Isolation and characterization of a myeloma–spleen-cell hybrid producing antibody to phenylalanine hydroxylase

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Application of the technique of myeloma–spleen-cell fusion [Kohler & Milstein (1975) *Nature (London)* **256**, 495–497] has allowed the isolation of a cell colony that produced a monoclonal antibody against monkey liver phenylalanine hydroxylase. The antibody exhibited cross-reactivity against hepatic phenylalanine hydroxylase from other mammalian species, including human, rat and mouse. Cross-reactivity was established by (a) enzyme-inhibition assay, (b) double-immunodiffusion reaction, and (c) two-dimensional polyacrylamide-gel-electrophoretic analysis of immunoprecipitate. The various properties of the monoclonal antibody and its use in the study of mammalian phenylalanine hydroxylase are presented.

Kohler & Milstein (1975) first described a technique that allows production of cell hybrids that secrete antibody to any preselected antigen. This involves isolation of the appropriate hybrid from fusion of a myeloma cell with spleen cells from an animal immunized with the desired antigen.

We were attracted to this method for the study of phenylalanine hydroxylase for two reasons: (a) the hybrid-antibody method (Kohler & Milstein, 1975) offers the ultimate elimination of repeated needs for purification of phenylalanine hydroxylase for repeated immunizations; (b) milligram quantities of pure antibody are available when the specific hybrid is grown as a tumour, thus the method also offers promise of production of antibody-specific affinity columns for the simple production of milligram quantities of phenylalanine hydroxylase.

In the present paper we describe the production and characterization of a mouse myeloma–spleen-cell hybrid that secretes an antibody to monkey liver phenylalanine hydroxylase. Use of the antibody for specific rapid immunoprecipitation of mammalian phenylalanine hydroxylase is described.

Materials and methods

Cell and culture media

The myeloma cell line NS1/1 Ag 4 is an azaguanine-resistant derivative (Kohler *et al.*, 1976) of a non-secreting line isolated in the course of a screening programme (Cotton *et al.*, 1973) of myeloma clones. Cells used for fusion were cultured

as described previously (Cotton & Milstein, 1973; Cotton *et al.*, 1973) in Dulbecco-modified Eagle's medium with 20% (v/v) inactivated foetal-calf serum and were not allowed to leave the exponential phase of growth.

Spleen from immunized mice (see below) were removed aseptically into Dulbecco's phosphate-buffered iso-osmotic salt solution, pH 7.4, containing 2% foetal-calf serum. The same solution was used to swell and disperse the spleen cells with the help of a syringe. The cell suspension was then centrifuged at 1000 rev./min ($r_{av.} = 16.41$ cm) for 5 min and the pellet resuspended in 5 ml of NH_4Cl -containing erythrocyte-removal buffer (0.14 M- NH_4Cl in 0.017 M-Tris/HCl, pH 7.2) at 37°C for 7 min. This was followed by the addition of a further 5 ml of 2%-foetal-calf serum/phosphate-buffered saline solution and re-centrifugation. The final cell pellet was resuspended in fresh 2%-foetal-calf serum/phosphate-buffered saline solution, determined for cell viability, and used for cell fusion. Cell viability was assessed as the ability of live cells to exclude the dye Trypan Blue (0.4% in 0.9% NaCl).

Immunization of mice

Highly purified monkey liver phenylalanine hydroxylase [prepared in a stabilizing buffer of 0.01 M-Tris (pH 7.6)/20% glycerol/10 mM-phenylalanine and 0.1 mM-EDTA] (Choo & Cotton, 1979) was used for immunization. Mice (strain 129 M) were immunized with the pure antigen by initial (subcutaneous at two sites) injection of 100 µg of

pure enzyme with complete Freund's adjuvant in 0.2 ml. A week later 10 µg of protein in incomplete Freund's adjuvant in 0.1 ml was injected (as above). A week later mice were eye-bled and tested for inhibition of phenylalanine hydroxylase activity (see below). If serum was positive compared with unimmunized control mice, another injection as for booster was given and the spleen taken 3 days later.

Cell fusion and hybrid isolation

This methodology is fairly standard and is essentially as described previously (Kohler & Milstein, 1975; Galfré *et al.*, 1977). Spleen cells and myeloma cells were fused with polyethylene glycol and parental myeloma cells were killed by 'HAT' medium (culture medium containing 100 µM-hypoxanthine, 4 nM-aminopterin and 16 µM-thymidine) in 96-well (0.2 ml) Linbro plates.

Detection of hybrids secreting phenylalanine hydroxylase antibody

Supernatants (approx. 150 µl) were taken from cups containing growing hybrids when they had grown to a cell number at which they were on the verge of cell death with at least 2 days of accumulation into the medium. These cells were then given fresh medium and would survive 1–2 days while the assay was performed.

Enzyme-inhibition assay

(a) *Culture supernatant screening.* Presence of antibody to phenylalanine hydroxylase was determined by antibody inhibition of enzyme activity in crude monkey liver extracts. Phenylalanine hydroxylase assay was based on the method previously described (Choo & Cotton, 1979), with the modifications described below.

Supernatant from a confluent hybrid-cell culture (0.035 ml) was mixed with 0.010 ml of crude monkey liver extract (Cotton & Grattan, 1975) in a 0.2 ml well of a 96-well Linbro plate. This was left at 4°C for 3 h. The reaction mixture (0.150 ml) was then added, followed by incubation at 37°C for 1 h. The standard reaction mixture was altered to contain 0.0675 mM-L-phenylalanine, 0.050 µCi of [¹⁴C]-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 terminated by the addition of 0.050 ml of 30% (w/v) trichloroacetic acid. A portion (2 µl) of this mixture was applied on to a t.l.c. plastic-sheet cellulose plate and treated as described previously (Choo & Cotton, 1979). Positive cultures were identified as ones that significantly decreased the phenylalanine hydroxylase activity [expressed as the ratio of tyrosine to total radioactivity (c.p.m.)] as compared with control tubes containing culture medium only. These cultures were

(a) frozen in liquid N₂ as soon as possible, (b) carried on in culture to detect any loss in antibody production, (c) subcloned at limiting dilution to eliminate any contaminating non-specific hybrids or any spontaneously arising non-secreting variants (Cotton *et al.*, 1973) and (d) grown as tumour and/or ascites (see below).

(b) *Precise assay of inhibition.* Precise enzyme-inhibition assay of phenylalanine hydroxylase by concentrated culture supernatant or serum from mice in which tumours were growing was performed by using a standard assay method as published for rabbit sera (Choo & Cotton, 1979). Culture supernatant was concentrated 50-fold with an Amicon PM10-membrane apparatus. Different amounts of the concentrated supernatant or serum were used in the reaction tubes, and residual volumes were adjusted by using a similarly concentrated supernatant from an inhibition-negative hybrid [AA8 (see later)] or a solution of bovine serum albumin containing 50 mg/ml respectively.

Tumour serum production

Cells (10⁷) were washed twice in serum-free medium, resuspended in 0.4 ml of this medium, and injected subcutaneously at two sites in 129 M × Balb/c F₁ hybrids. The mice were at least 8 weeks old. When tumours were fully grown, mice were killed by exsanguination and tumour cells either re-assayed or frozen in liquid N₂. Serum was stored at -70°C.

Ascites-fluid production

The mouse strain and age of mouse used were as for tumour-serum production. The animal was injected with 1 ml of pristane (2,6,10,14-tetramethylpentadecane) (ICN Pharmaceuticals, Plainview, NY, U.S.A.) intraperitoneally 3 weeks before injecting with cells. Cells (10⁷) were washed twice in serum-free medium, resuspended in 0.4 ml of the same medium, and injected intraperitoneally. Ascites fluid (2–4 ml/animal) was collected after 3 weeks and stored at -70°C.

Neutralization of antibody

A test supernatant was preincubated with IgG Sorb (10% cell suspension; Enzyme Centre, Boston, MA, U.S.A.) for 10 min at 4°C, or with rabbit anti-mouse immunoglobulins (Dako-immunoglobulins Ltd.), for 3 h at 4°C. The mixture was then centrifuged at 2000 g for 10 min and the supernatant assayed for enzyme inhibition of phenylalanine hydroxylase. Neutralization of antibody is indicated by decrease or absence of enzyme inhibition over control tubes in which IgG Sorb or anti-mouse immunoglobulin had not been added.

Extract preparation

Crude liver extracts for immunodiffusion and double-immunoprecipitation were prepared by homogenization at 4°C with a Caframo homogenizer (type RZRI-64) in 0.1M-K₃PO₄ buffer, pH 7.4, containing 10mM-phenylalanine; 1 g of liver tissue was used for 3 ml of buffer. The homogenates were centrifuged for 45 min at 27000g and 4°C. The supernatant was poured through gauze to remove lipid before it was frozen (-70°C) until use.

Double-immunodiffusion analysis

This was carried out as previously described (Choo & Cotton, 1979).

Karyotype analysis

Giemsa-stain chromosome banding was performed by Dr. G. Webb of the Cytogenetics Department of this Institution.

Immunoprecipitation of radiolabelled antibody

Radiolabelling of the cell cultures was carried out as described by Cotton *et al.* (1973), except that radioactive L-[U-¹⁴C]leucine (352 mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was used in place of radioactive lysine. Immunoprecipitation of radiolabelled culture supernatants with rabbit anti-mouse immunoglobulins (Dako) and subsequent sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were carried out as described by Choo *et al.* (1979). After electrophoresis the gel was fixed in 3.5% (w/v) HClO₄ and fluorographed (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Immunoprecipitation and two-dimensional polyacrylamide-gel electrophoresis

Immunoprecipitation of phenylalanine hydroxylase was carried out as reported previously (Choo *et al.*, 1979) with the following modifications. A portion (30–40 µl) of monoclonal antiserum from a mouse was mixed with 250–500 µl of a crude liver extract solution containing 6–12 mg of protein. The mixture was incubated for 17 h at 4°C. The resulting precipitate was collected by centrifugation. This was then washed and treated for two-dimensional gel electrophoresis as described previously (Choo *et al.*, 1979).

Results

Immunization, fusion, screening and cloning

Several mice were immunized with pure monkey liver phenylalanine hydroxylase and the sera obtained were tested for inhibition of enzyme activity in crude monkey liver extract. Serum from an immunized mouse was used in control tubes to account for

any possible non-specific inhibiting activity present in serum. Two mice showing significant inhibition by the serum were used to prepare spleen cells for two different fusions. One fusion showed no 'positive' hybrid cultures out of the 160 tested. The second fusion showed one positive out of 154 tested. This hybrid was named 'PH α 1-0'. Hybrid PH α 1-0 was readily detected by the screening test used, as it showed 70–80% inhibition of phenylalanine hydroxylase activity in monkey liver extract (Fig. 1).

A negative hybrid, AA8 (Fig. 1), was retained for control purposes.

Hybrid PH α 1-0 was cloned by limiting dilution. The fastest-growing most active clones were ranked, named PH α 1-1, PH α 1-2, etc. The inhibition by the supernatants of the subclones was tested from time to time to look for instability in the expression of the specific antibody. Subclone PH α 1-1 appeared stable over many months of cultivation (as was the parental hybrid clone PH α 1-0), and was used for further studies.

Karyotype of hybrids

Karyotypic analysis confirmed that PH α 1-1 cells were chromosomally hybrids. The myeloma cell line

Fig. 1. Screening assay

Results obtained with 26 different cell-hybrid-culture supernatants are shown. Phenylalanine hydroxylase activity was expressed as the ratio of tyrosine to total radioactivity (c.p.m.). 'PH α 1' designates a hybrid line that showed significant inhibition of enzyme activity. 'AA8' is a hybrid line that showed no significant inhibition activity and was retained as a control.

NS1/Ag 4 had a 55–68 chromosome range whereas PH α 1-1 cells showed 92–106.

Immunoprecipitation of radioactively labelled medium protein

Analysis of an immunoprecipitate of radio-labelled PH α 1-1 cell culture medium by using rabbit anti-mouse immunoglobulins on a sodium dodecyl sulphate/polyacrylamide gel demonstrated the presence of heavy and light immunoglobulin chains (results not shown). This is also seen in two-dimensional gel analysis of an immunoprecipitate (see Fig. 4 below).

Inhibition of phenylalanine hydroxylase

Titration results for the inhibition of monkey liver phenylalanine hydroxylase activity by a concentrated supernatant of PH α 1-1 cells and by a serum from a mouse carrying PH α 1-1-cell tumours are shown in Figs. 2(a) and 2(b) respectively. As can be seen, in both cases progressive inhibition of enzyme activity occurred, a final 70–80% inhibition being achieved. From Fig. 2 it could be estimated that the

Fig. 2. *Enzyme-inhibition titration*

Inhibition of phenylalanine hydroxylase activity in crude monkey liver extracts by (a) a 50-times-concentrated preparation of hybrid PH α 1-1 culture supernatant or (b) a serum from a mouse carrying PH α 1-1 tumor. Enzyme inhibition is expressed as a percentage of activity present in a tube containing antibody over a control containing no antibody.

inhibiting activity of the serum from a mouse carrying PH α 1-1 cells is approx. 500 times greater than that of an unconcentrated supernatant from PH α 1-1 cells.

The serum from a mouse carrying PH α 1-1 cells was also tested against other mammalian phenylalanine hydroxylase species. Progressive inhibition of activity was observed with the hepatic human, rat and mouse enzymes, approximate final inhibitions of 90, 60 and 60% respectively being observed (results not shown). When this serum was preincubated with IgG Sorb or rabbit anti-mouse immunoglobulins, its enzyme-inhibition property was completely eliminated. This indicates that the inhibition property was due to an immunoglobulin.

When the inhibiting activities of ascites fluids from mice carrying PH α 1-1 cells were tested, they were found to be generally two to three times greater than that shown by the antiserum.

Double-immunodiffusion analysis

Double-immunodiffusion reactions were carried out with sera from mice carrying the hybrid-cell tumours. The sera were tested against purified monkey liver phenylalanine hydroxylase that contained less than 0.3% of any contaminant protein. In addition, the sera were tested against purified rat liver phenylalanine hydroxylase (approx. 75% homogeneous; K. H. Choo & I. G. Jennings, unpublished work) and purified human liver phenylalanine hydroxylase (approx. 95% homogeneous) (Choo *et al.*, 1979). Fig. 3 shows that serum from a mouse carrying PH α 1-1 cells (and also serum from a mouse carrying PH α 1-0 cells) reacted with the enzyme from these sources to form a single precipitin line. Further, the line formed between the three enzyme species was a line of identity (i.e. no spurring or crossing was observed).

Fig. 3. *Double-immunodiffusion reactions*

(a) Well 1, purified monkey liver phenylalanine hydroxylase ($\sim 3 \mu$ g); well 2, purified rat liver phenylalanine hydroxylase ($\sim 4 \mu$ g); well 3, 1-in-2 dilution of serum from a mouse carrying PH α 1-1 cells; (b) well 1, purified monkey liver phenylalanine hydroxylase ($\sim 3 \mu$ g); well 2, purified human liver phenylalanine hydroxylase ($\sim 3 \mu$ g); well 3, 1-in-2 dilution of serum from a mouse carrying PH α 1-1 cells.

In double immunodiffusion, serum from mice carrying PH α 1-1 cells cross-reacted with crude liver extracts of monkey, human, rat and mouse to form a single precipitin line; the line formed was a line of identity between the four mammalian enzyme species (results not shown).

Additional evidence for specific immunoprecipitation of phenylalanine hydroxylase by PH α 1-1 antibody comes from two-dimensional polyacrylamide-gel analysis of double immunoprecipitates (see below).

Immunoprecipitation and two-dimensional polyacrylamide-gel-electrophoretic analysis

Figs. 4(a) and 4(b) show results of two-dimensional gel electrophoretograms of immunoprecipitate obtained by allowing PH α 1-1 antiserum to react with a crude monkey liver extract (see the Materials and methods section). As Fig. 4 shows, the serum contained specific antibody that cross-reacted with phenylalanine hydroxylase. That the polypeptide spots seen in Figs. 4(a) and 4(b) were phenylalanine hydroxylase was established by co-

Fig. 4. Two-dimensional gel-electrophoretic analysis of immunoprecipitate from a crude monkey liver extract using PH α 1-1-tumour serum

(a) The whole two-dimensional gel shows the positions of phenylalanine hydroxylase (in box) and of the heavy and light immunoglobulin chains (solid and open arrows respectively). The latter was established from a second analysis (results not shown) performed identically, except that non-immune (control) serum replaced PH α 1-1 tumour serum and in which none of the phenylalanine hydroxylase polypeptides was detected. A small amount of phenylalanine hydroxylase protein remained insoluble at the top of the isoelectric-focusing gel (enclosed by a circle). The positions for molecular-weight markers are indicated: CT (catalase, mol.wt. 57 000) and OA (ovalbumin, mol.wt. 45 000). The basic end of the first-dimensional isoelectric-focusing ('IEF') gel where sample was loaded is on the left. The second dimension was carried out in SDS/polyacrylamide (9%) gel ('SDS'). (b) Close-up of (a) showing the phenylalanine hydroxylase region. The higher-molecular-weight phenylalanine hydroxylase polypeptides are designated 'H', whereas the lower-molecular-weight polypeptides are designated 'L'.

precipitation with an enzyme preparation that had been purified to contain less than 0.3% of contaminants (Choo *et al.*, 1979) (Figs. 5a–5c). None of the polypeptide spots designated as phenylalanine hydroxylase could be detected on gels in similar experiments carried out with non-immune control serum (results not shown). The two subunit molecular weights (designated H and L in Fig. 4) were estimated to be approx. 50000 and 49000 respectively by using a low-molecular-weight-marker calibration kit obtained from Pharmacia. At least three 'H' polypeptides and four 'L' polypeptides differing in charges could be observed. Thus on two-dimensional polyacrylamide-gel electrophoresis under dissociating conditions, monkey phenylalanine hydroxylase purified by immunoprecipitation (or by affinity column) revealed multiple polypeptide forms that could be resolved into two size classes and different charge forms. A similar polypeptide pattern has been observed for human liver phenylalanine hydroxylase (Choo *et al.*, 1979).

When analysed on two-dimensional gels, PH α 1-1 antibody has also been demonstrated specifically to immunoprecipitate phenylalanine hydroxylase from crude extracts of human and rat livers (results not shown). It should be mentioned that when a similar

procedure was carried out with an extract from a phenylketonuric liver [sample designated as M in Choo *et al.* (1979)], none of the polypeptide species with molecular weight corresponding to those of the normal human enzyme nor any altered mutant products could be detected on two-dimensional gels. This result is in agreement with that of previous studies using rabbit antiserum, which showed the absence of cross-reacting material in this phenylketonuric liver sample (Choo *et al.*, 1979; 1980). It also provides independent evidence that hybrid PH α 1-1 produces a specific antibody against normal phenylalanine hydroxylase.

Discussion

A stable line of a myeloma-spleen cell hybrid producing antibody against monkey liver phenylalanine hydroxylase has been isolated in the present study. The frequency (1 in 314) of antibody-producing clones is rather low compared with those reported by other workers. A possible explanation is that our screening method is not sufficiently sensitive to detect clones producing antibodies that cross-react with the enzyme but result in little or no alteration to enzyme activity. Our observed frequency may, therefore, be an underestimation.

Evidence to indicate that hybrid PH α 1-1 secretes antibody directed against phenylalanine hydroxylase has come from the following experiments.

(a) In enzyme-inhibition assays, a progressive loss of phenylalanine hydroxylase activity was observed when extracts of monkey, human, rat and mouse livers were incubated with increasing amounts of the antibody; this activity loss could be completely blocked if the antibody-containing solution was preincubated with IgG Sorb or rabbit anti-mouse immunoglobulins.

(b) In double-immunodiffusion experiments, hybrid-PH α 1-1 antibody reacted with both purified and crude liver phenylalanine hydroxylase to form a single precipitin line. The joining of the precipitin lines formed by the four mammalian enzyme species suggests the existence of a common antigenic determinant on these enzyme species that is recognizable by the monoclonal antibody.

(c) In immunoprecipitation experiments, the presence of a great excess of non-specific proteins from a crude liver extract (of monkey, human or rat) did not interfere with the formation of specific phenylalanine hydroxylase-antibody complex as seen by the results of two-dimensional gel-electrophoretic analysis. The presence of multiple charge and size species of mammalian phenylalanine hydroxylase was shown previously (Choo *et al.*, 1979) in the study of the human liver enzyme. In the present

Fig. 5. Close-up of phenylalanine hydroxylase region on two-dimensional gels

The samples are immunoprecipitates of: (a) pure monkey liver phenylalanine hydroxylase alone ($\sim 14 \mu\text{g}$); (b) crude monkey liver extract alone ($\sim 17 \text{mg}$); and (c) pure enzyme mixed with crude extract, indicating that the phenylalanine hydroxylase polypeptides immunoprecipitated from the crude extract are identical with those of enzyme purified by an affinity column. Designations for 'H' and 'L' are as in Fig. 4.

study, the use of a monoclonal antibody that enables the immunoprecipitation of the various 'subunit' charge and size species provides further evidence that these molecular species co-exist as multimers in the undissociated enzyme (Choo *et al.*, 1979).

(d) In immunoprecipitation experiments where PH α 1-1 antiserum was allowed to react with a phenylketonuric-liver extract, none of the polypeptide spots designated as phenylalanine hydroxylase in normal extracts, nor any altered mutant polypeptides, could be detected on two-dimensional gels. The lack of cross-reacting material in this phenylketonuric liver was reported in previous studies using polyclonal antibody preparations (Choo *et al.*, 1979, 1980).

These properties of the PH α 1-1 antibody should make it a very useful tool for the study of mammalian phenylalanine hydroxylase. In particular, its use in immunoprecipitation and two-dimensional polyacrylamide-gel-electrophoretic analysis offers speed and specificity of enzyme purification to enable direct studies on the enzyme subunit structure with a small amount of extract materials. In addition, the stability of the antibody-producing property of PH α 1-1, plus the relative ease with which milligram quantities of antibody could be produced in tumour serum or in ascites fluid, have permitted us successfully to synthesize a specific antibody column for the

purification of mammalian phenylalanine hydroxylase (I. G. Jennings & K. H. Choo, unpublished work).

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