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Research Article

Evaluation of ^{99m}Tc-immunoglobulins for imaging infection in the rat

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Summary

Three immunoglobulin molecules were evaluated as infection imaging agents in a rat model of *S. aureas* infection: ^{99m}Tc-infliximab, ^{99m}Tc-human immunoglobulin (HIG) and ^{99m}Tc-rat immunoglobulin (RIG). Infliximab is a chimeric monoclonal antibody specific for human tumour necrosis factor alpha (TNFα). ^{99m}Tc-HIG was chosen as an exogenous protein and ^{99m}Tc-RIG as an endogenous marker. Each immunoglobulin was treated with 2-mercaptoethanol and the reduced antibody was isolated by size exclusion chromatography. In combination with Sn^{II}-methylenediphosphonic acid, cold kit formulations were prepared. Native and reduced infliximab were tested for rat TNFα binding ability *in vitro*. A focal intramuscular infection of *S. aureus* (1×10^8 colony forming units) was induced in the left thigh muscle of rats, that developed for 24 h. In separate experiments each tracer was administered by intravenous injection, then whole body scintigraphic imaging and biodistribution studies were performed at 1 and 4 h later. ^{99m}Tc-infliximab, ^{99m}Tc-HIG and ^{99m}Tc-RIG were prepared with $\geqslant$95% radiochemical purity from stable cold kits. Results from the organ assay gave infected (target) to non-infected (control) muscle ratios for ^{99m}Tc-infliximab as 5.7 ± 0.8, 7.1 ± 1.2, ^{99m}Tc-HIG gave 3.1 ± 1.1, 7.8 ± 1.2, and ^{99m}Tc-RIG 7.9 ± 0.3, 12.5 ± 1.5 at 1 and 4 h, respectively. Infliximab and Sn^{II}-infliximab did not bind to rat TNFα by the *in vitro* assay. Although lacking specific affinity for TNFα, ^{99m}Tc-infliximab accumulated at infectious sites *in vivo*. ^{99m}Tc-infliximab gave similar infection uptake ratios to ^{99m}Tc-HIG at 1 and 4 h, but these proteins were inferior in comparison to ^{99m}Tc-RIG, and is likely to be due to

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increased clearance associated with the foreign protein structure.

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Introduction

The imaging and diagnosis of infection or inflammation in nuclear medicine currently employs radiolabelled white cells and ^{67}Ga-citrate as radiotracers. Where the ^{67}Ga-complex has a lower sensitivity for infected lesions, ^{111}In- and ^{99m}Tc-labelled leukocytes are still regarded as the gold standard markers at the clinical level. The radiopharmaceutical agents used to label autologous patient leukocytes are ^{111}In-oxine,[1] ^{99m}Tc-HMPAO[2] and ^{99m}Tc-tin fluoride colloid.[3] ^{99m}Tc-colloid-labelled-leukocytes are particularly useful for the assessment of inflammatory bowel disease.[4] Despite their routine clinical application, radiolabelled cells do have practical disadvantages for procedures performed *ex vivo*, such as the lengthy radiolabelling and isolation steps, as well as possible contamination of the operator and/or the blood sample.

Generally, two other classes of non-cellular infection imaging agents have been investigated in preclinical and human studies. The first includes macromolecules or large particles of biological origin such as ^{99m}Tc-polyclonal human immunoglobulin (HIG),[5] ^{99m}Tc-HSA, ^{99m}Tc-interleukin-8,[6] ^{125}I-interleukin-1,[7] ^{99m}Tc-C5a,[8] ^{99m}Tc-PEG-liposomes[9] and the ^{99m}Tc-anti-CD15 IgM antibody Leutech.[10] A second class of agents comprises smaller synthetic molecules such as the inflammatory mediator peptides ^{99m}Tc-fMLPL,[11] ^{99m}Tc-ubiquicidin,[12] ^{99m}Tc-E-selectin IMP-178,[13] human neutrophil peptide-1,[14] an ^{111}In-/^{99m}Tc-leukotriene antagonist,[15] ^{99m}Tc-sulesomab (F_{ab} fragment, Leukoscan),[16] and the pharmaceutical-based ^{99m}Tc-alafosfalin,[17] ^{99m}Tc-fluconazole[18] or ^{99m}Tc-ciprofloxacin.[19] Some of the radiolabelled macromolecules of biological origin have shown potential in animal models but with mixed success in humans. This is mainly due to carrier material at very low concentration in the dose that results in either active biological function *in vivo* or elicits an immunological response by the host. Smaller protein fragments and synthetic agents are generally better tolerated, and they provide high target infection to control ratios.

In many radiotracer experiments conducted with rodents, ^{99m}Tc-HIG has been used as a large control molecule. There is permeability of vessels to the site of inflammation allowing accumulation of macromolecules to an extravascular inflammation site. This observation was explained by a 'non-specific' mechanism of retention due to binding with Fc receptors on local

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monocytes and other cells.[20] Whether ^{99m}Tc-HIG should be regarded as a control molecule is ambiguous, as is the significance of using a radioactive immunoglobulin in a different genetic host. The aim of this study was to examine three ^{99m}Tc-immunoglobulins for imaging a focal *S. aureas* infection in the rat, to distinguish any differences in uptake. In this species two exogenous proteins, ^{99m}Tc-HIG of human origin and the human/mouse chimeric ^{99m}Tc-infliximab were used, as well as ^{99m}Tc-rat immunoglobulin representing an endogenous protein marker.

Materials and methods

General

Infliximab (Remicade) was provided at no cost (Centocor Inc; Malvern; PA). Polyclonal human immunoglobulin, polyclonal rat immunoglobulin (RIG), human serum albumin (HSA), bovine serum albumin (BSA), o-phenylenediamine (o-PD) and 2-mercaptoethanol (2-ME) were obtained (Sigma-Aldrich; St Louis; Missouri) without further purification. All experiments were performed in triplicate unless otherwise specified. Cold kits were manufactured in a Biohazard Safety Cabinet (BH 120 Class II; Gelman Sciences; Australia) that was sterilised with aqueous ethanol (70%) before and after use. Sterile normal saline (0.9% w/v) and water for injection (WFI) were used for dilutions. ^{99m}Tc-pertechnetate was obtained from the daily milking of a ^{99}Mo/^{99m}Tc-generator (Gentech; Australian Radioisotopes; Sydney; Australia). Staphylococcus aureus ATCC 25923 (*S. aureus*) diluted with saline (1×10^9 colony forming units/ml) was used as the stock solution for inducing focal infections in rats.

Preparation of cold kits

Infliximab (INFX), HIG and RIG cold kits were prepared by a modification of a literature procedure.[21] 2-Mercaptoethanol in water (111 mg/ml; 0.15 ml; 0.21 mol) was added to the immunoglobulin (10 mg/ml; 3 ml; 66 nmol) in a sterile vial (10 ml), and the solution was mixed by rotation ($\sim$40 rpm) at room temperature for 30 min with a rotation apparatus (RSM6 suspension mixer; Ratek Instruments; Victoria; Australia). The reduced protein was purified using a size exclusion column (PD-10; Amersham Pharmacia Biotech AB, Uppsala; Sweden) and sterile phosphate buffered saline as the solvent. Eluate samples (0.5 ml) were collected to isolate protein (fractions 7–12) from 2-ME (fractions 13–20). The presence of protein in fractions was verified using a UV spectrophotometer (DU 640i; Beckman Instruments; San Diego; USA) with absorption of $\lambda_{max} = 278$ nm in each spectrum range 200–400 nm. A methylenediphosphonate kit (MDP; RAH Radiopharmacy; Adelaide; Australia) containing stannous chloride (1.68 mg/ml; 0.5 ml) was diluted with

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WFI (to 5 ml), and an aliquot (1.5 ml) was added to the combined protein fractions in a sterile vial (20 ml). The solution was further diluted with WFI (to 15 ml), nitrogen gas was gently bubbled through it (5 min) to provide an inert atmosphere, and then it was filtered (0.2 μm; Millex-GV; Millipore) into another sterile nitrogen-filled vial. The stock solution was dispensed (1 ml) into sterile nitrogen-filled vials (10 ml) and stored in a low temperature (−70°C) freezer prior to experimentation. Samples of each batch of immunoglobulin cold kit were tested by National Analytical Testing Authority-approved facilities to be both sterile and pyrogen-free.

Radiolabelling and quality control analysis

Thawed cold kits were reconstituted with ^{99m}Tc-pertechnetate (100–700 MBq) in saline (1.0 ml), and then allowed to stand at room temperature for 20 min. Radiochemical purity (RCP) of the ^{99m}Tc-immunoglobulin was determined by using instant thin layer chromatography (ITLC) with silica gel (SG) impregnated glass fibre strips (Gelman Sciences, Ann Arbor, USA) or albumin-coated ITLC-SG strips as the stationary phase. The radioactive sample was developed on ITLC-SG in the mobile phases acetone, saline and albumin coated-ITLC-SG/ethanol:ammonia:water (AEW) [2:1:5], to assay for the respective impurities ^{99m}Tc-pertechnetate, ^{99m}Tc-MDP and $^{99m}TcO_2$.[22] Albumin-strips were prepared by soaking ITLC strips in HSA solution (5 mg/ml in saline) for 30 min, rinsing them with WFI (2 × 10 ml), and then storage at 4°C after drying in air. One cm sections of each strip were counted in a gamma counter (Packard Auto-Gamma 5650, Hewlett Packard) over a Technetium[^{99m}Tc] window (70–210 keV). Percentage RCP of ^{99m}Tc-immunoglobulin was calculated as 100% − (% ^{99m}Tc-pertechnetate + % ^{99m}Tc-MDP + % $^{99m}TcO_2$). ^{99m}Tc-infliximab was tested for % RCP at 4 h post-reconstitution (± 'aged' AEW), and stability of the cold kit was assessed over the next 4 months in the same way. AEW solvent unsealed at room temperature for 4 h, allowing ammonia gas to escape, was defined as 'aged'. Reproducibility of the quality control method was defined by accuracy and precision. The accuracy of each method (testing ^{99m}Tc-INFX kits on multiple occasions) was calculated as a percentage standard deviation of the mean value ($n = 11$). The precision of each assay (multiple testing of the same kit) was calculated using the same formula above ($n = 3$).

Binding assays

Specificity of reduced-INFX. The binding specificity of INFX and 2-ME-reduced-INFX to rat/human TNF-α (Serotec; Oxford; UK) were each determined by an ELISA assay in duplicate. Rat TNFα in PBS (2 μg/ml; 50 μl/well) was added to a well tray (96 wells; Corning #3590), and PBS

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(50 µl/well) to another tray. After incubating overnight at 4°C, the solid material coated each well. Each tray was washed with Tween 20 in PBS (0.05% w/v; 3 × 0.2 ml/well), blocked by incubation with BSA in PBS (1% w/v; 150 ml/well) for 1 h at 37°C, then washed again with Tween 20 in PBS (3 × 0.2 ml/well). INFX or 2-ME-reduced-INFX or no antibody (control) in 1% w/v BSA in PBS (20 µg/ml; 0.1 ml/well) was added to the first row of wells on a tray, then a diluted sample to the second row (4 µg/ml; 0.1 ml/well) and so on for every row accepting a five fold dilution of the previous sample until the eighth row (26 ng/ml; 0.1 ml/well). Trays were incubated (37°C/1 h) and then washed with Tween 20 in PBS (3 × 0.2 ml/well). Rabbit anti-human immunoglobulin conjugated to horse radish peroxidase (400 ng/ml; 100 µl/well) was added to all wells, trays were incubated (37°C/1 h) and then washed with PBS (3 × 0.2 ml/well). o-PD (Sigma Fast tablet) was dissolved in WFI (20 ml) and an aliquot (0.1 ml) was added to each well. Trays were allowed to stand at room temperature in the dark for 15 min before the reaction was terminated by the addition of sulphuric acid (2.5 M; 14 µl). The absorbance of each solution was measured at $\lambda = 490$ nm. The limit of sensitivity of the assay was 1 ng/ml. Baseline or no binding was defined as twice the mean of the negative control values above the sensitivity threshold.

Binding capacity of ^{99m}Tc-INFX. The binding capacity of ^{99m}Tc-INFX was determined according to a literature procedure[23] based on its interaction with excess antigen (TNFα) expressed on stimulated human lymphocytes. Briefly, to PHA-activated lymphocytes (1×10^7 cells; 1 ml) in a series of tubes was added ^{99m}Tc-INFX (0.25 ml) containing the following levels of cold INFX (2.50-, 1.25-, 0.62-, 0.31-, 0.16-µg, 78.1-, 39.1-, 19.5-, 9.8-, 4.9-, 2.4-ng). BSA in PBS (1% w/v; 1 ml) replaced the cells as the control condition. The tubes were incubated at 37°C with shaking (OM6 platform mixer; Ratek Instruments; Victoria; Australia) at 60 rpm. After 30 min, each sample was filtered via a cellulose acetate membrane (0.8 µm) on a polypropylene holder (Advantec MFS; CA; USA) plus needle (19G) inserted into a vial under continuous vacuum. The filtered cells were washed with saline (2 × 0.5 ml), then the filter and filtrate were counted in a large volume counter linked to a multichannel analyser (Model 3100; Canberra Industries Inc; USA) over a ^{99m}Tc-window (70–210 keV) to determine the relative distribution of counts. Results are expressed as % bound (filter activity) versus mass of INFX in ^{99m}Tc-INFX.

Rat infection model

Experiments performed with the rats complied with '*The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes NHMRC*' and according to protocols approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science, Adelaide. Sprague-Dawley rats

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(female; 180–200 g) were anaesthetised by inhalation of a continuous flow of nitrous oxide (0.7 l/min), oxygen (0.3 l/min) and halothane (2% w/v) over 5–10 min, and then each rodent was injected into the left thigh muscle with a bolus *S. aureus* dose (1×10^8 colony forming units in 0.1 ml saline). The infection and abscess was allowed to develop for 24 h before qualitative and quantitative studies.

Quantitative rat biodistribution studies

For every group of three rats, ^{99m}Tc-immunoglobulin ($\sim$5 MBq; 0.2 ml) was injected intravenously into the tail vein. After 1 and 4 h each group was sacrificed by Halothane asphyxiation, then whole body images were obtained *post mortem* before the autopsy. For *S. aureas* infected rats, blood was also obtained from the descending aorta in the abdominal cavity, weighed and counted. Both thighs plus other organs of interest were excised, blotted dry, weighed and counted in the large volume counter. The urine, carcass and organs were related to a standard (dose equivalent), and the % injected dose/organ (% id) was calculated as a fraction of that standard. The % injected dose/gram for each organ was calculated by dividing % id by the respective organ weights. The ratio of infected muscle (target) to non-infected muscle (control) was calculated by dividing the % id/g of the left thigh muscle by that of the right thigh muscle respectively.

Scintigraphic studies

Planar static images were acquired for 5 min on the collimator of a gamma camera (Starcam 300M; GE) with a group of rats in the anterior position at 1 and 4 h post-radiotracer injection.

Statistical analyses

Results are reported as mean $\pm$ standard error. Statistical analyses were performed with ANOVA (single factor) to compare tracer uptake (% id/g) for target and control regions, and 1 versus 4 h. A paired sample *t*-test was used to compare the ratios between tracers at 1 and 4 h. Statistical significance was defined as a *p*-value less than 0.05.

Results

Radiolabelling and quality control analyses

Initial experiments to radiolabel infliximab with tartaric acid (0–2 mg) or mannitol (1.5 mg) instead of Sn^{II}-MDP in the formulation, resulted in $77.6 \pm 2.9\%$ RE (tartrate) or even lower by 30% for mannitol. Also, an attempt to isolate ^{99m}Tc-INFX prepared with Sn^{II}-MDP by size exclusion chromatography failed because the radiotracer oxidised in air. The optimum

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cold kit formulation contained mercaptoethanol-reduced infliximab (1 mg) and stannous chloride (5 μg) in WFI (1 ml). The process to prepare this formulation was applied to HIG and RIG, such that all cold kits had the same level of immunoglobulin in it, and the same ligand to stannous ratio. Radiochemical purity of ^{99m}Tc-INFX was $95.0 \pm 1.0\%$, $90.6 \pm 0.2\%$ at 20 min and 4 h post-reconstitution respectively from a cold kit prepared at $t = 0$ months. After cold storage at -80°C for 4 months % RCP was $96.0 \pm 1.5\%$, $95.5 \pm 1.6\%$ at 20 min and 4 h post-reconstitution, respectively. 'Aged' AEW decreased % RCP by $\sim 5\%$, from the $t = 0$ months at 4 h, versus all other time points. The quality control methods were performed with good reproducibility in terms of their accuracy ($\leqslant 2.4\%$) and precision ($\leqslant 1.7\%$). The % RCP of ^{99m}Tc-HIG and ^{99m}Tc-RIG exceeded 90% ($94.3 \pm 1.5\%$) for up to 3 h post-reconstitution.

Binding assays

Specificity of reduced-INFX. Reduced (*r*) INFX was bound to human TNFα to the same extent as native INFX ($p = 0.211$), but rINFX and INFX did not bind to any rat TNFα (Figure 1). The control condition where PBS replaced the antigen, defined the lower limit of absorbance at $\lambda = 490$ nm as ~ 0.13.

Binding capacity of ^{99m}Tc-INFX. ^{99m}Tc-INFX gave low binding ($\sim 5\%$) when 2.5 μg of 'cold' or unlabelled INFX was present, but it increased as the ratio of cold to ^{99m}Tc-INFX decreased (Figure 2). The assay assumes that expressed

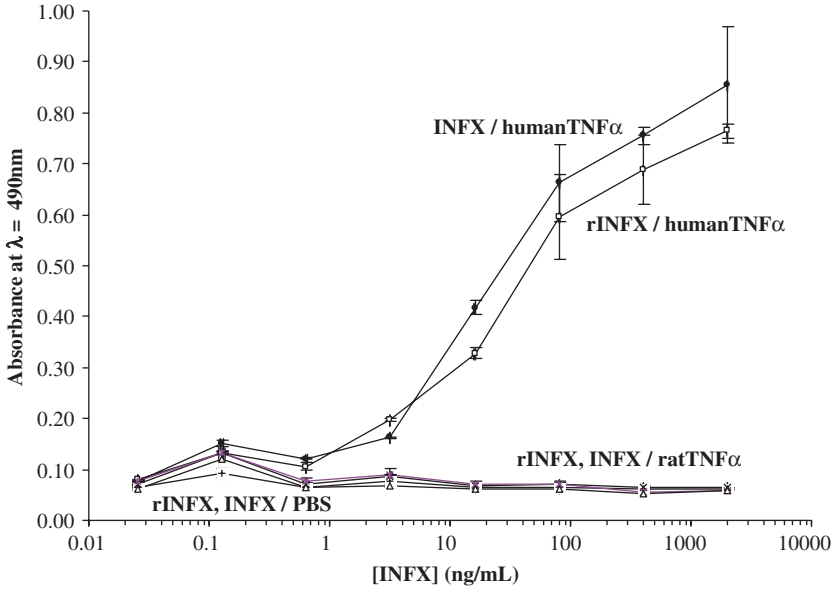

Figure 1. Graph of reduced INFX versus INFX binding to human and rat TNFα

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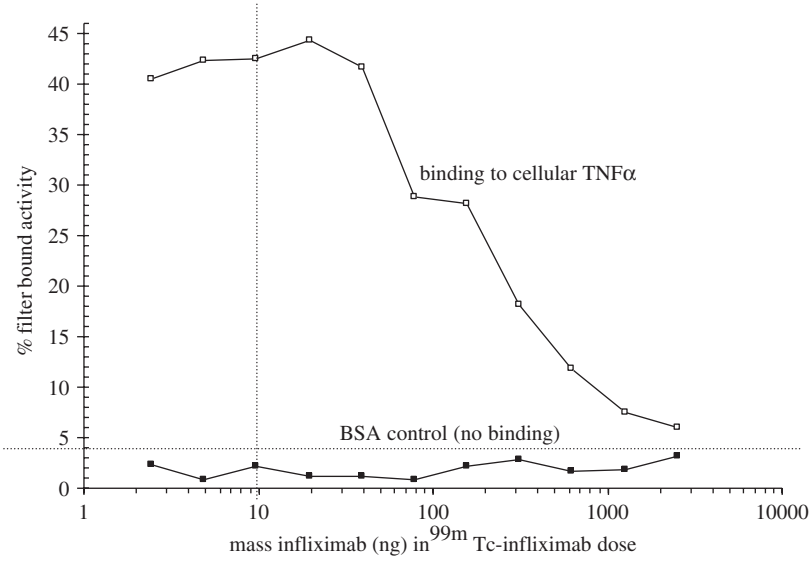

Figure 2. Graph of ^{99m}Tc-INFX binding to localised TNFα on human lymphocytes

TNFα is localised on the cell membrane, and this amount of surface antigen exceeds that of the INFX in the ^{99m}Tc-INFX dose. A maximum level of 44% binding was detected at 20 ng INFX, but below this value the low filter counts (300 cts) had reached the limit of counting sensitivity (> 5% error). The control condition where BSA replaced the cells defined the lower limit of non-specific activity detected.

Infection model

Results of the biodistribution of three ^{99m}Tc-immunoglobulins are shown in Table 1. There was prolonged high blood retention for all tracers with excretion predominantly via the kidneys. Of the intra-abdominal organs, ^{99m}Tc-INFX had slightly lower blood, liver and spleen values than ^{99m}Tc-HIG or ^{99m}Tc-RIG, the latter two agents giving similar values between them. An abscess was clearly visible at the site of *S. aureas* infection after 24 h. The three tracers were distinct in their target and control muscle uptake, and T/C ratios. ^{99m}Tc-INFX resulted in 0.4% id/g uptake at 1 h post-injection (pi) and remained unchanged to 4 h ($p = 0.67$). A similar trend was seen with the other immunoglobulins, such that ^{99m}Tc-HIG was higher (0.6–0.7% id/g; $p = 0.15$) and ^{99m}Tc-RIG accumulated further (0.9–1.0% id/g; $p = 0.05$) over the same time period. When the control samples were accounted for, the T/C ratios were for ^{99m}Tc-INFX 5.7 and 7.1 ($p = 0.07$), ^{99m}Tc-HIG 3.1 and 7.8 ($p = 0.01$), ^{99m}Tc-RIG 7.8 and 12.5 ($p = 0.03$) at 1 and 4 h, respectively. At 4 h T/C ratios

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Table 1. Biodistribution of ^{99m}Tc-immunoglobulins

	%id/g ^{99m}Tc-INFX		%id/g ^{99m}Tc-HIG		%id/g ^{99m}Tc-RIG	
Organ	1 h*	4 h*	1 h*	4 h*	1 h*	4 h*
Blood	3.37 ± 0.82	1.53 ± 0.35	5.11 ± 0.95	2.98 ± 0.19	5.56 ± 0.10	3.10 ± 0.21
Liver	1.23 ± 0.29	0.57 ± 0.15	2.10 ± 0.33	1.29 ± 0.02	1.96 ± 0.13	1.12 ± 0.12
Intestines	0.21 ± 0.03	0.27 ± 0.04	0.25 ± 0.02	0.32 ± 0.03	0.32 ± 0.03	0.30 ± 0.03
Spleen	0.52 ± 0.19	0.37 ± 0.08	1.50 ± 0.27	1.08 ± 0.04	1.17 ± 0.09	0.93 ± 0.05
Kidneys	3.59 ± 0.42	6.65 ± 0.45	4.27 ± 0.35	6.19 ± 0.15	5.01 ± 0.15	5.65 ± 0.33
target muscle	0.40 ± 0.04	0.38 ± 0.05	0.60 ± 0.05	0.71 ± 0.08	0.89 ± 0.07	1.11 ± 0.09
control muscle	0.07 ± 0.01	0.05 ± 0.01	0.22 ± 0.07	0.09 ± 0.01	0.11 ± 0.01	0.09 ± 0.01
Urine	19.8 ± 1.7	35.0 ± 1.8	8.1 ± 4.2	30.4 ± 1.1	14.2 ± 3.6	34.5 ± 2.1
Carcass	39.3 ± 1.0	30.1 ± 0.6	44.6 ± 4. 8	27.6 ± 1.3	37.9 ± 2.0	27.8 ± 1.1
T/*C* ratio	5.72 ± 0.83	7.14 ± 1.25	3.11 ± 1.09	7.84 ± 1.17	7.87 ± 0.27	12.53 ± 1.51

*$n = 3$.

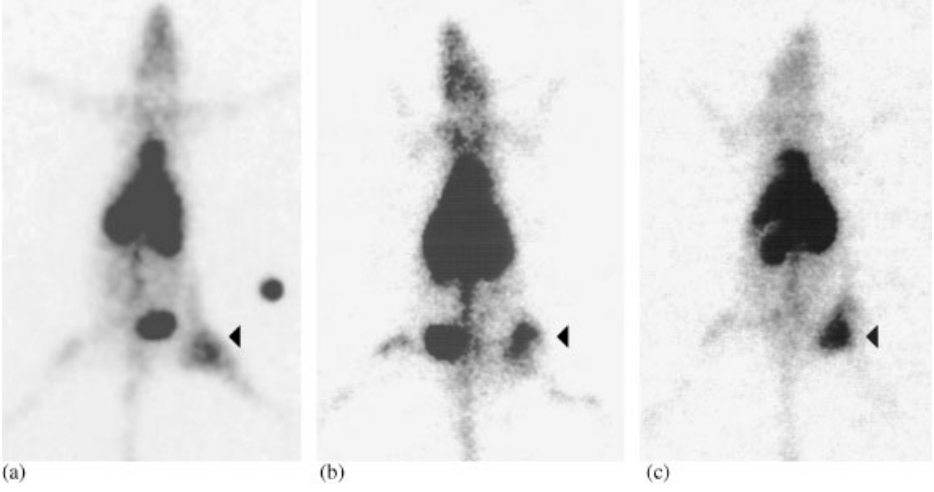

Figure 3. Whole body images of rats with a *S. aureas* focal infection (arrow) in the left thigh after administration with: (a) ^{99m}Tc-infliximab; (b) ^{99m}Tc-HIG or (c) ^{99m}Tc-RIG at 4 h

were the same between ^{99m}Tc-INFX and ^{99m}Tc-HIG ($p = 0.23$), whereas ^{99m}Tc-RIG had a significantly higher ratio than either of these molecules ($p < 0.05$).

Scintigraphic studies

For the infection model, all the ^{99m}Tc-immunoglobulins showed the site of *S. aureas* abscess in the thigh at 1 and 4 h (Figure 3). Significant blood pool activity for all tracers indicated slow clearance. Kidneys and bladder activity indicated the mode of excretion.

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Discussion

The ^{99m}Tc-immunoglobulins used in this study were prepared by a different method than previously reported,[24] with an improved formulation and conveniently presented as cold kits. Each immunoglobulin was initially exposed to 2-mercaptoethanol that partially reduced disulphide bonds in the hinge area of the structure,[25–27] and then the raw material was isolated by size exclusion chromatography. Stannous ions in the form of Sn^{II}-MDP were combined with the reduced immunoglobulin under an inert atmosphere, the stable complex proving to facilitate ^{99m}Tc-pertechnetate reduction. Preliminary experiments with infliximab indicated MDP was a superior ligand over tartaric acid or mannitol, and it was ultimately used in the final INFX, HIG and RIG cold kit formulations. The extent of ^{99m}Tc coordination to protein was determined to be 90–95% by the ITLC paper method, via three strips that assayed ^{99m}Tc-pertechnetate, $^{99m}TcO_2$ and ^{99m}Tc-MDP levels for up to 3–4 h. The infliximab cold kit particularly was found to be stable during storage at -80°C for 4 months, and yielded $\geqslant$95% RCP of the radiotracer after ^{99m}Tc-pertechnetate reconstitution.

Radiolabelled infliximab was previously investigated in *S. aureas* infected mice based on its TNFα binding ability,[24] but the affinity of this monoclonal antibody for TNFα from another rodent, the rat, was unknown. The results here have indicated that native INFX and the reduced protein both bound to human TNFα, but not to rat TNFα. The lack of specific binding of infliximab with rat TNFα, contrasts with a recent report regarding the therapeutic response to experimental colitis in Wistar rats.[28] Also, ^{99m}Tc present in the molecular structure resulted in 44% detectable binding with the human antigen, although this value may be higher with a more sensitive assay method. Provided the radiometal is exclusively coordinated to the hinge area of any immunoglobulin structure, then the interaction between antigen and the V_H/V_L sites in the molecule, is principally unaffected. Thus like ^{99m}Tc-human IgG, ^{99m}Tc-INFX was used in these rat distribution studies as an exogenous protein tracer of similar size ($\sim$150 000 Da) but absent of TNFα specificity. Although RIG raw material was not isolated from autologous rat blood, ^{99m}Tc-RIG was considered here as a radioactive representative of an endogenous immunoglobulin.

In the rat infection model, each of the three immunoglobulins resulted in significantly different T/C ratios at 1 h post-injection. ^{99m}Tc-HIG gave a lower ratio than ^{99m}Tc-INFX (3/1 versus 6/1, respectively) and ^{99m}Tc-RIG was 8/1 at 1 h. At 4 h post-injection, ^{99m}Tc-HIG and ^{99m}Tc-INFX ratios had both increased to 7–8 to 1 and were not significantly different, whereas ^{99m}Tc-RIG had accumulated to 13/1. The ^{99m}Tc-immunoglobulins examined in this study have resulted in higher T/C ratios than other reported agents including

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^{99m}Tc-alafosfalin, ^{99m}Tc-ciprofloxacin[29] and radioactive liposomes[30] in rat models of infection. However the ratios are lower than ^{99m}Tc-tin colloid-labelled-leukocytes,[17] indicating that these cells actively participate over a longer duration than immunoglobulins in the inflammatory process. This is one of the main reasons why radioactive leukocytes still feature prominently in the routine diagnostic imaging of infection and inflammation.

As shown in the whole body images, ^{99m}Tc-INFX, ^{99m}Tc-HIG and ^{99m}Tc-RIG clearly identified infected lesions in rats, and the endogenous marker ^{99m}Tc-RIG provided the best uptake of the group. These observations correlated with the target to background ratios derived from the quantitative biodistribution studies. The mechanism of concentration of immunoglobulins in the *S. aureas* infection is still uncertain[31] but has been proposed to involve Fc receptor binding with local inflammatory cells, or other 'non-specific' retention processes such as increased permeability. Increased permeability is unlikely to explain the tracer retention mechanism because the non-specific small molecule ^{99m}Tc-DTPA in the same rat model was reported to give lower T/C ratios than these immunoglobulins as 3.5–1.9 over 1–4 h,[17] or the larger soluble polymer ^{99m}Tc-dextran gave T/C of $\sim$2–3.5 (versus ^{99m}Tc-DTPA with $\sim$1–3.5) over 1–6 h in a mouse model of sterile inflammation.[32]

The Fc portion of antibodies is known to bind with Fcγ receptors on the surfaces of monocytes, macrophages and neutrophils to initiate immuno-modulatory functions such as phagocytosis, release of inflammatory mediators, antigen expression and generation of oxygen radicals.[33] The Fc receptor binding hypothesis of radioactive immunoglobulins was initially favoured[20] and then reassessed after experimentation with carbohydrate-modified ^{111}In-human IgG in *E coli*-infected rats.[31] The latter investigation did however report a reduction in Fc receptor binding that was consistent with deglycosylation. The glycosylation pattern in the Fc domain of the immuno-globulin structure differs between species. Two of the three immunoglobulins employed in this study were obtained from different species – HIG of human origin, and infliximab the human:mouse [75:25] chimeric antibody. Human IgG contains oligosaccharides with *N*-acetyl neuraminic acid (NANA), mouse IgG contains sugars with *N*-glycolylneuraminic acid (NGNA) and rat IgG contains both NANA and NGNA.[34] The variability of oligosaccharides at this site are known to affect biological functions such as resistance to proteases, interaction with the complement component C1q, feedback immunosuppression of IgG synthesis and the circulatory half-life *in vivo*.[35–37] The heterogeneous glycosylation arrangement between the three immunoglobulin structures can explain lower ligand specificity of Fc receptors on inflammatory cells for the exogenous proteins versus the endogenous ^{99m}Tc-rat IgG.

A decreased accumulation of ^{99m}Tc-INFX and ^{99m}Tc-HIG at the infected site, indicated that these molecules either had a decreased migration to the

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extravascular space or, increased proteolysis at the site of infection, or they transited the area and re-entered into the circulation. The amount of circulating ^{99m}Tc-INFX was lower than ^{99m}Tc-RIG at 4 h, and ^{99m}Tc-HIG had a similar blood value to the radioactive-endogenous molecule. The slightly elevated kidney uptake of ^{99m}Tc-INFX and ^{99m}Tc-HIG compared to ^{99m}Tc-RIG at 4 h, is consistent with the former antibodies undergoing more rapid metabolism and excretion, and ultimately the radioactivity deposited at the *S. aureas* infection is reduced. Enhanced elimination of exogenous immunoglobulins has been observed with murine based monoclonal antibodies from the human circulation, due to a lack of binding with human Fc receptors.[38]

In contrast to the other ^{99m}Tc-immunoglobulins used here, ^{99m}Tc-rat immunoglobulin appeared to participate in the inflammatory response, giving high infection to background ratios over 4 h and clearly identifying the *S. aureas* lesions in rat thighs. From this data, ^{99m}Tc-HIG in humans is expected to preferentially localise at inflammatory sites via an Fc receptor binding mechanism, because the raw material immunoglobulin is of human origin and the glycosylated-specific structure would allow its participation in the inflammatory process. ^{99m}Tc-INFX is also a promising tracer for human studies due to its specificity for TNFα, which is elevated at inflammatory sites.

Conclusion

In this study, cold kits of the three immunoglobulins INFX, HIG and RIG were successfully prepared. The ^{99m}Tc-agents were examined in *S. aureas* infected rats and found to give infection to control ratios exceeding 7/1 at 4 h post-radiotracer injection. ^{99m}Tc-RIG in particular gave the highest ratio of 13/1 at this time and uptake was clearly visible on the whole body images, making it the best radiotracer of the three in rats for imaging a focal muscle infection. The increased excretion as well as lower T/C ratios of ^{99m}Tc-INFX and ^{99m}Tc-HIG compared to ^{99m}Tc-RIG, suggest the former tracers are more rapidly removed from the circulation. ^{99m}Tc-INFX and ^{99m}Tc-HIG immunoglobulins are anticipated to be more useful as infection imaging agents in human studies than rodent models because of their host compatible molecular structures. An extension of this concept is currently being investigated, employing ^{99m}Tc-infliximab and ^{99m}Tc-polyclonal human immunoglobulin in patients with inflammatory disorders.

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