99m-TECHNETIUM LABELLING OF A TUMOR ASSOCIATED MURINE MONOCLONAL ANTIBODY FOR IMMUNOSCINTIGRAPHIC STUDIES IN MAN

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SUMMARY

The present study refers to the preparation of a 99m-Technetium labelled murine monoclonal antibody for clinical application. The monoclonal antibody was incubated with a 20fold molar excess of 2-iminothiolane. The free thiol groups created, were capable of binding reduced technetium. Labelling took place through an exchange reaction with 99m-Technetium-Glucoheptonate. The labelling conditions were studied extensively. Diverse chromatographic methods (ITLC, HPLC) were developed in order to study the

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radiochemical behaviour of the labelled antibody and evaluate the labelling efficiency and the in vitro stability. Results on the labelling technique and on the quality control methods are presented, as well as preliminary immunoscintigraphic data.

Key words: 2-iminothiolane, technetium-99m, monoclonal antibodies, immunoscintigraphy, cancer.

INTRODUCTION

Technetium-99m (^{99m}Tc) labelled monoclonal antibodies (MoAbs) are increasingly becoming valuable tools in clinical diagnosis, although they have been introduced to the Nuclear Medicine practice rather recently (1,2). Technetium labelling is usually performed either by direct binding of the radiometal to the aminoacid side chains of the protein -direct labelling methods (3,4,5)-or by conjugating the antibody to a synthetic chelator, capable to complexing the radionuclide -bifunctional chelate approach (6,7). Although the two labelling systems have been studied extensively by several research groups and many details regarding their advantages and disadvantages are known, few publications refer to the radiochemical behaviour of the complexes formed between technetium and the MoAbs. In the present work we investigated the ^{99m}Tc-labelling of HMFG1, an epithelium specific, tumour associated mouse monoclonal antibody (8). The Tc-99m labelling system which we have applied was based on the modification of the structure of the protein by the use of 2-iminothiolane (9,10). The latter reacted with the amine groups of the lysine molecules of the protein; thus free thiol groups were introduced, avoiding cleavage of the proteinic disulphide bridge. Incubation of the modified antibody with a preformed weak technetium chelate led to exchange of the radionuclide from the transfer ligand to the protein. A series of different chromatographic systems was developed in order

to study the labelling efficiency and to evaluate the in vitro stability of the ^{99m}Tc-complex. The immunoreactivity was also determined by immunoenzymatic and immunoassay techniques. Results on the labelling technique and on the quality control methods applied to the ^{99m}Tc-HMFG1 are presented. Immunoscintigraphic and kinetic data on one patient with Transitional Cell Carcinoma of the bladder (TCC), who received the radiolabelled conjugate intravesically for diagnostic purposes, are also reported.

MATERIALS AND METHODS.

Monoclonal Antibody

HMFG1 is a mouse monoclonal IgG1 which recognizes a tumor associated mucin, expressed on the cell-surface of most epithelial carcinomas (8). The MoAb was kindly provided to us in sterile and free of viruses and pyrogens form by Unipath Limited (U.K.).

Radiolabelling

A 20fold excess of a 0.07 M solution of 2-iminothiolane in triethanolamine (3.5×10^{-4} mmol), was added to a solution of the MoAb in 0.1 M Phosphate Buffer, pH=7.4 (1.0 mg of protein, 7.0×10^{-6} mmol). Following a reduction time, which ranged between 15 min and 24 hrs, 0.1 ml of a Glucoheptonate kit (N.C.S.R."Demokritos") containing 2.04×10^{-3} mmol Glucoheptonate and 0.53×10^{-7} mmol $SnCl_2$ was added, followed by $Na^{99m}TcO_4$ (N.C.S.R."Demokritos). The preparation was left at room temperature for a period ranging from one to five hours. It was then passed through a Sephadex G50 column (Pharmacia, Sweden), which was eluted with Phosphate Buffer Saline 0.1M, pH=6.8 purged with nitrogen. One ml fractions were collected and counted. One peak, corresponding to the radiolabelled MoAb, was obtained.

Radiochemical Quality Control

The determination of the labelling efficiency and the in vitro stability of the ^{99m}Tc-labelled MoAb HMFG1 was examined by Instant Thin Layer Chromatography (Gelman ITLC-SG USA) and by High Performance Liquid Chromatography (HPLC Waters Ass., USA).

The systems used for analysis were as follows:

A. I T L Chromatography.

Silica gel strips (SG, Gelman Chem. Co.) were used as stationary phase, while the following solvent systems were applied as mobile phases:

System 1. Methanol:10% ammonium acetate 1:1 (V/V)

System 2. First run: -Solvent: acetone
-Developing distance: 20 cm
Second run: -Solvent: Sodium chloride
-Developing distance: 10 cm

System 3. First run: -Solvent: methylethylketone (MEK)
-Developing distance: 20 cm
Second run: -Solvent: Aqueous solution of 5% glycine
-Developing distance: 10 cm

In the double runs the first run lasted for about 15 min and the second for 3 min, while between the two runs, the strips were air-dried for 3 min.

B. H P L Chromatography.

A Waters Ass. USA, model 6000A pump, linked to a U6K injector was used for the HPL Chromatography. Three different size exclusion columns were used in the present study namely:

a. μ-Bondagel E-125, 3.9mm (I.D.) x 30cm, Waters Ass., USA

b. μ-Bondagel E-500, 3.9mm (I.D.) x 30cm, Waters Ass., USA and

c. TSK-Gel G-2000SW, 7.8mm (I.D.) x 30cm, TosoHaas, USA.

A volume of 10 μL of the preparation was injected onto the column and eluted with a mobile phase containing a mixture of phosphate buffer, sodium sulfate and sodium azide (as stabilizer), at pH 6.7.

The column eluent was connected to a U.V. detector (detection wavelenth 240 nm) and then to a standard sodium iodide scintillation detector (Harshaw, Holl.) connected to a chart recorder (Houston Instr., USA). The flow rate was 1 mL/min and the operating pressures were in the range of 1100-1300 psi.

In vitro stability studies.

Stability studies of the labelled MoAb were performed at 2, 4, 12, and 24 hours, at both 4°C and room temperature.

Immunoreactivity

Following modification by 2-iminothiolane, the immunoreactivity of the HMFG1 was tested using an ELISA technique. The purified antigen, a peptide, was kindly provided by Dr. J. Taylor-Papadimitriou from ICRF, London, U.K. The peptide was disolved in water at a concentration of 0.2 ng/ml and was plated out in a 96 well plate (Costar, U.K.) at a volume of 25 µl/well. The plate was left to dry out at 37 °C overnight. It was then incubated at room temperature for 60 min with PBS, containing 0.05% Tween 20 (Sigma, U.K.). The modified test antibody was added at a concentration of 1µg/well and compared to an equivalent amount of the original unmodified antibody. All MoAb preparations were diluted 1:2 down to 0.008 µg/well. Following a 60 min incubation, at room temperature, the plate was washed with PBS/Tween 20 and incubated with rabbit antimouse immunoglobuline peroxidase-linked, at 1:500 dilution, at room temperature. Sixty minutes later the plate was washed with PBS/Tween 20 and the ortho-phenyl-diamine substrate (Sigma, U.K.) was added. The absorbance of each well was measured at 450 nm using a microplate photometer (Anthos, Austria). Following the addition of technetium, the immunoreactivity of the radiolabelled HMFG1 was measured by a direct RIA technique. A 96 well plate was prepared as above. The plate was incubated with 3 µg/well of ^{99m}Tc-labelled

HMFG1 followed by 1:2 dilution, with and without 3 μg/well of "cold" HMFG1. Sixty minutes later the plate was washed with PBS/Tween 20, each well was cut out and the radioactivity was counted at a γ-counter (Cobra 5002, Packard, USA).

Biodistribution studies in animals

The biodistribution pattern of the ^{99m}Tc-HMFG1, was studied in normal male Swiss mice, following i.v. injection of the radiolabelled compound in the tail vein. Animals were sacrificed by ether anesthesia, and the main organs were removed, weighed and counted in a γ-scintillation counter (Packard USA, Minaxi 5000). The percent dose per gram was calculated, at various time intervals post injection, in comparison to a standard.

Immunoscintigraphic study.

In one patient with TCC of the bladder, ^{99m}Tc-HMFG1 was administered intravesically for diagnostic purposes. Prior to the administration the patient's written consent was obtained. One mg of HMFG1 was injected, radiolabelled with 3 mCi of ^{99m}Tc. One and a half hours post administration, immunoscintigraphy of the bladder (SPECT) was performed on a rotating γ-Camera (Siemens, Orbiter). The data were reconstructed by filtered back projection, without attenuation correction. Twenty hours post administration the patient underwent cystoscopy and transurethral resection of the tumor. Biopsy specimen were taken from the tumor, as well as from macroscopically appearing normal tissue and were weighed and counted in a well type γ-counter before the histological examination. Blood samples were taken at 1, 4, and 20 hours post intravesical administration and were also counted in a γ-counter (Packard USA, Minaxi 5000). Cumulative urinary excretion at 20 hours was estimated on the basis of urinary collection.

RESULTS

Radiolabelling

The chromatographic systems, applied, indicated a high labelling yield. It was found, however, that the sequence by which the reagents were added, their volume, as well as, the reaction time for the modification of the antibody, were important to the final yield. Thus, it was found that 10 min incubation with 20 fold excess of 2-iminothiolane and 30 min incubation with ^{99m}Tc are the optimal conditions. The final volume should not exceed 0.5 mL. Under the above conditions the labelling efficiency ranges between 92 and 98%.

Radiochemical Quality Control

By ITLC (Fig 1.) with the mobile phase 1, the labelled MoAb

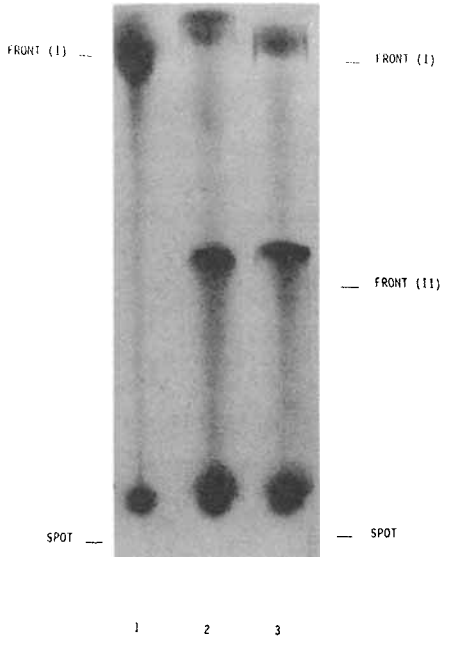

Figure 1. Autoradiography patterns of ITL Chromatographs obtained from analysis of labelled ^{99m}Tc-MoAb.

remained at the origin as one spot , while free pertechnetate moved

with the solvent front.

The R_f values were:

System 1. -R_f values: 0.00 (spot) ^{99m}Tc-MoAb

0.90-1.00 (solvent front) $^{99m}TcO_4^-$

With the mobile phases 2 and 3, two radiolabelled species of the MoAb were revealed. One of them remained at the origin, while the other moved with the solvent front of the second solvent. Free pertechnetate moved with the front of the first solvent.

The corresponding R_f values on these systems are as follows:

System 2. -R_f values: 0.00 (spot) ^{99m}Tc-MoAb

0.90-1.00 (solvent front) $^{99m}TcO_4^-$

-R_f values: 0.00 (spot) ^{99m}Tc-MoAb

0.90-1.00 (solvent front) ^{99m}Tc-MoAb (second species)

System 3. -R_f values: 0.00 (spot) ^{99m}Tc-MoAb

0.90-1.00 (solvent front) $^{99m}TcO_4^-$

-R_f values: 0.00 (spot) ^{99m}Tc-MoAb

0.90-1.00 (solvent front) ^{99m}Tc-MoAb (second species)

The results of HPLC analysis in the cases of μ-Bondagel columns, show that the labelled MoAb appeared as one peak - under the analysis standards used - with a retention time from 4.0 min to 4.8 min, while free pertechnetate had a retention time of 13.5 min.

In the case of the TSK-Gel G 2000SW column two peaks appeared (Fig. 2) with retention times 5.2 and 6.0 min. The retention time of free pertechnetate was 13.5 min.

Stability Studies.

^{99m}Tc-HMFG1 was found stable for 24 hours post preparation when stored at 4°C, while storage at room temperture after 2 hours resulted in a 10% content of free pertechnetate.

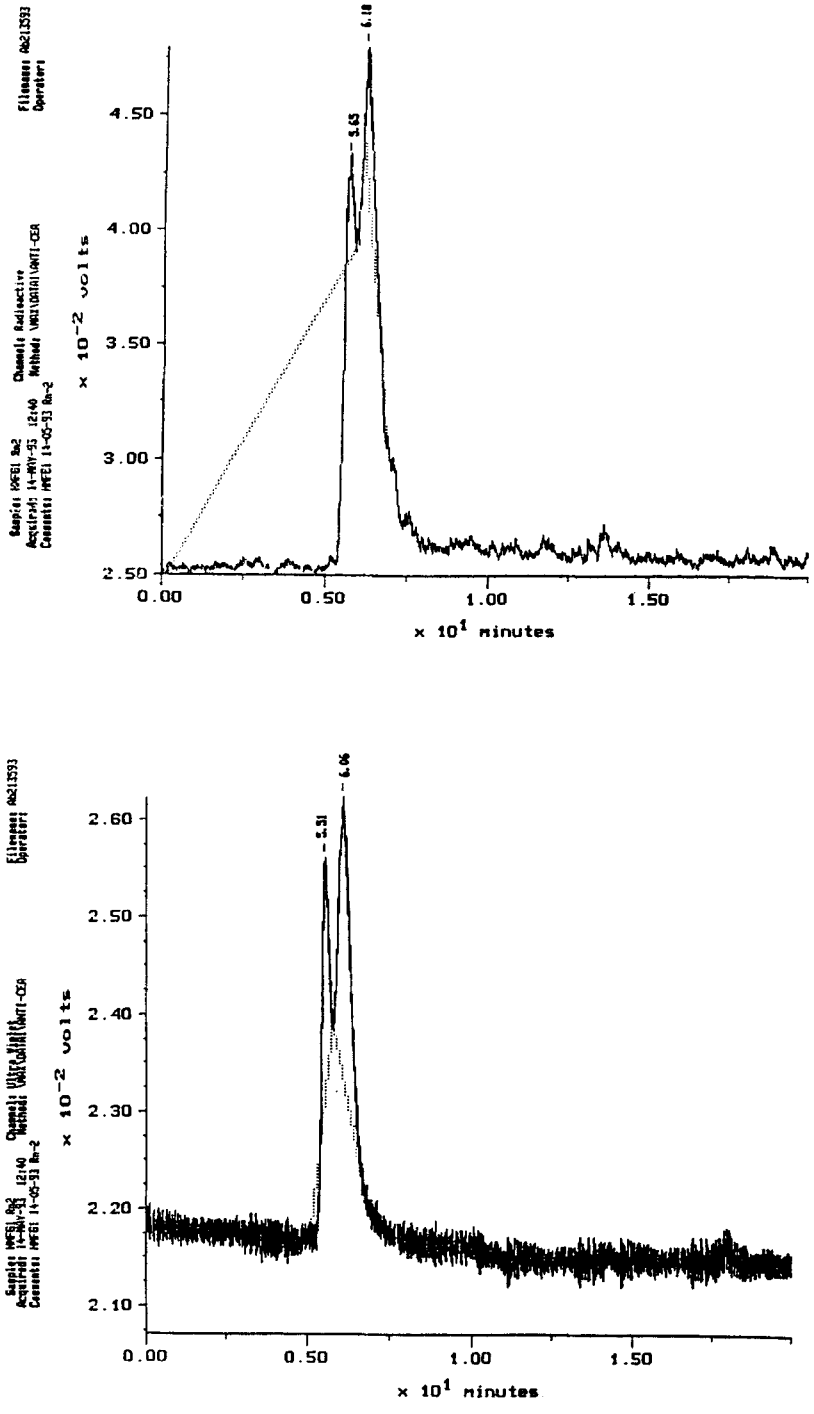

Figure 2. HPLC patterns on UV (Upper) and radiometric (lower) detectors.

Immunoreactivity

Figure 3, shows the binding capacity of the MoAb HMFG1, as measured by ELISA and expressed as absorbance at 450 nm, before and after radiolabelling with ^{99m}Tc. There is no statistical difference between the two preparations.

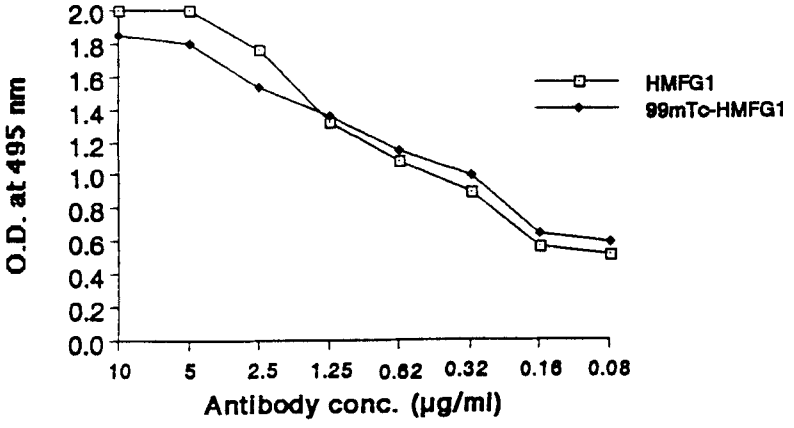

Figure 3. Immunorectivity of the unlabelled (HMFG1) and the ^{99m}Tc-Labelled (^{99m}Tc-HMFG1) monoclonal antibody as measured by ELISA. No statistical significant difference was found between the two preparations. The results are expressed as absorbance (O.D.) at 495 nm.

Figure 4, shows the binding capacity of the ^{99}Tc-labelled HMFG1 expressed in cpm. Competition with an equimolar amount of "cold" HMFG1 as measured by direct RIA, resulted in approximately 50%-100% reduction of the radiolabelled HMFG1 binding.

Biodistribution studies in animals

The biodistribution of the ^{99m}Tc-HMFG1in normal mice at 2, 4 and 24h p.i. is presented in Table 1. The radiolabelled antibody was rapidly excreted in the urine. Study of the kinetics did not show

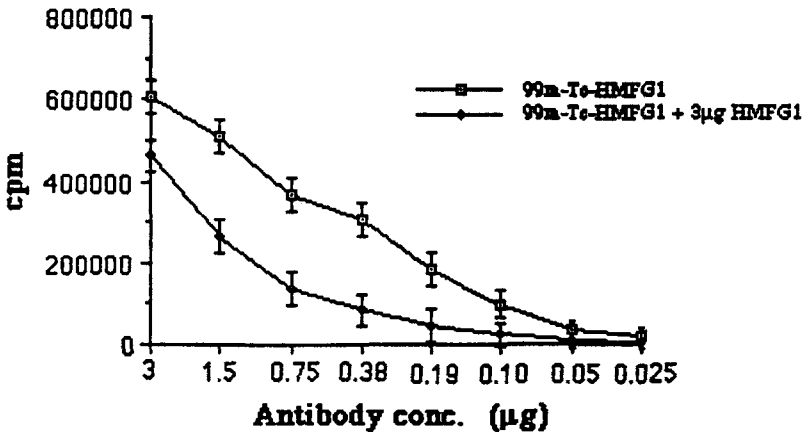

Figure 4. Immunorectivity of the ^{99m}Tc-Labelled HMFG1 (^{99m}Tc-HMFG1) monoclonal antibody as measured by direct radioimmunoassay. The results are expressed in cpm of the antigen bound radiolabelled HMFG1 and of the radiolabelled HMFG1 in competition with "cold" HMFG1.

any specific localization at any organ or tissue in healthy mice. The main labelled fraction collected from the HPLC showed a similar biodistribution pattern.

Immunoscintigraphic and kinetic results in a patient with TCC of the bladder

Figure 5, shows the results of tomographic imaging of the bladder at 90 min post administration. The areas of involvement were easily distinguishable and corresponded to cystoscopic findings. Biopsy specimens from the tumor areas showed a papillary and partially solid bladder carcinoma of transitional epithelial type grade II. No muscle infiltration was found in the biopsy specimen. Uptake

TABLE 1. % DOSE PER GRAM OF TISSUE*

ORGAN/TIME	2h	4h	24h
BLOOD	15.884 ± 1.84	9.503 ± 0.62	1.481 ± 0.10
LIVER	8.573 ± 1.63	5.238 ± 0.34	1.312 ± 0.08
KIDNEYS	16.651 ± 3.18	11.651 ± 0.83	3.152 ± 0.42
STOMACH	3.667 ± 1.10	1.439 ± 0.44	0.172 ± 0.04
INTESTINES	9.800 ± 1.40	4.344 ± 1.50	0.207 ± 0.01
SPLEEN	1.978 ± 0.89	1.591 ± 0.22	0.467 ± 0.07
MUSCLE	0.813 ± 0.15	0.641 ± 0.13	0.169 ± 0.04
LUNGS	5.546 ± 0.79	4.198 ± 0.39	0.680 ± 0.09
URINE**	33.773 ± 4.43	45.688 ± 4.27	

* Each value is the average of five experiments

**% DOSE/ORGAN

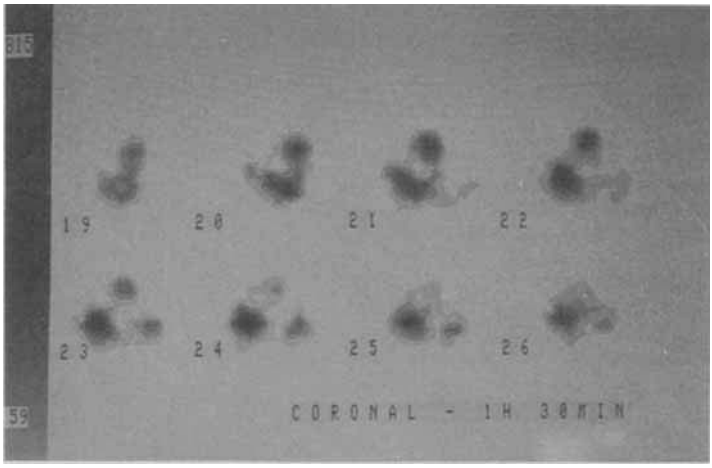

Figure 5. Coronal views of the bladder taken 90 minutes after intravesicaly administration of ^{99m}Tc-HMFG1. Tumor involvement appear as areas of increased uptake.

values of the monoclonal antibody by the tumor ranged up to 2.05 % of the administered dose/kg of tissue. Biopsy specimen from microscopically and histologically confirmed normal urothelium gave an uptake value of at most 0.4% of the injected dose/kg of tissue. Blood samples showed practically no radioactivity present in the circulation. Cummulative urinary excretion at 20 hours did not exceed 6%, taken into consideration that 47% of the administered dose was already excreted with the first washing of the bladder one hour after the intravesical administration.

DISCUSSION AND CONCLUSIONS

The MoAb HMFG1 was modified by the use of 2-iminothiolane. The minimum time required for the modification reaction was found to be ten minutes. Longer time intervals neither improved the final yield, nor destroyed the antibody. The thiol groups, thus introduced, were capable of binding reduced technetium through an exchange reaction with a preformed ^{99m}Tc-Glucoheptonate complex.
A thirty-minutes incubation time was found sufficient for obtaining a high labelling yield (95%). Best results were obtained when glucoheptonate and pertechnetate were added simultaneously to the modified antibody.
The monoclonal antibody retained its immunoreactivity both after 2-iminothiolane modification and technetium addition, as shown by ELISA and RIA (Figs 3,4). Quality control methods, (ITLC and HPLC), suggested the existence of two radiolabelled species, easily separable by both Thin Layer Chromatography and High Performance Liquid Chromatography. The hypothesis that two radiolabelled species of MoAb exist, as it comes out from the ITLC results, is in good agreement with the results of the HPLC analysis with the TSK-Gel G 2000SW column. In the latter case two peaks were separated, with retention times (RT) 5.6 and 6.2 min respectively. Under these experimental conditions, in which lower M.W. species

have higher RT, the peak with RT=5.6 min probably represents a polymer form which can be considered insignificant. Glucoheptonate and free pertechnetate had higher retention times (11.6 and 13.5 min respectively), under the same analysis standards. Preliminary biodistribution studies in normal mice showed that the two labelled species presented similar in vivo behaviour. Further investigation on the formation, equilibrium and biodistribution of the two species is in progress.

The immunoscintigraphy and kinetic data for this study referred to just one patient with grade II superficial TCC. Tumor localization was achieved with immunoscintigraphy performed 90 min post administration. HMFG1 as well as HMFG2 and AUA1 MoAbs have been used to monitor antigenic expression of Transitional Cell Carcinomas (TCC) of the bladder by immunohistochemical and immunocytological methods (11,12). Monoclonal antibody AUA1 labelled with In-111 (13) and Iodine-131 (14) was administered intravesically in patients with TCC prior to cystoscopy and transurethelial resection; and it was found that the uptake of AUA1 by TCC was correlated well with the grade of the tumor. In our study tumor to non tumor ratio of HMFG1 was about 5, which is an encouraging observation, taking into consideration that tumors of higher grade are expected to have a higher uptake. Finally, the fact that practically no blood radioactivity was detected is of great importance for radioimmunotherapy of bladder tumor.

In conclusion, we have optimized a practical, simple radiolabelling method of MoAbs with ^{99m}Tc for clinical use. Our preliminary clinical data are encouraging for the use of ^{99m}Tc-labelled MoAbs in bladder cancer diagnosis and therapy.

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