

Labelling, quality control and clinical evaluation of monoclonal antibodies for scintigraphy

*Final report of a co-ordinated research programme
1991–1996*



INTERNATIONAL ATOMIC ENERGY AGENCY

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**LABELLING, QUALITY CONTROL AND CLINICAL EVALUATION OF
MONOCLONAL ANTIBODIES FOR SCINTIGRAPHY**

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FOREWORD

The discovery of monoclonal antibodies spurred considerable interest in their applications in medical diagnostic techniques soon after their availability. They had been and still continue to be the subject of innumerable publications. They are now extensively used in kits for in vitro immunodiagnosics including radioimmunometric assays.

Monoclonal antibodies also were perceived to offer excellent carriers for radioisotopes for specific in vivo targeting of selected tissues, virtually as a long awaited 'magic bullet'. But the in vivo applications posed many technical challenges including purification of monoclonal antibodies of mouse origin to standards needed for human parenteral use, immune response in humans to mouse immunoglobulin, labelling the radioisotopes, particularly $^{99}\text{Tc}^{\text{m}}$, to the antibody molecule without significantly altering its immune properties, slow in vivo kinetics of labelled antibody, its non specific localisation and transchelation of $^{99}\text{Tc}^{\text{m}}$ and other metallic radionuclides by other serum proteins in vivo. These were, no doubt, formidable challenges but have provided an area of intense and interesting research and development spanning almost a decade. The results of these efforts have contributed significantly to the understanding of the co-ordination chemistry of technetium, synthesis of bifunctional chelates of immunoglobulins to label radionuclides, development of sensitive analytical methods for labelled antibodies and to the overall capabilities of radiopharmaceutical scientists to manipulate bioactive molecules for radiolabelling. Now a few ^{111}In and $^{99}\text{Tc}^{\text{m}}$ labelled monoclonal antibody preparations are already available for regular diagnostic use in nuclear medicine.

Realising the potential of labelled monoclonal antibodies for in vivo diagnosis and therapy and the interest in many developing Member States for acquiring expertise in this field the IAEA initiated a co-ordinated research programme in 1991 focusing on $^{99}\text{Tc}^{\text{m}}$ labelling of antibodies, their quality control and scintigraphic evaluation. Twelve laboratories from Asia, Latin America, Europe and North America participated in this programme which was concluded in 1996. During this programme the participants investigated the $^{99}\text{Tc}^{\text{m}}$ labelling of a murine anti-CEA antibody using the method of chelating $^{99}\text{Tc}^{\text{m}}$ with the free sulfhydryl groups generated by reaction with reducing agents such as mercapto ethanol. During the later part of the programme this method was also extended to $^{99}\text{Tc}^{\text{m}}$ labelling of hIgG. All the participating laboratories could gain valuable experience in $^{99}\text{Tc}^{\text{m}}$ antibody labelling techniques and formulation of kits. Many of them have been used in patients by collaborating nuclear medicine specialists with satisfactory results. This report is a compilation of the detailed results obtained by the participating laboratories and includes a summary and assessment of the achievements of the CRP.

The IAEA wishes to thank all the scientists who contributed to the success of the CRP.

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1. INTRODUCTION AND SUMMARY OF RESEARCH RESULTS

1.1. OBJECTIVES OF THE CO-ORDINATED RESEARCH PROGRAMME

The Co-ordinated Research Programme (CRP) on Labelling, Quality Control and Clinical Evaluation of Monoclonal Antibodies for Scintigraphy was organized as a follow-up of the recommendations of a consultants meeting organized by the IAEA in Vienna in August 1988. It was generally concluded in the meeting that radiolabelled monoclonal antibodies could be potentially useful for the scintigraphic detection of malignancies and labelling methods reported at that time showed sufficient promise for further development and absorption by laboratories in developing countries. The other recommendations made by the consultants with regard to the CRP were:

- (a) Methods for ^{131}I labelling of monoclonal antibodies (MoAb) are fairly well developed and could form part of the proposed CRP. However, considering the availability, cost and imaging properties, $^{99}\text{Tc}^{\text{m}}$ is universally recognized as the superior radiolabel for most imaging studies. Methods for $^{99}\text{Tc}^{\text{m}}$ labelling were under development in many laboratories and promising results were being reported, particularly using the antibody reduction method. These developments should be carefully watched and suitably incorporated in the proposed CRP work plan;
- (b) It was recognized that monoclonal antibody against carcino embryonic antigen (CEA), which is elevated in about 90% of colorectal cancer cases and also in significant numbers of other malignancies including breast, gastric, lung and medullary thyroid carcinomas could be chosen for radiolabelling under the CRP since it may lead to an agent potentially useful for their diagnosis and follow up. Anti-CEA monoclonal antibodies had also the advantage in their availability from several sources;

Twelve scientists from reputable laboratories from both developing and developed countries participated in the CRP organized during the period 1991–1996. During the course of the CRP four research co-ordination meetings were held where the participants presented and discussed the outcome of their investigations. The location and dates of the meetings were: Kuala Lumpur, Malaysia, 9–13 September 1991, Cambridge, United Kingdom, 15–19 March 1993, Sydney, Australia 19–22 October 1994 and Athens, Greece, 10–14 June 1996.

Monoclonal antibodies

In the beginning of the CRP an anti-adenocarcinoma murine IgG antibody of Biomira (Edmonton, Canada) designated as MoAb 170 was used. Although not an anti-CEA antibody, MoAb 170 was reported to be useful in the detection of gynecologic adenocarcinomas, breast carcinoma, and lung carcinoma.

Valuable experience was gained by all participants using MoAb 170 even though some unexpected problems came in the way of labelling and evaluation of this antibody. The antigen specifically reacting with MoAb 170 was not available, presumably because of its rapid degradation following isolation. Hence it would not be possible for participants in the CRP to determine the immunoreactivity of their labelled MoAb 170. Furthermore, although the MoAb 170 was reported to be radiolabelled successfully with $^{99}\text{Tc}^{\text{m}}$ by colleagues at Biomira using a proprietary labelling method, it proved difficult to label this antibody with the mercaptoethanol reduction method. In order to achieve labelling, the concentration of mercaptoethanol had to be raised by about a factor of five. Possibly because of these harsh reducing conditions, the immunoreactivity of the labelled MoAb 170 was reduced to about 16% of that of the native antibody. Because of these problems, MoAb 170 was given up and a decision was made to look for another monoclonal antibody.

A murine anti-CEA monoclonal antibody of the Center of Molecular Investigation, Havana, Cuba designated as ior-CEA-1 was made available subsequently. Ior-CEA-1 is an IgG1 MoAb

secreted by the F6/1B2-D2 hybridoma obtained from the fusion of the P3/X63 Ag8 653 murine myeloma with splenic lymphocytes of a Balb/c mouse immunized with CEA purified from a liver metastases of a colon adenocarcinoma. The ior-CEA-1 MoAb is purified from mouse ascitic fluid by affinity chromatography in protein A sepharose. This antibody was provided in several lots and in sufficient quantities with which labelling methods were standardized and in many cases, clinical trials were performed.

In addition to the anti-CEA antibody, participants were also provided with an inexpensive human IgG preparation (Sandoglobulin) for simulated labelling experiments.

Labelling methods

At the start of the CRP as stated above, a novel direct labelling method for $^{99}\text{Tc}^{\text{m}}$, which employed mercaptoethanol as antibody reducing agent was reported. This method was refined by one of the participating laboratories in the CRP and was shown to be reliable and reproducible. Although many other similar direct labelling methods were subsequently described in the literature, they offered no clear advantage to the mercaptoethanol method. Accordingly, this method was adopted for the CRP and proved to be very effective. The reduced $^{99}\text{Tc}^{\text{m}}$ presumably as $\text{Tc}(\text{v})$ is widely believed to be coordinated by two sulfhydryl (-SH) groups produced by the reduction of disulfide bonds in the hinge region of the antibody molecule. There is considerable indirect evidence for this hypothesis even though it has not been directly validated.

Quality assurance

It was considered essential that (a) radiochemical purity of the labelled antibody be routinely assessed, (b) the immunoreactivity be assessed initially and occasionally thereafter, (c) the stability of the label on the antibody be evaluated in human serum at 37°C, and (d) the biodistribution in mice be evaluated. It was also considered important that these, and other quality control procedures be performed in a uniform manner by all participants such that the results could be readily compared. Accordingly, detailed methods for antibody reduction and radiolabelling, for radiochemical purity determination, immunoreactivity measurements, for cysteine challenge and serum stability measurements and for animal biodistribution measurements were compiled and became a useful reference for participants. Based on the experience of the participants, the procedures recommended for labelling and quality control for use by these interested in preparing $^{99}\text{Tc}^{\text{m}}$. Antibodies are given in the Annex.

The results obtained by the participants are described in the individual reports included in this publication. The preparation procedure has resulted in >95% $^{99}\text{Tc}^{\text{m}}$ labelling efficiency and radiochemical purity. The other tests for evaluating the $^{99}\text{Tc}^{\text{m}}$ antibodies were primarily aimed at assessing their usefulness in predicting the in vivo stability. The results obtained by various participants for these stability tests are comparable. Further correlation with biological and clinical studies using different antibodies would be needed to arrive at acceptable cut-off values for these tests. However, test results reported by various participants can be used as guidelines by scientists working on $^{99}\text{Tc}^{\text{m}}$ antibody labelling.

Kit preparation

Initially, it was proposed that kits could be prepared and used based on storing the reduced antibody frozen. This in-house kit was found to work in the hands of several participants and remain stable for long periods. Later on, several participants also developed methods for the preparation of freeze-dried kits which were suitable for human use. Based on the experience of the participants, the procedures recommended the labelling and quality control for use by these interested in preparing $^{99}\text{Tc}^{\text{m}}$. Antibodies are given in the Annex.

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Clinical trials

Although not a goal of the CRP, as a result of the effort and initiative of participants of the CRP, several clinical trials were performed in collaboration with nuclear medicine specialists using $^{99}\text{Tc}^{\text{m}}$ labelled ior-CEA-1 antibody developed under this CRP.

Additional studies

On their own initiative, the participants also performed a number of separate but related research projects. It is expected that the results of these investigations will ultimately be the subject of several research reports appearing in the scientific literature.

1.2. PRINCIPAL OUTCOMES AND CONCLUSIONS OF THE CO-ORDINATED RESEARCH PROGRAMME

This CRP was established for development of methods for radiolabelling monoclonal antibodies for potential use in scintigraphy and for their quality control. As the programme progressed the efforts were focused on $^{99}\text{Tc}^{\text{m}}$ labelling of anti-CEA antibody and evaluation of the integrity of the labelled antibody for possible use in scintigraphic imaging of CEA secreting tumours. Considerable volume of useful data was collected during the course of the CRP on labelling methodology, quality control procedures, biodistribution studies and kit formulation by all the participants.

General outcome

The programme has provided opportunity for many participants to gain hands on experience in antibody labelling and in house development of a new range of skills and techniques covering their preparation, quality control storage and use. This expertise is a valuable resource which can be extended to development of other antibody based radiopharmaceuticals. In fact a number of centres have already applied the protocols developed in this programme to other antibodies which have either been developed in their own institutes or obtained through collaboration with other centres. In future this experience will be very useful for the laboratories to undertake radiolabelling and evaluation of other biomolecules such as other recombinant proteins, peptides and oligonucleotides which hold promise for future radiopharmaceutical development.

The programme has also provided opportunities for the participants to meet and interact at periodic intervals to share information on results obtained, discuss technical problems and derive solutions. The participants in this CRP had also an opportunity to interact with nuclear medicine specialists participating in a CRP organized by the Nuclear Medicine Section on Immunoscintigraphy using $^{99}\text{Tc}^{\text{m}}$ -labelled Anti-CEA Monoclonal Antibodies for the Detection of Colonic Cancer at two joint research co-ordination meetings. These joint meetings helped each group to better understand the viewpoints and requirements of the other. The feed back on the clinical use of labelled antibody obtained in these joint meetings helped many participants to stimulate interest and awareness of their clinical colleagues in their own centres and has led to use of $^{99}\text{Tc}^{\text{m}}$ labelled ior-CEA-1 antibody in patients in a number of centres. The programme has also provided a stimulus for undertaking the production of new monoclonal antibodies for

immunoscintigraphy in a number of associated institutes in participating countries. Several new radiopharmaceuticals based on these reagents are now under development. The CRP has therefore provided an important catalyst for new areas of collaboration for the participants particularly from developing countries both at national and international level. Exchange of reagents and materials amongst the participants has been an important feature of this CRP and it is expected that this collaboration will continue even after the conclusion of the CRP.

Scientific developments

After the initial establishment of the working protocols in each laboratory, the participants have used these techniques to perform some valuable comparisons between a number of labelling techniques such as the mercaptoethanol reduction technique, iminothiolane conjugation, ascorbate reduction etc. The effect of these labelling chemistries on the antibodies have been studied with chromatographic and electrophoretic techniques, antigen-binding assays and biodistribution studies. New labelling techniques based on the use of novel bifunctional chelators such as hydrazino nicotinamide and MAG3 and photoactivation have also been evaluated. These scientific developments have resulted in the publication of several papers as described in the individual reports. In addition many of the standard techniques employed have been further developed and refined. Although not the primary goal of this CRP, technetium-labelled anti-CEA antibody has been used in clinical trials in many participating countries. As a part of the programme human immunoglobulin was also labelled, initially as a model antibody, but subsequently for possible application for imaging infection and inflammation in patients. This latter application has been particularly successful. A summary of the level of clinical application is shown in Table I.

TABLE I. CLINICAL EVALUATION OF $^{99}\text{Tc}^{\text{m}}$ LABELLED hIgG AND ior-CEA-1 BY THE RESPECTIVE PARTICIPANT COUNTRIES UNDER THE CRP

Country	Product	Formulation	Number of patients	Number of institutions using the kit
Hungary	ior-CEA kit		10	3
	hIgG kit	Lyophilized	100	5
	Mab 47 kit		100	5*
India	ior-CEA kit	Lyophilized	0	
	hIgG kit		68	2
Portugal	ior-CEA kit	Lyophilized	9	2
	hIgG kit		3	
Greece	ior-CEA kit	Lyophilized	0	1
	hIgG kit		30	
Argentina	ior-CEA kit	Lyophilized	10	3
	hIgG kit		25	8
Cuba	ior-CEA kit	Frozen	> 70	5
	hIgG kit		> 60	5
Thailand	ior-CEA kit	Frozen	30	1
	hIgG kit		18	1

*in Switzerland.

China: none at present.

Reports from Malaysia and Czech Republic not received.

1.3. PROTOCOLS OF THE CO-ORDINATED RESEARCH PROGRAMME

At the beginning of this project a number of protocols were proposed for labelling of the antibody and subsequent evaluation. These protocols have been refined during the progress of the programme and have been fully evaluated by all the participants. The protocol based on

mercaptoethanol reduction detailed in this report has been found to be reliable and reproducible in the hands of the participating scientists and can therefore be recommended as a general method for $^{99}\text{Tc}^{\text{m}}$ labelling of antibodies. Methods for radiochemical analysis involving thin-layer chromatography, liquid chromatography and SDS-PAGE are also described. For antigen-binding studies, a competitive binding assay which is simple and can be used in any laboratory is described here. This method can be used for evaluation of any labelled antibody provided the antigen is available as was the case with CEA. For in-vivo evaluation, a normal animal model namely Balb/c mice, with a single four-hour measurement point was chosen since this was considered to provide the most reproducible model for inter-laboratory comparison. The limited number of clinical studies performed in different centres suggests that the results obtained with the ior-CEA-1 antibody and those with a commercially available anti-CEA antibody are very similar. However more clinical data may be needed to arrive at recommended imaging protocols.

2. SUMMARY REPORTS OF PARTICIPATING COUNTRIES

2.1. ARGENTINA



Research contract No. 6282/RB

Title of the Project : Development of Radiolabelling Techniques of
Anti-CEA Monoclonal Antibody

Chief Scientific Investigator : S.G. de Castiglia
Comisión Nacional de Energía Atómica,
Centro Atómico Ezeiza, Buenos Aires

2.1.1. Introduction

Different radionuclides have been tried for radiolabelling of antibodies in order to localize small tumours [1]. $^{99}\text{Tc}^m$ is the most popular radionuclide for clinical imaging because it has ideal nuclear properties, i.e. a single photon energy of 140 KeV, a half-life of 6 h and it is readily available from a ^{99}Mo - $^{99}\text{Tc}^m$ generator. Antibodies labelled with $^{99}\text{Tc}^m$ should be useful in tumour detection if localization and blood clearance are rapid enough to take advantage of the short half-life. There are different routes for labelling antibodies with $^{99}\text{Tc}^m$ directly, that is reacting endogenous sulphhydryl groups generated within the antibody [2] and indirectly, either before or after attachment of an exogenous chelator [3-4] to the antibody to bind reduced $^{99}\text{Tc}^m$. Recently, more attention has been paid to the direct labelling method due to the possibility of an "instant kit" formulation. In most cases the direct method is a combination of two sequential steps: the reduction of disulfide groups in the antibody and the use of a ligand capable of transferring the reduced technetium to the sulphhydryl groups of the protein [5-7]. Normally a purification step is necessary in order to remove the excess of reductant. Sykes et al. [8] have developed a photoactivation method based on irradiation of antibodies with ultraviolet light in order to reduce disulphide bonds. The advantage of this method is that no purification step is needed.

In indirect methods, technetium is coordinated by a synthetic chelating agent which may be conjugated to the immunoglobulin either before, or after, the radiolabelling process. The hydrazinonicotinamide system developed by Abrams and co-workers [9] has been successfully used for preparation of specifically labelled proteins and peptides with high specific activities. The chelator is covalently attached and labelling is accomplished by transchelation from $^{99}\text{Tc}^m$ glucoheptonate (GH) and $^{99}\text{Tc}^m$ tyrosine to produce $^{99}\text{Tc}^m$ products.

Recently, Winnard et al. [10] have developed a simple synthesis of the N-hydroxysuccinimide derivative of MAG_3 (NHS- MAG_3) in which a different protecting group (acetyl) from MAG_3 has been used. labelling with $^{99}\text{Tc}^m$ is achieved at room temperature by transchelation from $^{99}\text{Tc}^m$ glucoheptonate.

The purpose of this work was to label monoclonal and polyclonal antibodies with $^{99}\text{Tc}^m$ such as the ior-CEA-1 antibody and polyclonal IgG using a direct method, to check the radiochemical and biological behavior of labelled products, to prepare it under sterile and apyrogenic conditions as a lyophilized kit and to employ it in clinical trials. In addition, a photoactivation method was used to label polyclonal IgG with $^{99}\text{Tc}^m$ and to compare with the established method using mercaptoethanol (2-ME) as the reducing agent. Finally polyclonal IgG was labelled using an indirect method in which a chelator was covalently attached to the protein and the $^{99}\text{Tc}^m$ added as a glucoheptonate complex. The properties of $^{99}\text{Tc}^m$ when labelled with monoclonal and polyclonal antibodies by different methods were assessed by in vitro and in vivo studies.

2.1.2. Materials and methods

2.1.2.1. Direct labelling of ior-CEA-1 antibody

Labelling and quality control

Ior-CEA-1 monoclonal antibody (MoAb), produced by the Centro de Inmunología de la Habana, Cuba, which recognizes the carcinoembryonic antigen (CEA), was prepared at a concentration of 10 mg/mL in 0.1 M PBS, pH7.0 and labelled with $^{99}\text{Tc}^{\text{m}}$ using a direct method in order to be used in immunoscintigraphy. Monoclonal antibody purity was assessed before and after labelling by polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion HPLC. 2-ME was used as a reducing agent for the protein and a methylenediphosphonate (MDP) kit and $^{99}\text{Tc}^{\text{m}}$ for the labelling step. Several molar ratios (2-ME:MoAb) were tested and 1000:1 ratio was used to reduce the protein for 30 min. The solution was then purified on a Sephadex G50 column (Pharmacia, Uppsala, Sweden) and eluted with nitrogen purged phosphate buffer 10 mM pH6.0. Fractions were collected and measured at 280 nm and concentration fractions higher than 0.5 mg/mL were pooled. An aliquot (40 μL) of the MDP kit [containing 1.0 mg of MDP and 68 μg of SnF_2 per mL after reconstitution] was added followed by 187.5 MBq $^{99}\text{Tc}^{\text{m}}\text{O}_4$. Labelling efficiency was measured by instant thin layer chromatography (ITLC) developed in MEK and saline. In addition, the radiochemical purity of the labelled antibody was analyzed by size exclusion HPLC using a Protein Pak 300 (Waters) column with 0.1M phosphate buffer eluent pH7.0 at a flow rate of 1 mL/min. Recovery of the radioactivity was routinely determined.

Labelled antibody was tested for stability towards cysteine. A solution of cysteine (27 mg/mL, 100 mM) in PBS was prepared and diluted to a cysteine concentration of 0.1, 1, 5, 10, 32 and 64 mM. Each cysteine solution was added to a tube followed by a fixed volume of the labelled protein and tubes were incubated at 37°C for one hour. The cysteine: antibody molar ratios ranged from 700:1 at 100 mM of cysteine to zero in the absence of cysteine. At the end of the incubation each solution was spotted on ITLC and developed in saline and MEK.

Biodistributions in normal Balb/c mice were performed at 4 h after injection of 750 kBq of labelled antibody.

A competitive binding assay comparing the reduced antibody with unreduced antibody was carried out using CEA as antigen. Increasing amounts of native and reduced antibody were added to CEA coated tubes followed by a fixed volume of labelled antibody.

In addition an ELISA was performed comparing the binding of the reduced and native antibody with the purified antigen. Microwell plates were coated with the antigen and saturated with PBS-5% BSA. Serial dilutions of native and reduced antibody were added to each well and the binding was revealed with a rabbit antimouse antibody labelled with peroxidase. After washing the plates, the enzymatic reaction was developed with a chromogenic substrate and stopped by addition of H_2SO_4 . The optical density reading was at 492 nm.

2.1.2.2. Lyophilized kit of IgG

Labelling and quality control of IgG

The protein reduction step was achieved following the antibody reduction protocol and two molar ratios of 2-ME and protein were tried, 500:1 and 1000:1. The concentration of unreduced protein was 20 mg/mL. Lyophilized kits of reduced IgG (1 mg) were performed with the addition of 40 μL of a fresh MDP kit (1.0 mg of MDP and 68 μg of SnF_2 per mL) per mg of reduced protein. At the end of the experiments, the kit was labelled with 562.5 MBq of $^{99}\text{Tc}^{\text{m}}$ and labelling efficiency was measured by chromatographic methods: size exclusion HPLC and ITLC developed in MEK and saline. According to the obtained results, a bulk reduction of protein (50 mg) was performed at a

500:1 molar ratio. After purification aliquots containing 1 mg of reduced purified protein and MDP/SnF₂ were lyophilized. The composition of the kit was: 1 mg of reduced protein, 3 µg of SnF₂, 40 µg of MDP and saccharose as lyoprotectant. The kit was reconstituted with 2 mL of saline and the required activity of ^{99m}Tc was added.

Biological studies

An animal model was developed injecting 40 µL of turpentine in the posterior left thigh of Balb/c mice weighing approximately 25 gm. They were left for 48 h in normal conditions to develop the inflammation foci. Mice were injected with 7.5 MBq of IgG labelled with ^{99m}Tc and images were taken at 4 and 24 h with a gamma camera equipped with a medium energy collimator and 70 000 counts were preset using a 256 × 256 matrix. Biodistributions in normal Balb/c mice and in mice bearing a promoted inflammation foci were carried out at 4 and 24 h post injection of 1.875 MBq. of labelled IgG. The total injected dose was calculated by measuring syringes before and after injecting each animal. Five animals from each group were sacrificed by cervical dislocation at 4 and 24 h after injection. Samples of blood were taken and organs and tissues of interest were dissected, rinsed, dried and placed into pre-weighed tubes. The activity of all samples was counted together with appropriate dilutions of the labelled IgG and results were expressed as % ID/gm.

Stability towards storage was examined performing labelling tests at 15 days, 1, 2, 3, 4, 6 months after lyophilization. SDS PAGE was performed at the beginning and at the end of the experiment.

2.1.2.3. Lyophilized kit of ior-CEA-1 antibody

Similar experiments were done in order to obtain a lyophilized kit of reduced antibody. In this case a molar ratio of 800:1 was used and a concentration of unreduced antibody of 10–15 mg/mL. Within the quality assurance procedure the radiochemical purity, stability, sterility, apyrogenicity, immunoreactivity and biodistribution in normal Balb/c mice were studied.

2.1.2.4. Labelling of IgG by photoactivation

Labelling and quality control of photoactivated IgG

Experiments were carried out with this method to find the formulation of optimal binding of ^{99m}Tc to polyclonal IgG. In addition animal studies in normal mice and in mice bearing a promoted inflammation foci were performed.

A photochemical reactor Rayonet RMR 3000 with eight UV lamps was used. A typical labelling procedure for IgG was as follows: 100 µL of a solution of IgG in PBS (5 mg/mL) was injected into a 10 mL vial and purged with nitrogen. A fresh solution containing stannous fluoride (18/100 µL) and MDP was purged with nitrogen for 10 min and 100 µL of this solution was injected into the vial containing the protein. The vial was irradiated for 20 min in a photochemical reactor. Sodium [^{99m}Tc] pertechnetate was added to the vial and the solution was incubated for 30 min at RT. The labelled product was analyzed by size-exclusion HPLC and ITLC in saline and MEK systems.

Different parameters of the labelling reaction were evaluated such as: a) amount of tin, b) protein concentration, c) effect of storage on labelling efficiency. Labelling experiments were carried out to assess the binding of ^{99m}Tc to photoactivated IgG with different amounts of tin and to photoactivated IgG of different concentrations and the same amount of tin. Irradiation time and incubation time were kept constant in all the experiments. A bulk irradiation was performed in a vial containing 10 mg of IgG (10 mg/mL) and 2 mL of a solution of MDP/tin (43 µg of tin/mg of protein). One aliquot was immediately labelled with the radionuclide and the other aliquots were stored at -20°C and -70°C and labelled at different time intervals.

Biodistribution studies were carried out in Balb/c mice and in mice with a sterile turpentine induced inflammation in the left thigh. Both groups were injected intravenously in the tail vein with 100 μ L of photoactivated IgG (20 μ g) and results were obtained according to the protocol described above. In addition a group of four normal Balb/c mice was injected intravenously with 100 μ L of labelled IgG and each mouse was counted within a well-type ionization dose calibrator at 1 h intervals over the next 4–5 h. Loss of whole body radioactivity not accounted for by physical decay was assumed to be due to urinary excretion.

Transchelation to cysteine was performed at cysteine concentration ranging from 1000 μ M to 0 μ M. A comparison was performed between the labelled photoactivated IgG and the labelled IgG via the 2-ME reduction method.

2.1.2.5. Indirect labelling of IgG

Conjugation, labelling and quality control

Human polyclonal IgG (Sandoz) was conjugated with the NHS-MAG₃ chelator (a gift from Dr. Hnatowich, USA) according to the following protocol: IgG at a concentration of 10 mg/mL in PBS 10mM pH7.4 was conjugated at pH8.5 in bicarbonate buffer. A solution of NHS-MAG₃ (10 mg/mL) in dry DMSO was added at a molar ratio of 10:1 MAG₃:IgG. The mixture was incubated at room temperature for 30 min and purified on a 0.7 \times 20 cm column of Sephadex G-50 using 0.2M ammonium acetate buffer, pH5.2. Protein fractions were collected and concentrations measured by UV absorbance at 280 nm. The protein was radiolabelled with ⁹⁹Tc^m using glucoheptonate as transchelator. Pertechnetate was added to provide about 370–1850 MBq/mg of IgG. A freshly prepared solution (40 mg/mL) of sodium glucoheptonate (GH) in 1M bicarbonate, 0.25M ammonium acetate and 0.175 M ammonium buffer, pH8.5–9.0 was added in a sufficient volume to provide a final glucoheptonate concentration of 5.0–5.5 mg/mL. Finally, stannous ion was added from a fresh 0.5 mg/mL solution in ClH (1 μ g of Sn per 5–200 μ g of IgG and 1.875–18.75 MBq ⁹⁹Tc^m). The labelling solution was incubated at RT for 30–40 min and purified using a centrifugal filter (Centricon 30).

The labelled product was evaluated for radiochemical purity by high performance liquid chromatography analysis (HPLC) using a size exclusion column Protein Pak SW300 eluted with PBS 10 mM. Recovery was routinely determined. The product was also checked with ITLC in MEK and saline systems.

Labelled IgG was incubated at room temperature for 24 h. At various time points, aliquots of the sample were removed and analysed by HPLC and ITLC.

A cysteine challenge was performed with the labelled product. A fresh cysteine solution in phosphate buffer 0.4M, pH7.0 was prepared (0.082M). To 12 μ L of five diluted cysteine solutions, 90 μ L of the labelled protein (1.3 μ M) was added. Final concentrations of cysteine were 1000, 500, 50, 5 and 0.5 μ M. The highest molar ratio of cysteine to antibody was 908:1. After incubation at 37°C for one hour the percent of dissociation was measured by ITLC in saline.

Biodistribution studies were carried out in Balb/c mice and in mice with a sterile turpentine induced inflammation in the left thigh. Both groups were injected intravenously in the tail vein with 100 μ L of technetium-99m labelled to IgG via the bifunctional chelator NHS-MAG₃ (20 μ g) and results were obtained according to the protocol described above.

In addition a group of four normal Balb/c mice was injected intravenously with 100 μ L of labelled IgG and each mouse was counted within a well type ionization dose calibrator at 1 h intervals over the next 4–5 h.

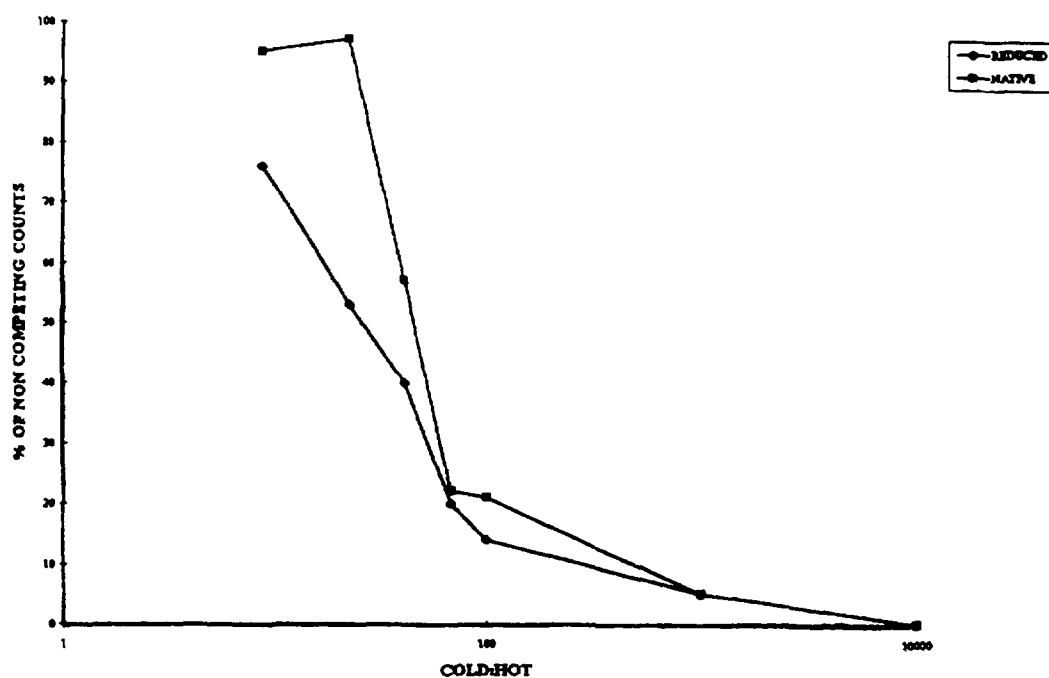


FIG. 1. Competitive binding assay comparing the reduced antibody with unreduced antibody.

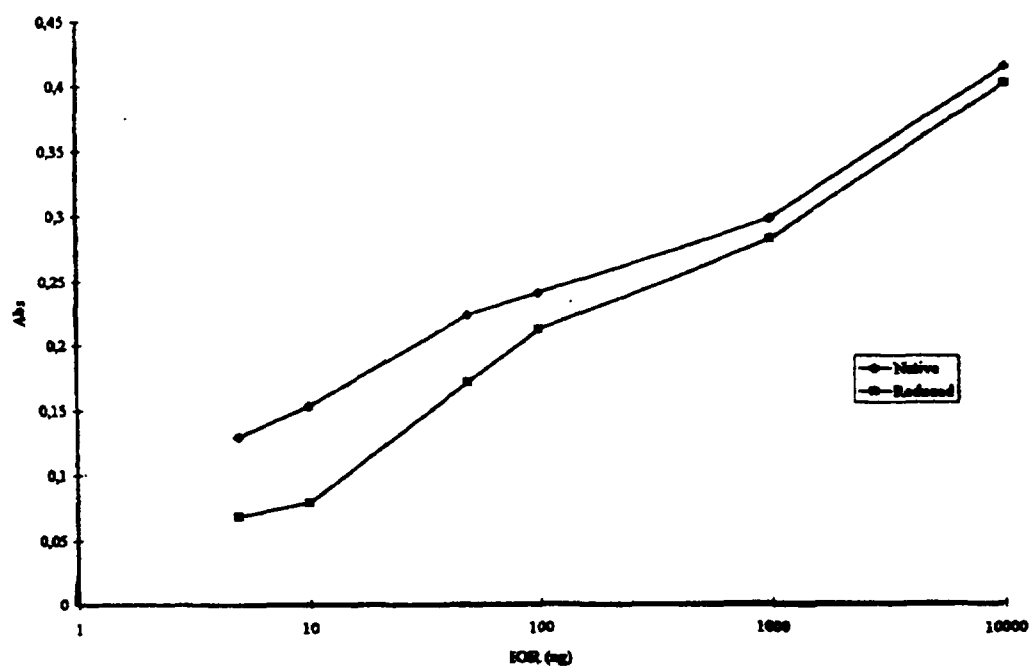


FIG. 2. ELISA comparing the binding of the reduced and native antibody with the purified antigen.

2.1.3. Results

2.1.3.1. Direct labelling and quality control of ior-CEA-1

Labelling efficiency values were higher than 95%. The HPLC radiochromatogram profile showed a single peak at 7.65 min (96%, recovery 100%) without fragments, aggregates or colloids. SDS PAGE under nonreducing conditions showed a fragmentation of the protein. 2-ME reduction followed by denaturing SDS PAGE produced fragments and comparison with the markers indicated one peak at 116.5 kd and the other one at 49.5 kd and the absence of high molecular weight species. The label is clearly not on IgG but is distributed among three lower molecular weight species and autoradiographies showed that the majority of the radioactivity was associated with the 116.5 kd peak. The instability to cysteine challenge is dependent on the temperature and this antibody showed a similar behavior towards cysteine relative to other ME reduced antibodies. Perhaps it would be necessary to perform this challenge at lower cysteine concentrations, to which the antibody may be expected to be exposed to, in vivo. Biodistribution studies showed the highest value for kidney levels (3.29 ± 0.44). Levels in blood and in other tissues tended to be lower probably in concordance with the instability showed to the cysteine challenge. Figure 1 shows the results of the competitive binding assay. Comparison of the curve shapes showed that the reduced antibody competes with binding as efficiently as the non reduced antibody. Figure 2 shows a similar curve for the reduced and native antibody with the ELISA assay.

2.1.3.2. Labelling and quality control of lyophilized IgG kit

Values for labelling efficiency of labelled IgG were higher than 95% SDS PAGE was performed in all cases and showed fragmentation of the protein. There was a slight difference between the two molar ratios. When the 500:1 ratio was used the radioactivity associated with the 116.5 kd peak was even higher. Lyophilized preparations were labelled and the labelling efficiency was higher than 95% (2% of aggregates). Biodistribution results in normal mice and in the animal model are listed in Table I and Table II respectively.

TABLE I. BIODISTRIBUTION (% ID/gm) IN NORMAL BALB/c MICE AT 4 AND 24 h POST INJECTION OF $^{99}\text{Tc}^m$ LABELLED IgG (KIT)

ORGAN	% I.D./gm at 4 h	% I.D./gm at 24 h
Blood	28.1 ± 3.0	14.5 ± 2.6
Liver	11.7 ± 1.8	6.3 ± 1.9
Spleen	7.7 ± 1.6	4.4 ± 1.6
Kidneys	22.4 ± 2.8	13.2 ± 0.9
Intestine	4.1 ± 0.9	1.2 ± 0.3
Lung	8.4 ± 1.8	5.3 ± 0.9
N.T.	4.5 ± 0.8	2.3 ± 0.5

TABLE II. BIODISTRIBUTION (% ID/gm) IN INDUCED INFLAMMATION MICE AT 4 AND 24 h POST INJECTION OF $^{99}\text{Tc}^m$ LABELLED IgG (KIT)

ORGAN	% I.D./gm at 4 h	% I.D./gm at 24 h
Blood	30.5 ± 2.5	16.3 ± 1.8
Liver	14.8 ± 1.6	8.2 ± 1.2
Spleen	10.3 ± 1.2	6.1 ± 0.9
Kidneys	27.3 ± 1.8	5.2 ± 1.3
Intestine	8.3 ± 0.9	2.5 ± 0.5
Lung	9.5 ± 1.4	5.8 ± 1.8
I.T.	10.8 ± 1.3	4.9 ± 0.8
N.T.	4.6 ± 0.5	2.6 ± 0.3

Biodistribution results showed higher kidney levels than liver levels and the ratio of I.T./N.T. was 2.3 at 4 h and 1.9 at 24 h.

Stability towards storage gave labelling efficiencies higher than 95% in all cases and no differences were observed between the two SDS PAGE autoradiographies.

2.1.3.3. Labelling and quality control of lyophilized ior-CEA-1

Lyophilized preparations of ior-CEA-1 were labelled with the required activity of $^{99}\text{Tc}^{\text{m}}$ after reconstitution of the kit with 2 mL of saline. Values for labelling efficiency were higher than 95%. Figure 3 shows a competitive binding assay comparing the lyophilized antibody with the native one.

2.1.3.4. Labelling and quality control of photoactivated IgG

Figure 4 shows the effect of the amount of tin per 500 μg of protein on the labelling efficiency. Higher amounts than 23 μg of tin gave between 87 and 93% labelling. Controls where IgG and MDP/tin were not irradiated but labelled after 20 min. and quality control tested after 30 min. gave between 7 and 10% labelling. Other controls were IgG was irradiated alone and MDP/tin was added to the antibody after irradiation gave very low labelling yields but better labelling was obtained when MDP was added after the irradiation of IgG and tin. It seems that tin is required during the UV irradiation. A typical photoactivation experiment was performed in two vials but the labelling was delayed 30 min in one case and 2 h in the other one. Labelling yield higher than 95% was obtained when the photoactivated IgG was labelled after 2 h. It seems that a pretinning approach was achieved but with a low amount of tin.

Figure 5 shows the effect of the IgG concentration on the labelling efficiency. Concentrations between 1.5 and 5 mg/mL gave 90% labelling yields but more reproducible results were obtained at concentrations higher than 5 mg/mL.

Labelling efficiencies higher than 95% were obtained for bulk irradiation and were more reproducible than single vial irradiation. Aliquots from bulk irradiation stored at -20°C could be labelled at high labelling efficiencies (>95%) for upto 10 days and for upto 1 month when the vials were stored at -70°C .

Figure 6 shows the cysteine challenge of two labelled IgG. Stability of the $^{99}\text{Tc}^{\text{m}}$ labelled photoactivated IgG was similar to that of the $^{99}\text{Tc}^{\text{m}}$ labelled mercaptoethanol reduced IgG.

Biodistribution results in normal Balb/c mice are listed in Table III.

Biodistribution results in mice with a sterile turpentine induced inflammation are listed in Table IV.

TABLE III. BIODISTRIBUTION (% ID/gm) IN NORMAL BALB/c MICE AT 4 AND 24 h POST INJECTION OF $^{99}\text{Tc}^{\text{m}}$ LABELLED PHOTOACTIVATED IgG

ORGAN	% ID/gm at 4 h	% ID/gm at 24 h
Blood	18.5 \pm 2.2	8.1 \pm 1.1
Liver	10.8 \pm 0.9	4.5 \pm 0.3
Spleen	4.8 \pm 1.6	1.1 \pm 0.1
Kidneys	25.0 \pm 2.7	8.7 \pm 1.3
Intestine	5.5 \pm 0.7	1.6 \pm 0.5
Lung	6.6 \pm 0.8	1.0 \pm 0.3
N.T.	3.3 \pm 1.1	1.9 \pm 0.3

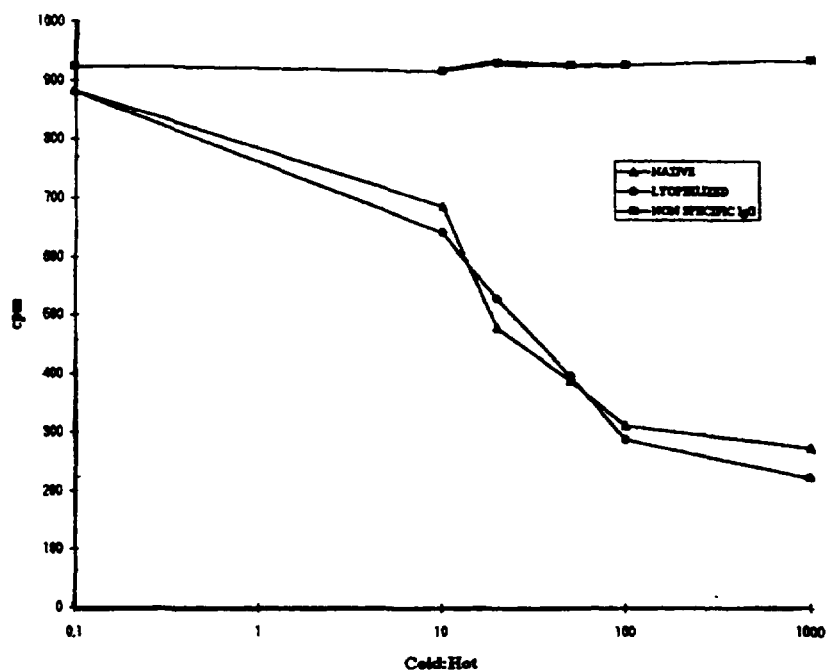


FIG. 3. Competitive binding assay comparing the lyophilized reduced antibody with the native and non-specific IgG.

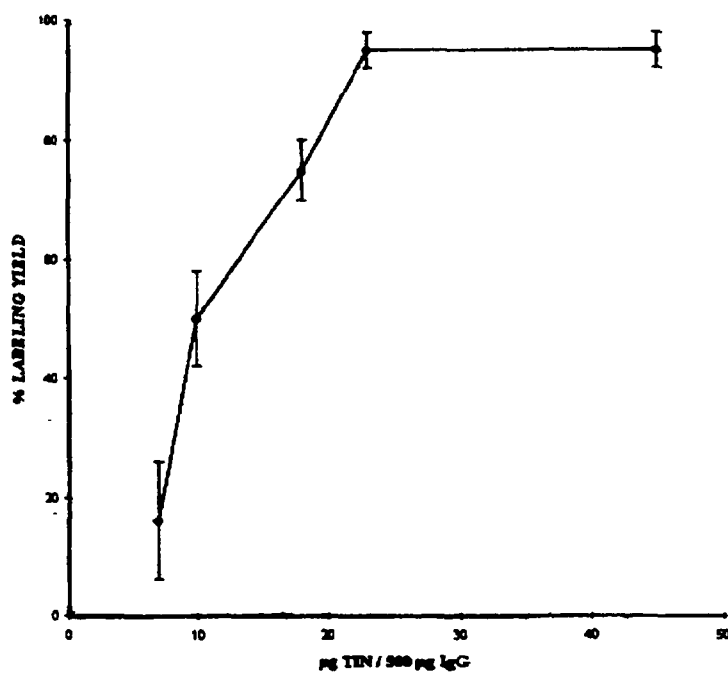


FIG. 4. The effect of the tin-antibody ratio on labelling efficiency.

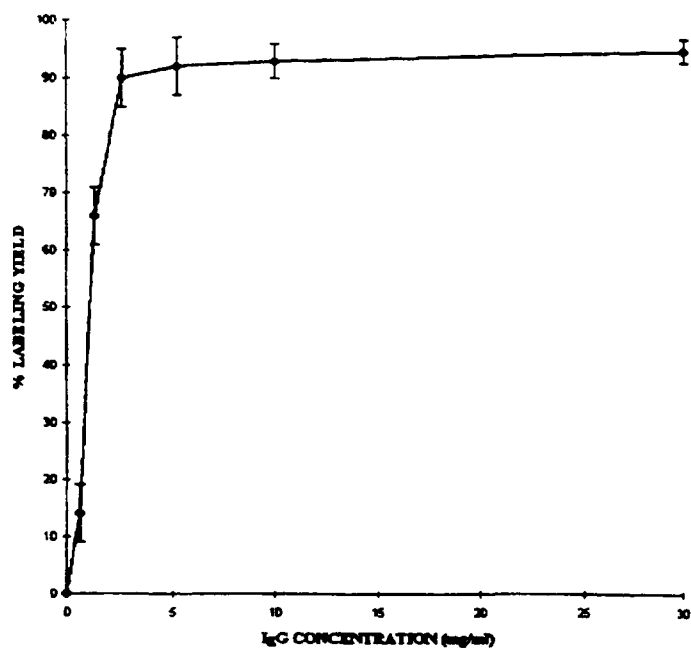


FIG. 5. The effect of antibody concentration on labelling efficiency.

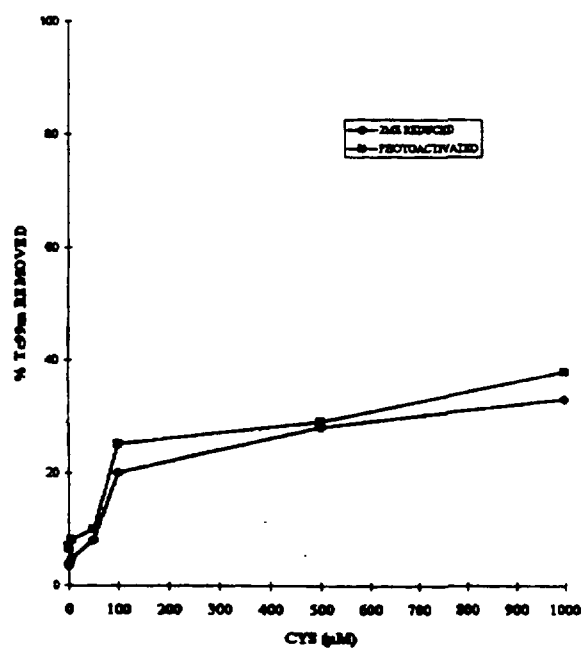


FIG. 6. Percent of $^{99}\text{Tc}^m$ removed from IgG versus cysteine concentration at 37°C for photoactivated IgG and 2-ME reduced IgG.

TABLE IV. BIODISTRIBUTION (% ID/gm) IN INDUCED INFLAMMATION MICE AT 4 AND 24 h POST ADMINISTRATION OF $^{99}\text{Tc}^{\text{m}}$ LABELLED PHOTOACTIVATED IgG

ORGAN	% ID/gm at 4 h	% ID/gm at 24 h
Blood	20.7 \pm 2.6	9.4 \pm 1.5
Liver	9.5 \pm 1.2	4.6 \pm 0.7
Spleen	4.9 \pm 1.2	3.5 \pm 0.3
Kidneys	26.2 \pm 3.7	11.5 \pm 1.8
Intestine	5.1 \pm 1.3	1.1 \pm 0.1
Lung	9.3 \pm 2.4	3.9 \pm 1.2
N.T.	2.4 \pm 0.5	1.4 \pm 0.2
I.T.	5.5 \pm 0.8	2.5 \pm 0.4

Kidney levels were higher than liver levels in both animal models and there was no significant difference in uptake of most tissues between the two groups. Results were similar to that obtained from distribution of $^{99}\text{Tc}^{\text{m}}$ labelled mercaptoethanol reduced IgG (control). However, the label cleared slightly faster from blood and some tissues than the control. The clearance in blood between the two points was 2.4 and 1.9 for labelled photoactivated IgG and the control respectively. The percentage for whole body retention during 5 h was 70 \pm 5.0 for labelled photoactivated IgG and 76 \pm 6.7 for the control. This difference is also an evidence of the more rapid clearance for the photoactivated IgG. Because of the short physical half-life of $^{99}\text{Tc}^{\text{m}}$, this clearance may be an advantage if there is no reduction in the uptake in tumour. In this case the label cleared from the inflammation site with a similar rate of 2.2 for labelled photoactivated IgG and even greater (2.2) than blood clearance (1.9) for the control. The ratio between IT/NT was similar and independent of the method of labelling.

2.1.3.5. Indirect labelling of IgG

Experiments were carried out to conjugate the protein at different molar ratios 0:1, 10:1 and 50:1 (NHS-MAG₃:IgG) and at different pH, in order to obtain the proper conditions for conjugation. Labelling of these complexes were achieved before and after removing the uncomplexed chelator. The control for non-specific labelling was the 0:1 molar ratio. In addition identical labelling experiments were performed to assess the binding of $^{99}\text{Tc}^{\text{m}}$ to glucoheptonate and to NHS-MAG₃ by ligand exchange from the labelled GH. Radiochemical purity was evaluated by HPLC and ITLC.

Labelling yields for the different controls were 98% for glucoheptonate, 95–98% for the NHS-MAG₃ labelled by ligand exchange from glucoheptonate and 2–3% for native IgG (non-specific labelling). Conjugation of IgG with the NHS MAG₃ chelator and labelling after purification was better achieved at a molar ratio of 50:1. Typically 1–2 mg of protein was conjugated and different amounts of conjugated protein (100–500 ug) with a concentration of approximately 0.5 mg/mL were used for labelling procedures. Labelling efficiency was usually 70–80% and purification of the labelled product was carried out using a centrifugal filter. Radiochemical purity as determined by HPLC and ITLC was 95–98% with 100% recovery. The radiochromatographic profile after labelling showed one prominent peak of labelled IgG, small amounts of radiolabelled aggregates of high molecular weight (3%) and radiolabelled species of low molecular weight (20%). This last percentage was in agreement with that obtained by ITLC in saline. As $^{99}\text{Tc}^{\text{m}}$ -glucoheptonate is retained by the HPLC column and recovery was nearly 100%, radiolabelled species of low molecular weight would be rather different than labelled glucoheptonate and they were equally removed after purification.

Transchelation to cysteine was studied at five molar ratios, cysteine: IgG ranging from 908:1 to 0.45:1 and the percentage of dissociation ranged from 6.2 to 1.2% respectively. The percentage of $^{99}\text{Tc}^{\text{m}}$ removed from labelled IgG via 2-ME reduction varied from 35 to 3.6% for the lower molar ratio and from 38 to 6.5% in the case of $^{99}\text{Tc}^{\text{m}}$ labelled photoactivated IgG. These two direct labels were more susceptible to transchelation than the indirect label. Results are shown in Fig. 7.

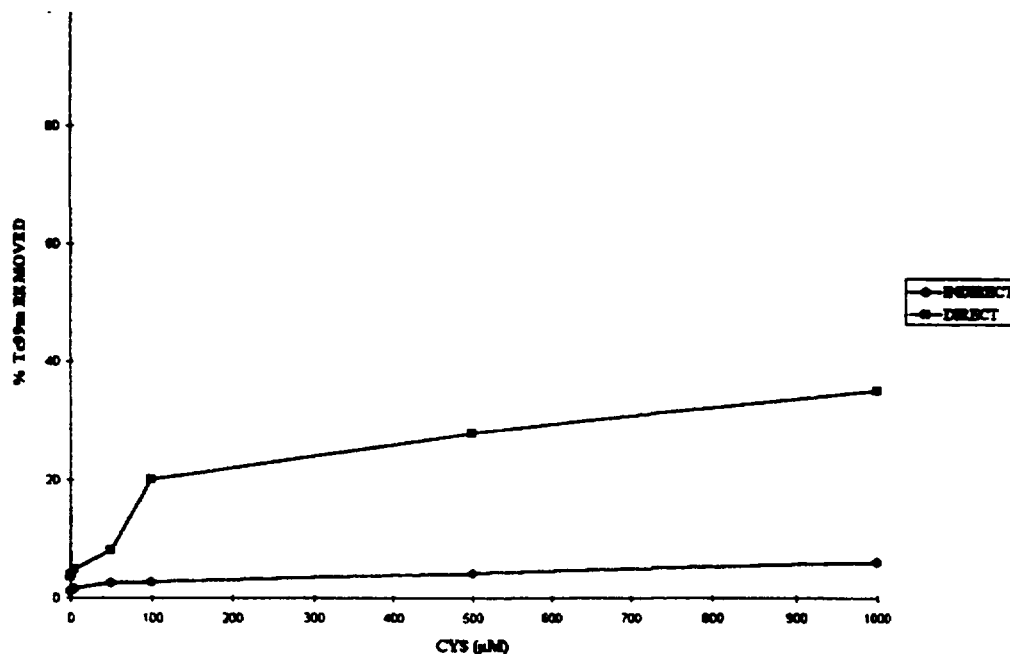


FIG. 7. Percent of $^{99}\text{Tc}^m$ removed from IgG versus cysteine concentration at 37°C for directly and indirectly labelled IgG.

Biodistribution results in normal mice at 4 and 24 h are listed in Table V. No significant differences were observed in uptake of the proteins by any organ in both animal models. Liver levels were slightly higher than kidney levels. Comparison in biodistribution at the same points with IgG labelled via 2-ME reduction were carried out in both animal models. Differences with labelling methods were evident, liver levels were lower and kidney levels were higher for the 2-ME relative to the NHS MAG₃ labelled IgG. The label cleared more rapidly from blood and tissues after direct labelling. The whole body activity levels measured in normal Balb/c mice at 1 h intervals were different. The percentage of the injected activity remaining in the animal at 4 h was 76 ± 6.7 for directly and 82.4 ± 2.6 for indirectly labelled IgG. This simplified conjugation procedure for acetyl-protected.

NHS-MAG₃ may provide an alternative (if chelator is available) for radiolabelling antibodies and other amines with $^{99}\text{Tc}^m$.

TABLE V. BIODISTRIBUTION (% ID/gm) IN NORMAL BALB/c MICE AT 4 AND 24 h POST ADMINISTRATION OF INDIRECTLY LABELLED $^{99}\text{Tc}^m$ -IgG

ORGAN	% ID/gm at 4 h	% ID/gm at 24 h
Blood	22.3 ± 1.3	15.9 ± 2.6
Liver	9.5 ± 2.3	7.4 ± 1.1
Spleen	7.2 ± 1.5	5.1 ± 0.2
Kidneys	8.4 ± 1.5	7.7 ± 1.6
Intestine	4.8 ± 0.1	2.4 ± 0.5
Lung	9.5 ± 3.4	7.5 ± 1.3
N.T.	3.1 ± 1.0	2.6 ± 0.8

TABLE VI. BIODISTRIBUTION (% ID/gm) IN INDUCED INFLAMMATION MICE AT 4 AND 24 h POST ADMINISTRATION OF INDIRECTLY LABELLED $^{99}\text{Tc}^{\text{m}}$ -IgG

ORGAN	% ID/gm at 4 h	% ID/gm at 24 h
Blood	25.2 \pm 2.9	17.8 \pm 0.74
Liver	8.7 \pm 0.8	8.2 \pm 1.1
Spleen	7.8 \pm 1.7	3.6 \pm 0.9
Kidneys	8.2 \pm 2.2	6.1 \pm 0.1
Intestine	4.9 \pm 0.5	1.4 \pm 0.1
Lung	9.0 \pm 1.1	5.6 \pm 0.2
N.T.	2.9 \pm 0.1	2.0 \pm 0.7
I.T.	5.8 \pm 0.3	3.7 \pm 0.4

2.1.4. Conclusions

Two direct labelling methods and one indirect method for radiolabelling monoclonal and polyclonal antibodies with $^{99}\text{Tc}^{\text{m}}$ were used. The principal advantage of the indirect method is that it can be employed with a wide range of biomolecules in contrast to direct methods which can be used only for labelling large molecules. Many chelating agents are generally not available and purification steps are required for indirect methods. The main advantage of the direct approach is its simplicity and the possibility of developing kit formulations that can be labelled in any Nuclear Medicine Center for diagnostic studies.

From the results described above it is clear that the ior- CEA-1 antibody can be directly labelled with $^{99}\text{Tc}^{\text{m}}$ using 2-ME as the reducing agent with a high radiochemical purity and without damage to the antigen-binding ability of the antibody. It is also clear that a lyophilized, sterile, pyrogen-free preparation under nitrogen atmosphere and ready for one step labelling of ior-CEA-1 and IgG with $^{99}\text{Tc}^{\text{m}}$ was achieved.

Further studies are required to establish the properties of photoactivated as well as the indirect labelling techniques for tumour imaging.

REFERENCES

- [1] HNATOWICH, D.J., Recent Developments in the Radiolabelling of Antibodies with Iodine, Indium and Technetium, *Semin. Nucl. Med.* **20** (1990) 80–91.
- [2] RHODES, B.A., ZAMORA, P.O., et al., Technetium-99m Labelling of Murine Monoclonal Antibody Fragments, *J. Nucl. Med.* **27** (1986) 685–93.
- [3] ARANO, Y., YOKOYAMA, A., et al., Synthesis and Evaluation of a New Bifunctional Chelating Agent for $^{99}\text{Tc}^{\text{m}}$ Labelling Proteins: p-carboxyethylphenylglyoxaldi(N-methylthio-semicarbazone), *Nucl. Med. Biol.* **12** (1986) 425–430.
- [4] FRITZBERG, A.E., ABRAMS, P.G., et al., Specific and Stable Labelling of Antibodies with Technetium-99m with a Diamide Dithiolate Chelating Agent, *Proc. Natl. Acad. Sci. USA.* **85** (1988) 4025–4029.
- [5] MATHER, S.J., ELLISON, D., Reduction-mediated Technetium-99m Labelling Monoclonal Antibodies, *J. Nucl. Med.* **31** (1990) 692–697.
- [6] SCHWARZ, A., STEINSTRASSER, A., A Novel Approach to Tc-99m-Labelled of Monoclonal Antibodies, *J. Nucl. Med.* **29** (1987) 721 (abstract).
- [7] THAKUR, M.L., DE FULVIO, J., et al., Technetium-99m Labelled Monoclonal Antibodies: Evaluation of Reducing Agents, *Nucl. Med. Biol.* **18** (1991) 227–33.

- [8] SYKES, T.R., WOO, T.K., et al., Direct Labelling of Monoclonal Antibodies with Technetium-99m by a Novel Photoactivation Process, *J. Nucl. Med.* **43** (1993) 100P (abstract).
- [9] ABRAMS, M.J., JUWEID, M., et al., Technetium-99m-Human Polyclonal IgG Radiolabelled via the Hydrazino Nicotinamide Derivative for Imaging Focal Sites of Infection in Rats, *J. Nucl. Med.* **31** (1990) 2022-2028.
- [10] WINNARD, P., RUSCKOWSKI, M., et al., A Simplified Synthesis of MAG₃-Coupled Oligonucleotides for Technetium-99m Labelling, *J. Nucl. Med.* **36** (1995) 2035.

Research contract No. CPR-6284/CF

Title of the Project : Studies on Radiolabelling of Monoclonal Antibodies with $^{99}\text{Tc}^m$ and other Radionuclides for Scintigraphy

Chief Scientific Investigator : Li Yongian
Department of radiopharmaceuticals,
Shanghai Institute of Nuclear Research,
Shanghai

2.2.1. Introduction

This work performed on the development of radiolabelling of monoclonal antibodies for scintigraphy can be summarized as follows: direct $^{99}\text{Tc}^m$ labelling and other radiolabelling methods of monoclonal antibodies with In-111 [1], Ga-67 or Ru-103.

2.2.2. Materials and methods

2.2.2.1. Direct $^{99}\text{Tc}^m$ labelling

Studies on influences of various factors in direct $^{99}\text{Tc}^m$ labelling [2] using mild reducing agent 2-mercaptoethanol (2-ME) [3–5] recommended by this co-ordinated research programme (CRP) were carried out. Monoclonal anti-CEA 170, ior-CEA-1 and hIgG provided by IAEA and IA5 were used in these experiments.

Influence of thiol group/antibody (-SH/MAb) ratio on labelling rate

Antibodies (MAb) were incubated with 2-ME at different molar ratio of 2-ME:MAb from 100:1 to 5000:1 at 20°C for 30 min. The reduced antibody was purified by Sephadex G-50 filtration. The antibody concentration was determined by its UV absorption at 280 nm and the thiol group concentration by Ellman method. Values of -SH/MAb were calculated corresponding to related 2-ME:MAb. At the same time the antibody reduced by different 2-ME:MAb composition was labelled through exchange reaction with MDP kit for 20 min at 20°C. The labelling rate was determined by ITLC.

Influence of SnCl_2 used in MDP kit and MDP/ SnCl_2 ratio on labelling rate

The reduced antibody was labelled with $^{99}\text{Tc}^m$ using MDP kit with different amounts of SnCl_2 (Figs 1 and 2). Labelling rate was determined by ITLC (Figs 3 and 4).

Influence of pH on labelling rate

The reduced antibody was labelled under similar conditions but at different pH (Fig. 5).

Influence of time course on labelling rate

The antibody was labelled under similar conditions but with different time course (Fig. 6).

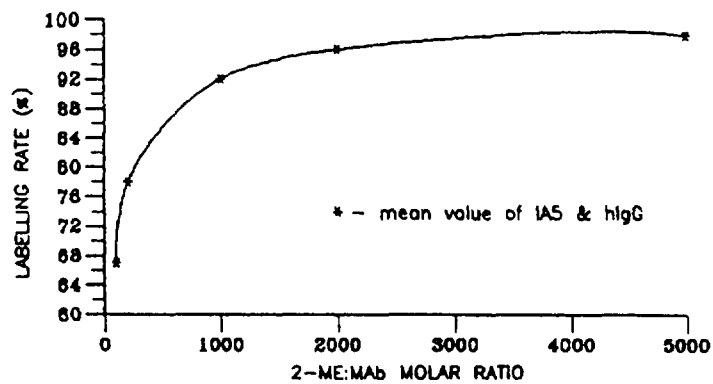


FIG. 1. Influence of 2-ME: MAb molar ratio on labelling rate.

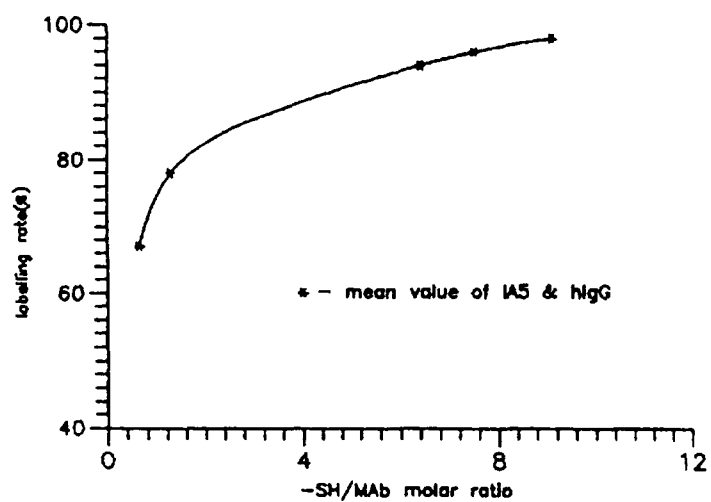


FIG. 2. Influence of thiol group/antibody (-SH/MAb) ratio on labelling rate.

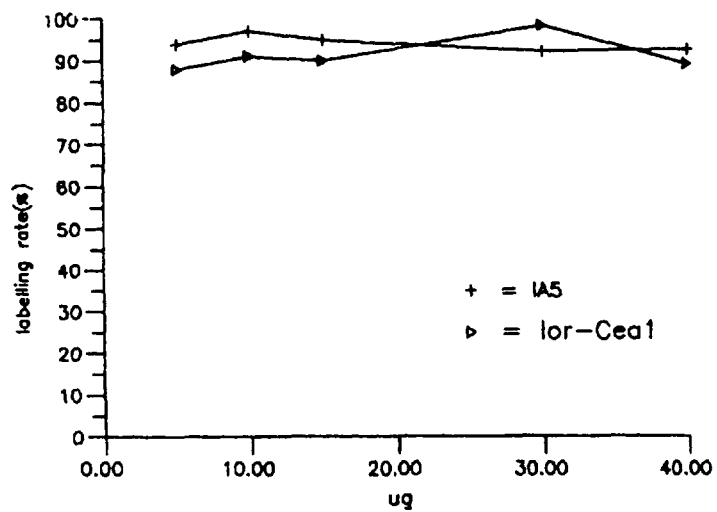


FIG. 3. Influence of SnCl_2 (wt) used in MDP kit on labelling rate.

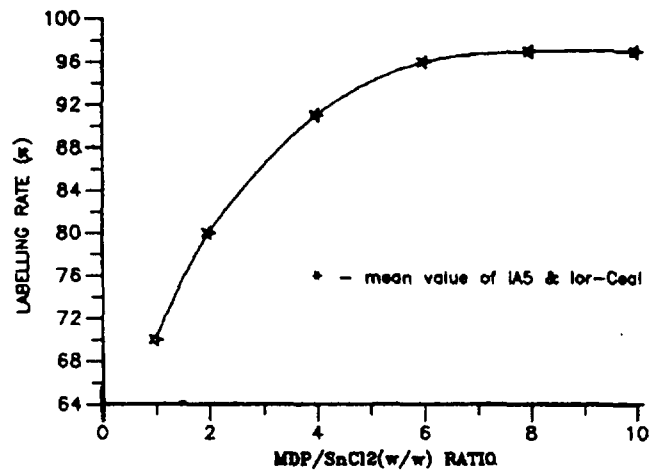


FIG. 4. Influence of MDP/SnCl₂ (w/w) ratio on labelling rate.

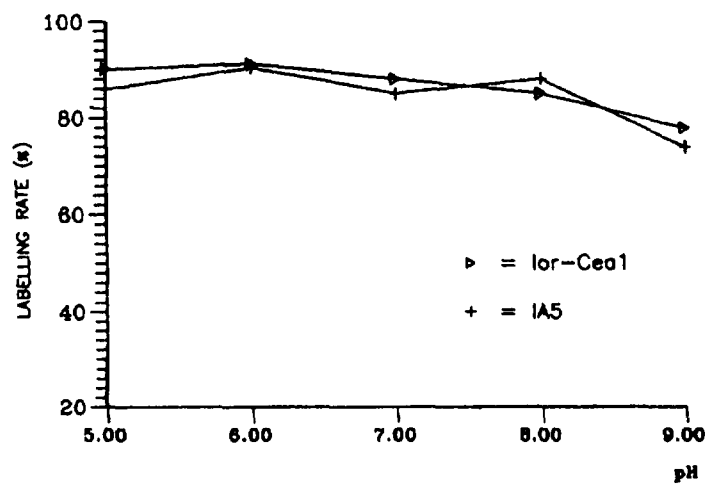


FIG. 5. Influence of pH on labelling rate.

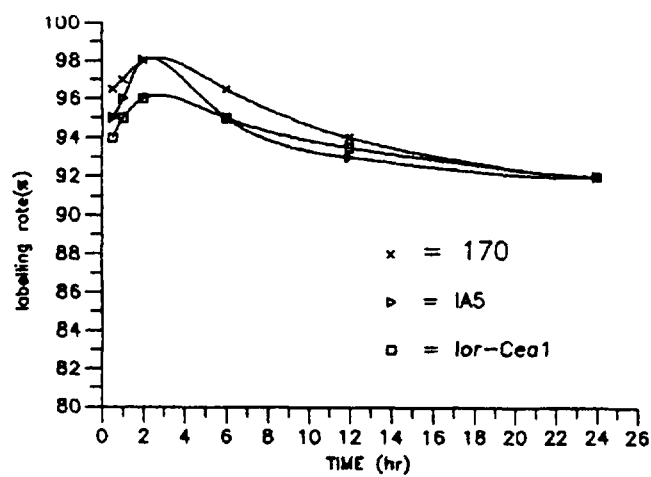


FIG. 6. Influence of time course on labelling rate.

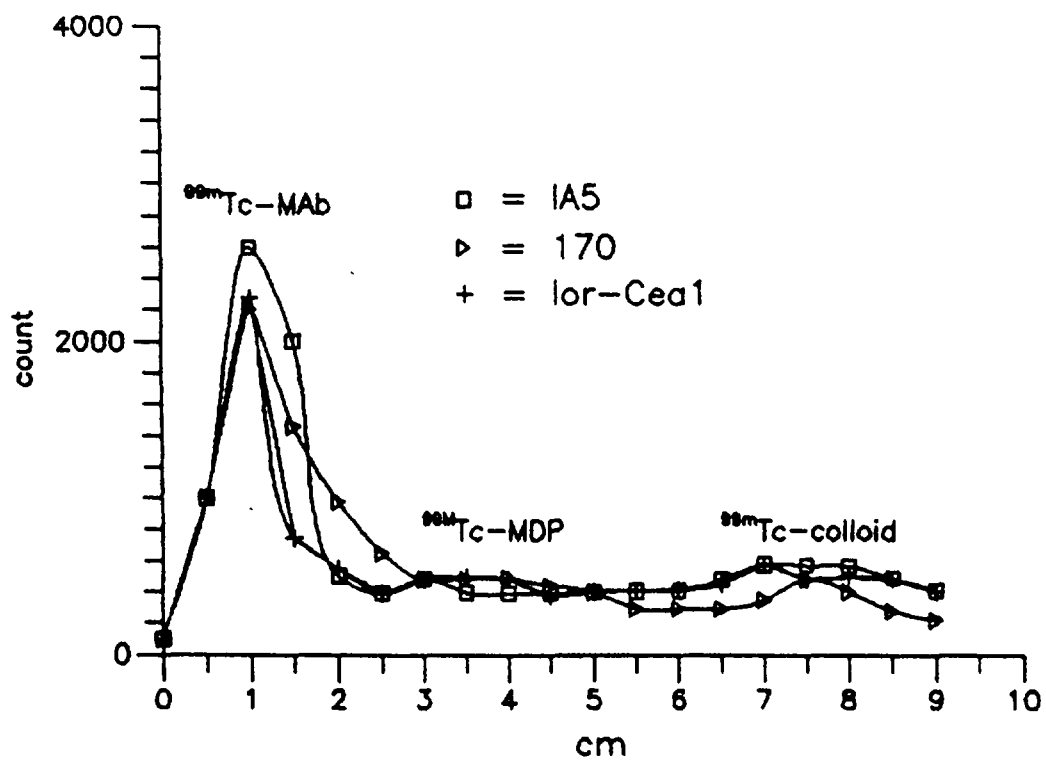


FIG. 7. Radiochromatography of MAb.

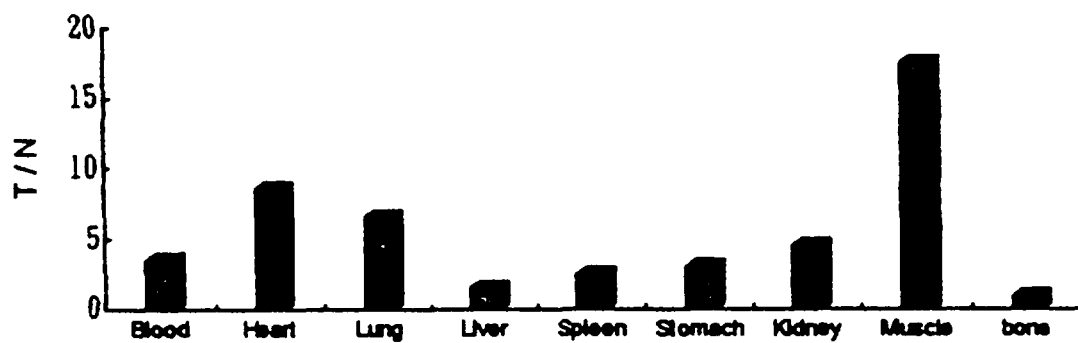


FIG. 8. Target to non-target ratio of $^{99m}\text{Tc}^m\text{-LC-IF(ab)2}$ ($n=4$).

TABLE I. EFFECT OF 2-ME REDUCTION ON LABELLING RATE (%)

	Antibody			
	ior-CEA-1	MoAb 170	hIgG	IA5
Reduced	98.5	97.5	98.5	97.0
Unreduced	18.5	28.5	32.0	27.5

TABLE II. EFFECT OF WEAK LIGAND ON LABELLING RATE (%)

	Antibody			
	ior-CEA-1	MAb 170	hIgG	IA5
MDP	98.5	97.5	98.5	97.0
Tartrate	70.0	72.5	62.5	63.0
Citrate	52.5	59.0	50.0	45.5
Blank	25.5	18.0	40.0	27.5

The effects of antibody reduction [6-7] and transition weak ligand [9] used on labelling rate were tested. 2-ME in 0.05 M PBS solution (pH7.4) was added to antibody PBS solution with 2-ME: MoAb ratio of 1000:1, swirled and incubated for 30 min at room temperature (20°C) in nitrogen atmosphere. The reduced antibody was purified on Sephadex G-25 column (1.5 cm × 7 cm) previously equilibrated with 0.1% BSA, PBS rinsed and eluted with N₂ purged 0.05 M PBS containing 1 mM 2-ME and monitored by UV detector. The protein containing fractions were pooled together, divided into aliquots and frozen immediately at -70° C, until use. A domestic MDP kit vial (containing 5 mg SnCl₂ and 5 mg MDP) was reconstituted with 0.05 mL nitrogen purged saline and 20 µL of it was added to every 0.2 mg reduced or unreduced antibody in 0.05 M PBS. ⁹⁹Tc^m-pertechnetate solution of 74 MBq was added, diluted with equal volume of saline, swirled and after 30 min at room temperature (20°C) purified over Sephadex G-50 using saline as eluent. Labelling rate was analyzed by paper chromatography [10] using saline as mobile phase (Fig. 7). The labelling obtained from reduced antibody was evidently higher than that from unreduced antibody ($P \leq 0.01$) (Table I). The tartrate solution reconstituted from a kit vial (containing 2 mg SnCl₂ and 20 mg tartaric acid) with 0.2 mL nitrogen purged saline; citrate solution made by dissolving 5 mg SnCl₂ and 50 mg citric acid in 0.5 mL nitrogen purged saline. SnCl₂ without ligand (as blank) was used in parallel with the above MDP solution. Each solution containing same amount of stannous ion (20 µg) was added to 0.15 mg of reduced antibody. 74 MBq ⁹⁹Tc^mO₄⁻ added to 0.15 mg of reduced antibody and made to equal volume with saline. After 30 min at room temperature (20°C) samples were analyzed. The results showed that the weak ligand had very significant effect on labelling rate, and MDP was the best choice among the three ligands (Table II).

The same method was also applied to label the fragment F(ab')₂ [11] of a home-made anti-lung cancer monoclonal antibody LC-1-IgM. SPECT imaging and comparative biodistribution studies were performed in Balb/c nude mice bearing human lung cancer LAX-83. Each mouse was injected with 50 µL (37 MBq) labelled antibody via tail vein and scanned at different time intervals, then finally assayed for tissue distribution at 40 h post i.v. administration. Among these scanings the most clear imaging was obtained at 20-24 h after administration and obvious accumulation of radioactivity in lung tumour could be seen. From the values of tissue distribution the uptake of ⁹⁹Tc^m-labelled antibody in lung tumour appeared higher than that of some major organs. The target to non-target ratio was higher than 5 (Fig. 8).

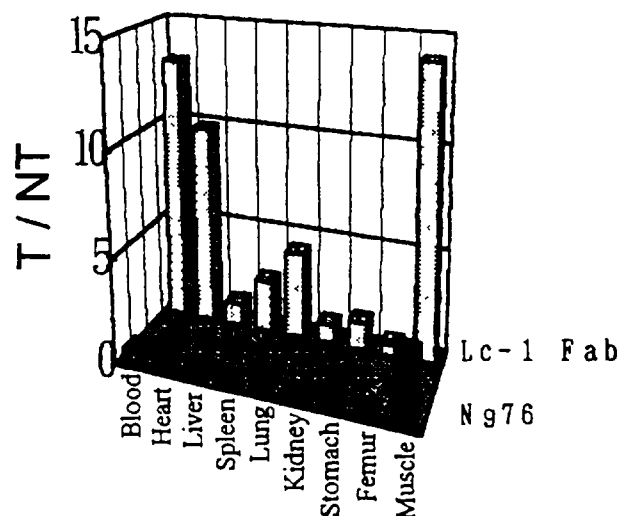


FIG. 9. Comparison of antibody T/NT ratio.

$^{99}\text{Tc}^m$ -antibody labelled without 2-ME treatment [12–14] in scintigraphy was tested. Anti-lung cancer antibody LC-1 fragment (LC-1 Fab) and anti-melanoma antibody Ng76 (as control) were used. 200 μL of MDP solution containing 100 μg stannous ion was added separately to 2 mg of LC-1 Fab and Ng76 in 0.05 M saline solution. About 740 MBq of $^{99}\text{Tc}^m$ -pertechnetate was added and after 30 min incubation at room temperature, their labelling rate was $\sim 85\%$. Each of the labelled antibodies was administered intravenously to lung cancer bearing Balb/c nude mice with dose of 15 μL (~ 80 MBq). Biodistribution and scintigraphic imaging of animals were performed at 24 h post injection. The distribution of LC-1 Fab had higher tumour uptake over other tissues except kidneys and bone. Its animal imaging also exhibited good tumour concentration. While as control Ng76 had different distribution and no significant tumour concentration (Fig. 9).

2.2.2.2. ^{111}In -labelling based on biotin-avidin conjugation for pre-targeting localization

Anti-CEA MoAb was biotinylated with N-hydroxysuccinimide ester of biotin (NHS-biotin) at MAb:NHS-biotin molar ratio of 1:15 in alkaline medium. The biotinylated MoAb (Bt-MAb) analyzed by HABA titration was found to contain 5–8 molecules of biotin per molecule of MoAb which were adequate to bind well with streptavidin (Av) without any significant change in immunoactivity.

MAb and Av were radioiodinated by iodogen method to form ^{125}I -MAb and ^{131}I -MAb. ^{125}I -MAb had immunoactivity of 74–82% and labelling efficiency of 56–84%. Biotin was labelled with ^{111}In through DTPA-biotin (DTPA-Bt) to form ^{111}In -DTPA-Bt with labelling efficiency of 91–98.7%.

Blood kinetics

The blood clearance of labelled biotin and avidin was studied in rabbits. Both of them showed biphasic blood clearance with $T_{1/2B}$ 6.3 h, $T_{1/2AV}$ 18 h.

Pre-targeting study

The study was carried out in Balb/c mice bearing human colon mucous adenoma xenograft. Two pre-targeting modes were studied: (1) 2-step mode [16]: Each mouse was administered intravenously (i.v.) 50 μg of Bt-MAB and after 24 h intraperitoneally administering 1480 MBq (10 μg ^{131}I -Av). Scanning and biodistribution were studied at time intervals of 24, 48, 96, 120 h post

administration. Mice injected with biotinylated IgG and ^{131}I -Av were used as control (2) 3-step mode [17]: Each mouse was administered i.v. 50 μg of Bt-MAb, 20 μg of Av at 24 h afterwards and i.p. 50 MBq (2 μg) of ^{111}In -DTPA-Bt after further 24 h. Scanning and biodistribution were studied at time intervals of 2, 24, 48 h post the last injection. The mice injected sequentially with biotinylated IgG, Av and ^{111}In -DTPA-Bt were used as control.

2.2.3. Results and discussion

The experimental results showed that for the 2-step mode, the observable radioactivity accumulation in tumour tissue began at 24 h post injection i.p., when its uptake (% ID/g) was 0.85 with tumour/blood ratio equal to 1.11, significant uptake of 1.48 with tumour/blood ratio 5.55 at 96 h and was very significant for scintigraphy at 120 h when tumour % ID/g was 1.70 with tumour/blood ratio equal to 8.55. While for the 3-step mode, evident positive scanning of tumour was obtained at 2 h post injection i.p. when % ID/g was 9.79 with tumour/blood ratio equal to 4.19 and significant at 48 h when tumour %ID/g was 3.74 with tumour/blood ratio of 5.57 (all were higher than control). Both modes could shift scanning to earlier time than no pretargeting and 3 step mode was more effective in reduction of time for accumulation in tumour.

It was also effective in applying these modes of pre targeting to inflammatory imaging with IgG. The imaging could be accomplished at 2 h post injection when target/non target ratio was equal to 2.7.

^{67}Ga labelling

Comparative evaluation of different indirect and direct methods for labelling antibody with ^{67}Ga were investigated. In the former the antibody was labelled via bifunctional chelating agents, diethylenetriaminepentaacetic cyclic anhydride (CA-DTPA) and deferoxamine mesylate (DFO), while in the latter antibody was used without modification.

In the indirect method of using CA-DTPA, antibody (IgG) conjugation was accomplished by adding to the protein solution (10 mg/mL, 0.1 mL PBS, pH7.4) 50 μL freshly prepared CA-DTPA/DMSO solution (5 mg/mL anhydrous dimethylsulfoxide). The solution was mixed and left for 1 h at room temperature. The conjugated antibody was separated by passing through a centrifuging Sephadex G-25 column (1 \times 7 cm). The number of chelator groups attached per antibody molecule were determined by reaction of the coupled antibody with standard radio-indium solution for 1 h at room temperature and assayed by HPLC UV elution measurement and counting. Radio-gallium labelling was accomplished by adding 50 μL ^{67}Ga -gallium chloride solution (185 MBq/mL) to 1.0 mL conjugated antibody solution (10 mg/mL, pH7.2). The solution was left for 2 h and passed through Sephadex column to separate labelled antibody and analysed by HPLC. Labelling efficiency of 86% with 99% radiochemical purity was obtained. Immunoreactivity was proved to be preserved well. Another indirect method was accomplished using DFO. The antibody (IgG and anti-human glioma) conjugation was achieved by adding to 1.0 mL protein solution (10 mg/mL and 2.0 mg/mL PBS) 30 μL DFO-glutaraldehyde solution (prepared by mixing 1.0 mL 60 mM/l DFO/PBS solution with 0.1 mL 25% glutaraldehyde for 10 minutes at room temperature) and incubated at 4°C for 1 h. Then it was added to the solution 0.5 mg of sodium borohydride (NaBH_4) and mixed at 4°C for 1 h. The conjugated antibody was purified through gel filtration. The DFO-coupled antibody was labelled by addition of 50 μL $^{67}\text{GaCl}_3$ solution (185 MBq/mL) and incubated at room temperature for 2 h. More than 97% labelling efficiency was attained.

The direct method was carried out by incubating 1.0 mL IgG/PBS solution (10 mg/mL) with 10 μL $^{67}\text{GaCl}_3$ solution for 2 h. The labelling efficiency after purification was only 20–24%.

^{103}Ru labelling

Both indirect and direct methods were used. In the former hIgG was labelled via bifunctional chelating agent, CA-DTPA while in the latter it was labelled with and without 2-ME reduction. Biodistribution of Ru-hIgG at different time intervals was done and compared with that of Ru-DTPA.

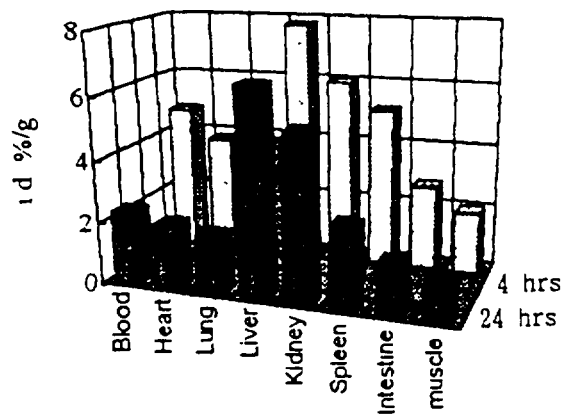


FIG. 10. Biodistribution of Ru-hIgG in mice at different time.

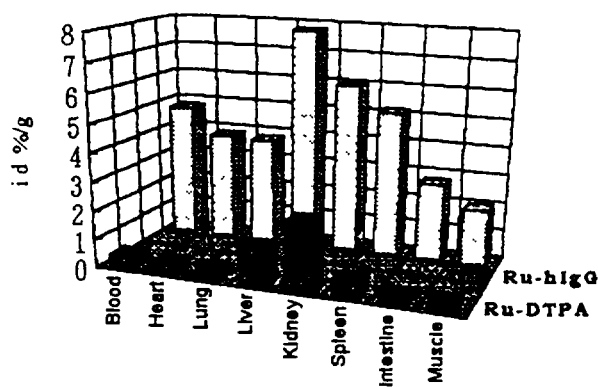


FIG. 11. Biodistribution of Ru-DTPA and Ru-hIgG in mice at 4 h.

TABLE III. BIODISTRIBUTION (% ID/gm) OF Ru-hIgG IN MICE AT DIFFERENT TIME INTERVALS

Organs	4 h	24 h
Blood	4.76 ± 1.39	2.35 ± 0.28
Heart	3.81 ± 1.39	2.06 ± 0.55
Lung	3.77 ± 0.87	1.84 ± 1.02
Liver	7.78 ± 1.73	6.56 ± 0.78
Kidney	5.97 ± 0.68	5.27 ± 0.37
Spleen	5.13 ± 1.92	2.59 ± 0.40
Intestine	2.72 ± 0.87	1.60 ± 0.76
Muscle	1.96 ± 0.43	1.28 ± 0.79

TABLE IV. BIODISTRIBUTION (% ID/gm) OF Ru-DTPA IN MICE AT DIFFERENT TIME INTERVALS

Organs	30 min	4 h
Blood	0.63 ± 0.04	0.43 ± 0.04
Heart	0.42 ± 0.16	0.34 ± 0.07
Lung	0.47 ± 0.23	0.28 ± 0.08
Liver	1.22 ± 0.47	1.27 ± 0.19
Kidney	2.81 ± 0.89	2.35 ± 0.27
Spleen	2.81 ± 0.89	0.53 ± 0.15
Intestine	0.58 ± 0.18	0.23 ± 0.07
Muscle	0.33 ± 0.18	0.25 ± 0.08

In the indirect method, conjugation was accomplished by adding to the hIgG solution (12.5 mg/mL, pH8) 5 µL of freshly prepared CA-DTPA/DMSO solution (5 mg/mL anhydrous dimethyl sulfoxide). The solution was mixed and left for 1 h at room temperature. The conjugated hIgG was separated by passing through a centrifuging Sephadex G-25 column (1 × 7 cm) at 1000 rpm for 3 min. Radio-ruthenium labelling was accomplished by adding 20 µL of ¹⁰³Ru-chloride solution (14 MBq/mL PBS, pH7) to 500 µL conjugated hIgG solution (10 mg/mL PBS, pH7). The solution was left for 2 h and passed through Sephadex column to separate labelled hIgG and analyzed by HPLC. Labelling efficiency of 86.2% with over 98% radiochemical purity was obtained.

The direct labelling was carried out by adding 20 µL of Ru-103-chloride solution (14 MBq/mL PBS, pH7) to 500 µL of hIgG solution (10 mg/mL carbonate buffer solution pH8) with and without previous reduction by 2-mercaptoethanol (with molar ratio up to 1:7500). Each solution was mixed and incubated overnight at room temperature and passed through the Sephadex column to separate hIgG and analyzed by HPLC. High labelling efficiency of 89.5% was obtained without previous reduction of hIgG, while the previously reduced hIgG produced lower efficiency and free 2-mercaptoethanol could deteriorate seriously the result.

Biodistribution of radoruthenium labelled hIgG (Ru-hIgG) was performed by administering i.v. to each normal mouse 37 kBq Ru-hIgG (2 mg/200 µL PBS, pH7). Data of uptake in various organs at 4 and 24 h post injection was presented in Table III and it was compared with biodistribution (Table IV) of radoruthenium labelled DTPA (Ru-DTPA) which was prepared by mixing 2 mL of CA-DTPA solution (10 mg/mL, pH8) with 20 µL of Ru-103-chloride solution and left for 1 h at 80°C.

REFERENCES

- [1] HNATOWICH, D.J., Recent Developments in the Radiolabelling of Antibodies with Iodine, Indium and Technetium, *Sem. Nucl. Med.* (1990) 80–91.
- [2] ALAUDDIN, M.M., KHAWLI, L.A., An Improved Method of Direct Labelling of Monoclonal Antibodies with Tc-99m, *Nucl. Med. Biol.* **19** (1992) 445–454.
- [3] SCHWARZ, A., STEINSTRASSER, A., A Novel Approach to Tc-99m Labelled Monoclonal Antibodies, *J. Nucl. Med.* **28** (1987) 721.
- [4] MATHER, S.J., ELLISON, D., Reduction Mediated Tc-99m-Labeling of Monoclonal Antibodies, *J. Nucl. Med.* **31** (1990) 692–697.
- [5] BLOCK, D., FEITSMA, R.I.J., et al., A New Method for Protein Labelling with Tc-99m, *Nucl. Med. Biol.* **16** (1989) 11–16.
- [6] DELIGY, C.L., GELSEMA, W.J., et al., The Influence of Experimental Conditions on the Efficiency of Labelling Human Serum Albumin with Tc-99m, using Sn(II) as the Reductant, *Int. J. Appl. Radiat. Isot.* **27** (1976) 351–356.

- [7] THAKUR, M.L., DeFULVIO, J., et al., Technetium-99m Labelled Monoclonal Antibodies: Evaluation of Reducing Agents, *Nucl. Med. Biol.* **18** (1991) 227–233.
- [8] PAIK, C.H., ECKELMAN, W.C., et al., Transchelation of Tc-99m from Low Affinity Sites to High Affinity Sites of Antibody, *Nucl. Med. Biol.* **13** (1986) 359–362.
- [9] LIN, M.S., Labelling Proteins with Tc-99m. In *Radiopharmaceuticals* (SUBRAMANIAN, G., RHODES, B.A., et al., Eds), Society of Nuclear Medicine, New York (1975) 36–48.
- [10] RHODES, B.A., ZAMORA, P.O., et al., Technetium-99m Labelling of Murine Monoclonal Antibody Fragments, *J. Nucl. Med.* **27** (1986) 685–693.
- [11] ECKELMAN, W.C., PAIK, C.H., et al., Three Approaches to Radiolabelling Antibodies with Tc-99m, *Nucl. Med. Biol.* **16** (1989) 171–176.
- [12] COLOMBETTI, L.G., GOLDMAN, J., et al., A Rapid Method for Labelling IgG with Tc-99m, *J. Nucl. Med.* **20** (1978) 652.
- [13] ZHANG, Z.M., BALLINGER, J.R., et al., Evaluation of Reduction-Mediated Labelling of Antibodies with Technetium-99m, *Nucl. Med. Biol.* **19** (1992) 607–609.
- [14] RUSCKOWSKI, M., FRITZ, B., et al., Localization of Infection Using Streptavidin and Biotin: an Alternative to Non-specific Polyclonal Immunoglobulin, *J. Nucl. Med.* **33** (1992) 1810–1815.
- [15] PAGNELLI, G., BELLON, C., et al., Two-step Tumour Targeting in Ovarian Cancer Patients Using Biotinylated Monoclonal Antibodies and Radioactive Streptavidin, *Eur. J. Nucl. Med.* **19** (1992) 322–329.
- [16] BHUMENTHAL, R.D., SHARKEY, R.M., et al., A Comparison of Direct Tumour Targeting with 3-step Avidin-Biotin Pretargeting, *J. Nucl. Med.* **35** (1994) 219.
- [17] LEE, J.T., GARMESTANI, K., et al., Evaluation of in Vivo Stability of three Ga-67 Chelator-MAb Conjugates in Mice, *J. Nucl. Med.* **35** (1994) 29.

Research contract No. 6480/R3/RB

Title of the Project : ior-CEA-1: Labelling, Quality Control and Clinical Evaluation

Chief Scientific Investigator : G.J. Pimentel
Department of Nuclear Medicine,
National Institute of Oncology and Radiobiology, Habana

2.3.1. Introduction

Within the Co-ordinated Programme on Labelling, Quality Control and Evaluation of Monoclonal Antibodies, the IAEA has made a great effort to expand efficient labelling methods, mainly those with radioisotopes which have been used for radioimmunoscintigraphy. In this sense, more recently $^{99}\text{Tc}^{\text{m}}$ has been mostly employed in the majority of the investigations [1–4] due to its ideal physical characteristics. Efficient labelling of monoclonal antibodies depends on a number of factors including the method and way of the label incorporation into the protein. During the last years several direct labelling approaches have been developed [2, 4, 5], which led to attain simple and inexpensive methods for medical practice, as well as safe and stable techniques which bring accurate and good quality images.

Accordingly, this paper describes the results obtained during last five years which come from the comparison among different labelling systems, passing through the quality control to test the labelled monoclonal stability and the protein bioreactivity, to continue in the clinical evaluation of ior-CEA-1, as well as the evaluation of other antibodies. Upto now we have evaluated more than 70 patients with the anti-CEA monoclonal antibody (ior-CEA-1), examined in different clinical assays such as: pilot [6], phase I-II [7] and extensive phase III-IV trials, whose results are encouraging. It confirms that the employed labelling approach was safe and adequate.

2.3.2. Materials and methods

2.3.2.1. Monoclonal antibody (MAb)

This is a murine IgG-1 antibody secreted by hybridoma clone K3/15 obtained from the cell fusion between P/X63-Ag 8-653 myeloma cells and spleen cells derived from Balb/c mice immunized with purified cells from the liver metastasis of a colonic adenocarcinoma. This IgG is highly specific against protein epitope on cell bound CEA and it is included in the gold 1 group according to Hedin's classification [8].

2.3.2.2. Labelling

MAb labelling was tested by different approaches using 2-mercaptoethanol (2-ME), $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, ascorbic acid (AA), dithionite (DT) as reductants.

The 2-ME approach was described elsewhere [3,4]. Briefly, ior-CEA-1 was reduced with a molar ratio of 2000:1 of 2-ME:Antibody and allowed to incubate at room temperature for 30 min. At the end of the incubation, the reduced IgG was purified by gel filtration on Sephadex G-50 (Pharmacia) using PBS solution as mobile phase. Aliquots of 0.5–1 mg of reduced antibody were used for labelling. After reconstitution of the AMERSHAM MDP kit with 5 mL of 0.9% saline, 50 μL of this solution was added to reduced monoclonal antibody and it was labelled with the corresponding activity (74 MBq or 1850 MBq) and allowed to react for 15 min.

For the evaluation of the other reducing agents two different systems were employed, on one hand Sn(II)/Ascorbate were used as reducing agents, while Ascorbate/DT were assessed on the other. For the analysis of these systems, two experimental designs were carried out (type 33 and 3 respectively).

In the first system the influence of Sn(II), AA as well as the tartrate on labelling yield was studied. The Sn(II) ions amounts were varied ranging from 5 to 20 μg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, according to the molar ratio in relation to MoAb of 3:1, 6:1 and 13:1. This interval was chosen since at lower stannous concentration $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ was present in the reaction mixture, while at higher concentrations an appreciable amount of $^{99}\text{Tc}^{\text{m}}$ -colloid could be observed. The ascorbate varied ranging from 0 to 500 μg for a molar ratio of 0–3500 in relation to MoAb and tartrate ranging from 50 to 200 μg . These three components were mixed according to corresponding amounts to conform a final volume of 70 μL of reducing solution. This solution was added to 250 μL of antibody solution. The mixture was incubated at room temperature for 30 min. and then the reduced antibody was immediately labelled with 74 MBq of $^{99}\text{Tc}^{\text{m}}$ and allowed to incubate for 15 min.

The third labelling approach is based on Thakur's method [5], where the ascorbate is added ranging in a molar ratio of 3500–35 000:1 in relation to antibody, and incubated for 1 h at room temperature. Then $^{99}\text{Tc}^{\text{m}}$ -pertechnetate was reduced over 5 min with a freshly dithionite solution at pH11 and added to the antibody solution previously treated with AA to conform a DT final concentration ranging from 0.4 to 4 mg/mL in a final volume of 182 μL . The pH was varied ranging from 4.5 to 6.9 and the mixture was incubated for 45 min at room temperature.

2.3.2.3. *Quality control*

Quality control was measured by means of a combination of paper and instant thin layer chromatography (ITLC) as well as FPLC or HPLC. Whatman paper no. 1 (10 mm \times 60 mm strips) was used for paper chromatography using methylethylketone as mobile phase. ITLC was carried out in 10 mm \times 75 mm strips (German Inc., USA) in 0.9% saline as solvent (protein bound R_f = 0.0, MDP and $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ R_f = 0.9 and 1.0 respectively). FPLC (Pharmacia system) was performed in a Superose 6 HR 10/30 column and 0.2 M phosphate buffer in 0.15 M NaCl, pH7.4 as eluent, a flow rate of 0.5 mL/ min in a Mono S HR 10/10 cation exchange column with a stepwise elution from 0.05 M acetate pH5.0 (solution A) to 0.5 M sodium + 0.5 M Tris-hydrochloride pH8.0 (solution B). The salt concentration increase (solution B) was of 0.2 fold in every step and the flow rate was 180 mL/h. HPLC was carried out in a Beckman system using a Dupont GF-250 gel filtration column using 254 nm UV and radioactivity flow detectors and 0.1 M phosphate pH7.0 buffer as mobile phase.

2.3.2.4. *Transchelation challenge test*

The samples were submitted to two different challenge media, serum and cysteine solution, in order to test the "in vitro stability". **Serum:** 10 μg of the labelled antibody is added to 1 mL of fresh human serum. After a mild shaking, the sample was analysed by paper and ITLC chromatography, as well as electrophoresis if considered necessary. **Cysteine:** Two cysteine solutions were added to a solution of the radiolabelled antibody, such that the final molar ratio were 0.5:1 and 500:1 in relation to MoAb. The protein concentration was 350 $\mu\text{g}/\text{mL}$. After incubations at room temperature and 37°C for over 4 hrs the solutions were analysed by a paper chromatographic system [9], whose mobile phase was PBS, pH7.2.

2.3.2.5. *Immunoreactivity studies*

Micro ELISA system

The immunoreactivity of reduced and labelled ior-CEA-1 was determined in a competitive binding assay against the native antibody by a micro ELISA system, described previously [10]. Polystyrene plates (high binding, COSTAR), were coated with ior-CEA-1 diluted in coating buffer

(10 µg/mL) with 50 µL per well. The plate was incubated for 18 h at 37°C and then washed with washing buffer (phosphate buffer saline (PBS) with 0.05% of Tween 20) three times with 200 µL of buffer per well. The modified MoAb (reduced and labelled) and control antibodies were serially diluted in dilution buffer (sheep serum at 5% washing buffer) to obtain concentrations ranging from 10–0.325 µg/mL. Two hundred microliter of CEA (2 µg/mL) and modified or control antibody were incubated in Eppendorf vials to a final volume at 200 µL and vortexed for 10 sec. Fifty microlitres were added to wells coated with ior-CEA-1 as described above. The plate was incubated at 37°C for 1 h and washed in the same way as before. A sheep conjugate polyclonal anti-CEA antibody was added to each well (50 µL/well), according to the glutaraldehyde method [11]. After incubation at 37°C for 1 h the plate was washed and a solution of p-nitrophenyl phosphate in diethanolamine buffer 1 M pH9.8 (1 mg/mL) was added to each well (50 µL/well). The color was developed in 30 min at room temperature and the reaction was stopped with NaOH 3 M (50 µL/well). The absorbance values were measured in a ELISA plate reader (Organon, Teknica) at 405 nm.

Immunohistochemistry

A biotin-streptavidin peroxidase complex system (Amersham) was used for detection of the tissue bound ior-CEA-1. Briefly, deparaffined and rehydrated sections were treated with 3% H₂O₂ (aqueous or methanol solution) for 30 min. to block endogenous peroxidase activity, rinsed in buffer (PBS), incubated with 0.02 mg/mL of ior-CEA-1 MAb, followed by biotin-conjugated sheep antimouse immunoglobulin (diluted 1:100) and finally with the biotin-streptavidin-peroxidase complex (diluted 1:500).

2.3.2.6. Animal biodistribution study

Balb/c female mice weighing 18–25 gm were used in the experiment. About 100 µL of labelled MAb, corresponding to ~3.7 MBq, were injected intraperitoneally. Four h later, the animals were sacrificed. Radioactivity in each organ was counted in a gamma counter and recorded as percentage of dose/g tissue.

2.3.2.7. Clinical studies

Up to now over 70 patients have been studied with an age range of 44–73 years. All patients had documented malignancies of prior resection of colorectal carcinoma and in whom there is a high clinical suspicion of recurrence. The patients signed informed consent and were studied with the approval of the National Co-ordination Centre of Clinical Trials.

Each patient received 1 mg of ior-CEA-1 labelled with 2.25 GBq of ^{99m}Tc^m. The preparation was intravenously administered over a period of 2–3 min. One pre-administration and several post-administration blood samples were obtained along with a complete urine collection throughout the period of study (24 h). Quantitation of radioactivity of liver, spleen and kidneys was determined through regions of interest at 10 min, 4 h and 24 h post-administration by imaging on a planer camera equipped with a medium energy collimator and the attenuation correction according to [12, 13].

2.3.3. Results

We have explored different alternative variants for the labelling of ior-CEA-1 in order to attain our own experience in the reducing mechanism and the role that each agent plays. The labelling efficiency by the 2-ME reduction approach was measured by a combination of different chromatographic methods, such as paper chromatography, ITLC and HPLC. Figure 1 shows the HPLC results, where yields over 98% were obtained.

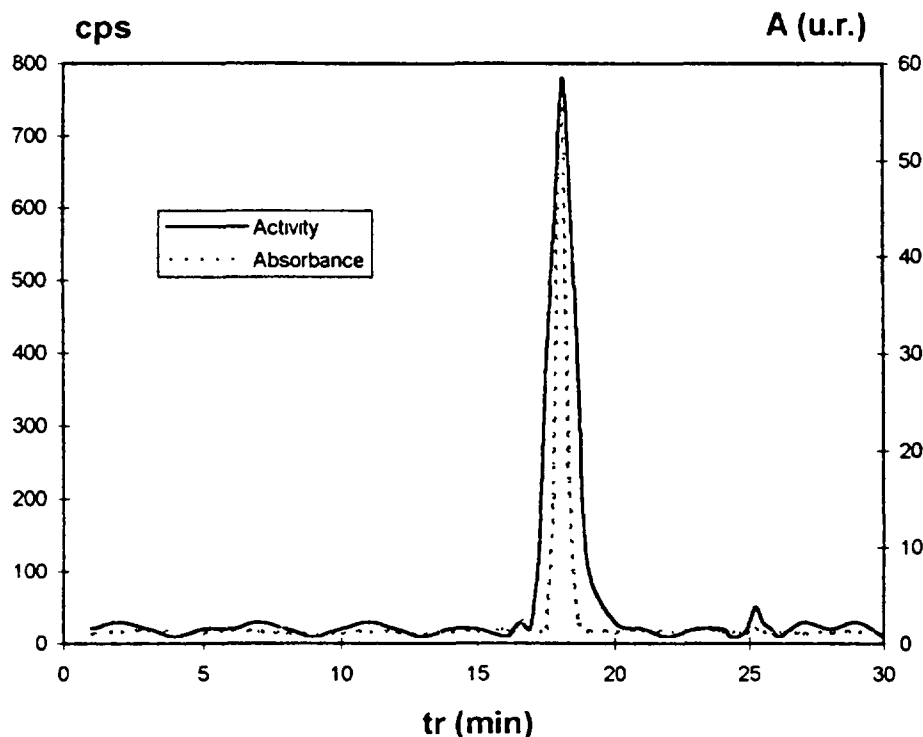


FIG 1. HPLC Analysis of $^{99}\text{Tc}^m$ -ior-CEA-1 antibody.

Figure 2a shows the influence of stannous ions, AA and tartrate in the Sn(II)/Ascorbate system. It can be observed that the effect of Sn(II) is not significant. However, as may be seen in Fig. 2a ascorbate influences positively upto a point, and is thereafter minimal. Tartrate was used in the system as an appropriate complexing agent for preserving $^{99}\text{Tc}^m$ in the required redox state [16]. Although the influence is almost minimal, we observed a certain negative trend in the labelling yield while increasing tartrate concentration. Quality control showed that free and colloidal technetium were at a relatively low level. Nevertheless, although part of the radioactivity was associated with the peaks of identical retention time and area of the protein, FPLC results (Fig. 2b) show a profile which do not correspond to that expected.

The experimental design for the AA/DT system demonstrated that ascorbate as well as pH played no significant role in antibody labelling. The results for the AA/DT system are given in Fig. 3a, where all labelling values for low concentrations of DT were under 25%. The HPLC elution profile shows results similar to those obtained by Sn(II)/AA reduction.

Labelling stability was tested in vitro in the presence and absence of serum, determining the percentage of radioactivity associated with the protein as a function of time. Table I shows the results of challenging with serum after labelling with the three different approaches. After incubating the samples in serum for 24 h at 37°C, they were analysed by electrophoresis suggesting that although the majority of the radioactivity was associated with the protein, certain label dissociation can be observed, presumably TcO_4^- , especially, in the Sn(II)/AA and AA/DT samples. The ior-CEA-1 labelled via 2-ME was submitted to cysteine challenge (Fig. 4). This labelling technique attained the best stability result in serum and its possible in vivo application.

The designed micro ELISA system allows the assessment of biological activity of modified monoclonal antibody against control. By means of an inverse competitive assay using a fixed amount of CEA (1 $\mu\text{g/mL}$) and serial dilution curves of MAb, the antigen will react with the coated antibody, so the color is developed when the modified antibody is not able to bind with the CEA. Fig. 5 shows the binding curve trends of the native, reduced, labelled ior-CEA-1 and a non-specific MoAb. By means of a linear regression line, the slope of each one was determined, which by the ratio from control and modified antibody binding curves allows to determine the immunoreactivity index, defined as a ratio between the slopes from modified antibody and control binding curves. The results are shown in Table II.

2a

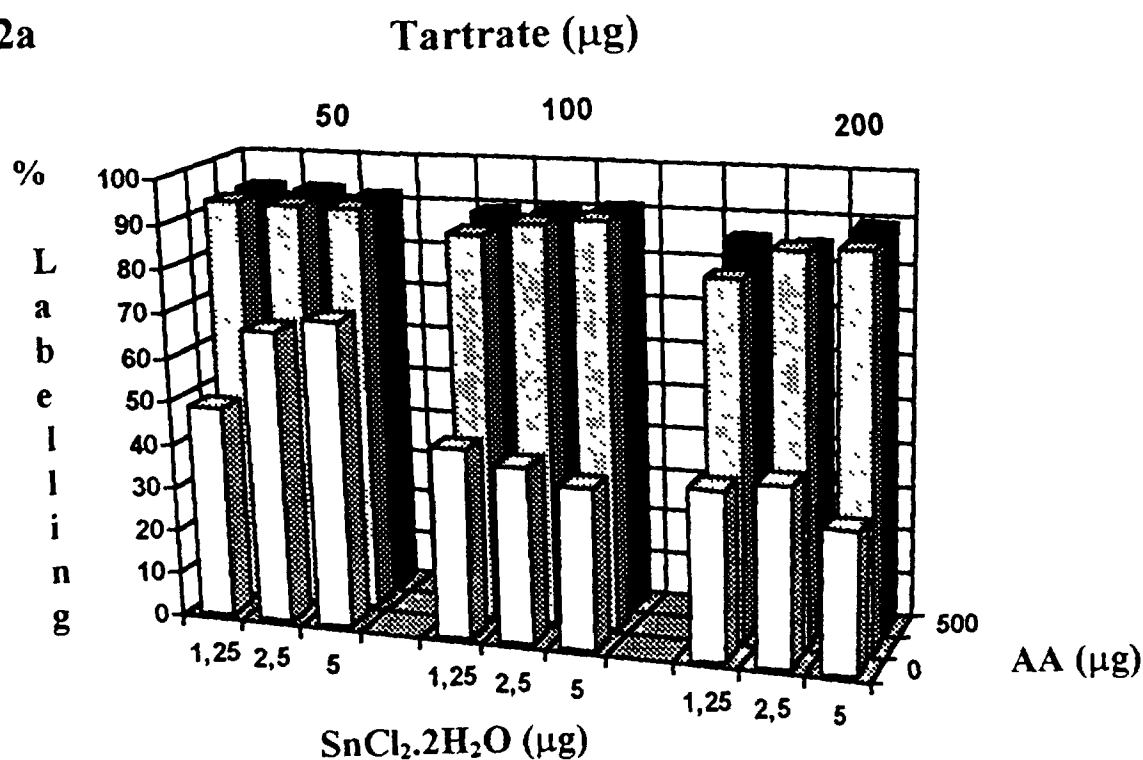


FIG 2a Influence of amount of reagents on $^{99}\text{Tc}^m$ labelling of antibody in Sn(II)/Ascorbate system

2b

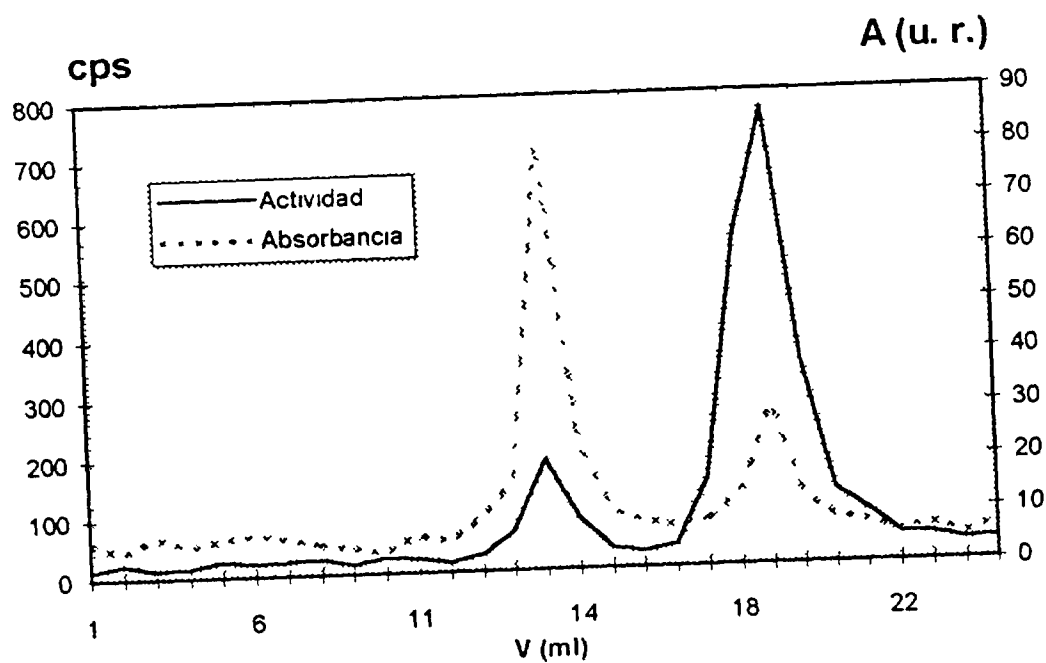


FIG 2b HPLC Analysis of $^{99}\text{Tc}^m$ labelled antibody

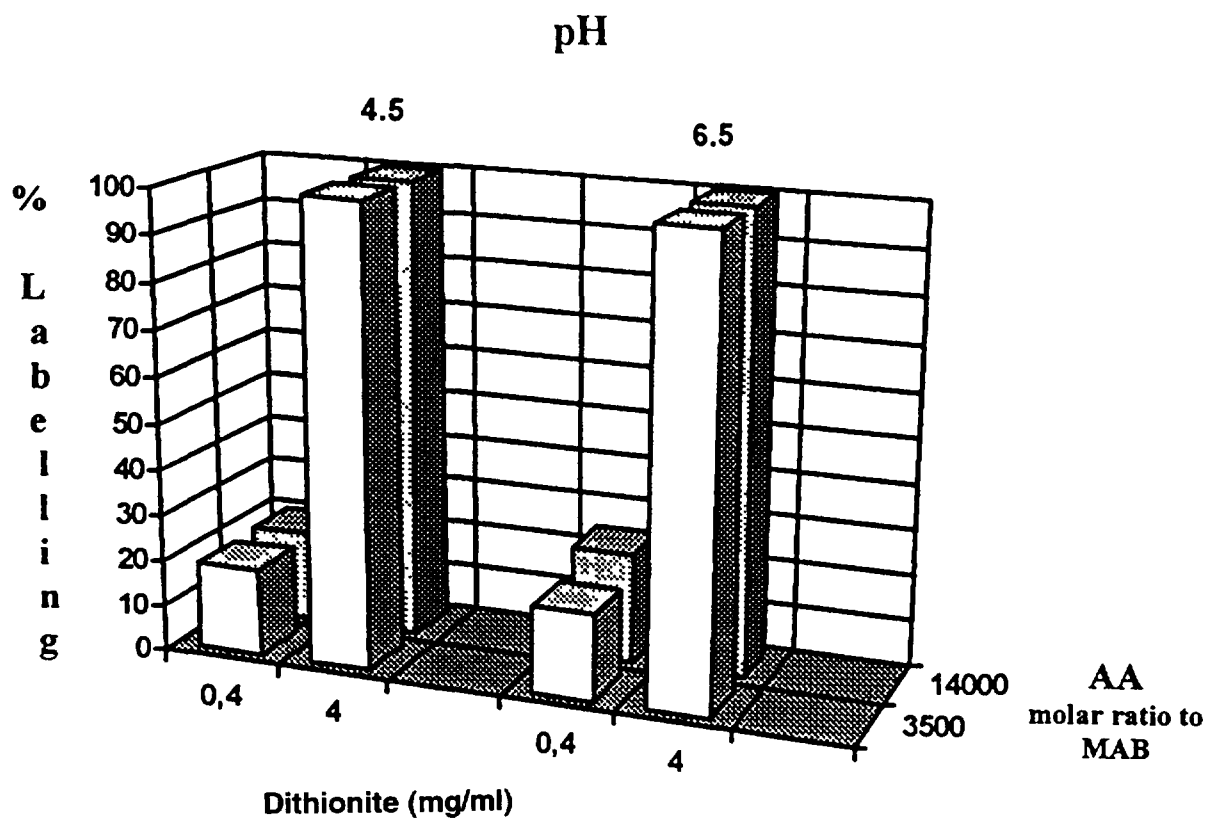


FIG 3 Effect of amount of reagents on $^{99}\text{Tc}^m$ labelling of antibody in AA/DT system

Stability in cysteine challenge of lor-CEA1

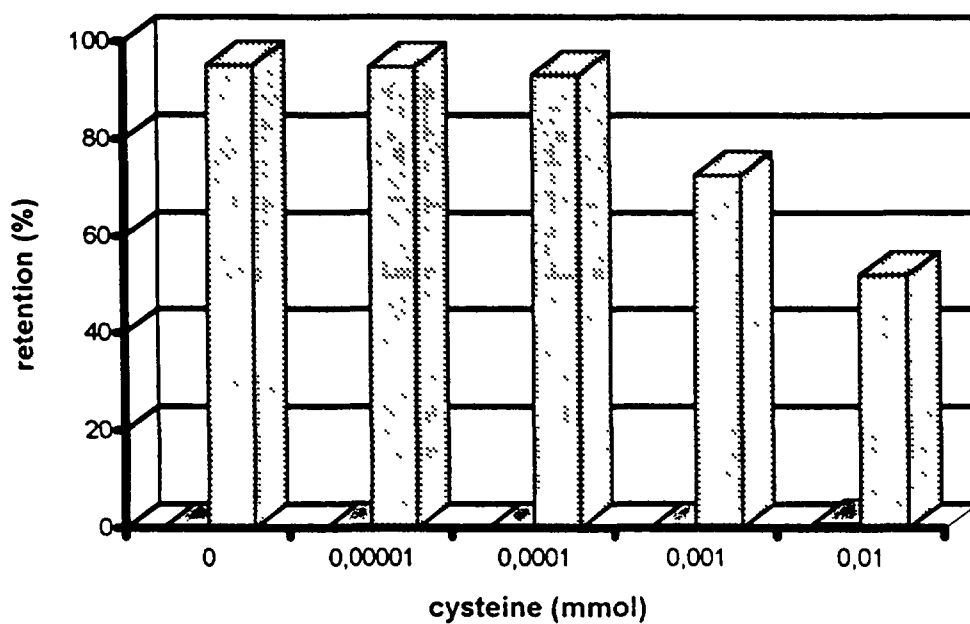


FIG 4 Cysteine challenge test of $^{99}\text{Tc}^m$ -lor-CEA-1 labelled using 2-ME method

Competitive binding assay of ior-CEA-1

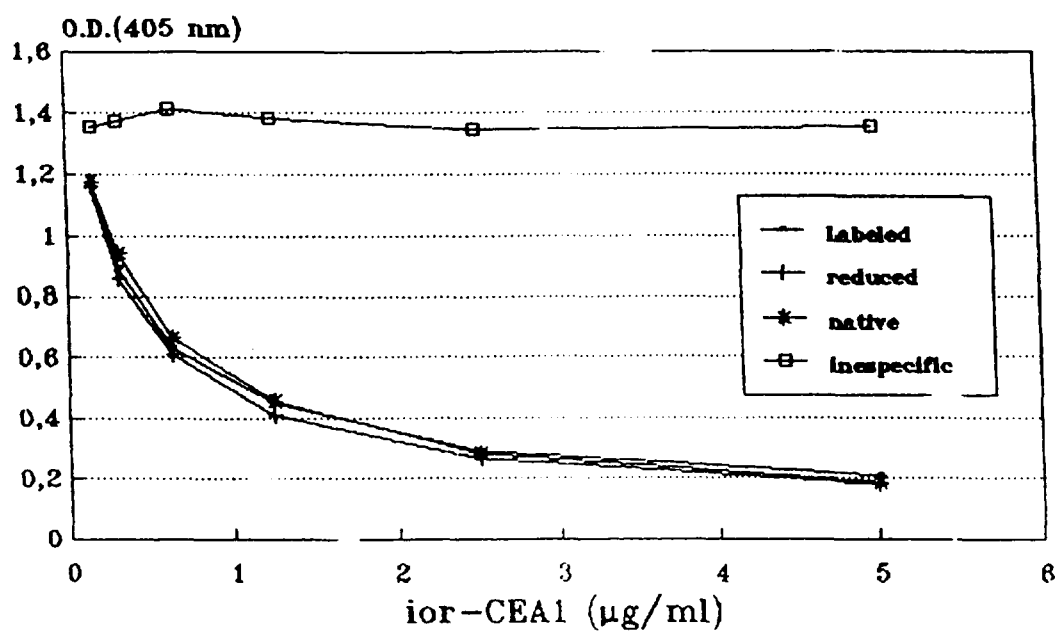


FIG. 5. Assessment of immunoreactivity of native and modified ior-CEA-1.

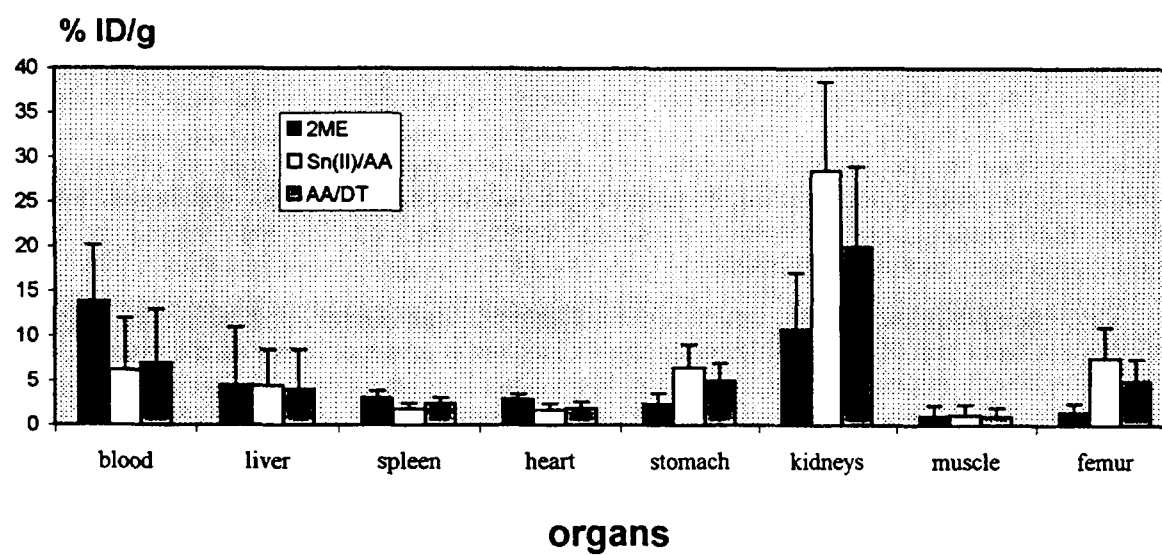


FIG. 6. Biodistribution of $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 prepared by different methods.

TABLE I. STABILITY IN SERUM OF ior-CEA-1 LABELLED BY DIFFERENT METHODS, EXPRESSED IN LABELLING PERCENTAGE

Method	15 min	1 h	2 h	4 h	11 h	24 h
2-ME	98.1	96.3	97.1	95.7	93.7	92.8
Sn(II)/AA	94.0	95.0	93.2	4.7	91.6	84.0
AA/DT	96.2	95.0	93.0	94.2	91.2	82.7

TABLE II. IMMUNOREACTIVITY INDEX OBTAINED FROM BINDING CURVES BY COMPETITIVE ASSAY

MAB	Intercept	Slope	R	Immunoreactivity Index
non-specific	0.32	0.004	-0.449	0.012
labelled	-0.015	-0.354	-0.924	0.985
reduced	-0.12	-0.239	-0.919	0.914
native	-0.08	-0.359	-0.936	1.00

As CEA antigen was not available in sufficient amounts, immunohistochemical studies were carried out in order to compare the immunoreactivity affection by the labelling process. In case of Schwarz's method it can be observed that all conjunctive tissue is clear and only the tumour areas remained hot, so labelled ior-CEA-1 retained the same recognition capacity as that of native MoAb. This suggested that the immunoreactivity of the MoAb was preserved during the labelling procedure. However, although immunohistochemical studies indicated that $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 labelled by means of the Sn(II)/AA method maintains the same recognition pattern as the native MAB, a relatively high background was observed. This indicates an increase of non-specific binding sites.

The results obtained in the animal biodistribution by the Schwarz labelling approach, suggested that there is no significant accumulation in non-critical organs, the excreting organs behaved as normal (Fig. 6). However, since in vitro studies by Sn(II)/AA and AA/DT labelling suggested some structural changes in the protein, the biodistribution study could play a role in evaluating the catabolism at the sites of antibody localization [17] or an in vivo instability. The results reflect a relatively high accumulation in stomach, femur and kidneys (Fig. 6). The high activity in kidneys suggests some lower molecular structure.

The immunogammagraphic results were correlated with those of CAT, US, cytology, endoscopy, surgery and clinic, in order to obtain the sensitivity and specificity of the method, which are 86.2% and 69% respectively. Each injection was tolerated without any secondary effect. No acute toxicity was observed, whereby the preparation was assimilated without significant change. Figures 7 and 8 show some picture of patients with recurrence and metastasis of the colorectal lesion. Radioactivity levels in liver, kidneys and spleen for $^{99}\text{Tc}^{\text{m}}$ at 24 h post administration are shown in Fig. 9 for some representative patients, which were corrected for radioactivity decay values for spleen and kidneys showed no significant differences in all patients, though were lower than those of liver (Fig. 9). Cumulative urinary excretion of radioactivity was $10 \pm 3\%$ ID ($n = 7$) at 24 h (Fig. 10), while the activity level in both kidneys was $3.5 \pm 0.8\%$ ID ($n = 8$).

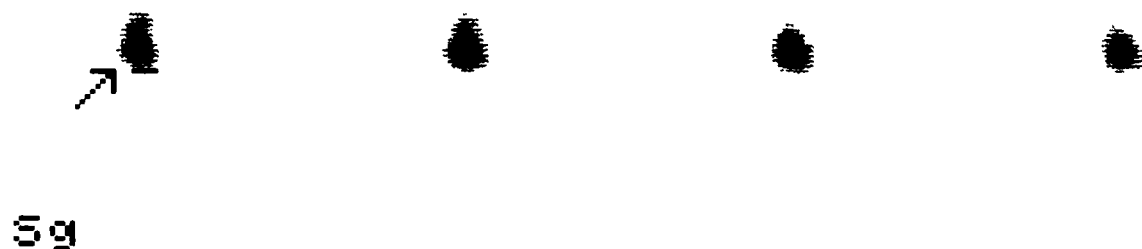
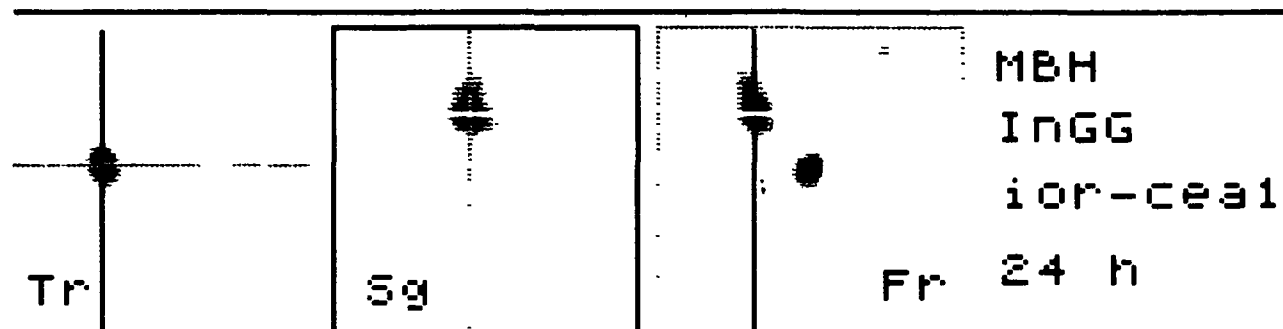
National Institute of Oncology

and Radiobiology (INOR)

Nuclear Medicine Dpt

NAME: InGG ior-cea1

DATE: 06/06/96



Recidiva de un T. de colon

FIG. 7. Spot anterior image of lower abdomen obtained at 24 h postadministration of ior-CEA-1 labelled by 2-ME approach.

National Institute of Oncology

and Radiobiology (INOR)

Nuclear Medicine Dpt

NAME: colombo

DATE: 06/06/96

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Liver metastases ("ring-type")

FIG. 8. Liver metastasis image (ring-type) obtained 22 h postadministration of ior-CEA-1 labelled by 2-ME approach.

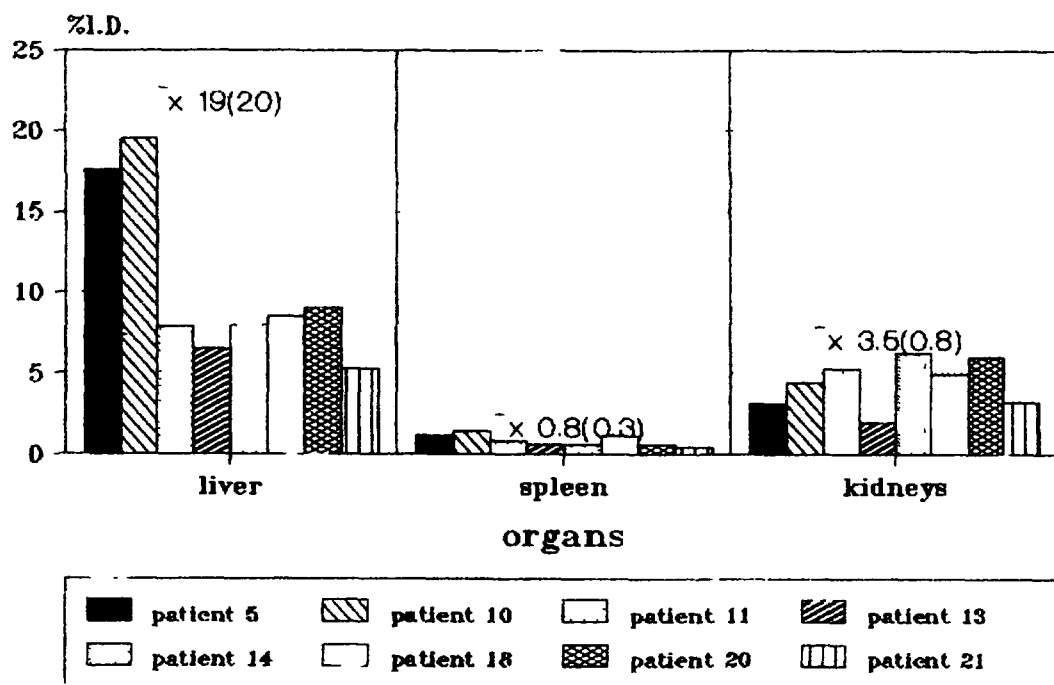


FIG. 9. Radioactivity levels in patents 24 h after administrations of $^{99}\text{Tc}^m\text{-ior-CEA-1}$

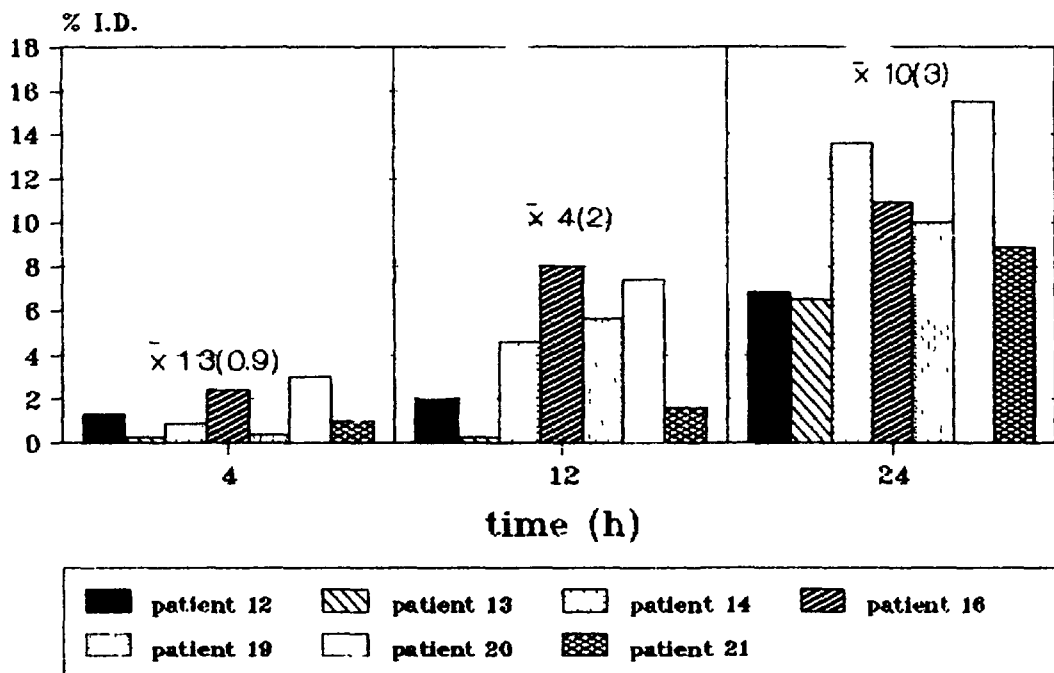


FIG. 10. Cumulative urinary excretion of radioactivity 24 h after administration of $^{99}\text{Tc}^m\text{-ior-CEA-1}$.

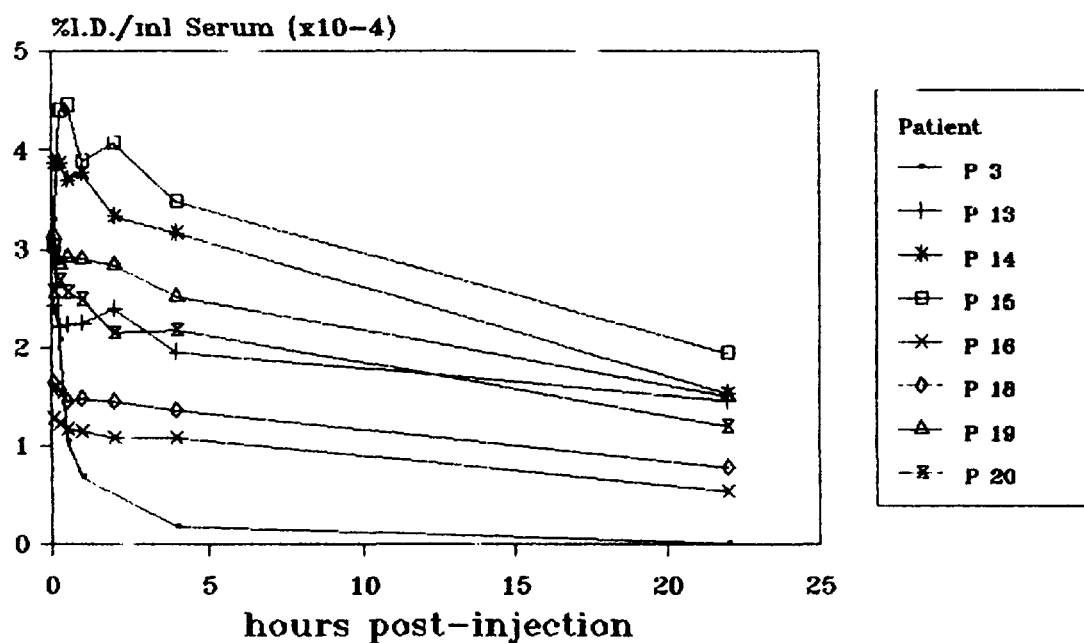


FIG. 11. Blood clearance of $^{99}\text{Tc}^m\text{-ior-CEA-1}$ in patients.

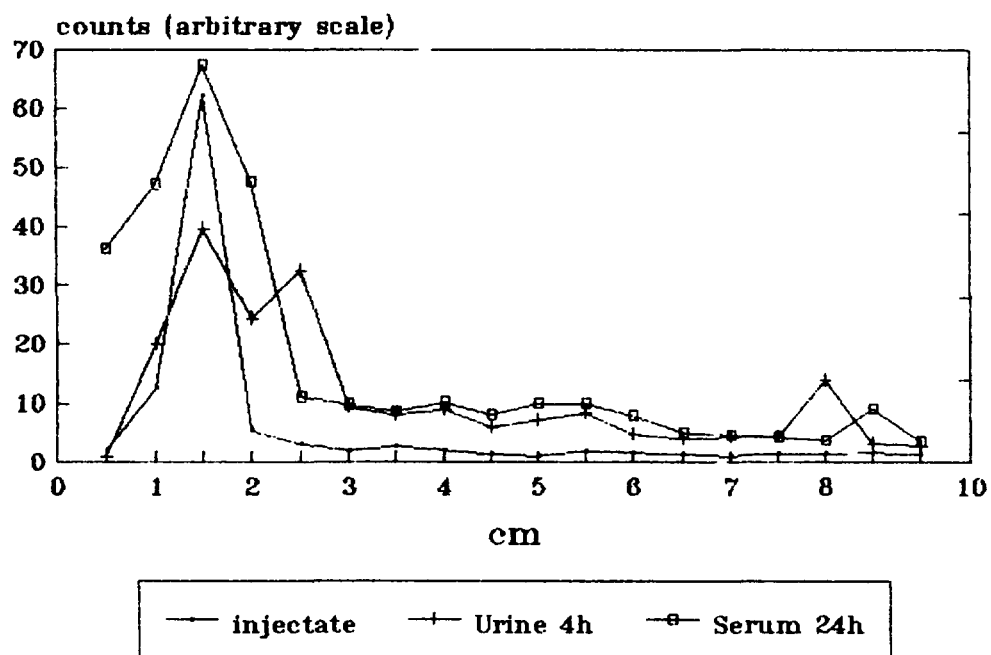


FIG. 12. SDS-PAGE analysis of serum and urine samples of patients.

TABLE III. PHARMACOKINETICS OF $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1

Dose	1 mg, (1875 MBq $^{99}\text{Tc}^{\text{m}}$)
Cl (% ID mL/h)	0.0093(0.004)
V_d	3.5(1.8)
$T_{1/2a}(\text{h})$	1(0.6)
$T_{1/2b}(\text{h})$	22(4)

Blood clearance of the injected ior-CEA-1 is shown in Table III. The results were calculated by means of the FARMAC program. Clearance of radioactivity from blood was biexponential in most cases. A rapid decrease is observed in the percentage of serum radioactivity (Fig. 11), followed by a second, much slower decline. In some patient curves, a slight early increase can be observed, so that the initial values were sometimes re-established. Thus, the clearance was determined mainly by second decline.

Radiochromatograms obtained by analysis on FPLC and electrophoresis of serum samples, show a complex radiochromatogram with multiple peaks, which correspond with some peaks of the UV detection. Nevertheless the majority of radioactivity remained associated to the labelled antibody, as a proof of label stability. Significant additional peaks not present in the native antibodies analysis shows the presence of high molecular weight species which in some cases increase slightly with time (Fig. 12). Likewise several peaks were observed in urine sample radiochromatograms being its radiochromatographic profile much more complex. Part of $^{99}\text{Tc}^{\text{m}}$ activity ran near to the front, suggesting the formation of low molecular weight species, presumably cysteine.

2.3.4. Discussion

In this investigation three direct labelling methods, which are simpler and more easy to perform for the daily practice were evaluated. From our results, obtained by the different quality controls, may be observed that the 2-ME approach gave the best results to be considered for the clinical application. The Sn(II)/AA system as well as AA/DT showed some change in the protein during the labelling process.

With regard to the role played by each agent in the reduction process, no evidence was found to suggest that ascorbate was capable of reducing the antibody. Additional studies of this investigation employing the Schwarz procedure, but using ascorbate as reducing agent for 1 h attained a labelling efficiency of 3.9% only for a 3500:1 ascorbate to antibody molar ratio and 8.3% for 35000:1.

On the other hand, ior-CEA-1 was treated with dithionite but not ascorbate, gave labelling efficiencies over those obtained with ascorbate alone (72%). If these results are compared with the AA influence in the Sn(II)/AA system, it could be concluded, that although the ascorbate presence plays a insignificant role in the reductant process, emphasizes the reducing action of the other partner agent.

Another important aspect of the $^{99}\text{Tc}^{\text{m}}$ -labelling methods is the protein stability. No noticeable changes were obtained for the labelled ior-CEA-1 by the 2-ME approach in the serum challenge (Table I). However, cysteine challenge experiments indicated that up to the molar ratio of 50 (cysteine to MAb) the cysteine influences significantly. Likewise, taking into account the immunohistochemical studies, the results reflected an increase of the non-specific binding sites by the Sn(II)/AA reducing system, while labelled ior-CEA-1 by the 2-ME approach retained the same recognition pattern as that of native MAb, suggesting that the effects on monoclonal biological activity due to reduction and labelling process was insignificant, coinciding so with that obtained with the micro ELISA assay.

We selected the 2-ME reducing method as an adequate labelling technique to be applied in human trials, since it accomplished with the required parameters to obtain a competent radiopharmaceutical, giving an injectable preparation of high radiochemical purity and unimpaired immunoreactivity.

The immunoscintigraphy has been able to detect not only the clinically suspected recurrent and metastatic lesions, but also unsuspected lesions, like an asymptomatic patient with negative laboratory tests, as well as the US and NMR. The immunoscintigraphy with ior-CEA-1 indicated a recurrent lesion and liver metastasis.

Pharmacokinetic behavior of ior-CEA-1 was similar to that observed with other monoclonal antibodies [19, 20]. The majority of the patient's data were fitted to a two compartmental model. The biodistribution results revealed that the largest accumulation was in liver (19 ± 2.05 , $n=15$) of injected dose at 24 h post injection. However, excluding the patients who had liver metastases and high CEA level, due to correlation between high liver uptake and immune complex formation [21], the value for the liver uptake was 9 ± 4 % ID ($n=10$). At 24 h post administration activity in liver, spleen, kidneys and tumour accounts for about 26 % of the injected dose, remaining about 50% in circulating serum. Taking into account that the cumulative urinary excretion accounts about 10%, the remaining is distributed in the rest of the body, under the assumption that the major route of excretion is urine; faeces excretion was not measured (approximately 14% ID).

2.3.5. Conclusions

The FPLC chromatographic analysis of serum samples at different times did not show significant presence of $^{99}\text{Tc}^{\text{m}}$ -pertechnetate neither in vitro nor in vivo assays. The $^{99}\text{Tc}^{\text{m}}\text{O}_4^-$ levels remained under 9% at 24 h, which were confirmed by the SDS-PAGE analysis (Fig. 12). Similar results were obtained previously [19, 22]. From the figure it can be observed that most of the label remained associated to the protein, at least within 24 h. This, together with the antibody capacity to detect the tumour [6–8], gives a measure of the ior-CEA-1 sensitivity to recognize the lesion, as well as that the selected labelling method accomplished the proposed objectives.

REFERENCES

- [1] BLOCK, D., FEITSMA, R.U., et al., A New Method for Protein Labelling with $^{99}\text{Tc}^{\text{m}}$, Nucl. Med. Biol. **16** (1989) 11–16.
- [2] RHODES, B.A., ZAMORA, P.O., et al., Technetium-99m Labelling Murine Monoclonal Antibody Fragments, J. Nucl. Med. **27** (1986) 685–693.
- [3] SCHWARZ, A., STEINSTRASSER, A., A Novel Approach to $^{99}\text{Tc}^{\text{m}}$ Labelled Monoclonal Antibody, J. Nucl. Med. **28** (1987) 721 (Abstract).
- [4] MATHER, S.J., ELLISON, D., Reduction-Mediated Technetium-99m Labelling of Monoclonal Antibodies: Evaluation of Reducing Agents, Nucl. Med. Biol. **18** (1990) 227–233.
- [5] THAKUR, M.L., DEFLUVIO, J., et al., Technetium-99m Labelled Monoclonal Antibodies: Evaluation of Reducing Agents, Nucl. Med. Biol. **19** (1991) 227–333.
- [6] OLIVA, J., PERALTAR, R., et al., ior-CEA-1 Un Nuevo Anticuerpo Anti-CEA $^{99}\text{Tc}^{\text{m}}$ Para la Immunogammagrafia de Tumours Colorectales Resultados Preliminares, Rev. Esp. Med. Nucl. **13** (1994) 32–37.
- [7] OLIVA, J., PIMENTEL, G., et al., ior-CEA-1: Un Nuevo Anticuerpo Monoclonal Anti-CEA Marcado con $^{99}\text{Tc}^{\text{m}}$ Para la Immunogammagrafia de le Tumours Colorectales. Resultados Finales de un Ensayo Clinico Fase I-II, Rev. Esp. Med. Nucl. **14** (1995) 213–221.
- [8] TORMO, B.R., GAVILONDO, J.V., et al., CEA in Colonic Adenocarcinomas and Precancerous Lesions. An Immunohistochemical Study with a Novel Monoclonal Antibody, APMIS **97** (1989) 1073–1080.
- [9] HNATOWICH, D.J., VIRZI, F., et al., Investigations of Ascorbate for Direct Labelling of Antibodies with Technetium-99m, J. Nucl. Med. **35** (1994) 127–134.

- [10] VELANDIA, A., NUNEZ, G., et al., Ultramicroanalists Para la Cauntification de Antfigteno Carcino Embrionario en Suero, *Biotech. Appl.* **13** (1995) # 1.
- [11] AVRAMEA, S., TERNYNCK, T., The Cross-linking of Protein with Glutaraldehyde and its Use for the Preparation of Immunoabsorbent, *Immunochemistry* **6** (1969) 53
- [12] HNATOWICH, D.J., GRIFFIN, T.W., et al., Pharmacokinetics of an Indium-111-Labelled Monoclonal Antibody in Cancer Patients, *J. Nucl. Med.* **26** (1985) 849-858.
- [13] GRAHAN, L.S., NEIL, A., In Vivo Quantitation of Radioactivity Using the Auger Camera, *Radiology* **112** (1974) 441-442.
- [14] SANCHEZ, G., GONZALEZ, A., et al., Sistema Automatizado Para el Cilculo de Parametros Farmacocineticos, II Simposio Acerca del Desarrollo de la Matematica. Sep 1992, Habana, Cuba.
- [15] FOSS, S.D., A Method for Obtaining Initial Estimates of the Parameters in the Exponential Curve Fitting, *Biometrics* **25** (1969) 580-584.
- [16] RHODES, B.A., Direct Labelling of Proteins with $^{99}\text{Tc}^m$, *Nucl. Med. Biol.* **18** (1991) 667-676.
- [17] CHILDS, R.L., HNATOWICH, D.J., Optimum Conditions for Labelling of DTPA-Coupled Antibodies with Technetium-99m, *J. Nucl. Med.* **26** (1985) 293-299.
- [18] HNATOWICH, D.J., MARDIROSSIAN, G., et al., Directly and Indirectly Technetium-99m Labelled Antibodies. A Comparison of in Vitro and Animal in Vivo Properties, *J. Nucl. Med.* **34** (1993) 109-119.
- [19] HNATOWICH, D.J., MARDIROSSIAN, G., et al., Pharmacokinetic of the FO23CS anti-CEA Antibody Fragment Labelled with $^{99}\text{Tc}^m$ and ^{111}In : A Comparison in Patients, *Nucl. Med. Commun.* **14** (1993) 52-63.
- [20] BROWN, B.A., DEARBORN, C.B., et al., Pharmacokinetics of $^{99}\text{Tc}^m$ -Metallothionine B72.3 and its F(ab)₂ Fragment, *Cancer Res. (suppl.)* **50** (1990) 835s-839s.
- [21] HNATOWICH, D.J., RUSCHOWSKI, M., et al., Pharmacokinetics in Patients of an Anti-carcinoembryonic Antigen Antibody Radiolabelled with Indium-111 Using a Novel Diethylenetriamine Pentaacetic Acid Chelator, *Cancer Res.* **50** (1990) 7272-7278.
- [22] BAUM, R.P., HERTEL, A., et al., $^{99}\text{Tc}^m$ -Labelled anti-CEA Monoclonal Antibody for Tumour Immunoscintigraphy, First Clinical Results, *Nucl. Med. Commun.* **10** (1989) 345-352.

Research Contract No. GRE-6459/RB

Title of the Project : Radiolabelling of Antibodies with Technetium-99m and their Clinical Evaluation

Chief Scientific Investigator : A.D. Varvarigou
National Centre for Scientific Research "Demokritos",
Athens

2.4.1. Introduction

Our study referred mainly to the comparative evaluation of two different methods, one direct and the other indirect, for the labelling of MoAbs with $^{99}\text{Tc}^{\text{m}}$. With the direct method, disulphide bridges were cleaved by the use of 2-mercaptoethanol (2-ME), as reductant, while with the indirect method, the antibody was coupled to 2-iminothiolane (2-IM). The latter reacts with the amine groups of the protein's lysine molecules, thus creating free thiol groups, capable of binding reduced $^{99}\text{Tc}^{\text{m}}$. In both cases a preformed intermediate chelate was used for the $^{99}\text{Tc}^{\text{m}}$ exchange. The $^{99}\text{Tc}^{\text{m}}$ labelled species were analyzed by Instant Thin Layer Chromatography (ITLC), High Performance Liquid Chromatography (HPLC), and Polyacrylamide Gel Electrophoresis (PAGE). We investigated the influence of the labelling systems on MoAbs' integrity, as well as the ability of the labelled species to tag on human cancer cells. The biodistribution of the labelled derivatives was comparatively evaluated in normal male Swiss mice and the stability of labelling was measured by cysteine challenge. The in vitro stability was comparatively studied at different temperatures and by incubation with human serum for 24 h.

2.4.2. Materials and methods

The Monoclonal Antibody, ior-CEA-1 raised against Carcinoembryonic Antigen was produced by the "Centro de Immunologia Molecular", La Habana, Cuba. The antigen, was obtained from Scripps Lab. Human Immunoglobulin (Sandoglobuline), was a Sandoz product. All other reagents were purchased from commercial sources and were of analytical grade.

2.4.2.1. Radiolabelling

Mercaptoethanol reduction

The antibodies, when necessary, were concentrated by ultrafiltration (Centricon, Amicon, MA) to a final concentration of 10 mg/mL in 0.2 M phosphate buffered saline (PBS), pH7.4, and reduced by reaction with (2-ME), at a molar ratio of 1000:1. The reaction mixture was incubated at room temperature for 30 min and then was purified on a 2×6 cm G50 Sephadex column, by eluting with cold, nitrogen purged PBS. One mL fractions were collected and their optical density was measured in a UV/VIS spectrophotometer at 280 nm. The fractions containing the antibody were pooled together, divided in one mg aliquots and frozen.

Technetium-99m labelling was performed by radionuclide exchange with a preformed Technetium kit. Two diverse commercial kits, prepared in our Institute, were used comparatively as follows: 40 μL of a methylene diphosphonate kit, containing 30 μg of MDP and 1.44 μg stannous chloride were added to one mL of the reduced antibody in PBS (1.0 mg), followed by 37 to 370 MBq of Tc-99m-pertechnetate. The mixture was left at room temperature for 15 min. 100 μL of a Glucoheptonate kit, containing 2.0 mg glucoheptonate (GH) and 2.0 μg stannous chloride, were added to 1.0 mg of the reduced antibody, followed by the addition of pertechnetate as above. The mixture

was left at room temperature for 15 min. For the reduction of Biomira MoAb, at the ratio 1000:1, a reaction time of 45 min was found necessary.

2-Iminothiolane coupling

To a solution of 150 μL of an anti CEA solution (7.0 mg/mL in 0.2M phosphate buffer, (pH7.4) a volume of 10 μL of a 0.07 M solution of 2-IM in 1 mL triethanolamine, pH8.0, was added (molar ratio 100:1, MoAb to 2-IM). The mixture was incubated for 10 min, for the coupling of 2-IM with the protein and the product was used for labelling without further purification. 100 μL of a Glucoheptonate kit, prepared as described above, was added, followed by $^{99}\text{Tc}^{\text{m}}$ -pertechnetate. The preparation was left at room temperature for 30 min.

2.4.2.2. Radiochemical quality control

The labelling efficiency and the in vitro stability were measured by Instant Thin Layer Chromatography (ITLC) and Size Exclusion High Performance Liquid Chromatography, (HPLC). ITLC was performed on Silica Gel strips, (Gelman USA), using as mobile phases: (a) methanol: 10% ammonium acetate solution 1:1, (v/v), (b) acetone, followed by a second run of sodium chloride and (c) methylethylketone, followed by a second run of 5% glycine. In systems b and c, between the two runs, the strips were air-dried.

HPLC (Waters Ass. USA), comprised of a UV detector and a radioactivity detector. The column used was a TSK-Gel G-2000SW, 7.8 mm (I.D.) \times 30 cm. A volume of 10 μL of the labelled solution was injected into the column and eluted with a mixture of phosphate buffer, sodium sulphate and sodium azide, at pH6.7. The column eluent was monitored by UV detector at 280 nm and then by sodium iodide scintillation detector. Both were connected to a computer for data storage and processing. The flow rate was 1.0 mL/min and the operating pressure was in the range of 400 psi.

2.4.2.3. Polyacrylamide gel electrophoresis (PAGE), applied to ior-CEA-1

The radiolabelled species were analysed on polyacrylamide gels, in the presence of SDS, on a mini gel electrophoresis apparatus. Mini gel plates were 8 \times 10 cm in size and 0.5 mm thick, consisted of 7.5% acrylamide, 0.2% bis-acrylamide, 0.1% TEMED, 34 mM sucrose, 2.0 mM EDTA, 0.15% SDS in 0.38 M Tris-HCl, pH8.8, and were polymerized with 0.05% ammonium persulphate (APS). Stacking gels, consisted of 3.2% acrylamide, 0.085% bis-acrylamide, 0.27% TEMED, 2.0 mM EDTA, 0.15% SDS in 0.125 M Tris-HCl, pH6.8, and were polymerized with 0.13% APS. After lyophilization, samples were taken in a sample buffer consisting of 2.0% SDS, 0.3 M sucrose, 0.025% bromophenol blue in 50 mM Tris-HCl, pH7.6. The concentration of both $^{99}\text{Tc}^{\text{m}}$ labelled and "cold" antibody was 1.0 mg/1.5 mL in PBS. Aliquots of the above solutions were TCA precipitated and freeze-dried, prior to the running. In all cases 20 μg of each sample were loaded on to the gel. The electrode buffer was 25 mM Tris-HCl, 0.19 M glycine, pH7.6, containing 2.0% SDS. Electrophoresis was run with constant voltage of 150 V for 80 to 100 min. After the end of each run gels were stained with coomassie brilliant blue R-250.

2.4.2.4. Immunoreactivity test

The immunoreactivity of ior-CEA, labelled with $^{99}\text{Tc}^{\text{m}}$, after structural modification by 2-ME reduction or 2-IM coupling was tested by radioimmunoassay, using a method previously described. Briefly, 96-well microtiter plates (Costar, UK) were coated with 200 μL of a 1.25 $\mu\text{g}/\text{mL}$ CEA solution in PBS, and left overnight at room temperature. The plates were rinsed with PBS/Tween 20 (0.05%) and incubated with 200 μL of a 1.0% HSA at 37°C for one h. They were then washed with PBS/Tween 20 and the samples containing the $^{99}\text{Tc}^{\text{m}}$ labelled antibody were added at a concentration of 1.0 $\mu\text{g}/\text{mL}$, followed by 1:2 serial dilutions (final volume 200 μL). The radiolabelled antibodies were also incubated with 0.25 $\mu\text{g}/\text{mL}$ "cold" antibody for competitive purposes. The plates were

incubated at 37°C for one h, washed with PBS/Tween 20 and the radioactivity of each well was measured in a gamma counter.

2.4.2.5. Cell binding assay for ior-CEA

All cell binding assays were performed with the cell line Colo 205, which produces carcino-embryonic antigen. These cells were grown in 75 sq cm flasks as monolayers in RPMI medium 1640, supplemented with 10%, heat inactivated, fetal bovine serum, at 37°C and 5% carbon dioxide. Subculturing was effected after trypsinization with 0.25% Trypsin in EBSS at a split ratio of 1:3 every 2–3 days. Single cell suspensions were obtained from trypsinized cell layers by pipetting in fresh medium and the cell suspensions for the binding assays were centrifuged. The residue was washed twice with PBS and then resuspended in PBS, containing 1.0% BSA. Cells were used for the binding assays immediately after harvesting.

The assays were performed by incubation in Eppendorf tubes, while shaking, at room temperature for two hours. The labelled antibody solution was diluted in 2% BSA in PBS plus 0.02% sodium azide. Diluted antibody (100 μ L) was aliquoted in duplicate in Eppendorf tubes and 100 μ L of an appropriate cell dilution were added. An 100 μ L aliquot of each preparation was also counted to determine total antibody concentration. For Lineweaver-Burk analysis [19, 20] a fixed concentration of the antibody (150 ng) was incubated with varying numbers of target cells (10^5 – 10^7 cells/incubation), while for Scatchard analysis [21, 22] varying dilutions of antibody (50 μ g/200 μ L to 50 mg/200 μ L) were incubated with a fixed number of cells (10^5 cells/incubation). After incubation, the target cells were washed with PBS four times by centrifugation and the residue was counted for radioactivity.

2.4.2.6. Stability studies

The in vitro stability of the radiolabelled antibodies was studied at different temperatures and by incubation with human serum. Thus, samples of the $^{99}\text{Tc}^{\text{m}}$ -labelled antibodies were stored at room temperature and at 4°C. Free pertechnetate contents was calculated for both labelling systems 6 and 24 h later by ITLC and HPLC under the conditions previously mentioned. Serum stability was determined by incubation of the radioactive solutions with fresh human sera at 37°C for 24 h. Additionally, the cysteine challenge test was performed by incubation of the labelled species for one hour at 37°C, under agitation in the presence of cysteine, at a cysteine to labelled MoAb ratio ranging from 10 to 500.

2.4.2.7. Kit formulation

For both anti-CEA antibody and hIgG instant kits, based on the 2-ME reduction system, were formulated as follows: To a solution of 20.0 mg of the 2-ME reduced protein in 10 mL of 0.2 M PBS, a solution of a reconstituted MDP kit, containing 0.6 mg MDP and 2.9 μ g stannous chloride in 0.8 mL saline was added followed by a sugar solution as stabilizer. Up to now a series of sugars (dextrose, mannitol, saccharose) have been tried. The final volume was brought to 80 mL with water for injection. The solution was distributed in twenty vials, 4.0 mL each and lyophilized at -4°C for 48 h.

2.4.2.8. Radiochemistry of the $^{99}\text{Tc}^{\text{m}}$ -labelled derivatives

We have focused our attention on the radiochemical impurities, which can be formed during MoAbs labelling. Although in the labelling procedures their percentage remained very low, ranging from 1.0 to 3.0, we tried to isolate and identify these by-products, in order to avoid their formation, which if increased, might interfere in tumour imaging. Thus, we have experimentally reproduced in higher yield and studied the by-products formed by the labelling systems. For the $^{99}\text{Tc}^{\text{m}}$ -anti CEA formation we have applied the methods already reported for the radio metal exchange using two commercial formulated preparations, MDP and GH kits. In order to identify the impurities formed by

the two labelling systems, we have tried to apply extreme experimental conditions, which result to the production of these by-products.

2.4.2.9. Mercaptoethanol reduction

In order to investigate the influence of 2-ME presence on the protein structure and on the final labelling yield we have performed the following experiments: (a) incubation of the intact antibody with 2-ME from 1 to 24 h, (b) study the influence of an excess of 2-ME on the intermediate Tc-99m-MDP chelate and (c) study the influence of the 2-ME on the intermediate Tc-99m-GH chelate.

2.4.2.10. 2-Iminothiolane coupling

In this method we have investigated respectively: (a) incubation of the intact antibody solution with 2-IM from 1 to 24 h, (b) purification of the antibody solution with 2-IM from 1 to 24 h and (c) labelling by the use of MDP as $^{99}\text{Tc}^{\text{m}}$ exchange ligand.

2.4.2.11. Animal studies

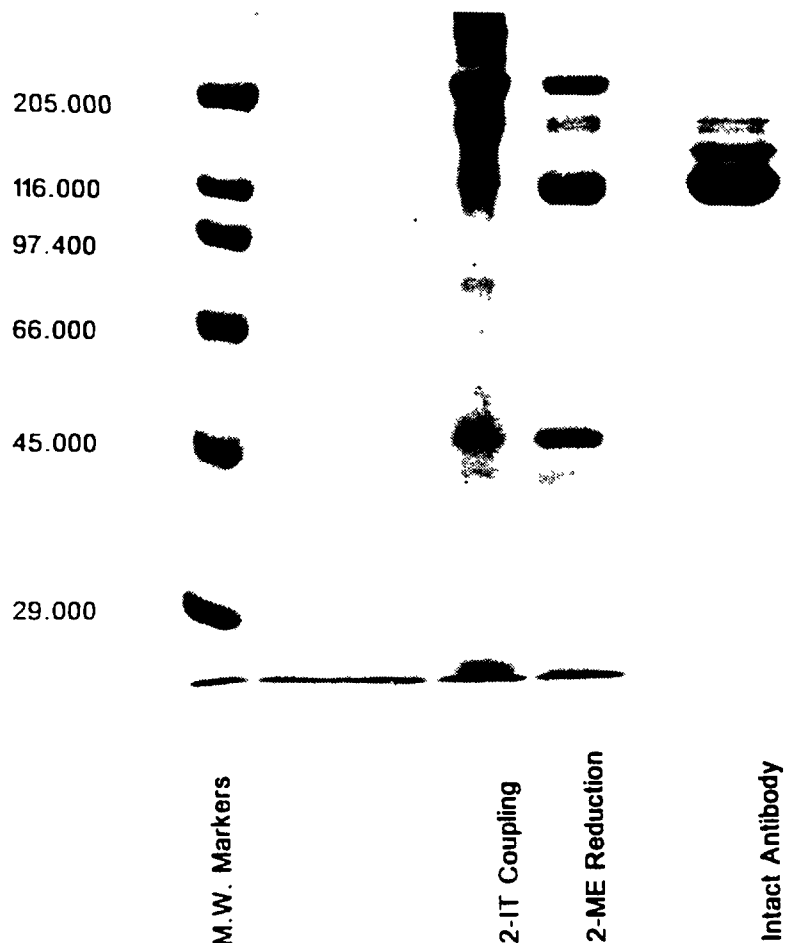
Comparative biodistribution of the $^{99}\text{Tc}^{\text{m}}$ labelled antibodies and of the $^{99}\text{Tc}^{\text{m}}$ labelled byproducts were performed in normal Swiss mice. A quantity of 0.2 mL of the labelled solution was injected in the tail vein. The animals were sacrificed, 4 h p.i. by anesthesia and the principal organs, as well as blood and muscle samples, were removed, weighed and counted in a gamma scintillation counter. The percent dose per gram of tissue was calculated and compared to a standard.

2.4.3. Results

For both labelling methods, ITLC indicated a labelling efficiency ranging from 96 to 98%. With the 10% ammonium acetate/methanol (1:1) system $^{99}\text{Tc}^{\text{m}}$ -antibodies had a R_f of 0.0, while the R_s for $^{99}\text{Tc}^{\text{m}}$ -pertechnetate and the exchange ligands were 1.0 and 0.9 respectively. In the other two chromatographic systems, in the case of 2-ME reduction a single radiolabelled species was obtained, which migrated with the solvent front of the second solvent. This may be due to denaturation of the protein, on the stationary phase, under the influence of the organic solvent. In the case of the 2-IM coupling two radiolabelled species were also observed, one of which remained at the origin and another, which also moved with the solvent front of the second solvent. We assume that, in this case, no additional denaturation was observed than that caused by the coupling agent since the percentage of each labelled species is in full agreement with the figures obtained by HPLC studies. As these double runnings are laborious in obtaining representative results, we have based our studies mainly on HPLC and PAGE findings.

HPLC resulted to a recovery of the radioactivity applied on the column greater than 90% and confirmed the ITLC findings. The respective patterns are presented in Fig. 1. The 2-ME reduction (Fig. 1a) gave one main peak, with a retention time of 6.0-6.5 min. The 2-IM coupling (Fig. 1b) resulted in two peaks, one corresponding to the protein (R_t 6.5) and another smaller peak, with a lower retention time (R_t 5.6), corresponding to some higher molecular weight specie. These components were revealed by both UV and radioactivity detectors. The HPLC findings were identical for all three antibodies studied.

SDS-PAGE results are presented in PIC. 1. As it can be observed, both methods led to partial modification of structure. However, while 2-ME reduction resulted in a limited formation of both lower and higher molecular weight species, 2-IM coupling caused disruption of the heavy chain and partial fragmentation of the antibody. Comparison with the molecular weight markers indicated the formation of a dimer. These results are in good agreement with the HPLC findings, which also indicated formation of two labelled species for the 2-IM coupling.



PICT. 1. Comparative SDS-polyacrylamide gel electrophoresis (PAGE)

Fig. 2 shows that the monoclonal antibody labelled by both systems retained its immunoreactivity. Nevertheless, the 2-IM coupling (Fig. 2b) led to a species with slightly different immunoreactivity as compared to 2-ME reduction (Fig. 2a). This is shown by the degree of competition with the cold antibody and by the absolute counts measured taking into account that the same antibody concentration was used.

Scatchard and Lineweaver-Burk analysis on the cancer cell binding showed a higher tagging in the case of 2-ME reduction of ior-CEA-1, as indicated in Fig. 3. Both labelling systems resulted in an in vitro stable labelled derivatives, when stored at 4°C. Storage at room temperature showed an approximately 15% liberation of pertechnetate, quite similar for the two methods applied. Serum stability studies indicated that for both antibodies, ior-CEA-1 and hIgG radioactivity bound to the protein two h after incubation was 85–90%. Cysteine challenge experiments for ior-CEA-1 indicated differences in stability between the labelled species obtained by the two methods. It can be observed from Fig. 4 that 2-ME reduction resulted in a more stable Tc-99m labelled derivative. Similar experiments for hIgG demonstrated a lower stability. Thus, 20% of transchelation was observed for a molar ratio of cysteine to antibody of 50. For the same molar ratio the transchelation for ior-CEA-1 was found to be about 10%.

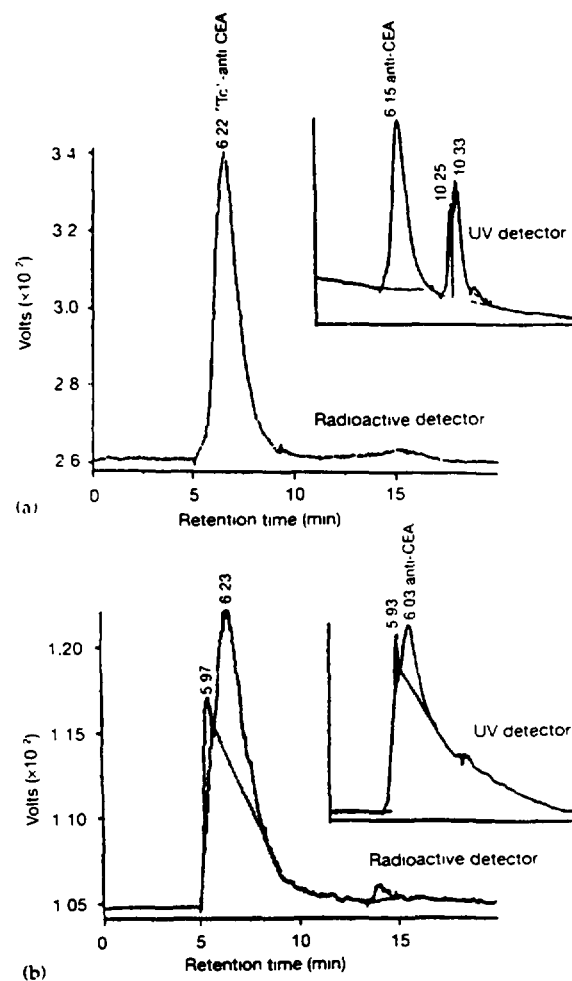


FIG. 1. HPLC Patterns of $^{99}\text{Tc}^m$ -ior-CEA-1. (a) Preparation by 2-mercapto-ethanol reduction; (b) Preparation by 2-iminothiolane coupling.

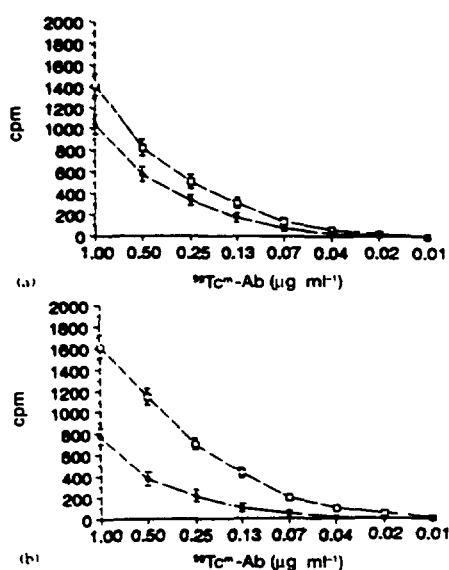


FIG. 2. Immunoreactivity of the $^{99}\text{Tc}^m$ -ior-CEA-1, as measured by direct radioimmunoassay. (a) Preparation by 2-mercapto-ethanol reduction; (b) Preparation by 2-iminothiolane coupling. The results are expressed in cpm of the radiolabelled ior-CEA-1 bound to the antigen and of the binding of radiolabelled ior-CEA-1 in competition with "cold" ior-CEA-1.

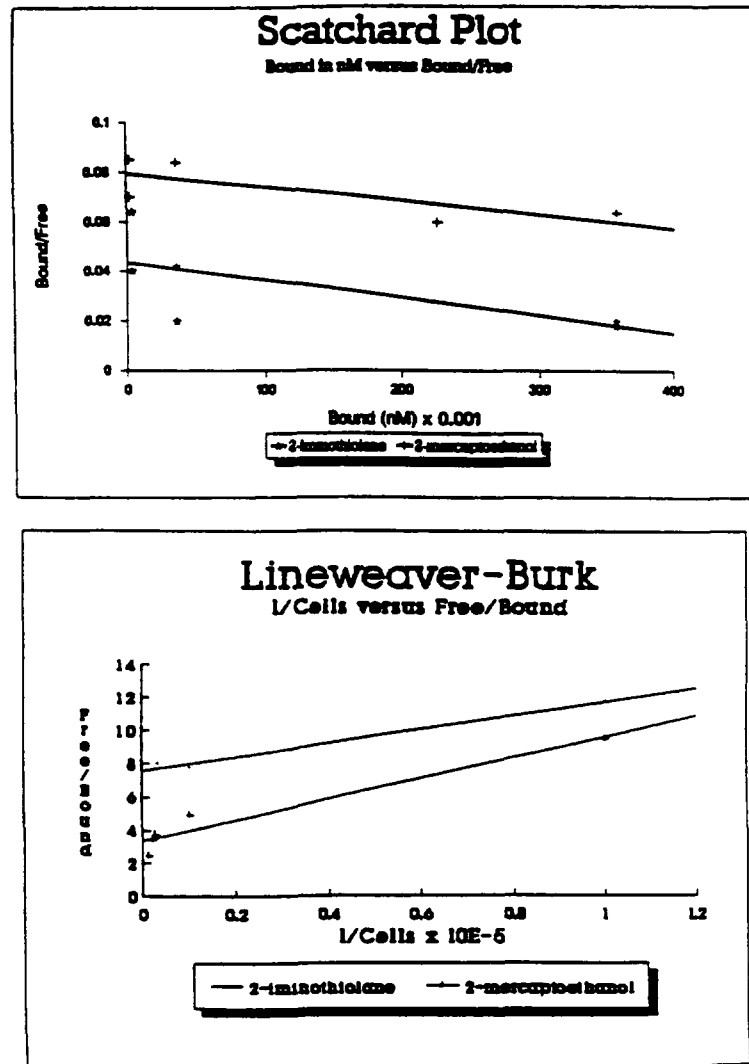


FIG. 3. Scatchard and Lineweaver-Burk analysis of cancer cell binding for both labelling methods.

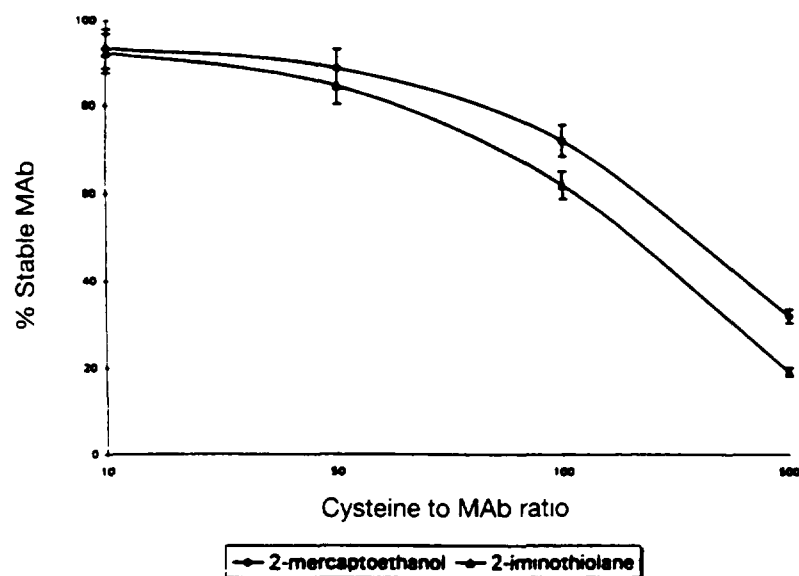


FIG. 4. Cystein challenge for labelled species obtained by the two methods.

With regard to the by-products, usually formed by the labelling systems applied, we have obtained the following results.

2.4.3.1. 2-Mercaptoethanol reduction

(a) When MDP was used for the reduced $^{99}\text{Tc}^{\text{m}}$ exchange, traces of 2-ME present in the reaction mixture led to the formation of a $^{99}\text{Tc}^{\text{m}}$ -chelate, formed among MDP, 2-ME and reduced $^{99}\text{Tc}^{\text{m}}$, with a retention time of 15.85 (Figs 5 and 6). (b) Prolonged incubation of ior-CEA-1 with 2-ME resulted to an extended formation of low molecular weight species (Fig. 7). (c) When GH was used as intermediate $^{99}\text{Tc}^{\text{m}}$ chelator any similar impurity was not obtained (Fig. 8).

2.4.3.2. 2-Iminothiolane coupling

(a) Prolonged incubation of anti-CEA with 2-IM resulted to a more extended formation of higher molecular weight species (Fig. 9). (b) Purification of the MoAb from the excess of the coupling agent before the addition of the exchange ligand and pertechnetate did not alter the radiochemical and radiobiological results. (c) The labelling of ior-CEA-1 by this method, using MDP as exchange ligand did not lead to the formation of any intermediate $^{99}\text{Tc}^{\text{m}}$ -labelled by-product (Fig. 10).

2.4.3.3. Animal studies

The comparative mice biodistribution studies of the labelled anti-CEA, as well as of the by-products formed by the labelling systems are presented in Table I. They indicate a different in vivo pattern in the case of 2-IM in which a dimer is formed. The last presented faster blood and Hepatobilliary clearance, while renal elimination remained practically unchanged. The respective biodistribution pattern of the by-product formed by the 2-ME reduction method showed very fast blood clearance and practically total urinary excretion. Biodistribution of hIgG kit ($^{99}\text{Tc}^{\text{m}}$ -Flegmon/Demoscan) was evaluated before and after lyophilization in normal mice. Results were practically identical and are presented in Table II. Biomira $^{99}\text{Tc}^{\text{m}}$ -MoAb 170 was also studied in normal mice by intravenous injection. Results are presented in Table III.

TABLE I. BIODISTRIBUTION IN NORMAL MICE

Organ	% Dose per gm, 4 h p.i.			
	$^{99}\text{Tc}^{\text{m}}$ -anti CEA		$^{99}\text{Tc}^{\text{m}}$ -Impurities	
	ME Reduction	IM Coupling		
		Monomer	Monomer & Dimer	
Blood	18.99 \pm 1.41	18.34 \pm 0.93	14.74 \pm 0.59	1.52 \pm 0.95
Liver	5.10 \pm 1.07	5.12 \pm 0.44	8.99 \pm 1.31	4.61 \pm 0.80
Kidneys	5.54 \pm 0.57	6.80 \pm 0.80	8.79 \pm 1.30	12.11 \pm 1.01
Intest.	2.69 \pm 0.33	2.51 \pm 0.36	2.68 \pm 0.28	4.89 \pm 1.17
Stomach	1.07 \pm 0.20	2.06 \pm 0.64	1.03 \pm 0.45	1.44 \pm 1.18
Urine *	8.95 \pm 1.26	12.53 \pm 2.02	8.14 \pm 2.30	70.21 \pm 13.90

Each value is the average of 5 animals.

* Data expressed as % dose per organ.

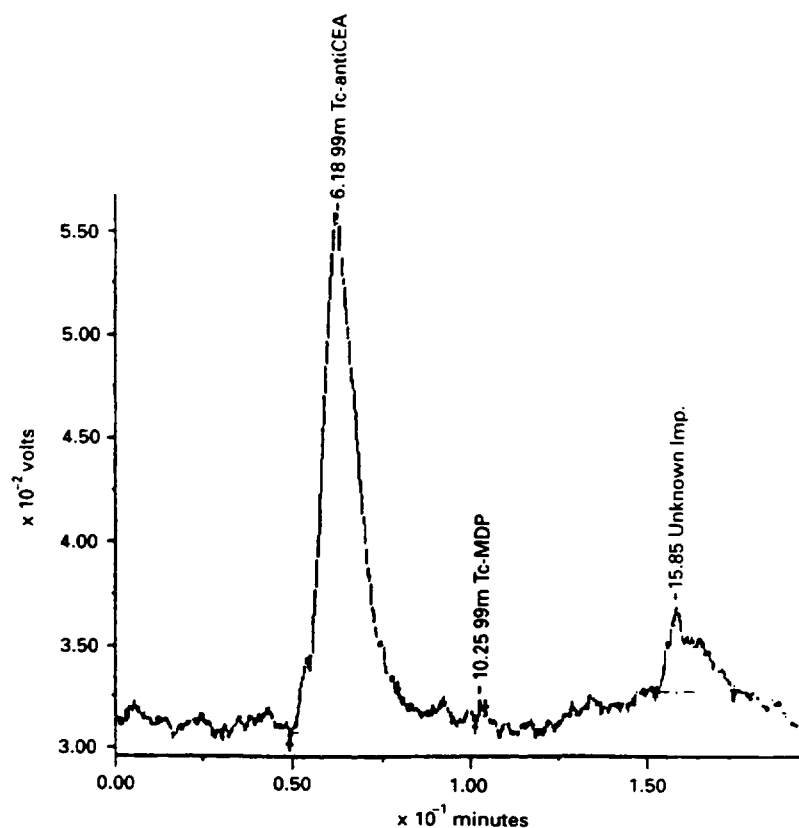


FIG. 5. HPLC Pattern of $^{99}\text{Tc}^m\text{-ior-CEA-1}$ showing by product formed by the 2-mercapto-ethanol reduction.

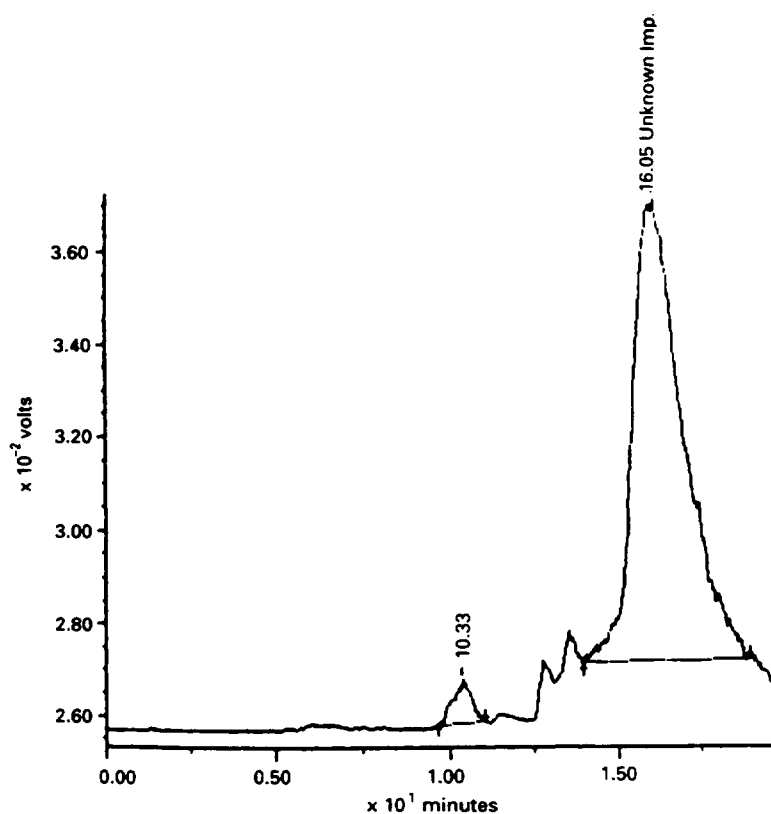


FIG. 6. Quantitative by-product preparation using excess of 2-mercapto-ethanol with MDP in the presence of reduced technetium (HPLC pattern).

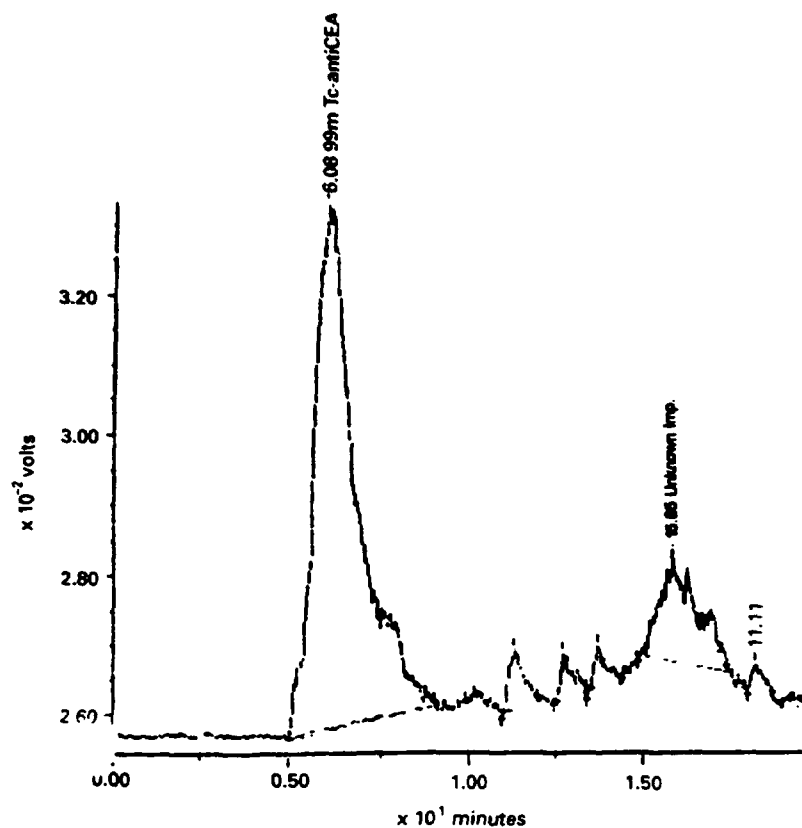


FIG. 7. Low molecular weight species formed by prolonged incubation of ior-CEA-1 with 2-mercaptoethanol as obtained by HPLC.

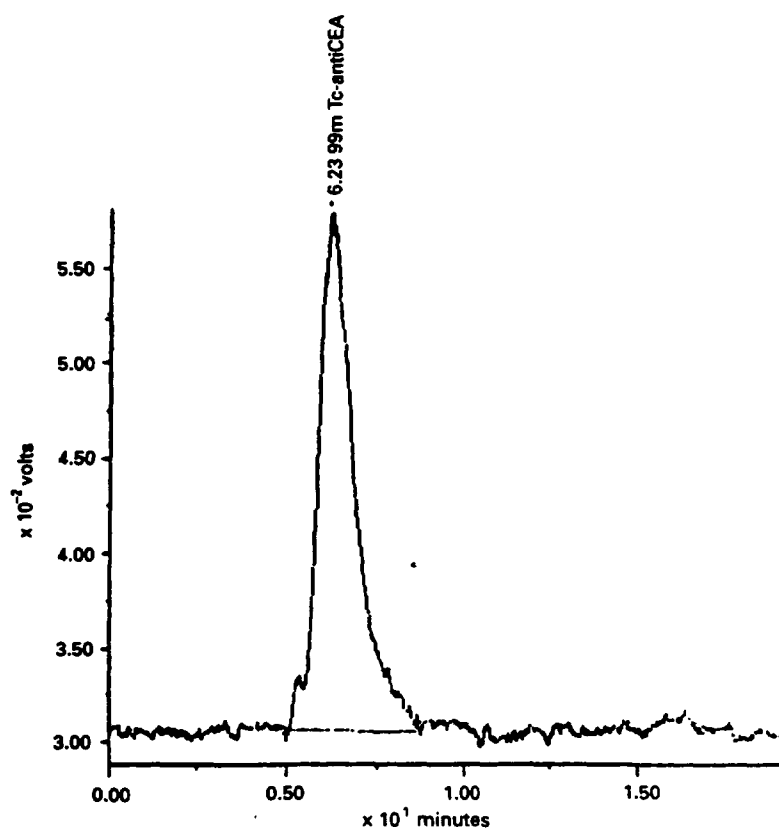


FIG. 8. Labelling of ior-CEA-1 by 2-mercapto-ethanol reduction using glucoheptonate as intermediate technetium chelator (HPLC pattern).

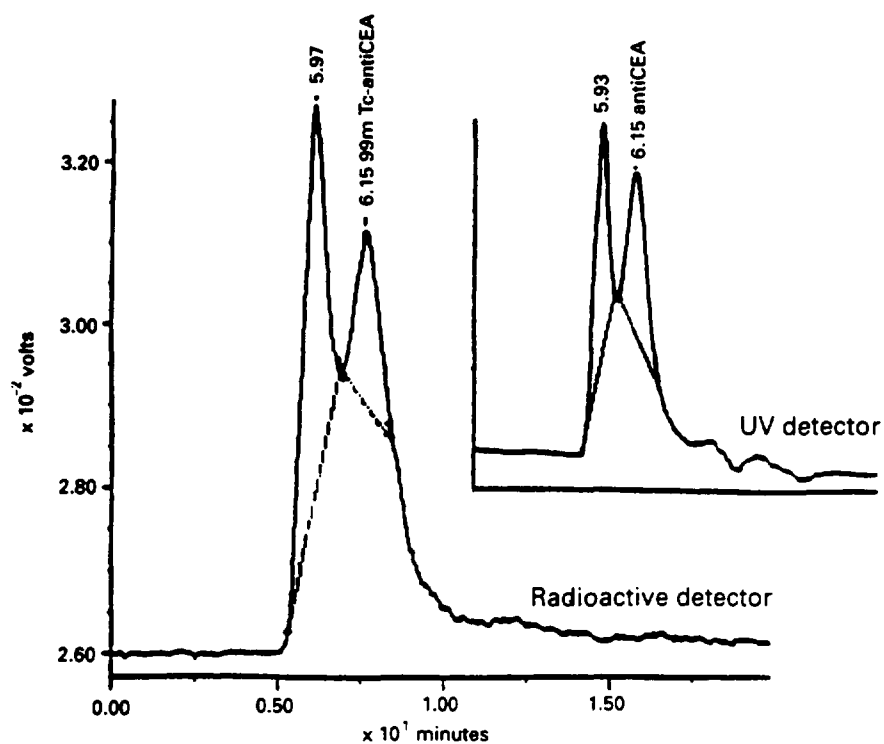


FIG. 9. HPLC Pattern of prolonged incubation of ior-CEA-1 with 2-iminothiolane.

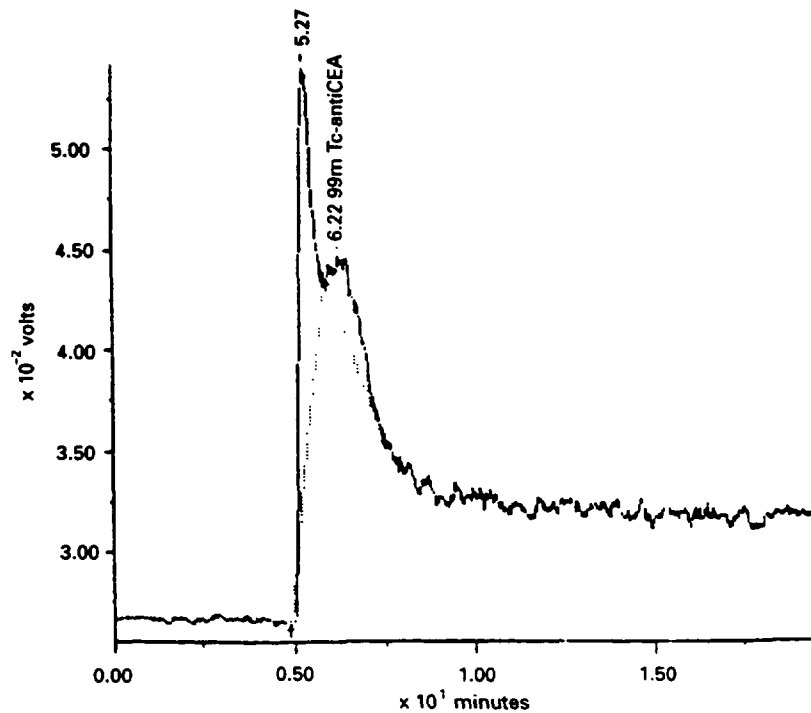


FIG. 10. HPLC Pattern of ior-CEA-1 labelled with $^{99}\text{Tc}^m$ using MDP and 2-iminothiolane. No impurities were observed.

TABLE II. $^{99}\text{Tc}^{\text{m}}$ -FLEGMON: BIODISTRIBUTION IN NORMAL MICE

Organ	% Dose per gm, 4 h p.i.	
	Before lyophilization	After lyophilization
Blood	10.45 \pm 1.79	11.28 \pm 1.02
Liver	3.93 \pm 0.49	3.11 \pm 0.48
Kidneys	19.34 \pm 2.08	15.96 \pm 1.31
Intestines	3.86 \pm 0.81	2.32 \pm 0.29
Stomach	1.95 \pm 0.76	1.53 \pm 0.32
Lungs	3.27 \pm 1.08	3.17 \pm 0.71
Urine *	26.21 \pm 3.90	16.59 \pm 0.64

Each value is the average of 5 animals.

* Data expressed as % dose per organ.

TABLE III. $^{99}\text{Tc}^{\text{m}}$ -MoAb 170: BIODISTRIBUTION IN NORMAL MICE

Organ	% Dose per gm, 4 h p.i.
Blood	24.41 \pm 2.55
Liver	12.14 \pm 2.04
Kidneys	15.14 \pm 2.72
Intestines	6.32 \pm 0.35
Stomach	3.81 \pm 1.03
Urine *	14.76 \pm 4.35

Each value is the average of 5 animals.

* Data expressed as % dose per organ.

2.4.3.4. Clinical evaluation of human immunoglobulin

For clinical use, each hIgG kit based on the 2-ME reduction system was reconstituted with 1.0–2.0 mL of pertechnetate. A dose of 555–1110 MBq was administered intravenously to each of twenty one patients, checked for endometriosis or tumours of the genitary system. Scanning was performed two to six h p.i. and twenty four h later. Planar, anterior images were obtained at the region of the pelvis. ROIs were estimated in the area of lesion as well as in nearby areas for comparison (background counting). Images were evaluated independently by two nuclear physicians. Findings were considered as positive when the ratio of the radioactivity in the lesion site to the adjacent normal tissues was greater than 1.5. Most representative results on the biodistribution of the radiopharmaceutical were obtained four h after the intravenous injection. Large vessels, heart, liver and spleen were delineated due to high blood pool activity. Kidneys were also visualized because of renal elimination of the radiopharmaceutical. Scintigraphic findings of patient imaging were compared to the results of other techniques and to the surgical findings as well. Thus, ten cases out of twenty one were considered as positive by scintigraphy, with a ratio of lesion to normal tissue greater than 1.5. Six of them indicated the existence of ovarian cancer, confirmed by surgery. Among the other four positive cases, post operative biopsy showed that one of them was a molar pregnancy, another was a fibroma, a third one a multicystic ovarian lesion and the last one was proved to be a cervix cancer with nearby metastases. The other eleven cases were considered as negative. In nine of them, ovarian cysts have been detected post-operatively, one was a vulvar neoplasia without metastasis, while for the last one a cystic endometrioma of the ovary was detected. Our studies indicated that this

radiopharmaceutical can be applied, not only for the scintigraphic localization of inflammation and/or infection foci, as referred to in literature, but also for the scintigraphic detection of several malignant and/or benign tumours of the female genital system. The exact trapping mechanism, by which the radiopharmaceutical is concentrated in tumours, remains to be clarified. We assume that the mechanism of tumour uptake is the same with that which permits the localization in inflammations, the inflammatory component being the determining factor in tumour uptake as well. The fact that ovarian neoplasms and some benign tumours, as fibromas, have inflammatory reactions, supports the hypothesis of the activation of particular cells, such as macrophages, lymphocytes, etc. These cells may be responsible for inflammatory and immunological responses as well. It is well established that macrophages are activated in inflammatory and tumour processes. Correlation of scintigraphic findings to the classification and determination of cell subpopulations, e.g. from tissue sections obtained from ovaries or uterus, will be the next topic of our studies.

2.4.4. Discussion

We comparatively evaluated two labelling methods for the preparation of $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1, $^{99}\text{Tc}^{\text{m}}$ -hIgG and $^{99}\text{Tc}^{\text{m}}$ -MoAb 170. The parameters which were taken into consideration in our investigation were: (1) the overall yield in pure labelled species, (2) the simplicity, the reproducibility and the cost of the method applied, (3) the immunoreactivity of the labelled derivatives and their ability to tag on human cancer cells and (4) the stability of the radioactive species obtained.

Our chromatographic results indicated that both 2-ME reduction and 2-IM coupling had an overall high labelling yield since practically no free pertechnetate was present in the final preparations. However, the comparative study by PAGE showed a partial modification of the protein structure leading to a limited formation of higher and lower molecular weight species in the case of 2-ME reduction, while in the case of 2-IM coupling a dimer was formed. The latter could also be observed in the HPLC profile and increased upto 20% under the experimental conditions applied.

Both methods resulted in immunoreactive labelled derivatives. Immunoreactivity was found slightly higher in the case of 2-IM coupling, while the ability to tag on human cancer cells was greater for the 2-ME reduction system. However, in the evaluation of the two labelling systems these differences can not be considered significant. Additionally, both yielded stable derivatives. On the other hand, by cysteine challenge, differences in stability were found for $^{99}\text{Tc}^{\text{m}}$ -labelled ior-CEA-1. The 2-ME reduction led to a more stable species in the presence of cysteine. The latter method always yields single radiolabelled species, regardless of the experimental conditions and of the age of the MoAb.

Our studies referring to the by-products and the radiochemical impurities formed during the labelling of MoAbs, indicated that, although both labelling systems had an overall high labelling yield and resulted to immunoreactive labelled derivatives, they created by-products, which if not kept in very low yield may influence the biodistribution of the radiolabelled antibodies. For the 2-ME reduction system a $^{99}\text{Tc}^{\text{m}}$ -labelled complex may be formed between the reducing agent and the intermediate exchange ligand, when MDP is used, while GH does not lead to a similar by-product. For the 2-IM coupling system, of greater importance is the formation of higher molecular weight species caused by polymerization of the protein. In both systems the intermediate exchange ligand and the reaction time play an important role on the formation of by-products and impurities. From the chelators studied, GH seems preferable since it does not create impurities.

2.4.5. Conclusions

Our detailed study on the labelling of MoAbs with $^{99}\text{Tc}^{\text{m}}$ indicates that the two comparatively studied methods resulted in high labelling yield. However, although the derivatives obtained by the two methods looked identical, ITLC, HPLC and PAGE revealed modification of the protein. The modification observed was greater in the case of 2-IM coupling and it was found to influence the biodistribution pattern as well.

We conclude that the method of labelling of MoAbs should be carefully selected and evaluated not only by ITLC but also by HPLC and PAGE, before starting clinical application. A strict labelling protocol should be developed and followed in order to minimize the effect on the protein.

Clinical evaluation of $^{99}\text{Tc}^{\text{m}}$ -hIgG showed that this radiopharmaceutical may be useful not only in inflammation and infection foci localization but also in benign and malignant tumour detection.

REFERENCES

- [1] VARVARIGOU, A.D., ARCHIMANDRITIS, S.C., BENDERMACHER, J.E., et al., Comparative Evaluation of Diverse Labelling Systems for the Preparation of $^{99}\text{Tc}^{\text{m}}$ -anti-CEA, Fifth European Symposium on Radiopharmacy and Radiopharmaceuticals, Cambridge, UK (1993).
- [2] VARVARIGOU, A.D., ARCHIMANDRITIS, S.C., SEKERI-PATARYAS, K., SOURLINGAS, T.G., SIVOLAPENKO, G., PASCHALI, E., et al., Radiochemical and Radioimmunological Data of $^{99}\text{Tc}^{\text{m}}$ -anti CEA Labelled by two Diverse Methods, Nucl. Med. Comm. 17 (1996) 80-88.
- [3] ARCHIMANDRITIS, S.C., SEKERI-PATARYAS, K., SIVOLAPENKO, G., VARVARIGOU, A.D., et al., Radiochemical Purity Studies on the Technetium-99m Labelling of Monoclonal Antibodies, The 12th International Hammersmith Conference on the Advances in the Applications of Monoclonal Antibodies in Clinical Oncology, Lesbos, Greece, 1995. Published in Tumour Targeting 2 (1996) 85-92.
- [4] VARVARIGOU, A.D., ARCHIMANDRITIS, S.C., STRIPSANELLI, A., ARKA, A., KOSTAMIS, P., et al., Technetium-99m Labelling of Human Immunoglobulin and Preliminary Clinical Evaluation in Tumour Patients, Sixth European Congress of Biopharmaceutics and Pharmacokinetics, Athens, Greece (1996).
- [5] ARKA, A., STRIPSANELLI, A., VARVARIGOU, A.D., ARCHIMANDRITIS, S.C., PAPADONIOU B., KOSTAMIS, P., et al., $^{99}\text{Tc}^{\text{m}}$ -Human Immunoglobulin (FLEGMON/DEMOSCAN) in the Detection of Tumours in the Female Genital System, submitted for publication.



Research contract No. HUN-6361/CF

Title of the Project : Direct Labelling of Monoclonal Antibodies with $^{99}\text{Tc}^{\text{m}}$.
Assessment of Labelling, Stability, Immunoreactivity and
Biodistribution

Chief Scientific Investigator : G.A. Janoki
Frederic Joliot Curie, National Research Institute for
Radiobiology and Radiohygiene, Budapest

2.5.1. Introduction

Reduction of disulfide bonds to sulfhydryl groups for direct radiolabelling of monoclonal antibodies for immunoscintigraphic application continues to be of significant interest. Reducing agents that have been used are the following: stannous ion [1, 2], 2-mercaptoethanol [3, 4], dithiothreitol, dithioerythritol [5], and ascorbic acid. The radiolabelling of the reduced and purified antibody is performed via Sn^{2+} reduction of pertechnetate in the presence of an excess of a low-affinity chelating ligand.

In a recent work the 2-mercaptoethanol (2-ME) reduction based method was studied by using different analytical and biological techniques. Human IgG (Sandoglobulin), anti-CEA MoAb (ior-1), and anti-granulocyte MoAb (MAK 47), were reduced with 2-ME at two different molar ratios. To determine the amount of contaminating mercaptoethanol which may have survived the gel-filtration step ^{14}C -ME was used. The number of the free endogenous sulfhydryl groups generated by reduction was determined by Ellman's reagent; absorbance was measured at 412 nm.

The integrity of MoAb samples after reduction was determined by non-reduced PAGE and size exclusion HPLC. Radiolabelling of reduced MoAbs with $^{99}\text{Tc}^{\text{m}}$ -pertechnetate was done by the use of stannous (II)-PYP. The stability of the $^{99}\text{Tc}^{\text{m}}$ -IgG and $^{99}\text{Tc}^{\text{m}}$ anti-CEA-MoAb were determined by cysteine challenge assay [6]. The radiochemical purity and stability were followed by radio-HPLC, TLC/ITLC or paper chromatography. The standard biodistribution of $^{99}\text{Tc}^{\text{m}}$ labelled IgG and $^{99}\text{Tc}^{\text{m}}$ anti-CEA MoAb in normal mice and the tumour localization of $^{99}\text{Tc}^{\text{m}}$ -anti-CEA MoAb in mice bearing adenocarcinoma xenografts were studied.

Based on our experimental results in the second part of the programme, freeze-dried formulations with additives were developed and tested. These kits developed were sterile and pyrogen free in nitrogen atmosphere and ready for one-step labelling with technetium-99m [7].

Within the quality assurance procedure of the 3 freeze dried kits the labelling efficiency, stability, pH, sterility, apyrogenicity, vial yield, syringe retention, filterable activity, free SH determination and animal distribution were studied again.

After receiving permission from local ethics committee pilot human studies were initiated. Study protocols were also approved [8–10].

2.5.2. Materials and methods

2.5.2.1. Antibody reduction and purification

During experiments anti-CEA MoAb (ior-1), human IgG (Sandoglobulin) and antigranulocyte MoAb (MAK 47) were reduced with 2-mercaptoethanol (2-ME) at molar ratios of 1000:1, 3000:1. After 30 minutes of treatment at room temperature the reaction mixtures were purified by column

chromatography. In separate experiments to measure non-specific 2-ME binding to protein 462 kBq ^{14}C -mercaptoethanol (Sigma Co.) was added to the reaction mixture. The mixture of treated MoAb's were applied to the top of the gel filtration column (Sephadex G-50, 30×2 cm). Column was eluted by cold N_2 purged PBS. One mL fractions were collected by ISCO fraction collector and O.D. of the collected fractions was measured at 280 nm by UV/VIS spectrophotometer. The 0.5–1.0 mL aliquots of the pooled MoAb samples were freeze-dried at -70°C or lyophilized and stored until use. Before freeze-drying Sn -pyrophosphate and glucose were added to reduced MoAb solution.

2.5.2.2. Radiolabelling

In a typical labelling reaction 1.0 mg of MoAb and 1–3 mL of $^{99}\text{Tc}^{\text{m}}$ as sodium pertechnetate (100–1000 MBq) obtained from commercial $^{99}\text{Tc}^{\text{m}}$ generator were mixed and incubated for 10 minutes at room temperature.

2.5.2.3. Chromatography

The starting MoAbs, 2-ME treated, gel filtered and radiolabelled MoAbs were analysed for molecular integrity by size exclusion high pressure liquid chromatography (HPLC). Chromatographic separation was performed on a 300×7.5 mm BIO-SIL TSK-250 column for molecular weight sizing. Samples (20 μL) were injected and eluted using 0.02 M Na_2SO_4 phosphate buffer (pH6.8) at a flow rate of 1.0 mL/min. Protein absorbance was monitored at 280 nm, 0.5–1.0 mL fractions were collected and radioactivity was measured in a gamma counter. TLC and ITLC were used to determine labelling efficiency and impurities.

2.5.2.4. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was done with disc electrophoresis apparatus. 40 μL volume of the radiolabelled compound was applied to 7.5% non reduced gel using 0.2 M phosphate buffer (pH6.0) as an electrode buffer. Electrophoresis was carried out at 4 mA per column. Each sample was tested on two parallel gels. The gels were stained, destained, and sliced into 2 mm sections and counted in order to determine the radioactivity distribution.

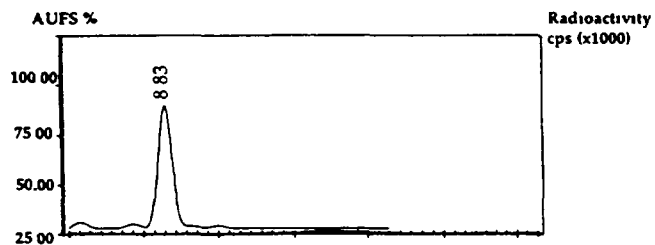
2.5.2.5. Animal biodistribution studies

50 μL of $^{99}\text{Tc}^{\text{m}}$ labelled MoAbs (30 μg , 11 MBq) were injected i.v. into the tail vein of CBA mice. The standard biodistribution was determined 1, 4 and 6 h after injection. The tumour localization of $^{99}\text{Tc}^{\text{m}}$ -MAK-47 was studied in immunosuppressed mice bearing CEA positive human colorectal adenocarcinoma xenografts (HT-29).

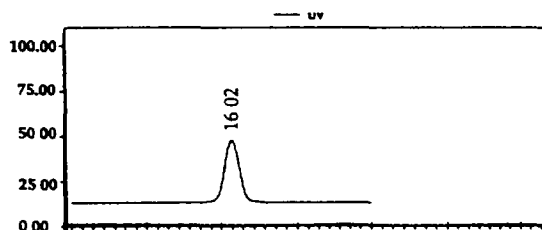
2.5.2.6. Determination of sulfhydryl concentration

The quantitation of sulfhydryl concentration was accomplished with Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid; DTNB) in which absorbance was measured at 412 nm. Ellman's reagent has been used extensively to measure sulfhydryl concentrations in solution of reduced antibodies. For analysis by Ellman's reagent the protein was diluted in 0.2 M TRIS-buffer, pH8.2 and was combined with an equal volume of 0.01 M solution of the reagent, freshly prepared by dissolving 99 mg DTNB in 25 mL of absolute methanol.

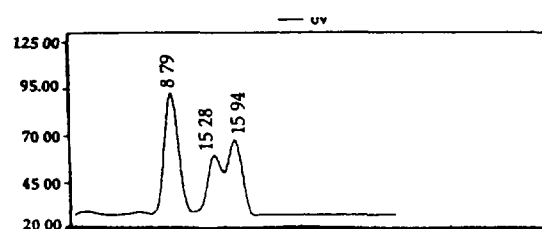
After 15 min at room temperature, the absorbance was measured using a recording UV spectrophotometer (HITACHI-220A UV-VIS spectrophotometer). The sulfhydryl concentrations of reduced antibodies were calculated based on calibration curve of glutathione.



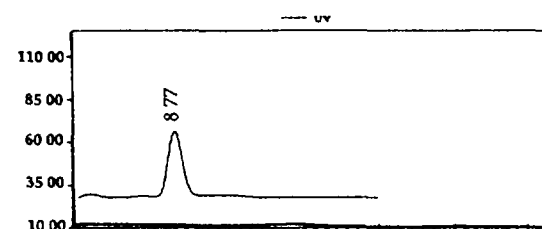
anti CEA



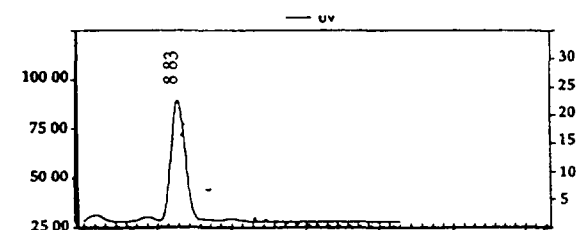
2-Mercaptoethanol(2-MEOH)



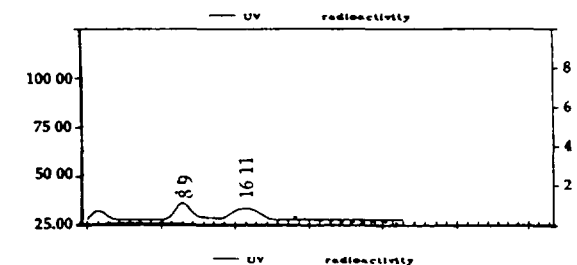
anti CEA + 2-MEOH



anti CEA after gel filtration



^{99m}Tc -anti CEA



^{99m}Tc -anti CEA + 2-MEOH

FIG 1 HPLC Analysis of starting anti CEA, 2-MEOH treated anti CEA, gel filtrated and radiolabelled anti CEA

2.5.2.7. Cysteine challenge assay

Cysteine challenge assay involved incubating aliquots of the $^{99}\text{Tc}^{\text{m}}$ -MAK-47 in 0.64, 1.6, 3.2, 6.4, 8.5, 12.8, 16, 32, 64 mM solution of cysteine-HCl in saline at room temperature. To 10 μL of each cysteine solution 90 μL of radiolabelled MoAb solution was added. The loss of activity from protein molecule was determined by paper chromatography (Whatman No. 1, 0.1 M PBS, pH7). In this system, labelled antibody remains at origin, while labelled cysteine migrates with R_f value of 0.78–1.0.

2.5.2.8. In vitro immunoreactivity determination of $^{99}\text{Tc}^{\text{m}}$ -MoAb with human granulocytes

Human granulocytes varying in concentration from 10×10^6 to 30×10^6 in 1 mL 0.9% sodium chloride were placed in test tube. 1–10 ng of antibody (1–5 μL) was added. Tubes were incubated at 28–30°C for 2 h with shaking (G-24 Incubator Shaker, New Brunswick Scientific Co., Inc.). After the samples were centrifuged, activities in both the cell pellets and supernatants were counted and the percentage of binding was calculated.

2.5.3. Results

2.5.3.1. Chemical-radiochemical analysis

The labelling efficiency determined by TLC showed in all cases that the levels of free $^{99}\text{Tc}^{\text{m}}$ pertechnetate in freshly reduced MoAbs were always less than 5%. The amount of radiocolloids (reduced/hydrolyzed $^{99}\text{Tc}^{\text{m}}$) in the same samples ranged between 0.5–5%. The in vitro serum stability study (24 h in fresh human serum at 37°C) showed that the loss of label from the antibodies varied between 1.5 and 7.2%. The HPLC studies are exemplified with the analysis of starting anti-CEA, 2-ME treated anti-CEA, gel filtered anti-CEA and radiolabelled anti-CEA. Results are shown in Fig. 1. The polyacrylamide gel electrophoresis showed (Figs 2 and 3) that at non reduced condition one major activity peak is seen with significant tailing. The results of cysteine challenge assay illustrate that above 1 mM cysteine concentration significant loss of label occurs (Fig. 4). The number of endogenous sulfhydryl group generated at two different molar ratios of MoAb /ME are shown in Table I. Standard curves supporting the SH group determination are given in Fig. 5.

2.5.3.2. Biological studies

The biodistribution of $^{99}\text{Tc}^{\text{m}}$ -anti-CEA-MoAb, $^{99}\text{Tc}^{\text{m}}$ -hIgG and $^{99}\text{Tc}^{\text{m}}$ -MAK-47 in normal mice at 1, 4, and 6 h after intravenous injection are shown in Tables II, III, and IV. Blood pool and well perfused organs showed the highest activity content.

The $^{99}\text{Tc}^{\text{m}}$ -anti-CEA MoAb showed the fastest kinetics. Tumour localization of $^{99}\text{Tc}^{\text{m}}$ -anti-CEA is shown in Fig. 6. The result of in vitro immunoreactivity of $^{99}\text{Tc}^{\text{m}}$ -antigranulocyte MoAb determined by using human granulocytes are presented in Table V.

TABLE I. THE NUMBER OF ENDOGENOUS SULFHYDRYL GROUPS GENERATED BY 2-ME REDUCTION (MOL. SH/MOL. MoAb)

	Before reduction	After reduction at different ratios	
		1000×	3000×
IgG	0.12	2.2	33.5
anti-CEA	0.15	3.4	7.6
Mab-47	0.15	6.3	7.6

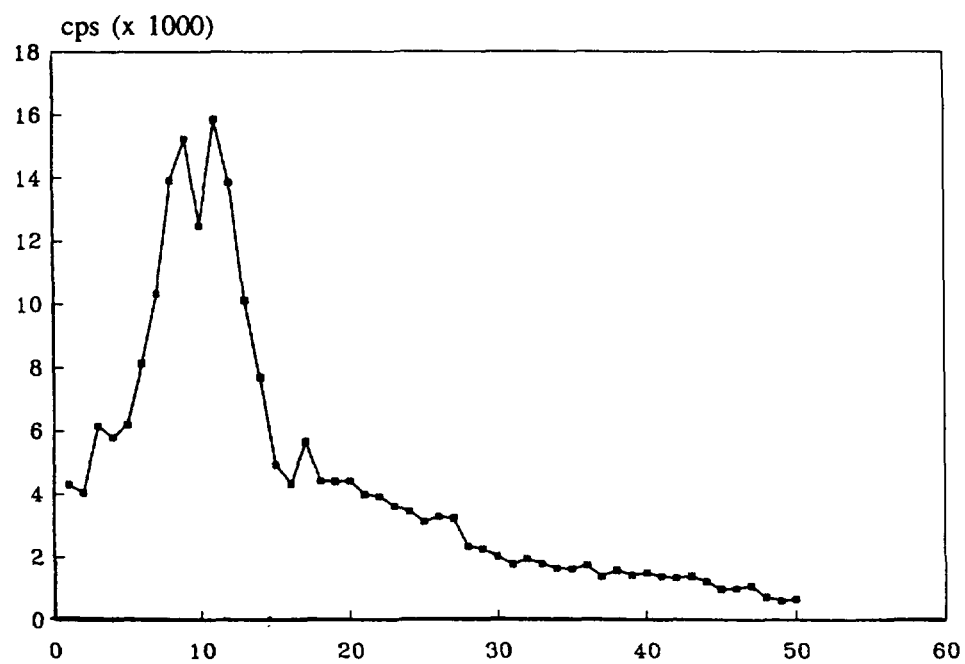


FIG. 2. Radioactivity distribution of $^{99}\text{Tc}^m$ anti-CEA in 7.5% polyacrylamide gel (non-reduced).

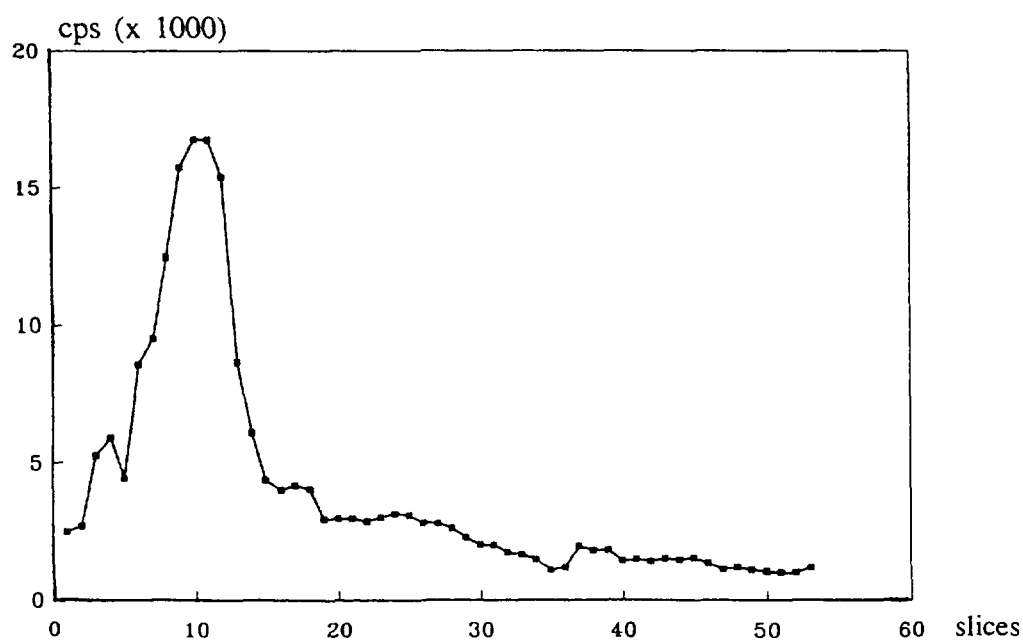


FIG. 3. Radioactivity distribution of $^{99}\text{Tc}^m$ -human IgG in 7.5% polyacrylamide gel (non-reduced).

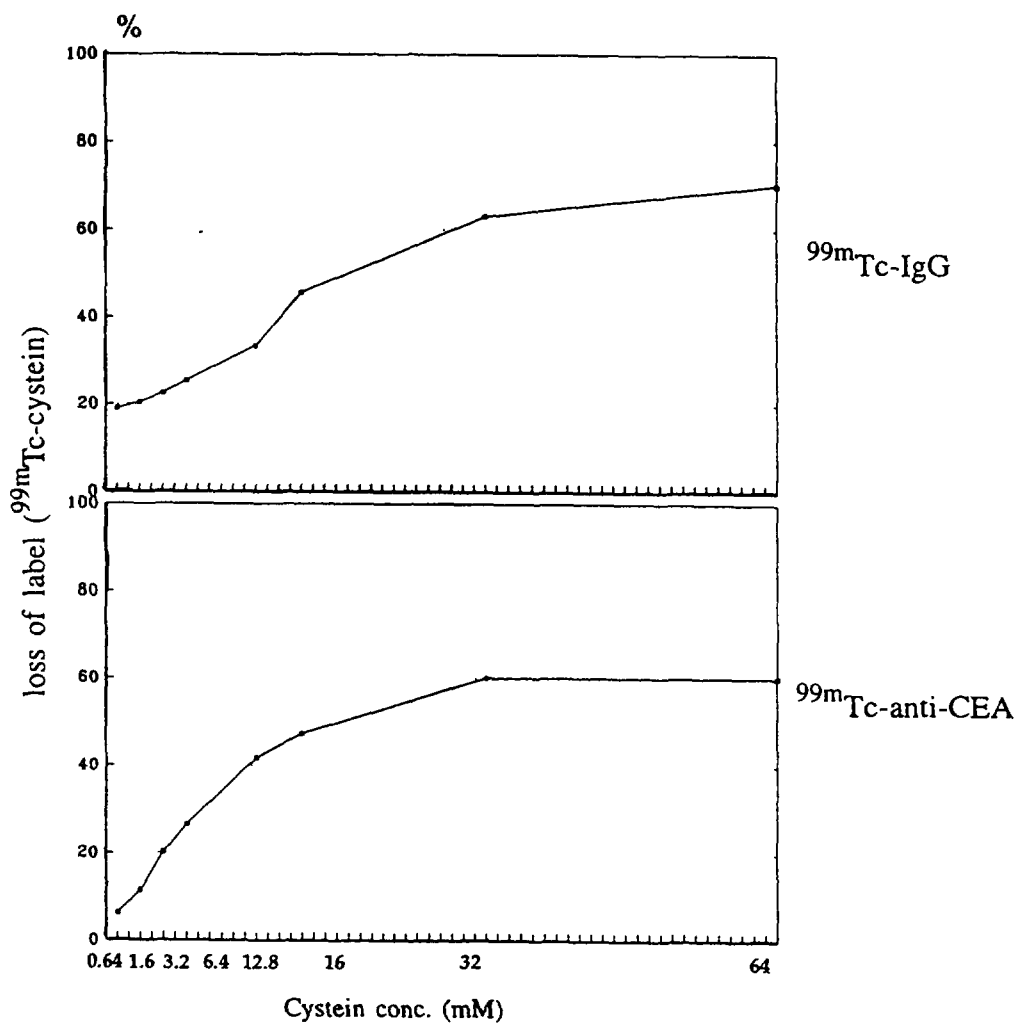


FIG. 4. Cystein challenge assay of ^{99m}Tc -IgG and ^{99m}Tc -anti CEA.

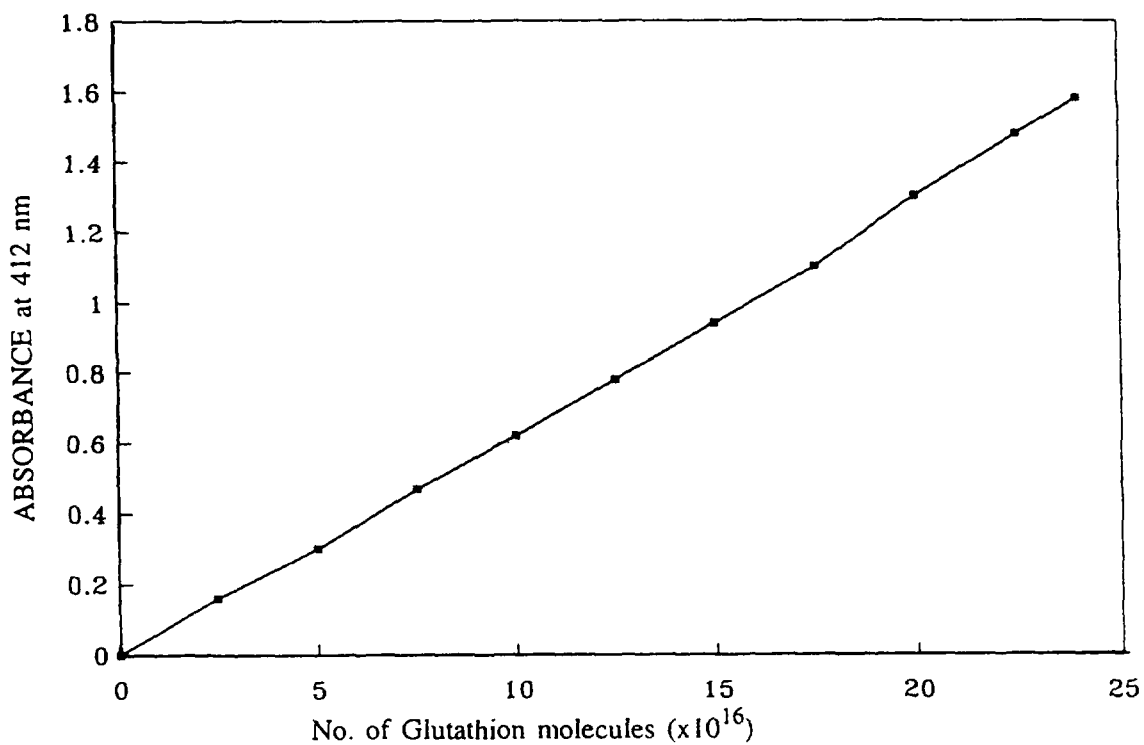
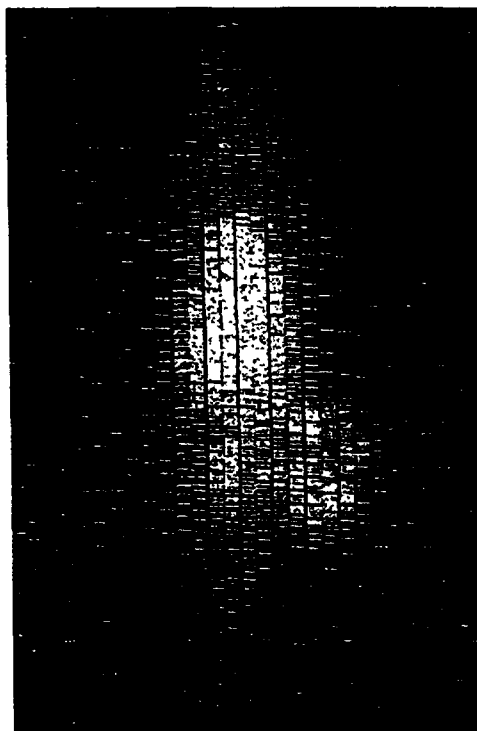
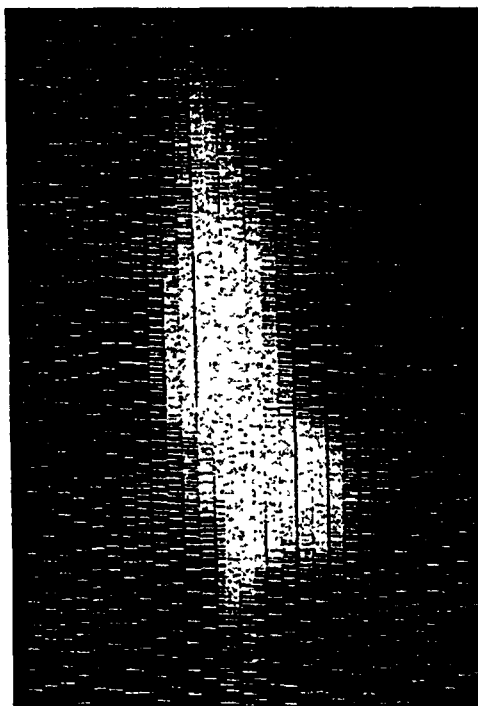


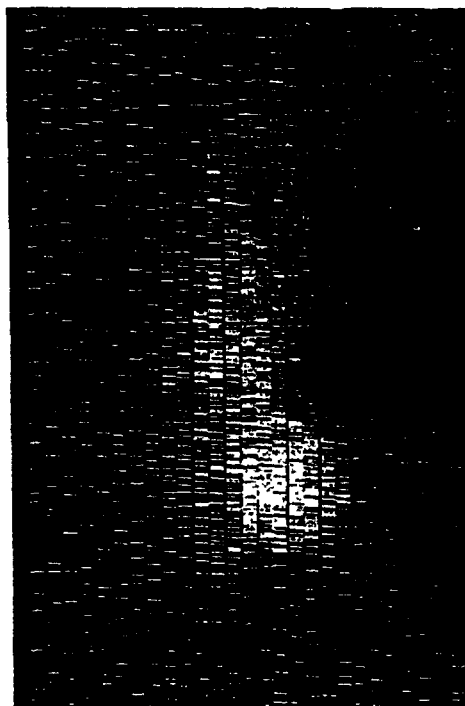
FIG. 5. Standard curve showing the relationship of Glutathion concentration and absorbance determined with Ellman's reagent.



1 h



6 h



24 h

FIG. 6. Distribution of $^{99}\text{Tc}^m$ -anti CEA (IOR1) in immunosuppressed mice bearing CEA positive human colorectal adeno carcinoma xenografts.

TABLE II. BIODISTRIBUTION OF $^{99}\text{Tc}^{\text{m}}$ -ANTI-CEA MoAb(ior-1) IN NORMAL MICE AT 1, 4 AND 6 h POST I.V. INJECTION (% ID/gm TISSUE \pm SD)

Organs	Duration		
	2 h	4 h	6 h
Blood	9.56 \pm 1.03	7.70 \pm 0.37	5.36 \pm 1.27
Muscle	0.50 \pm 0.21	0.34 \pm 0.01	0.29 \pm 0.01
Femur	1.29 \pm 0.16	0.96 \pm 0.18	0.79 \pm 0.16
Heart	2.98 \pm 0.45	1.56 \pm 0.33	1.11 \pm 0.12
Lung	3.89 \pm 1.04	3.98 \pm 0.38	3.47 \pm 1.49
Liver	10.58 \pm 1.96	8.37 \pm 1.85	4.52 \pm 1.34
Spleen	2.66 \pm 0.38	2.46 \pm 0.17	1.85 \pm 0.57
Kidneys	16.32 \pm 3.44	14.65 \pm 3.06	11.69 \pm 3.86
Stomach	1.92 \pm 0.89	1.25 \pm 0.56	0.71 \pm 0.33
Small bowel	5.63 \pm 0.74	2.91 \pm 0.79	1.59 \pm 0.51
Large bowel	2.56 \pm 1.22	13.60 \pm 5.41	7.20 \pm 2.29

TABLE III. BIODISTRIBUTION OF $^{99}\text{Tc}^{\text{m}}$ -hIgG IN NORMAL MICE AT 1,4 AND 6 h POST I.V. INJECTION (% ID/g tissue \pm SD)

Organs	Duration		
	2 h	4 h	6 h
Blood	22.80 \pm 4.45	16.40 \pm 2.86	11.70 \pm 2.28
Muscle	0.95 \pm 0.52	0.90 \pm 0.85	0.59 \pm 0.23
Femur	2.09 \pm 0.34	1.89 \pm 0.68	1.44 \pm 0.53
Heart	7.76 \pm 1.89	4.12 \pm 1.23	3.28 \pm 0.59
Lung	10.22 \pm 2.67	9.29 \pm 2.09	7.32 \pm 1.30
Liver	6.45 \pm 0.89	5.37 \pm 0.84	4.30 \pm 0.56
Spleen	4.95 \pm 1.44	3.81 \pm 1.18	3.91 \pm 0.55
Kidneys	16.70 \pm 3.31	6.82 \pm 2.88	14.54 \pm 4.40
Stomach	2.28 \pm 0.24	1.90 \pm 0.40	1.74 \pm 0.48
Small bowel	3.56 \pm 0.56	2.60 \pm 0.89	1.70 \pm 0.42
Large bowel	2.08 \pm 0.63	5.10 \pm 2.29	7.20 \pm 1.85

TABLE IV. BIODISTRIBUTION OF $^{99}\text{Tc}^{\text{m}}$ -MAK-47 IN NORMAL MICE

Organs	Duration		
	2 h	4 h	6 h
Blood	37.91 \pm 2.28	22.56 \pm 3.05	20.62 \pm 1.98
Heart	10.08 \pm 1.99	8.89 \pm 2.23	6.02 \pm 0.01
Lung	21.74 \pm 3.65	12.36 \pm 2.83	10.99 \pm 1.39
Liver	11.45 \pm 0.01	7.97 \pm 1.72	7.75 \pm 0.01
Kidney	13.93 \pm 1.12	15.56 \pm 2.12	17.65 \pm 3.41
Bone + bone marrow	3.98 \pm 0.36	3.21 \pm 0.29	2.83 \pm 0.11
Spleen	8.82 \pm 1.35	6.25 \pm 1.85	5.54 \pm 0.63
Muscle	1.65 \pm 0.45	1.36 \pm 0.23	2.83 \pm 0.25
Stomach	2.67 \pm 0.70	5.52 \pm 1.86	6.28 \pm 0.32
Small bowel	5.23 \pm 0.73	4.22 \pm 0.52	4.61 \pm 1.10
Large bowel	3.54 \pm 0.78	7.23 \pm 2.60	12.08 \pm 3.95

(n = 5 animals)

TABLE V. IMMUNOREACTIVITY OF $^{99}\text{Tc}^{\text{m}}$ LABELLED MAK-47 WITH HUMAN GRANULOCYTES

Amount of MAb 47	10×10^6	Cell No. 20×10^6	30×10^6
	in vitro immunoreactivity		
1 ng	67.94%	70.67%	72.55%
2 ng	66.33%	71.19%	71.68%
3 ng	66.24%	70.74%	–
$\bar{X} \pm \text{SD}: 69.66 \pm 2.47$			

TABLE VI. QUALITY PARAMETERS OF THE FREEZE DRIED FORMULATIONS

Test	hIgG	anti-CEA	MAK-47
Labelling efficiency (in %, TLC), 10 min	98.2 ± 0.9	97.8 ± 1.2	98.5 ± 1.1
4 h	97.8 ± 1.02	96.9 ± 1.1	95.8 ± 0.8
pH	7.2 ± 7.4	7.2 ± 7.4	7.2 ± 7.4
Colour	Clear colourless	Clear colourless	Clear colourless
Sterility apyrogenicity: (according to Hung Pharmacopoeia VII)	sterile non pyrogenic	sterile non pyrogenic	sterile, non pyrogenic
Vial yield	96.5%	97.3%	98.1%
Syringe retention (30 min)	5.0%	5.5%	6.1%
Filterable activity in $\%0.22 \mu\text{m}$	97.0	98.1	97.2
$0.1 \mu\text{m}$	87.5	88.0	85.4
Number of free SH	1.2 SH/molecule	3.4 SH/molecule	6.3 SH/ molecule
Group generated (% of total, 36 SH)	3.4%	9.4%	17.5%

2.5.3.3. *Quality control data of the freeze dried formulations*

The quality parameters of the three freeze-dried kit formulations are presented in Table VI. Results showed in all cases high labelling efficiency and stability. The additional practical tests (e.g. vial yield, syringe retention, etc.) showed very similar results in all cases. Vial yield ranged 96–98% and the syringe retention values were far below 10%. The amount of filterable activity was between 97–98% when 0.22 μm filter was used. In the case of 0.1 μm filter values dropped about 10%. The number of endogenous SH groups generated during reduction ranged between 1.2–6.3 SH/antibody molecule.

2.5.4. **Conclusions**

In any antibody labelling procedure, the aim is to produce a label with high stability using a procedure with minimal effect upon the biological activity of the antibody. When using short half-life radionuclides such as $^{99}\text{Tc}^{\text{m}}$, it is also desirable that the labelling procedure must be rapid and simple so it can be used in clinical centres with limited radiopharmaceutical expertise. Our attempt to radiolabel anti-CEA (ior-1), anti-MAK-47 and hIgG with technetium-99m using the developed method exhibited very encouraging results. From the results presented are well seen that the kits of three antibody showed:

- high labelling efficiency and stability.
- biodistribution and in vitro immunoreactivity study showed well preserved properties.
- the one step labelling kit is simple and allowed rapid labelling.
- the preliminary clinical evaluation of the three products showed promising results.

The method studied and developed during CRP can be used to label a number of monoclonal antibodies and hence hold a significant clinical promise.

REFERENCES

- [1] RHODES, B.A., Direct Labelling of Proteins with $^{99}\text{Tc}^{\text{m}}$, Nucl. Med. Biol. **18** (1991) 667–676.
- [2] PAK, K.Y., NEDELMAN, M.A., TAM, S.H., et al., Labelling and Stability of Radiolabelled Antibody Fragments by a Direct $^{99}\text{Tc}^{\text{m}}$ -Labelling Method, Nucl. Med. Biol. **19** (1992) 669–667.
- [3] SCHWARZ, A., STEINSTRASSER, A., A Novel Approach to $^{99}\text{Tc}^{\text{m}}$ Labelled Monoclonal Antibodies (Abstract), J. Nucl. Med. **28** (1987) 271.
- [4] MATHER, S.J., ELLISON, D., Reduction-Mediated Technetium-99m Labelling of Monoclonal Antibodies, J. Nucl. Med. **31** (1990) 692–697.
- [5] THAKUR, M.L., DeFULVIO, J., et al., Technetium-99m-Labelled Monoclonal Antibodies: Evaluation of Reducing Agents, Nucl. Med. Biol. **18** (1991) 227–233.
- [6] HNATOWICH, D.J., VIRZI, F., et al., Investigations of Ascorbate for Direct Labelling of Antibodies with Technetium-99m, J. Nucl. Med. **35** (1994) 127–134.
- [7] JANOKI, G.A., KOROSI, L., et al., Direct Labelling of Anti-CEA MoAb with $^{99}\text{Tc}^{\text{m}}$: Evaluation of the Degree of Fragmentation and in Vivo Stability, Eur. J. Nucl. Med. **21** (1994) 5208.
- [8] BAUM, R.P., HERTEL, A., et.al., $^{99}\text{Tc}^{\text{m}}$ -Labelled anti-CEA Monoclonal Antibody for Tumour Immunoscintigraphy: First Clinical Results, Nucl. Med. Comm. **10** (1989) 345–352.
- [9] STEINSTRASSER, A., OBERHAUSEN, E., Anti-CEA Labelling Kit BW 431/26 Results of the European Multicenter Trial, Nucl. Med. **34** (1995) 232–242.
- [10] BLAUENSTEIN, P., LOCHER, J.T., et al., Experience with the Iodine-123 and Technetium-99m Labelled Anti-Granulocyte Antibody MoAb 47: A Comparison of Labelling Methods, Eur. J. Nucl. Med. **22** (1995) 690–698.



Research contract No. IND-6512/CF

Title of the Project : Labelling of Monoclonal Antibodies with $^{99}\text{Tc}^{\text{m}}$, their Quality Control and Evaluation for Scintigraphy

Chief Scientific Investigator : U.P.S Chauhan
Institute of Nuclear Medicine and Allied Sciences, Delhi

2.6.1. Introduction

Several clinical studies have demonstrated that both the ^{111}In and $^{99}\text{Tc}^{\text{m}}$ labelled specific monoclonal antibodies and non specific human immunoglobulins can delineate variety of cancerous [1,2], infectious and inflammatory lesions [3–6]. However, due to practical advantages of $^{99}\text{Tc}^{\text{m}}$ over ^{111}In , $^{99}\text{Tc}^{\text{m}}$ labelled antibodies have exhibited distinct advantages for clinical investigations. Furthermore, unlike ^{111}In , universal availability of $^{99}\text{Tc}^{\text{m}}$ in the generator form makes immunoscintigraphy feasible even in developing countries which was not practicable with the use of ^{111}In for want of its availability. Although the most stable and specific method for labelling antibodies with $^{99}\text{Tc}^{\text{m}}$ is performed by the bifunctional approach [7], there has recently been an increased interest in direct labelling methods as a result of significant improvement in the stability of the radiolabel and its potential application in the form of kits which can produce $^{99}\text{Tc}^{\text{m}}$ -antibody complex at the time of use [8]. Direct methods based on the use of 2-mercaptoethanol [9, 10], ascorbic acid [11], borohydride [12] and boric acid buffer [13] appeared most promising. In addition, the method based on the use of iminothiolane to provide $^{99}\text{Tc}^{\text{m}}$ attachment site on the lysine has also been claimed a good practical method [14].

We have evaluated these methods for radiolabelling of antibodies with $^{99}\text{Tc}^{\text{m}}$ using human immunoglobulin (hIgG) and an anti CEA mouse monoclonal antibody (ior-CEA-1) by studying labelling efficiency, in vitro serum stability, relative radionuclide binding strength, immunoreactivity, blood kinetics, biodistribution, specific target scintigraphy and also for development of lyophilized kits. The study has led to the improvement of the method based on the use of 2-ME and development of a novel method which uses ascorbic acid (ASC) to label the antibodies with $^{99}\text{Tc}^{\text{m}}$ and the production of lyophilized kits which give quality $^{99}\text{Tc}^{\text{m}}$ -hIgG and $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 instantly when mixed with $^{99}\text{Tc}^{\text{m}}$ -pertechnetate. Both the products prepared from the kits have been found suitable for immunoscintigraphy.

2.6.2. Materials and methods

2.6.2.1. Preparation of $^{99}\text{Tc}^{\text{m}}$ -labelled hIgG and ior-CEA-1

Using 2-ME as protein reductant

The method was essentially as reported by us [10]. In brief, 10 mg of antibody and 4.2 μL 2-ME in a molar ratio of 1:1000 were dissolved in 100 μL 0.1 M phosphate buffered saline (PBS), pH 7.4 and incubated for 30 min at room temperature. The excess 2-ME was removed by passing through a Sephadex G-50 column using 0.1 M PBS as eluent. Protein fractions were pooled and the content was measured by UV spectrophotometer at 280 nm. To the above solution, 0.5 mL aliquot from indigenously developed MDP kit was added to give MDP and stannous chloride concentrations of 50 μg and 5 μg respectively per mg protein. ASC was mixed in the above solution to give 5 mM final concentration. One mg protein aliquots were frozen (-20°C) or lyophilized and stored at refrigerated temperature. At the time of use, the kit was brought to room temperature and mixed with requisite amount of $^{99}\text{Tc}^{\text{m}}$ -pertechnetate (200–2000 MBq) in 1–2 mL volume.

Using ascorbic acid as protein reductant

The details of the methodology have been reported by us recently [11]. An amount of 10 mg hIgG and 51 mg ascorbic acid in the molar ratio of 1:5000 were dissolved in 5 mL of 0.1 M PBS, pH 7.4 and incubated for 16–18 h at 4–7°C. Thereafter, the contents were treated with 0.5 mL solution containing 2.5 mg glucoheptonate and 50 µg stannous chloride from an indigenously developed GHA kit. The contents were then divided into 0.5 mL aliquots and either frozen at –20°C or lyophilized and stored at refrigerated temperature until used. At the time of use an aliquot of 1–2 mL of $^{99}\text{Tc}^{\text{m}}$ -pertechnetate (200–2000 MBq) was mixed after bringing the kit to room temperature.

Other methods

Methodologies based on the use of borohydride [12], borate buffer [13] and iminothiolane [14] were also standardized and used for radiolabelling of hIgG and ior-CEA-1 with $^{99}\text{Tc}^{\text{m}}$. The details of the protocols were followed as described in the respective references.

2.6.2.2. Labelling efficiency and stability of the labelled product

Labelling efficiency

It was measured by ascending instant thin layer chromatography (ITLC) using Gelman SG strips as the stationary phase. Free pertechnetate and GHA/MDP were moved away using 0.9% saline as the mobile phase, whereas using a EtOH: ammonium hydroxide: water (2:1:5, v/v) mixture as mobile phase on human serum albumin (HSA 0.1%) impregnated ITLC, the labelled hIgG and free pertechnetate moved together with the solvent front [15]. $^{99}\text{Tc}^{\text{m}}$ -antibody was calculated by subtracting the free pertechnetate activity from the total radioactivity of antibody and pertechnetate and expressed as per cent of total radioactivity used in the reaction. Reduced/hydrolyzed Tc products were calculated by measuring radioactivity remaining at the point of application on the second chromatogram.

Stability of $^{99}\text{Tc}^{\text{m}}$ -antibody

An aliquot of 0.2 mL of labelled product was mixed with 1.8 mL human serum and incubated at 37°C up to 24 h. Small aliquots were taken out during incubation at different time intervals and subjected to ITLC using normal saline as the mobile phase. Any increase in free pertechnetate was considered as the degree of the degraded products.

Transchelation using DTPA and cysteine

Aliquots of 0.5 mL of $^{99}\text{Tc}^{\text{m}}$ antibodies in 1.5 M protein concentration were treated with equal volumes of DTPA or cysteine solutions at concentrations of 25, 50 and 100 mM before mixing them with 1 mL $^{99}\text{Tc}^{\text{m}}$ -pertechnetate (100 MBq) and the contents were incubated at room temperature for 1 h. The effect of DTPA and cysteine on the labelling efficiency of hIgG/ior-CEA-1 was measured.

Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of native and reduced antibodies was carried out with 7% gel in non-reducing conditions at 10 mA for 2 h at room temperature.

High performance liquid chromatography (HPLC)

HPLC of the antibodies before and after radiolabelling was carried out on a size exclusion column using 50 mM PBS as mobile phase at the flow rate of 0.75 mL/min. The protein contents were monitored at 280 nm. Radioactivity was monitored by a radioactivity detector coupled in series with the UV monitor.

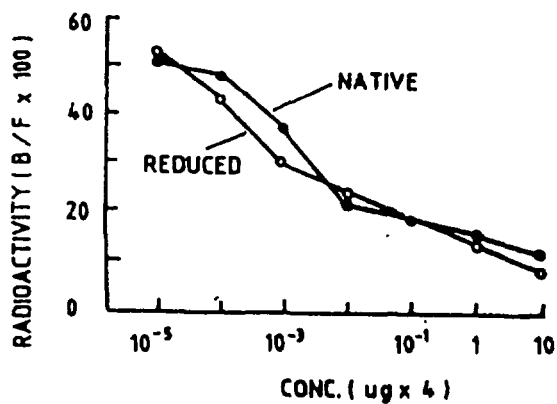


FIG 1 Immunoreactivity of 2-ME reduced hIgG.

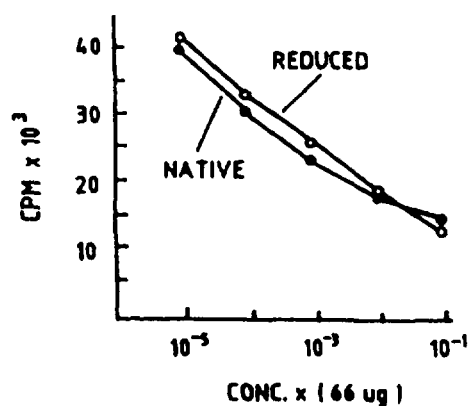


FIG 2. Immunoreactivity of ASC reduced hIgG.

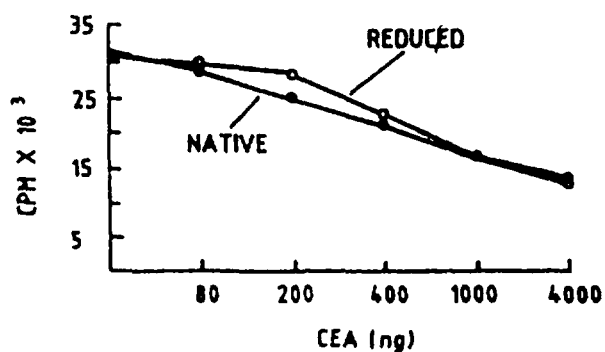


FIG 3. Immunoreactivity of 2-ME reduced ior.

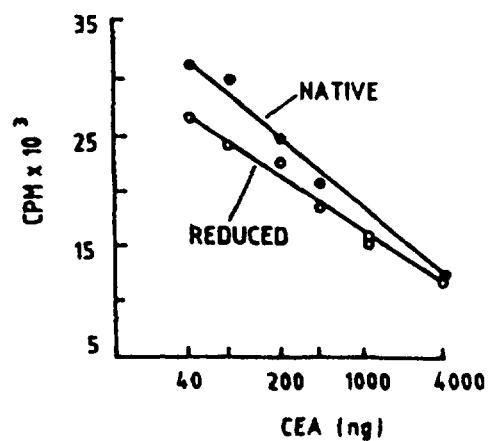


FIG 4. Immunoreactivity of ASC reduced ior.

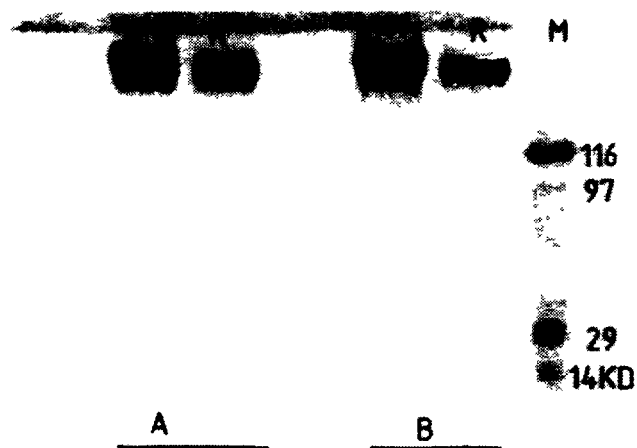


FIG 5. Page of native & ASC reduced hIgG. N = Native, R = Reduced, B = Double the protein concentration of A. M = Standard markers.

Immunoreactivity

The immunoreactivity of the antibodies before and after any treatment was measured by competitive binding radioimmunoassay using antihuman rabbit IgG and carcinoembryonic antigen (CEA) as binding agents for hIgG and ior-CEA-1 respectively. Whereas ^{125}I labelled hIgG was used as a radiotracer for hIgG, ^{125}I labelled ior-CEA-1 was used for ior-CEA-1. Enzyme linked immunosorbent assay (ELISA) modules were coated with different amounts of anti hIgG and CEA and unreacted binding sites were saturated with BSA. Amount of anti hIgG/CEA which gave 50% binding with the radiotracer in the absence of any cold hIgG/ior-CEA-1 were calculated. Subsequently, modules coated with these amounts were treated with same quantity of radiotracer in the presence of different concentration of native and reduced proteins. Bound radioactivity versus the amount of native or reduced proteins added in each well were plotted on a semilog scale to evaluate the effect of antibody reduction on immunoreactivity.

Blood clearance and biodistribution

Aliquots of 0.1 mL of radiolabelled hIgG/ior-CEA-1 (3.7 MBq) were intravenously administered through ear vein in rabbits of about 2.5 kg. Blood samples were withdrawn at various time intervals upto 24 h. Radioactivity was measured in blood samples and calculated per unit blood volume at each time point. Data were expressed as percent administered dose in whole body blood. For the biodistribution study, each mouse weighing about 25 g was intravenously administered through tail vein with 100 μL of the radiotracers (37 kBq). They were sacrificed at 4 and 24 h thereafter. Various organs were removed, weighed and radioactivity measured in a gamma counter. Data was expressed as per cent administered dose per whole organ.

Imaging of inflammatory and tumour lesions in animal models and patients

Whereas inflammatory lesions in Balb/c mice were produced by subcutaneously administering 0.2 mL turpentine oil in the hind leg thigh muscle, tumours were produced by intravenously administering Ehrlich Ascites tumour cells. Imaging was done for inflammation and tumour lesions at 24–48 h and 7 days respectively after the above treatments. Inflammatory lesions in patients resulting due to a variety of disorders were imaged 3–24 h after administering with $^{99}\text{Tc}^{\text{m}}$ -hIgG (200–800 MBq).

2.6.3. Results

The labelling efficiency of both the two methods based on the use of 2-ME and ASC irrespective of the nature of protein used was >97%. Labelled hIgG and ior-CEA-1 were stable in saline and human serum at 37°C, though the stability seems some what less in serum (Table I), which could possibly be due to presence of some proteases in the serum. However, only less than 5% radiolabel detached from the proteins, while incubating in serum, documents the suitability of both the methods for radiolabelling of antibodies with $^{99}\text{Tc}^{\text{m}}$ for in vivo use.

Data in Table II documents that the binding strength of the radionuclide with hIgG as well as ior-CEA-1 was adequate and of the similar order as even on incubating with 100 mM concentration of DTPA, not more than 5% of the radiolabel could be transchelated from the antibodies. Since cysteine is a strong chelator with respect to DTPA and at 100 mM level it was 12–14% irrespective of the protein used. This could be appreciated due to high -SH binding affinity for $^{99}\text{Tc}^{\text{m}}$.

Insignificant effect of hIgG reduction by 2-ME and ASC on immunoreactivity was evident by competitive binding assay in Figs 1 and 2. Both the native and reduced hIgG compete to the similar extent with the ^{125}I labelled hIgG for binding with anti-hIgG antibody. Though quantitative information about the binding affinity is lacking, it certainly infers that the treatment of hIgG with either of these reductants has little influence on its immunoreactivity. When the effect of 2-ME and ASC on ior-CEA-1 was examined a similar information was derived (Figs 3 and 4). In this case, however,

CEA was used as binding agent. Native and 2-ME/ASC reduced ior-CEA-1 were allowed to compete with ^{125}I labelled native ior-CEA-1 for ELISA modules bound CEA. No loss of immunoreactivity of the antibody irrespective of the nature of reductant was evident in this case as well.

Electrophoresis of native and reduced hIgG and ior-CEA-1 by using both the 2-ME and ASC was carried out. Results of native and ASC treated hIgG presented in Fig. 5 do not indicate any fragmentation of the protein. More or less similar patterns were found with 2-ME treated hIgG, ior-CEA-1 and ascorbic acid treated ior-CEA-1. Hence, it may be inferred that both 2-ME and ASC are safe reductants to generate -SH groups in the protein for Tc attachment. Further evidence on the effect of 2-ME on the protein intactness was gathered by size exclusion HPLC. Both the hIgG and ior-CEA-1 were treated with 2-ME and radiolabelled with $^{99}\text{Tc}^{\text{m}}$. HPLC of radiolabelled antibodies was carried out by monitoring the eluent at 280 nm and with radiometric detector (Figs 6a and 6b). Superimposable UV and radiopeaks confirm the inference drawn from electrophoresis data that the reduction of protein does not lead to fragmentation of the protein. A small peak seen in case of ior-CEA-1 suggests some impurity in the original sample. When HPLC was attempted with the ASC treated antibodies, several fold high concentration of ASC exhibited interference at 280 nm, ASC also gave high absorbance at 280 nm. However, its elution time was almost twice to that of proteins (data not shown).

TABLE I. STABILITY OF $^{99}\text{Tc}^{\text{m}}$ LABELLED hIgG AND ior-CEA-1

Time (h)	% Labelling efficiency							
	2-ME				Ascorbic acid			
	hIgG		ior		hIgG		ior	
	Saline	Serum	Saline	Serum	Saline	Serum	Saline	Serum
1	98.0	97.8	97.6	97.7	97.9	98.1	97.9	97.9
2	97.7	97.7	97.3	96.8	97.7	97.9	97.7	97.2
4	97.0	96.9	96.7	96.2	96.9	96.5	97.0	96.4
24	92.2	94.5	93.8	92.8	93.8	95.7	94.3	95.0

TABLE II. PERCENTAGE TRANSCHELATION OF $^{99}\text{Tc}^{\text{m}}$ FROM ANTIBODIES TO DTPA AND CYSTEINE

Chelating agent			Concentration (mM)			
			0	25	50	100
DTPA	2-ME	hIgG	2.1	2.4	5.0	7.4
		ior	3.0	4.1	4.7	6.5
	ASC	hIgG	1.5	1.7	3.2	7.1
		ior	2.0	2.5	4.0	6.5
Cysteine	2-ME	hIgG	2.1	7.8	11.5	15.5
		ior	3.0	7.0	11.0	14.2
	ASC	hIgG	1.3	7.0	10.1	13.3
		ior	2.5	10.0	13.5	16.5

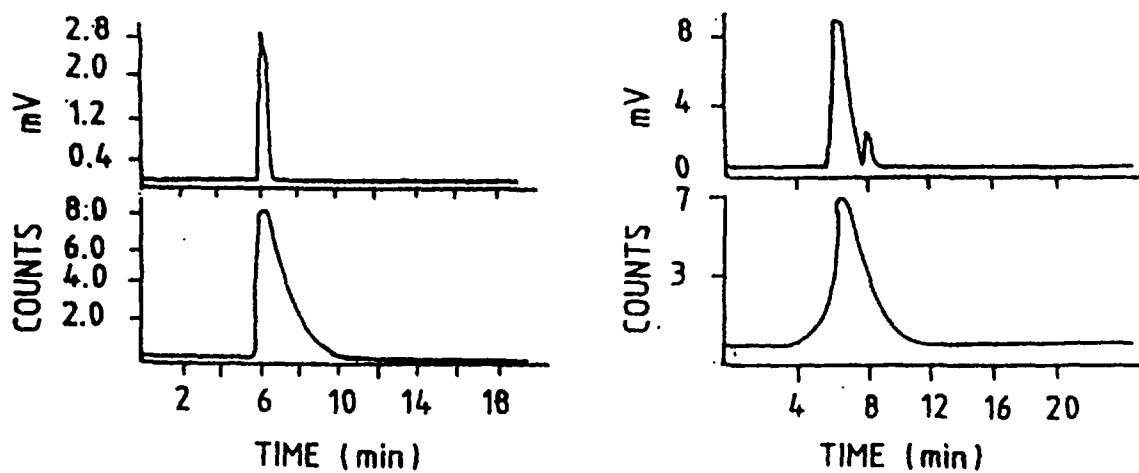


FIG. 6. Size exclusion HPLC of $^{99}\text{Tc}^m$ labelled 2-ME treated hIgG (6a) and ior (6b).

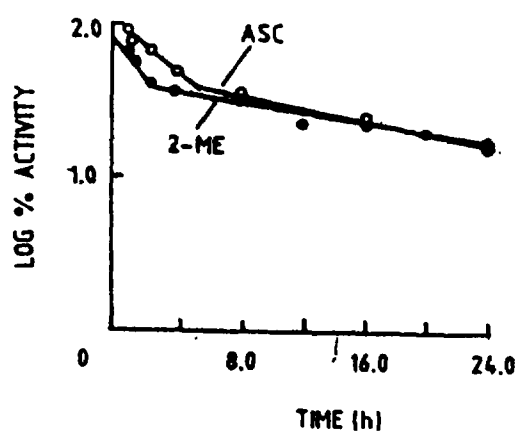


FIG. 7a. Blood clearance of 2-ME and ASC reduced ior.

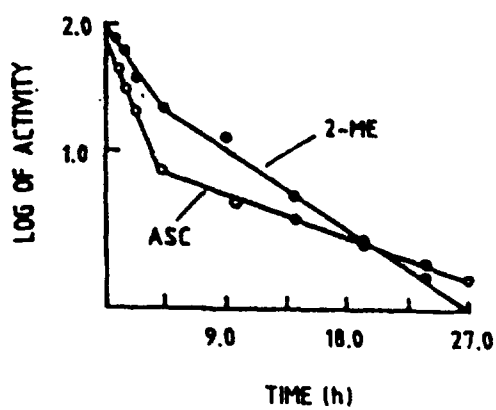


FIG. 7b. Blood clearance of 2-ME and ASC reduced hIgG.

TABLE III. BIODISTRIBUTION OF $^{99}\text{Tc}^{\text{m}}$ LABELLED hIgG AND ior-CEA-1 IN MICE AT 4 h (n = 4)

Organ	% ID per whole organ, 4 h p.i.			
	2-ME		Ascorbic acid	
	hIgG	ior-CEA-1	hIgG	ior-CEA-1
Heart	0.10 \pm 0.02	0.47 \pm 0.03	0.13 \pm 0.02	0.30 \pm 0.03
Liver	4.93 \pm 0.41	8.13 \pm 1.09	10.20 \pm 1.13	8.91 \pm 1.20
Kidneys	3.67 \pm 0.10	1.95 \pm 0.16	6.39 \pm 0.51	5.53 \pm 1.30
Intest.	2.12 \pm 0.21	6.14 \pm 0.57	7.23 \pm 0.51	16.50 \pm 2.40
Stomach	0.72 \pm 0.03	1.03 \pm 0.01	0.76 \pm 0.06	0.10 \pm 0.01
Muscle	0.70 \pm 0.32	7.20 \pm 1.20	3.90 \pm 0.30	2.70 \pm 0.32

Blood clearance of the radiolabelled hIgG and ior-CEA-1 prepared by using 2-ME and ASC and is shown in Figs 7a and 7b. It is evident from the pattern of both the antibodies that reducing agent does not contribute significantly in the blood kinetics of the antibody. The blood clearance exhibited, two components the fast ($T_{1/2}$) and the slow ($T_{1/2}$). A similar pattern but with different kinetics were seen with ior-CEA-1. Again no significant differences were noticed between the two preparations using 2-ME and ASC. However, in this case the fast $T_{1/2}$ (1.5 & 4.4) and slow $T_{1/2}$ (16.2 & 16.0) respectively, which are significantly different with respect to those obtained with hIgG.

Biodistribution data of $^{99}\text{Tc}^{\text{m}}$ -hIgG and $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 in mice at 4 h post administration are shown in Table III. In the case of both hIgG and ior-CEA-1 the protein reductant seems to have some influence on the biodistribution in different organs, though in both the cases the radiotracer seems to be excreted by hepatobiliary as well as renal routes. Low content in stomach suggests the high purity of the preparations. Another interesting observation was noticed that in the case of ior-CEA-1 hepatobiliary route was more predominant irrespective of the protein reductant used.

Imaging of turpentine induced inflammatory lesions in mouse and inflammatory lesions in 38 patients resulted due to infection and metabolic derangement have been carried out at various time intervals (1–24 hrs) using $^{99}\text{Tc}^{\text{m}}$ -hIgG prepared by using both the 2-ME and ASC as reductants. Two typical scintigrams of the patients are presented in Figs 8 and 9. The lesions of mastitis and osteoarthritis are clearly visible which show the merits of $^{99}\text{Tc}^{\text{m}}$ -hIgG prepared by the 2 methods developed here. The suitability of $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 prepared by the above methods would be evident from the Figs 10 and 11, where scintigrams of the mice implanted with Ehrlich tumour are presented. The good concentration of the labelled antibody irrespective of the methodology further validates the merits of the methodology developed for the radiolabelling of antibodies with $^{99}\text{Tc}^{\text{m}}$.

2.6.4. Discussion

In view of the greater potential of the direct labelling methods for kit preparation, the methods based on the use of 2-ME, ASC, sodium borohydride and borate buffer have been evaluated, in addition to iminothiolane based methodology. The two antibodies hIgG and anti CEA monoclonal antibody were selected for radiolabelling. For want of space detailed results on sodium borohydride, borate buffer and iminothiolane are not presented here though these methodologies also worked quite satisfactorily. However, the methodology based on borate buffer, was not found suitable for kit formation. In this investigation more emphasis was laid down on the use of 2-ME and ASC methods because of their superiority, simplicity and acceptability.

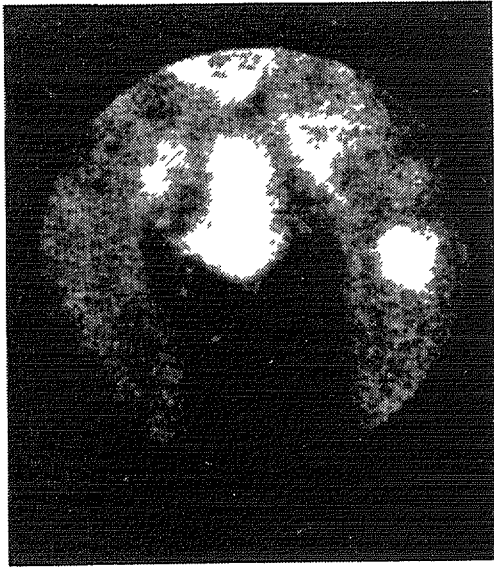


FIG 8 Scintigram of patient showing $^{99}\text{Tc}^m\text{-hIgG}$ accumulation in mastitis

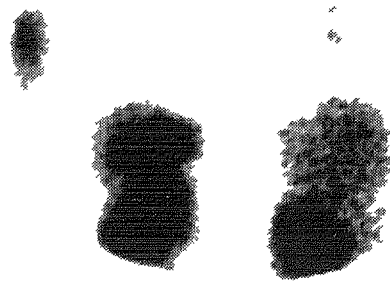


FIG 9 Scintigram of patient $^{99}\text{Tc}^m\text{-hIgG}$ accumulation in osteomyelitis



FIG 10 Scintigram At 4 h post injection of 2-ME reduced ior in ehrlich ascitis tumour bearing mouse

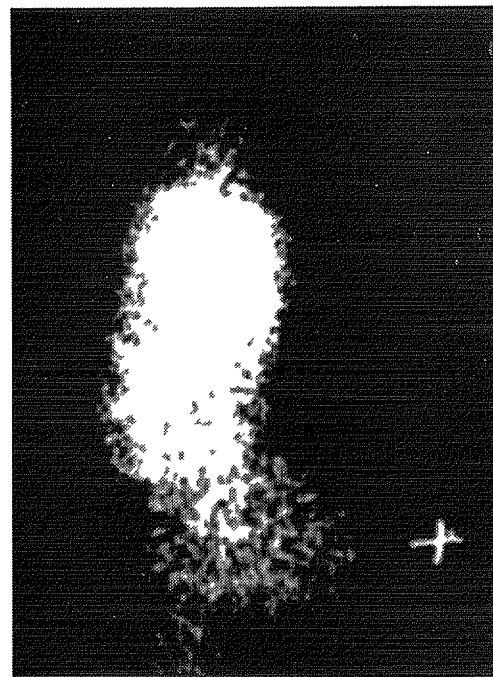


FIG 11 Scintigram at 4 h post injection of ASC reduced ior in ehrlich ascitis tumour bearing mouse

Ever since the report of Schwarz [8], 2-ME based methodology was highly acclaimed for quality radiolabelling of antibodies. However, the method when tailored for kit formation two major drawbacks were observed. First the lack of stability of 2-ME reduced antibody and difficulty in the adoption for a single vial kit. By the addition of ASC, we could improve the stability of the reduced antibody and by adding stannous and MDP before lyophilization the ingredient could be accommodated in a single vial. Thus first time it was made possible to produce single vial lyophilized kits of antibodies.

To obviate the necessity of post reduction purification of the 2-ME reduced antibody ASC has been successfully substituted for 2-ME which is milder and non-toxic reducing agent. Thakur et al. [16] have already used ASC as protein reductant. However, their methodology based on ASC exhibited major disadvantages as it needed highly concentrated sodium dithionite reduced $^{99}\text{Tc}^{\text{m}}$ and lack of its adaptability for kit development. We have successfully overcome these problems and produced the kits for hIgG and ior-CEA-1. The critical evaluation of the ASC concentration, temperature and time evolved ideal reaction conditions.

The labelling efficiency, stability, intactness of the protein and immunoreactivity of the products from both the methods are comparable. However, some differences have been observed for blood kinetics and biodistribution of both the products, as ASC treated $^{99}\text{Tc}^{\text{m}}$ -hIgG and $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 exhibited somewhat more liver uptake and intestinal secretion. However, these changes were noted only in animal experiments. In the case of $^{99}\text{Tc}^{\text{m}}$ -hIgG the behavior was similar in the patients irrespective of the methodologies adopted. So far 38 patients have been studied for the scintigraphy of inflammatory foci using $^{99}\text{Tc}^{\text{m}}$ -hIgG prepared from our kits. The agent has been found very useful specially in the detection of osteoarthritis and rheumatoid arthritic lesions with high sensitivity. $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1, although provides very exciting results in animal models in the detection of CEA expressing tumours, it is yet to be tested in patients which will be taken up soon subjected to approval from national nuclear medicine committee.

2.6.5. Conclusions

We have critically evaluated a variety of direct methods based on the use of 2-Mercaptoethanol, Ascorbic Acid, Sodium borohydride, Borate buffer and iminothiolane for radiolabelling of antibodies with $^{99}\text{Tc}^{\text{m}}$. The methodology based on the use of 2-Mercaptoethanol for radiolabelling of antibodies with $^{99}\text{Tc}^{\text{m}}$ have been suitably modified so as to be adopted for the preparation of single vial lyophilized kits. During this work, a novel method using ascorbate instead of 2-Mercaptoethanol as reductant has been developed which obviates the necessity of post reduction purification step in the earlier method using 2-Mercaptoethanol. The new method has been found to work satisfactorily for the preparation of cold lyophilized kits of antibodies. Kits of hIgG and an anti-CEA monoclonal antibody (ior-CEA-1) prepared by using both the procedures were evaluated for useful shelf life, labelling efficiency intactness immunoreactivity, transchelation, blood kinetics, biodistribution and immunoscintigraphy and found suitable for clinical use. The developed methodologies were also adaptable for antibody fragments F(ab')₂.

REFERENCES

- [1] HANI, A.N., RALPH, J., Clinical Application of Indium-111 Labelled Monoclonal Antibody Imaging in Colorectal Cancer Patients, *Sem. Nucl. Med.* **13** (1993) 99–113.
- [2] CHARLES, E., LINDA, C., et al., Monoclonal Antibodies in Ovarian and Prostate Cancer, *Sem. Nucl. Med.* **13** (1993) 114–126.
- [3] FRANS, M.M., CORSTENS, J.G., et al., Radioimmunoconjugates in the Detection of Infection and Inflammation, *Sem. Nucl. Med.* **13** (1993) 148–164.
- [4] ROBERT, M., et al., The use of Radiolabelled Non-specific Immunoglobulin in the Detection of Focal Inflammation, *Sem. Nucl. Med.* **14** (1994) 169–179.

- [5] BUSCOMBE, J.R., LUI, D., et al., $^{99}\text{Tc}^{\text{m}}$ -Human Immunoglobulin (hIgG), First Result of a New Agent for Localization of Infection and Inflammation, *Eur. J. Nucl. Med.* **16** (1990) 649–655.
- [6] BLOCK, D., et al., Detection of Inflammatory Lesions with Radiolabelled Immunoglobulins, *Eur. J. Nucl. Med.* **16** (1990) 303–309.
- [7] CHILDS, R.L., HNATOWICH, D.J., Optimum Conditions for Labelling of DTPA Coupled Antibodies with Technetium-99m, *J. Nucl. Med.* **26** (1985) 293–299.
- [8] SCHWARZ, A., STEINSTRASSER, A., A Novel Approach to $^{99}\text{Tc}^{\text{m}}$ Labelled Monoclonal Antibodies, *J. Nucl. Med.* **28** (1987) 721 (abstract).
- [9] MATHER, S.J., ELLISON, D., Reduction Mediated $^{99}\text{Tc}^{\text{m}}$ -Labelling of Monoclonal Antibodies, *J. Nucl. Med.* **31** (1990) 693–697.
- [10] SINGH, A.K., MISHRA, P., KASHYAP, R., CHAUHAN, U.P.S., A Simplified Kit for Instant Preparation of Technetium-99m Human-Immunoglobulin-G for Imaging Inflammatory Foci, *Nucl. Med. Biol.* **21** (1994) 277–281.
- [11] MISHRA, P., et al., A Novel Method for Labelling Human-Immunoglobulin-G with $^{99}\text{Tc}^{\text{m}}$ Suitable for Inflammation Scintigraphy, *Nucl. Med. Commun.* **15** (1994) 723–729.
- [12] PAUWELS, E.K.J., et al., The Labelling of Proteins and LDL with $^{99}\text{Tc}^{\text{m}}$: A New Direct Method Employing KBH-4 and Stannous Chloride, *Nucl. Med. Biol.* **20** (1993) 825–833.
- [13] ALAUDDIN, M.M., et al., An Improved Method of Direct Labelling of Monoclonal Antibodies with Tc-99m, *Nucl. Med. Biol.* **19** (1992) 445–454.
- [14] GOEDEMAN, S.W. Th., et al., A New Simple Method for Labelling of Proteins with $^{99}\text{Tc}^{\text{m}}$ by Derivation with 1 Imino-4-Mercapto Butyl Groups. In *Technetium and Rhenium in Chemistry and Nuclear Medicine* (Edited by Nicolini M, Bandoli G and Mazzi U) **3** (1990) 395–604. Cortina International, Verona, Raven press, New York.
- [15] THROLL, J.H., et al., Clinical Comparison of Cardiac Blood Pool Visualization with $^{99}\text{Tc}^{\text{m}}$ Red Blood Cells Labelled in-Vivo and with $^{99}\text{Tc}^{\text{m}}$ -HSA, *J. Nucl. Med.* **19** (1976) 796–803.
- [16] THAKUR, M.L., et al., $^{99}\text{Tc}^{\text{m}}$ Labelled Monoclonal Antibodies: Evaluation of Reducing Agents, *Nucl. Med. Biol.* **18** (1991) 227–233.



Research contract No. POR-6707/CF

Title of the Project : Technetium-99m Labelling of the ior-CEA-1 Monoclonal Antibody: Evaluation of Different Methods

Chief Scientific Investigator : L. Gano
Instituto Tecnológico Nuclear, Sacavem, Lisboa

2.7.1. Introduction

The chemistry by which antibodies are labelled with $^{99}\text{Tc}^{\text{m}}$ can be divided into two groups: (a) Direct method [1–6] in which endogenous disulfides are reduced to sulfhydryl groups which are thought to provide binding sites for $^{99}\text{Tc}^{\text{m}}$; (b) Indirect methods [7–12] by using exogenous bifunctional chelating groups or other derivatives which have a chelating moiety and an activated ester for antibody conjugation. An alternative indirect method has also been proposed [13–14] which involves the introduction of thiol groups in the protein by its conjugation with 2-iminothiolane (2-IM). The 2-IM reacts with amino groups of lysine molecules exposed outside, producing a thiolated derivative. In both methodologies following a subsequent purification the resulting reduced or conjugated antibody is labelled via stannous reduction of pertechnetate in presence of a weak competing ligand. Although radiolabelled antibodies have been used in patients, the advantages versus disadvantages of both direct and indirect methodologies are still a controversy.

In the present work, we evaluated the influence of experimental conditions on the characteristics of the radiolabelled ior-CEA-1 prepared by direct method, using 2-ME and stannous chloride as reductants and indirect method in which 2-IM is used to generate sulfhydryl groups in the protein. The following parameters were studied: number of free sulfhydryl groups of treated antibody, labelling efficiency, chemical and radiochemical composition of the radiolabelled antibodies as well as their immunological and biological properties. The properties of $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 obtained by 2-ME method were compared to the ones of the $^{99}\text{Tc}^{\text{m}}$ -monoclonal antibody obtained from a commercial kit (BW 431/26). Some clinical studies using $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 were also performed.

2.7.2. Materials and methods

2.7.2.1. Antibodies and antigens

Monoclonal antibody (MoAb) murine IgG ior-CEA-1 against carcinoembryonic antigen (CEA) was procured from the Center of Molecular Investigations (Havana, Cuba) supplied through the IAEA. ior-CEA-1 (1 mg/mL) was concentrated by ultrafiltration on Centricon-30 (Amicon, MA, USA) to approximately 5 mg/mL. Protein concentration was determined by absorbance at 280 nm (A_{280}) using a value for $E_{1\text{ mg/mL}}^{1\text{ cm}}$ of 1.4 for IgG. Kit for labelling the monoclonal antibody BW 431/26 (mouse origin) with $^{99}\text{Tc}^{\text{m}}$ obtained from Beringwerke AG, has been described as a diagnostic agent which reacts specifically with CEA bound to the cell membrane. MKN 45 is a human gastric cancer cell line which expresses CEA on the surface. The cells were grown in vitro in RPMI medium containing 10% foetal calf serum. CEA is from Scripps Laboratories, USA.

2.7.2.2. Radiolabelling methods

Ior-CEA-1 was radiolabelled by two direct methods using 2-ME and stannous chloride respectively as reductants. The indirect method involved the use of 2-IM for ior-CEA-1 coupling to generate free sulfhydryl groups.

Direct methods

(a) *2-ME reduction*: To a stirred solution of concentrated antibody in phosphate buffer saline (PBS) an appropriate volume of 2-ME was added to obtain a reaction mixture with molar ratios for 2-ME to ior-CEA-1 of 250:1, 750:1 and 1000:1. Each reaction mixture was kept for 30 min at room temperature. The reduced preparations were then purified on a 0.9×15 cm Sephadex G-50 column using nitrogen purged PBS as mobile phase. To ensure that an efficient purification was achieved the eluted fractions collected from the column were analyzed by spectrophotometry at 280 nm and 412 nm for protein and SH/Ellman detection respectively. Aliquots were taken to determine the reduction efficiency under different reduction conditions. Fractions of 1.0 mg of reduced protein (1000:1) were then frozen and/or lyophilized in the presence of a small amount of MDP/Sn solution (100 μ L of a MDP-tin kit previously reconstituted with 5 mL of 0.9% sodium chloride) corresponding to 1.3 μ g of tin (II) which provides the kit for antibody labelling. For labelling purpose 0.5 mL of $^{99}\text{Tc}^{\text{m}}$ -pertechnetate eluate was added. The solution obtained was allowed to stand for 10 min at room temperature. The use of MDP as pretinning weak chelate for labelling purpose was based on human immunoglobulin labelling efficiency studies performed when MDP, glucoheptonate and pyrophosphate kits were used [15].

(b) *Stannous reduction*: To ior-CEA-1 solution, appropriate volume of nitrogen purged solution of stannous tartarate-phthalate (pH5.6) was added to obtain a molar ratio (stannous chloride to antibody 500:1). The mixture was left to incubate overnight at room temperature. Then the preparation was aliquoted into vials (0.5 mg/vial) stored under nitrogen atmosphere and frozen at -20°C . For labelling to 0.5 mg of reduced MoAb, 0.5–1.0 mL of $^{99}\text{Tc}^{\text{m}}$ -pertechnetate eluate was added. The solution was allowed to stand for 10 min at room temperature and then purified on a 0.9×15 cm Sephadex G-50 column to remove free pertechnetate.

Indirect method

2-IM conjugation: Based on studies previously performed to optimize the experimental conditions for hIgG labelling [16] the following protocol was used: to a suitable volume of 2-IM solution, 1 mg of ior-CEA-1 in PBS (0.07 M), pH7.4 was added. The reaction mixture with molar ratio 260:1 (2-IM:ior-CEA-1) was incubated for 1 h at room temperature. For labelling purpose, 25 μ L of MDP solution containing 3.3 μ g of tin (II) was added followed by $^{99}\text{Tc}^{\text{m}}$ -pertechnetate eluate (1.5 mL). ior-CEA-1 conjugate was then purified on Sephadex G-50 column to remove the unreacted 2-IM. To assure that an efficient purification was achieved the eluted fractions collected from the column were analyzed by spectrophotometry at 280, 245 and 412 nm for protein, 2-IM and SH/Ellmans detection respectively. Fractions of conjugated ior-CEA-1 of 0.5 mg were nitrogen purged and stored at -20°C . Technetium labelling of conjugated antibody was achieved by adding an appropriate volume of $^{99}\text{Tc}^{\text{m}}$ -pertechnetate. BW 431/26 kit was radiolabelled according to the instructions supplied by producer.

Analytical methods

Determination of sulfhydryl groups

The determination of sulfhydryl groups was accomplished with Ellman's reagent (5-5'-dithio-bis-(2-nitrobenzoic acid), in which absorbance was measured at 412 nm. For analysis, equal volume of the reagent (0.3 mg/mL) freshly prepared in 0.1M phosphate buffer pH8.0 was added to the protein solution. Absorbance was measured using a UV spectrophotometer (412 nm). In the case of stannous chloride method the reduced reaction mixture was previously purified by Sephadex G-50 to avoid stannous interference. Free sulfhydryl groups were assayed with reference to a standard curve obtained from reaction with a series of known concentrations of free cysteine. The validity of the method was demonstrated by the study of linearity and precision. For this a series of standard cysteine solutions ranging between 0.05 mM to 2.0 mM were prepared. For each point 5 determinations by spectrophotometry at 412 nm were made. A linear regression analysis of the data gives the correlation coefficient.

2.7.2.3. Chromatographic systems

For labelling efficiency measurement and stability evaluation the following chromatographic systems were used: (a) ITLC-SG in saline. In this system labelled antibody and colloids remain at the origin while $^{99}\text{Tc}^{\text{m}}$ -MDP and pertechnetate moved with the solvent front. b) HPLC (Perkin Elmer) system with a TSK G 3,000 SW gel filtration coupled with a UV 280 nm and radioactive (Berthold) detectors. As mobile phase 0.1M phosphate buffer, pH7.0 at a flow rate of 1.0 mL/min was used. The recovery was determined by collecting the eluate from the HPLC column for 25 min from the time of injection and counting 0.5 mL aliquots in a gamma counter together with 0.5 mL aliquots of a suitable dilution of the injected solution. The HPLC column for 25 min from the time of injection and counting 0.5 mL aliquots in a gamma counter together with 0.5 mL aliquots of a suitable dilution of the injected solution. The HPLC column was calibrated using commercially available protein standards (LKB) with a MW range of 13–660 kD. The retention time corresponding to each standard was recorded and then plotted on a semi-log scale. A linear relationship was obtained.

2.7.2.4. Immunoreactivity

Immunoreactivity was evaluated by competitive binding assay and immunoreactive fraction determination.

(a) *Competitive binding assay*: It was used for the immunoreactivity study of each pretreated antibody for labelling. This was measured relatively to that of the native antibody using as antigen a suspension of MKN 45 cells or a CEA solid phase. When cells were used the following optimized protocol was carried out: to 100 μL of different solutions of native and pretreated ior-CEA-1 (0;250;1000;2500 and 10 000 ng/100 μL) 100 μL of I-125-ior-CEA-1 (20 ng) and 500 μL of suspension of cells ($5-7 \times 10^6$ cells/mL) were added. The reaction mixture was incubated for 1 h at room temperature and then the tubes centrifuged, the supernatant removed and the pellet washed twice with 500 μL of PBS/BSA. The radioactivity bound to the cells was counted. For solid phase, RIA tubes were coated with CEA in 0.1 M PBS (500 ng/200 μL) by incubating overnight at room temperature. The excess of CEA solution was aspirated. To minimize non-specific binding (NSB), 100 μL of 1% human serum albumin in PBS was added and incubated for 1 h at room temperature. To each coated tube, 100 μL of different concentration solutions of native and pretreated ior-CEA-1 were added (0,2.5, 5,10,50, 100, 250 ng/100 μL) followed by 100 μL (20 ng) of ^{125}I -ior-CEA-1 (labelled through iodogen method). The reaction was incubated for 2 h at room temperature and then the tubes were aspirated and washed twice with 2 mL of water and counted. The results were plotted as the percentage of ^{125}I -ior-CEA-1 bound to antigen versus the concentration of native, reduced or conjugated antibody.

(b) *Immunoreactive fraction*: The immunoreactivity of $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 preparations was also assessed with an antigen excess of MKN 45 cells binding. Serial dilutions from a 10^7 cells/mL were made. Each cell dilution was mixed with $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 at a final MoAb concentration of 20 ng/mL, incubated at 37°C for 1 h and then washed with PBS-1% BSA by centrifugation. The cell pellets were counted for radioactivity in a gamma counter. The immunoreactive fraction was calculated from double inverse plots, total to specific binding versus 1/cell concentration, extrapolated to infinite cell concentration [17]. The immunoreactive fraction of the $^{99}\text{Tc}^{\text{m}}$ labelled MoAb, is defined as the reciprocal of the y-intercept of the linear regression with the ordinate.

2.7.2.5. Polyacrylamide gel electrophoresis (SDS PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed under non-reducing conditions on the native, reduced or conjugated ior-CEA-1 as well as on the labelled antibody by the three labelling methods used in the study. Samples for assay were prepared at a SDS concentration of 0.5% and immersed in a boiling water bath for 3 min prior to gel loading. Molecular weight standards (LKB) were prepared in the same manner. Samples were loaded at 5–10 μg protein per lane and were resolved in a 2.5% acrylamide stacking gel and a 7.5% running gel

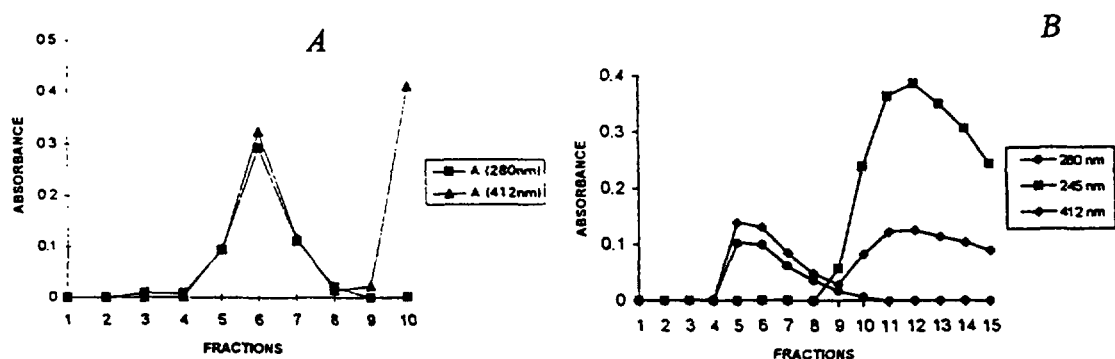


FIG. 1. Spectrophotometric analysis of elution fractions collected from Sephadex G-50, (A) 280 and 412 nm (protein and SH/Ellman detection), (b) 280, 245 and 412 nm (protein, 2-Im and SH-Ellman detection).

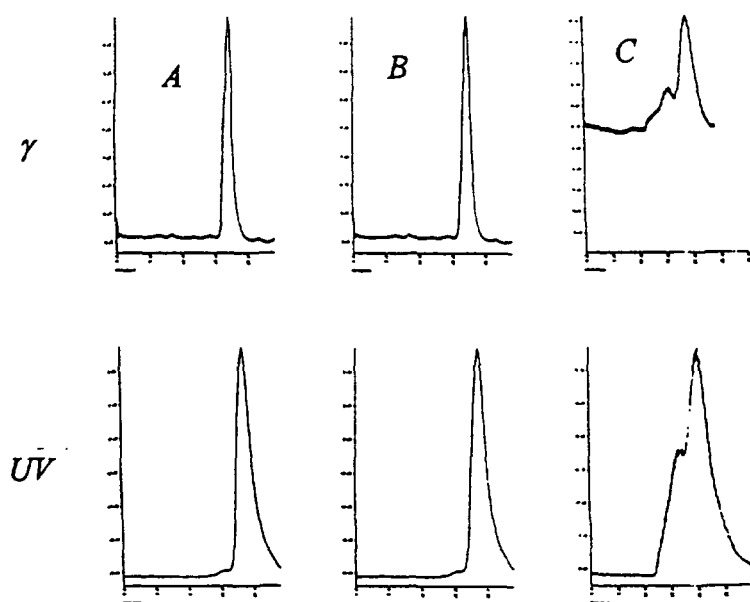


FIG. 2. UV and radioactive HPLC profiles of $^{99}\text{Tc}^m$ -ior CEA (A) 2-ME; (B) SnCl_2 ; (C) 2-Im. TSK G 3,000 column, flow rate 1.0 mL/min, mobile phase 0.1 M phosphate buffer, pH=7.0.

in a Pharmacia apparatus. Gels were run at 20–30 mA until the tracking dye reached 0.5–1.0 cm from the bottom of the gel. Each gel was then fixed and stained with Coomassie Brilliant Blue (0.1% w/v in 50% methanol and 10% acetic acid solution) and destained with a 7.5% acetic acid, 10% methanol and 3% glycerol solution until clear bands and transparent background are available. Radioactivity associated to each band was measured in a gamma counter.

2.7.2.6. Animal experiments

In vivo behavior of the $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 preparations was evaluated in normal mice when injected intravenously 100 μL of radioactive solution via the tail vein. The animals were killed 4 h post-injection and the organs were dissected and counted. The results were expressed as percent of injected dose per gram of tissue (% ID /g). The results of biodistribution were evaluated by analysis of variance. The level of significance was set at 0.05. For blood and muscle the activity was calculated assuming 7% and 40% of the total body weight respectively. Whole body gamma camera images at 6 and 24 h post injection were also acquired.

2.7.3. Results and discussion

Labelling yield determined on reaction mixtures with molar ratio for 2-ME to ior-CEA-1 of 250:1, 750:1, and 1000:1 revealed that for molar ratio 1000:1 labelling efficiency >98% was obtained. As the attempts to decrease the amount of reducing agent were not well succeeded this molar ratio was used for ior-CEA-1 kit preparation.

The determination of sulfhydryl groups generated was considered an important parameter to examine the efficacy of reducing agents and 2-IM coupling yield. On the other hand, such determination could eventually explain any undesirable effect detected on the immunological properties of antibodies upon which depends the success of their application in vivo. The number of free sulfhydryl groups determined on the pretreated antibodies (reduced and 2-IM conjugated) for radiolabelling purpose were 7.3, 3.1 and 9.8 for 2-ME, stannous chloride and 2-IM methods respectively. Thus the lowest value was found when stannous chloride was used as reducing agent. Similar number of SH groups was determined in frozen and lyophilized 2-ME ior-CEA-1 kits suggesting that the 2-ME reduced product is stable and that the lyophilization did not induce to the reassociation of chains to native molecule. The number of SH groups determined was not influenced by the presence of free 2-ME or 2-IM. The efficiency of Sephadex G-50 column to remove both the unreacted 2-ME and 2-IM is shown in Fig. 1.

Labelling efficiency and radiochemical purity monitored by ITLC were higher than 98% except when the stannous chloride was used as reductant. In this case labelling yield of 60–70% was found which implied necessity of post-labelling purification step. This lower labelling yield could be related to the lower number of free sulfhydryl.

UV and radioactive profiles obtained by HPLC analysis of labelled MoAbs from frozen formulation (stannous chloride) and lyophilized formulations (2-ME and 2-IM) are presented in Fig. 2. High radiochemical purity was found for radioactive MoAbs prepared by direct method. For these preparations single peak of identical retention time to native antibody (not shown), reduced and radioactive preparations was obtained. So no significant effect of the treatment and subsequent $^{99}\text{Tc}^{\text{m}}$ labelling was detected. However, a broad peak found at retention time (R_t) corresponding to the protein molecular weight which can be related to high polymeric and dimeric fractions when 2-IM was used. HPLC recoveries were essentially quantitative, although for 2-IM conjugated method high percentage of radioactivity is associated to dimeric and polymeric species. 2-IM method described as a simple method able to overcome the problem associated to the use of reductants, revealed to be the method for which high percentage of aggregated forms were detected. This could be related to the technetium complexation through SH groups of different MoAb molecules.

TABLE I. PERCENTAGE OF RADIOACTIVITY ASSOCIATED TO EACH BAND

MW (kD)	Radioactivity associated to each band (%)		
	2-ME	SnCl ₂	2-Im
150	15.2	70.5	57.0
116	58.7	16.7	33.9
90	3.3	5.0	3.9
54	18.5	2.9	4.6

Stability studies of radiolabelled antibodies evaluated by ITLC and HPLC have shown that the labelled antibody kept its radiochemical behavior at least upto a period of 4 h after labelling when stored either at room temperature or at 37°C.

SDS PAGE results indicate that not only intact IgG is present on pretreated antibody preparations. Other protein fractions of approximately MW 116, 90 and 50 kD are assigned in a variable percentage according to the methodology used (Fig. 3) The band intensities have shown the influence of the two reducing agents (2-ME and stannous chloride) as well as the 2-IM MoAb conjugation. As can be observed in 2-ME reduction the band corresponding to intact IgG revealed that more disulfide bridges were cleaved when compared to stannous and 2-IM treated antibodies. Similar SDS PAGE profiles were obtained for labelled antibodies. This led us to conclude that the MoAb treatment is the determinant factor for the final composition in terms of percentage of each protein fraction. The radioactivity associated to each one is presented in Table I.

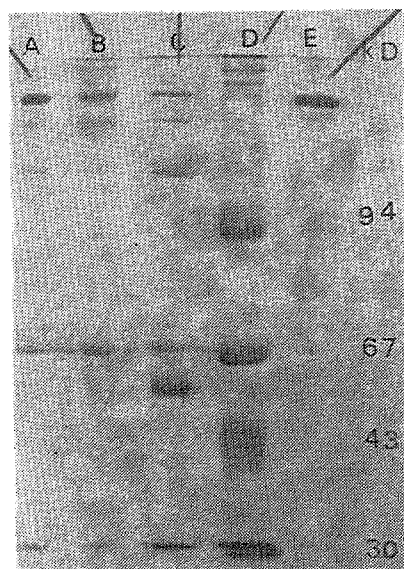


FIG. 3. SDS PAGE stained gels. (A) native MoAb; (B) (2-ME) MoAb; (C) (2-Im) MoAb (D) (SnCl₂) MoAb. (E) MW standards.

In the case of 2-ME, only 15.2% of the radioactivity was associated with IgG (MW 150 kD). However, significant high radioactivity is associated to fractions of 116 and 50 kD MW. For stannous and 2-IM labelled antibodies 70.5 and 57.0% of the radioactivity was associated to IgG bands. It was expected, mainly in direct method, that the disulfide bridge could be cleaved to yield a mixture of intact and fragmented antibodies as detected by SDS PAGE. Nevertheless no fragmentation was detected by HPLC, a frequently used method for antibody quality control. Therefore, we conclude that in our experimental conditions the HPLC has not enough resolution for antibody fragmentation detection or that the MoAbs treatment lead to labile molecules which undergo fragmentation even under nonreducing SDS PAGE procedure. So SDS PAGE seems a much more powerful tool. These results are in agreement with the observations reported by John [18] and Hnatowich [19]. In Figs 4 and 5 are presented the binding inhibition curves obtained when CEA and MKN 45 cells were used as antigens. The analysis of the inhibition curves led us to conclude that 2-ME and stannous reduction did not alter immunological reactivity of the antibody. However, 2-IM coupling decreased MoAb immunoreactivity by about 20%. In Fig. 6 are presented the curves of immunoreactive fractions obtained for the different labelled antibodies. Again for 2-IM method a relative immunoreactive fraction decreased by about 20%. The antibody fragmentation is often referred to destroy the immunospecificity. Despite this no correlation between SDS PAGE results and immunoreactivity studies was found. In spite of the higher fragmentation assigned for 2-ME reduction no decrease in the immunospecificity of 2-ME reduced and labelled antibody was detected. Although 2-ME acts as a strong reducing agent, it seems that under the experimental conditions chosen the cleaved intrachain disulfides did not affect the ior-CEA-1 immunoreactivity because the antibody chains are also held together by strong noncovalent forces in the absence of detergents such as SDS. The stannous reduced antibody showed low structural perturbation and high percentage of radioactivity associated with intact IgG (70.5%). Again no difference in immunoreactivity was registered. On the contrary a decrease of 20% in immunoreactivity was found for 2-IM conjugate, although an apparently low structural perturbation of IgG has been detected.

Biodistribution of different $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 preparations expressed as percentage of injected dose per gram of tissue (% ID /g) in healthy female mice 4 h after intravenous administration is presented in Fig. 7. The following urinary excretion data expressed as percentage of ID was obtained in a range from 9.8–23.8 (2-ME); 18.6–37.3 (stannous); 43.8–67.6 (2-IM).

As shown the $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 prepared via 2-IM revealed faster blood clearance, increased urinary excretion and higher kidney, stomach, gut and liver uptake when compared to the $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 obtained by 2-ME method. Biological profile of $^{99}\text{Tc}^{\text{m}}$ -(stannous)-ior-CEA-1, although is more similar to the $^{99}\text{Tc}^{\text{m}}$ -(2-ME)-ior-CEA-1, presented higher liver, gut, stomach and kidney uptake ($p < 0.001$). The higher kidney levels and urinary excretion could be explained by the more rapid clearance the kidneys of $^{99}\text{Tc}^{\text{m}}$ labelled fragments. However, these methods specially for stannous chloride method, high radioactivity was bound to IgG. Moreover, we admitted that the higher urinary excretion and kidney uptake registered is due to a low in vivo stability of 2-IM and stannous labelled MoAbs and then the majority of activity in urine is free $^{99}\text{Tc}^{\text{m}}$, although the chemical form has not been established. The in vivo instability that greatly influenced the biodistribution in mice, could be reflected on the quality of images in patients. The slow blood clearance for 2-ME labelled antibody was probably due to the high radioactivity (58.7%) associated to protein fraction corresponding to MW 116 kD, F(ab')_2 fragments which are known to have a different biokinetic when compared to IgG but yet a slow blood clearance.

Based on the high labelling yield, simplicity, reproducibility, radiochemical purity, immunoreactivity and on the favorable biodistribution in the animal model used, the $^{99}\text{Tc}^{\text{m}}$ -(2-ME)-ior-CEA-1 was considered the best preparation to use as tumour agent. In addition (2-ME)-ior-CEA-1 kit could be available in lyophilized form. Investigation was then performed to compare the properties of $^{99}\text{Tc}^{\text{m}}$ -(2-ME)-ior-CEA-1 and $^{99}\text{Tc}^{\text{m}}$ -antibody BW 431/26 (prepared from a commercial kit).

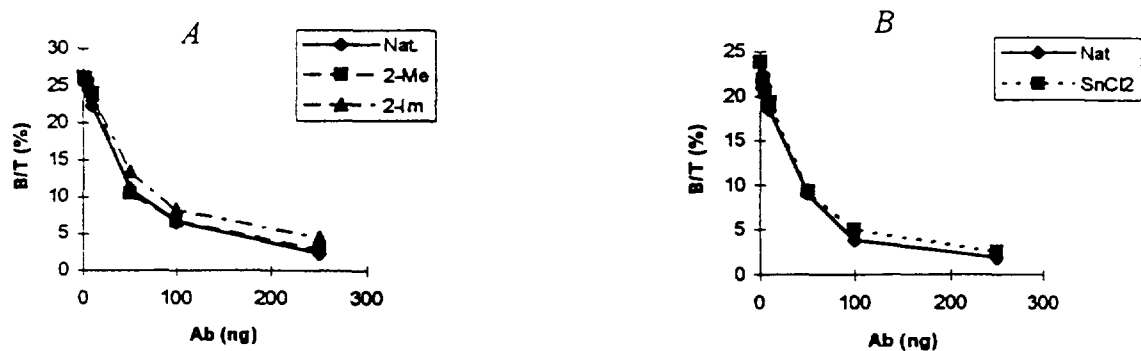


FIG. 4. Binding inhibition curves of 2-ME reduced, 2-Im conjugate (A) and SnCl₂ reduced (B) MoAbs compared with the native antibody using CEA as antigen.

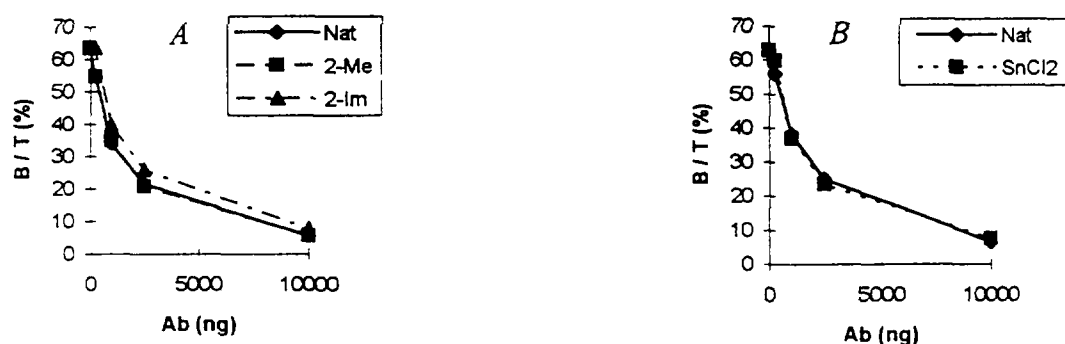


FIG. 5. Binding inhibition curves of 2-Me reduced, 2-Im conjugate (A) and SnCl₂ reduced (B) MoAbs compared with the native antibody using MKN 45 cells as antigen.

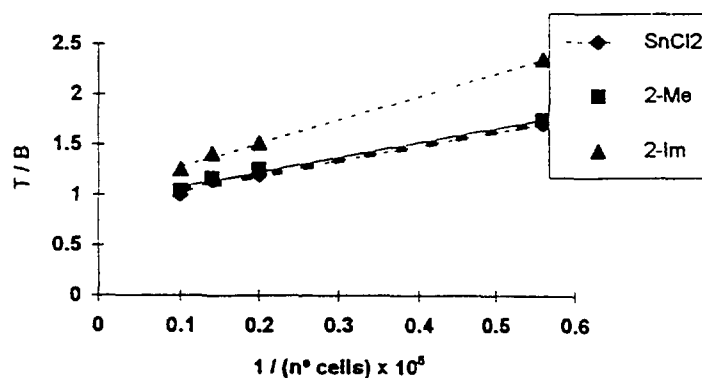


FIG. 6. Binding of ⁹⁹Tc^m-ior-CEA (2-ME, SnCl₂ and 2-Im). The figure shows a Lindmo plot of total/bound counts against 1/cell number.

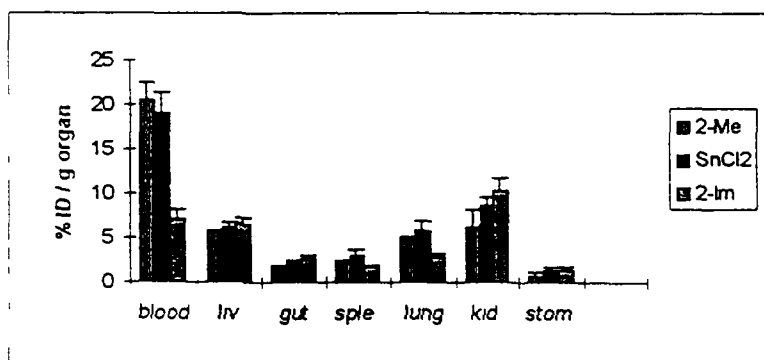


FIG. 7. Biodistribution data for ⁹⁹Tc^m-ior-CEA obtained via 2-ME and SnCl₂ reduction and 2-Im conjugation 4 h after i.v. administration in female mice.

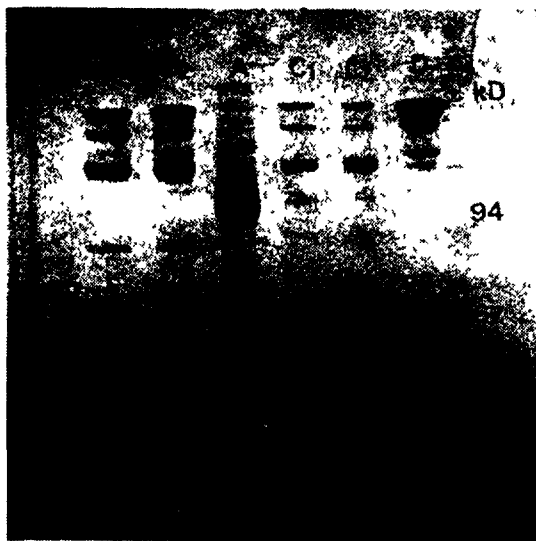


FIG 8 SDS PAGE stained gels. (A) MW standard, (B₁) lyophilized ior-CEA kit; (B₂) lyophilized BW 431/26 kit, (C₁) ⁹⁹Tc^m-ior-CEA; (C₂) ⁹⁹Tc^m-BW 431/26; (D) native ior-CEA.

ior-CEA-1 and BW 431/26 comparison

Studies were performed on ior-CEA-1 and BW 431/26 lyophilized kits and ⁹⁹Tc^m-labelled preparations for comparison purpose. The number of free SH groups determined in reduced antibodies was 7.3 and 6.4 for 2-ME and BW 431/26 respectively. For both ior-CEA-1 and BW 431/26 reduced formulations and labelled products similar UV and radioactive HPLC profiles were obtained. Also similar SDS PAGE profiles (Fig. 8) as well as the % of radioactivity associated to each protein fraction was found (Table II)

The immunoreactivity of BW 432/26 was only determined as the binding percentage of 20 ng of ⁹⁹Tc^m-BW 431/26 to CEA coated tube. The value found (30%) is comparable to the ones determined, under identical reaction conditions for ⁹⁹Tc^m-(2-ME)-ior-CEA-1 which indicates a similar immunoreactivity.

No significant differences on biological distribution determined as the percentage of ID/g of tissue was observed (Fig. 9) Planner gamma camera whole body images at 6 and 24 h p.i. are presented in Fig. 10.

TABLE II. PERCENTAGE OF RADIOACTIVITY ASSOCIATED TO EACH BAND

Radioactivity associated to each band (%)		
MW (kD)	ior-CEA-1	BW 431/26
150	15.2	19.4
116	58.7	4.1
90	3.3	—
50	18.5	12.1

TABLE III. INDEX ORGAN/BACKGROUND FOR BW 431/26 AND ior-CEA-1 ANTIBODIES

Organ	Index organ/ Background	
	BW 431/26	ior-CEA-1
Liver	7.23 ± 1.90	8.03 ± 0.76
Spleen	4.20 ± 1.31	5.17 ± 1.83
Lung	3.23 ± 0.67	3.63 ± 0.87
Heart	9.60 ± 3.34	0.22 ± 2.08
Kidney	3.87 ± 1.47	3.93 ± 0.85
Intestine	2.13 ± 0.15	2.24 ± 0.70
Stomach	3.30 ± 1.84	3.40 ± 2.31

The cumulative urinary excretion at 24 h p.i. represents almost 60% of the administered radioactivity for both labelled antibodies. The results obtained from in vitro studies and biological behavior in mice, provide evidence of the similarity of both labels $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 and $^{99}\text{Tc}^{\text{m}}$ -BW 431/26.

Clinical studies

In Nuclear Medicine Institute nine patients were submitted to immunoscintigraphic studies with 740 MBq $^{99}\text{Tc}^{\text{m}}$ labelled monoclonal antibodies. Whole body images were acquired at 6 and 24 h. Tomographic studies were performed in suspected regions. 6 patients were studied with ior-CEA-1 and 3 with BW 431/26. One positive antibody scan was registered by each patient group which were already known. From the negative scans one was a false negative and the other revealed a cold hepatic lesion. Both patients were studied with ior-CEA-1. To compare the MoAbs biodistributions the ratio organs/background were obtained in the 6 h images. For that purpose we considered a region of interest in the leg for background and the following other regions: spleen, kidneys, heart, liver, lung, intestine and stomach. We excluded 2 patients for the liver ratio because they had a great hepatic uptake: one had hepatic metastatic lesions and the other had a suspicious. The index values are presented in Table III. In the Nuclear Medicine Department of Portuguese Institute of Cancer 39 patients were submitted to immunoscintigraphy using BW 431/26 labelled with 1036-1110 MBq. Whole body images were acquired at 10 min, 6 and 24 h. SPECT of regions of interest were performed at 24 h p.i. Sensitivity of 94% for local recurrences and 83% for distant metastases were found. Accuracy was, respectively, 95 and 83%. ior-CEA-1 was used for immunoscintigraphy in 3 patients. One true positive and two false negative scans were obtained. The quality of images at 10 min, 6 h and 24 h p.i. were compared.

2.7.4. Conclusions

A comparative study of different radiolabelling methods for the monoclonal antibody ior-CEA-1 is described in this paper. Radiochemical purity and immunoreactivity assays of $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 preparations indicate that the direct method (2-ME and stannous) is less damaging. Despite the low percentage of radioactivity associated to the intact IgG, as determined by SDS PAGE, we conclude that the 2-ME is the most promising procedure due to its labelling efficiency, simplicity, reproducibility and in vivo stability. 2-IM method described as a simple method able to overcome the problem associated to the use of reducing agents, was the method for which the most poor properties of the label were found.

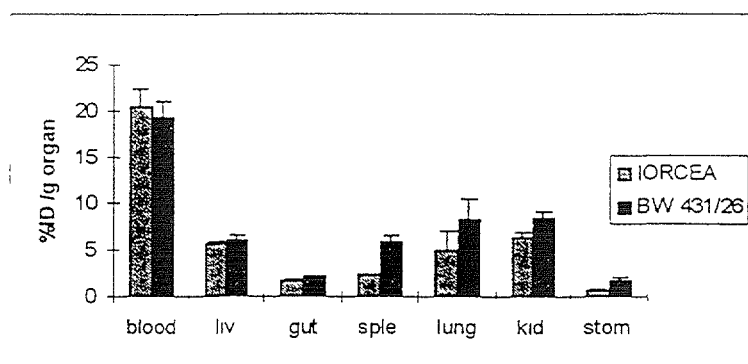


FIG 9. Biodistribution data for $^{99}\text{Tc}^m$ -ior-CEA obtained via 2-Me reduction and $^{99}\text{Tc}^m$ -BW 431/26 4 h after i.v. administration in female mice.

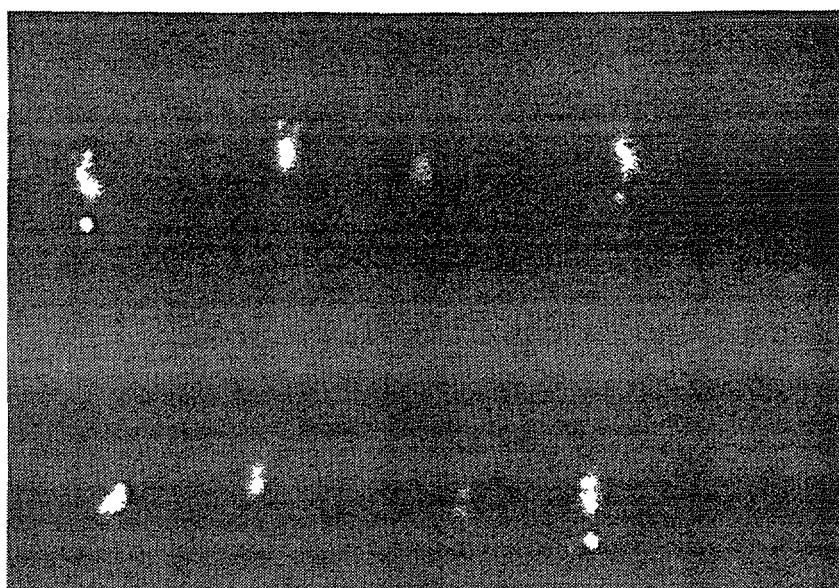


FIG. 10. Planar gamma camera images of mice injected (in duplicate) with $^{99}\text{Tc}^m$ -BW 431/26 (upper row) and $^{99}\text{Tc}^m$ -ior-CEA (bottom row) at 6 h (left side) and 24 h (right side) p.i.

Ior-CEA-1 and BW 431/26 comparison based on the free SH groups determination, HPLC and SDS PAGE analysis, immuno-reactivity and biodistribution in mice provide evidence of the similarity of both labels $^{99}\text{Tc}^{\text{m}}$ -ior-CEA-1 and $^{99}\text{Tc}^{\text{m}}$ -BW 431/26. Clinical studies have also revealed similar biodistribution profiles.

REFERENCES

- [1] RHODES, B.A., et al., Direct Labelling of Proteins with $^{99}\text{Tc}^{\text{m}}$, *J. Nucl. Med.* **27** (1986) 685–693.
- [2] PAIK, C.H., et al., The Labelling of High Affinity Sites of Antibodies with $^{99}\text{Tc}^{\text{m}}$, *Nucl. Med. Biol.* **13** (1986) 359–362.
- [3] SCHWARZ A., et al., A Novel Approach to $^{99}\text{Tc}^{\text{m}}$ -Labelled Monoclonal Antibodies, *J. Nucl. Med.* **28** (1986) 721.
- [4] MATHER, S.J., et al., Reduction-Mediated Technetium-99m Labelling of Monoclonal Antibodies, *J. Nucl. Med.* **31** (1990) 692–697.
- [5] THAKUR, M.L., et al., Technetium-99m Labelled Monoclonal Antibodies: Evaluation of Reducing Agents, *Nucl. Med. Biol.* **18** (1991) 227–233.
- [6] HNATOWICH, D.J., et al., Investigations of Ascorbate for Direct Labelling of Antibodies with Technetium-99m, *J. Nucl. Med.* **35** (1994) 127–134.
- [7] NAJAFI, A., et al., Coupling Antibody with DTPA — An Alternative to the Cyclic Anhydride, *Int. J. Appl. Radiat. Isot.* **35** (1984) 554–557.
- [8] BAIDOO, K.E., et al., $^{99}\text{Tc}^{\text{m}}$ Labelling of Proteins: Initial Evaluation of a Novel Diaminedithiol Bifunctional Chelating Agent, *Cancer Research* **50** (1990) 799s–803s.
- [9] NAJAFI, A., et al., Synthesis and Preliminary Evaluation of a New Chelate N_2S_4 for use in Labelling Proteins with Metallic Radionuclides, *Nucl. Med. Biol.* **18** (1991) 179–185.
- [10] KASINA, S., et al., Development and Biologic Evaluation of a Kit for Performed Chelate Technetium-99m Radiolabelling of an Antibody Fab Fragment Using a Diamide Dimercaptide Chelating gent, *J. Nucl. Med.* **32** (1991) 1445–1451.
- [11] QU, T., et al., Technetium-99m Labelling on Monoclonal Antibodies via N, N'-Ethylene-Bis-1-Cysteine, *Radiochim. Acta* **63** (1993) 209–212.
- [12] EISENHUT, M., et al., Bifunctional NHS-BAT Ester for Antibody Conjugation and Stable Technetium-99m Labelling; Conjugation Chemistry, Immunoreactivity and Kit Formulation, *J. Nucl. Med.* **37** (1996) 362–370.
- [13] GOEDEMANS, W.T.H., et al., Technetium and Rhenium in Chemistry and Nuclear Medicine, 3rd edn (Nicolini M., Bandoli G., Mazzi U., Eds), Verona (1990) 595–603.
- [14] JOIRIS, et al., A New Method for Labelling of Monoclonal Antibodies and their Fragments with Technetium-99m, *Nucl. Med. Biol.* **18** (1991) 353–356.
- [15] PATRICIO, L., et al., A Rapid and Efficient Method for Labelling Antibodies with $^{99}\text{Tc}^{\text{m}}$ Personal Communication, IAEA RCM, Kuala Lumpur (1991).
- [16] PATRICIO, L., et al., Radiochemical and Biological Properties of $^{99}\text{Tc}^{\text{m}}$ Human Immunoglobulin Prepared Via 2-Iminothiolane, *J. Nucl. Med.* **39** (1995) 74–75 (Abstract).
- [17] LINDMO, T., et al., Determination of the Immunoreactive Fraction of Radiolabelled Monoclonal Antibodies by Linear Extrapolation to Binding at Infinite Antigen Excess, *J. Imm. Method* **72** (1984) 77–89.
- [18] JOHN, E., et al., Structural Perturbations of Monoclonal Antibodies Following Radiolabelling: In Vitro Evaluation of Different Techniques, *Nucl. Med. Comm.* **15** (1994) 24–28.

Research contract No. THA-6363/RB

Title of the Project : Development of Instant Kits $^{99}\text{Tc}^{\text{m}}$ -Labelling of Anti-CEA Antibody and hIgG for Scintigraphy

Chief Scientific Investigator : V. Boonkitticharoen
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2.8.1. Introduction

$^{99}\text{Tc}^{\text{m}}$ -labelled monoclonal antibodies (MAbs) and human immunoglobulins (hIgG) have recently emerged as a new class of site specific radiopharmaceuticals [1–4]. The role of $^{99}\text{Tc}^{\text{m}}$ -labelled MAbs particularly anti-CEA IgG in tumour imaging has essentially established for early revealing of occult lesions in patients. Superiority of the method over X ray CT has been addressed for its capability in differentiating post-operation fibrosis from viable tumour [1–2].

On equal basis, radiolabelled hIgG has been repeatedly demonstrated for their effectiveness in detecting sites and delineating extent of disease involvement in a variety of infectious diseases and severe inflammatory processes [3, 5], such as, musculoskeletal infections, abscess, infections of vascular and orthopedic prostheses, etc. Most interesting of all is the infection detection in immunocompromised patients in whom signs and symptoms are often missing [6]. $^{99}\text{Tc}^{\text{m}}$ -labelled hIgG would continue to receive widespread acceptance for several reasons, namely: (a) The agent is as effective as labelled leukocytes in detection of infection/inflammation. (b) The method for radiolabelling of IgG is less technically demanding than blood cell labelling, (c) $^{99}\text{Tc}^{\text{m}}$ is the radionuclide emitting gamma radiation ideal for imaging. (d) IgG poses no threat to HIV contamination as is the case of handling human blood cells. (e) The molecule does not elicit HAMA like antibodies of murine origin.

At present date, instant kits for preparation of this particular class of radiopharmaceuticals can be obtained from commercial sources but unfortunately at high prices. This prohibits the use of these promising diagnostic agents in developing country like Thailand. Under the assistance from IAEA through a research coordinating program, we worked on the $^{99}\text{Tc}^{\text{m}}$ -radiochemistry in immunoglobulin labelling to establish the knowledge and acquire the knowhow in the development of in-house instant kits at low cost to serve the local nuclear medicine clinics in diagnosis of infectious and neoplastic diseases.

2.8.2. Materials and methods

2.8.2.1 Antibodies and human immunoglobulins

Three MAbs and two hIgGs as described in Table I were investigated where the technique of direct labelling [7] could successfully bring about high labelling efficiency.

2.8.2.2. Reduction of MoAb and hIgG

The MAbs and hIgGs were reduced with 2-mercaptoethanol (2-ME) at various molar ratios (2-ME:IgG) by procedure described in summary report of this CRP meeting at Malaysia in 1991. The reduced antibody solution after gel chromatography was adjusted to concentrations between 0.55–0.65 mg/mL and sterilized by 0.22 μm Durapore membrane filter. The solution was purged with sterile N_2 gas and stored under -60° until use.

TABLE I. ANTITUMOUR MONOCLONAL ANTIBODIES AND POLYCLONAL HUMAN IMMUNOGLOBULINS USED IN THE STUDY OF $^{99}\text{Tc}^{\text{m}}$ -LABELLING BY DIRECT TECHNIQUE

Designation	Clonal	Isotype/ subclass	Antigenic target	Source
M-170	Monoclonal	IgG1	Cytokeratin	BIOMIRA, (Canada)
ior-CEA	Monoclonal	IgG1	CEA	CIMAB, (Cuba)
EMD	Monoclonal	IgG2a	EGFR	Dr.Baum, (Germany)
Sandoglobulin	Polyclonal	IgG (95%)	Bacteria	Sandoz, (Switzerland)
Venoglobulin	Polyclonal	IgG (95%)	Bacteria	Alpha, (USA)

TABLE II. STANNOUS KITS FOR TECHNETIUM REDUCTION

Component	MDP-SnF ₂ kit	MDP-SnCl ₂ kit
MDP(mg/mL)	5	5
Sn(II)(mg/mL)	0.258	0.425
pH	between 6.5 and 7	between 6.5 and 7
Diluent	normal saline	normal saline
Storage	-20°C	-20°C
Shelf life	~1 year	~1 year

2.8.2.3. Stannous kits for $^{99}\text{Tc}^{\text{m}}$ reduction

Sn(II)-MDP (Medtronate) kits were used for $^{99}\text{Tc}^{\text{m}}$ reduction. Kit formulations are presented in Table II. Experiments were conducted to find out the optimal μg of Sn(II) in forms of SnF₂ and SnCl₂ that required for the conjugation of 37 MBq of $^{99}\text{Tc}^{\text{m}}$ into the antibody molecules.

2.8.2.4. Radiolabelling

Thaw the frozen antibody solution and allow it to warm up to room temperature. Add desirable mL of medronate kit into antibody vial and followed by predetermined activity of $^{99}\text{Tc}^{\text{m}}$. Swirl gently every 2–3 min for 15 min. Labelling efficiency was determined by instant thin layer chromatography (ITLC). For ITLC-SG using normal saline solution as developing agent, it yielded TcO_4^- and $^{99}\text{Tc}^{\text{m}}$ -MDP at the solvent front. While HSA-impregnated ITLC-SG developed by mixture of EtOH:NH₄OH:H₂O :: 2:1:5, it retained hydrolysed reduced Tc (HRTc) at the origin. After subtracting percentage TcO_4^- , $^{99}\text{Tc}^{\text{m}}$ -MDP and HR- $^{99}\text{Tc}^{\text{m}}$ from 100%, it yielded the percentage of radioactivity incorporated into the antibody molecules.

2.8.2.5. Immunoreactivity assay

Antibody immunoreactivity was assessed by ELISA technique using tumour cells expressing antigens of interest. A CEA-producing cell line, i.e. LS 174T (human colonic adenocarcinoma), and pharyngeal squamous cell carcinoma (FaDu) bearing epidermal growth factor receptor (EGFR) were used for investigating say ior-CEA and EMD respectively. Target cells for assay were prepared as follows: Plate fixed number of tumour cells (3.3×10^5 cells for LS174T with plating efficiency of 97.3% and 1.5×10^5 cells for FaDu with plating efficiency of 99.1%) into each well of the 96-well plate. Cells were cultured for 24 h and then fixed with 2% formalin for 1 h at room temperature. The fixed cells were stored under 4°C for antibody assay (shelf life ~1 year).

After saturating the non-specific binding sites with 1% bovine serum albumin, add 200 μL of MAbs with varied concentrations i.e. ior-CEA 0.1–1.0 $\mu\text{g/mL}$; EMD 0.009–0.15 $\mu\text{g/mL}$, to their respective target cells. Reactions were allowed to proceed at 4°C for 1 hour and target cells were washed 3 times with PBS. Target cells with bound antibodies were incubated with 200 μL of horse radish peroxidase (HRP)-conjugated rabbit antimouse IgG (Zymed Laboratory, USA). Quantitate HRP-conjugated IgG with 150 μL of o-phenylene diamine (OPD) and H_2O_2 (1mL of 0.1 M citrate buffer, pH5, contained 1.08 mg of OPD and 0.03% H_2O_2). The reaction was stopped with 50 μL of 2N H_2SO_4 after 15 min of incubation under subdued lighting at room temperature. The solution in each well was transferred to another plate for optical density (OD) measurement (EL311S Autoreader, Biotek, USA). Slope from the double inverse plot between antibody concentration and OD yield an index for immunoreactivity [8].

2.8.2.6. Cysteine challenge assay

$^{99}\text{Tc}^{\text{m}}$ -labelled MAbs and hIgGs were assayed for their stability towards cysteine transchelation at molar ratio (Cysteine:IgG) between 500:1, 50:1, 5:1, 0.5:1, 0:1. The procedure appeared in summary report of this CRP meeting at Australia in 1994.

2.8.2.7. Animal biodistribution study

Four h biodistribution of $^{99}\text{Tc}^{\text{m}}$ -labelled MAbs and hIgGs were measured using Balb/C female mice. The results were expressed as mean percentage of the injected dose per gm tissue with one standard error. Detailed methodology was described in summary report of this CRP meeting at Malaysia in 1991.

2.8.2.8. *Limulus amoebocyte lysate (LAL)* assay

Three lots of each reduced MoAb and hIg with the addition of MDP-SnF_2 were measured for endotoxin unit by a commercial agency licensed for performing LAL assay. The endotoxin unit was quantitated by chromogenic LAL method (Kinetic GCL System, Bio Whittacker, USA).

2.8.3. Results

2.8.3.1. Stoichiometry in $^{99}\text{Tc}^{\text{m}}$ -labelling of MAbs and hIgGs

The direct technique as suggested by Mather and Ellison [7] was adopted here for its technical feasibility and high labelling efficiency. The technique involves the use of 2-ME to reduce interchain disulfide bridges at hinge region to sulfhydryl groups for subsequent conjugation of $^{99}\text{Tc}^{\text{m}}$ which is presented in the form of weak complex with MDP. Efficiency of $^{99}\text{Tc}^{\text{m}}$ -labelling depended on a number of factors, namely: molar ratio for antibody reduction, concentrations of antibody for reduction and labelling, quantity of Sn(II) for $^{99}\text{Tc}^{\text{m}}$ reduction.

All types of molecules investigated here differed among each other in their susceptibility to 2-ME reduction. MAbs that were resistant to 2-ME reduction, i.e., M170 and EMD, required tin in form of SnCl_2 instead of SnF_2 for optimal reduction of $^{99}\text{Tc}^{\text{m}}$ for antibody incorporation (Table III). ior-CEA and M170 of the same IgG1 subclass or 2 preparations of hIgs which comprised similar IgG subclass distributions did not appear to share the same radiochemistry in labelling. Under optimal stoichiometry, however, all molecules could accommodate sufficient radioactivity for patient study.

TABLE III. STOICHIOMETRIC FACTORS IN ANTIBODY REDUCTION AND $^{99}\text{Tc}^{\text{m}}$ LABELLING

Antibody	Antibody reduction		$^{99}\text{Tc}^{\text{m}}$ -Labelling	
	Ab (mg/mL)	2-ME:Ab	Ab $\mu\text{g Sn(II)}/\text{mCi}^{99}\text{Tc}^{\text{m}}$ (mg/mL)	Attainable specific activity (GBq/mg)
M170	5	2000:1	>0.55 0.5, SnCl_2	>0.89
ior-CEA	7–9	1000:1	>0.55 0.1, SnF_2	>3.92
EMD	5	6000:1	>0.55 0.5, SnCl_2	>4.42
Sandoglobulin	10	500:1	>0.55 0.1, SnF_2	>2.96
Venoglobulin	10	1000:1	>0.55 0.1, SnF_2	>1.48

2.8.3.2. Characterization of $^{99}\text{Tc}^{\text{m}}$ -labelled MABs and hIgs

Immunoreactivity of $^{99}\text{Tc}^{\text{m}}$ -labelled MABs

Because of the lack of antigenic targets for some MABs and hIgs, this part of the study focused only on 2 clones of MABs which exhibited different degree of resistivity to 2-ME reduction, i.e. ior-CEA with molar ratio for reduction at 1000:1 and EMD at 6000:1. By ELISA technique, bindings of ior-CEA and EMD were tested using 2 respective cell lines, namely: LS174T (colonic adenocarcinoma) for ior-CEA and FaDu (Pharyngeal squamous cell carcinoma) for EMD. Change in antibody binding as a consequence of chemical modification or radiolabelling was expressed in terms of immunoreactivity index which was defined as the ratio of Line Weaver Burk slope of native antibody binding curve to that of the modified antibody [8]. The 2-ME has long been known to induce the opening of hinge region by reduction of the interheavy chain disulfide bridges [9]. Although the polypeptide chains remain associated by noncovalent bonding [9, 10], the question of how this alteration in hinge region would affect antibody immunoreactivity has never died out. In our study, we observed no change in immunoreactivity in reduced MABs no matter how high the reduction level was (Table IV). However, significant decline in immunoreactivity was recognized in $^{99}\text{Tc}^{\text{m}}$ -labelled ior-CEA at the specific activity as high as 3.92 GBq/mg. Whilst in EMD which was subjected to high level of reduction, i.e. 6000:1, adverse change started to manifest at specific activity as low as 1.10 GBq/mg. Our findings seemed to suggest that 2-ME reduction would generate abnormality in antibody molecule to an extent which depended on the degree of reduction. $^{99}\text{Tc}^{\text{m}}$ -conjugation appeared to behave as a means to precipitate the hidden abnormality to manifest as adverse change in antibody binding.

Stability to cysteine challenge

$^{99}\text{Tc}^{\text{m}}$ -labelled to MABs could be transchelated in vivo possibly to cysteine species [11]. Stabilities of $^{99}\text{Tc}^{\text{m}}$ -labelled MABs and hIgs to cysteine challenge in vitro were regarded as another criterion in MoAb or hIg characterization. ior-CEA, EMD, Sandoglobulin and Venoglobulin were exposed to cysteine at the molar ratio of 0, 0.5:1, 5:1, 50:1 and 500:1. The magnitude of radioactivity released as a consequence of cysteine transchelation was assayed by ITLC technique using 0.1 M phosphate buffer as developing agent. It happened that $^{99}\text{Tc}^{\text{m}}$ -labelled ior-CEA represented the most stable compound, followed by Sandoglobulin and then EMD, Venoglobulin, the least (Table V). Stability to cysteine transchelation seemed to reflect individuality in antibody conformation. Since it neither correlated to antibody clonality, monoclonal versus polyclonal, nor to number of disulfide bridges in antibody subclass, IgG1 versus IgG_{2a}. Our previous study also indicated M170 of IgG1 subclass was less stable to serum protein transchelation than ior-CEA.

TABLE IV. EFFECT OF $^{99}\text{Tc}^{\text{m}}$ -LABELLING ON ANTIBODY IMMUNOREACTIVITY

Antibody	Subclass	2ME:Ab	Specific activity (GBq/mg)	Immuno-reactivity Index	t-test
ior-CEA (anti-CEA)	IgG1	1000:1	0	1.061	p>0.5
			1.39	0.979	p>0.5
			2.62	0.976	p>0.5
			3.92	0.719	p<0.02
EMD (anti-EGFR)	IgG2a	6000:1	0	0.990	p>0.5
			0.55	0.842	p>0.5
			1.10	0.734	p<0.001
			2.29	0.745	p<0.005
			4.42	0.716	p<0.001

Level of significance: $p \leq 0.005$.

TABLE V. IN VITRO STABILITY TO CYSTEINE CHALLENGE OF DIFFERENT ANTIBODIES AND IMMUNOGLOBULINS

Cysteine:Ab	ior-CEA	EMD	Sandoglobulin	Venoglobulin
0	5.57±0.07	4.20±0.50	3.88±0.20	3.03±0.19
0.5:1	4.15±0.09	5.10±1.25	3.69±0.09	3.50±0.35
5:1	4.92±0.15	6.91±1.15	4.83±0.35	6.89±0.19
50:1	13.55±0.50	29.10±2.08	24.80±0.66	41.70±0.69
500:1	33.15±0.90	65.59±2.63	55.37±1.11	72.20±0.96

TABLE VI. THE FOUR-HOUR BIODISTRIBUTIONS OF $^{99}\text{Tc}^{\text{m}}$ -LABELLED MABs AND hIgs. THE DATA, MEAN \pm SE, ARE EXPRESSED AS PERCENTAGE OF INJECTED DOSE PER GRAM OF TISSUE

Organ	Monoclonal antibody			Polyclonal human immunoglobulin	
	M170	ior-CEA	EMD	Sandoglobulin	Venoglobulin
Blood	14.25±0.78	16.60±1.53	3.19±0.27	13.32±1.86	19.61±1.21
Liver	5.86±0.38	5.02±0.51	7.90±0.47	3.82±0.47	5.12±0.36
Spleen	5.41±0.42	4.95±0.34	1.35±0.19	4.50±0.43	5.63±0.18
Heart	3.92±0.23	4.78±0.42	1.12±0.11	4.45±0.28	7.79±0.34
Stomach	3.13±0.17	2.46±0.27	35.61±3.86	3.09±0.45	2.38±0.27
Kidney	8.88±0.52	8.47±0.57	6.27±0.30	23.24±2.27	16.00±0.83
Femur	2.04±0.33	1.79±0.17	1.10±0.07	1.47±0.20	2.10±0.56
Muscle	1.12±0.16	1.68±0.18	0.83±0.12	1.04±0.12	1.10±0.10

Biodistribution in mice

Four h biodistributions of $^{99}\text{Tc}^{\text{m}}$ -labelled MABs and hIgs were investigated using BALB/C female mice (Table VI). MABs, ior-CEA and M170, of the same IgG₁ subclass displayed similar organ distribution, i.e. high blood activity and relatively high kidney uptake. Polyclonal hIgs that showed different susceptibility to cysteine challenge exhibited the same patterns of organ distributions.

In Venoglobulin, there was 0.2 mg of human serum albumin (HSA) for every mg of IgG. In our initial work of HSA labelling, we noted that HSA could be effectively labelled with $^{99}\text{Tc}^{\text{m}}$ by direct technique. Slightly higher blood activity than what was observed for $^{99}\text{Tc}^{\text{m}}$ -labelled Sandoglobulin could be due partly to the presence of $^{99}\text{Tc}^{\text{m}}$ -labelled HSA which was known as a blood pool agent. In contrast to the rest of the proteins, EMD possessed low blood activity and extremely high uptake in stomach. Experiments were not conducted to verify whether the cause of high activity was the consequence of excretion of TcO_4 from liver or the expression of high level of EGFR in stomach.

2.8.3.3. Development of instant kits and assessment of kit performances

Because of the limited availability of some MAb, instant kits were developed for ior-CEA, Sandoglobulin and Venoglobulin.

Preparation of the instant kits

The instant kit was a two-component unit, i.e. reduced MoAb or reduced hIg and concentrated MDP-SnF₂ solution.

(a) Component A. Reduced MoAb or hIg: ior-CEA or Sandoglobulin or Venoglobulin was reduced under the stoichiometry as presented in Table III. After 30 minutes of reduction at room temperature, free 2-ME was removed by a sterile Sephadex column (2g, medium G-50) prechilled to 4°C. N₂ purged PBS (pH7.2–7.4) was used as eluent. By spectroscopy ($E^{1\%}_{1\text{cm}} = 14.3$ at 280 nm), the protein peak was collected and final concentration was adjusted to 0.55–0.65 mg/mL. The MoAb or hIg solution was sterilized by 0.22 μm membrane filter. Two mL aliquot of the MoAb or hIg solution was dispensed in each 5 mL sterile vial with rubber septum. Fill the head space with sterile N₂ gas. Kits were stored frozen under –60°C.

(b) Component B. MDP-SnF₂ solution: Each mL of 5x solution of MDP-SnF₂ contained 5 mg of MDP and 340 μg of SnF₂. The solution was stored under –20°C (shelf-life >1 year) until use. Before labelling, the frozen solution was thawed and diluted with N₂ purged normal saline to make 1 × solution. Each mCi (37 MBq) of $^{99}\text{Tc}^{\text{m}}$ required 0.15–0.20 μg of SnF₂ for optimal reduction of $^{99}\text{Tc}^{\text{m}}$.

Apyrogenicity

Apyrogenicity was determined by chromogenic LAL assay.

ior-CEA kit: less than 59.9 ± 42.4 EU/mg of endotoxin.

Sandoglobulin and Venoglobulin kits: less than 9.7 ± 0.2 EU/mg of endotoxin.

Endotoxin limit: FDA = 350 EU/mg; USP = 175 EU/mg

Radiolabelling

Thaw the frozen kit, i.e. components A and B, and allow the unit to warm up to room temperature. Add MDP-SnF₂ (0.05 mL for hIg and 0.1 mL for MoAb) into component A then followed by $^{99}\text{Tc}^{\text{m}}$ (740 MBq for hIgs and 1480 MBq for ior-CEA). Swirl gently every 2–3 minutes. The reaction was allowed to proceed at room temperature for 15 min. Labelling efficiency was determined by the two strip technique of instant thin layer chromatography (ITLC). ITLC-SG used normal saline solution as developing agent and HSA-impregnated ITLC-SG developed by mixture of EtOH:NH₄OH:H₂O=2:1:5. Subtracting the percentage of radiochemical impurities from 100%, we obtained the percentage of radioactivity incorporated into the MoAb and hIg molecules.

Stability of reduced MoAb and hIgs over storage

Reduced MoAb or hIg solution possessed a shelf life of no less than 1 year (Table VII).

TABLE VII. STABILITY OVER STORAGE OF INSTANT KITS ASSESSED BY $^{99}\text{Tc}^{\text{m}}$ -LABELLING EFFICIENCY

Month	Labelling efficiency (%)		
	ior-CEA	Sandoglobulin	Venoglobulin
1	98.36 \pm 0.08	94.05 \pm 1.09	97.81 \pm 0.81
3	98.35 \pm 0.08	98.42 \pm 0.81	97.82 \pm 0.02
6	97.06 \pm 0.13	97.37 \pm 0.16	–
9	96.25 \pm 0.62	99.15 \pm 0.74	–
12	98.84 \pm 0.12	97.01 \pm 0.19	–

Clinical trials

At present time, both the ior-CEA and Sandoglobulin kits are evaluated clinically. No adverse reactions had been reported in nearly 50 patient studies. Both radiopharmaceuticals could correctly localize lesions either known or unknown. Nevertheless, some false findings could be occasionally encountered, i.e. false positive for Sandoglobulin and false negative for ior-CEA. Studies are underway to compile solid evidences regarding the efficacy of this new class of radiopharmaceuticals.

2.8.4. Discussion

High count-rate from $^{99}\text{Tc}^{\text{m}}$ -labelled antibody allows the great ease in distinguishing regions of specific from non-specific uptake [12–14]. Granoska, et al. [15] have demonstrated the most accurate results in diagnosis of ovarian cancers from the use of $^{99}\text{Tc}^{\text{m}}$ -labelled antibody in comparing to other radiolabels, such as ^{111}In and ^{123}I . Extensive studies on radiochemistry in $^{99}\text{Tc}^{\text{m}}$ labelling of immunoglobulin molecules have been conducted ever since. There are several techniques for conjugation of $^{99}\text{Tc}^{\text{m}}$ with antibody molecule either by direct or indirect approach [16]. Schwarz technique in direct labelling [7] has gained widespread interest for its technical simplicity and its effectiveness in coupling $^{99}\text{Tc}^{\text{m}}$ atoms with stable binding sites, presumably the thiolate groups at hinge region of IgG molecule [7, 17, 18]. Besides, antibodies labelled by this technique are employed by many institutes in Europe in large number of patient studies which yield satisfactory outcomes [16].

In this study, Schwarz technique could successfully bring about labelling of 3 MAbs and 2 hIgs with $^{99}\text{Tc}^{\text{m}}$ to yield labelling efficiency of greater than 95%. This obviated the need for post labelling purification. The $^{99}\text{Tc}^{\text{m}}$ binding sites as generated by 2-ME reduction were sufficient for incorporation of $^{99}\text{Tc}^{\text{m}}$ to the level of specific activity that made the MoAb or hIg an imaging agent. In terms of antibody immunoreactivity, 2-ME reduction seemed to induce some degree of abnormality in population of reduced MoAb or hIgs molecules. Apparently, the abnormality thus induced was mild and required $^{99}\text{Tc}^{\text{m}}$ -complexation for manifestation. For antibody reduced at 1000:1 in molar excess, it required a specific activity as high as 3.92 GBq/mg for expression. This specific activity, however, was higher than the level adopted in routine preparation. In antibody, i.e. EMD, that required harsh reduction, 6000: 1 in molar excess, the abnormality (30% loss in immunoreactivity) appeared at lower level of specific activity, 1.10 GBq/mg. Nevertheless, no further loss in immunoreactivity could be recognized upon raising the specific activity up to 4.42 GBq/mg. This study suggested that hinge reduction, although generated some abnormalities, did not prevent the MAb, even the one that received harsh treatment from being a good tumour imaging agent. Since the increase in specific activity would enhance the detection signal to compensate some degree of loss in immunoreactivity.

Despite differences in labelling chemistry, similar biodistribution profiles in mice were observed among MAbs that belonged to the same IgG₁ subclass, or among hIg preparations that showed similar IgG subclass distribution. High blood and kidney activities as observed for molecules used in this study was not the unusual distribution characteristics for this class of $^{99}\text{Tc}^{\text{m}}$ -labelled radiopharmaceuticals.

Clinically, reduction-mediated labelling yields $^{99}\text{Tc}^{\text{m}}$ -labelled MABs or hIGs of high capability in disease diagnosis. Technically, the procedures in reduction and radiolabelling are simple enough for any hospital pharmacies to master and to develop their own in-house instant kits. In our experiences, the kits, even in form of frozen solutions, were highly stable with a shelf life of 1 year. The obvious advantage of the in-house kits rest not only on its readily availability but also on its low cost when compared to kits from commercial sources. The latter appears to be one of the critical factors which permits the application of this modern nuclear medicine technology in developing countries where expensive health cares are nonviable.

2.8.5. Conclusions

Direct $^{99}\text{Tc}^{\text{m}}$ -labelling of immunoglobulin by Schwarz technique could bring about high labelling efficiency in all MABs of different subclasses and hIGs from two commercial sources. Thiol reduction by 2-ME appeared to generate abnormality in antibody molecule where it remained concealed under the labelling scale performed in routine radiopharmacy. Almost all molecules labelled exhibited biodistribution profiles characterized as common distribution patterns observed in many $^{99}\text{Tc}^{\text{m}}$ -MABs and $^{99}\text{Tc}^{\text{m}}$ -hIGs. Reduced MABs and hIGs were highly stable for year even in the form of frozen solution. This vitalized the idea of in-house kit preparation not only for on-site use but also make nuclear medicine diagnosis by $^{99}\text{Tc}^{\text{m}}$ -hIGs an inexpensive technology.

REFERENCES

- [1] LARSON, S.M., Clinical Immunodetection, 1978–1988: Overview and Suggestion for Standardization of Clinical Trials, *Cancer Res. (Suppl.)* **50** (1990) 892S–898S.
- [2] GOLDENBERG, D.M., GOLDENBERG, H., et al., Clinical Studies of Cancer Radioimmunodetection with Carcinoembryonic Antigen Monoclonal Antibody Fragments Labelled with ^{125}I or $^{99}\text{Tc}^{\text{m}}$, *Cancer Res. (Suppl.)* **50** (1990) 909S–912S.
- [3] RUBIN, R.H., FISCHMAN, A.J., The Use of Radiolabelled Non-specific Immunoglobulin in the Detection of Focal Inflammation, *Semin. Nucl. Med.* **24** (1994) 169–179.
- [4] DATZ, F.L., The Current Status of Radionuclide Infection Imaging (Nuclear Medicine Annual, 1993), (Freeman L.M. ed.), Raven Press, New York (1993) 47–76.
- [5] DATZ, F.L., ANDERSON, C.E., et al., The Efficacy of Indium-111-Polyclonal IgG for the Detection of Infection and Inflammation, *J. Nucl. Med.* **35** (1994) 74–83.
- [6] RUBIN, R.H., FISHMAN, A.J., et. al., Indium-111-Labelled Non-specific Immunoglobulin Scanning in the Detection of Focal Infection, *N. Engl. J. Med.* **321** (1989) 935–940.
- [7] MATHER, S.J., ELLISON, E., Reduction-mediated Technetium-99m Labelling of Monoclonal Antibodies, *J. Nucl. Med.* **31** (1990) 347–359.
- [8] BOONKITTICHAROEN, V., LAOHATHAI, K., Analysis of Immuno-radiological Properties of Radiolabelled Monoclonal Antibody for Tumour Localization, *Nucl. Med. Commun.* **13** (1992) 342–348.
- [9] MECHAELEN, T.E., Alteration of the Conformation of Human IgG Subclasses by Reduction of the Hinge S–S bonds, *Molec. Immunol.* **25** (1988) 639–646.
- [10] BEHR, T., BECKER, W., et al., Structural Modifications of Monoclonal Antibodies following Direct Versus Indirect Labelling with $^{99}\text{Tc}^{\text{m}}$: Does Fragmentation Really Occur? *Nucl. Med. Commun.* **15** (1994) 865–870.
- [11] MARDIROSSIAN, T., WU, C., et al., The Stability of $^{99}\text{Tc}^{\text{m}}$ Directly Labelled to an Fab Antibody via Stannous Ion and Mercaptoethanol Reduction, *Nucl. Med. Commun.* **13** (1992) 503–512.
- [12] GRANOSKA, M., MATHER, S.J., et al., $^{99}\text{Tc}^{\text{m}}$ Radioimmunosintigraphy of Colorectal Cancer, *Br. J. Cancer* **62** (Suppl. X) (1990) 30–33.
- [13] GRANOSKA, M., BRITTON, K.E., et al., Radioimmunosintigraphy with Technetium-99m-Labelled Monoclonal Antibody, SM₃, in Gynaecological Cancer, *Eur. J. Nucl. Med.* **20** (1993) 483–489.
- [14] GRANOSKA, M., BRITTON, K.E., et al., Radioimmunosintigraphy with Technetium-99m Labelled Monoclonal Antibody, IA3, in Colorectal Cancer, *Eur. J. Nucl. Med.* **20** (1993) 690–698.

- [15] GRANOSKA, M., MATHER, S.J., et al., Radiolabelled Stripped Mucin, SM3, Monoclonal Antibody for Immunoscintigraphy of Ovarian Tumours, *Int. J. Biol. Markers* 5 (1990) 89–96.
- [16] REILY, R.M., Immunoscintigraphy of Tumours using ^{99m}Tc -Labelled Monoclonal Antibodies: A Review, *Nucl. Med. Commun.* 14 (1993) 347–359.
- [17] ECKELMAN, W.C., STEIGMAN, J., Direct Labelling with ^{99m}Tc , *Nucl. Med. Biol.* 18 (1993) 3–6.
- [18] RHODES, B.A., Direct Labelling of Proteins with Tc-99m, *Nucl. Med. Biol.* 18 (1991) 667–676.

Annex
RECOMMENDED PROCEDURES FOR RADIOLABELLING OF ANTIBODY
(ior-CEA-1) WITH $^{99}\text{Tc}^{\text{m}}$ AND ITS EVALUATION

Antibody reduction

- (a) Concentrate the antibody to 5–10 mg/mL.
- (b) Prepare a solution of phosphate buffered saline (PBS) pH7.4. Purge the buffer with nitrogen and chill to 4°C.
- (c) Prepare a column of Sephadex G-50 or equivalent. The column volume should be at least 20 times the volume of antibody solution used. Wash the column with at least 3 volumes of cold N_2 -purged PBS. Keep the column cold until required for use.
- (d) To an aliquot of concentrated antibody add 2-mercaptoethanol (2-ME) at a molar ratio of 1000:1 (equivalent to 0.47 μL of 2-ME per mg of antibody). (If necessary for accurate measurement, freshly dilute 2-ME 10 fold in water before use.).
- (e) Incubate 2-ME/antibody mixture at room temperature (20°C) approximately for 30 min.
- (f) Take a small aliquot (about 100 μL of the reaction mixture) and chill to 4°C. Apply the rest of the mixture to the top of the gel filtration column and elute with cold N_2 purged PBS. Collect 1 mL fractions and keep in closed tubes on ice.
- (g) Measure the OD at 280 nm of fractions until the protein peak is obtained. Pool all fractions with a protein concentration of > 0.5 mg/mL. Divide the pooled antibody into suitable aliquots (100–500 μL) and store at –70°C until used for radiolabelling.

In case of the preparation of lyophilized kits, add stannous-MDP solutions containing 5 μg stannous chloride, 50 μg MDP and 10 mg glucose per mg antibody before dividing the pooled antibody into aliquots containing 1 mg antibody. Freeze vials at –70°C and introduce into precooled freeze dryer. Cool the condenser till temperature reaches about –50 °C. Turn on the vacuum pump and at 100 μ vacuum in the drying chamber start heating the shelf until the temperature reaches 30°C. At a vacuum of about 10 μ freeze drying of the antibody samples is completed. Isolate the drying chamber and fill it up with N_2 gas of high purity. Close the vials inside the drying chamber using an automatic sealing system. The lyophilized material should not contain more than 1% residual moisture and should be stored refrigerated.

Radiolabelling of antibody

- (a) Thaw a reduced antibody aliquot at room temperature.
- (b) Reconstitute a MDP standard kit (5 mg MDP and 1 mg stannous chloride) with 5 mL saline solution.
- (c) Add to the antibody an aliquot of MDP kit solution (~50 μL) containing 5 μg stannous chloride and 50 μg MDP per mg antibody.
- (d) Add up to 1 GBq $^{99}\text{Tc}^{\text{m}}$ pertechnetate (in <3 mL) solution and mix. Incubate for 10 min at room temperature to complete the reaction. For radiolabelling of freeze dried antibody follow the protocol from (d) onwards.
- (e) Measure radiolabelling efficiency chromatographically using Whatman No.1 paper or ITLC-SG strip (Gelman, USA) as a stationary phase and 0.9% saline as a mobile phase. Free pertechnetate moves away (R_f 1.0), leaving the protein at the point of application.

Cysteine challenge assay

Make up a fresh 0.083M solution of L-cysteine (store cysteine desiccated in the refrigerator) in 0.4 M phosphate buffer, pH7. By successive dilutions of 100 μL of this solution with 900 μL of the phosphate buffer, make up four solutions from 0.0083 M to 0.0000083 M. Add to these four a fifth solution of buffer alone, i.e. zero cysteine concentration. Obtain about 0.5 mL of a 2.4 μM solution of antibody. To 12 μL of each of the five cysteine solutions add 90 μL of the antibody solution. Thus the most concentrated cysteine solution has 1 mM concentration. The remaining solutions are 0.1, 0.01,

0.001 and zero mM cysteine concentrations, whereas the antibody concentration in all solutions remains $90/102 \times 2.4 \mu\text{M}$ or $2.1 \mu\text{M}$. Thus the highest molar ratio of cysteine to antibody is $1000 \mu\text{M}/2.1 \mu\text{M}$ or approximately 500:1. Incubate each solution at 37°C for an h. Thereafter, analyse in triplicate each solution by paper chromatography using Whatman No. 1 paper and 0.1M phosphate buffer pH7.0 as mobile phase. Only labelled cysteine and pertechnetate will move away. After the chromatogram development each strip may be cut in two halves and counted separately.

Serum stability

This assay requires that samples of fresh human serum be incubated, preferably with mild shaking, in a 37°C incubator for 24 h. The labelled antibody is added to the serum at a concentration which approaches that to be expected in vivo ($1\text{--}10 \mu\text{g/mL}$). It is necessary to ensure that the pH of the serum does not change significantly during incubation. After 24 h analyse the mixture size by exclusion HPLC (in which case the in-line detector will probably be too insensitive and fractions will need to be collected for external counting), gel electrophoresis, paper or ITLC. Any loss of label from the antibody will be evidence of instability.

Animal biodistribution

An aliquot of $100 \mu\text{L}$ of the radiolabelled antibody solution containing $10\text{--}30 \mu\text{g}$ protein and 2 MBq radioactivity in normal saline or 0.05 M phosphate buffer, pH7.0 is administered intraperitoneally/intravenously to a minimum of 5 normal Balb/C mice of approximately 25 g each fed with food and water ad libitum. After 4 hours the animals are sacrificed (by spinal dislocation) and samples of blood ($50\text{--}100 \mu\text{L}$), the entire liver, spleen, heart, stomach with contents, femur, both kidneys and samples of thigh muscle free of fat are removed. All tissues are rinsed in cold saline immediately after their removal, dried in filter paper folds and counted in a sodium iodide crystal well type counter calibrated for $^{99}\text{Tc}^{\text{m}}$ against a standard of the injectate. The results are expressed as mean percentage of the injected dose per gm (or per mL in case of blood) with standard deviation.

Immunoreactivity

Prepare a coating solution containing $0.25 \mu\text{g}$ of carcino-embryonic antigen (CEA) per $200 \mu\text{L}$ of PBS and with enough volume so that $200 \mu\text{L}$ may be added to each tube to be coated. Since the analysis is to be performed in triplicate, 24 (8×3) tubes will be required for any single antibody to be tested and 24 more for the analysis of the native control. Add $200 \mu\text{L}$ of the coating solution to each $12 \times 75 \text{ mm}$ polystyrene test tube and leave it undisturbed at room temperature over night. Aspirate the coating solution and save it (since it will still contain part of the unbound CEA). Block the free binding sites of each test tube by incubating for 1 h at 37°C or over night at room temperature after the addition of $500 \mu\text{L}$ of 1% of HSA solution in PBS. Aspirate the blocking solution just prior to beginning the assay. Do not let the tubes dry out. Using any standard radioiodination methodology, label $100 \mu\text{g}$ of the native antibody with ^{125}I at a specific activity of $185\text{--}370 \text{ MBq}$. Purify with a Sephadex G50 or similar column and dilute in 0.1% HSA in PBS to a concentration of 160 ng/mL . $24 \times 2 \times 25 \mu\text{L}$ will be required for each assay. Prepare serial 1:1 dilutions in 0.1% HSA in PBS of the $^{99}\text{Tc}^{\text{m}}$ labelled and native antibodies with concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.031, 0.015 and $0 \mu\text{g/mL}$. Add $50 \mu\text{L}$ of each dilution in triplicate to CEA coated tubes. Add $25 \mu\text{L}$ of ^{125}I labelled antibody. Mix well and incubate, preferably with gentle agitation at room temperature for at least two hours. Count the ^{125}I activity in each tube in a sodium iodide well type counter to obtain the total counts. Decant the tubes, wash twice with 0.1% HSA/PBS and count again to obtain the bound counts. Calculate the percentage of total counts bound in each tube and plot a graph of percentage bound against antibody concentration. The loss of immunoreactivity as a result of the $^{99}\text{Tc}^{\text{m}}$ labelling procedure can be determined by comparing the concentrations of $^{99}\text{Tc}^{\text{m}}$ labelled antibody and native antibody able to inhibit 50% of the binding of ^{125}I antibody at $0 \mu\text{g/mL}$ antibody concentration.

BIBLIOGRAPHY

- ABDEL-NABI, H., MARCHETLI, D., ERB, D., et al., Imaging of Ovarian Carcinoma Patients with Tc-99m-88 Bv59 a Totally Human Monoclonal Antibody, *J.Nucl. Med.* **37** (1996) 9 (Abstract).
- ABRAMS, M.J., JUWEID, M., TENKATE, C.I., et al., Technetium-99m-Human Polyclonal IgG Radiolabelled via the Hydrazino Nicotinamide Derivative for the Imaging Focal Sites of Infection in Rats, *J. Nucl. Med.* **31** (1990) 2022-2028.
- ADAMS, R.L.P., WORK, T.S., BURDON, R.H., (Eds), In: *Cell Culture for Biochemists*, (1980) 1-179.
- ALEXANDER, C., VILLENA-HEINSEN, C.E., TRAMPERT, L., et al., Radioimmunoscintigraphy of Ovarian Tumours with Technetium-99m Labelled Monoclonal Antibody-170: First Clinical Experiences, *Eur. J. Nucl. Med.* **22** (1995) 645-651.
- ARCHIMANDRITIS, S.C., MALAMITSI, J., BELKAS, E., et al., 99m-Tc-labelling of the MoAb HMFG1 to be used for bladder cancer detection, *J. Lab. Comp. Radioph.* **34** (1994) 323-337.
- BALABAN, E.P., WALKER, B.S., COX, J.V., et al., Radionuclide Imaging of Bone Marrow Metastases with a Tc-99m Labelled Monoclonal Antibody to Small Cell Lung Carcinoma, *Clin. Nucl. Med.* **16** (1991) 732-736.
- BECKER, W., DOLKENEYER, U., GRAMATZKI, M., et al., Use of Immunoscintigraphy in the Diagnosis of Fever of Unknown Origin, *Eur. J. Nucl. Med.* **20** (1993) 1078-1083.
- BERBERICH, R., HENNES, P., ALEXANDER, C., et al., Detection of Inflammatory Lesions and HAMA Response after Administration of the Monoclonal Antibody BW 250/183, *J. Nucl. Med.* **31** (1992) 70-73.
- BIERSACK, H.J., OVERBECK, B., OH, G., et al., Tc-99m Labelled Monoclonal Antibodies Against Granulocytes (BW 250/183) in the Detection of Appendicitis, *Clin. Nucl. Med.* **18** (1993) 371-376.
- BISCHOF-DELALOYE, A., DELALOYE, B., BUCHEGGER, F., et al., Clinical Value of Immunoscintigraphy in Colorectal Carcinoma Patients: A Prospective Study, *J. Nucl. Med.* **30** (1989) 1646-1656.
- BLOCK, D., FEITSMA, R.I.J., WASSER, M.N., et al., A New Method for Protein Labelling with 99m-Tc, *Nucl. Med. Biol.* **16** (1989) 11-16.
- BOIS, DE, M.H.W., ARNDT, J.W., TAK, P.P., et al., 99m-Tc Labelled Polyclonal Human Immunoglobulin-G Scintigraphy before and after Intra-articular Knee Injection of Triamcinolone Hexacetonide in Patients with Rheumatoid Arthritis, *Nucl. Med. Commun.* **14** (1993) 883-887.
- BOUBAKER, A., BISCHOF-DELALOYE, A., BLANC, C.H., et al., Immunoscintigraphy with Antigranulocyte Monoclonal Antibodies for the Diagnosis of Septic Loosening of Hip Prostheses, *Eur. J. Nucl. Med.* **22** (1995) 139-147.
- BREE, R.DE., ROOS, J.C., QUAKE, J.J., et al., Clinical Imaging of Head and Neck Cancer with Technetium-99m Labelled Monoclonal Antibody E 48 IgG or F(ab)₂, *J. Nucl. Med.* **35** (1994) 775-783.
- CALANE, W., FEITSMA, H.I.J., ENSING, G.J., et al., Detection of a Local Staphylococcal Injection in Mice with Technetium-99m-Labelled Polyclonal Human Immunoglobulin, *J. Nucl. Med.* **32** (1991) 468-474.
- CHILDS, R.L., HNATOWICH, D.J., Optimum Conditions for Labelling of DTPA-coupled Antibodies with Technetium-99m, *J. Nucl. Med.* **26** (1985) 293-299.

- CHOI, C.W., CHUNG, J.K., LEE, D.S., et al., Development of Bone Marrow Immunoscintigraphy Using a Tc-99m Labelled Anti NCA-95 Monoclonal Antibody, *Nucl. Med. Biol.* **22** (1995) 117-123.
- CORSTENS, F.H.M. and CLAESSENS, R.A., Imaging Inflammation with Human Polyclonal Immunoglobulin: Not Looked for but Discovered, *Eur. J. Nucl. Med.* **19** (1992) 155-158.
- DAHLQUIST, F.W., The Meaning of Scatchard and Hill Plots, *Methods in Enzymology*; XLVIII (1978) 271-299.
- DENNIS, W.W., JACK, I.E., WILL, W.J.R., Detection of Acute Infection/Inflammation with Tc-99m Labelled Intact Polyvalent Human IgG, *Nucl. Med. Biol.* **22** (1995) 513-519.
- DOMINGUEZ-GADEA, L., MARTIN-CURTO, L.M., CALLE, H., et al., Diabetic Foot Infections: Scintigraphic Evaluation with 99m-Tc-Labelled Antigranulocyte Antibodies, *Nucl. Med. Commun.* **14** (1993) 212-218.
- ECKELMAN, W.C., PAIK, C.H., STEIGMAN, J., Three Approaches to Radiolabelling Antibodies with 99m-Tc, *Nucl. Med. Biol.* **16** (1989) 171-176.
- EISENHUT, M., et al., Bifunctional NHS-BAT Ester for Antibody Conjugation and Stable Technetium-99m Labelling: Conjugation Chemistry, Immunoreactivity and Kit Formulation, *J. Nucl. Med.* **37** (1996) 362-370.
- FRITZBERG, A.R., ABRAMS, P.G., BEAUMIER, P.L., et al., Specific and Stable Labelling of Antibodies with Technetium-99m with a Diamide Dithiolate Chelating Agent, *Proc. Natl. Acad. Sci. USA*, **85** (1988) 4025-4029.
- GOEDEMANS, W. TH., PANEK, K.J., ENSING, G.J., et al., A New Simple Method for Labelling of Proteins with $^{99}\text{Tc}^{\text{m}}$ by Derivatization with imino-4-mercaptobutyl Groups, In: NICOLINI, M., BANDOLI, G., MAZZI, U., (Eds), *Technetium and Rhenium in Chemistry and Nuclear Medicine 3*, New York: Raven Press (1990) 595-603.
- GOH, A.S.W., AW, S.E., SUNDRAM, F.X., et al., Imaging of Focal Inflammation with $^{99}\text{Tc}^{\text{m}}$ -Labelled Human Polyclonal Immunoglobulin G, *Nucl. Med. Com.* **11** (1990) 843-856.
- GRANOWSKA, M., BRITTON, K.E., MATHER, S.J., et al, Radioimmunosintigraphy with Technetium-99m Labelled Monoclonal Antibody IA3, in Colorectal Cancer, *Eur. J. Nucl. Med.* **20** (1993) 690-698.
- GRANOWSKA, M., BRITTON, K.E., MATHER, S.J., et al., Radioimmunosintigraphy with Technetium-99m-Labelled Monoclonal Antibody SM3 in Gynaecological Cancer, *Eur. J Nucl. Med.* **20** (1993) 483-489.
- GRUNDALD, F., HOTZE, A., BRIEK, B., et al., Planar and SPECT Imaging with Technetium Labelled Antigranulocyte Antibodies in Bone and Soft Tissue Infection, *Clin. Nucl. Med.* **18** (1993) 587-589.
- HIRANO, T., TOMIYOSHI, K., WATANABE, N., et al., Technetium 99m Labelled Anti Fibrin Monoclonal Antibody Accumulation in an Inflammatory Focus, *J. Nucl. Med.* **33** (1992) 1181-1182.
- HNATOWICH, D.J., Recent Developments in the Radiolabelling of Antibodies with Iodine, Indium and Technetium, *Sem. Nucl. Med.* **20** (1990) 80-91.
- HNATOWICH, D.J., MARDIROSSIAN, G., RUSOKOWSKI, M., et al., Directly and Indirectly Technetium-99m-Labelled Antibodies: A comparison of in Vitro and Animal in Vivo Properties, *J. Nucl. Med.* **34** (1993) 109-119.

HNATOWICH, D.J., VIRZI, F., FOGARSI, M., et al., Can a Cysteine Challenge Assay Predict the in Vivo Behaviour of $^{99}\text{Tc}^m$ -Labelled Antibodies?, *Nucl. Med. Biol.* **21** (1994) 1035–1044.

HNATOWICH, D.J., et al., Investigations of Ascorbate for Direct Labelling of Antibodies, *J. Nucl. Med.* **35** (1994) 127–134.

HOTZE, A.L., BRIELE, B., OVERBECK, B., et al., Technetium 99m Labelled Anti Granulocyte Antibodies in Suspected Bone Infections, *J. Nucl. Med.* **33** (1992) 526–531.

JOIRIS, E., BASTIN, B., THORNBACKE, J.R., A New Method for Labelling Monoclonal Antibodies, their Fragments and other Proteins with Technetium 99m, In: NICOLINI, M., BANDOLLI, G., MAZZI, U., (Eds.), *Technetium and Rhenium in Chemistry and Nuclear Medicine 3*, New York: Raven Press (1990) 609–613.

JUWEID, M., STRAUSS, H.W., YAOITA, H. et al., Accumulation of Immunoglobulin G at Focal Sites of Inflammation, *Eur. J. Nucl. Med.* **19** (1992) 159–165.

KASINA, S., et al., Development and Biologic Evaluation of a Kit for Preformed Technetium-99m Radiolabelling of an Antibody Fab Fragment using o-Diamide-Dimercaptide Chelating Agent, *J. Nucl. Med.* **32** (1991) 1445–1451.

KRAUSE, B.J., BAUM, R.P., STAIBSEBLER, E., et al., Human Monoclonal Antibody Tc-99m-88 Bv 59: Detection of Colorectal Cancer, Recurrent or metastatic disease and Immunogenicity Assessment, *J. Nucl. Med.* **37** (1996) 9 P (Abstract).

LEE, K.H., CHUNG, J.K., CHOI, C.W., et al., Technetium-99m Labelled Antigranulocyte Antibody Bone Marrow Scintigraphy, *J. Nucl. Med.* **36** (1995) 1800–1805.

LINBDO, T., BOVEN, E., CUTTITTA, F., et al. Determination of the Immunoreactive Fraction of Radiolabelled Monoclonal Antibodies by Linear Extrapolation to Binding at Infinite Antigen Excess, *J. Immun. Meth.* **72** (1984) 77–89.

LIND, P., LECHNER, P., ARIAN-SCHAD, K., et al., Anti-carcinoembryonic Antigen Immunoscintigraphy (Technetium-99m-Monoclonal Antibody BW 431/26) and Serum CEA Levels in Patients with Suspected Primary and Recurrent Colorectal Carcinoma, *J. Nucl. Med.* **32** (1991) 1319–1325.

LIND, P., SMOLA, M.G., LEICHNER, P., et al., The Immunoscintigraphic Use of Tc-99m-Labelled Monoclonal Anti CEA Antibodies (BW 431/26) in Patients with Suspected Primary Recurrent and Metastatic Breast Cancer, *Int. J. Cancer.* **47** (1991) 865–869.

MACH, J.P., FORMI, M., RITSCHARD, J., et al., Use and Limitations of Radiolabelled Anti-CEA Antibodies and their Fragments for Photoscanning Detection of Human Colorectal Carcinomas, *Onc. Biol. Med.* **1** (1980) 49–69.

MALAMITSI, J., ZORZOS, Z.J., VARVARIGOU, A.D., et al., Immunolocalization of Transitional cell Carcinoma of the Bladder with Intravesically Administered Technetium-99m Labelled HMFGI Monoclonal Antibody, *Eur. J. Nucl. Med.* **22** (1995) 25–31.

MATHER, S.J., ELLISON, D., Reduction-mediated Technetium-99m Labelling of Monoclonal Antibodies, *J. Nucl. Med.* **31** (1990) 692–697.

MUNZ, D.L., MORGUET, A.J., SANDROCK, D., et al., Radioimmunoimaging of Subacute Infective Endocarditis Using a Technetium-99m Monoclonal Granulocyte Specific Antibody, *Eur. J. Nucl. Med.* **18** (1991) 977–980.

MURTHY, S., SHARKEY, R.M., GOLDENBERG, D.M., et al., Lymphoma Imaging with a New Technetium-99m Labelled Antibody LL2, *Eur. J. Nucl. Med.* **19** (1992) 394–401.

- MUXI, A., SOLA, M., BASSA, P., et al., Radioimmunosintigraphy of Colorectal Carcinoma with ^{99m}Tc -Labelled Anti CEA Monoclonal Antibody (BW 431/26), Nucl. Med. Commun. **13** (1992) 261–270.
- NAJAFI, A., et al., Synthesis and Preliminary Evaluation of a New Chelate N_2S_4 for use in Labelling Proteins with Metallic Radionuclides, Nucl. Med. Biol. **18** (1991) 179–185.
- PAIK, C.H., et al., The Labelling of High Affinity Sites of Antibodies with ^{99m}Tc , Nucl. Med. Biol. **13** (1986) 359–362.
- PATRICIO, L., et al., Radiochemical and Biological Properties of ^{99m}Tc -Human Immunoglobulin Prepared via 2-Iminothiolane, J. Nucl. Med. **39** (1995) 74–75 (Abstract).
- PELTIER, P., PATEL, G., LEVAT, E., et al., Detection of Lung and Bone Infection with Anti-granulocyte Monoclonal Antibody BW 250/ 183 Radiolabelled with ^{99m}Tc , Nucl. Med. Commun. **14** (1993) 766–774.
- PONS, F., MOYA, F., HERRANZ, R. et al., Detection and Quantitative Analysis of Joint Activity Inflammation with ^{99m}Tc -Polyclonal Human Immunoglobulin G, Nucl. Med. Commun. **14** (1993) 225–231.
- PONS, F., SANMARTI, R., HERRANZ, R., et al., Scintigraphic Evaluation of the Severity of Inflammation of the Joints with ^{99m}Tc -hIgG in Rheumatoid Arthritis, Nucl. Med. Commun. **17** (1996) 523–528.
- QU, T., et al., Technetium- 99m Labelling of Monoclonal Antibodies via N, N-ethylene-bis-l-cysteine, Radiochimica Acta **63** (1993) 209–212.
- REILLY, R.M., Immunoscintigraphy of Tumours Using ^{99m}Tc Labelled Monoclonal Antibodies: A Review, Nucl. Med. Commun. **14** (1993) 347–359.
- RHODES, B.A., Direct Labelling of Proteins with ^{99m}Tc , Nucl. Med. Biol. **18** (1991) 667–676.
- SANDROCK, D., VERHEGGEN, R., HELWIG, A.T., et al., Immunoscintigraphy for the Detection of Brain Abscesses, Nucl. Med. Commun. **17** (1996) 311–316.
- SCHWARZ, A., STEINSTRASSER, A., A Novel Approach to ^{99m}Tc -Labelled Monoclonal Antibodies, J. Nucl. Med. **28** (1987) 721 (Abstract).
- SCIUK, J., BRANDAN, W., WOLLET, B., et al., Comparison of Technetium- 99m Polyclonal Human Immunoglobulin and Technetium- 99m Monoclonal Antibodies for Imaging Chronic Osteomyelitis, Eur. J. Nucl. Med. **18** (1991) 401–407.
- SENDA, M., FISCHMAN, A.J., WEISSE, S., et al., Regional Perfusion, Oxygen Metabolism, Blood Volume and Immunoglobulin-G Accumulation at Focal Sites of Infection in Rabbits, Eur. Jour. Nucl. Med. **19** (1992) 166–172.
- SENIOR, R., BHATTACHARYA, S., MANSPEAKER, P., et al., ^{99m}Tc -Antimyosin Antibody Imaging for the Detection of Acute Myocardial Infarction in Human Beings, Am. Heart. J. **126** (1993) 536–542.
- SHIMURA, N., KOZINA, S., KUBODERA, A., et al., Radioimmuno-detection of Human Colon Cancer Using ^{99m}Tc -MDP-Moab-A7 in Mice, Nucl. Med. Biol. **19** (1992) 105–109.
- SIRISRIRO, R., KIM, E.E., PODOLOFF, D.A., et al., Radioimmunosintigraphy in the Differential Diagnosis of Hepatic Mass Lesion, Eur. J. Nucl. Med. **22** (1995) 385–388.
- SPINELLI, F., MILELLA, M., SARA, R., et al., The Value of ^{99m}Tc -Labelled Human Immunoglobulin Scanning in the Evaluation of Crohn's Disease, Eur. J. Nucl. Med. **16** (1990) 436.

STOEGER, A., MUR, E., PENZ-SCHNEEWEISS, et al., Technetium-99m Human Immunoglobulin Scintigraphy in Psoriatic Arthropathy: First Results, *Eur. J. Nucl. Med.* **21** (1993) 342–344.

SYKES, T.R., WOO, T.K., QI, P., BAUM, R.P., et al., Direct Labelling of Monoclonal Antibodies with Technetium-99m by a Novel Photoactivation Process, *J. Nucl. Med.* **43** (1993) 100 P (Abstract).

TAILLEFER, R., BOUCHER, L., LAMBERT, R., et al., Technetium-99m Antimyosin Antibody (3–48) Myocardial Imaging: Human Biodistribution, Safety and Clinical Results in Detection of Acute Myocardial Infarction, *Eur. J. Nucl. Med.* **22** (1995) 453–464.

THAKUR, M.L., DE FULVIO, J., RICHARDS, M.D., et al., Technetium-99m Labelled Monoclonal Antibodies: Evaluation of Reducing Agents, *Nucl. Med. Biol.* **18** (1991) 227–233.

VANSANT, P.J., JOHNSON, D.H., O' DONNELL, D.M., et al., Staging Lung Carcinoma with a Tc-99m Labelled Monoclonal Antibody, *Clin. Nucl. Med.* **17** (1992) 431–438.

WONG, D.W., MANDAL, A., Technetium-99m IgG Immunodetection of Acute Appendicitis in Rats, *Nucl. Med. Biol.* **23** (1996) 473–478.

PUBLICATIONS RESULTING FROM THE PROJECT WORK

LEA, J.T., GARMESTANI, K., BRACHPIEL, M.W., et al., Evaluation of in-vivo stability of three Ga-67 chelator – MoAb conjugates in mice, *J. Nucl. Med.* **35** (1994) 29 (Abstract).

PIMENTEL, G., OLIVA, J., Anticuerpo monoclonal ior-CEA-1: comparison de dos methods de marcaje can Tc-99m, *Rev. Esp. Med. Nucl.* 1996 (in press).

OLIVA, J., PERALTAR, R., CHOY, J., PIMENTEL, G., et al., ior-CEA-1 un nuevo anticuerpo anti-CEA Tc-99m, para la inmunogammagrafia de tumours colorectales: Resultados prelimanares, *Rev. Esp. Med. Nucl.* **13** (1994) 32–37.

OLIVA, J., PIMENTEL, G., CASCANTE, L., et al., Un nuevo anticuerpo monoclonal anti-CEA marcado con Tc-99m para la inmunogammagrafia de los tumours colorectales: Resultados finales de unensayo clinico fares I-I, *Rev. Esp. Med. Nucl.* **14** (1995) 213–221.

VARVARIGOU, A.D., et al., Radiochemical and Radioimmunological data of Tc-99m anti CEA labelled by two diverse methods, *Nucl. Med. Commun.* **17** (1996) 80–88.

ARCHIMANDRITES, S.C., et al., Radiochemical purity studies on the technetium-99m Labelling of monoclonal antibodies, *Tumour Targetting*, 1996 (in press).

JANOKI, G.Y, KOROSIL, L., BODO, K., BALOUGH, L., Direct Labelling of anti-CEA MoAb with Tc-99m. Evaluation of the degree of fragmentation and invivo stability, *Eur J. Nucl. Med.* **21** (1994) 5208 (Abstract).

SINGH, A.K., MISHRA, P., KASHYAP, R., CHAUHAN, U.P.S., A simplified kit for instant preparation of technetium-99m humanimmunoglobulin-G for imaging inflammatory foci, *Nucl. Med. Biol.* **21** (1994) 277–281.

MISHRA, P., SINGH, A.K., CHAUHAN, M., BHATNAGAR, A., KASHYAP, R., CHAUHAN, U.P.S., A novel method for Labelling human immunoglobulin-G with Tc-99m suitable for inflammation scintigraphy, *Nucl. Med. Commun.* **15** (1994) 723–729.

CHAUHAN, U.P.S., MISHRA, P., SINGH, A.K., et al., A new approach of a CEA specific monoclonal antibody (ior-CEA-1) Labelling with technetium using ascorbic acid as protein reductant and its characterization, *Eur. J. Nucl. Med.* **21** (1994) 525 (Abstract).

PATRICIO, L., et al., Radiochemical and biological properties of Tc-99m-human immunoglobulin prepared via 2-iminothiolane, *Quat. J. Nucl. Med.* **39** (1995) 74–75 (Abstract).

ABBREVIATIONS

MoAb/MAb	monoclonal antibody
MAG3	mercapto acetyl triglycine
GH	glucoheptonate
MDP	methylene Diphosphonate
EtOH	ethanol
MEK	methyl Ethyl Ketone
HSA	human serum albumin
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
PBS	phosphate buffered saline
hIgG/IgG	human immunoglobulin/immunoglobulin
ELISA	enzyme immuno assay
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
HPLC	high performance liquid chromatography
FPLC	fast protein liquid chromatography

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