Isolation of a phenylalanine hydroxylase-stimulating monoclonal antibody by rat-myeloma–rat-spleen-cell fusion

K. H. CHOO,* J. MYER,† R. G. H. COTTON,* J. CAMAKARIS† and
D. M. DANKS*

*Genetics Research Unit, Royal Children's Hospital Research Foundation, Flemington Road, Parkville, 3052, Australia, and †Department of Genetics, University of Melbourne, Parkville, 3052, Australia

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A monoclonal antibody directed against monkey liver phenylalanine hydroxylase was produced by using a rat-myeloma–rat-spleen-cell-fusion system. This antibody showed the interesting property of increasing mammalian phenylalanine hydroxylase activity more than 2-fold. Perhaps monoclonal antibodies with this effect on other enzyme or proteins could be developed.

The demonstration that hybrids between two immunoglobulin-producing cells of heterologous (Cotton & Milstein, 1973) or homologous origin (Kohler & Milstein, 1975) produced both parental immunoglobulin molecules provided the background for a technique that allows the production of monoclonal antibodies of predefined specificity in cell culture. Microgram-per-millilitre quantities of a standardizable antibody directed against a specific antigenic determinant can be produced by growing hybrid cells *in vitro* in massive cultures. Alternatively, milligram-per-millilitre quantities of the antibody can be obtained from serum or ascites fluid in host animals that have been injected with the hybrid cells. Currently, laboratories adopting this new method have used cells of Balb/c mouse origin. However, Galfré *et al.* (1979) have described production of monoclonal antibodies against mouse immunoglobulin G in a rat myeloma system, which has obvious advantages, owing to the larger size of the animal. We describe here the use of such a system in the production of a monoclonal antibody directed against monkey liver phenylalanine hydroxylase. This antibody showed the interesting property of increasing the enzymic activity of mammalian phenylalanine hydroxylase.

Experimental and results

The myeloma cell line R210.RC.TG1 is a thioguanine-resistant derivative of R210.RC cells produced from the rat tumour S210 (Cotton & Milstein, 1973), which produces a K light chain only (Querijnje & Milstein, 1972). A previous study (Galfré *et al.*, 1979) used R210.RCY3.AG1.2.3 derived from the same cell line. Spleen cells were from

an immunized Wistar rat. We used monkey liver phenylalanine hydroxylase as immunogen. Presence of specific-antibody-producing hybrid cells was determined by alteration in enzyme activity in crude monkey liver extracts after incubation with the supernatant of each hybrid. Of 60 hybrid wells tested, one (B5) consistently resulted in a significantly elevated phenylalanine hydroxylase activity (Fig. 1) in the extract after incubation with supernatant. Activation of enzyme activity was linear with increasing amounts of the hybrid medium, reaching a 2.5-fold maximum activation. An activation of similar magnitude was observed with enzymes present in human liver extract, whereas with enzymes present in rat liver extract a 4.0-fold maximum activation was observed (result not shown). The enzyme-activation property of B5 remained stable over more than 3 months of cultivation. When subcloned by limiting dilution, more than 90% of the clones were positive. Although results reported here used the parent B5 cell population, identical results were true for subclones B5-1, B5-2 and B5-3. Karyotype analysis of these subclones confirmed that the cells were chromosomal hybrids. The karyotype for the Wistar rat, R210.RC.TG1, B5-1, B5-2, and B5-3 were 42 (Levan, 1974), 39, 67, 63 and 67 respectively.

The phenylalanine hydroxylase-activation factor produced by B5 has been characterized in the following six ways: (1) B5 culture medium alone did not exhibit enzyme activity; (2) its phenylalanine hydroxylase-activating property was completely abolished by boiling; (3) the immunoglobulins secreted by B5 contained K-chain from the parental myeloma cells in addition to new heavy (G) and light (L) chains (Fig. 2); these new immunoglobulin

Fig. 1. Isolation of an anti-(phenylalanine hydroxylase)-antibody-secreting cell hybrid

Male Wistar rats were immunized with 100 μ g of highly purified monkey liver phenylalanine hydroxylase [prepared in 0.01 M-Tris buffer, pH 7.6, 20% (v/v) glycerol, 10 mM-phenylalanine and 0.1 mM-EDTA (Choo & Cotton, 1979)] in complete Freund's adjuvant in 0.25 ml by subcutaneous injection at two sites. A second injection of 100 μ g of antigen in incomplete Freund's adjuvant in 0.1 ml was carried out 1 week later. After a further 7 days, another injection as booster was given and the spleen removed for fusion 3 days later. A 10-fold excess of spleen cells over myeloma R210.RC.TG1 cells was fused with poly(ethylene glycol) (Galfré *et al.*, 1977) and parental myeloma cells were killed by HAT medium (Kohler & Milstein, 1975) in 192 $\times$ 0.2 ml Linbro wells. Growth medium (Dulbecco-modified Eagle medium with 20% (v/v) inactivated foetal-calf serum, 30 μ l) from confluent hybrid cultures was mixed with 10 μ l (310 μ g) of crude monkey liver extract (Choo & Cotton, 1979) in 0.2 ml wells of a 96-well Linbro plate. After 3 h incubation at 4°C, 150 μ l of a reaction mixture was added followed by incubation at 37°C for 1 h. The standard reaction mixture (Choo & Cotton, 1979) was modified to contain 0.0675 mM-L-phenylalanine, 0.05 μ Ci of [14 C]phenylalanine and 0.5 mM-2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine (replacing 0.025 mM-5,6,7,8-tetrahydrobiopterin). The reaction was stopped by addition of 50 μ l of 30% (w/v) trichloroacetic acid. The precipitate was then centrifuged down (2000 rev./min, 10 min) and 2 μ l of supernatant applied on to a t.l.c. plastic-sheet cellulose plate and treated as described previously (Choo & Cotton, 1979). B5 designates a hybrid, culture medium from which a significant elevation of phenylalanine hydroxylase activity (expressed as nmol of [14 C]tyrosine formed) was found over media from other hybrid lines (enclosed by a large square bracket), which showed no significant effect on enzyme activity after incubation; one of the latter lines (F3) was retained as a control for some experiments. CM designates a point obtained when culture medium in which no hybrids had been grown

chains were presumably derived from the spleen parental cell for the specific anti-(phenylalanine hydroxylase) antibody; (4) the presence of such specific antibody in B5 culture medium is established in Fig. 3, which shows complete abolition of enzyme activation by a sheep anti-(rat immunoglobulin) preparation; (5) specific antibody was further established in two ways by using a highly purified preparation of monkey liver phenylalanine hydroxylase: (a) the formation of a precipitin line between the antibody and the purified enzyme preparation in double-immunodiffusion reaction (result not shown), and (b) the formation of antibody-(phenylalanine hydroxylase) complex in radioimmunoassay experiments (Fig. 4); (6) subcutaneous injection of B5 cells into a Wistar rat led to tumour production and the appearance of specific antibody in the serum.

Discussion

The above results confirm that monoclonal antibody to a predefined antigen can be produced by using the rat-myeloma-rat-spleen-cell-fusion system. Although here we have used a myeloma cell line that produces a K-light chain, it should be reasonably simple to isolate a non-producing variant cell line for fusion. Use of the variant cell line for fusion should yield hybrids producing only the specific antibody chains. The isolation of an activating antibody in the present study must be due to chance, and it should be possible to isolate hybrid lines producing different antibodies with other effects on the enzyme molecule.

Production of an antibody that stimulates the activity of phenylalanine hydroxylase has not previously been reported, although a phenylalanine hydroxylase-stimulating protein was isolated previously (Huang *et al.*, 1973). Also, in other enzyme systems (e.g. Pollock *et al.*, 1965), polyclonal antibodies that stimulate activity have been reported. Apparent stimulation of activity by an antibody could be the result of enzyme stabilization, but this appears unlikely in the present study because maximum increase in activity occurred almost

was used. PH1-1 designates a hybridoma clone isolated by the standard mouse-myeloma-mouse-spleen-cell fusion method (Kohler & Milstein, 1975; Galfré *et al.*, 1977) that produces a monoclonal antibody showing phenylalanine hydroxylase-inhibiting activity (Cotton *et al.*, 1980); this was included to show that the assay method resolved hybrids whose growth medium significantly altered the enzyme activity either by an increase or a decrease.

Fig. 2. Fluorography of immunoprecipitates of labelled components secreted by the parental myeloma R210.RC.TG1 and hybrid B5 cells lines analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Near-confluent cultures in 2 ml wells of a 24-well Linbro culture plate were labelled by using 0.2 ml of leucine-free medium (supplemented with 10% dialysed foetal-calf serum) containing 25 μ Ci of [14 C]leucine (352 μ Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) for 24 h at 37°C. Labelled B5 medium (20 μ l) was mixed with 20 μ l of a 40-fold concentrated unlabelled B5 medium. After this, 20 μ l (170 μ g) of sheep anti-(rat γ -immunoglobulins) (a gift from Dr. P. Bartlett) was added and the mixture left overnight at 4°C. The resulting precipitate was collected by centrifugation (4000 rev./min, 10 min) and treated for gel electrophoresis as previously described (Choo *et al.*, 1979). A similar treatment was carried out for the parental myeloma labelled medium, except that 20 μ l of rabbit anti-sheep immunoglobulins (OTPS; Behringwerke A.G., Marburg-Lahn, Germany) was added 2 h after the addition of sheep anti-rat immunoglobulins to assist formation of visible precipitate. The positions of the different immunoglobulin chains are indicated. (a) R210.RC.TG1 K-chain and (b) B5 immunoglobulins (G-, K- and L-chains). D designates electrophoretic-dye front.

Fig. 3. Abolition of phenylalanine hydroxylase activation by sheep anti-rat immunoglobulins

A series of tubes each containing 30 μ l of a culture medium from B5 (●) or from a control hybrid line F3 (■) (see Fig. 1) was preincubated with increasing amounts of a solution of sheep anti-rat immunoglobulins (8.5 μ g/ μ l) for 17 h at 4°C. After this, 10 μ l (310 μ g) of crude monkey liver extract was added to each tube, followed by incubation and enzyme assay as described in Fig. 1. The F3 slope shows that the anti-rat immunoglobulin preparation caused some apparent enzyme inhibition. The B5 slope indicates that the 2.5-fold activation of phenylalanine hydroxylase by B5 culture medium was progressively abolished by increasing anti-rat immunoglobulins. The effect of anti-rat immunoglobulins on elevated enzyme activities generated by an increased input of crude monkey liver extract (without B5) was also used as a control (▲).

instantaneously after incubation of enzyme with antibody at 4°C (result not shown). In addition, we have found that, over a 1 h period of incubation at 4°C in the absence of antibody, there was no significant loss of phenylalanine hydroxylase activity in the extract. The B5 antibody reported here should be a useful tool for the understanding of the mechanism of activation of the enzyme. It may also prove useful for the study *in vitro* of possible restoration of residual normal or mutant enzyme proteins in livers from patients with classical phenylketonuria or hyperphenylalaninaemia. Results of preliminary experiments have indicated the usefulness of the antibody for the synthesis of a specific affinity column for the purification of mammalian phenylalanine hydroxylase (J. Myer & K. H. Choo, unpublished work).

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Fig. 4. Radioimmunoassay for anti-(phenylalanine hydroxylase) activity

Cooke 96-well poly(vinyl chloride) microtitre plates were used. All dilutions were carried out in a phosphate-buffered iso-osmotic saline solution (8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4 , 0.2 g of KH_2PO_4 in 1 litre of water), pH 7.4. Individual wells were first precoated with 50 μl of a 20 $\mu\text{g}/\text{ml}$ solution of highly purified monkey liver phenylalanine hydroxylase (Choo & Cotton, 1979) for 1 h under humidified conditions at room temperature. After removal of the solution, 100 μl of a solution of 0.1% bovine serum albumin was added. This was incubated for a further 1 h. The wells were then rinsed once in phosphate-buffered saline before 50 μl of growth media from confluent hybrid cultures was added. After incubation for 4 h, the wells were rinsed three times in phosphate-buffered saline + 0.05% Tween 20, and twice in phosphate-buffered saline. This was followed by the addition of 50 μl of a solution containing ^{125}I -labelled (20000 c.p.m.) rabbit anti-mouse immunoglobulins (kindly provided by Mr. F. Fargair). After 17–20 h incubation the wells were rinsed five times as described above before they were air-dried and cut out for counting of ^{125}I radioactivity. The anti-(mouse

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immunoglobulin) preparation was suitable for use as a second antibody, since it showed sufficient cross-reactivity with rat immunoglobulins (see below). The designations for B5, F3 and R210 were as described in Fig. 1. It can be seen that B5 culture medium bound significantly more radioactivity than the control media (F3 and R210) and media from other negative hybrid lines. (These hybrid lines were derived from two other fusion experiments different from that from which B5 and F3 were derived). This result indicates the presence of antibody in B5 medium that specifically recognized phenylalanine hydroxylase.