PRODUCTION AND CHARACTERISATION OF ANTI-CARDIAC TROPONIN-I MONOCLONAL ANTIBODIES

KH.H. HAIDER* AND W.H. STIMSON†

*Faculty of Pharmacy, University of the Punjab, Lahore, Pakistan

†Department of Immunology, University of Strathclyde, The Todd Centre, Glasgow, U.K.

SUMMARY

Cardiac troponin-I (cTn-I) was isolated from bovine left ventricular tissue and used as immunogen. Sixteen murine hybridoma lines were produced with two of them, 1D12 and 5F4, showing a high specificity for cTn-I; both of these monoclonal antibodies (McAbs) were isotypized as IgG₁ with kappa - light chains. The specificity of the McAbs for cTn-I was confirmed by ELISA, western blotting and by the ability of the antibodies to block actomyosin ATPase inhibition by cTn-I. The McAbs may be useful for human *in vivo* imaging of myocardial infarcts and other pathological conditions related to cardiac myocyte damage.

KEY WORDS Cardiac troponin-I Monoclonal antibodies Myocardial infarcts
 Myocardial damage

INTRODUCTION

The advent and advancement of thrombolytic therapy has significantly affected the management of patients with acute myocardial infarction (AMI) (Verstraete, 1992). The familiar triad of clinical history, ECG and serum enzyme analysis, is still of some significance in the diagnosis of MI (Willerson, 1989). However, none of these criteria reveal quantitative and precise information regarding the extent and location of the infarct. Also, although studies have been made to correlate infarct size with changes in the blood concentrations of the marker molecules released from necrosing myocytes they have met with little success (Serrano *et al.*, 1990; Vaananen *et al.*, 1990; Doran and Fleck, 1990; Prager *et al.*, 1992).

The physical destruction of the dying cardiac cells is an irreversible phenomenon which causes a loss in the ability of the cells to maintain the ionic balance with the surrounding tissue. The leakage of macromolecular contents from cells, which have lost membrane integrity, has become an area of interest in the identification of cardiac specific markers. Amongst these macromolecules, the contractile and regulatory proteins of the myocardium are considered to be of particular significance (Cummins *et al.*, 1987). This is due to the expression of the cardiac-specific isoforms of these proteins, their higher intracellular concentration (Swynghedauw, 1986) and continuous release from the infarcting myocardium (Leger *et al.*, 1985, Katus *et al.*, 1992). In the past, polyclonal antibodies (Khaw *et al.*, 1976; Cummins *et al.*, 1987)

Correspondence to: Department of Immunology, University of Strathclyde, The Todd Centre, 31 Taylor Street, Glasgow. G4 0NR, U.K.

and affinity purified antibodies (Cummins *et al.*, 1990), raised against cardiac proteins, have been examined *in vitro* as well as in experimentally-induced myocardial infarctions.

Since the advent of monoclonal antibody technology (Kohler & Milstein, 1975), this type of antibody has been produced against a number of contractile and regulatory proteins of heart muscle (Khaw *et al.*, 1984; Katus *et al.*, 1989; Jin *et al.*, 1990; Ladenson *et al.*, 1990; Larue *et al.*, 1992, 1993). Following these results, we report the development of unique McAbs against Tn-I that are highly specific for its cardiac isoform.

Troponin-I is the inhibitory component of the troponin complex (Tsukui and Ebashi, 1973). It has been reported to exist in three isoforms, encoded by three different genes (Hunkler *et al.*, 1991) with distribution of the cardiac specific form, mol wt 24Kd, only in the cardiac muscle. cTn-I is considered to be of significance for both the *in vitro* as well as *in vivo* diagnosis of MI due to its cardiac specificity, uniform distribution in the cardiac muscle, unique location in myofibrillar structure and release characteristics from the necrotising cardiac tissue in the event of MI (Cummins *et al.*, 1987, 1990; Haider and Stimson, 1993).

MATERIALS AND METHODS

Purification of cardiac proteins

The extraction method described by Syska *et al.* (1974) was modified for the purification of cardiac proteins from bovine heart. Cardiac tissue was obtained 1-10 h after death and stored at -70°C until required. Bovine, ovine, human and rabbit cardiac tissue, 5-50g was homogenised in 10 volumes of buffer containing 9M urea, 1mM CaCl₂, 60mM 2-mercaptoethanol and 5mM Tris/HCl, pH 8.0 at 4°C in a Warring blender and centrifuged at 10,000 rpm for 20 min. The supernatant was passed through glass wool and adjusted to 35% (NH₄)₂SO₄ saturation by the slow addition of saturated ammonium sulphate solution with constant stirring at 4°C for 1h. The precipitate was then removed by centrifugation at 10,000 rpm for 20 min and the supernatant adjusted to 50% (NH₄)₂SO₄ saturation. After stirring for 1 h, it was centrifuged at 10,000 rpm for 20 min and the precipitate redissolved in 0.1M citrate buffer, pH 6.0. This **crude cardiac extract** was dialysed overnight against the same buffer, centrifuged at 20,000 rpm and concentrated by ultrafiltration (PM10 membrane, Amicon). Finally, the was purified on a Sephadex G75-SF column (1.6 x 95cm) with 0.1M sodium citrate buffer, pH 6.0, as eluent. One millilitre fractions were pooled separately for cTn-I and used for further experimentation.

Actomyosin ATPase inhibition assay

The inhibitory activity of purified cTn-I on skeletal actomyosin ATPase (Sigma) activity, in the presence of an equal concentration of cardiac tropomyosin (cTm), was determined as described by Brekke and Greaser (1976). The assay was performed with skeletal actomyosin (0.3mg) in 1ml 25mM Tris/HCl buffer, pH 7.4, containing 25mM KCl, 5mM MgCl₂ and 10mM CaCl₂ at 25°C. The reaction was initiated by the addition of 50µl 0.1M ATP solution and after 10 min it was stopped with 1ml 20% trichloroacetic acid. The inorganic phosphate released during the reaction was determined by the Fiske Subarow method (Mark and Zimmer, 1967).

Monoclonal antibody production

Immunisation of 6-8w, female BALB-c/NZB F1 hybrid mice was performed with pure cTn-I (10µg per injection) and monoclonal antibody production was carried out as described previously (Islam and Stimson, 1987). Hybridoma cell lines were propagated in pristane-primed histocompatible mice at a cell density of $5 \times 10^6/0.5\text{ml}$ RPMI 1640 culture medium, for ascites production. Antibodies were purified from ascites by 50% $(\text{NH}_4)_2\text{SO}_4$ fractionation and then by affinity chromatography using Protein A. These were conjugated with horse radish peroxidase (HRP) as described by Nkane and Kawaoi (1974) for use in a direct ELISA for cTn-I. Isotype analysis of the antibodies was carried out by ELISA using isotype-specific HRP-conjugated goat anti-mouse antisera (BioRad).

ELISA for screening hybridomas

Plates were coated with crude bovine cTn-I (5µg/ml - 100µl/well) in 10mM Tris/HCl buffer, pH 9.0, and incubated overnight at room temperature (RT). Plates were washed x4 with 20mM Tris/HCl wash buffer, pH 7.4, containing 0.05% Tween 20 and incubated at 37°C for 1h with 250µl/well blocking solution (10% newborn calf serum in wash buffer); these were again washed x4 with the buffer and then incubated at 37°C for 1 h with 100µl hybridoma cell supernatant/well. Following treatment with wash buffer (x4) 100µl anti-mouse IgG-HRP conjugated antibody was added to each well for 1h at 37°C. Finally, plates were washed x4 and enzyme activity assessed by incubation at RT with TMB substrate (250µl stock TMB solution in 25ml sodium acetate buffer and 4µl 30% H_2O_2 solution) for 30 min in the dark. The reaction was stopped by the addition of 50µl, 10% (v/v) H_2SO_4 /well and the A_{450} measured.

ELISA on the fractions eluted from the gel filtration column

The eluted fractions from the Sephadex G75-SF column were adjusted to pH 9.0 with 0.1M NaOH solution. An ELISA plate was coated with 100µl of each fraction per well, in duplicate. The immunoassay was then carried out using the 1D12 or 5F4 anti-cTn-I specific McAbs as described above.

Inhibitory effect of the anti-cTn-I McAbs on cTn-I inhibition of actomyosin ATPase activity

cTn-I and cTm, in a 1:1 ratio were mixed with 30µl purified 5F4 or 1D12 McAbs and left for 1 h at RT. The actomyosin ATPase inhibition assay was then carried out as before. Increasing concentrations of cTn-I and cTm were used in the presence of the antibodies in volumes of 30, 60, 120, 180, 240 and 300µl.

Electrophoresis and Western blotting:

Crude cardiac proteins or pure cTn-I were resolved on one dimensional SDS-PAGE gels containing 10%T and 2.6%C with a discontinuous buffer system (Lammeli, 1970). For running the gel, 5-10µg protein samples were boiled in 100-200µl Lammeli's sample buffer for 5 min. They were applied at a loading concentration of 2.5-5µg protein/50µl sample buffer per well. Electrophoresis was carried out at 100V for 8 h using 0.1M Tris/glycine buffer, pH8.3.

For Phast Gel SDS-PAGE, a pre-cast homogeneous gel, 45mm thick containing 12.5%T and 2%C (Pharmacia) was used with SDS-buffer strips (Pharmacia). Proteins

samples, 1 μ l, were loaded using a sample applicator (8 slots with 1 μ l capacity per slot) at a loading concentration of 0.5-1 μ g protein/ μ l of sample buffer for each track. For visualisation of proteins, the gels were stained either with Coomassie blue or silver, as described by Morrissey, 1981.

For immunoblot analysis, proteins from SDS-PAGE were transferred to a nitrocellulose membrane using 0.1M Tris/glycine buffer, pH 8.3, at a constant current of 80V for 16-18 h in a Transblot apparatus (BioRad), as described by Towbin *et al.*, 1979. After transfer of proteins the membrane was incubated at RT for 1 h with 5% casein solution in 50mM Tris/HCl, pH 7.4, (wash buffer). The blot was treated x3 with wash buffer and incubated with anti-cTn-I McAb in PBS containing 1% BSA. After extensive washing, the blot was incubated for 1 h at RT with sheep anti-mouse IgG HRP-conjugated antibody (1:200) in PBS containing 25% (v/v) sheep serum. Finally, the blot was washed x3 in wash buffer and soaked for 2 min in substrate solution (6mg diaminobenzidine/10ml 50mM Tris/HCl buffer, pH 7.6, 1ml 0.3% CoCl₂ solution in H₂O and 10 μ l 30% (v/v) H₂O₂.

RESULTS

Purification of cTn-I

Preparation of cTn-I and cTm was carried out by gel filtration following initial urea buffer extraction and partial purification by ammonium sulphate fractionation. Partial purification by salt fractionation (30-50% saturation) resulted in a relatively pure cTn-I preparation. This was further purified on a Sephadex G75-SF column and a plot of the elution profile of the cardiac proteins (Figure 1) shows that the elution volumes for cTn-I and cTm were 106-110ml and 81-85ml respectively. These fractions were pooled, concentrated and used in later experimentation. The average yield of cTn-I and cTm obtained was 10-15mg/50g cardiac tissue. For determination of purity, the cTm and cTn-I preparations were analysed by SDS-PAGE. The actomyosin ATPase inhibition assay showed that the cTn-I purified by this method retained its biological activity and caused 50-60% inhibition at 80 μ g/0.3 μ g actomyosin concentration (Figure 2). Skeletal muscle troponin I (sTn-I), prepared by the same procedure, was employed as a positive control.

Antibody production

Six separate cell fusions were performed before stable, anti-cTn-I secreting hybridoma cell lines were obtained; these resulted in 70-80% of the wells seeded showing positive hybrid cell growth. However, sixteen cell lines were finally chosen and cloned by limiting dilution with two lines showing a particularly high specificity for cTn-I. These cell lines, 1D12 and 5F4, were selected for further characterisation and isotype analysis revealed that both were IgG₁ kappa. The 1D12 and 5F4 cell lines were grown successfully as ascites in pristane-primed mice and the McAbs purified by 50% (NH₄)₂SO₄ fractionation before conjugation to HRP for the direct ELISA (Islam and Stimson, 1987). A higher purification level of the 1D12 and 5F4 McAbs was achieved by Protein A affinity chromatography.

ELISA on gel filtration fractions

The results of the indirect ELISA carried out on the fractions obtained by gel filtration showed that the 1D12 and 5F4 McAbs reacted only with fractions containing cTn-I

Figure 1. Elution profiles of the bovine cardiac protein extract and marker proteins on Sephadex G75-SF; (1) – cTn-I, (2) – cTn-I. Molecular weight markers: a – dextran blue, b – bovine serum albumin (66kd), c – carbonic anhydrase (29kd), d – cytochrome C (12.5kd), e – fluorescein.

Figure 2. Inhibitory activity of cTn-I and sTn-I on skeletal muscle actomyosin ATPase. Results are shown as the mean $\pm$ SD of 3 separatedeterminations.

(fractions 106-110) (Figure 3a,b). The reactivity of the antibodies for the other column fractions was found to be negligible (background levels). For confirmation of the validity of these results, the indirect 'Fraction ELISA' was carried out under the same conditions but using an unrelated antibody, 1D11 (Figure 3c).

Inhibitory effect of 5F4 and 1D12 McAbs on ATPase inhibitory activity of cTn-I

The inclusion of the 1D12 or 5F4 McAbs in the actomyosin ATPase inhibition assay by cTn-I completely blocked the inhibitory effect of cTn-I on actomyosin ATPase, thus confirming their specificity of the antibodies for cTn-I (Figure 4).

SDS-PAGE and Western blotting

ELISA showed that both the 1D12 and 5F4 McAbs recognised cTn-I. To confirm that these McAbs were specific for cTn-I, the binding of McAbs was analysed by SDS-PAGE of crude as well as pure cTn-I followed by immunoblot analysis; all the major cardiac protein components of the myocardium were resolved under these conditions. When the blots were probed with the 2 McAbs, reaction with a single protein band of 24KD was observed. This protein had previously been identified as cTn-I on the basis of its characteristics, viz. elution profile from the gel filtration column, mol.wt analysis on SDS-PAGE and the actomyosin ATPase inhibition assay. No reactivity of these McAbs was observed with any other cardiac protein. Figure 5 shows that McAb 5F4 identified only one component in crude cardiac extracts from human, bovine, ovine and rabbit tissues; identical results were obtained with 1D12, using SDS-PAGE followed by Western blotting.

Figure 3a

Figure 3b

Figure 3c

Figure 3. Indirect ELISA of fractions obtained from gel filtration of the crude bovine cardiac protein extract. Column effluents were monitored at A_{280} while aliquots of the fractions (100 μ l/well) were adsorbed onto microtitre plates and detected by ELISA at A_{450} using (a) 1D12 (b) 5F4 (c) an unrelated McAb (1D11).

Figure 4. Blocking of the inhibitory activity of cTn-I on the ATPase activity of skeletal actomyosin by the anti-cTn-I McAbs, ID12 and 5F4.

Figure 5. Western blotting of crude cardiac protein extracts using the 5F4 anti-cTn-I McAb. Lane 1 – human, Lane 2 – bovine, Lane 3 – ovine, Lane 4 – rabbit.

DISCUSSION

Recently, various groups of research workers have reported McAbs with specificity for cTn-I (Bodor *et al.*, 1990; Larue *et al.*, 1992; Landenson *et al.*, 1992;). Most of these McAbs have been used for development of cTn-I specific immunoassays for the *in vitro* diagnosis of MI by estimation of serum cTn-I in patient blood samples. We are reporting here the development of cTn-I specific McAbs which have been successfully used in a brain death model in Landrace pigs (unpublished).

Considering the fact that cTn-I in mammalian cardiac tissue shows definite inter-species relationship (Cummins and Cummins, 1987), bovine cardiac tissue was used for extraction of cTn-I for use as immunogen because of its easy availability compared to human myocardium. Most of the procedures described in the literature for the purification of cTn-I involve the initial purification of the Tn-complex, with subsequent fractionation into individual components (Tsukui and Ebashi, 1973; Brekke and Greaser, 1976; Potter and Johnson, 1982). The relatively long isolation times used with multiple steps often lead to degradation of the component proteins, especially cTn-I, which is particularly susceptible to endogenous cathepsins (Katagiri, 1981). The method used in this study for purification of cTn-I was short, simple and less labour-intensive and it gave a high level of purity. In addition the cTn-I prepared by this method retained full biological activity.

The initial selection of the hybridoma cell lines was based on the assessment of cell culture supernatants by the indirect ELISA of cTn-I. Once cell lines had been established, antibodies were analysed for antigen specificity by ELISA and immunoblotting, which showed the specificity of the 1D12 and 5F4 McAbs for cTn-I from bovine, ovine, rabbit and human cardiac tissue. Both of these McAbs belong to the IgG₁ class, an isotype which is preferred for *in vivo* imaging.

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