Characterization of a monoclonal antibody to bovine xanthine oxidase

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The isolation of a hybridoma cell line, C-41, secreting monoclonal antibody to bovine xanthine oxidase (EC 1.2.3.2), is described. The specificity of this antibody was determined by solid-phase immunoassay, immunoblotting procedures, affinity chromatography, immunoelectrophoresis and precipitation techniques. The results are compared with those obtained in similar specificity studies on a previously described monoclonal antibody secreted by hybridoma cell line A-94 [Mather, Nace, Johnson & Goldsby (1980) *Biochem. J.* **188**, 925–928]. This latter antibody appears to bind to xanthine oxidase only when the enzyme is immobilized on a solid support such as a plastic plate or nitrocellulose paper. Potential problems in the determination of the specificity of monoclonal antibodies, especially towards membrane proteins of unknown biological activity, are discussed.

Xanthine oxidase (EC 1.2.3.2) is present in comparatively large quantities in the mammary tissue and milk of many species (Jenness, 1974; Heid, 1983). A fraction of the enzyme in milk is associated with milk-fat globules in both a soluble and a membrane-bound form (Briley & Eisenthal, 1974; Mather *et al.*, 1977; Bruder *et al.*, 1982). In mammary tissue a large proportion of the enzyme is soluble (Bruder *et al.*, 1982; Mather *et al.*, 1982), and by using immunofluorescence procedures can be detected in both secretory epithelial and capillary endothelial cells (Jarasch *et al.*, 1981; Bruder *et al.*, 1982). In all other tissues examined (e.g., heart, lung, liver, intestine), xanthine oxidase appears largely concentrated in capillary endothelial cells (Jarasch *et al.*, 1981; Bruder *et al.*, 1982).

Since immunofluorescence techniques are qualitative, a solid-phase immunoassay was devised to measure the levels of xanthine oxidase in various tissues and cultured cell lines (Bruder *et al.*, 1983). This assay required two monospecific antibodies raised in different species to the antigen. For con-

venience we therefore elected to use both polyclonal rabbit antisera and monoclonal murine antibodies to bovine xanthine oxidase. Previously described monoclonal antibodies to xanthine oxidase (Mather *et al.*, 1980b) were found to be of questionable specificity. We therefore isolated a new hybridoma cell line secreting monoclonal antibodies to the enzyme and rigorously determined the specificity of this antibody. The present paper describes the results of these studies and discusses in more general terms potential problems in the determination of the specificity of monoclonal antibodies, especially to membrane proteins of unknown biological activity.

Materials and methods

Materials

All materials for electrophoresis, chromatography and the isolation of hybridoma cell lines were as described previously (Mather *et al.*, 1980b, 1982). In addition, acrylamide and bisacrylamide were obtained from Serva (Heidelberg, Germany), and nitrocellulose paper for immunoblotting procedures was from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

All antibody preparations were from Miles-Yeda Ltd. (Rehovot, Israel, or Elkhart, IN, U.S.A.), except for some samples of goat or guinea-pig anti-(mouse IgG) serum used for immunoblotting, which were obtained from Behring

Abbreviations used: SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

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Werke (Marburg, Germany) and Sigma Chemical Co. (St. Louis, MO, U.S.A.) respectively. Protein A labelled with ^{125}I was obtained from New England Nuclear Corp. (Dreieich, Germany) and protein-A-containing cells of *Staphylococcus aureus* were from Sigma Chemical Co.

Methods

Assays. Xanthine oxidase was assayed spectrophotometrically by measuring the conversion of xanthine to uric acid at 295 nm. Incubation conditions were as described previously (Mather *et al.*, 1980a). A unit of enzyme activity was defined as the amount of enzyme required to oxidise 1 μmol of xanthine to uric acid under the conditions of assay. Xanthine oxidase in immunoprecipitates in agarose gels was detected by a histochemical procedure (Mather *et al.*, 1980a).

Antibody to xanthine oxidase was measured by a solid-phase enzyme-linked immunoassay employing alkaline phosphatase-conjugated rabbit antibody to mouse IgG, essentially as described by Mather *et al.* (1982).

Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Electrophoresis. Samples were separated in slabs of SDS/polyacrylamide gel by the method of Laemmli (1970). M_r standards, from Bio-Rad Laboratories, were as follows: myosin (EC 3.6.1.3, M_r 200 000), β -galactosidase (EC 3.2.1.23, M_r 116 000), phosphorylase *b* (EC 2.4.1.1, M_r 92 500), bovine serum albumin, M_r 68 000, ovalbumin, M_r 45 000, carbonic anhydrase (EC 4.2.1.1, M_r 31 000), soya-bean trypsin inhibitor, M_r 21 500, and lysozyme (EC 3.2.1.17, M_r 14 400). Proteins were stained with Coomassie Blue as described by Mather *et al.* (1980a), and carbohydrate-containing components were stained with the periodic acid/Schiff reagent by the procedures of Fairbanks *et al.* (1971).

Immunization of mice. Xanthine oxidase was purified from bovine milk as described by Sullivan *et al.* (1982). Female BALB/cJ mice (14 weeks old) were primed by the intraperitoneal injection of 100 μg of purified xanthine oxidase that had been emulsified with Freund's complete adjuvant. Mice were given booster injections of 100–200 μg of purified xanthine oxidase, without adjuvant, at 3½, 5, 6, 8, 12, 13 and 14 months after the primary injection. On the last two of these occasions the enzyme was injected as a precipitate adsorbed on $\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$. A final booster injection of soluble xanthine oxidase was administered 3 days before the hybridization.

Hybridization and culturing techniques. Spleen cells from two immunized mice (2.09×10^8 lymphocytes) were fused with mouse myeloma

cells (7.34×10^7) of the SP-2/0 cell line (Shulman *et al.*, 1978), with 52% (w/v) poly(ethylene glycol) as the fusing agent.

After hybridization of the spleen and SP-2/0 cells, the cells were centrifuged and resuspended in 65 ml of hypoxanthine/aminopterin/thymidine (HAT)-supplemented medium (Littlefield, 1964) to obtain selective growth of hybrid cells. The basal medium consisted of Dulbecco's modified Eagle's medium (containing 4.5 mg of glucose/ml), RPMI-1640 (Gibco, Grand Island, NY, U.S.A.) and foetal bovine serum (9:9:2, by vol.), supplemented with glucose (4.5 mg/ml), NaHCO_3 (2.85 mg/ml), 2 mM-L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg /ml). Part of the cell suspension was further diluted to one-third and one-ninth of the initial cell density, and each of these suspensions (representing three different cell densities) was distributed in 0.1 ml portions into the wells of three 96-well tissue-culture plates. To each well of the 96-well plates was also added 0.1 ml of a 'feeder' suspension of normal BALB/cJ mouse spleen cells (10^6 cells/ml) in basal medium. Cells were maintained in a humidified atmosphere of CO_2/air (1:19).

When colonies of cloned hybridomas became visible, supernatants from these cultures were assayed for the presence of monoclonal antibody against xanthine oxidase by solid-phase enzyme-linked immunoassay, as described above. Of 180 clones tested, one cell line (C-41) was isolated which remained stable and continued to secrete anti-(xanthine oxidase) antibody during passage in culture. This cell line was subcloned three times by limiting dilution and grown as solid tumours in BALB/cJ mice. C-41 monoclonal antibody was harvested from supernatants of cell cultures and from the plasma of mice bearing tumours of the C-41 cell line. Antibody C-41 was determined to be IgG_{2a} (κ -light chain) by immunodiffusion in agarose gels.

Procedures for the isolation of clones A-94, -113, -155 and -166 were described previously (Mather *et al.*, 1980b). The isotype of the antibodies secreted by all these clones was determined to be IgG₁ (κ -light chain).

Isolation of fractions. Bovine milk-fat-globule membrane was isolated either by the method of Jarasch *et al.* (1977) or Mather (1978). Soluble fractions from bovine milk-fat globules and bovine liver, lung and mammary tissue were obtained as described by Jarasch *et al.* (1981).

Determination of specificity of isolated monoclonal antibodies. Immunoblotting procedures. Samples of SDS-solubilized protein were separated by electrophoresis in 1.5 mm-thick slabs of polyacrylamide gel. After electrophoresis the gels were rinsed with distilled water and incubated for 2 h in

10mM-Tris/HCl buffer, pH7.5, containing 4M-urea, 2.2mM-EDTA, 50mM-NaCl and 0.1mM-dithiothreitol. After washing for 30min in 25mM-Tris/glycine buffer, pH8.9, the separated proteins were electrophoretically transferred in the same buffer to sheets of nitrocellulose paper, essentially as described by Towbin *et al.* (1979). The paper was then incubated overnight in phosphate-buffered saline (10mM-KH₂PO₄/Na₂HPO₄ buffer, pH7.4, in 150mM-NaCl), containing 1% (w/v) bovine serum albumin. The nitrocellulose was then incubated for 1 h at room temperature with antibody (as specified in the legend to Fig. 2) in 10ml of phosphate-buffered saline containing 2% (w/v) bovine serum albumin. Excess antibody was removed by 30min washes in phosphate-buffered saline containing consecutively 0.5% (v/v) Triton X-100, 0.1% (v/v) Triton X-100, 0.5M-NaCl, no additions, and then 0.1% (w/v) bovine serum albumin. Monoclonal antibody bound to the paper was detected by incubating for 2 h with anti-mouse antibodies from rabbit, goat, or guinea pig in phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin. Unbound antibody was removed by washing with phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin (for 30min), 0.1% (v/v) Triton X-100 (for 30min), 0.5% (v/v) Triton X-100 (overnight), 0.5M-NaCl (for 30min) and finally twice with phosphate-buffered saline for 30min each time. The anti-mouse second antibody was then detected by incubating for 2 h with ¹²⁵I-labelled protein A (1μCi) in 0.1% (w/v) bovine serum albumin. Unbound protein A was removed by the same washing cycle as above (from the second antibody onwards). The paper was then dried and exposed at -80°C to X-ray film (Kodak safety film, ARO; Eastman Kodak Co., Rochester, NY, U.S.A.). After appropriate exposure times the X-ray film was developed by standard procedures.

In some experiments the initial washing step with buffered urea solutions was omitted, and for some immunoblots either ¹²⁵I-labelled monoclonal antibodies were used directly in a one-step procedure or the anti-mouse second antibody was labelled with ¹²⁵I and the protein A step omitted. Antibodies were labelled with ¹²⁵I by the procedure of Hunter & Greenwood (1962).

Affinity chromatography. Monoclonal antibodies A-94 and C-41 were purified from mouse plasma by preparative electrofocusing essentially as described by Mather *et al.* (1980a), with the omission of Triton X-100 from both the samples and the bed of Ultradex. The purified antibodies (3–4mg) were coupled to CNBr-activated Sepharose 4B as described previously (Mather *et al.*, 1982) and the immunoadsorbents equilibrated with 50mM-Tris/HCl, pH7.5, at 4°C. To determine the

specificity of these immobilized antibodies, samples of bovine milk-fat-globule membrane solubilized with Triton X-100 (Mather *et al.*, 1980a) were fractionated on the immunoadsorbents. The columns were washed with 70ml of 50mM-Tris/HCl, pH7.5, containing 154mM-NaCl and 0.1% (v/v) Triton X-100 and specifically bound material eluted with 3M-NaSCN. After removal of the NaSCN by dialysis overnight against 50mM-Tris/HCl, pH7.5 at 4°C, the samples were analysed by electrophoresis in SDS/polyacrylamide gels.

Immunotitration. Portions of purified xanthine oxidase (100μg) were incubated with various dilutions of plasma from control animals or from mice bearing tumours of cell lines A-94 or C-41 in 50mM-Tris/HCl, pH7.5, containing 154mM-NaCl, 0.1% (w/v) bovine serum albumin and 0.1% (w/v) NaN₃ in a final volume of 1.5ml. The mixtures were incubated at 37°C for 60min and then for 36 h at 5°C. After centrifugation at 12000g for 30min at 5°C, 200μl samples of the supernatants were assayed for xanthine oxidase activity. Goat anti-(mouse IgG) serum (Miles-Yeda, 3.3mg/ml) was then added in a final ratio (v/v) of mouse plasma to goat serum of 20:1. Mixtures (1.5ml final volume) were incubated at 37°C for 60min and then for 24 h at 5°C. A suspension of *S. aureus* cells containing protein A (10mg of dried cells in 100μl of phosphate-buffered saline) was then added and the mixtures incubated at 37°C for 60min and at 5°C for 5h. After centrifugation as described above, 290μl portions of the supernatants were taken for enzyme assay.

Gel filtration. Samples of purified xanthine oxidase (133μg, 0.63 unit) in 0.4ml of 10mM-Tris/HCl, pH7.5, containing 154mM-NaCl and 0.01% (w/v) NaN₃ were mixed with 0.4ml of either control or plasma samples as specified in the legend to Fig. 5. The mixtures were incubated at 37°C for 30min and then separated on a column (100cm × 1.8cm) of Ultrogel AcA 22 equilibrated with the above-mentioned Tris-buffered saline at 4°C. The eluate was collected in 2.0ml fractions, and 0.2ml samples were assayed for xanthine oxidase activity.

Immuno-electrophoresis. 'Rocket' immuno-electrophoresis with or without an intermediate gel containing additional antibodies was performed as described by Weeke (1973) and Axelsen (1973). Samples were analysed in 1% (w/v) agarose gels of 1.5mm thickness on glass plates (1.5mm × 100mm × 100mm). The gels contained 0.25% (v/v) Triton X-100 and 20mM-Tris/50mM-glycine buffer adjusted to pH8.9 with NaOH. Electrophoresis was conducted overnight at 1–2V/cm in a Bio-Rad immuno-electrophoresis cell (model 1400). Immunoprecipitates in the agarose gels were stained histochemically for xanthine

oxidase activity (see above) and then counter-stained with Coomassie Brilliant Blue R-250 (Weeke, 1973).

Results

We previously described the isolation of four hybridoma cell lines that secreted monoclonal antibodies which bound to xanthine oxidase immobilized on plastic plates (Mather *et al.*, 1980b). These cell lines were derived by the fusion of SP-2/0 cells with spleen cells from mice immunized with unfractionated milk-fat-globule membrane. Clones secreting antibodies apparently specific for xanthine oxidase were subsequently selected by using a solid-phase immunoassay with purified xanthine oxidase as the test antigen. These monoclonal antibodies (from cell lines A-94, -113, -155 and -166; Mather *et al.*, 1980b) also apparently bound to xanthine oxidase after separation of milk-fat-globule-membrane proteins by electrophoresis in SDS/polyacrylamide gels (C. S. Kaetzel & I. H. Mather, unpublished work). Further tests, however, conducted largely with antibody A-94, gave equivocal results.

A second hybridization was undertaken by using spleen cells from mice immunized with purified xanthine oxidase. The purified antigen was also used in the solid-phase immunoassays employed for the selection of potentially positive clones. One such clone, C-41, was stabilized and the specificity of the antibody secreted by this cell line compared with that of antibody A-94.

Immunotitration of plasma from mice bearing tumours of cell lines C-41 and A-94 showed that both monoclonal antibodies bound to purified xanthine oxidase (Fig. 1). In comparison, monoclonal antibody A-143, which is specific for a glycoprotein of approx. M_r 100000 (C. S. Kaetzel & I. H. Mather, unpublished work), showed no reaction.

The specificity of antibodies C-41 and A-94 was examined further by immunoblotting procedures after transfer of electrophoretically separated mixtures of proteins to nitrocellulose paper (Fig. 2). Antibody C-41 bound to purified xanthine oxidase (Fig. 2d, lane 2) and a component of similar M_r present in bovine milk-fat-globule membrane (Fig. 2d, lane 1). A similar result was obtained with antibody A-94 (Fig. 2c). Both antibodies also bound to a protein of approx. M_r 155000 in immunoblots of the postmicrosomal supernatants from liver, lung and lactating or non-lactating mammary tissue (results not shown). If the gels were not pretreated with 4M-urea solutions before electrophoretic transfer of the proteins to nitrocellulose, antibody A-94 bound to xanthine oxidase and a second component with an approx.

Fig. 1. Immunotitration of plasma from mice carrying antibody-secreting tumours C-41, A-94 and A-143

Plasma samples from mice bearing tumours of cell lines C-41 (●), A-94 (○) and A-143 (▲) were diluted down to 1:256000 and various dilutions were tested by solid-phase enzyme-linked immunoassay using plastic plates coated with purified xanthine oxidase (50 μ l/well; 100 μ g/ml). Samples were incubated with substrate for 2h before terminating the reaction.

M_r of 200000 (Fig. 2e). This material was apparently present in trace amounts even in our purified preparations of xanthine oxidase (compare lanes 2 of Figs. 2a and 2e). We also observed occasional binding, at low levels of antibody C-41, to the same component. Essentially similar results were obtained if the protein A step was omitted and 125 I-labelled A-94 and C-41 antibodies were used directly or if 125 I-labelled anti-(mouse Ig) antibodies were used as a second antibody (results not shown).

Interpretation of immunoblots was complicated by the binding of control IgG to xanthine oxidase (Fig. 2b). This observation could be explained by the presence of autoantibodies against xanthine oxidase in all the sera tested from a number of species (e.g. mouse, guinea pig, goat and human; G. Bruder, unpublished work). Interference from these antibodies was particularly marked with immunoblotting procedures coupled with autoradiography, owing to the sensitivity of the methods employed.

Fig. 2. Specificity of antibodies C-41 and A-94 determined by immunoblotting procedures

SDS-solubilized protein samples were separated by electrophoresis in SDS/(6%, w/v)-polyacrylamide gels and transferred to nitrocellulose paper. The proteins adsorbed to the nitrocellulose paper were incubated with either (b) a 200-fold dilution of IgG from control mice, (c) a 100-fold dilution of A-94 antibody, (d) a 400-fold dilution of C-41 antibody or (e) a 60-fold dilution of A-94 antibody. Bound antibodies were detected with anti-(mouse IgG) (all subclasses) diluted from 1- to 2-fold more than the first antibody, followed by ^{125}I -labelled protein A (approx. $1\ \mu\text{Ci}$). Gels (b)–(d) were washed in buffered 4M-urea solutions before transfer of the proteins (see the Materials and methods section). In (e) this step was omitted. Samples were: 1, bovine milk-fat-globule membrane ($180\ \mu\text{g}$); 2, purified xanthine oxidase ($10\ \mu\text{g}$). A Coomassie Blue-stained gel of these fractions is shown in (a). M_r values for the two major components detected by the antibodies (arrowheads) were determined, by reference to protein standards, to be approx. 200000 and 155000.

Because of these ambiguous data we fractionated detergent-solubilized samples of bovine milk-fat-globule membrane on immunoabsorbents prepared by covalently linking either antibody C-41 or A-94 to CNBr-activated Sepharose 4B. Specifically absorbed material was eluted with a chaotropic agent and the eluates analysed by electrophoresis in SDS/polyacrylamide gels (Fig. 3). Antibodies specific for xanthine oxidase should recognize a protein in detergent extracts with an M_r of 155000 (black arrowhead in Fig. 3; see also Waud *et al.*, 1975) which does not stain with the periodic acid/Schiff reagent (see, e.g., Sullivan *et al.*, 1982). This result was obtained with antibody C-41 (Fig. 3, lanes 5 and 6). Since we used denaturing conditions (3M-NaSCN) for elution of specifically bound material from the immunoabsorbents, we were unable to detect xanthine oxidase activity in the collected fractions. However, this material, as would be expected, bound to affinity-purified rabbit antibodies to xanthine oxidase in solid-phase assays (results not shown). In contrast with these results, antibody A-94 bound to a periodic acid/Schiff-positive protein of higher M_r which did not stain with Coomassie Blue (open arrowhead in Fig. 3, lanes 3 and 4).

The specificity of antibody C-41 was confirmed

by further tests which depended on the detection or measurement of enzyme activity after the addition of antibody. Titration of antibody C-41 against purified xanthine oxidase resulted in precipitation of the enzyme (Fig. 4). Xanthine oxidase activity could be completely removed from the supernatant by addition of goat anti-(mouse IgG) serum as a second antibody, followed by protein A-containing *S. aureus* cells. That the enzyme was removed from the supernatants and not merely inactivated in solution by the C-41 antibody was confirmed by rocket immunoelectrophoresis (results not shown). Under similar conditions, no precipitation of xanthine oxidase was observed with A-94 antibody.

To confirm that, in solution, antibody A-94 does not bind to purified xanthine oxidase, the antibody was incubated with enzyme and the mixture separated by filtration through a column of Ultrogel. Xanthine oxidase activity was eluted in the same volume of buffer as that for enzyme previously incubated either with plasma from control mice or with buffer solutions containing no plasma (compare Figs. 5a, 5c and 5d). In contrast, enzyme activity was eluted in a smaller volume after incubation of antibody C-41 with xanthine oxidase (Fig. 5b). Apparently, binding of antibody

Fig. 3. Immunoadfinity chromatography of detergent-solubilized milk-fat-globule-membrane proteins on C-41 and A-94 antibodies covalently linked to Sepharose 4B

Peak fractions eluted from the immunoadsorbents with 3M-NaSCN were analysed by electrophoresis in SDS/polyacrylamide gels (10%, w/v) and compared with total milk-fat-globule membrane. Gels were either stained with the periodic acid/Schiff reagent (lanes 1, 3 and 5), or with Coomassie Blue (lanes 2, 4 and 6). Samples were as follows: milk-fat-globule membrane (lanes 1 and 2); eluate from A-94-Sepharose 4B (lanes 3 and 4); eluate from C-41-Sepharose 4B (lanes 5 and 6).

to enzyme led to the formation of enzymically active aggregates of higher M_r than that of the free enzyme.

The ability of antibody C-41 to bind to xanthine oxidase was further demonstrated by immunoelectrophoresis in which the purified enzyme, at appropriate concentrations, was forced into agarose containing C-41 antibodies. Two precipitates were formed, both of which contained xanthine oxidase activity (Fig. 6). Under similar conditions antibody A-94 did not precipitate the enzyme (results not shown). We therefore subjected detergent-solubilized milk-fat-globule-membrane proteins to immunoelectrophoresis against A-94 antibodies contained in an intermediate gel between the antigen and a second antibody consisting of rabbit anti-(mouse IgG). Soluble complexes between the antigen and A-94 antibodies formed in

Fig. 4. Immunotitration of purified xanthine oxidase. Samples of purified xanthine oxidase (100 μ g) were incubated with various dilutions of plasma from either control mice or mice bearing tumours of cell lines A-94 or C-41 in 50mM-Tris/HCl, pH 7.5, containing 154mM-NaCl, 0.1% (w/v) bovine serum albumin and 0.1% (w/v) NaN₃ in a final volume of 1.5ml. Samples were subsequently incubated with goat anti-(mouse IgG) serum and protein A-containing cells of *S. aureus*. Values given in the Figure are the averages for duplicate determinations for enzyme activity remaining in the supernatant. ●, Plasma from control mice; ○, A-94 plasma; △, C-41 plasma.

this intermediate gel and were subsequently precipitated by the rabbit anti-(mouse IgG) antibodies (Fig. 7b). These precipitates, however, contained no detectable xanthine oxidase activity (Fig. 7a). Similar results (not shown) were also obtained with the other putative monoclonal antibodies to xanthine oxidase secreted by clones A-113, -155 and -166 (Mather *et al.*, 1980b).

Discussion

Monoclonal antibodies have several advantages over standard polyclonal antisera, including specificity for a single epitope and the potential for unlimited production of well-characterized antibody over a number of years [see Milstein (1981) and Edwards (1981) for reviews]. Mixtures of many proteins can be used as immunogen, because the

Fig. 5. Gel filtration of purified xanthine oxidase after incubation with control mouse plasma or plasma from mice carrying cell lines C-41 or A-94

Purified enzyme (133 μ g, 0.63 unit) was incubated at 37°C for 1 h with (a) 10 mM-Tris/HCl buffer, pH 7.5, containing 154 mM-NaCl and 0.01% (w/v) NaN_3 ; (b) a 20-fold dilution of plasma containing antibody C-41; (c) a 5-fold dilution of plasma containing antibody A-94, or (d) a 5-fold dilution of plasma from control mice. The mixtures were then separated by gel filtration on Ultrogel AcA 22. Fractions were assayed for xanthine oxidase activity.

Fig. 7. Immuno-electrophoresis of detergent-solubilized milk-fat-globule-membrane proteins against antibody A-94

Triton X-100-solubilized milk-fat-globule membrane (12.5 μ g), contained in the well indicated by a circle in the Figure, was subjected to immuno-electrophoresis into agarose containing a 1:20 dilution of rabbit anti-(mouse immunoglobulin) (all isotypes). An intermediate gel, indicated by a black rectangle in the Figure, contained a 1:3 dilution of plasma from mice carrying cell line A-94. Membrane proteins were forced through this intermediate gel and formed soluble complexes with the mouse antibody, which precipitated in the second agarose gel containing polyclonal rabbit antibodies. Immunoprecipitates were stained for xanthine oxidase activity (a) and then counterstained with Coomassie Blue (b).

Fig. 6. Immuno-electrophoresis of purified xanthine oxidase against antibody C-41

Purified xanthine oxidase was subjected to immuno-electrophoresis into agarose containing a 500-fold dilution of plasma from mice carrying cell line C-41. Immunoprecipitates were stained for xanthine oxidase activity (a) and then counterstained with Coomassie Blue (b). Wells 1-3 contained respectively 2.5, 5.0 and 10 μ g of purified enzyme.

because of the practical difficulties experienced in purifying hydrophobic membrane proteins (see, e.g., Williams *et al.*, 1977; Hughes & August, 1981). However, even if the immunogen used is apparently 'homogeneous', trace contaminants of highly immunogenic material may stimulate mitogenesis of lymphoid cells in the immunized donors that secrete antibody of unwanted specificity. Even with careful cloning and screening procedures, therefore, the specificity of the antibody secreted by selected hybridoma cell lines has to be rigorously determined.

Specificity of antibody A-94 was originally determined by solid-phase immunoassay using both unfractionated milk-fat-globule membrane and highly purified xanthine oxidase either before (Mather *et al.*, 1980b) or after the separation of antigen mixtures by SDS/polyacrylamide-gel electrophoresis (C. S. Kaetzel & I. H. Mather, unpublished work). When we used gels containing a

cloning and screening procedures employed allow the selection of cell lines secreting antibody specific solely for the antigen of interest. This has proved especially useful in membrane biology,

lower percentage of acrylamide and immunoblotting procedures (Towbin *et al.*, 1979), a second component, with an approximate M_r of 200000, was detected which also bound to antibody A-94 (Fig. 2e). Binding of antibody to this component was variable, and if the gels were pretreated with buffered solutions of 4M-urea, binding was almost completely abolished, although the antibody still recognized xanthine oxidase (Fig. 2c).

From solid-phase assays (Fig. 1) and immunoblotting procedures under certain conditions (Fig. 2c), antibody A-94 appears to be specific for xanthine oxidase. These tests are the most frequently used methods for determining the specificity of monoclonal antibodies, especially with membrane proteins of unknown biological activity; however, they can give very misleading data. When we used affinity chromatography or tests relying on the detection or estimation of enzyme activity it became obvious that antibody A-94 is not specific for xanthine oxidase. This antibody binds to xanthine oxidase when the enzyme is fixed to a solid phase, e.g., plastic plates or nitrocellulose paper, but apparently not when in solution. The specificity of antibody A-94 therefore remains uncertain, although chromatography of detergent-solubilized milk-fat-globule-membrane proteins on A-94-Sepharose 4B indicated that the antibody binds to a glycoprotein of unknown biological activity (Fig. 3). This is possibly the same as the component of M_r 200000 detected by immunoblotting under certain conditions (Fig. 2e).

In contrast, antibody C-41 bound to xanthine oxidase in all the tests described, including solid-phase assays, immunoblotting procedures and methods designed to facilitate the precipitation of antibody-antigen complexes from solution. The recent successful use of this antibody for the estimation of xanthine oxidase in various cells and tissues (Bruder *et al.*, 1983) also underscores the specificity of this antibody.

Interestingly, two precipitates formed when purified xanthine oxidase was subjected to immunoelectrophoresis against antibody C-41 (Fig. 6). The formation of a precipitate between monoclonal antibodies and xanthine oxidase is possible because the enzyme is a dimer of similar, if not identical, monomers [for discussion of this, see Bray (1975) and Coughlan (1980)]. There should therefore be at least two copies of each epitope on the native protein, thus facilitating the formation of immunoprecipitates. The formation of two distinct precipitates or rockets, however, implies the existence of different variants of the enzyme (for other evidence, see Krenitsky & Tuttle, 1978; Jarasch *et al.*, 1981; Sullivan *et al.*, 1982). Alternatively the enzyme may be present in solution in polydisperse aggregates which react

differently with the antibody. Multiple immunoprecipitates between xanthine oxidase and conventional affinity-purified polyclonal antibodies have been described previously (Sullivan *et al.*, 1982).

On the basis of our experience with the above monoclonal antibodies, we would recommend the use of as many different techniques as possible for the determination of antibody specificity. Although this conclusion may seem obvious, many investigators rely on a limited number of techniques (e.g. immunoassay and immunoblotting procedures), especially with antigens of unknown biological activity. However, this does not preclude the use of immunoaffinity chromatography and precipitation techniques. For the detection of antigens of unknown activity in immunoprecipitates, radiolabelling techniques can be used, followed by solubilization of the labelled precipitated antigen in SDS. The antigen can then be identified by SDS/polyacrylamide-gel electrophoresis and autoradiography (see, e.g., Hughes & August, 1981).

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