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Abzyme Generation Using an Anti-Idiotypic Antibody as the "Internal Image" of an Enzyme Active Site

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ABSTRACT

Since the two reports published in 1986 by the laboratories of R. Lerner and P. G. Schultz, it has been clearly established that antibodies may be induced to act as catalysts in numerous chemical reactions. In all cases, catalytic antibodies were elicited using a substrate-based approach. In the present article, we propose an alternative and complementary enzyme-based approach to generate catalytic antibodies. This approach uses the properties of anti-idiotypic antibodies to generate internal images of enzyme active sites. Experimental results are discussed for polyclonal and monoclonal anti-idiotypic antibodies.

Index Entries: Anti-idiotypes; catalytic antibodies; cholinesterase activity.

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INTRODUCTION

A number of investigators have employed the "molecular mimicry" phenomenon exhibited by idiotypes and anti-idiotypes in the study of antibodies (Abs) as pseudo-receptors and pseudo-ligands (*1–4*). However, these remarkable properties of internal image had never been used to induce on Ab-combining site not only binding properties mimicking receptor proteins, but also more sophisticated properties like catalysis.

The catalysis of a chemical transformation by an enzyme molecule results from the addition of several contributions that can be summarized as:

1. The covalent binding of an intermediate with specific amino acids of the active site;
2. The key role of polarizing groups (Lewis acids, Lewis bases, nucleophiles, and so on) able to polarize a chemical bond;
3. The role of some amino acids that create microenvironmental conditions making solvatation substitutions easier;
4. The entropy and geometric effects allowing the proximity and orientation of reactive catalytic groups; and
5. The structural complementarity of the active site to the transition state as suggested by Linus Pauling in the 1940s (*5*).

Enzymes have been perfecting their skills during evolution to reach structures capable of very high efficiencies by optimizing these contributions. Several examples of catalysis by monoclonal antibodies (mAbs) have been demonstrated since pioneer reports published in 1986 (*6–8*). In all cases, catalytic antibodies were elicited using a substrate-based approach, either to provide the stabilization of the transition state of the reaction, to act as entropy traps, or to present specific functional catalytic groups using hapten complementarity. Alternatively, we propose to develop an enzyme-based approach that could take advantage of the structural solutions selected during evolution by inducing Abs mimicking at least in part the structure of enzymatic sites.

This approach follows the same experimental steps as for receptor mimicry (Fig. 1). A first Ab (idiotypic Ab, Ab1) is raised that recognizes the active site of an enzyme, in a fashion that the combining site of Ab1 has structural features complementary to those of the enzyme. The selected first Ab, which is characterized by its inhibitory potency against the enzymatic reaction, is a mirror image of the target site, both in structural and electronic senses. After production and purification of this idiotypic Ab, a second set of Abs (Ab2) is produced against the Ab1-combining site. Among these second-generation, or anti-idiotypic Abs, some are internal images of the enzymatic site (Ab2β). Abs not only possessing the binding function for the substrate of the enzyme used as immunogen, but also able to catalyze its transformation may thus be screened.

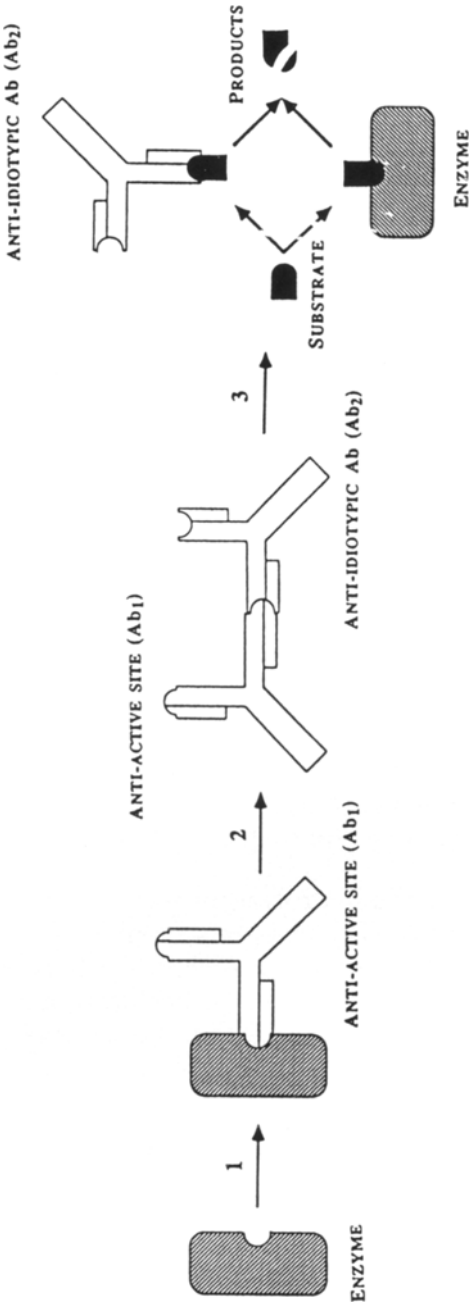

Fig. 1. Isolation of a catalytic anti-idiotypic antibody. (1) Animals immunized with an enzyme generate antienzyme Ab. Some of them are isolated that recognize the catalytic active site (Ab1). (2) Ab1 is usedas immunogen to induce anti-Ab1 Abs. Among these second-generation Abs, anti-idiotypic Abs (Ab2β) are elicited. (3) Ab2β are screened using immunological and biochemical criteria, and their catalytic efficiency is tested.

POLYCLONAL ANTI-IDIOTYPIC CATALYTIC ANTIBODIES

In first attempt, polyclonal catalytic anti-idiotypic Abs were produced. Polyclonal Abs offer a very rapid way for the evaluation of catalytic activities produced by immunization by an Ab1, and conclusions reached with polyclonal Abs concerning the feasibility of the anti-idiotype approach could be of more general value than with isolated monoclonal Abs.

The first experimental results were obtained with acetylcholinesterase (EC 3.1.1.7; AChE) as the antigenic enzyme (*9*). A monoclonal inhibitory Ab of AChE, AE-2, was produced by Fambrough et al. (*10*) against human erythrocyte AChE, and the HB-73 cell line secreting this IgG was available at the American Type Culture Collection; mAb AE-2 has been shown to inhibit the AChE activity (*11,12*), and although the epitope has not been completely characterized, several studies, using both specific inhibitors and synthetic peptides, strongly suggest that it covers at least in part the anionic subsite of the AChE active site (*13*).

Rabbits were immunized with protein A-Sepharose-purified AE-2. Serum was titrated on its immunoglobulin content to AE-2-$(Fab')_2$ fragments with a solid-phase enzyme immunoassay, and a titer of 1/16,000 was found. Binding of mAb AE-2 to AChE was inhibited by immune serum, but not by a nonimmune serum. Anti-idiotypic Abs (against both combining site and noncombining site idiotopes) were thus isolated following sequential adsorption of the serum to immunoadsorbent columns, protein A-Sepharose, then AE-2-Sepharose, and subsequent elution of Abs from the AE-2-Sepharose column. The catalytic activity of AE-2-Sepharose-purified polyclonal Abs was assayed with acetylthiocholine as the substrate. The curves indicate linearity between the amount of adsorbed anti-AE-2 Abs and AChE activity, although no activity was detected in control preparations.

When studied in solution, the hydrolysis of acetylthiocholine by anti-AE-2 Abs follows characteristic Michaelis-Menten kinetics. In contrast to human erythrocyte AChE and rabbit serum AChE, which exhibit an usual excess substrate inhibition above 1 m*M* acetylthiocholine, the Lineweaver-Burck transformation of the data does not deviate from linearity. The kinetic parameters are summarized in Table 1.

The calculated kinetic constants indicate a very high value when compared with values generally obtained with catalytic Abs. On the other hand, the Michaelis constant value differs by more than 500-fold from the values obtained with AChE from different species. Similar results were obtained with specific inhibitors of AChE. However, all the values obtained for the different constants may be composite owing to the unknown composition of our polyclonal preparation. To determine further the kinetic properties of Ab molecules in catalysis of acetylcholine, a monoclonal preparation was needed.

Table 1
Kinetic Constants for the Hydrolysis of Acetylthiocholine by Human Erythrocyte (HE) AChE, Rabbit Serum (RS) AChE, and anti-IdAbs

Catalysts	k_{cat}, s^{-1}	K_m, m*M*	K_i, m*M*	k_{cat}/k_{uncat} $\times 10^{-8}$
HE-AChE	8000	0.13	22	410
RS-AChE	3470	0.12	18.3	178
Anti-IdAbs	17.5	65	n.i.	0.9

k_{cat} values were determined assuming a mol wt of 75,000 per Ab active site. The K_i value is the inhibition constant for excess substrate. n.i., not inhibited. The rate acceleration (k_{cat}/k_{uncat}) was calculated by using a value of $1.95\ 10^{-7}\ s^{-1}$ for the uncatalytic constant of hydrolysis of acetylthiocholine in our experimental conditions.

MONOCLONAL ANTI-IDIOTYPIC CATALYTIC ANTIBODIES

Biozzi mice, a strain selected for its high immunological responsiveness (Institut Curie, France), were immunized ip with purified $F(ab')_2$ fragment of AE-2 and given booster injections at 2-wk intervals. Six weeks after the first injection, the mice were bled, and the serum was tested for anti-AE-2 Abs. Spleen cells were then fused with Sp2/0 nonsecreting myeloma cells, and the hybridoma cells were screened for anti-idiotypic Abs by ELISA. Only 1 of 600 wells screened was found positive for both the immunological anti-idiotypic activity and for hydrolytic activity against acetylthiocholine. Stable subclones were established by limiting dilution. The catalytic activity exhibited by one subclone (9A8) differed significantly from that obtained with subclones negative for anti-idiotypic activity and with unrelated mAbs prepared following the same procedure, and was inhibited by $F(ab')_2$ fragment of AE-2. The isotype of mAb 9A8 was determined to be IgM. To characterize mAb 9A8 properties, cells were injected into mice to generate ascites. Monoclonal IgM 9A8 was then purified from ascites, after precipitation in distilled water, by FPLC gel filtration followed by ion-exchange FPLC on Mono Q, and was further studied (*14*).

The catalytic properties of IgM 9A8 were determined in solution in 10 m*M* phosphate buffer, pH 7.4. We first checked that the measured maximum velocity varied linearly with the amount of Ab. As for the polyclonal preparation, the hydrolysis of thiocholine esters by mAb 9A8 follows characteristic Michaelis-Menten kinetics, which saturate at high substrate concentration (Fig. 2), in contrast to AChE, which is inhibited by substrate concentration above 1 m*M*. The kinetic constants derived from experimental results with mAb 9A8, human erythrocyte AChE, which is the immunogen used to induce the production of mAb AE-2,

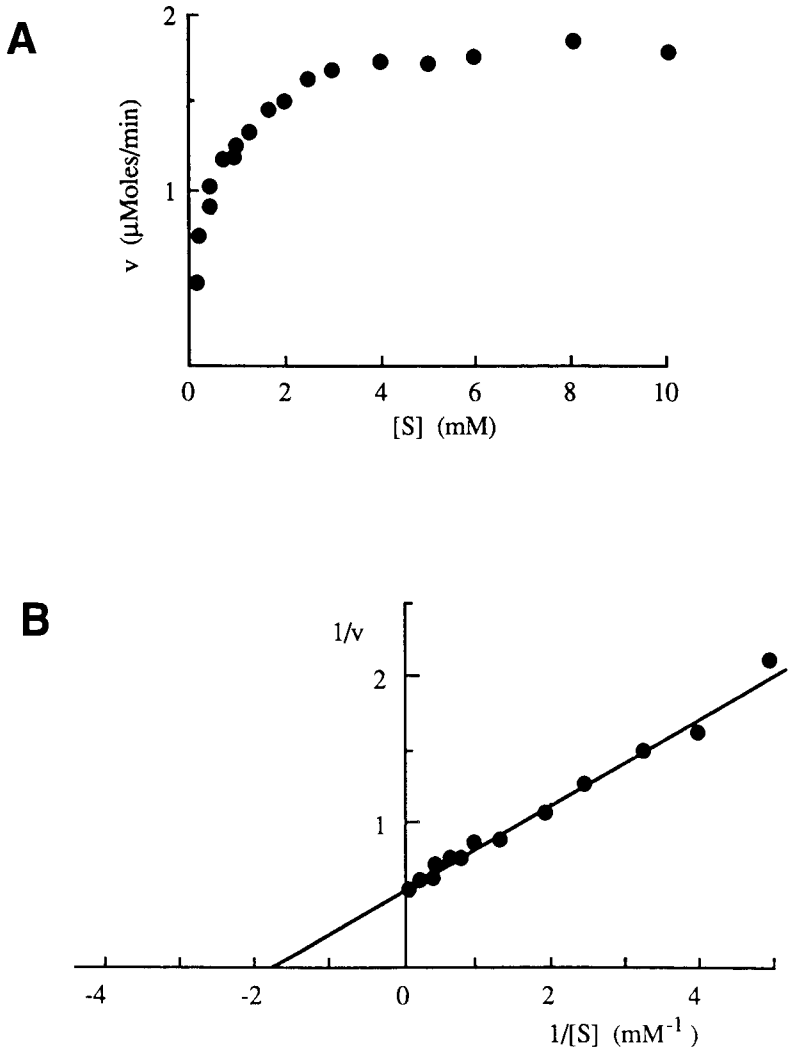

Fig. 2. Kinetics of acetylthiocholine hydrolysis catalyzed by 35 µg/mL mAb 9A8 at pH 7.4. (**A**) Activity as a function of substrate concentration from 0.1 to 10 m*M* and (**B**) Lineweaver-Burk plot of the data.

and fetal bovine serum AChE, which is present in the culture medium, are summarized in Table 2. Specificity of hydrolysis of various choline esters and of a neutral ester (*p*-nitrophenyl acetate) differs both from specificities of both AChE and butyrylcholinesterase (E.C. 3.1.1.8, BuChE), which is the other enzyme catalyzing the hydrolysis of choline esters in mammals (Table 3). The mAb 9A8 looks like a relaxed specificity from AChE. Similar conclusions were reached with irreversible (DFP, echothipate) and reversible (BW 284-C-51, ethopropazine) inhibitors of AChE and BuChE (*14*). It is interesting to note that specific inhibitors of AChE (gallamine, propidium), directed against an anionic peripheral site,

Table 2
Kinetic Constants for the Hydrolysis of Acetylthiocholine by mAb 9A8, Human Erythrocyte (HE) AChE, and Fetal Bovine Serum (FBS) AChE

Catalysts	k_{cat}, s^{-1}	K_m, m*M*	K_i, m*M*	k_{cat}/k_{uncat} $\times 10^{-8}$
mAb 9A8	81	0.60	n.i.	4.15
HE-AChE	8000	0.13	22	410
FBS-AChE	5800	0.11	18	297

k_{cat} values were determined assuming a mol wt of 90,000 per Ab active site. K_i constant for excess substrate. n.i., not inhibited.

Table 3
Relative Rate of Hydrolysis of Various Choline Esters and of a Neutral Ester by mAb 9A8, Human Erythrocyte (HE) AChE, and BuChE

	Thiocholine esters			Neutral ester
Substrate	Acetyl-	Propionyl-	Butyryl-	*p*-nitrophenyl acetate
mAb 9A8	1	1.06	0.67	0.46
HE-AChE	1	0.45	0.04	0.08
BuChE	1	2.1	5.6	0.02

distinct from the active site, fail to inhibit mAb 9A8 catalytic activity. All these results on catalytic properties of mAb 9A8 with respect to both substrate and inhibitor specificities and kinetic constants cannot be explained by the presence of a natural cholinesterase. Different experimental results argue against a possible contamination of our preparation by an enzyme. First, mAb 9A8 activity is inhibited by $F(ab')_2$ fragment of the Ab1 AE-2. IgG AE-2 has been shown to be a specific Ab for AChE from mammals (*10*), and no inhibition of BuChE activity or of nonspecific esterases has been obtained or reported by others. Moreover, when studied on solid phase by adsorption of $F(ab')_2$ fragment, the catalytic activity varies linearly with the amount of bound mAb 9A8. Various mAbs positive for anti-idiotypic activity and purified by the same procedures never showed detectable catalytic activity against acetylthiocholine. Finally, different preparations of mAb 9A8, with various cycles of purification, always exhibit the same catalytic constant.

An important question related to the structural properties of active sites of catalytic Abs produced by the anti-idiotypic approach concerns the conservation of structures of the enzyme active site taken as immunogen. Crystallographic analysis of AChE has confirmed the existence of a catalytic triad (Glu, Ser, His) in its active site (*15*). A preliminary answer to this question is brought by chemical modification experiments of mAb

9A8. On one hand, the Ab catalytic activity is irreversibly inhibited by phosphorylating agents. Although no spontaneous reactivation of phosphorylated mAb 9A8 was observed by dilution, 1 m*M* 2-PAM (2-pyridine aldoxime methiodide), a classical reactivator of phosphorylated AChE, restored 65% of the initial activity. On the other hand, incubation of mAb 9A8 with diethyl pyrocarbonate, a modifying agent of histidine residues, results in total inhibition of the catalytic activity. More than 85% of the initial activity can be protected against inhibition by addition of BW 284-C-51, a reversible inhibitor of AChE, during incubation with diethyl pyrocarbonate. These results strongly suggest the presence of essential Ser and His residues in the active site of mAb 9A8.

MAIN IMPLICATIONS OF THE ANTI-IDIOTYPIC APPROACH FOR PRODUCING CATALYTIC ANTIBODIES

The kinetic properties of polyclonal anti-idiotypic Abs and of mAb 9A8 are characterized by a high catalytic constant value when compared with other chemical reactions catalyzed by antibodies and by a relaxed specificity toward substrates when compared with the enzyme used as immunogen. This relaxed specificity may be useful in biotechnological applications when a catalyst exhibiting a wide spectrum of specificity is needed, in depollution processes or in biosensor technology for instance. For therapeutic applications, the potential induction of human catalytic anti-idiotypic Abs by immunization with an Ab1 could be alternative to the use of enzymotherapy.

In the field of basic research, the described strategy using internal imagery could be a general method for studying enzyme molecules. The possibility of isolating catalytic Abs by using the properties of the idiotypic network opens routes in future approaches to study structure–activity relationships of enzymatic activities and to produce new catalytic Abs using the same enzyme active site as antigen. These different copies possessing similar structural information, but with different "chemical alphabets" would allow one to assess the degree of freedom in the primary sequence to obtain not only the binding of a given molecule, but also a specific catalytic activity. Moreover, the specificity of enzymatic activities may be modified by chemical modifications of amino acids. The production of anti-idiotypic Abs from these chemically modified enzyme active sites would result in purely proteic copies of modified catalysts. The information contained in the chemical modification would be integrated into the genetic material of the cells producing these catalytic Abs. The results obtained by the anti-idiotypic approach raise the question of the involvement of the idiotypic network in the presence of catalytic human autoantibodies in various diseases as described for peptidase and DNA-nicking activities (*16,17*).

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DISCUSSION

A. Friboulet

Green: Do have some data on the Fab fragment or the sequence of the antibody?

Friboulet: No. We are now beginning the work to prepare the Fab and F_v fragments, and to sequence antibody.

Green: I have followed your work now for a few years, and it is impressive to see the progress. Concerning the effort to eliminate the possibility of enzyme contamination, you have concentrated on possible acetylcholinesterase or butylcholinesterase contamination. Of course, the

contaminant could be some other enzyme. Have you considered other approaches, like carefully determining specific activity using two or three substrates as a function of many operations, for example, Fab fragment purification, and demonstrating that the specific activity remains essentially identical?

Friboulet: Thank you for your comments. The isotype of mAb 9A8 is IgM, and we have met many difficulties in the preparation of Fab fragments by enzymatic digestion. Until now, we have not obtained reproducible results.

Gabibov: What is the turnover number for cholinesterase? It is a fast enzyme, I think.

Friboulet: It is one of the fastest enzymes. The turnover is between 5000 s^{-1} and 10,000 s^{-1}.

Gabibov: Fast enzymes can cause contamination artifacts. Did you check if your activity is reactive with protein-G?

Friboulet: Yes. The antibodies were also purified on a protein-G-Sepharose column.

Paul: It is somewhat ironic that trivial explanations, like contamination with enzymes, are mostly offered when efficient catalysis of difficult reactions is observed in antibody preparations. In fact, the greatest danger of artifacts, including the enzyme contamination artifact, exists in the case of low-level catalysis of reactions with a high background rate. When new paradigms begin to develop, as may be the case with Dr. Friboulet's work, the data ought to be evaluated very carefully. Nevertheless, a set of standards need to be agreed upon whereby catalytic activities observed in antibody preparations can be accepted as being the result of the antibody. The kinetics and DFP-titration experiments in your studies appear to be a convincing demonstration of lack of contamination artifacts.

Friboulet: To overcome the problem of contamination, we are currently studying β-lactamase as a model enzyme. This enzymatic activity has been chosen because there is no background β-lactamase activity in the serum of mammals. Catalytic activities measured both in purified preparations or in the serum of immunized animals could not be the result of a contamination by a serum enzyme.

Paul: Have you compared the levels of background activity owing to conventional enzymes in blood and in tissue-culture supernatants or ascites? What are the relative amounts of the contaminants that you start with?

Friboulet: Serum from immunized rabbits has a twofold higher activity than serum from nonimmune rabbits. Culture medium containing monoclonal antibodies has a very high activity. Medium from cells grown without fetal bovine serum cholinesterase has no activity. In this case, we have no possible contamination coming from the medium. The cells can still produce enzymes, of course.

Zouali: You have shown that Ab2 is able to mimic the acetylcholinesterase. One of your slides showed rabbits running away. Did you see any clinical signs in animals that produce the Ab2.

Friboulet: Yes. We find some clinical symptoms over long-term immunization of mice, similar to symptoms of intoxication by organophosphorus inhibitors of acetylcholinesterase, but this has to be studied further.

Zouali: What uses can be made of the Ab2 that you have produced?

Friboulet: The main use of this work is for basic research. We want to study the degrees of freedom of living systems to produce structures capable of a given activity. To do that, we have to prepare several catalytic antibodies, which we plan to do.

Rodkey: This is an elegant demonstration of the use of the idiotype network to perform a new function—to make an enzyme. In your studies of modification of Ser and His, did you do protection experiments to make sure you were not modifying a residue outside the binding site?

Friboulet: Yes, we see protection with BW 284-C-51, which is a specific reversible inhibitor of the active site of cholinesterase and inhibits the catalytic activity of our antibody.

Rodkey: Is Ab1 cleaved by either Ab2-β or by enzyme?

Friboulet: We have not checked this.