# Catalytic Antibodies Generated By *in Vitro* Immunization

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In this communication we report a new method for the generation of catalytic antibodies. The method of *in vitro* immunization was used to generate antibodies that catalyzed the hydrolysis of a carbonate ester. This is the first report of a catalytic antibody that has been produced and fully characterized[1] from an *in vitro* immunization.

All of the catalytic antibodies reported in the literature so far have been produced using either *in vivo* immunization[2] or selection from a phage library[3a] or have been found in the serum of hospital patients.[3b] Use of *in vitro* immunization[4] offers significant advantages over *in vivo* methods for the creation of catalytic antibodies. These include an immunization protocol that takes 3–5 days rather than the 3 months plus required by *in vivo* methods; use of transition-state analogs (antigens) without conjugation to carrier proteins, reducing the possibility of key hapten functionality being concealed by the carrier surface;[5] the creation of antibodies against antigens that are weakly antigenic or of low stability or exhibit high toxicity *in vivo*; the possibility of creating human catalytic antibodies using peripheral blood lymphocytes;[4] no requirement for direct handling and maintenence of live animals; and a need for much smaller quantities of antigen (1 ng to 50 $\mu$g), advantageous if the synthesis of the transition-state analog is difficult and/or the synthetic precursors are expensive.

The *in vitro* immunization procedure followed gives rise to class switching from IgM to IgG and IgA class antibodies such that the latter are produced as up to 45% of the total antibody content.[4]

By using *in vitro* immunization techniques the total time taken from the immunization of the mouse splenocytes to growth of sufficient monoclonal antibody to screen for catalysis takes only 4–6 weeks. This allows the efficacy of many transition-state analogs to be determined within the period necessary for monoclonal antibodies to be produced from one *in vivo* immunization.

In this paper we report the creation and characterization of monoclonal catalytic antibodies that have been generated against 4-nitrophenyl 4′-(3-aza-2-oxoheptyl)phenyl hydrogen phosphate (**1**) (Figure 1), a compound first used by Gallacher *et al.*[6a,b] to create polyclonal antibodies. This compound mimics the structure of the transition state for the hydrolysis of 4-nitrophenyl 4′-(3-aza-2-oxoheptyl)phenyl carbonate (**2**). This particular compound was chosen because it had previously been shown to produce several polyclonal antibody mixtures that displayed high catalytic activity for both carbonate and amide hydrolysis,[6a–c] but had not yet been used to generate monoclonal antibodies.

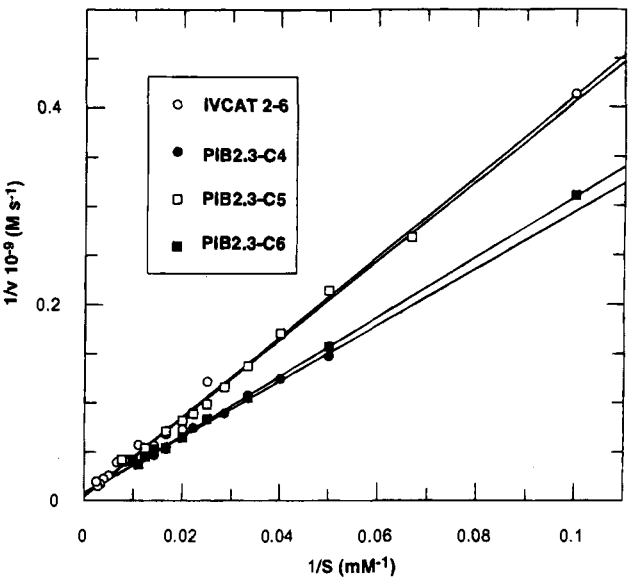
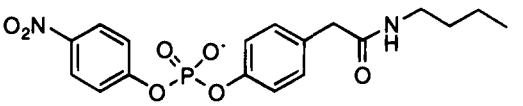
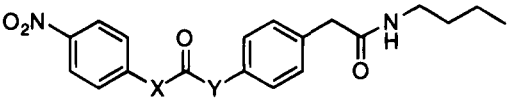


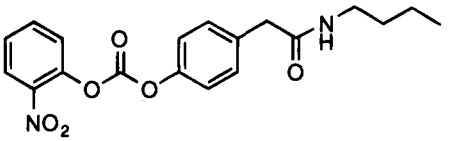

**Figure 1.** Representative Lineweaver–Burk plots for the catalytic reactions of the four antibodies elicited against hapten **1** and generated by an *in vitro* immunization method. The substrate was the carbonate **2**, and the reactions were performed at 30 °C in 50 mM sodium phosphate buffer at pH 8.0.



**1**



**2** X = Y = O, **3** X = NH, Y = $CH_2$



**4**

The details of the *in vitro* immunization procedure used in our experiments are given below.[7] Mouse spleen cells (isolated from a BALB/c strain mouse) were immunized with unconjugated transition-state analog **1** for a period of 3 days. Hybridoma cells for the production of monoclonal antibodies were then created by fusing immunized spleen cells with a myeloma cell line (SP2/0-Ag14) using standard protocols.[8]

Supernatants from the hybridomas created were then screened for binding to the transition-state analog **1** by ELISA. Several of the high-affinity hybridomas were selected, and antibodies were purified from 1-L portions of their supernatants by affinity (protein L-G)[9] chromatography and isoelectric focusing.[10] From the first immunization performed, six reactive hybrids were identified from 1920 wells screened, and these were then tested for catalytic activity against substrate **2**. The antibodies gener-

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**Table 1.** Catalytic Constants for the Catalytic Antibodies Generated by *in Vitro* Immunization (A)[a] and Kinetic Data on Carbonate Antibodies That Have Been Created by *in Vivo* Immunization Methods (B)

| | $K_M$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/k_{uncat}$ | $k_{cat}/K_M$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|---|
| A. Antibodies Generated by *in Vitro* Immunization | | | | |
| IVCAT2-6 | 0.98 | 1.2 | 5500 | 1200 |
| PIB2.3-C4 | 0.36 | 0.6 | 2500 | 1700 |
| PIB2.3-C5 | 1.04 | 1.3 | 5300 | 1250 |
| PIB2.3-C6 | 0.55 | 0.8 | 3400 | 1500 |
| B. Other Carbonate-Hydrolyzing Antibodies Generated by *in Vivo* Methods | | | | |
| MOPC167[16] | 0.21 | 0.007 | 770 | 3.4 |
| 6D4[17] | 0.002 | 0.027 | 960 | 14000 |
| 48G7-4A1[18] | 0.36 | 0.43 | 7300 | 1200 |
| 7K16.2[19] | 3.33 | 0.0005 | 969 | 1.56 |

[a] The substrate was 4-nitrophenyl 4′-(3-aza-2-oxoheptyl)phenyl carbonate (**2**); reactions were performed at 30 °C in a 50 mM sodium phosphate buffer at pH 8.0.

ated by hybridoma IVCAT2-6 were found to have significant catalytic activity. The immunization was repeated an additional two times with the same hapten, and catalytic activity was observed for several of the hybridomas produced from these immunizations (PIB2.3-C4, PIB2.3-C5, PIB2.3-C6) (Table 1).

The activity of the catalytic antibodies followed Michaelis–Menten saturation kinetics. Hydrolysis of the carbonate ester **2** was catalyzed up to 5500 times faster than the background reaction ($k_{uncat} = 2 \times 10^{-4}$ $s^{-1}$) at 30 °C. The antibody preparations used for the kinetic studies were electrophoretically pure and found to be specific for substrate **2**, with the four antibodies displaying no reactivity toward 2-nitrophenyl 4′-(3-aza-2-oxoheptyl)phenyl carbonate (**4**) or an amide substrate analog (**3**).[11] The antibodies IVCAT2-6, PIB2.3-C4, and PIB2.3-C6 were found to be of the immunoglobulin G (IgG) class by molecular weight determination on native gels.

The catalytic antibody IVCAT2-6 was competitively inhibited by the transition-state analog **1** with $K_i = 200$ $\mu$M. This demonstrates that the antibodies generated *in vitro* bind the antigen used during the immunization procedure. To rule out hydrolysis due to enzyme contamination, $F_{ab}$ fragments of IVCAT2-6 were produced and purified from whole antibody.[13] The specific activity of $F_{ab}$ fragments was compared directly to the specific activity of intact immunoglobulin in a parallel rate measurement, which showed that the $F_{ab}$ fragments had the same specific activity as their parent immunoglobulin.

A remarkable feature of the IVCAT2-6 antibody is the high catalytic constant ($k_{cat}$) of 1.2 $s^{-1}$, which gives it $k_{cat}/k_{uncat}$ and $k_{cat}/K_M$ values that are up to an order of magnitude higher than those of most other carbonate hydrolyzing antibodies (Table 1).[12]

The dissociation constant of **1** ($K_D = 65$ $\mu$M) from antibody IVCAT2-6 was determined by the fluorescence quench method.[14] Antigen and substrate binding is only moderately strong in this instance. This may reflect the fact that the affinity maturation of antibodies *in vitro* operates via antigenic selection of high-affinity B cells alone. *In vivo* immunization would also involve a considerable period of somatic mutation. The immunization period of *in vitro* methods is too short for somatic mutation to contribute significantly to the yield of high-affinity antibodies.[4] On first impression this may indicate that the catalytic activity of antibodies created by *in vitro* methods will be compromised when compared with their counterparts generated *in vivo*. However, in the few cases where a direct comparison of catalytic efficiency and hapten affinity has been made,[15] it has been demonstrated that catalytic activity does not appear to correlate directly with high binding affinity. This could be due to the fact that the majority of antibody–antigen contacts in a high-affinity antibody may be directed toward portions of the antigen structure that are not critical to its role as a transition-state analog, *i.e.*, those groups used for attaching the hapten to the carrier protein, or alternatively to a poor kinetic profile brought about by product inhibition. Certainly in the example reported here the antibody IVCAT2-6 has superior catalytic activity for carbonate hydrolysis to antibodies generated by *in vivo* methods using similar hapten structures (see Table 1).

We have shown for the first time that it is possible to generate catalytic antibodies using the *in vitro* immunization technique. This method is simple to use, and it is advantageous for the specific generation of catalytic antibodies as it allows a greater number of fusions to be performed, gives antibodies against a wider range of antigen structures, and reduces the total time for the production of antibodies from a minimum of 3 months to 3–4 weeks. This method offers researchers a rapid means of testing both new transition-state analog structures and better methods of screening for catalytic activity. Improvements in our understanding of these two areas will be crucial to the production and identification of superior antibody catalysts.

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**Supplementary Material Available:** Details of the protocols for antibody purification, $F_{ab}$ generation, and kinetic measurements (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(7) An unprimed 10–12-week-old mouse of the BALB/c strain was sacrificed, the spleen was removed, and the splenocytes were dissociated by pressing through a sterile mesh filter. The cells were then placed in a 10 mL sterile tube, spun down, and resuspended in an immunization medium (5 mL) based upon the α-modification of modified Eagle's medium (Gibco) without oxyriboses and deoxyriboses, and with 10% fetal calf serum. The resuspended cells were then added to a culture flask containing further immunization medium (25 mL) and the antigen **1** (30 $\mu$g). The flask was incubated for 3 days at 37 °C in an atmosphere of 5% $CO_2$. The immunized spleen cells were subsequently harvested and fused with $10^7$ SP2/0-Ag-14 myeloma cells following a method based on that of Kohler and Milstein[8] which uses polyethylene glycol. Fused cells were plated out in 20 × 96 well culture plates (Costar, Cambridge, MA) in a growth medium containing 100 $\mu$M hypoxanthine, 400 nM aminopterin, 16 $\mu$M thymidine (HAT), and Doma Drive, a "feeder supplement" (Immune Systems Ltd., U.K.). The plates were left undisturbed in an incubator (5% $CO_2$ at 37 °C) for 12 days. All culture wells were examined for hybridoma growth and the supernatants screened by ELISA.[13] Materials and protocols for immunization and fusion are available from Immune Systems Ltd. (U.K.) as the Cell Prime HP-2 Kit.

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