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Radiolabelled Monoclonal Antibodies (McAb): An Alternate Approach to the Conventional Methods for the Assessment of Cardiomyocyte Damage in an Experimental Brain-Death Pig Model

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The present study was carried out to determine the possible use of cTn-I in the cardiac myofibrillar architecture, as a potential target for *in vivo* radioimmunodetection of cardiac damage in a brain death pig model. Radioiodiantion of the anti-cTn-I 5F4 McAb was carried out by lactoperoxidase method. The percentage iodine incorporation achieved was 70~75%. The radioiodinated McAbs were purified on Sephadex G-25 column and characterised by Paper chromatography, Phast Gel electrophoresis and electroimmunoblotting. Radioiodinated anti-cTn-I 5F4 McAbs were employed alongside Pyrophosphate (Tc^{99m}-PPi) and Thallium^{201} chloride (Tl^{201}) in 24 landrace pigs (brain-dead=18 & sham-operated=6). The percentage cardiac uptake of the radiolabelled antibody injected dose was significantly higher in the brain dead animals (0.196%) as compared to that of sham-operated animals (0.11%). Specific *in vivo* localization of radiolabelled McAbs in the infarcted cardiac tissue was confirmed by computer-aided reconstruction of 3-D images of the isolated heart. The preliminary results of the study revealed preferential uptake of radiolabelled antibody at the site of myocyte damage resulting from artificially induced brain death.

Key words : Brain death, Cardiac Troponin-I, Monoclonal antibodies, Radioimmunoimaging, Radioiodination

INTRODUCTION

The irreversible global damage to the myocardium results from the pathophysiological events that follow brain death possibly in response to the sympathetic surge resulting from body's compensatory mechanism (Novitzky *et al.*, 1986 & 1987). The failure to correlate cardiac graft functioning post-transplant with clinical parameters such as ECG, donors blood pressure etc., has given rise to the need for some non-invasive but specific method to identify irreversibly damaged heart with poor function. The myocyte damage to the cardiac muscle results in expected release and exposure of structural and regulatory proteins of the muscle at the damaged site (Cummins *et al.*, 1979; Katagiri *et al.*, 1981; Editorial, Lancet, 1991). Several groups of researchers have reported the development of antibody based probes specific for structural and regulatory proteins of myocardium for *in vivo* localization and imaging of the cardiomyocyte damage in myocardial infarction and related pathologies (Caputo *et al.*, 1989; Cummins *et al.*, 1989 & 1990; Hoberg *et al.*, 1988; Tamaki *et al.*, 1990).

cTn-I is one of the constituent proteins of the troponin complex (Ebashi, 1972) having three principle isoforms, cardiac, slow skeletal and fast skeletal in the humans (Cummins *et al.*, 1978) and contributes to the functional differences of the various muscle types. They are reported to be encoded by three separate genes (Vallins *et al.*, 1990; Hunkler *et al.*, 1991) and are muscle specific (Dhoot *et al.*, 1978) but species non-specific (Haider *et al.*, 1994). High muscle specificity, unique location site in the muscle architecture and an even distribution throughout the atrial and ventricular muscle has created interest in its use as a cardiac specific biochemical marker (Cummins *et al.*, 1978 & 1990; Russel *et al.*, 1989). Many reports have been published in the recent past related to the production of McAb specific for cTn-I in relation to the development of cTn-I specific immunoassay (Ladenson *et al.*, 1990; Bodor *et al.*, 1991 & 1992; Larue *et al.*,

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1992). However, no attempt has been made to make use of these McAbs for cTn-I specific imaging. We report here cTn-I specific McAb which have been successfully used to visualize and localize cardiomyocyte damage in a pig brain death model.

MATERIALS AND METHODS

Bovine Serum Albumin (SIGMA), ELISA Plate (Dynatech), Iodobead (PIERCE), Iodogen (SIGMA), Lactoperoxidase (SIGMA), Nitrocellulose membrane (BDH), Phast Gel (Pharmacia), Radioiodine (Amersham), Sephadex G-25 (Pahrmacia).

The bovine cTn-I specific 5F4 McAbs were produced (Haider *et al.*, 1994). After purification and characterisation, they were radioiodinated with I^{125} for use in a brain-dead pig model.

Radioiodination of McAbs

Radioiodination of anti-cTn-I 5F4 McAbs was carried out by lactoperoxidase method (Marchalonis, 1969). Anti-cTn-I 5F4 McAb, 20 ug in 20 ul 0.5 M phosphate buffer, pH 7.4, was added to a reaction vial containing 0.5 mCi carrier free NaI^{125} and 10 ul lactoperoxidase. The reaction was initiated by the addition of 10 ul 30% hydrogen peroxide solution. After 15 min incubation at room temperature, 10 ul hydrogen peroxide was added again. The reaction was terminated after 15 min by diluting the reaction mixture with 250 ul reaction buffer. Unreacted iodine was removed from the iodinated product by gel filtration using Sephadex-G25 column.

Characterization of radioiodinated McAb paper chromatography

Paper chromatography strips (1.5×15 cm) were cut and marked 1.5 cm from one end of the origin. Aliquots of 2 ul were taken from the reaction mixture and applied on to the origin point of the paper strips and air dried. The paper strips were then developed up to 10 cm by ascending chromatography in a chromatography chamber containing 10% trichloroacetic acid (TCA). The developed chromatogram was air dried and the distribution of radioactivity was determined by cutting paper strips into 1 cm pieces and counting them individually in a well-type counter to determine percentage incorporation of radioactivity.

Gel filtration using Sephadex-G25 column

Upon completion of iodination reaction, the reaction mixture was diluted with 1 ml, 0.1 M phosphate buffer pH 7.4 containing 0.1% BSA. It was applied on to a precalibrated Sephadex G-25 column (1×20 cm). The column was run at a flow rate of 1 ml/min and 2 ml elution fractions were collected. The radioactivity contents of each fraction were determined in a well-type counting chamber and the elution profile was plotted.

ELISA for immunoreactivity determination

The void volume fractions from Sephadex G-25 gel filtration column were pooled together and 100 ul of this was diluted serially by two-fold dilution in PBS in a 96-well transfer plate. For comparison, 100 ul of un-iodinated anti-cTn-I 5F4 McAb adjusted to approximately the same dilution was also diluted by two fold dilution separately. The serial dilutions of the iodinated and un-iodinated anti-cTn-I 5F4 McAb were used in an indirect ELISA for immunoreactivity determination after iodination. Both these assays were carried out, in duplicate, on the same microtitre ELISA plate, previously coated with bovine cTn-I as described by Corney *et al.*, 1989.

SDS-PAGE analysis of radioiodinated McAb

SDS-PAGE analysis of the radioiodinated anti-cTn-I 5F4 McAb was carried out using PHAST-gel system (Pharmacia). A pre-cast gel SDS-polyacrylamide gel, 0.45 mm thick containing 12.5% T and 2.5% C and pre-cast SDS buffer strips (Pharmacia) were used. The protein samples were heated for 5 min at 100°C in the sample buffer and 1 ul of the sample at a loading concentration of 0.5~1 ug/ul was applied on the gel near the cathode. Separation was allowed to take place until the dye front has reached the required distance. The gel, mounted in a plastic cover, was exposed to X-ray film in a sealed cassette lined with an intensifier screen for 5~10 min. The film was later developed as per manufacturer's instructions.

Immunoblotting and autoradiography

cTn-I from bovine, pig and human cardiac muscle (Haider *et al.*, 1994) was electrophoresed on a 40% T and 2.6% C separating polyacrylamide gel using 25 mM Tris, 192 mM glycine pH 8.3 electrophoresis buffer containing 0.1% SDS (Lammeli, 1970). The proteins were transferred to nitrocellulose membrane (Schleicher and Schull, BDH), using 25 mM Tris and 192 mM glycine transfer buffer, pH 8.5 (Towbin *et al.*, 1979). The electroblot was probed using radioiodinated 5F4 McAbs to assess their specificity for cTn-I post-iodination reaction. The immunoblot was incubated with the iodinated McAbs for 1 hour and washed ×3 with 50 mM Tris/HCl buffer, pH 7.4 wash buffer. The immunoblot was exposed to X-ray film for autoradiography which was later developed as per manufacturer's instruction.

Brain death model

The acute non-survival model of brain death was pro-

duced in 24 young, male, Landrace pigs each weighing 20~30 kg (sham operated=6 and brain dead=18). The animals were anaesthetized using halothane and intubated. Anaesthesia was maintained with 0.5% isoflurane, 70% nitrous oxide and intravenous morphine. Full ventilation was maintained with respiratory pump. Vascular access was gained through cannulation of femoral vein by a triple catheter for the infusion of fluids and drugs and the femoral artery catheter for sampling and arterial pressure measurement. Brain activity was monitored using Elemar Schonander EEG recorder with two channels in the occipital and temporal areas using six intradermal needles, four active and two neutral. ECG was monitored continuously with special emphasis on ST-changes and T-wave inversion. A vertical incision was made though the skin and temporalis muscle in the temporal region of the skull followed by the insertion of Foley's catheter with 30cc balloon inside the cranial cavity.

Routine sternotomy was performed to expose the heart and the intracardiac catheters such as Gaeltec left ventricular pressure monitoring catheter and Swan Ganz catheter were sited. The baseline measurements of the cardiovascular parameters were also recorded. At the same time, the first injection of radioactive microspheres was administered for the measurement of blood flow. Full thickness biopsies were taken from the left ventricle and frozen in the liquid nitrogen for histochemical, microscopic and birefringent studies. Brain death was induced by inflating the balloon of a Tienmann catheter by slow infusion water using infusion pump at 1 ml/min and the pressure changes were recorded. After infusion of 20 ml, the pressure inside the balloon was maintained by slow and continuous fusion of water at 0.1 ml/min.

I^{125}-labelled anti-cTn-I 5F4 McAb (50 ug labelled with 500 uCi I^{125}) and Tc^{99}m-PPi (2~2.5 mCi) were injected 4 hour after the brain death. Five minutes after Tl^{201} injection, the animals were sacrificed. For euthanasia, 5 ml intracardiac injection of K^+ was administered. The vena cava and pulmonary blood vessels were ligated, aorta was cross-clamped and cardioplaegia was induced. The major blood vessels were then divided and heart was excised and flushed extensively to remove any blood.

Post-operative protocol

The excised heart was imaged as a whole at different angles and exposing its chambers, for Tc^{99m}-PPi and Tl^{201} uptake. The ventricular myocardium was divided into 1 cm^2 sections and then subdivided in to epi- and endocardial sections. Each of the section was weighed and counted for Tl^{201}, Tc^{99m}-PPi and I^{125}-anti-cTn-I 5F4 McAb. The sections of myocardium were then kept in formalin for 3~4 weeks for Tl^{201} and Tc^{99m} decay and recounted for I^{125} and microsphere distribution. The counts for I^{125} were normalised for one gram of cardiac tissue and were processed for computer-aided reconstruction of images for I^{125}-anti cTn-I 5F4 McAb distribution.

RESULTS

The percentage of radioactivity incorporation achieved using lactoperoxidase method was 70~75%. The void volume fractions eluted from Sephadex G-25 column containing highest radioactivity counts were pooled together and used during later experimentation. Parallel titration by indirect ELISA of the radioiodinated anti-cTn-I 5F4 McAb using un-iodinated anti-cTn-I 5F4 McAb as control gave a semi-quantitative estimate of the immunoreactivity retention by the antibody molecules post iodination (Fig. 1). The SDS-PAGE analysis of radioiodinated anti-cTn-I 5F4 McAb on Phast Gel showed very high purity of the product. No free radioactivity was found in the preparation, with all of the radioactivity bound to the light and heavy chains of the antibody molecules as is apparent from the autoradiograph of SDS-PAGE gel (Fig. 2). Immunoblotting followed by autoradiography of cTn-I in the cardiac protein extracts from bovine, pig, and humans, using radioiodinated anti-cTn-I 5F4 McAb showed no change in their reactivity and specificity (Fig. 3). These radiolabelled antibodies were used in animal studies.

Eighteen animals survived full length of experiment. At the end of each experiment, the animals were killed and their hearts were excised, washed and imaged for Tc^{99m}-PPi and Tl^{201} uptake. Intensely hot areas of

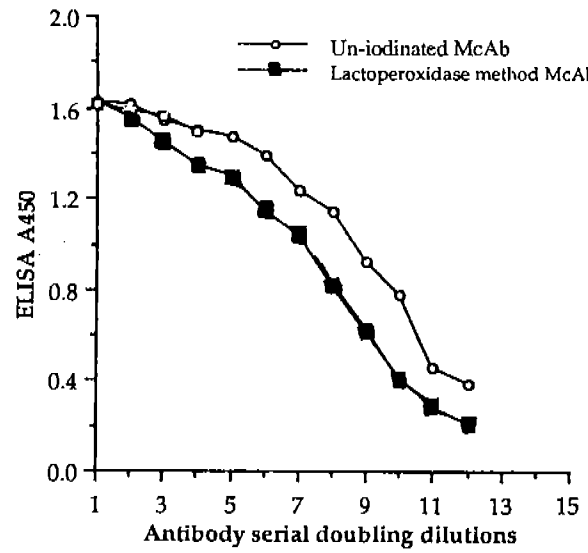

Fig. 1. Effect of radioiodination on immunoreactivity of anti-cTn-I 5F4 McAb determined by indirect ELISA using underivatised anti-cTn-I 5F4 McAb as control.

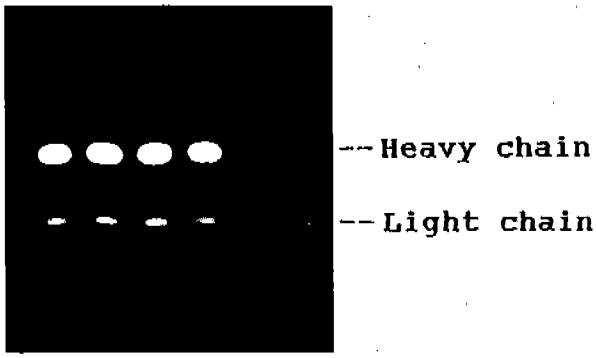

Fig. 2. Autoradiograph of a PHAST-gel (Pharmacia) with radiolabelled anti-cTn-I 5F4 McAb, after purification on Sephadex G-25 column. The void volume fractions containing radioiodinated McAb were electrophoresed under reducing conditions.

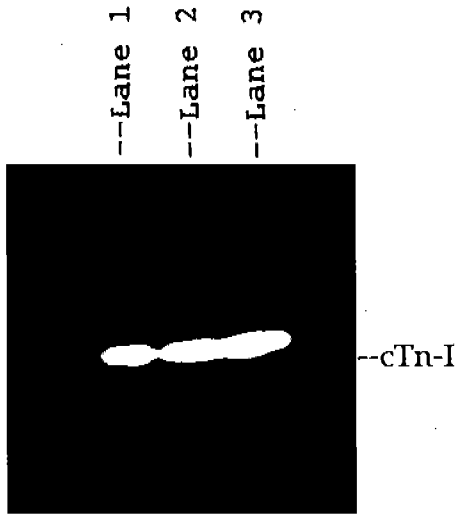

Fig. 3. Autoradiograph of cTn-I immunoblot probed by using radioiodinated anti-cTn-I 5F4 McAb. Lane 1: Bovine cardiac protein extract. Lane 2: Pig cardiac protein extract. Lane 3: Human cardiac protein extract.

Tc^{99m} uptake are clearly visible in the scintiscans of brain dead animals (Fig. 4) as compared to markedly reduced, scanty uptake in sham operated animals (Fig. 5). The normalised radiation counts for I^{125} were found to be upto 20 times greater in the individual slices from the damaged myocardium as compared to those for normal myocardium. The computer-aided reconstruction of the images from the normalised counts show a high and specific uptake of the radiolabelled antibody by the damaged myocytes (Fig. 6a,b,c). The 3D images constructed using the normalised counts for I^{125}-anti-cTn-I 5F4 McAbs uptake show a very specific pattern of distribution of the radiolabelled antibody with bright crests in the Figures representing the areas of high uptake and the darker troughs representing no antibody uptake. The banding pattern between the peaks and the background shows the antibody localization in relation to the extent of dam-

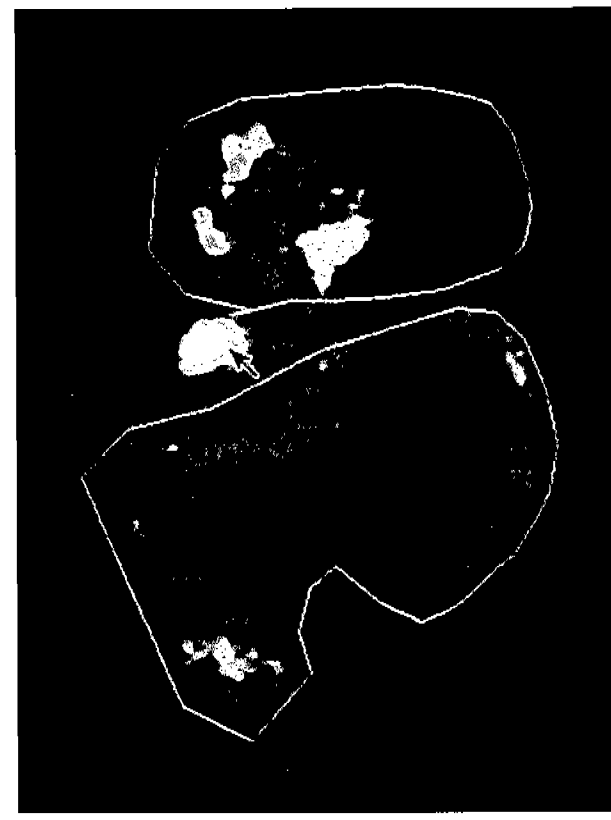

Fig. 4. A typical gamma scintiscan of an isolated heart from a brain dead pig. The scintiscan shows extensive uptake of Tc99m-PPi in the damaged myocardium.

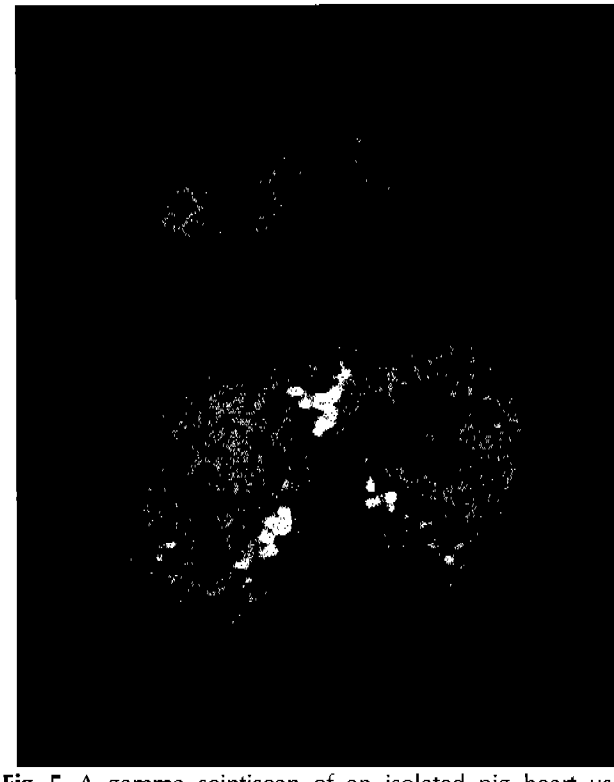

Fig. 5. A gamma scintiscan of an isolated pig heart using Tc99m-PPi shows slight uptake of radioactivity in LV. The animal was sham operated and no biopsy samples were taken from the cardiac tissue during experimentation.

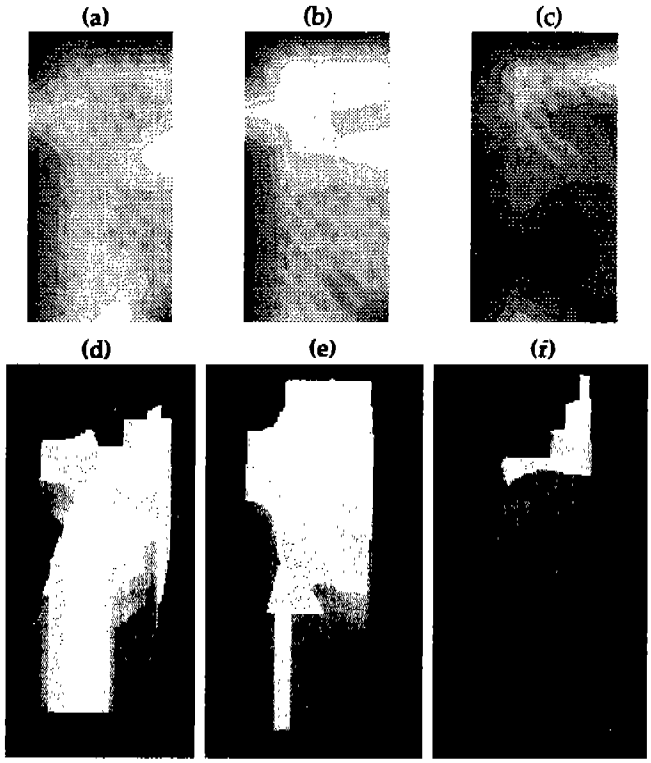

Fig. 6. Tissue distribution of a) Tl201 b) Tc99m-PPi and c) I 125 anti-cTn-I 5F4 McAb in the left ventricular (LV) endocardium of a brain dead pig. The LV was cut into equal slices and each of the slice was differentially counted for respective isotpe value. The degree of the specific uptake of the respective isotope was represented on grey scale of 8 of equal ranges from clear to dark (Clear=highest uptake; Dark =lowest uptake). a, b & c=Tissue distribution of Tl201, Tc 99m-PPi and I125 anti cTn-I 5F4 McAb in the epicardium of LV respectively. d,e & f=3-D projection of the images in Figures a, b & c respectively showing gradation in the levels of radioactivity localization.

age induced to the muscle and hence exposure of cTn-I for antibody binding (Fig. 6d,e,f). The total uptake of I^{125} anti-cTn-I 5F4 McAbs in the cardiac tissue in the brain dead animals, on average was two to three folds more than in the control animals. The percentage uptake of the injected antibody dose was found to be 0.196% for the brain dead animals as compared to 0.11% for the sham operated animals.

DISCUSSION

The heart of a brain dead patient considered for donation is assessed for its function prior to acceptance. This assessment is mostly made on the basis of clinical parameters such as ECG donors blood pressure etc. However, these clinical parameters poorly correlate with graft function following transplantation. Thus there is requirement to develop a non-invasive mean of investigation to distinguish and identify irreversibly damaged hearts with poor function from those which have poor function but can be improved by active intervention.

Myocardial damage resulting from pathophysiological events following brain death is irreversible and global, scattered all over the myocardium and may involve upto 8% cardiomyocytes. Of the two brain death models reported by Novitzky *et al.* (1988), the one employed in the present study closely mimics the sequence of events that occur after acute intracranial haemorrhage. However, it does not exactly duplicate the clinical situation. The haemodynamic changes observed in this case reflect the bodys attempt to compensate for the intracranial changes taking place during 'coning'. The large but the transient surge of catecholamines at this stage results in ECG changes and ventricular arrythmia thus resulting in damage to the myocardium. The actual mechanism is, however, multifactorial (Novitzky *et al.*, 1986).

In the present study, I^{125} was employed for radiolabeling of anti-cTn-I 5F4 McAb due to its compatible energy of gamma emission with that of Tc^{99m} (140 Kev), Tl^{201} (60~80 Kev) and chromium radioisotopes. The use of radiometals such as In^{111} for antibody labelling suffer from the complex chemistry of the labelling reaction and they cannot be directly tagged with proteins to produce a stable complex (Eckelman *et al.*, 1989). From amongst the iodine group of radioisotopes, dual isotope imaging using I^{123} in the presence of Tc^{99m} is difficult due to their similar energy of gamma emission (159 Kev and 140 Kev respectively). Furthermore, I^{131} with major component of gamma emission of 364 Kev is unsuitable for use with modern day imaging devices. Its high energy gamma radiations give of scatter towards lower energy levels thus interfering with the scintillation procedure when used concomitantly with lower energy gamma emitting radioisotopes. Hence the choice for radiolabel was restricted to I^{125} and the study was designed and modified to accommodate the use of I^{125} as a radiolabel for anti-cTn-I 5F4 McAb. After a comparative study of the various radioiodination procedures (Haider *et al.*, 1996), lactoperoxidase method was found to be the method of choice for radioiodination of anti-cTn-I 5F4 McAb due to its milder reaction conditions. Moreover, the radioiodinated product retained its immunoreactivity (Fig. 1 & 3). Tc^{99m}-PPi was used as a standard marker to elucidate damaged myocardium and distribution pattern of Tl^{201} in the heart muscle revealed the viable tissue (Saha *et al.*, 1992).

For successful radioimmunoimaging, the ratio of the radiolabelled McAb uptake between the normal and necrotic tissue is imperative, together with the absolute injected dose of the radiolabelled antibody gaining access to the target site. A mean uptake of 0.196% of the injected dose of I^{125} anti-cTn-I 5F4 McAb in the myocardium at 1~1.5 hours post injection time is highly significant when considered in relation to 0.098% uptake per gram of the infarct tis-

sue, 48 hours post injection using Fab fragments of affinity purified anti-cTn-I specific antibodies in a canine infarct model (Cummins *et al.*, 1990). However, there is no report in the literature where cardiac troponin-I has been targeted *in vivo*, using cTn-I specific radiolabelled McAb to assess myocardial damage in a brain death model. While the uptake of the administered dose of I^{125}-anti-cTn-I 5F4 McAb is significantly higher as compared to the previously reported studies, the present study does not fully elaborate the imaging characteristics of anti-cTn-I 5F4 McAb. Hence, some more experimental studies are required to fully explore the potential of this McAb as radioimaging agent specific for cardiac necrotic tissue.

Examination of the preliminary data from this study reveals a reciprocal distribution pattern of I^{125}-anti-cTn-I 5F4 McAb and Tc^{99m}-PPi with respect to the distribution of Tl^{201} (Fig. 6a,b,c). Contrarily, the former two show good relative uptake in the necrotic tissue, with each other although the mechanisms of Tc^{99m}-PPi and I^{125}-anti-cTn-I 5F4 McAb uptake are quite unrelated. A comparison of the images of Tc^{99m}-PPi and I^{125}-anti-cTn-I 5F4 McAb reveals more specific uptake of the radiolabelled antibody and shows a more diffused pattern of distribution in the myocardium. Analysis of the three dimensional images (Fig. 6d,e,f) reveals the site specific uptake of the antibody. However, Tl^{201} also showed uptake at the biopsy wounds alongside Tc^{99m}-PPi and I^{125}-5F4 McAb. The possible explanation for this phenomenon is that the resulting infarct from the pathophysiologic mechanism is different from the one which was created by the biopsy stabs. In case of pathophysiologically generated infarcts the necrotic tissue is devoid of its blood supply in the centre of the infarct with a gradation of ischaemic, potentially ischaemic and live cells in the peripheral tissue. Hence, Tc^{99m}-PPi gets deposited in the tissue where the blood supply has been considerably reduced as Tc^{99m}-PPi shows a biphasic uptake with blood flow (Beller *et al.*, 1979). Contrararily, Tl^{201} which is a flow marker (Gibson, 1983) can only deposit in the live cells with a functional Na^+/K^+ pump. Deposition of both these radioisotopes at the biopsy sites indicates the presence of dead cells at the site of insult but at the same time, the surrounding tissue is normal with considerable blood flow in the region to carry Tl^{201}. The uptake of the radiolabelled McAb in this area reveals the exposure of cTn-I as a result of destruction of the fibrillar organization of the cardiac tissue.

To conclude, the study clearly demonstrates the preferential localization of the radiolabelled anti-cTn-I 5F4 McAb in the necrotic myocardium. However, there are several areas where improvements could be made to develop this McAb into a dependable marker of choice for myocardium related pathologies. First and foremost is the use of superior radiolabel such as I^{123}, Tc^{99m} or In^{111}. Secondly, the use of $F(ab)_2$ and Fab fragments instead of whole antibody molecule may improve its imaging characteristics. Moreover, it can also be tested in an myocardial infarction model.

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