A monoclonal antibody against the skeletal muscle enzyme, creatine kinase

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1. INTRODUCTION

Creatine kinase (CK) is often used as an index of differentiation in cultures of embryonic muscle cells [1] and its presence in serum is also used to monitor muscle damage in disease [2,3]. Immunological assays of CK can be made specific for any one of the CK isoenzymes and polyclonal rabbit antisera have been used for the study of muscle-specific CK (MM-CK) accumulation and distribution in differentiating myoblast cultures [4,5]. A monoclonal antibody (McAb) for these assays would have the added advantages of providing an unlimited supply of antibody with defined properties which can be selected for during McAb preparation.

We have prepared clonal mouse cell lines secreting antibody against chick muscle creatine kinase using a polyethylene glycol fusion method [6]. The properties of one of the McAbs, 'CK-JOE', are described here. It has been purified by affinity chromatography, using MM-CK bound to Sepharose, and has also been obtained in high titre by growing intraperitoneal tumours in mice.

Unlike polyclonal anti-CK sera, CK-JOE antibody both fails to precipitate MM-CK in an Ouchterlony double diffusion test and also fails to inhibit CK enzyme activity. In an ELISA assay, it does not cross-react with rabbit MM-CK or with BB-CK from chick. On 'Western'-type blots of SDS-PAGE separations of total SDS-extracted proteins from either early (fusing) or late (myotube) muscle cell cultures, the McAb binds only to a single band co-migrating with authentic MM-CK. Thus, although some McAbs have been found to show unexpected cross-specificities (see [7] for review), McAb CK-JOE does appear to be suitable for measuring MM-CK levels in muscle cell cultures.

2. MATERIALS AND METHODS

2.1. *Immunization*

MM-CK was prepared from 19-day embryonic chick muscle by the method of [8]. Balb/c mice were given 100 μg of MM-CK intraperitoneally in complete Freunds adjuvant, followed by 80 μg subcutaneously in incomplete adjuvant after 4 weeks. Between 2 and 8 months later, and 3 days before fusion, one mouse was given 120 μg of MM-CK in saline, partly intravenously through the tail-vein and partly intraperitoneally. As expected, considerable variation in final antibody titre was observed between individual mice, CK-JOE being obtained from a relatively-low titre mouse.

2.2. *Fusion*

The NS1-Ag 4/1 myeloma line was maintained in RPMI medium with 20% foetal calf or horse serum (selected for high cloning efficiency by limiting dilution without feeder layers), glutamine (2 mM), pyruvate (1 mM), penicillin and streptomycin.

The spleen was removed from the mouse and the cells were removed from the capsule by scraping, washed and resuspended. Spleen cells (10^8 nucleated cells) were mixed with 10^7 NS1 cells in mid-growth phase and treated with 50% polyethylene glycol (PEG 1500; BDH, Poole, Dorset, UK) by the method of [6]. The cells were washed once and incubated in growth medium overnight. They were then resuspended in HAT selective me-

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dium [6] distributed at 0.1 ml per well between the 96 wells of a round-bottomed microtitre plate (NUNC). Fresh growth medium was added on days 8 and 12. By day 17, macroscopic clones were visible in most wells and all wells were screened on day 18.

2.3. *ELISA screening*

Microtitre plates (Dynatech, Billingshurst, Sussex, UK) were coated with chick MM-CK in 0.1 M $NaHCO_3$ (pH 9.6) (15 μg/ml) and blocked with 1% ovalbumin. After exposing the plate to culture supernatants, bound antibody was detected with peroxidase-labelled rabbit anti-mouse Ig (DAKO Antisera) followed by *o*-phenylenediamine and H_2O_2 as substrates.

2.4. *Cloning*

In the fusion which produced CK-JOE, 7 wells were highly positive (20–100 × background) in the ELISA screen. Only two were also positive in an immunofluorescence microscopy test and only these two continued to produce antibody as stable cell lines.

These hybridomas were cloned by limiting dilution in 96-well microtitre plates (NUNC flat-bottomed) on a 1–4 day old feeder layer of Porton mouse peritoneal cells (2×10^4 cells/well), obtained by rinsing out the peritoneal cavity with saline. All wells positive for clonal growth were also positive in the ELISA test. Eight clones were recloned in the same way and two subclones (CK-JOE and CK-ART, one from each of the two original fusion wells) were selected for antibody production.

2.5. *Antibody production*

2.5.1. *Culture supernatants*

Cell cultures were slowly expanded by dilution with growth medium in large glass bottles (200 ml/bottle). They grew to densities of $4–6 \times 10^5$ cells/ml. Culture supernatants, containing about 30 μg/ml of specific antibody, are stored either at 4°C with azide or at −70°C.

2.5.2. *Affinity purification*

MM-CK was bound to CNBr-activated Sepharose 4B as described by the manufacturers (Pharmacia). Culture supernatants were loaded directly onto the column, which was then washed with phosphate-buffered saline before eluting the bound antibody with 0.1 M glycine (pH 2.2).

2.5.3. *Tumour serum and ascites fluid production*

Balb/c mice were injected intraperitoneally with 0.3 ml pristane (Aldrich Chemical Co.) and given $1–2 \times 10^6$ CK-JOE cells intraperitoneally 7–10 days later. Solid tumours were evident after 3 weeks and both serum and ascites fluid contained anti-CK Ig levels of over 2 mg/ml.

3. RESULTS

Analysis of affinity-purified CK-JOE Ig by SDS-PAGE [9] revealed only two bands which co-migrated with a bovine Ig standard (H and L chains). The Ig concentration was obtained by direct protein determination [10]. Anti-CK Ig levels in other McAb preparations were determined by comparison with this purified Ig in an ELISA assay as illustrated in fig.1. An approximate estimate

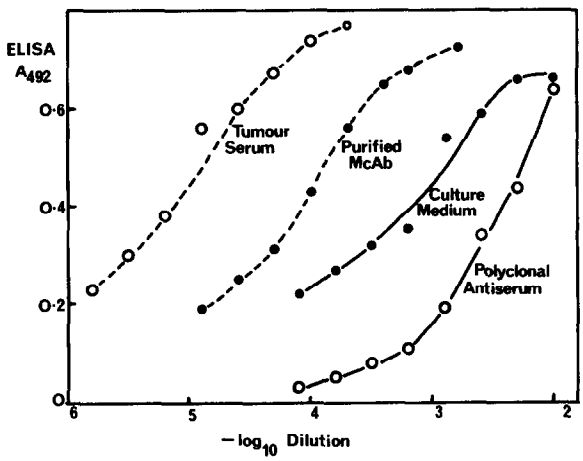

Fig.1. ELISA assay of anti-CK Ig. The assay was carried out as in Methods using serial dilutions of: (a) serum from a mouse carrying a CK-JOE tumour; (b) affinity-purified CK-JOE antibody containing 230 μg/ml Ig; (c) medium from a high-density culture of the CK-JOE clone; and (d) antiserum from a CK-injected mouse similar to that used for the original cell fusion. The 0.15 ml reaction mixture was stopped by the addition of 0.05 ml of 3 M H_2SO_4 and diluted to 2 ml with water. The A_{492} obtained is shown on the abscissa axis. Relative Ig concentrations were estimated by comparing dilutions required to give the same A_{492} reading, using the steepest parts of the curves.

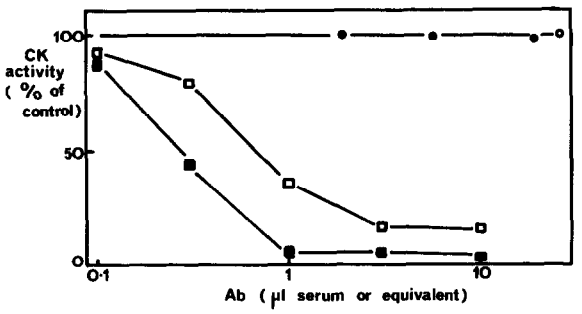

Fig.2. Effect of antibodies on CK enzymic activity. All sera were heated to 56°C for 10 min to inactivate endogenous CK activity. Corrections were applied for any remaining endogenous CK, if necessary. 10 ng of chick MM-CK in 10 μl of assay buffer [17] + 0.05% bovine serum albumin were pre-incubated for 30 min at 25°C with 10 μl of various dilutions of different antibody preparations: CK-JOE tumour serum (open circles), affinity-purified CK-JOE Ig (filled circles), serum from the CK-injected mouse used to prepare CK-JOE cell line (open squares) and a polyclonal rabbit anti-MM-CK serum (filled squares). To make the comparison more meaningful, ELISA data of the type shown in fig.1 were used to normalize McAb concentrations and express them as mouse serum equivalents. CK enzyme assay was as in [17].

of anti-CK Ig levels in polyclonal mouse sera can also be made by assuming their affinities are the same as CK-JOE antibody. Thus, specific antibody levels in serum from the tumour-bearing mouse in fig.1 were over 100× higher than in the culture supernatant and 10× to 500× higher than in polyclonal mouse serum, depending on the individual mouse.

Figure 2 shows that CK-JOE antibody does not inhibit CK enzyme activity, even when applied in considerable excess over the amounts of rabbit or mouse polyclonal antisera required for almost complete inhibition. Even a 1000-fold excess of CK-JOE antibody produced less than 5% inhibition of enzyme activity (results not shown), whereas 50% inhibition of 10ng of CK required less than 6 ng of anti-CK Ig from the polyclonal mouse serum (an approximate calculation based on figs.1 and 2 and with no allowance for affinity differences). Extension of the antibody–antigen preincubation period by 24 h at 4°C also failed to reveal any specific enzyme inhibition by CK-JOE antibody. In fig.2, ELISA antibody titres from fig.1 were used to normalize the ordinate axis to that of mouse antiserum, except for the rabbit antiserum, the higher inhibitory activity of which may be due to a higher titre.

Figure 3 shows that CK-JOE antibody does not precipitate MM-CK in an Ouchterlony double diffusion test, in contrast to both rabbit anti-CK serum and polyclonal antiserum from the mouse used for the fusion.

To show that the McAb is directed against CK (rather than some contaminant of the antigen) and *only* against CK, purified antigen and SDS-extracts of muscle cell cultures at various stages of differentiation were separated by SDS-slab gel electrophoresis and transferred to nitrocellulose sheets by a Western blotting method [11]. CK-JOE antibody binding was detected by peroxidase-labelled second antibody and was found only in the

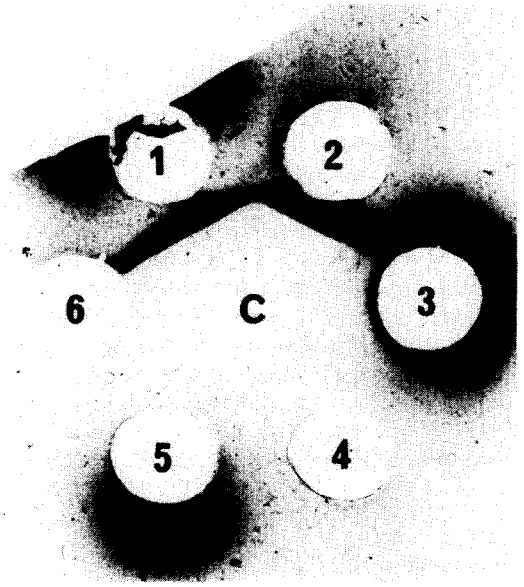

Fig.3. Ouchterlony double-diffusion precipitation tests. Centre well, C: 2.5 μg of purified chick MM-CK. Outer wells: (1) polyclonal mouse anti-CK serum (from mouse used for fusion); (2) polyclonal rabbit anti-CK serum; (3) serum from mouse carrying CK-JOE tumour; (4) 1 in 7 dilution of well 3; (5) ammonium sulphate-concentrated McAb from a different clone, CK-ART; and (6) 1 7 dilution of well 5. 1% agarose gels were incubated at 4°C for 48 h, washed in phosphate-buffered saline for 48 h, dried and stained with Coomasie Blue.

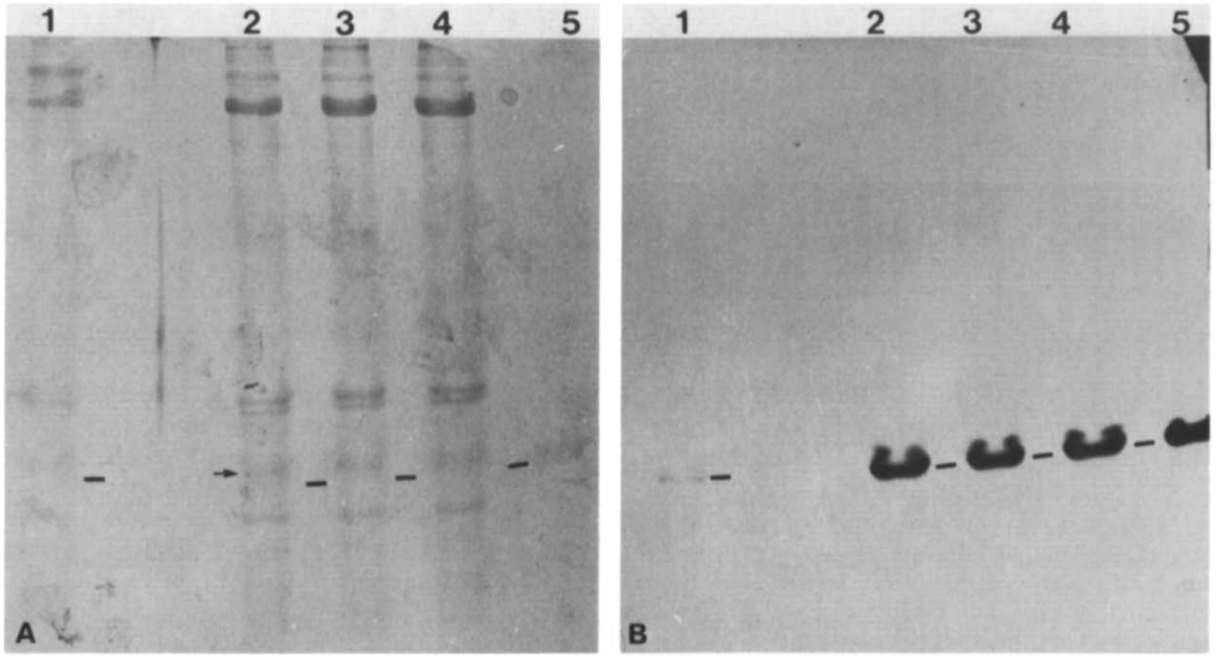

Fig.4. Specificity of CK-JOE antibody binding to proteins in muscle cell extracts. Muscle cell cultures prepared as in [18] were taken at different stages of development, rinsed in saline and frozen at −20°C. Protein was extracted with 0.2 ml of 9 M urea, 1% SDS, 5% 2-mercaptoethanol, 0.1 mM phenylmethylsulphonylfluoride (PMSF). The extracts were centrifuged at 200 000 × *g* for 1 h to remove DNA and, after sampling for protein estimation [10], boiled for 2 min and adjusted with 8× sample buffer for SDS-PAGE [9]. Equal amounts of total protein were loaded onto a 10% polyacrylamide slab gel with a 3% stacking gel as follows: lane (1) 48 h culture, (2) 96 h, (3) 120 h, (4) 144 h, (5) 1 μg of chick MM-CK standard. After electrophoresis, the gel was blotted for 36 h onto nitrocellulose paper (Schleicher and Schull) with a 4 M urea step as in [11]. The blot was then incubated at 25°C for 30 min with a 1/50 dilution of CK-JOE culture supernatant in phosphate-buffered saline (pH 7.2), containing 0.3% bovine serum albumin, washed 3× with the same buffer, incubated for 30 min with 1/1000 dilution of peroxidase-labelled rabbit anti-mouse Ig (DAKO Antisera), washed again and incubated with 0.4 mg/ml diaminobenzidine, 0.012% H_2O_2 in phosphate/citrate buffer (pH 5.0). The original gel, stained with Coomasie Blue after blotting, is shown in (A), alongside the nitrocellulose blot (B). Except that very high molecular weight bands are transferred less efficiently, the stained gel pattern in (A) is similar to that of a duplicate blot stained with amido black as in [11]. In (B), the large increase in MM-CK between 48 h and 96 h is characteristic of the muscle differentiation occurring over this period [1,12]. Bars in (A) and (B) indicate the position of MM-CK and the arrow in (A), lane 2, indicates the actin position.

band co-migrating with the 43 000 dalton M-CK subunit, just ahead of the 45 000 dalton actin band on the stained gel (fig.4). This method probably depends on the refolding of the proteins on SDS-gels into their native conformations and CK is evidently able to do this. The possibility that CK-JOE cross-reacts with a protein which is unable to refold in this way has been investigated by performing the same blotting experiment as fig.4 with Triton X-100 cell extracts and non-denaturing isoenzyme gels described in [12]. In this case, CK-JOE antibody bound only to MM-CK and to the MB intermediate isoenzyme which also contains the M-subunit [12], together with some diffuse high molecular weight staining which could be due to CK aggregation. Since less than 40 ng of MM-CK on the original slab gel can be detected by this method and 60 μg of total protein were loaded onto each lane in fig.4, any other cross-reacting antigenic determinant of similar affinity must constitute less than 0.06% of the total protein.

To test for cross-reaction with rabbit MM-CK (Boehringer) and chick BB-CK isoenzyme, prepared from chick brain and partially-purified to

the Mg^{2+} precipitation step in [8], these proteins were bound to ELISA microtitre plates at concentrations of 15 μg/ml and 50 μg/ml respectively. No specific binding of CK-JOE antibody to these proteins was observed.

4. DISCUSSION

Recent reports have shown that McAbs can display unexpected cross-reactions, often with apparently quite unrelated antigens [7], leading to concern that McAbs may not be as useful as research tools as had originally been hoped. Although we have not tested every conceivable antigen, the results presented here have not revealed any such problems with our McAb against chick CK, at least insofar as its use in measuring M-CK in muscle extracts is concerned. In 'Western' blots of muscle cell extracts (fig.4), CK-JOE antibody was detectably bound to the M-subunit of CK only, showing that the McAb does not cross-react with any protein constituting more than 0.06% of the total extract. It remains possible either that some antigens are not extracted, even by 1% SDS, or that they do not refold into the necessary conformation for antibody binding, or that they transfer to the nitrocellulose paper much less efficiently than CK. The refolding problem has been avoided by using non-denaturing gels, though presumably at the expense of less complete extraction from the cells. A further observation in favour of specificity is that comparison of CK-JOE antibody and rabbit anti-CK serum in the staining of myoblast cultures by immunofluorescence microscopy has so far given identical results, under a variety of conditions, in terms of the pattern of staining both between cell types (failure to stain fibroblasts, etc.) and within myotubes (unpublished results).

It is not surprising that our monoclonal antibody did not cross-react with chick BB-CK, since the two isoenzymes differ considerably in amino acid composition and polyclonal rabbit antisera against the two isoenzymes do not cross-react with each other [4,5,13]. The lack of cross-reaction with rabbit CK is rather less predictable, since amino acid differences between species are generally less than between MM and BB [14]. Thus, an anti-rabbit MM-CK serum has been described which does not react with avian CK [15], whereas some anti-chick MM-CK sera do appear to react with mammalian CKs [13].

Antibodies specific for a single determinant on a protein will not precipitate it, since there is no opportunity for antibody–antigen lattice formation. MM-CK, however, is a dimer with identical subunits [12], so precipitation by a McAb is at least possible. That it does not occur in practice (fig.3) can be explained by conformational or steric restrictions on antibody-binding or lattice formation.

Conformation changes in CK are known to occur and the enzyme is thought to undergo a conformational change on binding its substrates [14]. Since CK-JOE antibody does not inhibit CK activity (fig.2), then either the presence of bound antibody does not prevent this conformational change or substrate-binding is able to reverse antibody-binding. It is known that substrate-binding will at least protect CK against inhibition by certain polyclonal antisera [16], but reversal of antibody-binding would have to be remarkably rapid and complete to explain the present results.

One possibility that must be considered is that the ELISA screening method is selecting for McAbs with the properties described here. However, when we tested 96 hybridoma supernatants (two of which were ELISA-positive) for inhibition of CK activity, we found no inhibition and, when 21 hybridoma supernatants from a different fusion were checked by immunofluorescence microscopy on muscle cell cultures, only those which were positive in the ELISA test gave positive immunofluorescence. Although not exhaustive, there is no indication from these observations that ELISA might fail to detect certain McAbs.

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