form of hapten than to the phenolic form. We address here the question of whether affinity maturation accompanies in the fine specificity of these antibodies by analyzing the interaction between NP_1-, NIP_1-, or NNP_1-hen egg lysozyme and anti-NP antibodies that possess different association constants to NP using a surface plasmon resonance biosensor. We measured interactions at various pH values and found that heterocliticity as well as preferential binding to the phenolate form of hapten were most prominent in a germline antibody having immature affinity and that fine specificity becomes less evident, i.e., anti-NP antibodies become more specific to the immunizing antigen, NP during the process of affinity maturation.

1. Introduction

Antibodies have been widely used as tools such as in developing biopharmaceuticals and biosensor material due to their ability to specifically recognize antigens by means of antigen-combining sites constructed mainly of amino acid residues in complementarity determining regions (CDRs) (Trilling et al., 2013; Dingjan et al., 2015). Primary antibodies produced at an early stage of immunization are characterized by low antigen affinity, while those secreted at a late stage possess a higher affinity, which is referred to as affinity maturation (Eisen and Siskind, 1964) and which has been shown to be induced by somatic hypermutation (SHM) (Milstein and Rada, 1995; Azuma, 1998). As a biosensor material, antibodies must possess high sensitivity in detection of antigen, which is attained by raising their affinity through affinity maturation. However, antibodies are known to bind to antigens with different structures (James et al., 2003; Kamatari et al., 2014), which is referred to as cross-reactivity. It has not been known whether SHMs introduced for raising affinity induce a change in specificity indicative of cross-reactivity to antigens.

Anti-(4-hydroxy-3-nitrophenyl) acetyl (NP) antibodies obtained from C57BL/6 mice have unique structural properties; they are encoded by the canonical gene segments, *V186.2*, *DFL16.2*, and *JH2* for the heavy chain and *Vλ1* and *Jλ1* for the light chain (Bothwell et al., 1981). Therefore, anti-NP antibodies are rather homogeneous in terms of the usage of their gene segments. However, even though they are encoded by canonical genes, these antibodies are heterogeneous in sequences of their 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 Ig gene rearrangement (Benedict et al., 2000). By preparing hybridomas, we previously demonstrated two distinct anti-NP antibody populations that were characterized by the amino acid residue at position 95 (Kabat et al., 1991) corresponding to the V_H–D_H junction in CDRH3. These two groups have different strategies for raising affinity. One harbors Tyr95 ($V186.2^+Tyr95^+$) and appears at the early stage of immunization, and its affinity was found to increase ~10-fold with the introduction of an amino acid replacement of Trp33 with Leu ($Leu33^+$) by means of SHM (Cumano and Rajewsky, 1986). The other harbors Gly95 ($V186.2^+Gly95^+$)

Abbreviations: NP, (4-hydroxy-3-nitrophenyl)acetyl; NIP, (4-hydroxy-3-iodo-5-nitrophenyl)acetyl; NNP, (4-hydroxy-3,5-dinitrophenyl)acetyl; CDR, complementarity determining region; SHM, somatic hypermutation; HEL, hen egg lysozyme; K_a, equilibrium associa tion constant; k_{on}, association rate constant; k_{off}, dissociation rate constant; Cap, caproic acid; SPR, surface plasmon resonance; RAMFc, rabbit anti-mouse Fc; RU, response unit.

* Corresponding author. Fax: +81 75 703 5673.
E-mail address: oda@kpu.ac.jp (M. Oda).

http://dx.doi.org/10.1016/j.molimm.2015.12.003
0161-5890/

instead of Tyr95. Gly95$^+$ antibodies have approximately 100-fold higher affinity than that of V186.2$^+$Tyr95$^+$Leu33$^+$ antibodies due to the induction of multiple SHMs in both heavy and light chains (Furukawa et al., 1999). In the present experiments, we selected three representative anti-NP antibodies, N1G9, B2, and C6. N1G9 is a germline antibody possessing Tyr95, which is a dominant component of primary antibodies (Cumano and Rajewsky, 1986). B2 is an affinity-maturated antibody possessing Tyr95 with a few residues different from N1G9 (Taketani et al., 1995). C6 is an affinity-maturated antibody having Gly95 and multiple SHMs (V186.2$^+$Gly95$^+$SHM$^+$). We attempted to determine whether there are any differences in fine specificity between Tyr95 and Gly95 in affinity-maturated antibodies (see below).

Anti-NP antibodies provide unique tools for studying the relationship between affinity and specificity to antigens, since the affinity maturation of these antibodies has been studied at the molecular level (Mariuzza and Strand, 1981; Azuma et al., 1987; Taketani et al., 1995; Sagawa et al., 2003) and since they have been shown to have unique specificity to antigens, referred to as heterocliticity (Imanishi and Mäkelä, 1973; Reth et al., 1978). Heterocliticity allows anti-NP antibodies to bind to antigen (hapten) analogues such as (4-hydroxy-3-iodo-5-nitrophenyl) acetyl (NIP) and (4-hydroxy-3,5-dinitrophenyl) acetyl (NNP) with higher affinity than to the autologous hapten, NP. Anti-NP antibodies were also shown to exhibit preferential binding to the phenolate form compared to the phenolic form of NP (Azuma et al., 1987). It is still controversial whether the affinity maturation is accompanied by a change in specificity (Haynes et al., 2005), therefore, we examined the interaction between anti-NP antibodies and hapten or hapten analogues in detail using a surface plasmon resonance (SPR) biosensor, which enabled us to describe changes in fine specificity in terms of the change in the association constant (K_a) and Gibbs free energy (ΔG). Previously, we measured the hapten–antibody interaction by SPR using NP-hen-egg lysozyme (NP-HEL), NP-bovine serum albumin, or NP-chicken γ-globulin and showed that carrier proteins having high molecular weight cause a steric hindrance in hapten–antibody interactions, while this effect was not observed in the case of NP-HEL (Oda et al., 2004). In addition, we were able to prepare hapten–HEL conjugates having a single hapten conjugated per HEL (NP_1-, NIP_1- and NNP_1-HEL), which enabled us to measure monovalent hapten–antibody interactions using Biacore with little interference from the carrier proteins.

In the present study, we measured K_a as well as rate constants of association (k_{on}) or dissociation (k_{off}) for interactions between anti-NP antibodies and NP_1-, NIP_1-, or NNP_1-HEL at various pH values. We show here that the fine specificity characterized by preferential binding to an ionized form of NP (phenolate ion) and heteroclitic binding to NIP or NNP both became less evident in affinity-maturated antibodies, suggesting that they had become more specific to the immunizing antigen, NP, during immunization.

2. Materials and methods

2.1. Antigen and antibody preparation

The hydroxysuccinimide ester of the haptens, NP, NIP, and NNP, was coupled to ε-amino-caproic acid (Cap) or HEL (Wako Pure Chemical Industries) as described previously (Azuma et al., 1987). The homogeneous monovalent antigens, NP_1-HEL, NIP_1-HEL, and NNP_1-HEL, were purified from the hapten–HEL conjugate, a mixture of HELs with different numbers of haptens, by reverse-phase high-pressure liquid chromatography on a Cosmosil 5C18-AR-300 column (4.6 mm × 15 cm, Nakarai Tesque), as described previously (Oda et al., 2004). The hapten concentrations were determined from the absorption at 430 nm, 430 nm, and 445 nm, respectively (Brownstone et al., 1966).

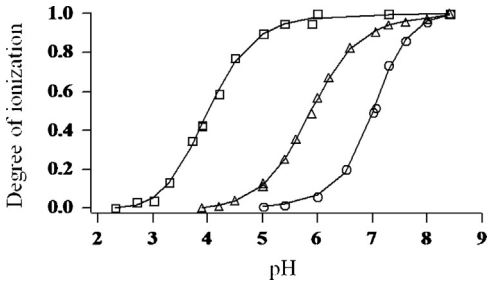

Fig. 1. Spectrophotometric titration of hapten-Caps. Degrees of ionization of NP-Cap (○), NIP-Cap (Δ), and NNP-Cap (□) were plotted against pH.

The anti-NP antibodies, B2 and C6, were prepared as described previously (Azuma et al., 1987). N1G9 was kindly provided by Dr. K. Rajewsky (Cumano and Rajewsky, 1986). The K_a values of N1G9, B2, and C6 to ε-NP-aminocaproic acid (NP-Cap), as determined by isothermal titration calorimetry, are $2.9 \times 10^5\ M^{-1}$, $3.4 \times 10^6\ M^{-1}$, and $3.3 \times 10^7\ M^{-1}$, respectively (Torigoe et al., 1995; Furukawa et al., 1999).

2.2. Spectrophotometric titration of haptens

The antigens, NP-Cap, NIP-Cap, and NNP-Cap, in solution at various pHs were prepared and their absorbance was measured. The ratio of absorbance at 430 nm to 360 nm for NP-Cap and NIP-Cap and that at 445 nm to 360 nm for NNP-Cap were plotted as a function of pH, and pK values of haptens were determined.

2.3. SPR measurements

The Biacore experiments were performed as described previously (Oda and Azuma, 2000). A solution of 0.1 μM anti-NP antibody in phosphate-buffered saline containing 0.005% Tween20 was applied to the rabbit anti-mouse Fc (RAMFc) antibody chip at a rate of 20 μl/min over a course of 30 s, which resulted in the capture of about 1000 response units (RU) of anti-NP antibody. Antigens were diluted at various concentrations (0.05–3.2 μM) and applied over the sensor chips coupled with anti-NP antibodies bound to RAMFc antibody, at a rate of 20 μl/min for 3 min. The K_a values were determined from both the kinetic rate constants and the equilibrium levels obtained on sensorgrams, using Scatchard analysis (Oda et al., 2006). Kinetic data were analyzed using BIA evaluation 3.2 software, which was supplied with the Biacore system. In this program, a global fitting method was used to determine the association and dissociation rate constants, k_{on} and k_{off}, using a model of 1: 1 Langmuir binding.

3. Results

3.1. Spectrophotometric titration of haptens

We first examined the pH-dependent ionization state of hapten hydroxyl groups, since it was expected that electrostatic forces would affect the hapten–antibody interactions. Although phenolic forms of hapten molecules showed spectra with a peak at 360 nm, the phenolate form of NP and NIP provided an absorbance that peaked at 430 nm and that of NNP with a peak at 445 nm. Using the absorbance ratios of 430 nm or 445 nm to 360 nm at different pHs, degrees of ionization at respective pHs were determined, and plotted as a function of pH (Fig. 1). pK values for the hydroxyl groups of NP, NIP, and NNP were determined to be 7.15, 5.89, and 3.99, respectively.

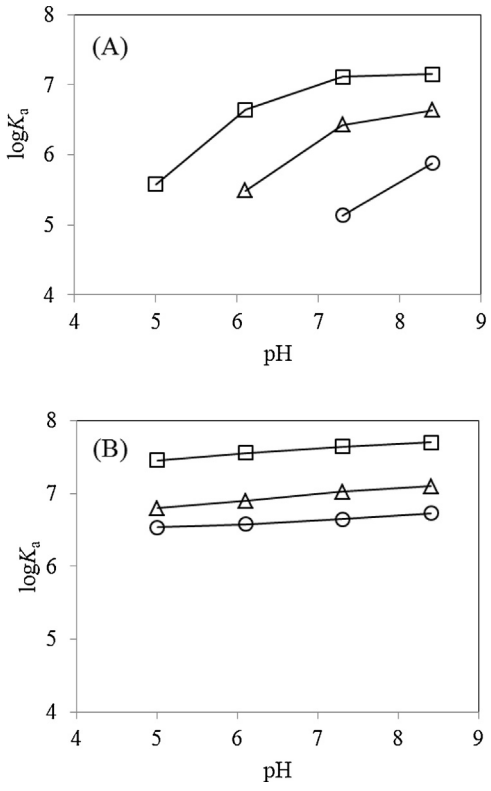

Fig. 2. Binding affinities of anti-NP monoclonal antibodies to NP_1-HEL (A) and NNP_1-HEL (B). The log K_a values for N1G9 (○), B2 (Δ), and C6 (□) were plotted against pH.

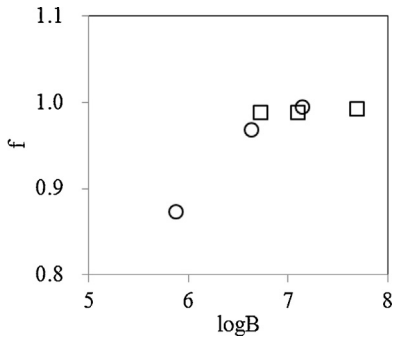

Fig. 3. Preferential binding to the phenolate form. The *f* values for NP_1-HEL (○) were plotted against logB. Those for NNP_1-HEL (□) were also shown for comparison.

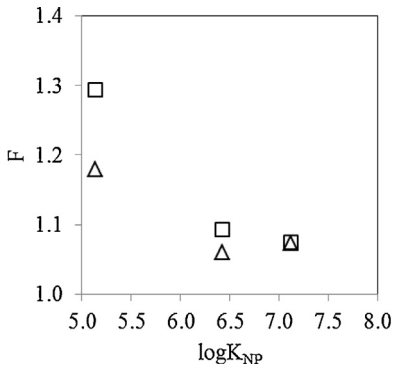

Fig. 4. Changes in the heteroclitic interaction. The *F* values for NIP_1-HEL (Δ) and NNP_1-HEL (□) were plotted against log K_{NP} .

3.2. pH-Dependent antigen binding to anti-NP antibodies

The SPR biosensor Biacore was used to determine binding kinetics of hapten–HEL conjugates to N1G9, B2, and C6, respectively. We first examined the effect of hydroxyl ionization of NP molecules on the interaction with anti-NP antibodies. The K_a values were obtained from the ratio of k_{on} and k_{off} ($K_{a,kin}$) or from Scatchard plots of equilibrium values of response units ($K_{a,Sca}$). These two values were in good agreement with each other and we used $K_{a,kin}$ as the K_a value for further analysis. The amount of NP_1-HEL binding to the antibodies increased with increasing pH values between pH 5.0 and 8.4 (Fig. 2A). The K_a for the binding of NP_1-HEL to C6 at pH 8.4 was about 100 times higher than that at pH 5.0 (Fig. 2A), while those of NNP_1-HEL at pH 8.4 and 5.0 were similar (Fig. 2B), indicating that the antibodies bind to the ionized form of NP with higher affinity and that a pH-dependent interaction reflects the ionization of NP, since this dependence was not observed in the interaction with NNP whose ionization state does not change above pH 5 (Fig. 2B). However, since essentially no information is available for the binding of N1G9 and B2 at pH 5 due to the essential lack of signal changes in SPR measurements, we measured binding at pH 7.3, at which pH all antibodies showed binding, and approximately 60% of NP groups were in the phenolate form.

In order to examine whether the preferential binding to the phenolate form was altered as affinity maturation was processing, we compared the Gibbs free energy (ΔG) for the interaction between pH 7.3 and 8.4,

$$\Delta G_A = -2.303RT\log A \quad (1)$$

$$\Delta G_B = -2.303RT\log B \quad (2)$$

in these equations; *R*, gas constant; *T*, absolute temperature; *A*, K_a at pH 7.3; B, K_a at pH 8.4. The ratio of $\Delta G_A/\Delta G_B$, referred to as *f*, is defined by Eq. (3).

$$f = \frac{\Delta G_A}{\Delta G_B} = \frac{\log A}{\log B} = \log_B A \quad (3)$$

$$A = B^f \quad (4)$$

The *f* values of the respective antibodies were plotted against log *B* as in Fig. 3, where corresponding *f* values for the interaction with NNP_1-HEL are also shown for comparison. The *f* values for NP_1-HEL increased with an increase in log *B*, suggesting that preferential binding to the phenolate form is the most remarkable and is characteristic of the germline antibody, N1G9, while it becomes less so in affinity-maturated antibodies.

3.4. Changes in the heteroclitic interaction

We measured the binding of anti-NP antibodies to NP_1-, NIP_1-, and NNP_1-HEL at pH 7.3 and calculated the *F* values using the K_a of the interaction with NXP (K_{NXP}) and NP (K_{NP}), according to Eq. (5).

$$F = \frac{\Delta G_{NXP}}{\Delta G_{NP}} = \frac{\log K_{NXP}}{\log K_{NP}} \quad (5)$$

$$K_{NXP} = (K_{NP})^F \quad (6)$$

where NXP represents either NIP or NNP. The *F* values thus obtained were plotted against log K_{NP}, as shown in Fig. 4, and are a measure of heterocliticity. By definition, larger *F* values represented a higher degree of heteroclitic binding and no such interaction was observed when $F = 1$. The interaction of N1G9 with NNP_1-HEL or NIP_1-HEL provided the highest *F* value, while the *F* values decreased with a rise in K_{NP}, and reached a value of 1, indicating that germline antibodies are the most heteroclitic and that heterocliticity is lost as a

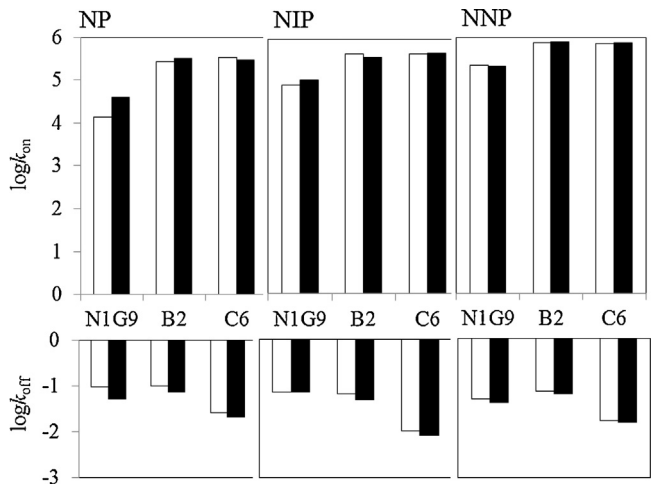

Fig. 5. Binding kinetics of anti-NP monoclonal antibodies to NP_1-HEL, NIP_1-HEL, and NNP_1-HEL. The k_{on} and k_{off} values at pH 7.3 (white bars) and 8.5 (black bars) were indicated in the log phase.

result of affinity maturation. Although a similar plot of F vs log K_{NP} was obtained at pH 8.4, the differences in F values between N1G9 and other antibodies was smaller than those observed at pH 7.3 (data not shown), suggesting that heterocliticity is more remarkable at a lower pH at which NP is predominantly in the phenolic form.

In order to gain insight into the preferential binding to the phenolate form as well as the heteroclitic interaction with NXP, we carried out a kinetic analysis (Fig. 5). Results showed that the stronger binding to the phenolate form and the heteroclitic interaction are both due to the faster association rate and the slower dissociation rate.

4. Discussion

Throughout the immune response to T cell-dependent antigens, antibody affinity increases with time post immunization, which is referred to as affinity maturation (Eisen and Siskind, 1964), a process which was shown to arise from induction of SHMs in V_H and V_L domains (Milstein and Rada, 1995). We attempted to address the question of whether the affinity maturation is accompanied by a change in specificity, since it was reported that anti-HIV antibodies secreted from patients suffering from chronic HIV infection bind to antigens with different structures (Haynes et al., 2005). For this purpose, we focused on the unique specificity of germline anti-NP antibody, which is characterized by its preferential binding to the phenolate form of NP and heteroclitic binding to hapten analogues, and examined whether these specificities are altered in the affinity-maturated antibodies B2 and C6. Previously, we showed that two groups of anti-NP antibodies were produced during immunization; one with Tyr95 at the junction of V_H–D_H gene segments and which had gained higher affinity by SHM of Trp33 with Leu (Trp33Leu), and the other possessing Gly95 instead of Tyr95 with increased affinity as a result of multiple SHMs other than Trp33Leu. B2 is representative of the former and C6, the latter.

To examine the change in the preferential binding to the phenolate form of NP, we compared the ΔG in binding to the phenolic (pH 5.0) vs the phenolate form (pH 8.4). Since the binding of N1G9 and B2 to NP at pH 5.0 was under the limit of detection, we measured binding at pH 7.3, at which 40% of NP groups are in the phenolic form, judging from the pK value (Fig. 1). The ΔG ratio at pH 7.3 vs pH 8.4, referred to as f, was 0.873 for N1G9, 0.988 for B2, and 0.993 for C6. It is evident that N1G9 binds preferentially to the phenolate form. The preference for the phenolate form would be due to salt bridges of phenolate oxygen within the side-chains of Arg50H and Lys58H, as observed in the crystal structure of N1G9 Fab complexed with NP (Mizutani et al., 1995). This would result in the higher k_{on} and the lower k_{off} values (Fig. 5), because electrostatic interactions contribute to faster association and stable complex formation (Oda and Nakamura, 2000). By definition, the f value would be 1 when an equal binding of the phenolate and phenolic forms occurred. Although B2 and C6 still prefer to bind to the phenolate form, the f values reached 1 with an increase in K_a values, indicating that they tend to bind to both the phenolic and phenolate forms (Fig. 3).

Similar ΔG ratios were employed for describing the change in heteroclitic binding. In the case of N1G9, the ΔG ratios, referred to as F in Eq. (5), for the binding of NNP or NIP relative to that of NP were calculated to be 1.29 or 1.18, respectively. The F values decreased with an increase in K_{NP} (Fig. 4), suggesting that anti-NP antibodies become more specific to the immunizing antigen, NP. In the crystal structure of N1G9 Fab complexed with NP (Mizutani et al., 1995), the C-5 atom of NP, to which iodine and nitro groups are attached in NIP and NNP, respectively, is located within 4 Å of the side-chains of Trp33H and Trp91L, indicating that these amino acid side-chains provide van der Waals contacts with the iodine of NIP or the nitro group of NNP. Namely, the antigen combining sites of anti-NP germline antibodies were constructed to accommodate NNP or NIP with a bulky residue at position C-5 and an ionized hydroxyl residue at position C-4. Since the NP molecule has a hydrogen atom at C-5 and non-ionized hydroxyl residue at C-4, it binds less efficiently to N1G9 than does NNP or NIP. On the other hand, the combining sites of B2 or C6 gained binding free energy so as to increase complementarity to NP, which resulted in lower heterocliticity, i.e., the antibodies became more specific to NP. In other words, an increase in affinity is accompanied by an increase in specificity to the immunizing antigen in both B2 and C6, although these antibodies were shown to employ different strategies for affinity maturation depending on the amino acid residue at position 95 (Tyr or Gly) (Murakami et al., 2010).

In the present study, we showed changes in the fine specificity of antibodies toward the immunizing antigen during immunization by monitoring the preferential binding to the phenolate form of NP as well as heteroclitic interactions of anti-NP antibodies with hapten analogues. Heteroclitic recognition was first reported in anti-bacteriophage antibodies (Mäkelä, 1965), and was then reported in other antibodies against various haptens and proteins (Chitarra et al., 1993; Minnerath et al., 1995; Guo et al., 1997). Therefore, it might be a common phenomenon and the findings obtained from analyses of anti-NP antibodies may be applicable to other antigen systems in general. Although our results suggest that the affinity maturation of antibodies is accompanied by alteration of the fine specificity to attain better binding to the immunizing antigen, it was reported that an anti-dinitrophenyl antibody, SPE-7, was able to bind multi-specifically to antigens with different structures, i.e., plural specificities (James et al., 2003). SPE-7 was obtained by immunization with dinitrophenyl-chicken γ-globulin after treatment with anti-idiotype antibodies. In addition, patients with chronic HIV infection were found to secrete anti-HIV antibodies with binding activity to self-components (Haynes et al., 2005). Since specificity is likely to be tuned toward the immunizing antigens, it is as yet uncertain whether different immunization protocol or simultaneous virus infection during immunization would result in the production of antibodies different from those obtained by conventional immunization.

5. Conclusions

Although the affinity maturation of antibodies has been studied extensively, whether specificity is also changed along with

affinity change has not been well understood. Here, we showed that an increase in affinity was brought about by tuning the complementarity of the antibody combining sites to the structure of the immunizing antigen, which modulated the fine specificity. Namely, we found that anti-NP antibodies that gained higher affinity became capable of binding to both forms of NP antigen, although germline antibodies had a preference for only the ionized phenolate form. Since NP groups are in a state of equilibrium in terms of ionization of their phenolate and phenolic forms, antibodies capable of binding to both forms have an advantage in being able to capture NP antigens under physiological conditions. In addition, although the heteroclitic interaction was explained as stabilization by van der Waals contact between bulky substituents such as nitro or iodine groups of NIP or NNP and the hydrophobic side chain of antibodies in addition to contact with the backbone structure of NP, it was most prominent in germline low affinity antibodies and less evident in affinity maturated antibodies. This was explained in terms of a decrease in the contribution of these van der Waals contacts to the total binding energy in the high affinity antibodies. Based on these observations, we concluded that affinity maturation is accompanied by the maturation of fine specificity to the immunogen.

Acknowledgments

The authors thank Dr. Klaus Rajewsky for providing N1G9 and Dr William Campbell for English editing of the manuscript.

References

Azuma, T., Sakato, N., Fujio, H., 1987. Maturation of the immune response to (4-hydroxy-3-nitrophenyl)-acetyl (NP) haptens in C57BL/6 mice. Mol. Immunol. 24, 287–296.

Azuma, T., 1998. Somatic hypermutation in mouse λ chains. Immunol. Rev. 162, 97–105.

Benedict, C.L., Gilfillan, S., Thai, T.H., Kearney, J.F., 2000. Terminal deoxynucleotidyl transferase and repertoire development. Immunol. Rev. 175, 150–157.

Bothwell, A.L.M., Paskind, M., Reth, M., Imanishikari, T., Rajewsky, K., Baltimore, D., 1981. Heavy chain variable region contribution to the NP^b family of antibodies: somatic mutation evident in a γ2a variable region. Cell 24, 625–637.

Brownstone, A., Mitchison, N.A., Pitt-Rivers, R., 1966. Chemical and serological studies with an iodine-containing synthetic immunological determinant 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) and related compounds. Immunology 10, 465–479.

Chitarra, V., Alzari, P.M., Bentley, G.A., Bhat, T.N., Eisele, J.L., Houdusse, A., Lescar, J., Souchon, H., Poljak, R.J., 1993. Three-dimensional structure of a heteroclitic antigen-antibody cross-reaction complex. Proc. Natl. Acad. Sci. U. S. A. 90, 7711–7715.

Cumano, A., Rajewsky, K., 1986. Clonal recruitment and somatic mutation in the generation of immunological memory to the hapten NP. EMBO J. 5, 2459–2468.

Dingjan, T., Spendlove, I., Durrant, L.G., Scott, A.M., Yuriev, E., Ramsland, P.A., 2015. Structural biology of antibody recognition of carbohydrate epitopes and potential uses for targeted cancer immunotherapies. Mol. Immunol. 67, 75–88.

Eisen, H.N., Siskind, G.W., 1964. Variation in affinities of antibody during immune response. Biochemistry 3, 996–1008.

Furukawa, K., Akasako-Furukawa, A., Shirai, H., Nakamura, H., Azuma, T., 1999. Junctional amino acids determine the maturation pathway of an antibody. Immunity 11, 329–338.

Guo, W.X., Burger, A.M., Fischer, R.T., Sieckmann, D.G., Longo, D.L., Kenny, J.J., 1997. Sequence changes at the V-D junction of the V_H1 heavy chain of anti-phosphocholine antibodies alter binding to and protection against *Streptococcus pneumoniae*. Int. Immunol. 9, 665–677.

Haynes, B.F., Fleming, J., St Clair, E.W., Katinger, H., Stiegler, G., Kunert, R., Robinson, J., Scearce, R.M., Plonk, K., Staats, H.F., Ortel, T.L., Liao, H.X., Alam, S.M., 2005. Cardiolipin polyspecific autoreactivity in two broadly neutralizing HIV-1 antibodies. Science 308, 1906–1908.

Imanishi, T., Mäkelä, O., 1973. Strain differences in the fine specificity of mouse anti-hapten antibodies. Eur. J. Immunol. 3, 323–330.

James, L.C., Roversi, P., Tawfik, D.S., 2003. Antibody multispecificity mediated by conformational diversity. Science 299, 1362–1367.

Kabat, E.A., Reed-Miller, M., Perry, H.M., Gottesman, K.S., 1991. Sequences of Proteins of Immunological Interest, 5th ed. Department of Health and Human Services, Public Health Service, National Institutes of Health, Bethesda, MD.

Kamatari, Y.O., Ohta, S., Inoshima, Y., Oda, M., Maruno, T., Kobayashi, Y., Ishiguro, N., 2014. Identification and characterization of a multispecific monoclonal antibody G2 against chicken prion protein. Protein Sci. 23, 1050–1059.

Mäkelä, O., 1965. Single lymph node cells producing heteroclitic bacteriophage antibody. J. Immunol. 95, 378–386.

Mariuzza, R., Strand, M., 1981. Chemical basis for diversity in antibody specificity analyzed by hapten binding to monoclonal anti-4-hydroxy-3-nitrophenacetyl (NP) immunoglobulins. Mol. Immunol. 18, 847–855.

Milstein, C., Rada, C., 1995. The maturation of the antibody response. In: Honjo, T., Alt, F.W. (Eds.), Immunoglobulin Genes. , 2nd ed. Academic Press, London, pp. 57–81.

Minnerath, J.M., Wakem, L.P., Comfort, L.L., Sherman, F., Jemmerson, R., 1995. The BALB/c mouse B-cell response to pigeon cytochrome c initiates as a heteroclitic response specific for the self antigen mouse cytochrome *c*. Proc. Natl. Acad. Sci. U. S. A. 92, 12379–12383.

Mizutani, R., Miura, K., Nakayama, T., Shimada, I., Arata, Y., Satow, Y., 1995. Three-dimensional structures of the Fab fragment of murine N1G9 antibody from the primary immune response and of its complex with (4-hydroxy-3-nitrophenyl) acetate. J. Mol. Biol. 254, 208–222.

Murakami, A., Takahashi,Y, Nishimura, M., Shimizu, T., Azuma, T., 2010. The amino acid residue at position 95 and the third CDR region in the H chain determine the ceiling affinity and the maturation pathway of an anti-(4-hydroxy-3-nitrophenyl) acetyl antibody. Mol. Immunol. 48, 48–58.

Oda, M., Nakamura, H., 2000. Thermodynamic and kinetic analyses for understanding sequence-specific DNA recognition. Genes Cells 5, 319–326.

Oda, M., Azuma, T., 2000. Reevaluation of stoichiometry and affinity/avidity in interactions between anti-hapten antibodies and mono- or multi-valent antigens. Mol. Immunol. 37, 1111–1122.

Oda, M., Sato-Nakamura, N., Azuma, T., 2004. Molecular characterization of monovalent and multivalent hapten–protein conjugates for analysis of the antigen-antibody interaction. Anal. Biochem. 333, 365–371.

Oda, M., Uchiyama, S., Robinson, C.V., Fukui, K., Kobayashi, Y., Azuma, T., 2006. Regional and segmental flexibility of antibodies in the interaction with antigens of different size. FEBS J. 273, 1476–1487.

Reth, M., Hammerling, G.J., Rajewsky, K., 1978. Analysis of the repertoire of anti-NP antibodies in C57BL/6 mice by cell fusion. Eur. J. Immunol. 8, 393–400.

Sagawa, T., Oda, M., Ishimura, M., Furukawa, K., Azuma, T., 2003. Thermodynamic and kinetic aspects of antibody evolution during the immune response to hapten. Mol. Immunol. 39, 801–808.

Taketani, M., Naitoh, A., Motoyama, N., Azuma, T., 1995. Roles of conserved amino acid residues in the comlementarity determining regions on hapten–antibody interaction of anti-(4-hydroxy-3-nitrophenyl) acetyl antibodies. Mol. Immunol. 32, 983–990.

Torigoe, H., Nakayama, T., Imazato, M., Shimada, I., Arata, Y., Sarai, A., 1995. The affinity maturation of anti-4-hydroxy-3-nitrophenylacetyl mouse monoclonal antibody. A calorimetric study of the antigen–antibody interaction. J. Biol. Chem. 270, 22218–22222.

Trilling, A.K., Beekwilder, J., Zuilhof, H., 2013. Antibody orientation on biosensor surfaces: a minireview. Analyst 138, 1619–1627.