# PREPARATION OF MONOCLONAL ANTIBODIES AGAINST CARDIAC MYOSIN AND SOME RADIOLABELLING STUDIES

K. BAPAT, M. VENKATESH, M.R.A. PILLAI
Radiopharmaceuticals Section, Isotope Division


H.D. SARMA
Radiation Biology and Biochemistry Division

K.B. SAINIS
Cell Biology Division

Bhabha Atomic Research Centre,
Mumbai, India

**Abstract**

Monoclonal antibodies were raised against myosin, a specific indicator of myocardial infarction and labelled with $^{125}I$ and $^{99m}Tc$. Human cardiac myosin was isolated from normal human heart and was used for raising the monoclonal antibodies by the hybridoma technique. Antibody producing clones were identified by ELISA and cloning was done by the limiting dilution technique. Of the 13 clones obtained, 4 were deemed suitable for further studies. The antibodies were grown in ascites, purified, isotyped and their cross reactions with other forms of myosin were estimated. All the clones showed negligible cross reaction with rabbit myosin, but reacted to different extents with bovine skeletal myosin. The most avid antibody Mab-4G4 was chosen for further labelling studies.

Mab-4G4 was labelled with $^{125}I$ using different oxidising agents such as iodogen, chloramine-T and lactoperoxidase. Purified radioiodinated antibody with radiochemical purity >95% could be obtained by gel filtration. Immunoreactivity was retained as tested by binding to myosin immobilised on a solid support. Mab-4G4 was also labelled with $^{99m}Tc$ using stannous tartrate as the reducing agent. Radiolabelling yield was ~60%, the purity was >95% and the immunoreactivity was retained. Both the labelled preparations were tested for bio-distribution in normal and infarcted rats. The activity accumulation in the infarcted region was ~ 1.5 and 3.5 times as that in normal heart muscle for $^{125}I$ and $^{99m}Tc$ labelled Mab-4G4 respectively. The major problem with the iodinated antibody was the *in vivo* deiodination resulting in very high

percentage of activity in the thyroid. Although the fraction of the total activity associated with the infarcted heart is not very impressive, the fact that the activities with the infarcted and normal hearts are significantly different is heartening. With further optimisation of labelling and use of $F(ab)'_2$ fragments, better delineation of the infarct sites is aspired.

## 1. INTRODUCTION

Myocardial ischemia leading to damage and necrosis of myocytes or acute myocardial infarction (MI) is one of the major causes of morbidity. Myocardial ischemia and infarction are often monitored *in vitro* by the presence of certain serum proteins/enzymes released from the damaged myocytes [1]. Cardiac myosin is one such intracellular protein which is exposed due to sarcolemmal disruption occurring in MI. Immunoradiometric assays (IRMA) for light and heavy chains of myosin have been used for *in vitro* detection of MI. Heavy chains of myosin can serve as specific markers to detect MI by immunoscintigraphy [2-3]. These can be accomplished using anti-myosin antibodies and monoclonal antibodies specific for human cardiac myosin (HCM) would be ideal for this purpose. Attempts were therefore made to generate monoclonal antibodies against HCM and their suitability for immunoscintigraphy was assessed. Of the several clones obtained, four were selected and used for further studies on radiolabelling. Of these four, the monoclonal antibody Mab-4G4 which had the maximum relative affinity was used for *in vivo* studies in Wistar albino rats.

## 2. MATERIALS AND METHODS

All chemicals and biochemicals for hybridoma work, bovine serum albumin (BSA), lactoperoxidase, chloramine-T, Tween-20, iodogen, goat-antimouse polyvalent

antibody conjugated with alkaline phosphatase and Sephadex-G 25 were purchased from Sigma Chemical Co., USA. Hybridoma subtyping kit was from M/s. Boehringer Mannheim, Germany. All chemicals and salts for buffer preparations were purchased from M/s. Sarabhai Chemicals, India. $^{99m}TcO_4^-$ was eluted from a column generator. $Na^{125}I$ (NEZ 033A) was from Dupont Canada. All the plastic wares used for the hybridoma work was from Nunc Inc., Denmark.

All the major equipment such as -80°C freezer, $CO_2$ incubator etc. in the hybridoma lab were from Forma Scientific Inc., USA. NaI(Tl) scintillation counter was used for measurement of radioactivity.

BALB/c mice were bred in the animal house of our institute. Human heart was obtained from the morgue of K.E.M. Hospital, Mumbai, India.

## 2.1. Isolation of human cardiac myosin(HCM)

Myosin was isolated from normal human hearts as per reported procedure [4]. The heart was stored at -70°C before processing and processed at 4°C. The purity of the myosin preparation was checked by 12.5% SDS polyacrylamide gel electrophoresis.

## 2.2. Anti-myosin monoclonal antibodies

### *2.2.1. Hybridoma technique*

In brief, male BALB/c mice (10-12 weeks old) were immunised with 100 μg of HCM emulsified with Freund's complete adjuvant injected intraperitoneally. A single booster dose was given after a fortnight with the same amount of antigen emulsified in Freund's incomplete adjuvant. Three days after the booster, the splenocytes from the immunised mice were used for generation of Mab as per the reported method [5].

Spleen cells ($1\times10^6$) and Sp2/0 myeloma cells ($2\times10^6$) were centrifuged together and were fused by using 30% PEG as the fusogen. Fused cells were suspended in complete DMEM with HAT (5 mM hypoxanthine, 20 μM aminopterin, 800 μM thymidine). Supernatant of Sp2/0 culture was added (25%) to the medium instead of feeder cells [6]. The cells were initially plated at the cell density of $10^5$ cells/mL. After observing the growth of the hybrids the cells producing antimyosin antibodies were identified by ELISA technique. In brief, Immulon-1 microtitre wells were coated with 5 μg of HCM in 0.05 M Tris-KCl buffer, pH 8.5 for 18 h at 4°C. After washing the reaction wells with 0.05 M phosphate buffered saline, pH 7.5 (PBS) containing 0.05 % tween-20, the wells were blocked with PBS, 1% BSA for one hour at 25°C. The wells were again washed as before. 100 μL supernatant from each of the plated wells was added to a corresponding HCM coated well and was allowed to incubate for 3 h at 25°C. After repeating the washing step, 0.1 mL 1:500 goat-antimouse polyvalent Ig conjugated with alkaline phosphatase was added to each well and incubated for 18 h at 4°C. These wells were once again washed as before and 0.1 mL (100 μg) p-nitrophenylphosphate was added and the O.D. measured at 410 nm. The cells from the positive wells were pooled and were cloned by limiting dilution (0.3 cells /well). The individual clones were identified, tested for production of specific antibodies, and further recloned by limiting dilution to confirm the monoclonality.

### *2.2.2. Antibody production from ascites*

The identified clones were propagated in BALB/c mice and the antibodies were collected from the ascitic fluids. One week prior to the injection of the desired hybridoma cells, the mice were primed (*i.p.*) with 0.5 mL of Freund's incomplete adjuvant [7]. The antibody from the ascitic fluid was purified using caprylic acid followed by ammonium sulphate precipitation [8].

The monoclonal antibodies were characterised for their isotype, titre, cross reactivities and relative affinities towards HCM. Isotyping was done by using the isotyping kit. Essentially the antibodies in the culture supernatant and ascites were captured on sheep-antimouse antibody coated wells and were identified by peroxidase labelled mouse subclass specific antibodies. Cross reactions of these antimyosin monoclonal antibodies with bovine skeletal myosin (BSM), rabbit myosin light (RMLC) and heavy chains (RMHC) were estimated by the ELISA technique. The relative affinity of Mabs for myosin was estimated by a competitive assay in terms of amounts of myosin required for 50 % inhibition of binding of the Mab to the solid phase bound myosin.

## 2.3. Radiolabelling of Mab with $^{125}I$

The purified monoclonal antibodies were labelled with $^{125}I$ using different oxidants such as chloramine-T, iodogen and lactoperoxidase [9, 10]. 30 μL of 0.1 M phosphate buffer, pH 7.5 and ~30 μg of antibody solution were taken in a glass tube and 18–30 MBq (500–800 μCi) of $Na^{125}I$ was added and the contents were mixed thoroughly. In the case of iodogen, the reaction was carried out in a glass tube coated with 10 μg iodogen for 5 minutes and stopped by removing the solution from the tube for purification. In case of chloramine-T, 25 μg of chloramine-T (2.5mg/mL) was added, mixed for 2 minutes and arrested by the addition of 25 μL of sodium metabisulphite (7.6 mg/mL). In the case of lactoperoxidase, 4 μg lactoperoxidase with 2 μL 1:10,000 $H_2O_2$ was used. After 2 minutes at ambient temperature, the reaction was arrested by dilution with buffer. In all the cases, purification was carried out by gel filtration over Sephadex G-25 column (1×25 cm) using phosphate buffer (0.025 M, with 0.25% BSA, pH 7.5) as the eluant. The reaction yield and radiochemical purity were estimated by paper

electrophoresis using Whatman- 3 chromatography paper, in phosphate buffer (0.025 M, pH 7.5) for 1 h at 10 V/cm. The immunoreactivity of the peak fractions was checked.

One of the monoclonal antibodies raised, Mab-4G4, assessed to be the best of the lot, was labelled with $^{125}I$ using chloramine-T and bio-distribution studies in Wistar albino rats were carried out.

## 2.4. Radiolabelling of Mab with $^{99m}Tc$

Mab-4G4, was labelled with $^{99m}Tc$ using stannous tartrate as the reducing agent. 16 MBq (~420 μCi) of $^{99m}Tc$ was mixed with 0.1 mL of a 5 mg/mL solution of stannous tartrate. To this was added ~80 μg of antibody in normal saline and reacted for 30 minutes. The radiolabelling was also attempted after reduction of the antibodies with 2-mercaptoethanol [11]. The radiochemical yield was determined by paper chromatography using saline as the mobile phase. The mixture was purified through Sephadex G-25 (1×10 cm) column. The immunoreactivity of the peak fractions was tested and the appropriate fraction was used for bio-distribution studies in Wistar albino rats.

## 2.5. Bio-distribution of $^{99m}Tc$ and $^{125}I$ labelled Mab-4G4

Myocardial infarction was induced in Wistar albino rats by two subcutaneous injections of isoproterenol (75 mg/kg) on two consecutive days[12]. About 4 hours after the second injection, radiolabelled Mab-4G4 (4–5 μCi, ~1 μg/rat ) was intravenously injected. After 24 hours, the animals were sacrificed and radioactivity in different organs was determined.

## 3. RESULTS AND DISCUSSION

The HCM obtained was highly pure and was very similar to the BSM as confirmed by the SDS PAGE pattern. HCM was stored in 0.6 M KCl diluted with equal volume of glycerol at -20°C.

**TABLE I : ISOTYPES, TITRE, AFFINITY AND CROSS REACTIVITY OF HCM MONOCLONAL ANTIBODIES**

| Clone (Mab) | Isotype | Titre | Relative Affinity - $IC_{50}$* | % Cross reaction with BSM |
|---|---|---|---|---|
| 4G4 | IgG2a, λ | 1:780 | 4.9 | 117 |
| 8G5 | IgM, κ | 1:450 | N.D. | 107 |
| 1G4 | IgG2b, λ | 1:50 | 3 | 143 |
| 8C4 | IgG1, λ | 1:70 | 1 | 115 |

** Relative affinity was measured in terms of amount of myosin in μg/mL required to achieve 50% ($IC_{50}$) inhibition of the antimyosin activity assessed by ELISA,*

*N.D.- Not carried out since it was IgM*

All the immunised BALB/c mice exhibited the presence of antibodies against HCM after the booster. After fusion and initial plating in 131 wells, antimyosin antibody activity was detected in the culture supernatants of 46 wells. After the two limiting dilution steps, 13 clones could be identified. Of these, four clones were selected on the basis of their reasonable titre values. Table I shows the isotypes, titres, affinities of the different antibodies and their cross reaction with bovine skeletal myosin (reaction with HCM is taken as 100%) of the selected antibodies. Barring one clone of IgM type, the clones were of IgG type, belonging to different subtypes. Being pentavalent, IgM was not used for further studies. The titres of these antibodies however, are not too high, perhaps because myosin is a complex sequestered protein of all vertebrates. None of these clones reacted with murine Coxsackie B virus, B1 to B6, or the light and heavy chains of rabbit myosin. It is seen that all the antibodies exhibited strong cross reactivity against bovine skeletal myosin, some even stronger than that for HCM. Several epitopes on HCM may

be "self" epitope for mouse and only a few epitopes may get exposed. It has been reported that complex antigens result in low affinity antibodies as compared to small antigens[13]. Such moderate affinity combined with the striking similarity between HCM and BSM could have resulted in high cross reactions for BSM. The affinity for HCM expressed in terms of HCM required to inhibit the solid phase binding by 50 % is seen to vary for the Mabs. The greater the amount required to inhibit the binding, the better the affinity and Mab-4G4 had the maximum affinity for HCM as well as maximum titre value. Mab-4G4 was hence used for labelling with $^{125}I$ and $^{99m}Tc$.

The ascitic fluids were purified satisfactorily by a two step method using caprylic acid precipitation to remove the non-IgG fractions, followed by the precipitation of the IgG fractions with ammonium sulphate and dialysis to remove salt.

$^{125}I$-Mab-4G4 was prepared and studied for its localization with a view to explore the possibility of using $^{123}I$-Mab-4G4 as an immunoscintigraphic agent for infarct localisation. Irrespective of the oxidant used, the labelling yield of Mab-4G4 with $^{125}I$ was ~ 60% by paper electrophoresis and the radiochemical purity of the labelled antibody purified over G-25 gel was >95%. Similar results were obtained with other anti-HCM Mabs also. Although there was no significant difference when different oxidants were used in the quality of labelled Mab obtained in terms of purity, shelf life etc., the yields were consistently around 60% with chloramine-T while it fluctuated with the other two. A typical gel chromatography pattern is seen in Fig.1.

Radiolabelling yield with $^{99m}Tc$ was observed to be ~ 61-65%. The use of other transchelating agents such as citrate or reduction of antibodies with 2-mercaptoethanol did not improve the yield. Despite the variations in the amounts of stannous tartrate used for reduction of $TcO_4^-$, the yields were around 60%. On the other hand, similar experiments with purified immunoglobulins gave >90% labelling yield.

**TABLE II: BIO-DISTRIBUTION OF $^{125}$I-Mab-4G4 IN WISTAR RATS (n=3)**

| Organ | % Activity/organ ± s.d. | | % Activity/g ± s.d. | |
|---|---|---|---|---|
| | Normal | Infarcted | Normal | Infarcted |
| Blood | 20.2 ± 5.2 | 25.8 ± 6.2 | 1.2 ± 0.4 | 1.2 ± 0.25 |
| Heart(perfused) | 0.3 ± 0.02 | 0.51 ± 0.1 | 0.3 ± 0.03 | 0.5 ± 0.1 |
| Lungs | 0.8 ± 0.2 | 0.6 ± 0.1 | 0.4 ± 0.2 | 0.5 ± 0.2 |
| Liver | 5.5 ± 2.1 | 3.5 ± 1.1 | 0.4 ± 0.1 | 0.3 ±0.1 |
| Spleen | 0.4 ± 0.2 | 0.5 ± 0.1 | 0.6 ± 0.2 | 0.9 ± 0.2 |
| Kidneys | 3.0 ± 0.3 | 2.9 ± 0.1 | 2.2 ± 0.3 | 1.8 ± 0.1 |
| Muscle | - | - | 0.3 ± 0.1 | 0.5 ± 0.3 |
| Thyroid | 74 ± 8 | 81 ± 0.5 | - | - |
| Carcass | 2.7 ± 1.2 | 3.8 ± 0.3 | 0.02 ± 0.01 | 0.01 ± 0.001 |

*Only % act./g was taken in the case of muscle primarily to compare with the accumulation in other organs; while in the case of thyroid only % activity in the whole organ was alone calculated as a measure of in-vivo deiodination..*

**TABLE III: BIO-DISTRIBUTION OF $^{99m}$Tc-Mab-4G4 IN WISTAR RATS (n=3)**

| Organ | % Activity/ organ ± s.d. | | % Activity/ gram ± s.d. | |
|---|---|---|---|---|
| | Normal | Infarcted | Normal | Infarcted |
| Blood | 6.9 ± 2.2 | 3.3 ± 1.2 | 0.8 ± 0.3 | 0.35 ± 0.15 |
| Heart (perfused) | 0.1 ± 0.05 | 0.5 ± 0.03 | 0.2 ± 0.1 | 0.7 ± 0.04 |
| Lungs | 0.4 ± 0.3 | 1.1 ± 0.6 | 0.6 ± 0.4 | 1.1 ± 0.1 |
| Liver | 45 ± 7.8 | 57 ± 6.8 | 8.6 ± 2.0 | 6.9 ± 0.4 |
| Spleen | 1.8 ± 0.2 | 3.9 ± 0.6 | 2.4 ± 0.4 | 4.8 ± 1.6 |
| Kidneys | 24.3 ± 5.5 | 14.1 ± 2.0 | 15.0 ± 3.1 | 8.9 ± 1.4 |
| Intestine | 9.7 ± 4.6 | 12.2 ± 4.1 | 0.7 ± 0.4 | 0.7 ± 0.3 |
| Muscle | - | - | 0.03 ± 0.01 | 0.13 ± 0.1 |
| Stomach | 7.7 ± 2.8 | 2.3 ± 0.6 | 1.1 ± 0.2 | 0.4 ± 0.1 |
| Carcass | 11.6 ± 0.6 | 8.6 ± 3.5 | 0.05 ± 0.03 | 0.07 ± 0.04 |

*Only % act./g was taken in the case of muscle primarily to compare with the accumulation in other organs.*

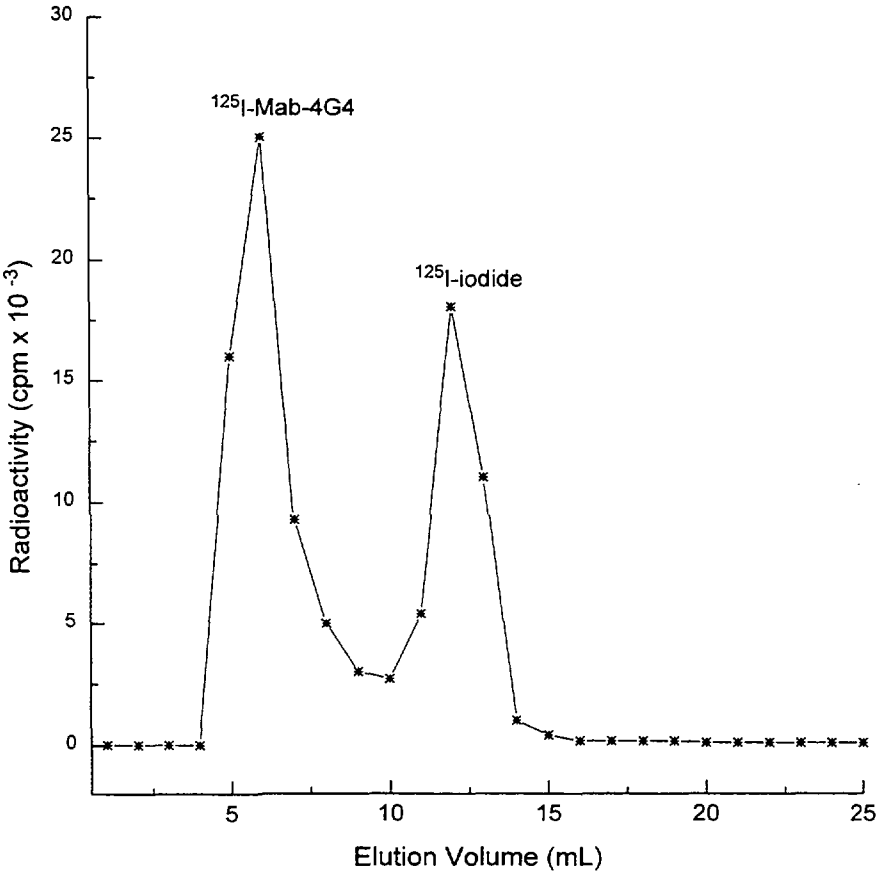


Fig. 1. Elution pattern of radioiodinated Mab-4G4 over sephadex G-25 column

Table II and III show the bio-distribution pattern of the labelled Mab-4G4, in normal and MI induced rats when $^{125}$I-Mab-4G4 and $^{99m}$Tc-Mab-4G4 were used respectively. The induction of MI was confirmed by histopathological examination and the damage to the myocardium could be clearly visualised. The net total activity retained at the end of 24 hours was taken as the total activity, since the prime aim was to study the distribution pattern of the retained activity in various organs. From the table II, it can be seen that severe *in-vivo* deiodination of $^{125}$I-Mab-4G4 has occurred leading to accumulation of ~ 70-80% activity in the thyroid in 24 hours. It was rather surprising to note that the amount of activity accumulated in the thyroid is much higher than even a normal thyroid uptake. High thyroid uptake has been a major problem with radioiodinated antibodies, especially when the tyrosyl moiety is labelled [14-16] and

alternative methods have been suggested [17]. Attempts are now being made to radioiodinate the Mabs through modified linkers such alpha methyl tyrosine. In this study, it is encouraging to find a 1.5 fold increase in activity accumulation in the infarcted heart compared to the normal heart, although increased uptake is seen in other organs such as spleen, lungs, muscles etc. of the infarcted animal. It is not known if this is due to the effects of isoproterenol, the agent used for artificially producing the infarct. We are also attempting to produce infarcts in experimental animals by other means. But, use of a radioiodinated antibody for scintigraphy requires a lot of efforts to improve the performance at this stage.

In the case of $^{99m}$Tc-Mab-4G4, 3.5 fold increase in activity was seen in hearts where MI was induced. In this case, the activity has also accumulated in several non-target organs such as liver, kidneys and spleen to a considerable extent. The increased liver uptake in the case of $^{99m}$Tc-Mab-4G4 which is not seen in the case of $^{125}$I-Mab-4G4 is perhaps an indication of aggregation of antibody molecules. In a similar manner, the activity in stomach and intestines were also appreciable for $^{99m}$Tc-Mab-4G4 while they were negligible for $^{125}$I-Mab-4G4 (not tabulated). Attempts to label this antibody with $^{99m}$Tc through a suitable bifunctional chelating agent are also being made. As mentioned earlier, use of isoproterenol for infarct induction could be causing changes in the organs such as spleen, muscles etc. On comparing the two labelled antibodies, it is seen that in the case of radioiodinated antibody, the activity retained in thyroid and blood are much higher while that in liver, kidneys and carcass are much lesser than those for $^{99m}$Tc-Mab-4G4. As discussed before, these effects could be due to multiple reasons such as *in-vivo* deiodination, aggregation etc.

In conclusion, of the various Mabs tested, Mab-4G4 had high affinity for HCM. Bio-distribution studies using $^{99m}$Tc-Mab-4G4 and $^{125}$I-Mab-4G4 in Wistar albino rats

with induced MI have given results that are encouraging but needing improvement. Although a product that could be used for infarct imaging has not been achieved in the present studies, monoclonals specific to HCM could be prepared and their use for development of *in vitro* or *in vivo* diagnostic agents appears possible. We propose to extend this work by using $F(ab)_2$ fragment of the Mabs.

## ACKNOWLEDGEMENTS

The authors are thankful to Dr. S.M. Rao, Head, Isotope Division and Dr. D.D. Sood, Director, Radiochemistry and Isotope Group, Bhabha Atomic Research Centre for the encouragement and support.

## REFERENCES

[1] AHUMADA, G., ROBERTS, R., SOBEL, B. E., Evaluation of myocardial infarction with enzymatic indices, Prog. Cardiovasc. Dis., **18** (1976) 405-420.

[2] WILLERSON , J. T., BUJA , L. M., $^{99m}$Tc-pyrophosphate and $^{111}$In-antimyosin-antibody scintigraphy appear to be more comparable methods for infarct detection. J. Am. Coll. Cardiol., **17** (1991) 527-529.

[3] LEGER, J. O. C., et al., Levels of ventricular myosin fragments in human sera after acute myocardial infarction determined with monoclonal antibodies to myosin heavy chains, Eur. J. Clin. Invest., **15** (1985) 422-428.

[4] MARGOSSIAN, S. S., LOWEY, S., Preparation of myosin and its sub-fragments from rabbit skeletal muscle, Meth. Enzymology, **85** (1982) 55-71.

[5] KHAW, B.A., MATTIS J.A., et al., Monoclonal antibody to cardiac myosin: Imaging of experimental myocardial infarction, Hybridoma, **3** (1984) 11-23.

[6] MOUDGIL, K.D., QUADRI, A., GUPTA, S.K., Mouse myeloma (Sp2/0-Ag-14) culture supernatant as a substitute for feeder cells in mouse×mouse and rat×mouse hybridomas, Ind. J. Expt. Biol., **24** (1986) 578-580.

[7] MUELLER, H., HAWES, C.S., JONES, W.R., Monoclonal antibody production by hybridoma growth in Freund's adjuvant primed mice, J. Immun. Meth., **87** (1986) 193-196.

[8] RUSSO, C., et al., Purification of IgG monoclonal antibody by caprylic acid precipitation, J. Immun. Meth., **65** (1983) 269-271.

[9] KHAW, B. A., et al., Imaging with antibodies, "The heart and the cardiovascular system" ( FOZZARD et al. eds), Raven press, N. Y., chap. **23**, 423-451(1986).

[10] SEIGFRIED, M., HENNING, K., METTE, N., Iodination of monoclonal IgG antibodies at a sub-stoichiometric level: Immunoreactivity changes related to the site of iodine incorporation, Nucl. Med. Biol., **14** (1987) 451-457.

[11] MATHER, S.J., ELLISON, D., Reduction mediated technitium-99m labeling of monoclonal antibodies, J. Nucl. Med., **31** (1990) 692-697.

[12] TEERLINK, J. R., PFEFFER, J. M., PFEFFER, M. A., Progressive ventricular remodelling in response to diffuse isoproterenol induced myocardial necrosis in rats, Circ. Res., **75** (1994) 105-113.

[13] KUBY, J., "Immunology", Chapter 4, 73-97, W.H. Freeman & Co. Publ., (1992).

[14] ZHENPING, Z., et al., Immunoreactivity, stability, pharamcokinetics and bio-distribution of a monoclonal antibody to human leukemic B cells after three different methods of radioiodination. Nucl. Med. Biol., **21** (1994) 873-882.

[15] SEIGFRIED M., HENNING, K., METTE, N., Iodination of monoclonal IgG antibodies at a sub-stoichiometric level: Immunoreactivity changes related to the site of iodine incorporation, Nuc. Med. Biol., **14** (1987) 451-457.

[16] HNATOWICH, D.J., Antibody radiolabelling : Problems and promises, Nucl. Med. Biol., **17** (1990) 49-55.

[17] ZALUTSKY, M.R., NARULA, A.S., A method for radiohalogenation of proteins resulting in decreased thyroid uptake of radioiodine. Appl. Radiat. Isot., **38** (1987) 1051-1055.