Dissociation of indium from indium-111-labelled diethylene triamine penta-acetic acid conjugated non-specific polyclonal human immunoglobulin G in inflammatory foci

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Abstract. Several investigators have reported retention of indium-111 in infectious foci after intravenous injection of 111In-labelled immunoglobulin G (IgG). With this study we intended to test the hypothesis that, upon administration of 111In-diethylene triamine penta-acetic acid (DTPA-IgG), 111In is retained in the infectious foci after dissociation from IgG. Therefore we measured the tissue distribution of double-labelled 111In-DTPA-IgG-(carbon-14) in rats with a focal infection and compared the results with corresponding data for DTPA-IgG-(14C). DTPA-conjugated IgG was labelled with 111In via citrate transchelation. 111In-DTPA-IgG and DTPA-IgG were labelled with 14C through methylation. High-performance liquid chromatography (HPLC) and instant thin-layer chromatography analysis were performed to test the in vitro stability of the labelled proteins. Young Wistar rats with a Staphylococcus aureus infection of the left calf muscle were injected intravenously with 0.2 ml of a solution containing either 0.4 MBq 111In and 30 kBq 14C or 30 kBq 14C labelled to 80 µg IgG. Groups of five rats were sacrificed at 2, 6, 24, and 48 h p.i. Activity uptake was determined for plasma, urine, abscess, muscle and various other tissues. Averages and standard deviations were calculated for groups of five rats. HPLC analysis was performed on plasma and urine samples taken up to 48 h p.i. The radiochemical purity of the IgG preparations was >95%. The labelled preparations appeared stable in vitro. The 14C abscess activity decreased from 1.2% to 0.7% of the injected dose per gram (% I.D./g) between 2 and 48 h after injection and was linearly related to the 14C plasma concentration. However, the 111In concentration in the infectious foci remained constant over time (1.0% I.D./g) despite a decreasing concentration of 111In in plasma. Labelling with 14C did not influence the abscess uptake of 111In after administration of 111In-DTPA-IgG. On the other hand, conjugation with DTPA and labelling with In111 did not influence the tissue distribution of 14C-IgG either. Assuming that 14C-IgG behaves like native IgG, our results strongly suggest that in abscesses 111In is released from IgG with local retention of the 111In. The dissociation of 111In from IgG provides a new explanation for retention of 111In in sites of inflammation. This phenomenon might also be relevant to the explanation of non-specific tumour uptake of monoclonal antibodies labelled with 111In through DTPA.

Key words: Indium-111 – Immunoglobulin G – Inflammation – Infection – Mechanism


Introduction

Despite the extensive evidence of its clinical usefulness, the mechanism of uptake of 111In-labelled non-specific polyclonal human immunoglobulin G (IgG) in foci of inflammation and infection remains to be clarified. Several mechanisms have been proposed for the uptake of 111In-IgG in inflammatory and infectious foci. At the time of its introduction it was postulated that 111In-IgG might bind to leucocyte surface receptors through its Fc fragment after exudation through the leaking capillary walls at the site of inflammation into the locally expanded extravascular space [1]. However, the first part of this hypothesis had to be abandoned for several reasons. First, in an autoradiographic study the 111In in the interstitial...
space at the site of an infection appeared not to be inflammatory cell associated [2]. Second, in infectious foci 111In human serum albumin (HSA) performed very similarly to 111In-IgG [3]. Third, the uptake of 111In-IgG in infections in neutrophilic humans and rats was at least as high as in otherwise comparable cases with normal or elevated leucocyte counts [4].

Binding to Staphylococcus aureus protein A [5], monocytes [6] and rheumatoid factors [5] have been proposed to explain retention of radiolabelled IgG in inflammatory foci. However, none of these mechanisms provides an adequate explanation for the observed uptake and retention of 111In-IgG in sites of acute and chronic infections by a variety of micro-organisms as well as in sterile inflammatory foci [7].

In a pilot study using mixtures of 111In-IgG and carbon-14-IgG in rats with a focal S. aureus infection we were able to demonstrate that the concentration of 14C-IgG in a site of infection is linearly related to the plasma concentration, whereas 111In-IgG is retained in an infection independent of the plasma concentration [8]. It is the objective of this study to provide additional evidence of release of indium from indium-labelled IgG by using double-labelled 111In-DTPA-IgG-14C in a rat infection model.

Materials and methods

Radiopharmaceuticals

Indium-111-IgG. Diethylene triamine penta-acetic acid bicyclic anhydride (DTPA bicyclic anhydride, Sigma, St. Louis, Mo.) was conjugated to IgG (Sandoglobulin, Sandoz, AG, Nuernberg, Germany) according to the technique described by Hnatowich et al. [9]. The number of DTPA ligands conjugated to one protein molecule was determined by the method described in the same paper [9]. The DTPA-conjugated protein, purified by gel filtration on Sephadex G-25 (Pharmacia LKB, Uppsala, Sweden), was diluted to 2 mg/ml with 0.15 M acetate (pH = 6.5) and sterilized by membrane filtration (Millipore SA, Molsheim, France, Milllex-GV SLG025LS, 0.22 µm). Aliquots of 1 ml of the conjugate (2 mg IgG) were mixed with 0.1 ml 0.15 M citrate buffer (pH = 5) and radiolabelled with 10 MBq of 111In-indium chloride in 0.04 M hydrochloric acid solution (>2.4 GBq/mM; Mallinkrodt Medical, St. Louis, Mo.) and sterilized by membrane filtration. The radiochemical purity was determined by ITLC on Gelman ITLC-SG strips (Gelman Laboratories, Ann Arbor, Mich.) with 0.1 M citrate (pH = 5) as solvent.

An aliquot of the non-purified 111In-DTPA-IgG was incubated at room temperature for 24 h with a threefold excess of free DTPA (related to the amount of protein-bound DTPA) and then for 1 h with a 6000-fold excess of free DTPA. To estimate the amount of 11In liberated by the free DTPA, ITLC was performed on Gelman ITLC-SG strips with 0.1 M citrate (pH = 5) as solvent after 0, 1 and 24 h incubation with the smaller amount of DTPA and after 1 h incubation with the larger quantity of DTPA.

Double-labelled 111In-DTPA-IgG-14C. 111In-labelled IgG was labelled with 14C by methylation using 14C-formaldehyde and sodium cyanoborohydride [10]. Briefly: 1.6 ml of a solution of 111In-IgG [10 MBq 111In; 2 mg IgG; pH adjusted to 7 with approximately 0.5 ml 0.15 M bicarbonate buffer (pH = 8.2)] was added to 0.35 ml of a solution of 13 MBq 14C-formaldehyde (1.7 GBq/mM; 0.064% formaldehyde in water; NEN-Du Pont, Wilmington, Del.) in water. Then, 0.7 ml was added of a freshly prepared solution of 6 mg anhydrous sodium cyanoborohydride (Aldrich Chem. Co. Ltd., Gillingham, Dorset, UK) in 1 ml of 0.04 M phosphate buffer (pH = 7). The mixture was incubated for 24 h at room temperature. The labelled protein was purified by size exclusion chromatography on a Sephadex G-25 containing column (18x1 cm), incubated with 0.1 M acetate buffer (pH = 6). The protein fraction was collected and diluted to 10 ml with phosphate-buffered saline (PBS; pH = 7.4). The radiochemical purity was determined by ITLC on Gelman ITLC-SG strips with 0.1 M citrate (pH = 5) as solvent. The solution was sterilized by membrane filtration. Based on the estimated protein concentration the extent of methylation was calculated.

Aliquots of the purified 111In-DTPA-IgG-14C) were incubated at room temperature for 48 h with equal volumes of either PBS or rat plasma. After incubation samples were diluted 1:5 with PBS and analysed by high performance liquid chromatography (HPLC) using a PROTEIN PAK 300 SW column (7.8x300 mm; Millipore-Waters, Milford, Mass.) and PBS (pH = 7.4) as mobile phase (flow rate 1.0 ml/min). The outflow from the HPLC column was monitored by measurement of the optical density at 280 nm (OD280) and by measurement of the radioactivity. For measurement of the beta and gamma radioactivity 0.25-ml fractions were collected. In addition, samples of untreated IgG from the same batch as used for labelling were diluted with PBS and analysed by HPLC. In this system the void volume is approximately 5 ml, the retention volume of IgG and albumin is approximately 7 and 8 ml respectively, whereas the retention volume of 111In-DTPA is approximately 12 ml.

Single-labelled DTPA-IgG-14C. The labelling procedure was similar to the procedure for labelling of 111In-DTPA-IgG-14C, except that no 111In was added. Briefly: 0.5 ml of a solution of DTPA-IgG [0.67 mg IgG; pH adjusted to 7 with 0.15 M bicarbonate buffer (pH = 8.2)] was added to 0.1 ml of a solution of 4 MBq 14C-formaldehyde (1.7 GBq/mM; 0.064% formaldehyde in water). Then, 0.2 ml was added of a freshly prepared solution of 6 mg sodium cyanoborohydride in 1 ml of 0.04 M phosphate buffer (pH = 7). The mixture was incubated for 24 h at room temperature. The labelled protein was purified by size exclusion chromatography on a Sephadex G-25 containing column (18x1 cm), incubated with 0.1 M acetate buffer (pH = 6). The protein fraction was collected, 1 ml DTPA-IgG [1.3 mg IgG; pH adjusted to 7 with 0.15 M bicarbonate buffer (pH = 8.2)] was added, and the mixture was diluted to 10 ml with PBS. The radiochemical purity was determined by ITLC on Gelman ITLC-SG strips with 0.1 M citrate (pH = 5) as solvent. The solution was sterilized by membrane filtration.

The protein dose per rat was 80 µg; the 14C dose was 30 kBq per rat; the 11In dose per rat was either 0 or 0.4 MBq.

Infection model and study design

After ether anaesthesia a calf muscle abscess was induced in young, male, randomly bred Wistar rats (weight 200–220 g) with approximately 2x10^8 colony-forming units of S. aureus in 0.1 ml 50%-50% suspension of autologous blood and saline. The animals were randomly divided into groups of five. Twenty-four hours after inoculation of S. aureus in the muscle, when swelling of the muscle was apparent, the respective radiopharmaceuticals were injected in the tail vein.
To collect tissues, rats were killed with 30 mg intraperitoneally injected phenobarbital, followed by cervical dislocation at either 2, 6, 24 or 48 h after injection of the respective radiopharmaceutical. Both double-labelled $^{111}$In-DTPA-IgG-(14C) and single-labelled DTPA-IgG-(14C) were evaluated in five animals at each time point. Samples of bone marrow (taken from the right femur), blood and urine were collected. The infected left calf muscle, the right calf muscle, the liver, the spleen, one kidney, one lung, the duodenum and the right femur were collected and blotted dry. Blood, collected in a heparin-containing tube, was centrifuged and a plasma sample was taken. Plasma and urine samples taken at 6, 24 and 48 h were 1:5 diluted with PBS and analysed by HPLC, as described above.

The gamma-activity in the tissues and samples was measured in a shielded well-type gamma counter. To correct for radioactive decay and permit calculation of the uptake of the radiopharmaceuticals in each organ as a fraction of the administered dose, aliquots of the respective doses were counted simultaneously. The measured activity in tissues and samples was expressed as percentage of injected dose per gram.

$^{14}$C activity was measured by liquid scintillation counting after dissolving the tissue samples in 1-ml aliquots of Soluene-350 (Packard, Meriden, Conn.) at 50°C and decolourizing the tissue solutions by incubating overnight with 0.3 ml of 30% hydrogen peroxide. Then, 0.2 ml isopropanol was added to stabilize the scintillator suspension and 10 ml of Hionic-Fluor (Packard, Meriden, Conn.) was added as liquid scintillator. To correct for quenching, 10000 dpm $^{14}$C activity was added as an internal standard. To permit calculation of the uptake of $^{14}$C-IgG in each organ as a fraction of the administered dose, aliquots of the respective doses were counted simultaneously. The results were expressed as a percentage of the administered dose per gram of tissue, plasma or urine. Care was taken to prevent contribution of $^{111}$In activity to the $^{14}$C counting results. Therefore the liquid scintillation counting was performed at least 1 month after the $^{111}$In measurements. By repeated liquid scintillation counting with 3-day intervals it was confirmed that no significant contribution of $^{111}$In activity to the $^{14}$C counting results was left.

**Statistical analysis**

All mean values are given ± the standard deviation (SD). Significance was set at 0.05 (two-sided). Before carrying out the statistical testing, all measured values were logtransformed in order to obtain homoscedasticity (lognormal distribution). In order to evaluate the differences between $^{111}$In and $^{14}$C a closed testing procedure [11] was carried out in the following way: Student's $t$ test was performed on the samples taken at 48 h after administration. Then, only for those tissues which showed a significant difference at a $t$ test carried out on the 24-h samples. Again, only if this result proved significant were the 6-h samples tested, and so on. This procedure corresponds to the intuitive idea that one should first check the long-term effects, and then – if these prove significant – proceed to establish from which time onwards these effects can be shown. In this way the overall error rate per tissue remains at 0.05 (even under partial null hypotheses).

**Results**

**Labelling efficiency, radiochemical purity and stability in vitro**

Conjugation of DTPA to IgG resulted in two to three DTPA ligands per protein molecule. For $^{111}$In-labelling of DTPA-IgG the labelling efficiency was 95%. The efficiency of labelling of $^{111}$In-DTPA-IgG with $^{14}$C was approximately 5%. Approximately 16 amino groups per IgG molecule were dimethylated. The average number of $^{14}$C nuclei per IgG molecule was approximately 25[11a, 11b]. The efficiency of labelling of DTPA-IgG with $^{14}$C was approximately 15%. For both $^{111}$In-DTPA-IgG-(14C) and DTPA-IgG-(14C), after removing unbound $^{14}$C and $^{111}$In the radiochemical purity as determined by ITLC was better than 99%. Figure 1 presents the HPLC profiles for a sample of $^{111}$In-DTPA-IgG-(14C) incubated with PBS buffer at room temperature for 48 h. The OD$_{280}$ profile shows one major peak corresponding to IgG. In addition, two or three small peaks are observed with an elution volume slightly smaller than that of IgG, possibly representing polymers or aggregates (<10%), and another small peak with an elution volume similar to that of $^{111}$In-DTPA, representing a low molecular contaminant (<2%). The $^{111}$In and $^{14}$C profiles in Fig. 1 completely match with the OD$_{280}$ profile. The OD$_{280}$ profile of untreated IgG (not shown) also completely matches with the OD$_{280}$ profile for $^{111}$In-DTPA-IgG-(14C). After incubation with plasma for 48 h at room temperature the $^{111}$In and $^{14}$C profiles look very similar to the corresponding profiles obtained after incubation with buffer. After incubation of $^{111}$In-DTPA-IgG with free DTPA in threefold excess at room temperature the amount of "free" $^{111}$In was 4.7% at $t = 0$, 2.8% at $t = 1$ h, and 6.3% at $t = 24$ h. After incubation for one additional hour with 6000-fold excess of DTPA the amount of "free" $^{111}$In was 9.3%.

**HPLC analysis of plasma and urine samples**

Figure 2 presents the HPLC profiles for a plasma sample taken at 24 h after injection of double-labelled $^{111}$In-DTPA-IgG-(14C). The OD$_{280}$ profile shows various peaks and is identical to the OD$_{280}$ HPLC profile of a plasma sample of an untreated rat (not shown here). The $^{14}$C plasma profile is very similar to the corresponding HPLC profile obtained after incubation with buffer. After incubation of $^{111}$In-DTPA-IgG with free DTPA in threefold excess at room temperature the amount of "free" $^{111}$In was 9.3%.

**Conjugation of DTPA to IgG**

When the DTPA was added to activated IgG, two or three peaks are observed. In the case of DTPA-IgG-(14C) the labelling efficiency was 95%. The average number of $^{14}$C nuclei per IgG molecule was approximately 25[11a, 11b]. The efficiency of labelling of DTPA-IgG with $^{14}$C was approximately 15%. For both $^{111}$In-DTPA-IgG-(14C) and DTPA-IgG-(14C), after removing unbound $^{14}$C and $^{111}$In the radiochemical purity as determined by ITLC was better than 99%. Figure 1 presents the HPLC profiles for a sample of $^{111}$In-DTPA-IgG-(14C) incubated with PBS buffer at room temperature for 48 h. The OD$_{280}$ profile shows one major peak corresponding to IgG. In addition, two or three small peaks are observed with an elution volume slightly smaller than that of IgG, possibly representing polymers or aggregates (<10%), and another small peak with an elution volume similar to that of $^{111}$In-DTPA, representing a low molecular contaminant (<2%). The $^{111}$In and $^{14}$C profiles in Fig. 1 completely match with the OD$_{280}$ profile. The OD$_{280}$ profile of untreated IgG (not shown) also completely matches with the OD$_{280}$ profile for $^{111}$In-DTPA-IgG-(14C). After incubation with plasma for 48 h at room temperature the $^{111}$In and $^{14}$C profiles look very similar to the corresponding profiles obtained after incubation with buffer. After incubation of $^{111}$In-DTPA-IgG with free DTPA in threefold excess at room temperature the amount of "free" $^{111}$In was 4.7% at $t = 0$, 2.8% at $t = 1$ h, and 6.3% at $t = 24$ h. After incubation for one additional hour with 6000-fold excess of DTPA the amount of "free" $^{111}$In was 9.3%.

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Fig. 1. HPLC analysis of double-labelled $^{111}$In-DTPA-IgG-(14C) after incubation in PBS buffer for 48 h at room temperature. The upper curve represents the OD$_{280}$ profile. The lower curves represent the activity profiles of $^{111}$In and 14C.

Fig. 2. HPLC analysis of a plasma sample taken at 24 h after administration of double-labelled $^{111}$In-DTPA-IgG-(14C). The upper curve represents the OD$_{280}$ profile. The lower curves represent the activity profiles of $^{111}$In and 14C.

The tissue distribution of $^{111}$In-DTPA-IgG-(14C) is presented in Table 1. The tissue distribution of DTPA-IgG-(14C) is presented in Table 2.

Figure 3 shows the abscess uptake of both labels in double-labelled $^{111}$In-DTPA-IgG-(14C) as a function of time. The abscess uptake of indium is constant over time, whereas the 14C-uptake shows a decreasing trend. At 48 h after injection the difference between the abscess uptake of indium and of 14C is significantly different. In Fig. 4 the quotient of the abscess uptake and the plasma concentration of both labels in $^{111}$In-DTPA-IgG-(14C) is plotted as a function of time after injection. From 6 h after injection onwards, the abscess to plasma ratio of 14C is constant, whereas the abscess to plasma ratio of indium increases. From 24 h after injection onwards, there is a significant difference between the abscess to plasma ratio of $^{111}$In and 14C. The constant abscess to plasma ratio for 14C is in agreement with a 14C-abscess uptake linearly decreasing over time with the plasma concentration. By contrast, $^{111}$In is retained in the abscesses despite decreasing plasma concentrations of 111In.

Figure 5 compares the abscess uptake over time of the 14C label of double-labelled In-DTPA-IgG-(14C) and single-labelled DTPA-IgG-(14C). Except for the lung at 48 h after injection, no significant differences are observed between the 14C tissue distributions of $^{111}$In-DTPA-IgG-(14C) and DTPA-IgG-(14C) (cf. Tables 1, 2).

At 48 h after administration of double-labelled $^{111}$In-DTPA-IgG-(14C), the abscess uptake of the $^{111}$In label is significantly higher than the 14C-abscess uptake (cf. Table 1). From 24 h after injection onwards, there is a significant difference between the $^{111}$In and the 14C concentration in plasma, liver, bone marrow and bone. In plasma from 24 h onwards, the 14C concentration is higher. In liver, bone marrow and bone the $^{111}$In uptake is higher. In spleen, kidney and lung at all time points a signifi-
Table 1. Biodistribution of $^{111}$In-DTPA-IgG-($^{14}$C) (percentage of administered dose per gram; mean values ± standard deviation; $n = 5$)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>2 h</th>
<th>6 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abscess</td>
<td>$^{111}$In</td>
<td>1.0 ±0.1</td>
<td>1.0 ±0.1</td>
<td>1.0 ±0.1</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>1.2 ±0.3</td>
<td>0.8 ±0.3</td>
<td>0.7 ±0.4</td>
</tr>
<tr>
<td>Muscle</td>
<td>$^{111}$In</td>
<td>0.10±0.02</td>
<td>0.11±0.02</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>0.12±0.03</td>
<td>0.15±0.05</td>
<td>0.23±0.03</td>
</tr>
<tr>
<td>Plasma</td>
<td>$^{111}$In</td>
<td>5.9 ±0.5</td>
<td>3.8 ±0.3</td>
<td>1.8 ±0.2*</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>10 ±1</td>
<td>3.7 ±0.7</td>
<td>3.5 ±0.3*</td>
</tr>
<tr>
<td>Liver</td>
<td>$^{111}$In</td>
<td>0.91±0.07</td>
<td>0.70±0.08</td>
<td>0.76±0.1*</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>1.1 ±0.2</td>
<td>0.6 ±0.2</td>
<td>0.38±0.06*</td>
</tr>
<tr>
<td>Spleen</td>
<td>$^{111}$In</td>
<td>0.8 ±0.1*</td>
<td>0.63±0.03*</td>
<td>0.92±0.05*</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>1.0 ±0.2*</td>
<td>0.52±0.05*</td>
<td>0.38±0.06*</td>
</tr>
<tr>
<td>Kidney</td>
<td>$^{111}$In</td>
<td>2.4 ±0.3*</td>
<td>2.4 ±0.2*</td>
<td>2.6 ±0.2*</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>1.3 ±0.1*</td>
<td>0.69±0.08*</td>
<td>0.62±0.09*</td>
</tr>
<tr>
<td>Lung</td>
<td>$^{111}$In</td>
<td>1.1 ±0.1*</td>
<td>0.8 ±0.1*</td>
<td>0.53±0.09*</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>1.7 ±0.2*</td>
<td>1.2 ±0.1*</td>
<td>0.9 ±0.2*</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>$^{111}$In</td>
<td>1.5 ±0.3</td>
<td>1.1 ±0.1</td>
<td>0.98±0.05*</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>1.9 ±0.5</td>
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<tr>
<td>Bone</td>
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<td>0.20±0.04</td>
<td>0.20±0.04</td>
<td>0.24±0.02*</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>0.25±0.05</td>
<td>0.20±0.04</td>
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<tr>
<td>Duodenum</td>
<td>$^{111}$In</td>
<td>0.36±0.05</td>
<td>0.28±0.04</td>
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<tr>
<td></td>
<td>$^{14}$C</td>
<td>0.57±0.08</td>
<td>0.43±0.09</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>Urine</td>
<td>$^{111}$In</td>
<td>10 ±4</td>
<td>0.6 ±0.3</td>
<td>0.26±0.09</td>
</tr>
<tr>
<td></td>
<td>$^{14}$C</td>
<td>4 ±1</td>
<td>1.1 ±0.4</td>
<td>0.14±0.08</td>
</tr>
</tbody>
</table>

The plasma clearance is faster for $^{111}$In than for $^{14}$C, in agreement with the higher retention of $^{111}$In in most tissues. Moreover, there is a fast excretion of $^{111}$In into the urine and the urinary excretion of $^{111}$In at 2 h after injection seems higher than the urinary excretion of $^{14}$C at the same time point. The half-life for the second phase of the plasma disappearance curve of the $^{14}$C label is approximately 160 h. In liver and spleen the $^{111}$In activity shows an increase between 6 and 48 h after injection. By contrast in these tissues the $^{14}$C activity decreases over the same time period.

**Discussion**

In order to provide evidence of the release of $^{111}$In from IgG in infectious foci we used so-called double-labelled $^{111}$In-DTPA-IgG-($^{14}$C). In this study we introduced an external $^{14}$C label into IgG by $^{14}$C methylation of lysine ε-amino groups as a substitute for a stable internal label like S-35. The preparation of S-35-labelled human IgG requires an appropriate cell line and results in the production of monoclonal IgG. This does not necessarily

* Significant difference between $^{111}$In and $^{14}$C; paired t test; $P<0.05
Fig. 3. Abscess uptake over time of both labels of the double-labelled $^{111}$In-DTPA-IgG-($^{14}$C). Average values for $n = 5$. The vertical bars represent standard deviations.

Fig. 4. Ratio of abscess activity uptake over plasma concentration of both labels of the double-labelled $^{111}$In-DTPA-IgG-($^{14}$C) over time. Average values for $n = 5$. The vertical bars represent standard deviations.

Fig. 5. Abscess uptake over time of the $^{14}$C label of In-DTPA-IgG-($^{14}$C) and DTPA-IgG-($^{14}$C). Average values for $n = 5$. The vertical bars represent standard deviations.

behave exactly like the polyclonal human IgG used in clinical studies. From literature data it is known that reductive alkylation of proteins renders stable products without interfering too much with the protein’s properties [10]. The carbon–nitrogen bond introduced after methylation of lysine ε-amino groups is almost as strong as a single carbon–carbon bond and is approximately 40% stronger than the carbon–iodine bond in iodinated proteins [13]. Indeed, HPLC analysis of plasma samples in this study does not provide evidence of release of the $^{14}$C label from the $^{14}$C-methylated IgG (cf. Fig. 2). Moreover, the half-life of the second phase of the plasma disappearance curve of the $^{14}$C label on IgG (approximately 1 week) is in agreement with published normal values (cf. Table 1) [14].

By reaction with formaldehyde and reduction with cyanoborohydride mainly dimethyl derivatives of the lysine residues are formed [10]. Assuming the presence of 50 lysine residues per IgG molecule, our labelling results suggest that 30% of the lysine ε-amino groups are actually methylated. With an average number of 16 dimethyl amino groups and an average number of 25 $^{14}$C nuclei per IgG molecule, all IgG molecules may be assumed to be $^{14}$C labelled. So $^{111}$In-DTPA-IgG-($^{14}$C) is a real double-labelled molecule. Methylation of an amino group reduces its base strength. A decrease in the protein pK of 0.5 unit value as a result of $^{14}$C methylation has indeed been reported [10]. At least in one case $^{14}$C methylation caused the complete loss of an enzyme’s activity [10]. However, in our rat infection model $^{14}$C labelling of IgG appeared not to interfere with the tissue distribution of the indium label in the double-labelled $^{111}$In-DTPA-IgG-($^{14}$C) [3]. Moreover, labelling with $^{111}$In did not influence the tissue distribution of $^{14}$C-labelled IgG (cf. Tables 1, 2).

The results presented in Table 1 and in Figs. 2–4 demonstrate a partial dissociation between the $^{111}$In and the $^{14}$C label of double-labelled $^{111}$In-DTPA-IgG-($^{14}$C) in abscesses as well as in other tissues like liver and
spleen and in plasma and urine. The abscess to plasma ratio of the $^{14}$C-label of double-labelled $^{111}$In-DTPA-IgG-$^{14}$C, being constant over time (cf. Fig. 4), demonstrates the existence of a linear relationship between the $^{14}$C concentration in abscesses and the DTPA-IgG-$^{14}$C concentration in plasma. This is in agreement with the existence of an increased vascular permeability in foci of infection and the absence of retention of the $^{14}$C-label of double-labelled $^{111}$In-DTPA-IgG-$^{14}$C. By contrast, the abscess to plasma ratio of the $^{111}$In-label of $^{111}$In-DTPA-IgG-$^{14}$C increases over time, demonstrating retention of the $^{111}$In-label in the infectious focus despite decreasing plasma concentrations of $^{111}$In-DTPA-IgG-$^{14}$C. This phenomenon provides the basis for the observation made in patients of increasing contrast over time between indium in a site of inflammation and the indium in the blood pool.

In plasma the $^{111}$In/$^{14}$C ratio, being 1.0 at the time of injection, decreases to 0.5 at 24 h and 0.4 at 48 h after injection. Since at 48 h the $^{14}$C plasma concentration is approximately 30% of the initial plasma concentration, this means that between 18% and 60% of the plasma $^{111}$In is released from IgG during the course of the experiment. Release of $^{111}$In from IgG in plasma is also supported by the HPLC analysis of a plasma sample at 24 h p.i. (Fig. 2). Whereas all $^{14}$C activity in plasma shows the same profile on HPLC as the injected agent [111In-DTPA-IgG-$^{14}$C] (cf. Figs. 1, 2), there is evidence of transchelation of some $^{111}$In activity to a macromolecular species with a lower molecular weight than IgG (possibly transferrin). Also, some low molecular $^{111}$In activity is present in plasma at 24 h p.i.

The high level of urinary excretion of $^{111}$In at 2 h p.i., which seems higher than that of $^{14}$C (difference not significant), seems to support the suggestion by Davidson and co-workers that a part of the $^{111}$In is not tightly bound to the IgG [15]. In vitro, however, $^{111}$In-DTPA-IgG-$^{14}$C appears to be rather stable (cf. Fig. 1), since incubation in vitro of $^{111}$In-DTPA-IgG with an excess of DTPA does not remove more than 5% of the $^{111}$In activity from the IgG.

There are several possible explanations for the observation of dissociation of $^{111}$In from IgG in inflammatory foci. Two of the possible mechanisms of uptake will be discussed here.

1. The proteolytic environment in the interstitial fluid space of sites of inflammation may enzymatically catabolize labelled IgG and other proteins. Proteolytic enzymes in inflammatory foci are released by neutrophils and phagocytic cells. Free $^{111}$In-DTPA is expected to be rapidly excreted through the kidneys, unless it is locally retained. Endocytosis by inflammation cells with or without a receptor might provide an explanation for retention of $^{111}$In-DTPA in infectious foci. Intracellular retention of $^{111}$In after administration of an indium-labelled monoclonal antibody has indeed been described [16]. Intracellular uptake, however, is not in agreement with the observations by Morrel and co-workers, who found $^{111}$In only in the interstitial fluid space of foci of infection [2].

Part of the indium in an inflammatory focus may transchelate to transferrin, which has a higher affinity for indium than protein-conjugated DTPA. Transchelation of $^{111}$In is supported by the plasma data: HPLC analysis reveals that between 6% and 9% of the $^{111}$In activity in plasma has a slightly larger retention volume than IgG and is possibly bound to transferrin (see Fig. 2). Indium transferrin is supposed not to interact with transferrin receptors [17] and since it is soluble under physiological conditions, induction transferrin is expected not to be retained in an inflammatory focus. This was confirmed by a pilot study with $^{111}$In-transferrin, which demonstrated a decreasing trend in the abscess uptake of $^{111}$In during the first 24 h after injection of $^{111}$In-transferrin (Claessens et al., unpublished results). Transchelation of $^{111}$In to transferrin followed by release of indium transferrin from the focus of infection might explain the observed constant level of $^{111}$In uptake in infectious foci (cf. Table 1), whereas at the given plasma concentrations an increasing $^{111}$In uptake over time should be expected.

2. Hydrolysis and (co)precipitation of indium after release from $^{111}$In-DTPA-IgG might provide another explanation for dissociation of the two IgG labels and for retention of indium in sites of inflammation without the need for specific receptors. Recently Sasso and Weiner presented results from in vitro experiments showing that at slightly decreased pH and in the presence of certain mediators an appreciable amount of free $^{111}$In may be released from $^{111}$In-DTPA-IgG and that under the same conditions a smaller quantity of $^{111}$In is transchelated to lactoferrin [18]. In abscesses the pH is indeed lower than in normal tissues, having been reported to be as low as 6.7 [19] and even lower in diabetic patients [20].

Dissociation of $^{111}$In from double-labelled monoclonal antibodies has been reported previously. Koizumi et al. used selenium-75 as an internal label of monoclonal antibodies. Indium labelling was performed through DTPA. Significant differences between tumour and liver uptake of $^{111}$In and $^{75}$Se were observed [21].

Release of $^{111}$In from both IgG and HSA provides an explanation for the high ratio between the concentrations of $^{111}$In-IgG and $^{111}$In-HSA in the interstitial fluid space of normal muscle and in plasma, which was reported by Juweid and co-workers [22]. A higher protein concentration in the interstitial fluid space of normal muscle than in plasma is very unlikely, since it would cause oedema. However, high concentrations of free $^{111}$In in the interstitium of normal muscle do not interfere with normal physiological conditions.

Since tumours often show inflammatory reactions and increased vascular permeability, release of indium from a $^{111}$In-DTPA-monoclonal antibody and local retention might also contribute to the uptake of $^{111}$In in tumours. In cases where positive localization of indium has been reported without the local presence of specific receptors for the antibody involved, release of $^{111}$In from the l-
labelled antibody may provide an adequate explanation for non-specific accumulation of 111In in tumours [23].

In conclusion: Indium is released from indium-DTPA-IgG and retained free from IgG in infectious foci. Several mechanisms may explain this observation. Additional experimental work by our group has provided indications of retention of free In111-DTPA in sites of infection [24]. Part of the indium seems to be transchelated to transferrin and may thus leave the infection. How 111In-DTPA is retained after dissociation from IgG is not yet known. Further studies are necessary to elucidate the details of the mechanism of uptake of indium in sites of inflammation after administration of 111In-DTPA-IgG.

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References