EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 28, No 2, 71-76, June 1996

Bovine cardiac troponin-I specific monoclonal antibodies which show species cross reactivity

Kh. Husnain Haider[1,3] and W.H. Stimson[2]

1 Department of Pharmacy, University of the Punjab, The Mall, Lahore, Pakistan
2 Department of Immunology, Todd Centre, 31-Tayor Street, University of Strathclyde, Glasgow, G4 ONR, U.K.
3 Corresponding author

Accepted 28 February 1996

Abbreviations: Tn, troponin; McAb, monoclonal antibody; HRP, horseradish peroxidase;

Abstract

Cardiac troponin-I (cTn-I) specific monoclonal antibodies (McAb) were produced against bovine cTn-I. Six somatic cell fusions using polyethylene glycol (40%) between spleen cells from cTn-I immunized mice BALB/c-NZB F1 hybrid and a mouse myeloma cell line (P3.X63.Ag8.653) were carried out. Sixteen positive monoclonal cell lines were produced; two of them, 1D12 and 5F4, showing high specificity for cTn-I. Both of these McAbs were characterized for their antigen specificity, antibody titre in the culture supernatant as well as ascites, class, subclass and light chain analysis. Tissue specificity and species cross-reactivity for cTn-I of 5F4 and 1D12 McAb were evaluated and confirmed by enzyme linked immunosorbent assay (ELISA) and immuno-blotting. Direct competition ELISA revealed their partial competition for the same epitope on cTn-I. The specificity for 1D12 and 5F4 against cTn-I was further confirmed by the reversal action for the actomyosin ATPase inhibition in the presence of these McAbs.

Keywords: monoclonal antibodies; cardiac troponin-I

Introduction

Troponin (Tn) is a complex of regulatory proteins consisting of troponin-I (Tn-I), troponin-C (Tn-C) and troponin-T (Tn-T) (Ebashi, 1972). For Tn-I, three principle isoforms, cardiac, slow skeletal and fast skeletal, have been reported in humans (Cummins *et al.*, 1978) which contribute to the functional differences of various muscle types. These are reported to be encoded by three separate genes (Vallins *et al.*, 1990; Hunkler *et al.*, 1991) and are muscle specific but not species specific (Dhoot *et al.*, 1978). Several groups of researchers have reported the production of McAb specific for various contractile and regulatory muscle proteins for use in morphological and structural differentiation in myogenesis (Payne, 1984). Moreover, high muscle specificity, unique location site in the muscle architecture and an even distribution throughout the atrial and ventricular muscles (Humphreys *et al.*, 1984) has created considerable interest in their possible use as cardiac specific biochemical markers to assess cardiac myocyte damage and related pathologies (Russell *et al.*, 1989; Cummins *et al.*, 1987, 1990; Ladenson *et al.*, 1990; Larue *et al.*, 1992; Bodor *et al.*, 1991, 1992; Haider *et al.*, 1994). We are reporting here the production of McAb which are cTn-I specific and show species cross reactivity.

Materials and Methods

Monoclonal antibody production

Immunization of 6-8 week, female BALB-c/NZB F1 hybrid mice was performed with pure bovine cTn-I (10 μg per injection) and McAb production was carried out as described previously (Islam *et al.*, 1987). Hybridoma cell lines were propagated in pristane primed histocompatible mice at a cell density of 5 x 10^6/0.5 ml RPMI-1640 culture medium, for ascites production. Antibodies were purified from ascites by 50% ammonium sulfate fractionation followed by affinity purification using protein A. These were conjugated with horse-raddish peroxidase (HRP) as described by Nakane *et al.* (1974) for use in direct ELISA for cTn-I. The purified antibodies were characterized as described previously (Haider *et al.*, 1994).

ELISA for screening of hybridomas

Plates were coated with crude bovine cTn-I (5 μg/ml-100 μl/well) in 10 mM Tris/HCl buffer, pH 9.0 and incubated overnight at room temperature. Plates were washed four times with 20 mM Tris/HCl wash buffer, pH 7.4 containing 0.05% Tween-20 and incubated at 37°C for 1 h with 250 μl blocking solution (10% new born calf serum in wash buffer) per well; these were again washed four times with the buffer and incubated at 37°C for 1 h with 100 μl hybridoma cell super-

natant/well. After washing four times with wash buffer, 100 μl anti-mouse IgG- horseradish peroxidase (HRP) conjugated antibody was added to each well for 1 h at 37°C. Finally, the plates were washed four times and enzyme activity assessed by incubation at room temperature with TMB substrate (250 μl stock TMB solution in 25 ml sodium acetate buffer and 4 μl 30% H_2O_2 solution) for 30 min in the dark. The reaction was stopped by the addition of 50 μl 10% (v/v) H_2SO_4/well and A_{450} measured.

Competition ELISA for epitope specificity

ELISA competition assay for determination of epitope specificity (Juronen *et al.*, 1988) of the anti-Tn-I McAb 5F4 and 1D12, was carried out. ELISA plates were coated with 10 μg/ml cTn-I in coating buffer, Tris/HCl, pH 9.0, at room temperature for overnight. The antigen solution was removed by washing the plate four times with wash buffer, Tris/HCl pH 7.4 containing 0.05% Tween-20 and incubated at 37°C for 1 h with 250 μl/well blocking solution (10% newborn calf serum in wash buffer); these were again washed four times with buffer and then incubated at 37°C for 2 h with 100 μl/well, serial dilution of the unlabelled blocking antibody and 100 μl/well of the appropriate constant concentration of HRP-conjugated detection antibody. Finally, plate was washed four times and enzyme activity was assessed by incubation at room temperature with TMB substrate and A_{450} measured as described earlier.

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

The inhibitory activity assay of purified cTn-I on skeletal actomyosin ATPase activity, in the presence of equal concentration of cardiac tropomyosin (cTm) was performed as described by Brekke and Greaser (1976).

The assay was carried out with skeletal actomyosin (Sigma, St. Louis, U.S.A.) (0.3 mg) in 1 ml 25 mM Tris/HCl buffer, pH 7.4, containing 25 mM KCl, 5 mM $MgCl_2$ and 10 mM $CaCl_2$ at 25°C. The reaction was initiated by the addition of 50 μl 0.1 M ATP solution and after 10 min it was stopped with 1 ml 20% trichloroacetic acid. The inorganic phosphate released during the reaction was determined by the Fiske and Subarow method (Mark and Zimmer, 1967).

For determination of blocking effect of cTn-I specific McAb 1D12 and 5F4, on the inhibitory activity of cTn-I on actomyosin ATPase, cTn-I and cTm, in a 1:1 ratio were mixed with 30 μl purified 5F4 or 1D12 McAb and left for 1 h at room temperature. 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 300 μl.

Preparation of crude cardiac protein extracts from the heart muscle samples of different animals

The heart muscle specimens from different animals, i.e., mouse, rat, hamster, guinea pig, rabbit, bovine, landrace pig and human, were obtained immediately after the death of the animals. All the samples were kept frozen at -70°C until further use. For the cardiac protein extraction, 2-5 g cardiac muscle was homogenized in 10 ml, 9 M urea buffer, pH 8.0, containing 1 mM $CaCl_2$, 60 mM 2-mercaptoethanol and 5 mM Tris/HCl, using Ultra Turux. The homogenate was adjusted to 30% saturation using saturated ammonium sulfate solution. After stirring at 4°C for 30 min, it was centrifuged at 10,000 r.p.m. for 20 min and the supernatant was dialyzed overnight against 0.1 M tri-sodium citrate buffer, pH 6.0. The crude cardiac protein extract thus obtained was aliquoted and kept frozen at -70°C till further use.

Electrophoresis and immunoblotting

Crude cardiac proteins were resolved on one dimensional SDS-PAGE gels containing total monomer concentration of 10%T and crosslinking of 2.6%C, with a discontinuous buffer system (Lammeli, 1970). For running the gels, 5-10 μg protein samples were boiled in 100-200 μl Lammeli's sample buffer for 5 min. They were applied at a loading concentration of 2.5-5 μg protein/50 μl sample buffer per well. Electrophoresis was carried out at 100 V for 8 h using 0.1 M Tris/glycine buffer, pH 8.3.

For Phast Gel SDS-PAGE, a pre-cast homogeneous gel, 45 mm thick containing 12.5%T and 2%C was used with SDS-buffer strips (Pharmacia). Protein samples, 1 μl, were loaded using a sample applicator (8 slots with 1 μl capacity per slot) at a loading concentration of 0.5-1 μg protein/μl of sample buffer for each track. For visualization of proteins, the gels were stained either with Coomassie blue or silver, as described by Morrisey (1981).

For immunoblot analysis, proteins from SDS-PAGE were transferred to a nitrocellulose membrane using 0.1 M Tris/glycine buffer, pH 8.3 at a constant current for 80 V for 16-18 h in a Transblot apparatus (BioRad, Hercules, U.S.A.), as described by Towbin *et al.* (1979). After transfer of proteins the membrane was incubated at room temperature for 1 h with 5% casein solution in 50 mM Tris/HCl, pH 7.4 (wash buffer). The blot was treated three times with wash buffer and incubated with anti-cTn-I McAb in phosphate buffer saline (PBS) containing 1% bovine serum albumin. After extensive washing, the blot was incubated for 1 h at room temperature with sheep anti-mouse IgG-HRP conjugated antibody (BioRad) (1:200) in PBS containing 2.5% (v/v) sheep serum. Finally, the blot was washed three times in wash buffer and soaked for

2 min in substrate solution (6 mg diaminobenzidine/10ml 50mM Tris/HCl buffer, pH 7.6, 1 ml 0.3% $CoCl_2$ solution in water and 10 ul 30% (v/v) H_2O_2.

Results

Six somatic cell fusions were performed before stable, cTn-I specific McAb secreting hybridoma cell lines were obtained. The fusion of the immune splenocytes with 653 myeloma cells resulted in 70-80% wells seeded with cells showing positive hybrid growth. However, most of the hybrids were found unstable and stopped secreting antibody after 3-4 weeks in the culture. Sixteen cell lines were cloned by dilution cloning and two cell lines, 1D12 and 5F4, showing high specificity for cTn-I were obtained. These cell lines, 1D12 and 5F4, were selected for further characterization and used in brain death model in pigs (unpublishd data). Isotype analysis revealed that both 1D12 and 5F4 McAbs were IgG1 with κ-light chains. The 1D12 and 5F4 cell lines were grown as ascites in pristane primed BALB/c mice. McAbs, purified from respective mouse ascites showed extreme purity of the antibody preparation. Lanes 3 and 4 (Figure 1), each show the light and heavy chain dissociation fragments of the respective McAbs on a Phast-gel under reducing conditions.

The direct competition ELISA carried out using 1D12 and 5F4 McAbs revealed partial competition by the two antibodies for the epitope on cTn-I molecule (Figure 2a and 2b). This is obvious from the left side drift when 1D12 or 5F4 McAb was used as blocking antibody using a nonspecific anti-Salmonella McAb or PBS as negative controls. It was of interest to use the same McAb as blocking antibody as well as HRP-conjugated second antibody using PBS and anti-salmonella McAb as negative controls. The competition between the antibodies was found more pronounced when 5F4 was used as blocking antibody and 1D12-HRP conjugate as the detection antibody, as compared to the situation *vice versa*.

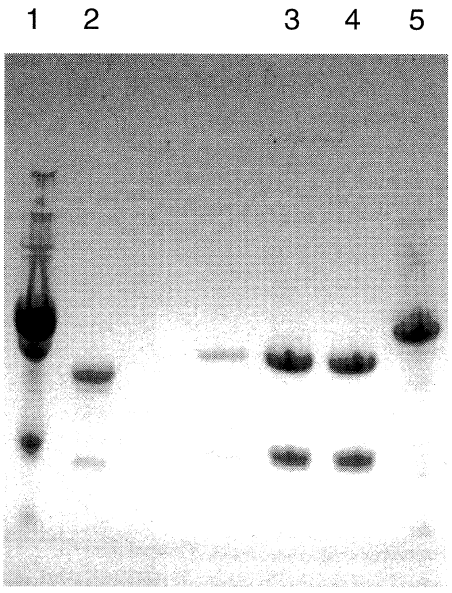


Figure 1. Assessment of purification of 1D12 and 5F4 anti-cTn-I McAb from ascites fluid using a protein A affinity column, was carried out by SDS-PAGE analysis on a 12.5% homogeneous precast gel under reducing conditions, with the Phast System (Pharmacia). The gel was fixed and stained with Comassie blue R-250. Lane 1, crude ascites fluid; lane 2, ovalbumin 44kDa and trypsinogen 24 kDa; lane 3, purified 5F4 McAb; lane 4, purified 1D12 McAb; lane 5, protein A column washings after sample loading of the column.

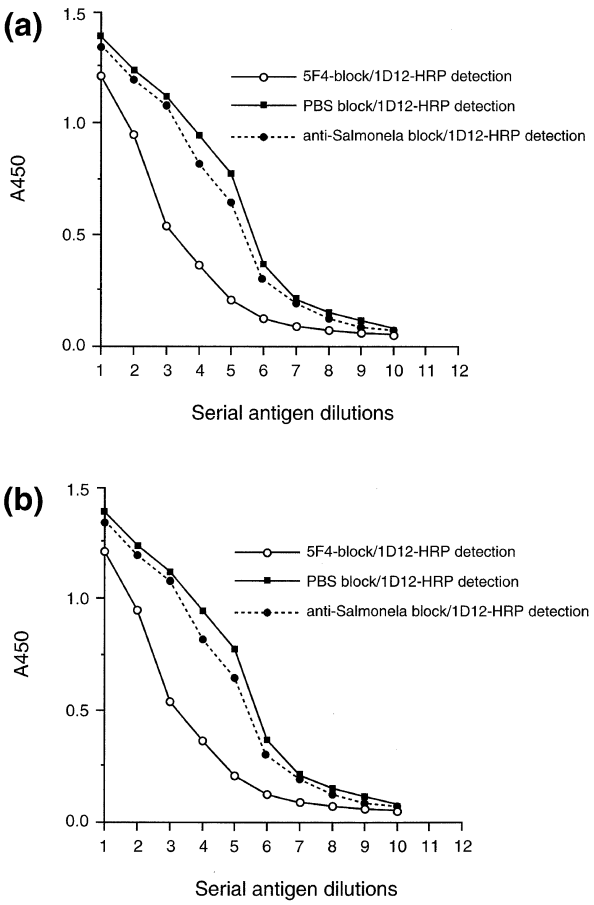


Figure 2. (a) The blocking effect of 5F4 anti-cTn-I McAb on the detection of cTn-I by 1D12-HRP conjugate in a direct competition ELISA. Anti-Salmonella McAb and PBS were used as negative controls. **(b)** The blocking effect of 1D12 anti-cTn-I McAb on the detection of cTn-I by 5F4-HRP conjugate in a direct competition ELISA. Anti-Salmonella McAb and PBS were used as negative controls.

When 1D12 and 5F4 McAb were included in cTn-I actomyosin ATPase inhibition assay, these antibodies completely blocked the cTn-I induced inhibition of actomyosin ATPase activity as shown in Figure 3.

Immunoblot analysis of crude cardiac protein extracts from different animals; mouse, rat, hamster, guinea pig, rabbit, bovine, pig and human was carried out to determine the tissue as well as species specificity of 1D12 and 5F4 McAb. Replicas of electroblots were also stained with Coomassie blue or by silver staining on the nitrocellulose membrane to visualize proteins for comparison and identification. When probed with 5F4 or with 1D12, the antibodies showed very strong species cross reactivity for cTn-I in the myocardium of various animals (Figure 4a and 4b).

Discussion

In the present study, we have reported the production of two bovine cTn-I specific McAb 1D12 and 5F4. Both McAb appear to be highly specific and reactive against cTn-I during *in vitro* analysis by ELISA and immunoblotting. They have shown broad species cross reactivity thus suggesting the presence of evolutionarily conserved epitope in cTn-I molecular structure. This common epitope may be functionally significant for cTn-I and incurrs functional characteristics to the molecule. Otherwise, the epitope might well be located on or near the part of cTn-I which is responsible for the inhibitory action of cTn-I. This assumption supported by the results of actomyosin ATPase inhibition assay

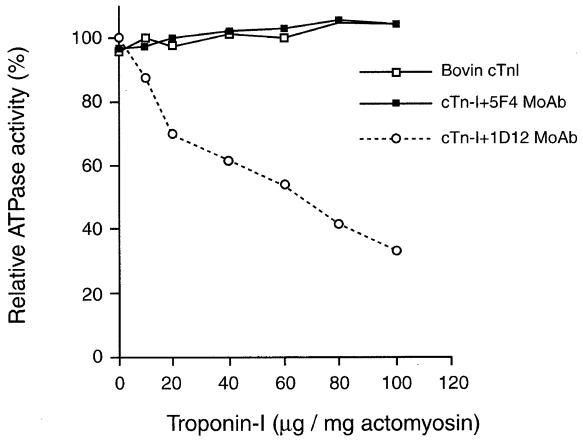
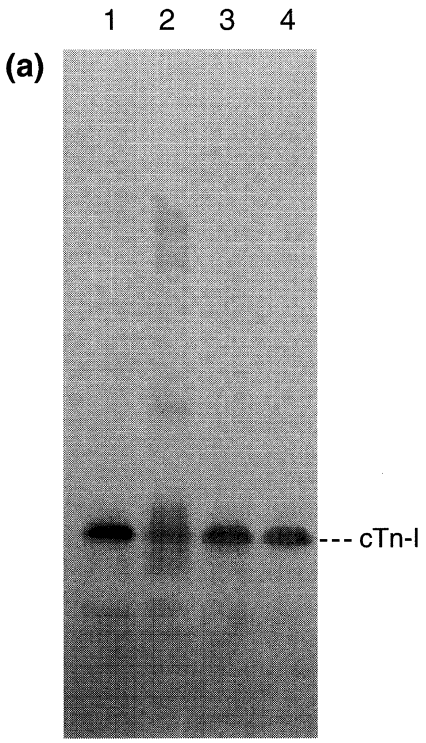


Figure 3. Blocking of the inhibitory activity of cTn-I on the ATPase activity of skeletal actomyosin by anti-cTn-I 5F4 and 1D12 McAbs.



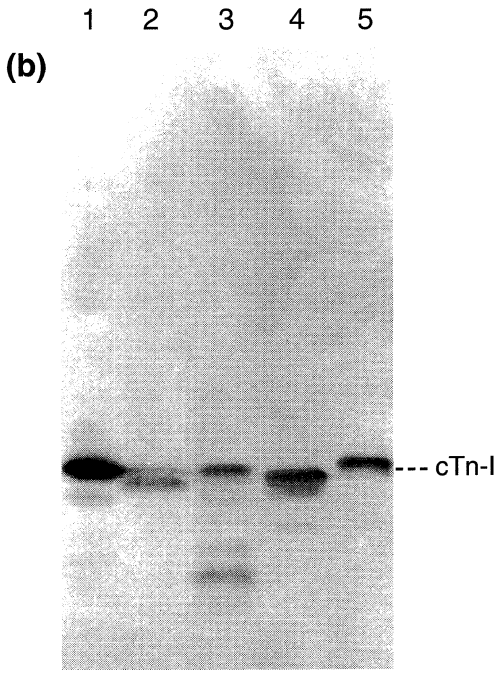


Figure 4. (a) Immunoblot of the crude cardiac protein extracts using the 5F4 anti-cTn-I McAb. Lane 1, human; lane 2, bovine; lane 3, ovine; lane 4, rabbit. **(b)** Immunoblot of the crude cardiac protein extracts using the 5F4 anti-cTn-I McAb. Lane 1, bovine; lane 2, hamster; lane 3, guinea pig; lane 4, rat; lane 5, mouse.

where in the inhibitory effect of cTn-I on actomyosin ATPase activity was completely blocked by the presence of 1D12 and 5F4 McAb (Figure 3). Once the antibodies have occupied their respective epitopes, cTn-I fails to interact with other proteins thus breaking the sequence of protein interaction responsible for the cTn-I dependent induction of actomyosin ATPase inhibition. These results are in accordance with the findings of Eyk *et al.*, (1988), which stated that amino acid sequence from 104-115 in the cTn-I molecule was the inhibitory peptide responsible to inhibit the activity of the cTn-I molecule. Moreover, the same sequence was important for the interaction of cTn-I with cTn-C and tropomyosin, and any alteration in the sequence will result in complete loss or reduction in the inhibitory activity of cTn-I.

The reaction of the 1D12 and 5F4 McAb is highly specific for cTn-I obtained from cardiac tissue of all the animals examined in the study (Figure 4a, b). One important aspect of the species cross reactivity analysis was that the whole homogenates of the cardiac tissue from various animals were employed as antigen instead of their purified cTn-I. As a result, the immunoblot analysis not only indicated the species cross reactivity of the McAb, but it also showed their monospecific nature for cTn-I. These results are very much in accordance with the findings of other investigators who have in the past shown the existence of such common epitopes shared amongst the cardiac isoforms of other proteins such as myosin heavy chain (Umeda *et al.*, 1989), tropomyosin (Yamane *et al.*, 1989), Tn-T (Maulof *et al.*, 1992) and cTn-I (Dhoot *et al.*, 1978).

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