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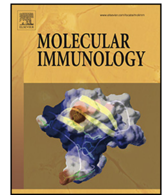

Affinity maturation of anti-(4-hydroxy-3-nitrophenyl)acetyl antibodies accompanies a modulation of antigen specificity

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ABSTRACT

Anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibodies bearing λ1 chains are known to possess fine specificity, referred to as heteroclicticity, 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 heteroclicticity 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.

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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.

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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

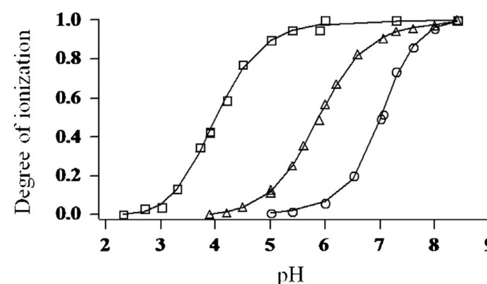

Fig. 1. Spectrophotometric titration of hapten-Caps. Degrees of ionization of NP-Cap (○), NIP-Cap (△), and NNP-Cap (□) were plotted against pH.

from the absorption at 430 nm, 430 nm, and 445 nm, respectively (Brownstone et al., 1966).

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.

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