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Tc-^{99m} Direct Radiolabeling of Monoclonal Antibody Ior egf/r3: Quality Control and Image Studies in Mice

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ABSTRACT

Monoclonal antibodies (Mabs) have been useful for immunoscintigraphic applications in clinical diagnosis since they were introduced in the practice of nuclear medicine. The ior egf/r3 (Centis, Cuba) is a murine monoclonal antibody against epidermal growth factor receptor (EGF-R) and has been widely used in the radioimmunodiagnosis of tumors of epithelial origin. Labeled with 99mTc, its main application in Nuclear Medicine is the follow up, detection and evaluation of tumor recurrences. The objective of this work is to describe the preparation of a lyophilized formulation (kit) for radiolabeling the Mab ior egf/r3 with 99mTc for immunoscintigraphic applications. Radiolabeling efficiency, effects on immunoreactivity, image studies and stability of the formulation are reported. The study demonstrated that the kit formulation can be labeled with 99mTc at high yields and can be used to visualize in vivo human tumors of epithelial origin by immunoscintigraphy studies.

Key words: Radiolabeling, technetium, monoclonal antibodies, nuclear medicine

INTRODUCTION

In 1975, Kohler and Milstein published the results of their work describing the production of "hybridoma" cell lines capable of producing monoclonal antibodies (Mabs): polyclonal antibodies are produced in animals repeatedly immunized with the target antigen; the antibody-producing B-lymphocytes are then isolated and immortalized by hybridization with a murine myeloma cell line. The rapidly developing technique soon entered clinical trials. In 1980 the first patient with relapsed lymphoma was treated (Stern et al., 2005).

Monoclonal antibodies can be used in a variety of assays as highly specific probes to correlate specific structural determinants with functional properties, making Mabs important tools for validating drug targets (Jia et al., 2004). They have been used for the diagnostic and therapeutic treatment of some types of tumors (Navarro et al., 2005).

Cancer diagnosis should lead directly to cancer treatment, and cancer management should be performed based on scientific evidence. In this sense, nuclear medicine is one the best tools among the diagnostic modalities (Fukuda et al., 2002).

The radionuclide technetium-99m (^{99m}Tc) is extensively used in radiodiagnostics in nuclear medicine (Alfassi et al., 2005). It has been used in the last decade to label many kits. Its nuclear features of 6-hour half-life and gamma energy of 140 keV make it one of the best radionuclides as

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regards imaging qualities, with a half-time long enough to permit complete studies without an unnecessary dose of radiation. It is easily available by elution in isotonic saline from a $^{99}Mo/^{99m}Tc$ generator (Marques et al., 2001; Faintuch et al., 2004).

The direct method for radiolabeling reduced Mabs with ^{99m}Tc and methylene diphosphonate (MDP) developed by Schwarz et al. (1987) and Mather et al. (1990) has been proved as a good procedure to obtain high labeling efficiency and a stable labeled Mab. The ^{99m}Tc-MDP method uses the reduction of disulfide bridges of the molecule with an excess of the reducing agent, 2-mercaptoethanol (2-ME). After purification to eliminate the excess of 2-ME, the reduced antibody is labeled with technetium via Sn^{2+} reduction of pertechnetate, using MDP as a weak competing ligand. Direct methods are efficient and adaptable to a kit type of radiolabeling procedure (Morales-Morales et al., 1998).

The human epidermal growth factor receptor (EGF-R) is a transmembrane glycoprotein with a molecular weight of 170 kDa. EGF-R overexpression has been found in a variety of malignant epithelial tumors arising in different organs such as breast, bladder, colon, lung and glioma (Iznaga-Escobar et al., 1998). The Mab ior egf/r3 is a murine IgG_{2a} antibody that recognizes the EGF-R (Morales-Morales et al., 2000; Duconge et al., 2004).

The present work describes the preparation of a lyophilized formulation for radiolabeling the ior egf/r3 Mab with ^{99m}Tc for immunoscintigraphic applications. Radiolabeling efficiency, image studies and stability of the formulation are reported.

MATERIALS AND METHODS

Materials

The Mab ior-egf/r3 was supplied by CENTIS (Cuba) provided by the International Atomic Energy Agency (IAEA). Vials containing 5 mL with an antibody concentration of 5 mg/mL were used.

Technetium-99m was obtained from an alumina-based $^{99}Mo/^{99m}Tc$ generator locally supplied by the Institute of Energetic and Nuclear Research (IPEN/CNEN) – São Paulo, Brazil.

Methods

Antibody Reduction

The Mab (10 mg) was reduced by reaction with 10 μL of 2-ME at room temperature for 30 min. The resulting solution was purified on a PD-10 column (Sephadex G-25M, Pharmacia) using phosphate buffered saline (PBS) (pH 7.4) purged with nitrogen as mobile phase and fractions of 1 mL were collected (12 fractions). The concentration of the reduced antibody was determined by optical density at 280 nm on a UV/visible spectrophotometer (Hitachi Instruments).

Sulphydryl Groups

The number of resulting free sulphydryl groups was assayed with Ellman's reagent (Sigma); 50 μL of the sample of reduced antibody was mixed with 50 μL of the solution containing 0.3 mg/mL of 5,5'-dithiobis (2-nitrobenzoic acid) and diluted to 3 mL with 0.1 mol/L phosphate buffer pH 8. The mixture was incubated at room temperature for 5 min and coloration (yellow color) measured in a UV/vis spectrophotometer at 409 nm. The number of thiols was obtained by comparison with a standard curve obtained by the assay of a series of cysteine standards ranging from 0.25 to 1.0 mmol/L. The result was expressed as sulphydryl groups per antibody molecule.

Kit Formulation

A methylene diphosphonate (MDP) bone-scanning kit (IPEN) containing 5 mg medronate, 1 mg stannous chloride, 0.1 mg ascorbic acid and 20 mg sodium pyrophosphate was dissolved in 5 mL of 0.9% sodium chloride solution purged with nitrogen. Secondly, 29 and 87 μL of this solution was added to 1 mL of reduced antibody solution (1 mg/mL). The mixture was sterilized by membrane filtration (Millipore, 0.22 μm), storage in pre-sterilized serum vials and frozen with liquid nitrogen. The frozen products were then lyophilized for 24 h, sealed under vacuum and kept in the freezer until use. Two lots were prepared: Lot A- 1 mg of the Ab, 29 μL of the MDP and 3 μg of the Sn^{2+}. Lot B- 1 mg of the Ab, 87 μL of the MDP and 8 μg of the Sn^{2+}.

Radiolabeling

Vials of lyophilized monoclonal antibody (kits) were reconstituted with perthecnetate (39.59 – 2301.4 MBq) eluted from the $^{99}Mo/^{99m}Tc$

generator system. The contents were dissolved by swirling the vial gently and left at room temperature to complete the labeling reaction.

Quality Control

The labeled product was subjected to ascending paper chromatography on Whatman 3MM paper as the stationary phase and 0.9% saline and acetone as the mobile phase to separate free pertechnetate and ^{99m}Tc-MDP, which moved with the solvent. Human serum albumin (HSA) (5%)-impregnated Instant Thin Layer Chromatography-Silica Gel (ITLC-SG; Gelman Science Inc.) strips were used as the stationary phase and ethanol:NH_4OH:water (2:1:5 [v/v]) as the mobile phase to separate radiocolloids that remained at the origin while the radiolabeled Mab and pertechnetate moved with the solvent front (colloid, Rf = 0.0, labeled ^{99m}Tc, Rf = 1.0).

Optimization of Radiolabeling

Optimization was achieved by varying parameters, including reaction time (0.25 and 1.0 hour), volume of a pertechnetate (1 - 5 mL) and variation of the ^{99m}Tc activity. The stability of the radioactive solution was determined 2 months after is preparation.

Cysteine Challenge

Known amounts of ^{99m}Tc-egf/r3 was challenged over a range of cysteine concentrations (0.0083 to 8.3 mmol/L) at 37°C for 1 hour. The percentage of ^{99m}Tc displaced by cysteine was determined by Whatman 3MM using 0.9% saline as mobile phase, and the amount of displaced radiolabel found at the solvent front was determined.

Image Studies in mice

A human pancreatic carcinoma cell line, AR42J (ATCC), used for the development of the tumors, was grown in RPMI-1640 medium supplemented with 10% fetal calf serum (Cultilab). Animals were used after 30 days to inoculate the AR42J cell suspension (10^6 cells in 1 mL) in the dorsal region of nude mice (IPEN).

Male athymic nude mice (normal and with tumor) were injected through a tail vein with 0.15 mL (510.23 MBq, 1 mg) of labeled antibody. Static images were obtained with a gamma camera after 24 hours post injection.

RESULTS AND DISCUSSION

Antibody Reduction

The average protein concentration was found to be 3.08 mg/mL (recovery average of the protein: 95.9% ± 5.2).

Sulphydryl Groups

An excellent linear correlation was found between the cysteine concentration and absorbance (r=0.9911). After reduction, the number of SH groups generated per molecule of antibody was calculated based on the cysteine standard curve (Table 1).

Table 1 - Number of endogenous sulphydryl groups generated by reduction with 2-ME.

Antibody	Before reduction	After reduction	% of total (36 SH/mol)
ior-egf/r3	0.3	12.5 ± 2.3	34.8

*média ± SD (n = 3)

Mouse IgG contains only six interchain and 12 intrachain disulfide bonds, giving an absolute maximum of 36 SH groups per antibody molecule (Mather et al., 1990). The determination of sulphydryl groups by reduction with 2-ME using Ellman's reagent (Table 1) demonstates the production of 12.5 ± 2.3 sulfydryl groups, which represents 34.8% of the total SH groups generated per molecule.

Griffiths et al. (1991), using 2-ME with intact IgG obtained from one to nine SH groups per IgG molecule using Ellman's reagent. Iznaga-Escobar et al. (1996), working with monoclonal antibody ior egf/r3 obtained 6.65 ± 0.69 SH groups (18.5% of the total SH groups generated per molecule). The results obtained in the present work were superior to those encountered in the literature.

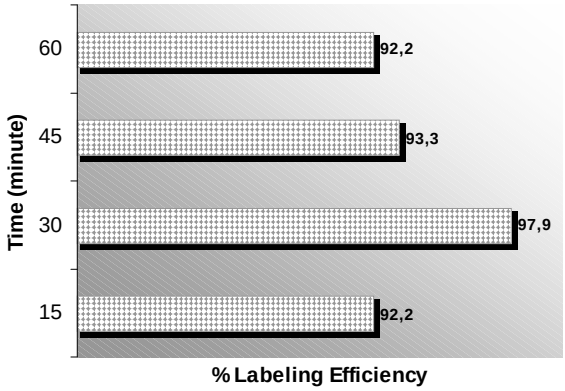

Figure 1 - Labeling yield with the variation of incubation time (Lot A).

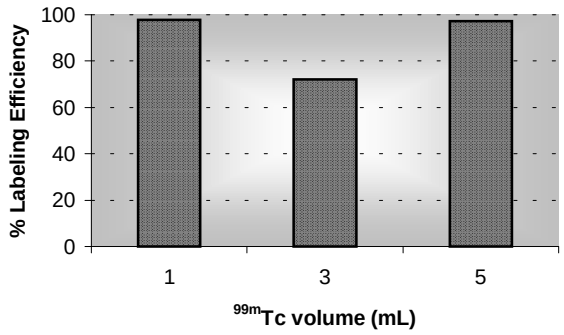

Figure 2 – Comparison of the labeling yield with the variation of the volume of ^{99m}Tc.

Radiolabeling studies

The parameters that were varied in the labeling procedures were volume, ^{99m}Tc activity and incubation time after the addition of ^{99m}Tc. The pH value of 7.0 was constant throughout the labeling procedures. Figure 1 shows the labeling yield obtained when the time of incubation was varied

The best incubation time of the antibody with ^{99m}Tc was 30 minutes. The mean value was 94 ± 2 % for all incubation times. Figura 2 shows the labeling yields when the volume of ^{99m}Tc required to dissolve the kit (Lot A) was varied.

The values for 1 and 5 mL were adequate, with a mean value of 97.5% ± 0.4. With the volume of three mL, the labeling yield (72.07%) was lower than recommended, indicating the presence of a high level of impurities.

The kits were tested using high ^{99m}Tc activities, very important for clinical application of the radiopharmaceutical. The Lot A and Lot B kits were tested. The results are presented in Table 2.

Table 2 - Labeling yield with the variation of ^{99m}Tc activity.

Lot	^{99m}Tc activity (MBq)	% Labeling efficiency
A	501.35	96.22
A	2171.9	91.11
B	1975.8	93.67
B	2301.4	91.06

Table 3 - Percent Labeling efficiency after 2 months of storage.

^{99m}Tc activity (MBq)	Time after labeling (h)	% Labeling efficiency
358.53	0.5	98.8
	24	96.4

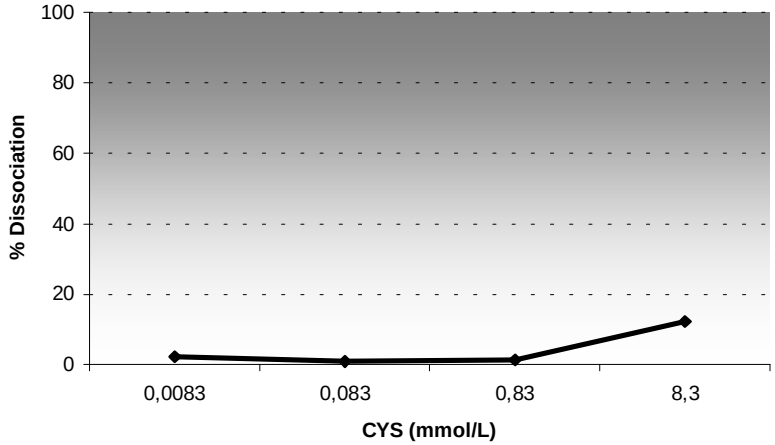

Figure 3 - Percent dissociation of ^{99m}Tc from antibody vs. Cys concentration.

All the results were very good, showing that the kits can be labeled with high activities. The labeling efficiency of the Lot A kit after 2 months of storage at 5-8 ^{o}C is presented in Table 3. According to the results, the kits are stable for at least 2 months of storage at low temperature. The value of the residual immunoreactivity of the Ab was also studied using the immunoaffinity chromatography method. The results indicate that the labeling conditions did not affect the immunoreactivity of the Ab (data not shown).

Cysteine Challenge

Transchelation studies evaluated the bond strength of the ^{99m}Tc-antibody complexes in the presence of cysteine in the body. Transchelation to cysteine was studied at four molar ratios (Fig. 3).

The maximum concentration of cysteine to which the peptide was exposed *in vivo* was 1 mmol/L (Cooper, 1983). Adjusting the values of Figure 3 to 1 mmol/L, 1.56% of dissociation was obtained, whereas 18% was observed by Pak et al. (1992) for monoclonal antibody fragments for the same concentration of cysteine. The high molar ratio necessary to cause displacement suggests that the bond strength of the ^{99m}Tc-egf/r3 was very high.

Image Studies in mice

Imagese of athymic nude mice by static imaging at 24 hours after injection of the labeled kit (Figure 4) were acquired with an acquisition time of 10 minutes. The animals were positioned sideways in relation to the gamma camera for better tumour visualization.

A large accumulation at the tumor site at 24 hours post injection can be seen. The quantification of the images showed 26% of accumulation in the tumor compared to the whole body counts. This number can not be compared to the dose injected, but the quality of the image is significant.

No selective accumulation was seen in normal organs, with the exception of salivary glands, thyroid, liver and stomach, in both normal and tumor mice. The visualization of salivary glands, thyroid and stomach can be explained by a small amount of free pertechnetate coming from the degradation of the labeled product, as observed by Ramos-Suzarte et al. (1999).

The liver showed a high uptake compared to other organs, suggesting that this is the major organ for catabolism and metabolism of labeled proteins and their degradation products (Iznaga-Escobar et al., 1997). The two routes of elimination for labeled antibodies are via the urine and the feces, with the latter predominating in this study.

Figure 4 - Static images obtained with a gamma camera of the normal mice (up) and with tumor (down) after 24 hours post injection iv of the antibody ior egf/r3 labeled with ^{99m}Tc. Tumor (T), thyroid (Ty) and liver (L).

According to Fritzberg (1987), in studies performed with antibodies fragments, a significant uptake in the tumor occurred four hours after injection, with a good clearance after 10 hours. This observation indicated a typical slow blood clearance of Mab. Taking the observations of this and other authors into account, the visualization of the tumor after 24 hours of injection was considered successful and the images proved the quality of the radiopharmaceutical prepared in this work.

CONCLUSION

The reduction of the antibody ior egf/r3 was efficient with a high recovery of the reduced protein. Labeling of antibody with ^{99m}Tc by a direct method was simple and efficient, with a high labeling yield (higher than 95%) and stability in the face of a cysteine challenge. There was no need for subsequent purification. ^{99m}Tc-ior egf/r3 was radiochemically stable for 24 hours and after two months of storage at low temperature, showing the efficiency of the kit preparation procedure.

The images obtained had very good quality with high accumulation in the tumor and no uptake in specific organs. Results indicated that the products can be obtained with high radiochemical yield, in a simple routine procedure, appropriate for further studies to assess their efficacy in radiodiagnosis.

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RESUMO

Anticorpos monoclonais (AcM) tem sido usado para aplicações imunocintilográficas no diagnóstico clínico desde sua introdução na prática da medicina nuclear. O ior egf/r3 (Centis, Cuba) é um anticorpo monoclonal murino contra o receptor do fator do crescimento epidérmico (EGF-R) e tem sido usado extensivamente no radioimunodiagnóstico de tumores de origem epitelial. Marcado com 99mTc sua maior aplicação em Medicina Nuclear é: acompanhamento, detecção e avaliação de recorrências tumorais. O objetivo deste trabalho é descrever a preparação da formulação liofilizada (kit) para radiomarcação do AcM ior egf/r3 com 99mTc para aplicações imunocintilográficas. A eficiência da radiomarcação, efeito sobre a imunorreatividade, estudos de imagem e estabilidade da formulação são relatados. O estudo demonstrou que a formulação do kit pode ser marcada com 99mTc com alto rendimento e pode ser usado para visualizar in vivo tumores humanos de origem epitelial por estudos imunocintilográficos.

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