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# Human Polyclonal Immunoglobulin Labelled with Technetium-99m via NHS-$MAG_3$: A Comparison of Radiochemical Behavior and Biological Efficacy with Other Labelling Methods

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**ABSTRACT. The aim of this study was to evaluate the radiochemical behavior, biological distribution, and localization in infection sites in mice of a human polyclonal immunoglobulin (HIG) labelled with $^{99m}$Tc by a novel $MAG_3$-labelling method. The resulting [$^{99m}$Tc]$MAG_3$-HIG was compared with [$^{99m}$Tc]HIG preparations radiolabelled directly via 2-mercaptoethanol (2-Me) or stannous ion (Sn) reduction and indirectly via 2-iminothiolane (2-Im) conjugation. All preparations showed similar UV and radioactivity HPLC profile to that of native HIG except for 2-Im-HIG, which showed aggregates. The stabilities of the label to challenge with cysteine were similar for all the preparations. By nondenaturing SDS-PAGE, all preparations other than $MAG_3$-HIG showed evidence of lower molecular weight fragments. The tissue distribution 4 and 24 h after intravenous administration of the four preparations were compared in mice previously administered with an isolate of *Staphylococcus aureus* in one thigh. The pharmacokinetics varied among the different preparations. When prepared via 2-Me, Sn, and 2-Im, both blood clearance and urinary excretion were faster than that of labelled $MAG_3$-HIG. The absolute uptake in the infected thigh at 24 h was significantly higher for HIG labelled via $MAG_3$ and 2-Me vs. the remaining methods. The infected thigh/normal thigh radioactivity ratios were similar at both time points for labelled HIG prepared via 2-Me, 2-Im, and NHS-$MAG_3$ methods but was significantly lower at 24 h for HIG prepared via Sn. The radioactive HPLC profiles of serum at 4 and 24 h were similar to that of the radiolabelled injectates. Based on these data we conclude that each radiolabelled HIG preparation studied showed increased localization in infectious foci although [$^{99m}$Tc]$MAG_3$-HIG showed superior radiochemical and biological characteristics under the conditions of this investigation.** NUCL MED BIOL 25;4:395–403, 1998. **© 1998 Elsevier Science Inc.**

**KEY WORDS.** Immunoglobulin, Infection model, $MAG_3$

## INTRODUCTION

Radiolabelled human nonspecific polyclonal immunoglobulin (HIG) has been proposed for the scintigraphic detection of focal sites of infection/inflammation (1–7, 9, 24, 26), and indium-111 ($^{111}$In)-labelled HIG has been found to be clinically useful in a wide range of infectious diseases. However, technetium-99m ($^{99m}$Tc), owing to its superior physical properties and availability, is usually the preferred imaging radionuclide. Several experimental studies and clinical applications with [$^{99m}$Tc]HIG have been reported recently. Despite occasional contradictory results concerning the clinical efficacy with the [$^{99m}$Tc]labelled preparations (2, 3, 14, 21, 26) and despite the availability of competing modalities (labelled leukocytes, monoclonal antibodies for granulocyte labelling, labelled chemotactic peptides and antibiotics), [$^{99m}$Tc]HIG may be the agent of choice for imaging a variety of clinical situations related to inflammatory processes.

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Recently, Hnatowich *et al.* introduced a new method for $^{99m}$Tc radiolabelling of proteins, oligonucleotides, and peptides by conjugation with a bifunctional chelator, an acetyl *S*-protected ester of $MAG_3$ (*i.e., N*-hydroxysuccinamidyl-*S*-acetylmercaptoacetyltriglycine [NHS-$MAG_3$]) (19). To evaluate this labelling method for the preparation of $^{99m}$Tc-labelled HIG, we compared the radiochemical properties, biological distribution, and localization in infection sites in mice of [$^{99m}$Tc]$MAG_3$-HIG with that of [$^{99m}$Tc]HIG prepared directly via 2-mercaptoethanol (2-Me) or stannous ion (Sn) reduction and indirectly via 2-iminothiolane (2-Im) conjugation.

## MATERIALS AND METHODS

A human nonspecific polyclonal immunoglobulin (HIG) for IV administration (Intraglobulin, Biotest Pharma GmbH, Germany) was used as received. A HIG solution was prepared in 0.01 M PBS buffer, pH 7.4, at a concentration of about 10 mg/mL, except in the case of stannous reduction, for which the HIG was diluted in saline to a final concentration of 3–5 mg/mL. Protein concentration was determined by absorbance at 280 nm using an absorbance value of 1.4 for IgG. NHS-$MAG_3$ was synthesized as previously described (27).

Pertechnetate was eluted from a $^{99}Mo/^{99m}Tc$ generator (Amersham, UK); the methylene diphorphonate (MDP)(Sn) solution was obtained by reconstitution with saline of a MDP radiopharmaceutical kit (ITN, Portugal). All reagents were analytically pure.

### *Antibody Modification and Radiolabelling*

The HIG was radiolabelled with $^{99m}Tc$ by two direct and two indirect methods. The direct methods used 2-Me and Sn reduction based on published procedures (20, 23, 25). One of the indirect methods was modified from published reports using 2-Im for HIG coupling to generate free sulfhydryl groups (12, 18). All three methods were compared with an indirect method using acetyl-protected NHS-$MAG_3$ (27).

2-MERCAPTOETHANOL REDUCTION. To a stirred solution of HIG at a concentration of approximately 10 mg/mL in 0.01 M phosphate buffer saline (PBS), pH 7.4, an appropriate volume of 2-Me was added to obtain a reaction mixture with molar ratio for 2-Me/HIG of 1000:1. The reaction mixture was kept for 30 min at room temperature. The reduced preparation was then purified on a 0.9 × 15 cm Sephadex G-50 column (Pharmacia, Uppsala, Sweden) using nitrogen-purged PBS as mobile phase. The efficiency of purification was assured by spectrophotometric analysis of eluted fractions at 280 nm and 412 nm for protein and SH/Ellman's detection, respectively (11). Aliquots of 0.5 mg of reduced protein were then frozen and/or lyophilised after adding 50 μL of a MDP kit previously reconstituted with 5 mL of saline (corresponding to 1.3 μg of Sn [II]). For labelling, 0.5–2.0 mL of [$^{99m}Tc$]pertechnetate eluate (37–148 MBq) was added to 0.5 mg of HIG and allowed to stand for 10 min at room temperature.

STANNOUS REDUCTION. To HIG saline solution at a concentration approximately 5 mg/mL, an appropriate volume of nitrogen-purged 50 mM solution of stannous tartrate-phthalate, pH 5.6, was added to obtain a molar ratio (Sn[II]/HIG) of 500:1. The mixture was left to incubate overnight at room temperature. The preparation was then aliquoted into vials (0.5 mg/vial) under nitrogen and frozen at −20°C. For labelling, 0.5–1.0 mL of [$^{99m}Tc$]pertechnetate eluant (37–148 MBq) was added to 0.5 mg of HIG, and the solution was allowed to stand for 10 min at room temperature. The preparation was purified on a 0.9 × 15 cm Sephadex G-50 column to remove free pertechnetate.

2-IMINOTHIOLANE CONJUGATION. Published procedures for labelling antibodies via 2-Im reduction were modified to optimize the experimental conditions for HIG labelling (22). The final protocol was as follows: to a solution of HIG at a concentration of approximately 10 mg/mL in 0.01 M phosphate-buffered saline (PBS), pH 7.4, a suitable volume of 0.07 M 2-Im solution in PBS was added. The molar ratio (2-Im/HIG) was therefore 260:1. After 1 h of incubation at room temperature, the HIG conjugate was purified on a Sephadex G-50 column to remove the unreacted 2-Im. The efficiency of purification was monitored by spectrophotometric measurement of each fractions at 245, 280, and 412 nm for 2-Im, protein, and SH/Ellman's concentration, respectively (11). Aliquots of conjugated HIG of 0.5 mg were nitrogen-purged and stored at −20°C. For labelling, 25 μL of the reconstituted MDP (Sn) kit (containing 3.3 μg of Sn [II] was added followed by 0.5–1.0 mL of [$^{99m}Tc$]pertechnetate eluant (37–148 MBq), and the solution was allowed to stand for 10 min at room temperature.

NHS-$MAG_3$ CONJUGATION. HIG was conjugated to *N*-hydroxysuccinamidyl-*S*-acetylmercaptoacetyl triglycine (NHS-$MAG_3$) as follows: A solution 10 mg/mL of HIG in 0.01 M PBS was added to an equal volume of 0.5 M HEPES buffer, pH 8.0. With vigorous shaking, a 1 mg/mL solution of NHS-$MAG_3$ in dry dimethyl formamide (DMF) was added to a molar ratio $MAG_3$/HIG of 10:1. The solution was incubated at room temperature for 15 min. Thereafter, the conjugated HIG was purified on a Sephadex G-50 column using as eluant 0.01 M PBS and 0.25 mM DTPA. Protein fractions were then collected and pooled. The efficiency of purification was assured by size-exclusion HPLC analysis. Aliquots of 0.5 mg were nitrogen-purged and stored at 4°C. Radiolabelling with $^{99m}Tc$ was accomplished by transchelation from labelled tartrate. An appropriate volume of [$^{99m}Tc$]pertechnetate eluate (37–148 MBq) was added to 0.5 mg HIG followed by 150 μL of sodium tartrate solution in 0.5 M sodium bicarbonate, 0.25 M ammonium acetate, 0.18 M ammonium hydroxide buffer (50 mg tartrate/mL buffer, pH 8.9), and finally 50 μL of a fresh 1 mg/mL $SnCl_2 \cdot 2H_2O$ in 0.1 N HCl. Final pH was about 7.5. The labelling solution was allowed to stand 30 min at room temperature. Each labelled preparation was purified on a Sephadex G-50 column using PBS as eluate.

The identical radiolabelling procedure was performed on the free NHS-$MAG_3$ and on the native, uncoupled HIG as controls. The stability of NHS-$MAG_3$ in dry DMF solution, stored at 4°C, was evaluated on the basis of conjugation yields and on radiolabelling efficiency of conjugated HIG preparations prepared using the DMF solution at 3 days, 6 days, and 3 months.

### *Analytical Methods*

DETERMINATION OF SULFHYDRYL GROUPS. The determination of free sulfhydryl groups on reduced and 2-Im-conjugated HIG preparations was accomplished with Ellman's reagent (5-5′-dithiol bis-[2-nitrobenzoic acid]) (8). To the purified protein solutions, an equal volume of the reagent (2 mg/mL) freshly prepared in 0.1 M phosphate buffer, pH 8.0, was added. Absorbance was measured using a UV spectrophotometer at 412 nm.

In the case of $MAG_3$-HIG, the free sulfhydryl groups were generated by a 1-h incubation at room temperature with 0.5 M hydroxylamine hydrochloride solution in phosphate buffer to deprotect the thiol. Determination of sulfhydryl groups was then achieved by reaction with 4,4′-dithiodipyridine (DTP) (13), and UV absorbance was read at 324 nm after 15 min. The difference in absorbance between conjugated and native HIG is contributed by the thiol groups of $MAG_3$.

Free sulfhydryl groups were assayed with reference to a standard curve obtained by analysis of a series of known concentrations of free cysteine. Standard cysteine solutions ranging between 0.05–2.0 mM (Ellman's method) and 5–100 μM (DTP method) were prepared. A linear regression analysis of the data gave correlation coefficients of 0.9999 for Ellman's and 0.9897 for DTP.

CHROMATOGRAPHIC SYSTEMS. Labelling efficiency and stability evaluations were accomplished by chromatography. The following chromatographic systems were used: (a) ITLC SG (Gelman Sciences, Ann Arbor, MI) with saline and acetone eluants. In saline, labelled HIG and colloids remain at the origin while [$^{99m}Tc$]-labelled MDP, tartrate, and pertechnetate migrate. In acetone, only labelled pertechnetate migrates. (b) HPLC (Perkin-Elmer, Norwalk, CT) system with a TSK G 3000 SW size-exclusion column fitted with a fixed UV (280 nm) and radioactive (Berthold, Wildbad, Germany) detectors. The mobile phase was 0.1 M phosphate buffer, pH 7.0, at a flow rate of 0.5 mL/min. Recovery was determined by collecting the total eluate for 30 min (2-Me, Sn, and 2-Im methods) or 40 min (NHS-$MAG_3$ conjugation) from the time of injection. Fractions (0.5 mL) were counted in a gamma

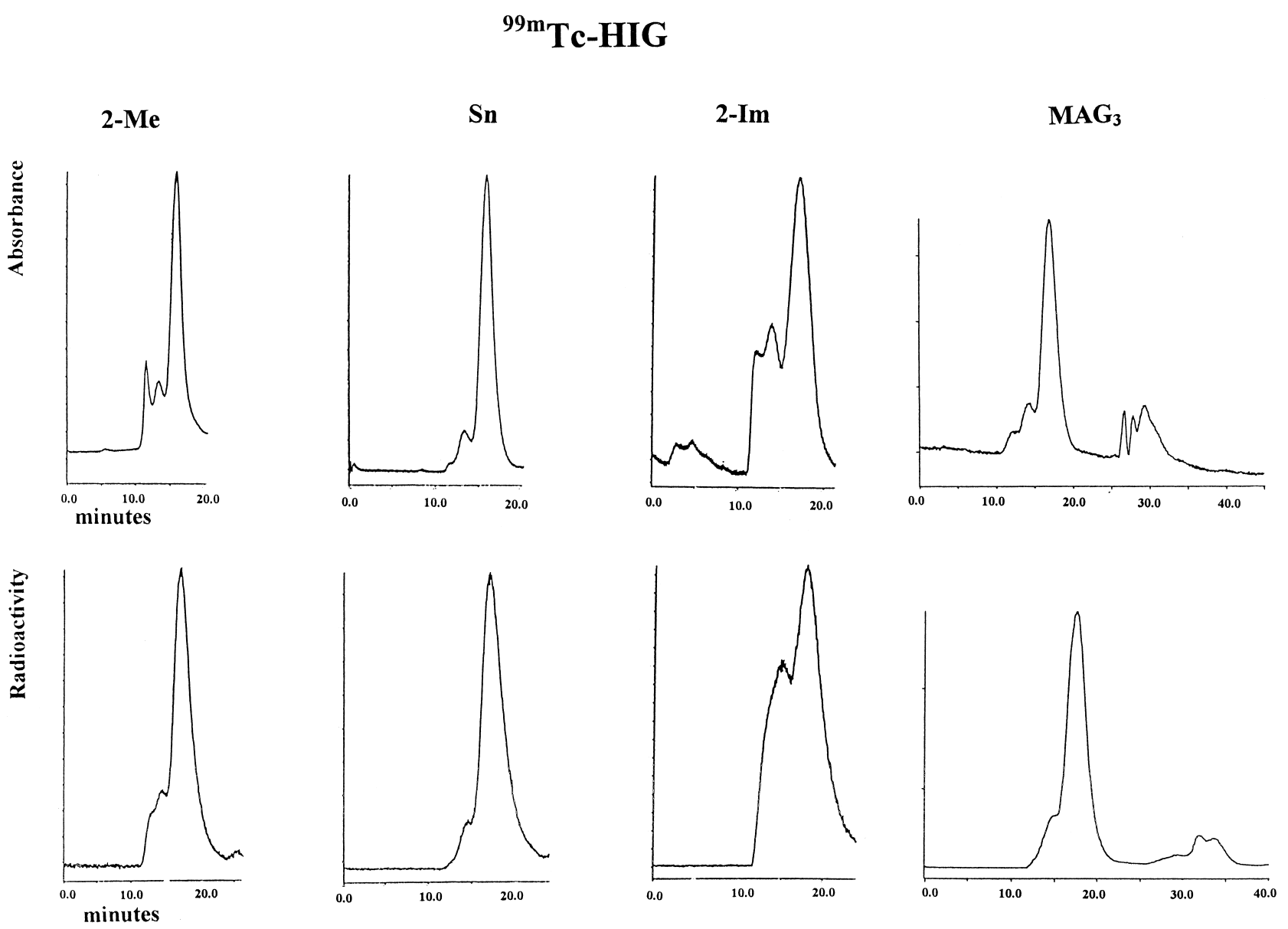


FIG. 1. UV and radioactive HPLC profiles of [$^{99m}$Tc]HIG obtained via (A) 2-Me; (B) Sn; (C) 2-Im; (D) NHS-MAG$_3$. Experimental conditions: TSK G 3000 column; flow rate 1.0 mL/min.; mobile phase 0.1 M phosphate buffer; pH = 7.0.

counter together with a suitable standard of the injectate. The HPLC column was calibrated for molecular weight using commercially available protein standards (Pharmacia Biotech, Milwaukee) in the range 13–660 kDa. The retention time corresponding to each standard was recorded and then plotted on a semi-log scale. A linear relationship was obtained.

STABILITY OF LABELLED HIG TO CHALLENGE WITH CYSTEINE. The stability of $^{99m}$Tc on each labelled HIG preparation toward cysteine was tested by an *in vitro* cysteine-challenge assay previously described (16). Cysteine solutions were freshly prepared with PBS by serial dilution to final concentrations of 1.0, 0.1, 0.01, and 0.001 mg/mL. Each cysteine solution was incubated for 1 h at 37°C with appropriate volume of each HIG preparation to achieve molar ratios cysteine: HIG ranged from 0.5:1 to 500:1. After incubation, each solution was analysed simultaneously by ITLC SG/acetone and Whatman N.1/saline. Each strip was cut in half, and each half was counted in a gamma counter. In acetone, only labelled pertechnetate migrates, while in saline both labelled pertechnetate and cysteine migrate.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under nonreducing conditions on the native, reduced, or conjugated HIG as well as on the HIG labelled by the four methods under investigation. 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 (Pharmacia Biotech, Milwaukee) 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 using a Pharmacia apparatus. The electrophoresis was run at 20–30 mA until the tracking dye reached 0.5–1.0 cm from the bottom of the gel. The SDS-PAGE analysis was performed in duplicate. One of the gels was measured for radioactivity using a radiochromatograph scanner (Berthold, LB 2723, Wildbad, Germany) and was used to plot the radioactive SDS-PAGE profiles. It 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 were available. The other gel was immediately fixed and stained.

### *Animal Experiments*

INFECTION MODEL. A single clinical isolate of *Staphylococcus aureus* from a biological sample was used to produce focal infection. Individual colonies were diluted to obtain turbid suspensions containing approximately $10^8$ organisms/mL. The optimization of these infectious conditions was previously established (10). Groups of six female CD-1 mice, weighting approximately 25 g each, were injected with 100 μL of bacteria suspension in the right lateral thigh muscle. Twenty-four hours later, when gross swelling was apparent in the infected thigh, each of the labelled HIG preparations was intravenously administered.

BIODISTRIBUTION. The *in vivo* behavior of the labelled HIG preparations was evaluated in groups of six infected female CD-1 mice (randomly bred, Charles River, Wilmington, MA), weighting approximately 25 g each. Animals were IV injected with 100 μL (5–10 MBq) of each preparation via the tail vein and were maintained on normal diet *ad libitum*. At 4 h and 24 h postinjection, the animals were killed by cervical dislocation. The radioactive dosage administered and the radioactivity in the sacrificed animal were determined by counting in a dose calibrator (AloKa, Curiemeter IGC-3, Aloka, Tokyo, Japan). The difference between the radioactivity in the injected and sacrificed animal was assumed to be due to urinary excretion. Blood samples were taken by cardiac puncture at sacrifice. The blood was then centrifuged and the serum separated for HPLC analysis as described, and tissue samples were then removed for counting in a gamma counter (Innotron, Hydragamma, Innotron Ltd., Oxford, UK). The results were expressed as percent of injected dose per organ (% ID/organ). The remaining activity in the carcass was also measured in a dose calibrator. Biodistribution results were evaluated by an analysis of variance. The level of significance was set at 0.05 (two-sided). For blood, the activity was calculated by assuming that this organ constitutes 7% of the total weight body. Both the infected and noninfected thighs were dissected and counted so that the infected/noninfected thigh radioactivity ratio could be evaluated.

IMAGING STUDIES. A separate set of mice (two per preparation) with the same infections induced as described were intravenously injected with 37 MBq of each radiolabelled preparation under study. Whole-body images of infected mice were obtained with a GE gamma camera connected to a Starcam 4000i computer. All the images were acquired in a 128 × 128 matrix at 4 and 24 h after administration of the radiolabelled HIG preparations.

## RESULTS

### *Antibody Modification and Radiolabelling*

For all methods, an efficient purification of the reduced or conjugated HIG from excess reducing agent or free 2-Im or $MAG_3$ was achieved. In the case of HIG conjugated with NHS-$MAG_3$, the UV profile obtained by size-exclusion HPLC before Sephadex G-50 purification presented two peaks at retention times of 16 and 30 min, which were assigned to HIG and free $MAG_3$, respectively. Recoveries of 2-Im or NHS-$MAG_3$-conjugated HIG after column purification averaged about 80%.

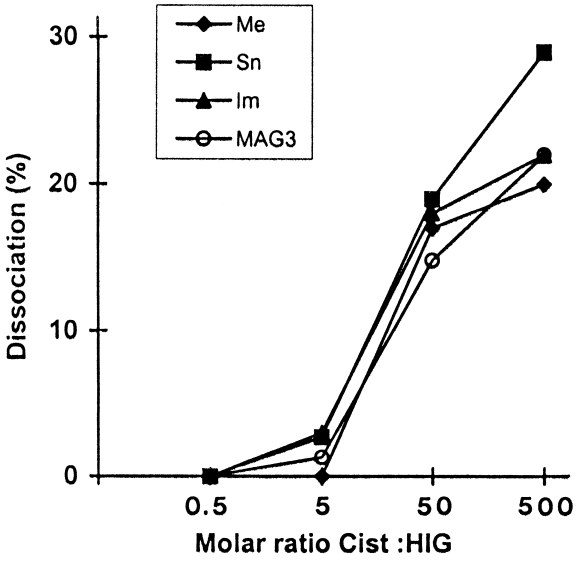


**FIG. 2. Dissociation of [$^{99m}$Tc] from labelled HIG obtained via 2-Me, Sn, 2-Im, and NHS-$MAG_3$ versus molar ratio cystein:HIG (0.5:1; 5:1; 50:1; 500:1), after 1-h incubation at 37°C.**

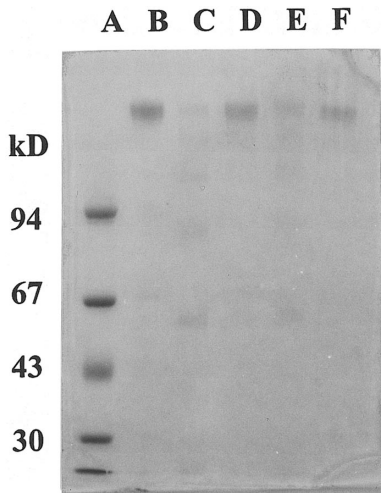


**FIG. 3. SDS-stained gels. (A) MW standards; (B) native HIG; (C) 2-Me reduced HIG; (D) Sn-reduced HIG; (E) 2-Im-coupled HIG; (F) NHS-MAG$_3$-conjugated HIG.**

The influence of time on protein conjugation with NHS-MAG$_3$ was studied over 15 to 60 min. The results indicated that no improvement in radiolabelling efficiency was achieved for incubation times longer than 15 min. Furthermore, labelling efficiencies ranging from 60% to 85% were obtained using either fresh DMF solutions of NHS-MAG$_3$ or solutions stored at 4°C for 3, 6, and 180 days. Thus, the NHS-MAG$_3$ solution appears to be stable at least for 3 months when stored at refrigerator temperatures.

### *Analytical Methods*

DETERMINATION OF SULFHYDRYL GROUPS. The average number of free sulfhydryl groups per molecule of reduced HIG was 6.1 and 6.4 for 2-Me and Sn methods, respectively. The number of sulfhydryl groups introduced per molecule of coupled HIG was 11.2 and 3.0 for the 2-Im and NHS-MAG$_3$ methods, respectively.

RADIOCHEMICAL PURITY AND *IN VITRO* STABILITY. Labelling efficiency and radiochemical purity monitored by instant thin layer chromatography (ITLC) was always higher than 98% for HIG preparations obtained by 2-Me reduction and 2-Im coupling. When Sn was used as reductant, labelling yields dropped to 70–90%. Labelling efficiencies for HIG conjugated with NHS-MAG$_3$ averaged 60–85%. Thus, both Sn-reduced and MAG$_3$-coupled HIG preparations required post-labelling purification. Radiolabelling efficiency percentages under identical conditions for native HIG and free NHS-MAG$_3$ controls were less than 5% and about 70%, respectively. Stability studies of radiolabelled HIG evaluated by ITLC and HPLC have shown that preparations were stable for at least 4 h after labelling (data not presented).

Figure 1 shows the UV (top row) and radioactivity (bottom row) profiles by HPLC analysis of labelled HIG preparations from 2-Me, Sn, 2-Im, and NHS-MAG$_3$ formulations. For both direct and

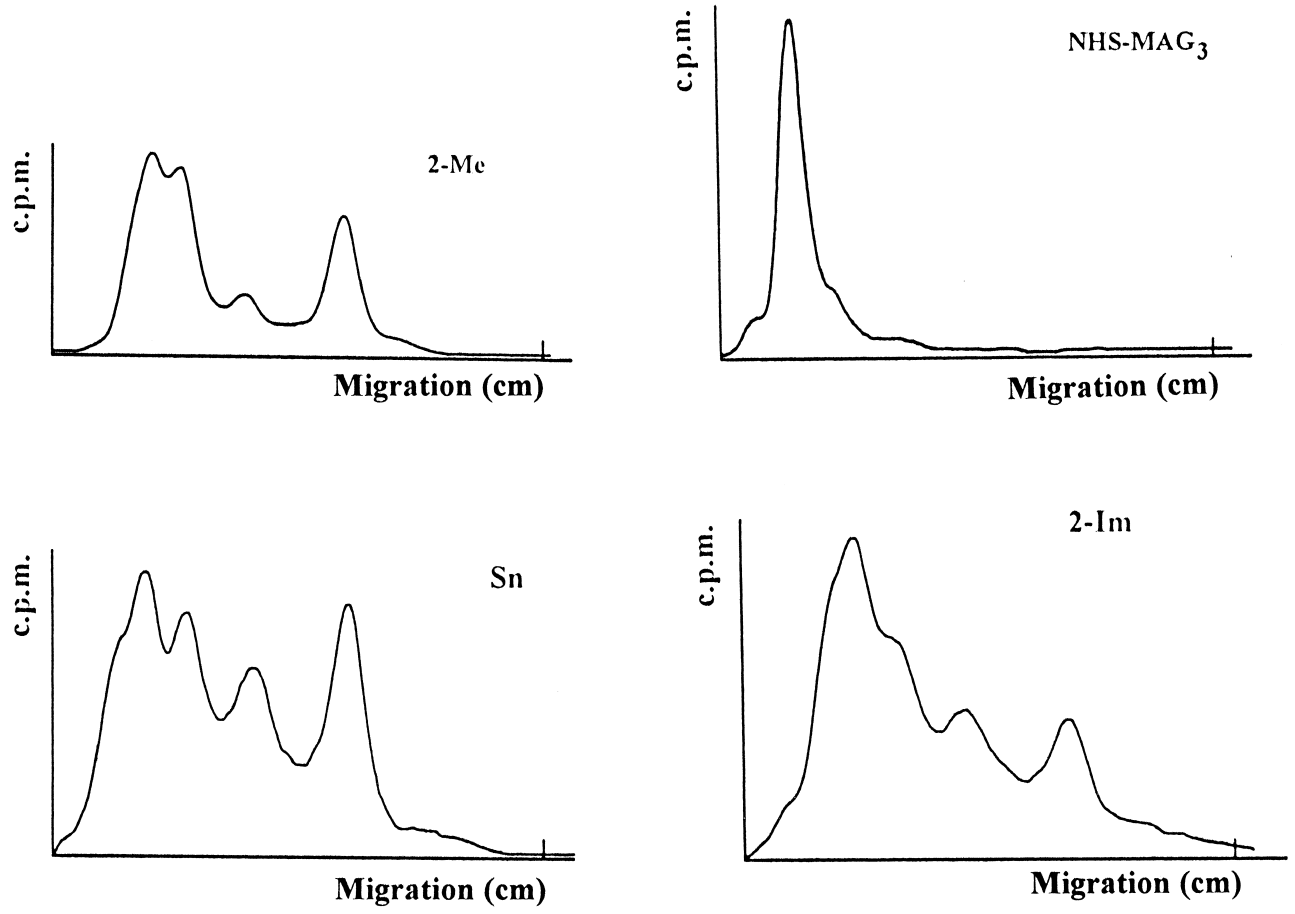


**FIG. 4. SDS-PAGE radioactive profiles of [$^{99m}$Tc]HIG obtained via 2-Me, Sn, 2-Im, or NHS-MAG$_3$.**

**TABLE 1. Biodistribution Data in Percent Injected Dose per Total Organ (Mean Values ± SD) for [$^{99m}$Tc]HIG Obtained Via 2-Me or Sn Reduction and Via 2-Im and NHS-MAG$_3$ Conjugation 4 H and 24 H after IV Administration in Infected Female Mice ($n$ = 4–8)**

| | HIG (2-Me) | | HIG ($SnCl_2$) | | HIG (2-Im) | | HIG (NHS-MAG$_3$) | |
|---|---|---|---|---|---|---|---|---|
| **Organ** | **4 h** | **24 h** | **4 h** | **24 h** | **4 h** | **24 h** | **4 h** | **24 h** |
| Blood | 19.4 ± 0.6 | 10.5 ± 2.0 | 30.3 ± 9.9 | 4.8 ± 0.2 | 23.1 ± 4.7 | 6.3 ± 0.8 | 27.5 ± 4.3 | 12.6 ± 1.0 |
| Liver | 9.3 ± 3.0 | 3.3 ± 0.6 | 8.1 ± 1.4 | 3.8 ± 0.4 | 10.0 ± 1.3 | 4.6 ± 0.7 | 9.7 ± 2.0 | 3.8 ± 0.3 |
| Gut | 5.5 ± 0.6 | 2.3 ± 0.3 | 5.2 ± 0.5 | 2.3 ± 0.2 | 6.5 ± 1.0 | 2.2 ± 0.1 | 6.7 ± 0.6 | 3.5 ± 0.2 |
| Spleen | 0.5 ± 0.2 | 0.2 ± 0.03 | 0.5 ± 0.1 | 0.3 ± 0.02 | 0.4 ± 0.06 | 0.2 ± 0.0 | 0.7 ± 0.08 | 0.2 ± 0.0 |
| Heart | 0.5 ± 0.1 | 0.2 ± 0.04 | 0.5 ± 0.1 | 0.2 ± 0.1 | 0.6 ± 0.1 | 0.1 ± 0.0 | 0.9 ± 0.2 | 0.3 ± 0.0 |
| Lung | 1.2 ± 0.2 | 0.7 ± 0.2 | 1.3 ± 0.3 | 0.6 ± 0.1 | 1.3 ± 0.3 | 0.3 ± 0.0 | 1.8 ± 0.4 | 1.2 ± 0.1 |
| Kidney | 3.7 ± 0.5 | 1.6 ± 0.2 | 4.4 ± 0.3 | 2.6 ± 0.4 | 4.2 ± 0.6 | 2.3 ± 0.3 | 2.5 ± 0.3 | 1.1 ± 0.1 |
| Stomach | 0.5 ± 0.05 | 0.4 ± 0.1 | 0.5 ± 0.03 | 0.4 ± 0.04 | 0.6 ± 0.1 | 0.2 ± 0.0 | 0.7 ± 0.1 | 0.4 ± 0.0 |
| Carcass | 29.4 ± 5.0 | 23.6 ± 0.6 | 29.2 ± 3.9 | 27.0 ± 1.2 | 30.6 ± 2.6 | 15.9 ± 0.9 | 48.4 ± 5.2 | 43.9 ± 0.9 |
| Normal thigh | 1.8 ± 0.6 | 0.9 ± 0.1 | 1.6 ± 0.2 | 0.7 ± 0.2 | 1.2 ± 0.2 | 0.5 ± 0.0 | 1.7 ± 0.3 | 1.2 ± 0.1 |
| Infected thigh | 6.0 ± 0.9 | 6.3 ± 0.6 | 5.6 ± 1.3 | 2.8 ± 0.7 | 5.3 ± 1.6 | 3.5 ± 0.6 | 7.8 ± 0.6 | 8.4 ± 0.7 |
| Urine | 33.5 ± 4.0 | 55.6 ± 3.6 | 34.2 ± 1.5 | 50.2 ± 2.9 | 36.3 ± 3.7 | 68.3 ± 5.6 | 9.5 ± 2.5 | 37.6 ± 4.9 |

NHS-MAG$_3$ labelling, the main UV peak showed an identical retention time to native HIG. In the 2-Im case, however, the UV and radioactive profiles showed distinct evidence of dimeric and polymeric species.

STABILITY OF LABELLED HIG TO CHALLENGE WITH CYSTEINE. Results of stability toward cysteine are presented in Figure 2. The percentage of dissociation was identical for all HIG preparations at each cysteine: HIG molar ratio. Instability increased with increasing molar ratios in each case.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS. SDS-PAGE stained gels of each HIG preparation, native HIG, and MW standards are shown in Figure 3. HIG conjugated with NHS-MAG$_3$ shows a pattern (Figure 3F) quite similar to that of the native HIG (Figure 3B). However, low molecular weight bands appear for each of the remaining preparations (Figure 3C–E). Using the molecular weight standards, the molecular weights of the low molecular weight bands are estimated as 115, 90, and 50 kDa. The correspondent radioactive SDS-PAGE profiles are presented in Figure 4 and show the same pattern of single peak for MAG$_3$-HIG and multiple low molecular weight peaks for the remaining preparations. A qualitative analysis of both SDS-PAGE profiles (colour-band intensity and radioactive distribution) indicates that some correlation can be established between each colour-band intensity and the radioactivity associated to it except for the Sn method. In fact, the stained SDS-PAGE profile of [$^{99m}$Tc]HIG preparation obtained via Sn procedure revealed few fragments of low molecular weight (90, 50 kDa MW) but a significant high radioactivity associated to those bands that were weakly stained.

### *Animal Experiments*

BIODISTRIBUTION. Tissue distribution data of different labelled HIG preparations expressed as percentage of injected dose per organ (%ID/organ) 4 h and 24 h after IV administration in infected female mice are presented in Table 1. It was observed that clearance of radioactivity from the carcass and from most tissues tended to be slower for MAG$_3$-HIG relative to the other preparations.

Table 2 presents the ratio of infected thigh to normal thigh radioactivity at 4 and 24 h postinjection. At 24 h, a significantly ($p < 0.02$) lower ratio of infected thigh/normal thigh was obtained with HIG prepared by Sn reduction. No significant differences were found ($p > 0.05$) among the remaining three preparations at either time point.

The radioactive profiles from HPLC analysis of mouse serum and urine samples collected at 4 and 24 h postadministration are presented in Figure 5. For comparison purposes, the radiochromatograms of the injected HIG preparations are also presented (top row). The serum profiles are very similar to those of the correspondent labelled HIG. The 4-h radiochromatograms of Me, Sn, and Im labelled HIG show a slight percentage of activity eluted at low molecular weight. As expected, in urine, the radioactivity is associated with low molecular weight species, possibly catabolism products such as cysteine (15) and possibly free pertechnetate.

GAMMA CAMERA IMAGING. Whole-body images of infected mice at 4 and 24 h postadministration of the different HIG preparations are presented in Figure 6. In all animals the infectious foci are clearly visible in the right thigh (right side in image) at both time points. The slow blood clearance of MAG$_3$-HIG is reflected in the high activity in the heart.

## DISCUSSION

This work was undertaken to investigate the *in vitro* and *in vivo* properties of $^{99m}$Tc labelled to HIG by means of a *S*-acetyl NHS-MAG$_3$ bifunctional chelator (27). Results were compared to that obtained with [$^{99m}$Tc]HIG prepared by three additional, well-established methods: 2-mercaptoethanol reduction, stannous reduction, and 2-iminothiolane coupling.

The determination of sulfhydryl groups generated during HIG reduction and conjugation showed that antibody modification was

**TABLE 2. Radioactivity Ratio Between the Infected Thigh and Noninfected Thigh Obtained at 4 ($n$ = 8) and 24 H ($n$ = 4) after Administration of 4 [$^{99m}$Tc]HIG Preparations**

| | Infected/noninfected | |
|---|---|---|
| **$^{99m}$Tc-HIG preparations** | **4 h** | **24 h** |
| HIG (2-Me) | 3.8 ± 0.8 | 7.3 ± 1.2 |
| HIG ($SnCl_2$) | 3.8 ± 0.7 | 4.0 ± 0.6 |
| HIG (2-Im) | 4.2 ± 1.0 | 6.0 ± 0.8 |
| HIG (NHS-MAG$_3$) | 4.6 ± 0.9 | 7.0 ± 1.2 |

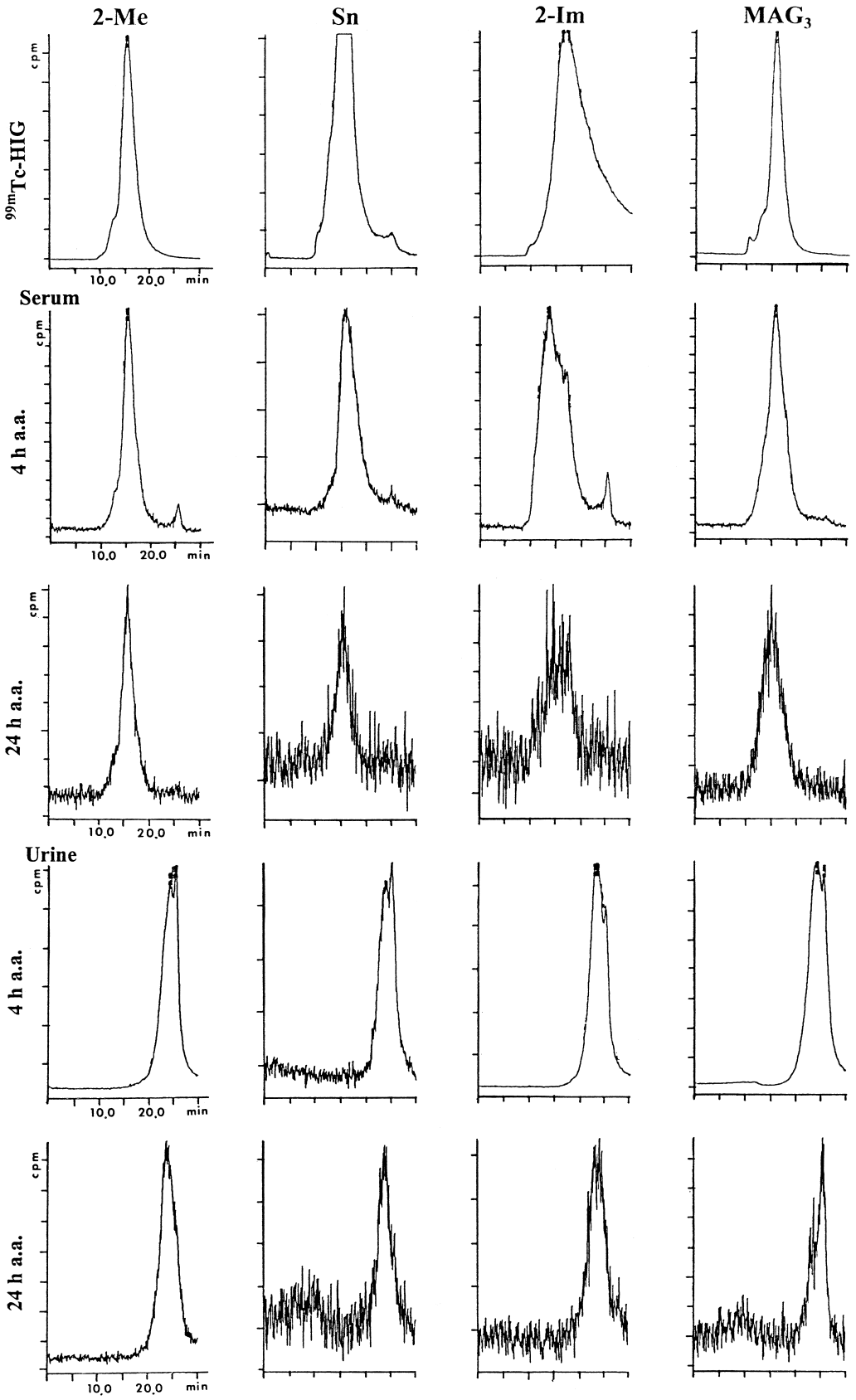


**FIG. 5. Size-exclusion HPLC radiochromatograms of [$^{99m}$Tc]HIG obtained via 2-Me, Sn, 2-Im, or NHS-MAG$_3$ in solution (top row) and in serum and urine obtained from mice at 4 and 24 h postadministration.**

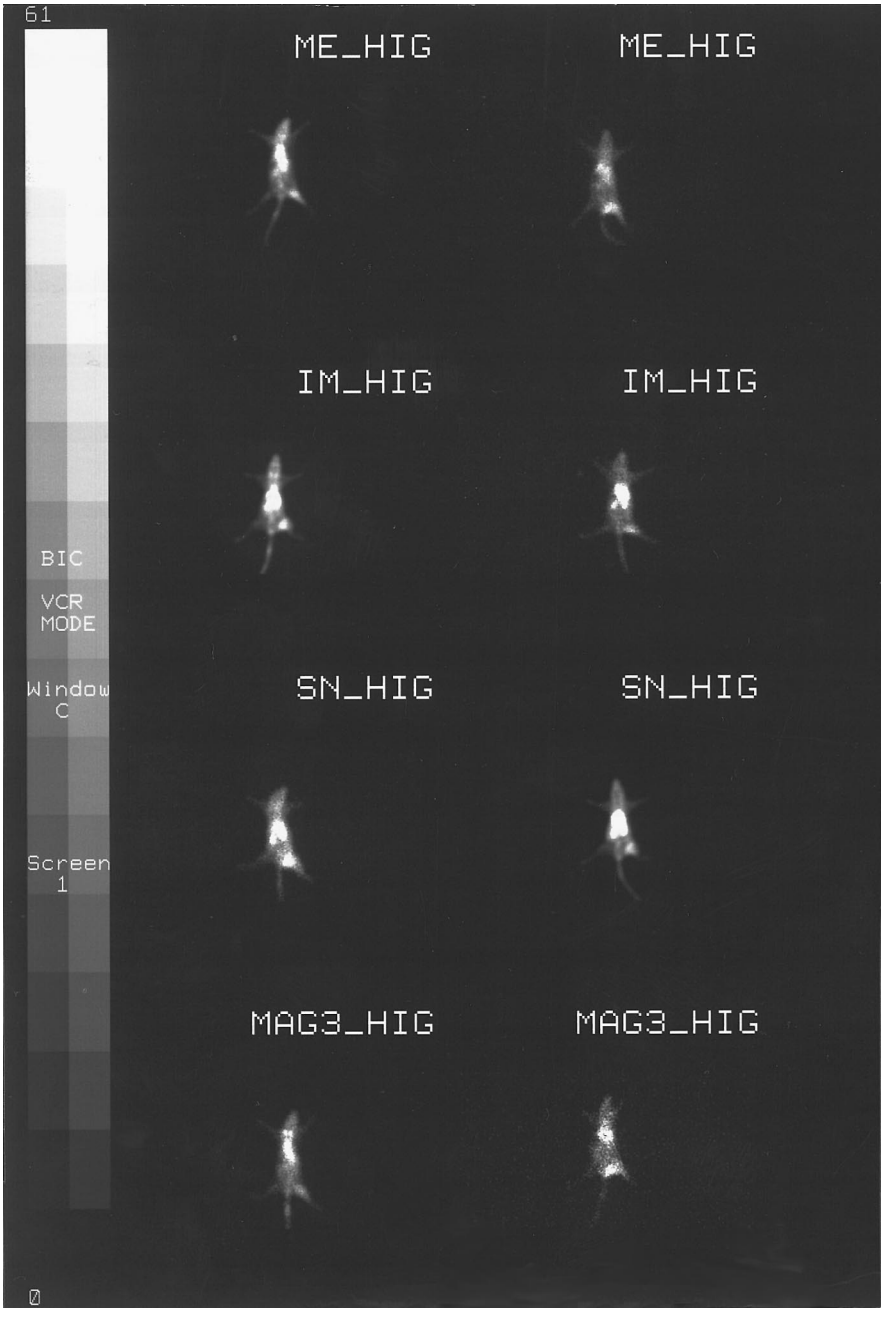


**FIG. 6. Planar whole-body gamma camara images of mice (infected in the right thigh) injected with [$^{99m}$Tc]HIG obtained via 2-Me, Sn, 2-Im, or NHS-MAG$_3$ at 4 (left column) and 24 h (right column) postadministration.**

successful in all cases. Reduction by 2-Me or Sn generated an average of about six free sulfhydryls per antibody molecule. The number of additional sulfhydryl groups added by conjugation with 2-Im or NHS-MAG$_3$ was found to be in the range expected. Whereas an average of three groups per molecule for MAG$_3$-HIG would not be expected to seriously alter antibody properties, it is possible that the 11 groups attached via 2-Im could have some deleterious effects (22). Overconjugation might explain the high molecular weight aggregates observed in the UV and radioactivity profiles for 2-Im-HIG (Fig. 1).

Under the condition of this investigation, radiolabelling efficiencies for 2-Me-reduced and 2-Im coupled HIG preparations were >98%. Radiolabelling efficiencies were lower for Sn-reduced and, especially, for MAG$_3$-conjugated HIG. Only low specific activity labelling was attempted in this research (about 72–296 MBq/mg of HIG), which should not have influenced the labelling efficiency. After purification, the radiochemical purity was higher than 98% in all cases.

Stability to cysteine transchelation did not present pronounced differences. All radiolabelled preparations exhibited higher dissociation at cysteine molar ratios above 5:1, corresponding to cysteine concentration, which may be not relevant *in vivo* (15).

SDS-PAGE analysis revealed the presence of other protein fractions of lower molecular weight on all labelled HIG preparations other than MAG$_3$-HIG (Figs. 3, 4). The presence of low molecular weight bands by SDS-PAGE analysis of 2-Me and Sn-reduced antibodies has been previously reported (15, 17). That 2-Im should also weaken intramo-

lecular disulfide bonds is not surprising since 2-Im is also a reducing agent. Antibody reduction should not and apparently did not result from NHS-$MAG_3$ conjugation as shown (Figs. 3, 4). The protein fragments detected by SDS-PAGE most probably were related to the harsh analysis conditions, in particular exposure to boiling water temperatures (15) associated with an increased liability of pretreated (2-Me, Sn, 2-Im) HIG molecules when compared to intact or conjugated HIG. These findings, therefore, do not necessarily imply that fragmentation also takes place *in vivo*. In fact, size-exclusion HPLC analysis of serum taken at 4 and 24 h postadministration of all the HIG preparations showed the radiolabel to be present predominantly as labelled HIG (Fig. 5).

The most striking difference in pharmacokinetics was the significantly slower blood clearance and urine accumulation with $MAG_3$-HIG. This may reflect an increased stability of the label when attached via $MAG_3$ over the other three methods, and is in accordance with SDS-PAGE results. Few other significant differences in biodistribution were observed at either time point among the preparations.

Accumulation of HIG in sites of infection/inflammation is reported to be due to increased blood flow in areas of infection or inflammation together with increased capillary permeability (9, 24). The uptake in the target thigh was significantly higher for labelled $MAG_3$-HIG relative to the other three preparations (Table 1). The target thigh/normal thigh radioactivity ratios for $MAG_3$-HIG at 24 h was statistically identical among all preparations other than Sn (Table 2).

In conclusion, no particular difficulty was experienced in radiolabelling HIG by any of the four methods considered in this research. Although SDS-PAGE analyses of protein and radioactivity profiles show protein fragmentation for all preparations other than $MAG_3$-HIG, the HPLC analysis in fresh mouse serum indicated that even for those preparations no significant fragmentation occurs *in vivo*. Probably owing to overconjugation, the 2-Im-prepared HIG showed obvious evidence of aggregate formation by HPLC although that also did not seem to alter significantly its pharmacokinetics. Each radiolabelled preparation was able to localize infectious focus induced with *Staphylococcus aureus* in mice. With absolute uptake in the target thigh significantly higher with $MAG_3$-HIG and pharmacokinetics indicative of improved label stability *in vivo*, it is possible that [$^{99m}$Tc]$MAG_3$-HIG may be the superior agent for infection/inflammation imaging among those agents investigated.

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