Catalytic Antibodies Generated via Heterologous Immunization

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The mechanism of a selected reaction constitutes a basis for the rational design of haptens which induce catalytic antibodies.[1-6] Our recent studies on esterolytic antibodies suggested that efficient catalysis could be achieved with the aid of both acidic and basic amino acid residues appropriately disposed in the combining site of an antibody, and that these residues would be induced to a zwitterionic hapten.[2] Normally, however, this type of hapten synthesis is not a simple task, and it was thought that a heterologous immunization strategy (*vide infra*) might serve as an alternative to the use of a zwitterionic hapten for the generation of catalytic antibodies. We describe herein the outcome of this strategy, as compared with that of the standard homologous immunization.

Upon heterologous immunization wherein an animal is immunized in succession with two different but structurally related haptens, the host crossreactively responds to the secondary antigen and produces antibodies which have affinity for the primary antigen, the secondary antigen, or both antigens.[7] Thus, the positively charged quaternary ammonium alcohol **1a** (Chart 1) and the negatively charged phosphonamidate **2a** were chosen as haptens with the hope that antibodies generated upon heterologous immunization would be capable of binding both functionalities as a result of the induction of more than one essential amino acid residue into an antibody combining site. Both **1a** and **2a** as well as **3**, the substrate to be used for hydrolysis, were readily available.[2,4,8]

Balb/c mice were immunized twice with **1a** conjugated to BSA (BSA-1) followed by either BSA-1 (homologous immunization) or BSA-2 (heterologous immunization), and monoclonal antibodies were prepared according to standard protocols.[9] Homologous immunization with **1a** produced 48 monoclonal antibodies having strong affinity to **1b** while only five antibodies among them bound weakly to **2b**.[10,11] Seven antibodies accelerated the hydrolysis of the ester **3** with a rate acceleration (k_{cat}/k_{un}) of $1-3 \times 10^3$, and their catalytic activities were effectively inhibited by the addition of free hapten **1b**. It is noted that none of the catalytic antibodies were inhibited by the addition of **2b**, which is consistent with the results of affinity measurements.[11] On the other hand, heterologous immunization provided 50 monoclonal antibodies with varied affinities for **1b** and/or **2b** (Figure 1A).[12] Among them, 19 antibodies proved to be catalytic (Figure 1B), and *nine* of these had significantly higher catalytic efficiency ($k_{cat}/k_{un} > 10^4$) than those derived from the homologous immunization protocol. Interestingly, the majority of catalytic antibodies possessed similar crossreactivities to haptens **1b** and **2b** (Figure 1B), and these catalytic activities were effectively inhibited by both haptens. The rate enhancement (k_{cat}/k_{un}) observed in the catalysis by antibody H5H2-42 was 1.5×10^5 at pH 6.6[13] (Table 1), which is approximately 2 orders of magnitude greater than that observed for antibody H5-67.

Two representative catalytic antibodies were selected from the pool of antibodies prepared via each of the two immunization protocols, and their affinities for amide substrate **4**, *p*-nitrophenyl (PNP), and the product acid were measured (Table 1). Despite the differences in catalytic activity and hapten specificity between the antibodies generated via the different immunization protocols, all of the antibodies exhibited similar dissociation constants (K_ds) for the compounds described above.[14] This result indicates that heterologous immunization did not alter the ability of the antibodies to recognize the ground-state molecules, but instead greatly enhanced their catalytic efficiency. Furthermore, the lack of strong product inhibition observed for all these catalysts (greater than 500 actual turnovers by H5H2-42 were observed) is characteristic and may be a reflection of the high product K_ds. The product inhibition and poor turnover have been problems with some previously reported antibodies generated by phosphonate haptens such as **2a**.[4-6,15] This striking contrast suggests that the production of the catalysts via heterologous immunization is probably not due solely to the single immune response to **2a**.

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(8) Both haptens **1** and **2** share common structural features with substrates **3** and **4** differing only in the moiety containing the scissile ester/amide bond. Therefore, this set of hapten designs is readily applicable to hydrolytic cleavage of many other ester and amide substrates.

(9) Kohler, G.; Milstein, C. *Nature* **1975**, *256*, 495. See also ref 2.

(10) The K_ds were determined by the modified procedure of competition enzyme-linked immunosorbent assay (ELISA), originally described in the following paper: Friguet, B.; Chaffotte, A. F.; Djaudi-ohaniance, C.; Goldberg, M. E. *Immunol. Methods* **1985**, *77*, 305. See also ref 2.

(11) For the distributions of antibodies and catalytic antibodies as a function of their dissociation constants for haptens **1b** and **2b**, see Figure 2, the in supplementary material. Some data for homologous immunization shown in this report were recorded earlier,[2] but are reproduced for the sake of comparison with those for the heterologous immunization.

(12) The distributions of monoclonal antibodies in Figure 1A might not necessarily represent those of antibodies originally induced *in vivo*, since they were artificially subcloned after the fusion.

(13) Studies of the pH dependent steady-state kinetics of H5H2-42 over the pH range 6.2–8.5 have revealed that (i) the antibody-catalyzed hydrolysis of **3** is virtually independent of the hydroxide ion concentration and (ii) a functional group with a pK_a of ~7 exists in the catalytic site.

(14) The K_ds for the product acid were found to be too weak to detect within its solubility limit (20 mM). We also determined K_ds of the representative noncatalytic antibodies for the above compounds (see Table 2, in the supplementary material); the K_d s were similar to those observed for the catalytic antibodies.

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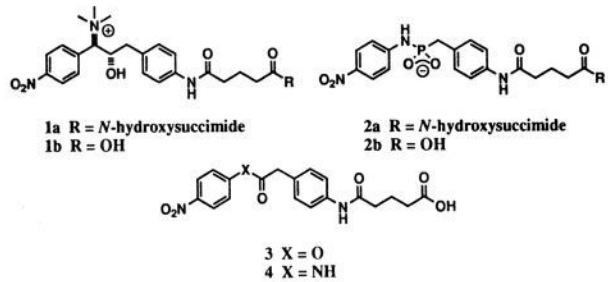
Table 1. Dissociation Constants and Michaelis–Menten Kinetic Parameters of Antibodies[a]

		K_d (10^{-2} M)						K_i (μM)		
immunization protocol	antibody	**1b**	**2b**	PNP[b]	**4**	k_{cat} (min^{-1})	K_m (μM)	**1b**	**2b**	k_{cat}/k_{un}[e] (10^3)
homologous	H5-67	1.6	>200	5.5	2.1	0.48 (0.11[c])	970	4	nd[d]	1.6 (1.5[c])
	H5-25	0.17	>200	9.4	4.1	0.79	1430	5	nd	2.7
heterologous	H5H2-42	35	3.1	4.8	2.9	12.5 (11.0[c])	240	300	22	68 (149[c])
	H5H2-187	12	8.5	9.8	4.6	3.5	820	322	150	19

[a] The K_d for all compounds were determined by competion ELISA in 50 mM Pipes, 80 μM NaCl, pH 7.0. The Michaelis–Menten kinetic parameters of H5-67 and H5-25 were determined in 50 mM Tris·HCl, 80 μM NaCl, pH 7.8 at 25 °C, and those of H5H2-42 and H5H2-187 were determined in 50 mM Pipes, 80 μM NaCl, pH 7.0 at 25 °C unless otherwise noted. All assays were performed in triplicate. [b] The K_ds could only be approximated due to the low affinity. [c] The kinetics were run in 50 mM Pipes, 80 μM NaCl, pH 6.6 at 25 °C. [d] The abbreviation nd denotes that inhibition in the presence of 1.0 mM **2b** was not detected. [e] The background hydrolysis rate constants (k_{un}) of **3** at pH 7.8, 7.0, and 6.6 were determined to be 3.0×10^{-4}, 1.8×10^{-4}, and 7.4×10^{-5} min^{-1}, respectively.

Chart 1. Structure of Haptens, Inhibitors, and Substrates



Control experiments were carried out to confirm the above supposition.[16] All monoclonal antibodies obtained by the single immunization of mice with hapten **2a** were found to be immunoglobulin M (IgM) molecules, while all antibodies isolated in the heterologous immunization protocol were found to be IgGs. This result strengthens the view that catalytic antibodies via heterologous immunization are derived through the unique stimulation of **1**-primed memory B-cells, but not through the primary response of virgin B-cells.[7h,17]

We have demonstrated that heterologous immunization of two structurally related haptens, each containing a different functional group, may represent a new general strategy for preparing antibodies which are crossreactive to these haptens and exhibit high catalytic activity. This strategy offers two distinct advantages: (i) the ability to recruit a repertoire of antibody combining sites expanded by the immunization protocol and (ii) a means of introducing catalytic residues into the active sites without resorting to complex hapten synthesis. Further investigation of this approach and its application to other chemical reactions are currently underway.

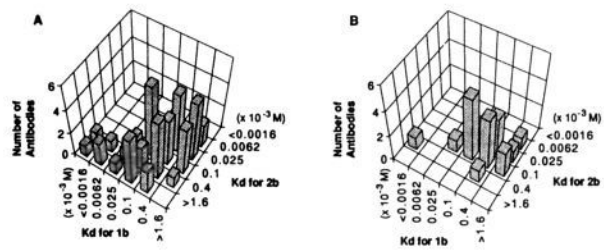


Figure 1. Distributions of antibodies (A) and catalytic antibodies (B), generated by the heterologous immunization protocol, *versus* the dissociation constants (K_d) of free hapten **1b** and **2b**. The K_d of each hapten was determined by competition ELISA.[2,1]10

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Supplementary Material Available: Listing of relevant immunization protocols, preparation of monoclonal antibodies, distributions of antibodies and catalytic antibodies generated by homologous immunization, and the dissociation constants of noncatalytic antibodies (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(15) A referee urged us to disclose information and comments on our experiments concerning antibodies homologously induced to **2a**. Of 48 monoclonal antibodies that were found to bind tightly to **2b**, none of them showed affinity to **1b**. Seventeen antibodies accelerated the hydrolysis of **3**, and the best catalyst found, H2-39, exhibited kinetic parameters, $k_{cat} = 5.8$ min^{-1} and $K_m = 900$ μM at pH 7.8, which led to $k_{cat}/k_{un} = 1.9 \times 10^4$ (the highest attainable in the pH range, 6.2–7.8). This ratio is somewhat better than that of the antibody reported by Chen *et al.*,[4b] but it is less than that of the extraordinarily efficient catalyst generated by Janda *et al.*[4a] Finally, as noted in the text for antibodies generated to phosphonate haptens, several of our catalytic antibodies induced to **2a** suffered product inhibition, resulting in the loss of more than half of the original activity after six to ten turnovers. Details of kinetic and thermodynamic properties of catalytic antibodies will be presented in a future full paper.

(16) We used two mice for the control experiments. A total of 11 hybridomas producing positive binders to Fib-**2** were examined. All antibodies exhibited very weak affinity (presumably K_ds $> 10^{-3}$ M) to Fib-**2** and no affinity to Fib-**1**. The antibody subclasses were determined by using monoclonal antibody isotyping kits purchased from Pierce (37501G) and Amersham (RPN29).

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