# Scintigraphic imaging of experimental colitis with technetium-99m-infliximab in the rat


## Abstract

The radiolabeled monoclonal antibody $^{99m}$Tc-infliximab was assessed as an inflammation imaging agent in a rat colitis model in comparison with $^{99m}$Tc-tin colloid-labeled-leucocytes. $^{99m}$Tc-infliximab and $^{99m}$Tc-tin fluoride colloid-labeled-leucocytes were administered to (n>3) rats previously exposed to 2,4,6-trinitrobenzenesulfonic acid by rectal instillation. Whole body scintigraphic images were acquired and physiological organ assays were performed to obtain quantitative data. Histological examination of colon samples was performed to assess the site and severity of the colitis. In the inflamed colon, $^{99m}$Tc-infliximab resulted in inflamed target to control colon tracer uptake ratios of 2.7±1.0 (n=5) and 2.6±0.3 (n=5) at 1 and 4 h post tracer injection respectively. $^{99m}$Tc-leucocytes gave higher ratios of 19.5±9.9 (n=3) and 41.2±16.1 (n=5) respectively. $^{99m}$Tc-leucocytes gave higher ratios of 19.5±9.9 and 41.2±16.1 at the corresponding time points. $^{99m}$Tc-infliximab accumulated at sites of inflammation in this rat model but not due to a specific tumor necrosis factor-α binding mechanism. Although the tracer uptake was lower than radioactive leucocytes, this easily prepared $^{99m}$Tc-monoclonal antibody may have some advantages in imaging inflammatory bowel disease in humans based on its biological activity.


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**Chris Tsopelas**[1], **Stan Penglis**[1], **Andrew Ruskiewicz**[2], **F. Dylan L. Bartholomeusz**[3]

1. *Royal Adelaide Hospital, Nuclear Medicine Department, RAH Radiopharmacy, Adelaide, Australia*
2. *Institute of Medical and Veterinary Sciences, Division of Human Pathology, Adelaide, Australia*
3. *Royal Adelaide Hospital, Nuclear Medicine Department, Adelaide, Australia*


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***Correspondance address:***

Dr Chris Tsopelas
RAH Radiopharmacy,
Nuclear Medicine Department,
Royal Adelaide Hospital,
North Terrace, Adelaide,
South Australia 5000,
Fax: +61 8 8222 5949,
E-mail:
ctsopela@mail.rah.sa.gov.au


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## Introduction

Inflammatory bowel disease (IBD) is characterised by chronic inflammation and ulceration of the intestine, Crohn's disease results in mucosal damage of the small and large bowel, and ulcerative colitis involves the colon. The etiology of IBD is not clear, although there is evidence in the literature of genetic [1, 2] and environmental influences, complex immunological reactions and changes to normal microintestinal flora [3] being implicated in its pathogenesis. The modes of treatment currently available include 5-aminosalicylic acid, corticosteroids, immunosuppressants, antibiotics, probiotics, and more recently: anti-tumor necrosis factor (TNF) alpha agents, other anti-inflammatory cytokines, etc [4]. Since IBD can be a relapsing disorder, accurate assessment of the extent and severity of this disease is important in guiding appropriate treatment.

Leucocytes radiolabeled with $^{111}$In or $^{99m}$Tc have found a routine clinical role to image and diagnose inflammatory bowel disease. $^{99m}$Tc-labeled leucocytes can be prepared using either $^{99m}$Tc-hexamethylpropyleneamine oxime or $^{99m}$Tc-tin fluoride colloid with peripheral whole blood. The choice of radiolabeling method depends on the availability of isotope or cold kits, diagnostic value and cost [5,6]. The commonly used radiopharmaceutical in Australia for this indication is $^{99m}$Tc-tin fluoride colloid-labeled-leucocytes. A sample of patient whole blood is labeled with radiocolloid ex vivo, via a mechanism based on surface attachment of predominantly neutrophils, that is the step prior to phagocytosis. Studies of this technique in patients with active Crohn's disease undergoing resection, confirmed the selective accumulation of $^{99m}$Tc-leucocytes in areas of inflamed bowel, and radioactive faeces were only found in the presence of active inflammation [7]. Immunoglobulins have been frequently used to image inflammation. In this particular study, the chimeric monoclonal antibody $^{99m}$Tc-infliximab was investigated for its ability to detect inflammation in an experimental colitis in rats, and compared with $^{99m}$Tc-tin colloid-labeled-leucocytes.

## Methods

### General

Inliximab (Remicade; Centocor Inc; Malvern; PA, USA) was provided at no cost. 2,4,6-trinitrobenzenesulfonic acid (TNBS) was obtained as an aqueous solution (1M; Fluka; Buchs,

Switzerland). Sodium $^{99m}$Tc-pertechnetate was obtained from a $^{99}$Mo/$^{99m}$Tc-generator (Gentech; Australian Radioisotopes; Sydney; Australia). $^{99m}$Tc-infliximab was prepared from cold kits reconstituted with $^{99m}$Tc-pertechnetate (100 MBq in 1 mL saline) and then assayed by paper instant thin layer chromatography to yield a radiochemical purity >95% [8]. Experiments were performed in triplicate unless stated otherwise.

### Animal studies

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 a protocol approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Sciences, Adelaide.

## Rat colitis model

A model of experimental colitis with TNBS in rats was used [5,9]. TNBS was diluted in ethanol to achieve a final 30% alcoholic TNBS solution (80 mg/mL). Groups (n>3) of rats (Sprague-Dawley; 200-250 g) were anaesthetized over 5-10 min by inhalation of a continuous flow of nitrous oxide (0.7 L/min), oxygen (0.3 L/min) and halothane (2% w/v). TNBS (1.0 mL) was slowly infused via the rectum into the colonic lumen using a winged infusion set (Terumo; Tokyo, Japan) without the needle. Rats were held upside-down by the tail for twenty seconds to minimize any outflow of the dose, and then placed in an open cage until recovery. The inflammation was allowed to develop over 96 h and then a histological examination of colonic samples was performed to determine the extent of disease.

### Radiotracer localisation

After the inflammation was allowed to develop, each rat was injected intravenously via the tail vein with $^{99m}$Tc-infliximab (5 MBq) diluted in saline (0.2 mL). Groups of rats were sacrificed using halothane asphyxiation at 1 h post radiotracer injection (pi) and at 4 h pi. Firstly, scintigraphic images were acquired for each animal (described below) and then an autopsy was performed. The autopsy involved removal of the large intestine (12 cm) that was cleansed of faecal matter with running water and then divided into the distal section (with rectum). The distal colon was usually macroscopically inflamed and the proximal non-inflamed colon was used as a control. Samples were blotted dry, weighed and counted in the large volume counter (Biosentry; AEI-EKCO, Australia) linked to a multichannel analyser (Model 3100; Canberra Industries Inc; USA) to calculate the injected dose (id)/g per rat as related to a dose equivalent standard. The ratios of inflamed target (T) colon to control (C) colon each at 1 and 4 h pi were calculated by dividing the respective % id/g values. The experiments were repeated with rat $^{99m}$Tc-tin colloid-labeled-leucocytes.

### Leucocyte radiolabeling procedure

Rat leucocytes in whole blood were radiolabeled as previously described [10]. A sample of rat blood was treated with $^{99m}$Tc-tin fluoride colloid prepared from a LWC Kit [A+B] (RAH Radiopharmacy; Adelaide; Australia) according to the manufacturer's instructions. Briefly, Kit A (1.25 mg/mL sodium fluoride in 5 mL WFI; 4 mL) was mixed with the contents of Kit B (0.64 mg stannous fluoride in 1 mL WFI) and then filtered (0.2 μm) into a sterile vial (10 mL). To $^{99m}$Tc-pertechnetate (800 MBq in 2.5 mL saline) was added filtered colloid (0.5 mL) and then the dispersion was mixed by rotation (~40 rpm) in a syringe (5 mL) for 60 min. Radiochemical purity of the $^{99m}$Tc-tin fluoride colloid was measured by instant thin layer chromatography as >98%. The radiocolloid (0.3 mL) was added to rat venous blood (2.7 mL) in another syringe (5 mL) containing porcine heparin (20 units) and then rotated for 50 min. The blood containing a stock of $^{99m}$Tc-labeled leucocytes was transferred into a sterile vial (10 mL) and used within 20 min of preparation in the animal studies.

### Scintigraphic images

Three rats with colitis were injected with $^{99m}$Tc-infliximab and then sacrificed at 1 and 4 h as above. Planar static images were acquired for five minutes on the collimator of a gamma camera (Starcam 300M; GE, USA) with rats in an anterior position and their legs out-stretched. This procedure was repeated for $^{99m}$Tc-leucocytes. An excised sample of the intestinal tract containing the TNBS-inflamed area was imaged for $^{99m}$Tc-infliximab activity as above.

### Pathology

Inflamed rat colon samples were excised at 1 h after radiotracer injection and stored in aqueous formalin (37% w/v) until histological examination the following day. The specimens were stained with haematoxylin and eosin (HE) then viewed by light microscopy (200-400x magnification) for interpretation as low, moderate or severe colitis. A digital image of an inflamed intestine was also taken.

### Statistical analyses

Results are reported as mean ± standard error. Statistical analyses were performed with the paired sample t-test to compare the uptake and uptake ratios between 1 and 4 hours. For $^{99m}$Tc-leucocytes, an average of the 1 h values (n = 3) supplemented the missing data at that time point to allow analysis of the same size data set with the 4 h values. Statistical significance was defined as a p value less than 0.05.

## Results

### Rat colitis model

The rectal infusion of TNBS resulted in the development of a moderate to severe distal colitis. Visual examination of the distal colon showed inflammation, mucosal congestion and extensive ulceration with intermittently discolored areas and an altered surface texture. The control tissue samples were of homogeneous texture and a native pink color. Microscopic examination of the specimens revealed marked inflammation with infiltration of neutrophils and mucosal ulceration (Fig. 1).

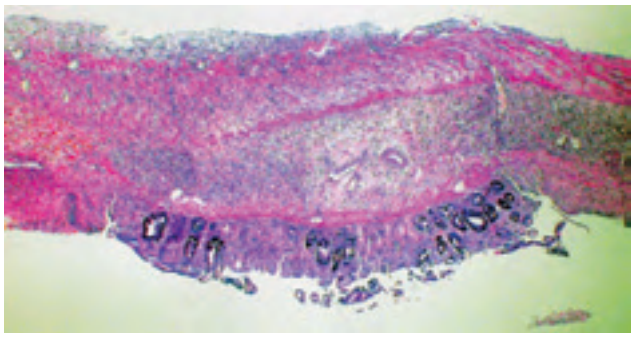

**Figure 1.** H&E section of an inflamed rat colon

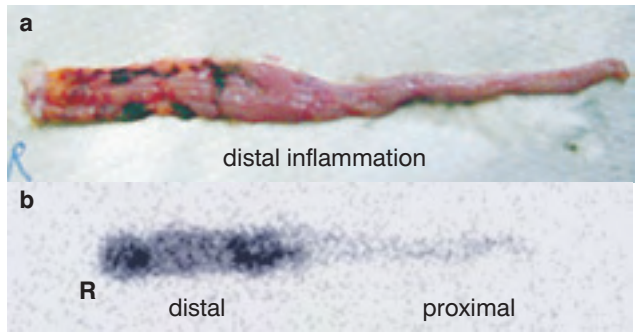


**Figure 3.** Images of the same resected rat colon (a) after TNBS exposure and (b) radiotracer distribution at four hours after $^{99m}$Tc-infliximab injection. R = rectum

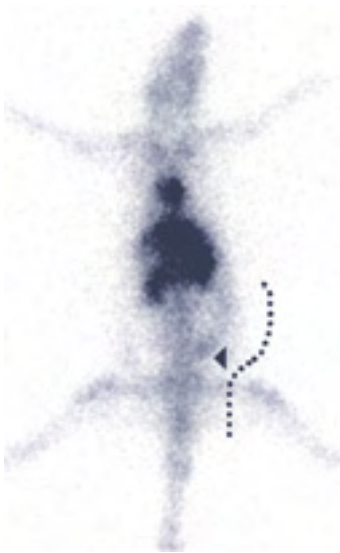

**Figure 2.** $^{99m}$Tc-infliximab distribution in a rat at 4 h pi. Annotated line represents the descending colon until the anus. There is increased tracer uptake in the distal colon (arrowhead)

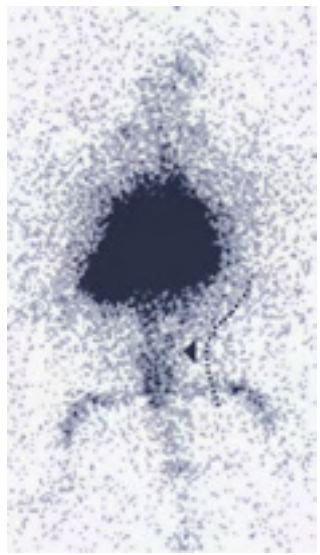

**Figure 4.** $^{99m}$Tc-leucocytes distribution in a rat at 4 h pi. Annotated line represents the descending colon until the anus. There is a bolus of high activity (arrowhead) in the distal colon

## Radiotracer localisation

Results of the quantitative rat physiological distribution assays with $^{99m}$Tc-infliximab and $^{99m}$Tc-leucocytes at 1 and 4 h are shown in Table 1. $^{99m}$Tc-infliximab uptake in inflamed colon reached 1% id/g at 1 h and it was maintained at 4 h (P=0.23). $^{99m}$Tc-leucocytes in inflamed colon increased markedly from 1 to 4 h (P=0.02). The level of either radiotracer in control colons was not statistically different between 1 and 4 h. The T/C ratios for $^{99m}$Tc-infliximab were substantially lower than $^{99m}$Tc-leucocytes. The T/C ratio for $^{99m}$Tc-infliximab remained unchanged (2.7 to 2.6; P=0.45) whereas the ratio for $^{99m}$Tc-leucocytes increased from 19.5 to 41.2 (P=0.03) over 1 to 4 h respectively.

## Scintigraphy

A scintigraphic image of an inflamed rat at 4 h post $^{99m}$Tc-infliximab injection is shown in Figure 2. There was high uptake by the kidneys and urinary tract indicating renal excretion. In both early and delayed scans activity accumulated at the rectum and distal colon where the ulceration was apparent (Fig. 3). Activity was more pronounced in the distal colon at 4 h.

The $^{99m}$Tc-leucocytes images showed high liver and spleen, with low bone marrow activity [11], as well as colon uptake that also increased over 1 to 4 h (Fig. 4). Despite the bone marrow uptake in the lower abdominal area, the accumulation of $^{99m}$Tc-leucocytes clearly identified the inflamed site.

**Table 1.** *Distribution of 99mTc-infliximab, 99mTc-leucocytes in rats with target (moderate to severe colitis) versus control bowel*

| Organ | % id/g $^{99m}$Tc-infliximab | | % id/g $^{99m}$Tc-leucocytes | |
|---|---|---|---|---|
| | 1 h* | 4 h* | 1 h | 4 h* |
| target colon | 1.04 ± 0.31 | 1.20 ± 0.15 | 0.54 ± 0.24 | 0.96 ± 0.59 |
| control colon | 0.40 ± 0.04 | 0.46 ± 0.06 | 0.03 ± 0.00 | 0.03 ± 0.02 |
| T/C ratio | 2.69 ± 1.01 | 2.64 ± 0.29 | 19.46 ± 9.91 | 41.22 ± 16.14 |

*n = 5 rats

## Discussion

The sudden presence of foreign materials such as chemicals, cells or particles in the human body, signals the immune system to commence an inflammatory response. The first line of defence is the appearance of naïve dendritic cells (derived from monocytes) that internalize some antigen to create major histocompatibility complex (MHC) molecules to be expressed on their outer membrane surface. Extracellular TNBS binds covalently with expressed MHC molecules [12]. The subsequent differentiation of naïve dendritic cells into a mature state occurs in the lymph nodes and involves toll-like receptor signalling for the purpose of inducing T lymphocyte immunity. Engagement of toll-like receptors results in the expression of proinflammatory cytokines such as TNF-α, interferon (IFN)γ, interleukin (IL) 12, IL-1, and chemokines that serve to recruit neutrophils and other macrophages from the circulation. At the endothelial cell barrier, neutrophils participate in rolling, adhesion and transmigration events into the extravascular site. The leucocyte milieu there, is immersed in a complex sequence of functions characterized by intense cell-cell communication, followed by numerous direct and indirect actions in an attempt to control the presence of antigen. Among those, macrophages and T helper cells become activated so that inflammatory mediators are amplified, or destructive enzymes such as myeloperoxidase, xanthine oxidase, and reactive oxygen metabolites such as superoxide anions, nitric oxide (NO), hydrogen peroxide and N-chlorinated analogues are enhanced. The over-expression of NO from NO synthase in neutrophils however, increases the oxidative insult to the colonic mucosa by enhancing vascular permeability and producing peroxynitrite that indiscriminantly oxidizes proteins and membrane lipids [13]. Increased NO levels have been detected in patients with ulcerative colitis and Crohn's disease [14]. Furthermore innate immunity is also part of the defense response, where the complement system is activated, anti-TNBS immunoglobulins are produced and chemotactic mediators such as C5a [15] are released to recruit neutrophils against any foreign material including displaced bacteria.

Some of the cellular and molecular mechanisms occurring during inflammation have been defined with radiolabeled agents. For example, $^{111}$In-labeled lymphocytes were used to characterize lymphocyte homing characteristics in a mouse model of colitis, and the radioactive uptake ratio was concluded to be a parameter of disease activity *in vivo* [16]. Similarly, $^{111}$In-dendritic cells were used to image the migration from extracellular tissue to local lymph nodes for antigen presentation to lymphocytes [17]. Endogenous molecules have also been radiolabeled to assess their control functions during the inflammatory response directly, including $^{125}$I-C5a [18], $^{99m}$Tc-HYNIC-IL-12 [19], $^{123}$I-IL-1 receptor antagonist [20], a $^{111}$In-leucotriene B4 antagonist [21], the human antimicrobial peptide fragment $^{99m}$Tc-ubiquicidin 29-41 [22], or indirectly, via an $^{125}$I-TNFα assay [23] and liposomes containing $^{125}$I-INFγ [24]. $^{99m}$Tc-HYNIC-IgG was compared with $^{99m}$Tc-HMPAO-granulocytes to show an experimental colitis in rabbits [25] or $^{111}$In-HIG in humans [26], and a rat $^{123}$I-antibody against the adhesion molecule VCAM-1 that is connected with polymorphism of neutrophils at the extracellular matrix [27].

In this study colonic mucosal injury was created in Sprague Dawley rats with TNBS, that was visible as a distal colitis after four days. Pathological examination of the inflammation revealed the extent of disease and confirmed it was a moderate to severe colitis. The underlying tissue ulceration and necrosis observed in the rodent model, is similar to an acute colitis. $^{99m}$Tc-infliximab was chosen to image the inflammatory lesion. This radiotracer is derived from infliximab, a monoclonal antibody that is an approved drug used in treating rheumatoid arthritis and Crohn's disease. The chimeric monoclonal antibody has a molecular weight of 149 kD and is composed of human constant and murine variable regions in the immunoglobulin structure. Its mode of action involves binding to TNFα that is elevated at inflammatory sites, to reduce the local concentration and consequently down regulate the positive autoregulatory loop involving the NF-κB pathway [28]. Infliximab binds to human TNFα, but not rat TNFα [8], and therefore the radiotracer was used here as a non-TNFα-active immunoglobulin. Immunoglobulins have been often used to image pathogenic infections in animals and humans. The retention mechanism is believed to involve binding between the Fc portion of the immunoglobulin G structure with Fcγ receptors on the surfaces of monocytes, macrophages and neutrophils. This interaction is known to initiate immuno-modulatory functions such as phagocytosis, release of inflammatory mediators, antigen expression and generation of oxygen radicals [29,30].

$^{99m}$Tc-infliximab was prepared from a convenient cold kit formulation, and following its administration to rats, there was high uptake (1% id/g) in inflamed distal colon after one hour that subsequently increased by 15% three hours later. As a comparison, $^{99m}$Tc-tin colloid-labeled-leucocytes accumulated to a similar level at the inflamed lesion over the same time period, although at a different rate than the $^{99m}$Tc-antibody. The uptake ratios of $^{99m}$Tc-leucocytes (>19/1) were superior to $^{99m}$Tc-infliximab (~3/1) at 1 and 4 hours after tracer injection. $^{99m}$Tc-infliximab resulted in lower T/C ratios than other agents such as $^{99m}$Tc-alafosfalin [5] and $^{99m}$Tc-ciprofloxacin [31], nevertheless, high uptake at the distal colon sufficiently distinguished the inflamed area from normal tissue. An isolated colon sample was imaged digitally and scintigraphically. The radioactive image highlighted more counts at the distal colon than the proximal region, the emphasized activity correlating with the inflamed area shown in the digital image (Fig. 3). Although the T/C ratio for $^{99m}$Tc-infliximab was lower than 99mTc-leucocytes, the significant uptake at the inflamed lesion was absent of a TNFα-antibody complex.

The favorable distribution of $^{99m}$Tc-infliximab allowed for the identification of experimental colitis in rats, and tracer accumulation was due to an Fc monocyte receptor binding mechanism rather than from TNFα binding. Based on this immunoglobulin retention mechanism, coupled with the ability of $^{99m}$Tc-infliximab to bind with human TNFα, it was con-

cluded that this tracer would be worth exploring in humans with inflammatory disease to evaluate its diagnostic potential. This nuclear medicine department is currently investigating the use of $^{99m}$Tc-infliximab in patients with certain inflammatory disorders.

*In conclusion*, $^{99m}$Tc-infliximab was examined in rats with TNBS-colitis and found to result in high distal colon uptake (1% id/g) with moderate target to control ratios of ~3 to 1 up to 4 h after injection. $^{99m}$Tc-tin fluoride colloid-labeled-leucocytes in comparison gave lower uptake but significantly higher ratios of >19 to 1 during that period, and the uptake was clearly visible on the whole body images. Although $^{99m}$Tc-infliximab had inferior ratios to the gold standard $^{99m}$Tc-leucocytes, a moderate to severe colitis was distinguishable in the whole body images, and this was also supported by *in vitro* evidence. The mechanism of retention is attributed to binding with Fc receptors on leucocytes participating in the local inflammation.

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