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=5) 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 $2.3\pm 1.0$ (n=5) and $2.6\pm 0.3$ (n=5) at 1 and 4 h post tracer injection respectively. $^{99m}$Tc-leucocytes gave higher ratios of $19.5\pm 9.9$ (n=3) and $41.2\pm 16.1$ (n=5) respectively. $^{99m}$Tc-leucocytes gave higher ratios of $19.5\pm 9.9$ and $41.2\pm 16.1$ 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-a 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.


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-leucocytes can be prepared using either $^{99m}$Tc-hexamethylpropyleneamine oxide 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 feces 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
Infliximab (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,
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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.

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).
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 $^{99m}$Tc-infliximab, $^{99m}$Tc-leucocytes in rats with target (moderate to severe colitis) versus control bowel

<table>
<thead>
<tr>
<th>Organ</th>
<th>% id/g $^{99m}$Tc-infliximab</th>
<th>% id/g $^{99m}$Tc-leucocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h*</td>
<td>4 h*</td>
</tr>
<tr>
<td>target colon</td>
<td>1.04 ± 0.31</td>
<td>1.20 ± 0.15</td>
</tr>
<tr>
<td>control colon</td>
<td>0.40 ± 0.04</td>
<td>0.46 ± 0.06</td>
</tr>
<tr>
<td>T/C ratio</td>
<td>2.69 ± 1.01</td>
<td>2.64 ± 0.29</td>
</tr>
</tbody>
</table>

* $n = 5$ 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 birds 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 (IFNγ), 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 increases the oxidative insult to the colonic mucosa by enhancing vascular permeability and producing peroxynitrite that indiscriminately 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, 111In-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, 111In-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 radio labeled to assess their control functions during the inflammatory response directly, including 125I-C5a [18], 99mTc-HYNIC-IL-12 [19], 123I-IL-1 receptor antagonist [20], a 111In-leucotriene B4 antagonist [21], the human antimicrobial peptide fragment 99mTc-ubiquicidin 29-41 [22], or indirectly, via an 125I-TNFα assay [23] and liposomes containing 125I-IFNγ [24]. 99mTc-HYNIC-IgG was compared with 99mTc-HMPAO-granulocytes to show an experimental colitis in rabbits [25] or 111In-HIG in humans [26], and a rat 123I-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. 99mTc-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 TNFs 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 TNFs, but not rat TNFs [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 Fcy receptors on the surfaces of monococytes, 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]. 99mTc-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, 99mTc-Tc colloid-labeled-leucocytes accumulated to a similar level at the inflamed lesion over the same time period, although at a different rate than the 99mTc-antibody. The uptake ratios of 99mTc-leucocytes (>19/1) were superior to 99mTc-infliximab (3/1) at 1 and 4 hours after tracer injection. 99mTc-infliximab resulted in lower T/C ratios than other agents such as 99mTc-salofasalin [5] and 99mTc-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 99mTc-infliximab was lower than 99mTc-leucocytes, the significant uptake at the inflamed lesion was absent of a TNFα-antibody complex. The favorable distribution of 99mTc-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 99mTc-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 99mTc-infliximab in patients with certain inflammatory disorders.

In conclusion, 99mTc-infliximab had inferior ratios to the gold standard with inflammatory disease to evaluate its diagnostic potential. This nuclear medicine department is currently investigating the use of 99mTc-infliximab in patients with certain inflammatory disorders.

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Bibliography