ACRIDINIUM ESTER LABELLING AND CHARACTERIZATION OF BOVINE CARDIAC TROPONIN (cTn-I) SPECIFIC MONOCLONAL ANTIBODIES

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SUMMARY: The use of monoclonal antibody (McAb) based immunoassays to detect serum concentration changes of cardiac specific structural and regulatory proteins, as biochemical markers, is gaining immense interest in diagnostic procedures for cardiomyocyte necrosis. Of the non-isotopic probes, chemiluminescent molecules such as acridinium esters (A.E.) are regarded as being of real value in routine clinical chemistry. In the present study, bovine cardiac troponin-I specific 5F4 McAb was purified by Protein 'A' and Mono-Q ion-exchange chromatography and conjugated with A.E. The method of purification hardly interfered with the conjugation procedure. The A.E. conjugated 5F4 retained its immunoreactivity post conjugation and was used to design direct and indirect immunoassays for the detection of cTn-I in vitro samples.

Key Words: Acridinium ester, Cardiac troponin-I, Monoclonal antibody.

INTRODUCTION

Chemiluminescent molecular probes are beginning to find favour as highly sensitive, stable and safe alternatives to radioimmunoassay (4,13,16). The commonly employed chemiluminescent molecules include A.E. luminol or its structural variants. However, A.E have shown to be more sensitive than luminol derivatives (12). These are derivative compounds of acridine with a quaternary nitrogen centre and derivastized to yield an ester moiety (14). The excited product of its reaction, N-methyl acridone, is cleaved from the parent compound by mild oxidation stimulated by dilute alkaline H_2O_2 and does not require the presence of a catalyst. Hence, photon emission takes place independent of any possible chemical modification in coupling the label to the protein and is less susceptible to background and interference effect (14). Chemiluminescence occurs when vibronically excited products of an exoergic chemical reaction reverts to the ground state with the emission of photons which are then detected by luminometer (15).

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Figure 1: Effect of acridinium ester conjugation on immunoreactivity of 5F4 anti-cTn-I McAbs determined by indirect ELISA using unconjugated 5F4 McAb as control.

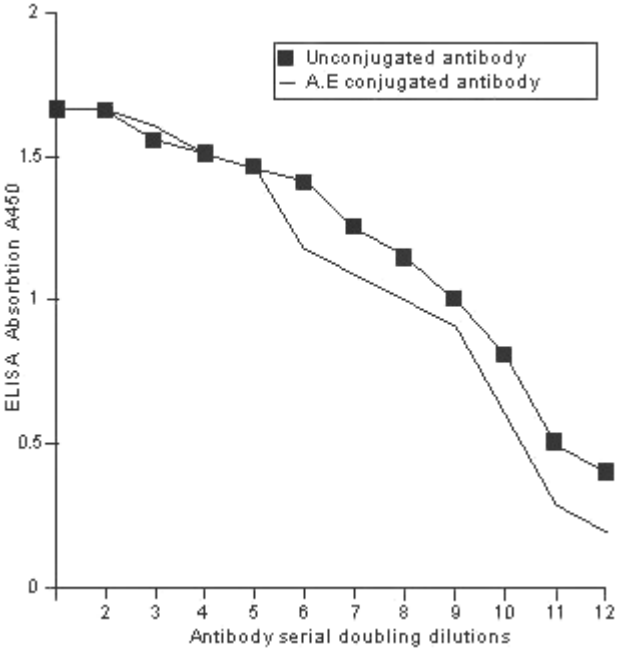
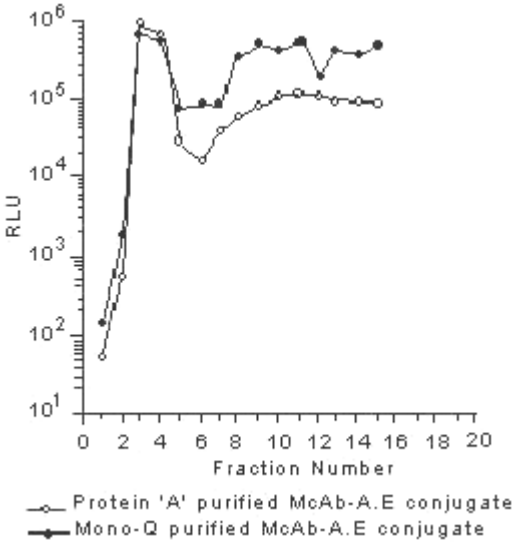


Figure 2: Incorporation of acridinium ester label into anti -cTn-I 5F4 McAb as measured by monitoring chemiluminescence in fractions from a PD-10 column. The antibody was purified by o Protein 'A' and o Mono-Q anion-exchange columns.



We are reporting here labelling of cTn-I specific 5F4 McAb (9) using an A.E (4-(2-succinimidyloxy carbonyl-ethyl) phenyl-10-methyl acridinium-9 carboxylate fluorosulfo-nate). The A.E-conjugated 5F4 McAb was used to develop an immunoassay for *in vitro* detection of cTn-I as a biochemical marker in patients with myocardial damage.

MATERIALS AND METHODS

Bovine cTn-I specific 5F4 McAb was produced and characterized for cTn-I specificity as described earlier (9).

Purification of Monoclonal Antibody

Purification of 5F4 McAb from ascites (9) was carried out by Affinity Purification using Protein 'A' column or by FPLC (Pharmacia) using Mono-Q anion exchange column.

Affinity Purification Using Protein 'A'

Ascitic fluid, 5 ml, was diluted 1:1 with sample dilution buffer containing 1M glycine and 0.3M NaCI pH 8.6 and left overnight at 4°C. It was centrifuged at 20.000 rpm. The supernatant was loaded onto Protein 'A' column and washed with five column volumes of the binding buffer containing 1M glycine and 0.15M NaCI, pH 8.6. A stepwise pH-gradient from pH 6.0 to 3.0 was applied using elution buffer containing 0.1M sodium citrate. The elution profile was monitored at 280 nm. The fractions containing 5F4 McAb were pooled and dialysed against phosphate buffer saline pH 7.4 and assessed for purity on Phast Gel System (Pharmacia) together with Coomassie blue staining (10).

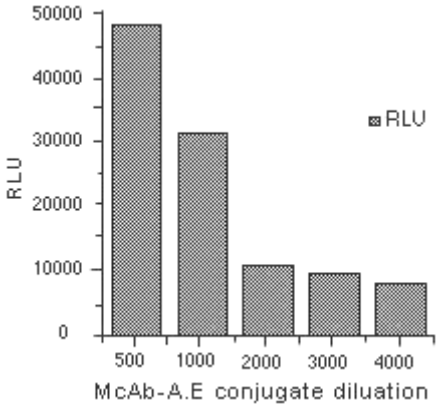
Ion Exchange Column Chromatography

Ascitic fluid containing 5F4 McAb was diluted using 20mM Tris/HCI buffer pH 7.7 elution buffer. Mono-Q column attached with FPLC (Pharmacia) was equilibrated using elution buffer. Two milliliter diluted ascitic fluid was loaded onto column through an automatic valve injector. The instrument was programmed to apply a salt gradient from 0-100% using 0.5M NaCI in elution buffer over a period of 60 minutes and at a flow rate of 1 ml/min. Elution fractions, 1ml, were collected and antibody containing fractions were pooled and run on a Phast Gel System (Pharmacia) for purity determination using coomassie blue staining.

Acridinium Ester Labelling

Purified 5F4 McAb from Protein 'A' and Mono-Q purifica-

Figure 3: Determination of optimum 5F4 McAb- acridinium ester conjugate dilution for use in chemiluminescent immunoassay of cTn-I.



tion methods, was dialysed using labelling buffer (0.2M sodium phosphate, pH 8.0). Fifty microgram 5F4 McAb (250mg/ml) was incubated at room temperature in a vial coated with A.E (Molecular Light Tech. Res. Ltd. UK), for 1hour in the dark. The reaction was stopped by adding 100μl quenching buffer (0.2M sodium phosphate and 10mg/ml lysine monohydrochloride). The A.E-conjugated 5F4 McAb was purified on PD-10 column (Pharmacia) using elution buffer (0.1M sodium phosphate, 0.15M sodium chloride, 0.1% bovine serum albumin and 0.05% sodium azide, pH 6.3). One milliliter fractions were collected. A comparison of the efficiency of labelling of the two products was performed by taking 1:100 dilutions, in elution buffer, for each of the collected fraction, in Greiner's assay tubes on Autolumat LB-953.

ELISA on gel filtration column Fractions

An indirect ELISA was carried out on the elution fractions containing from PD-10 gel filtration column (9). Each of the fraction was however, diluted 1:20 in PBS before being assessed in ELISA.

ELISA for immunoreactivity determination

The void volume fractions from PD-10 gel filtration column containing A.E conjugated McAb, were pooled together and 100 μl of this was diluted serially by two-fold dilution in PBS in a 96-well transfer plate (Dynatech). For comparison, 100 ml of unconjugated 5F4 McAb, adjusted to approximately the same dilution, was also diluted by two fold dilution separately. The serial dilutions of the A.E-conjugated and unconjugated 5F4 McAb were used in an indirect ELISA for immunoreactivity determination. Assay was carried out, in duplicate, on the same microtitre ELISA plate, previously coated with bovine cTn-I as previously described (10).

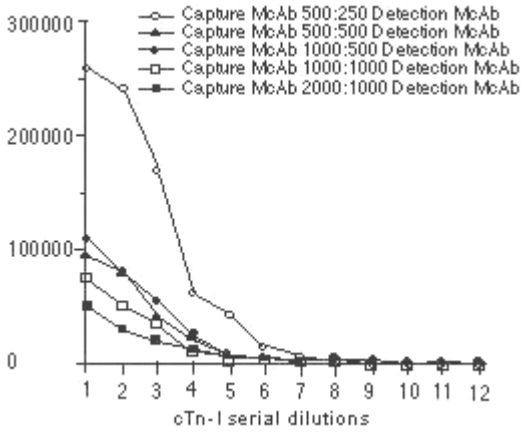
cTn-I Assays

To determine optimum concentration of A.E-conjugated 5F4 McAb for use in cTn-I assay, assay tubes were coated with 5ml/ml, 100μl/tube of crude cTn-I and incubated at 37°C for 1 hour with 1:500, 1:1000, 1:2000, 1:3000 and 1:4000 dilutions of antibodyester conjugate. The tubes were washed x3 with wash buffer, flick dried and read in Autolumat LB-953 luminometer.

For use in direct assay, the assay tubes were coated by incubation with 100μl/tube, 1:2 serial dilutions from 10μl/ml crude cardiac protein extract containing cTn-I for overnight. The tubes were washed x3 with wash buffer containing phosphate buffer saline pH 7.4 (PBS) and incubated with 100μl/tube, 1:500 dilution of the A.E-conjugated 5F4 McAb at 37°C for 1 hour. After washing with the wash buffer, the tubes were flick dried and read in the luminometer.

For cTn-I capture assay, the assay tubes were coated by incubation with varying dilutions; i.e., 1:500, 1:1000, and 1:2000, of 1D12 anti-cTn-I McAb, 5D8 anti-cTm-McAb, and rabbit anti-cTn-I polyclonal antiserum, separately (9). The tubes were washed using wash buffer and incubated with serial 1:2 dilutions of crude cardiac protein extract containing cTn-I for 1 hour at 37°C. After washing x3 with the wash buffer, they were incubated with 1:500 dilution, 100 μl/tube of A.E-5F4 McAb conjugate for 1hour. The tubes were then washed with wash buffer, flick dried and read in the luminometer.

Figure 4: Comparative study of the effect of anti -cTn-I rabbit anti serum, 5D8 anti-cTm specific McAb and 5F4 anti-cTn-I specific McAb as coating antibodies in the chemiluminescent antigen capture assay for cTn-I. The detection antibody used was 1D1 acridinium ester conjugate.



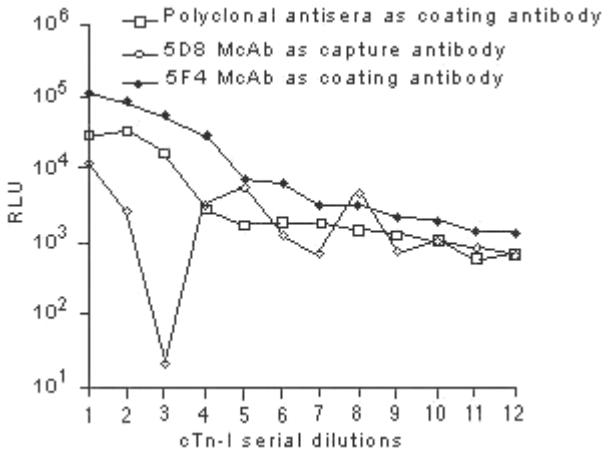
RESULTS AND DISCUSSION

Various myocardial regulatory and structural proteins such as myosin, tropomyosin, troponins etc., are released into blood circulation as a result of cardiac myocyte damage during myocardial infarction and related pathologies. Hence, these are considered to be potential biochemical markers for the diagnosis and assessment of myocardial damage (3,6,8,11). However, their existence as cardiac tissue specific isotypes has not been fully confirmed. In contrast, specificity of cTn-I isoform for cardiac tissue has been well established (5). Moreover, lack of polymorphism, uniform distribution and unique location in the cardiac tissue architecture make cTn-I a better candidate for use as a marker to assess cardiac myocyte damage.

Many researchers in the past have reported the development of immunoassays to detect and monitor serum level changes of cTn-I due to cardiac myocyte damage (2,7). We are reporting here the possible *in vitro* use of A.E-conjugated cTn-I specific 5F4 McAb for the detection of cTn-I. Apart from relatively improved sensitivity over enzymometric immunoassays, the ease of A.E conjugation and preservation of immunoreactivity by McAb post conjugation are the added advantages of chemiluminescent assay. cTn-I specific 5F4 McAb was purified (Figure 1) and successfully conjugated with A.E. The A.E-conjugated McAb was purified on PD-10 column and read in a luminometer. Figure 2 shows maximum chemiluminescent activity, measured as 'relative light units' (RLU), in the void volume fractions which contained the A.E conjugated McAb as revealed by the ELISA carried out on the PD-10 column elution fractions. Considering RLU as the labelling efficiency measure, the level of incorporation of A.E achieved for Protein 'A' purified McAb was found almost similar to that of Mono-Q purified 5F4 McAb. This shows that method of purification of McAb from ascites hardly interferes with the labelling efficiency (Figure 1).

Chemiluminescent assay carried out by direct coating of cTn-I on the assay tubes and detection made with various concentrations of A.E conjugated-5F4 McAb revealed that increased concentration of the conjugate in the assay enhanced the signal (Figure 3). However, the background noise signals were very high when A.E-McAb conjugate concentration was more

Figure 5: Effect of various concentrations of capture and detection antibodies on the sensitivity of the chemiluminescent assay for cTn-I, 1D12 McAb and 5F4-acridinium ester conjugate were used as capture and detection antibodies respectively.



than 1:500. For antigen capture chemiluminescent assay of cTn-I, a combination of 1D12 anti-cTn-I as capture antibody with A.E-conjugated 5F4 McAb was found superior to using 5D8 anti-cTm McAb and cTn-I specific polyclonal antiserum as capture antibodies in the same assay (Figure 4). The concentration of capture antibody 1:1000 (6mg/ml) and 1:500 of A.E conjugated 5F4 McAb was found the best combination for use in antigen capture chemiluminescent assay of cTn-I (Figure 5). The presence of human serum showed little effect on the assay sensitivity. However there was slight suppression in the signal intensity (unpublished data).

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