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

Roland A.M.J. Claessens[1], Emile B. Koenders[1], Otto C. Boerman[1], Wim J.G. Oyen[1], George F. Borm[2], Jos W.M. van der Meer[3], Frans H.M. Corstens[1]

[1] Department of Nuclear Medicine, University Hospital Nijmegen, Nijmegen, The Netherlands
[2] Department of Medical Statistics, University Hospital Nijmegen, Nijmegen, The Netherlands
[3] Department of Medicine, University Hospital Nijmegen, Nijmegen, The Netherlands

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Abstract. Several investigators have reported retention of indium-111 in infectious foci after intravenous injection of ^{111}In-labelled immunoglobulin G (IgG). With this study we intended to test the hypothesis that, upon administration of ^{111}In-diethylene triamine penta-acetic acid (DTPA-IgG), ^{111}In is retained in the infectious foci after dissociation from IgG. Therefore we measured the tissue distribution of double-labelled ^{111}In-DTPA-IgG-(carbon-14) in rats with a focal infection and compared the results with corresponding data for DTPA-IgG-(^{14}C). DTPA-conjugated IgG was labelled with ^{111}In via citrate transchelation. ^{111}In-DTPA-IgG and DTPA-IgG were labelled with ^{14}C 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 ^{111}In and 30 kBq ^{14}C or 30 kBq ^{14}C 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 ^{14}C 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 ^{14}C plasma concentration. However, the ^{111}In concentration in the infectious foci remained constant over time (1.0% I.D./g) despite a decreasing concentration of ^{111}In in plasma. Labelling with ^{14}C did not influence the abscess uptake of ^{111}In after administration of ^{111}In-DTPA-IgG. On the other hand, conjugation with DTPA and labelling with In111 did not influence the tissue distribution of ^{14}C-IgG either. Assuming that ^{14}C-IgG behaves like native IgG, our results strongly suggest that in abscesses ^{111}In is released from IgG with local retention of the ^{111}In. The dissociation of ^{111}In from IgG provides a new explanation for retention of ^{111}In in sites of inflammation. This phenomenon might also be relevant to the explanation of non-specific tumour uptake of monoclonal antibodies labelled with ^{111}In through DTPA.

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

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Introduction

Despite the extensive evidence of its clinical usefulness, the mechanism of uptake of ^{111}In-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 ^{111}In-IgG in inflammatory and infectious foci. At the time of its introduction it was postulated that ^{111}In-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 ^{111}In in the interstitial

Correspondence to: R.A.M.J. Claessens, University Hospital Nijmegen, Department of Nuclear Medicine, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands

space at the site of an infection appeared not to be inflammatory cell associated [2]. Second, in infectious foci ^{111}In human serum albumin (HSA) performed very similarly to ^{111}In-IgG [3]. Third, the uptake of ^{111}In-IgG in infections in neutropenic 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 ^{111}In-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 ^{111}In-IgG and carbon-14-IgG in rats with a focal *S. aureus* infection we were able to demonstrate that the concentration of ^{14}C-IgG in a site of infection is linearly related to the plasma concentration, whereas ^{111}In-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 ^{111}In-DTPA-IgG-^{14}C 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, Millex-GV SLGV025LS, 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 ^{111}In-indium chloride in 0.04 *M* hydrochloric acid solution (>2.4 GBq/µg indium; Mallinckrodt Medical, Petten, The Netherlands) via citrate transchelation. The radiochemical purity was determined by instant thinlayer chromatography (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 ^{111}In-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 ^{111}In 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 ^{111}In-DTPA-IgG-(^{14}C). ^{111}In-labelled IgG was labelled with ^{14}C by methylation using ^{14}C-formaldehyde and sodium cyanoborohydride [10]. Briefly: 1.6 ml of a solution of ^{111}In-IgG [10 MBq ^{111}In; 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 ^{14}C-formaldehyde (1.7 GBq/m*M*; 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 (18×1 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 ^{111}In-DTPA-IgG-(^{14}C) 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 (7.8×300 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 (OD_{280}) 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 ^{111}In-DTPA is approximately 12 ml.

Single-labelled DTPA-IgG-(^{14}C). The labelling procedure was similar to the procedure for labelling of ^{111}In-DTPA-IgG-(^{14}C), except that no ^{111}In 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 ^{14}C-formaldehyde (1.7 GBq/m*M*; 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 (18×1 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 ^{14}C dose was 30 kBq per rat; the ^{111}In 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 2×10^8 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-(^{14}C) and single-labelled DTPA-IgG-(^{14}C) 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 was 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[1] [11a, 11b]. The efficiency of labelling of DTPA-IgG with ^{14}C was approximately 15%. For both ^{111}In-DTPA-IgG-(^{14}C) and DTPA-IgG-(C14), 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-(^{14}C) 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-(^{14}C). 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-(^{14}C). 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 in Fig. 1. However, the ^{111}In profile shows a small shoulder with an elution volume similar to that

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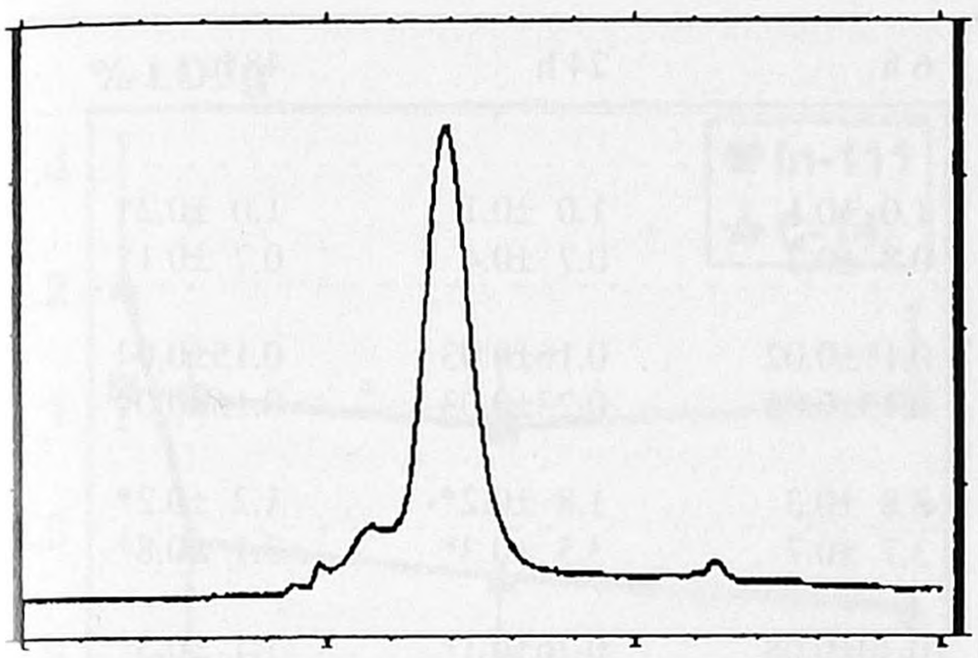
[1] 1. *Calculation of the average number of ^{14}C nuclei per IgG molecule*: The average number of disintegrations per time unit in radioactive material is linearly related to the number of radioactive nuclei present, according to Eq. 1 [11a]:

$$dN/dt = -\lambda \times N, \quad (1)$$

$$\text{where } \lambda = 0.693/T_{1/2} \quad (2)$$

For ^{14}C the half-life is 5730 years or 1.81×10^{11} s [11b]. Thus it follows from Eq. 2 that $\lambda = 3.83 \times 10^{-12}$ per second. Per rat a ^{14}C dose of 30 kBq was given with an IgG dose of 80 µg. HPLC analysis showed that all ^{14}C activity was bound to IgG. With $dN/dt = 30000$ (30 kBq), it follows from Eq. 1 that $N = 7.83 \times 10^{15}$ ^{14}C nuclei or, by dividing by Avogadro's number (6.02×10^{23}), $N = 1.3 \times 10^{-8}$ mole ^{14}C. 80 µg IgG (estimated molecular weight is 150 kDa) is 5.33×10^{-10} mole. The average number of ^{14}C nuclei per IgG molecule in our experiment is thus 24.4



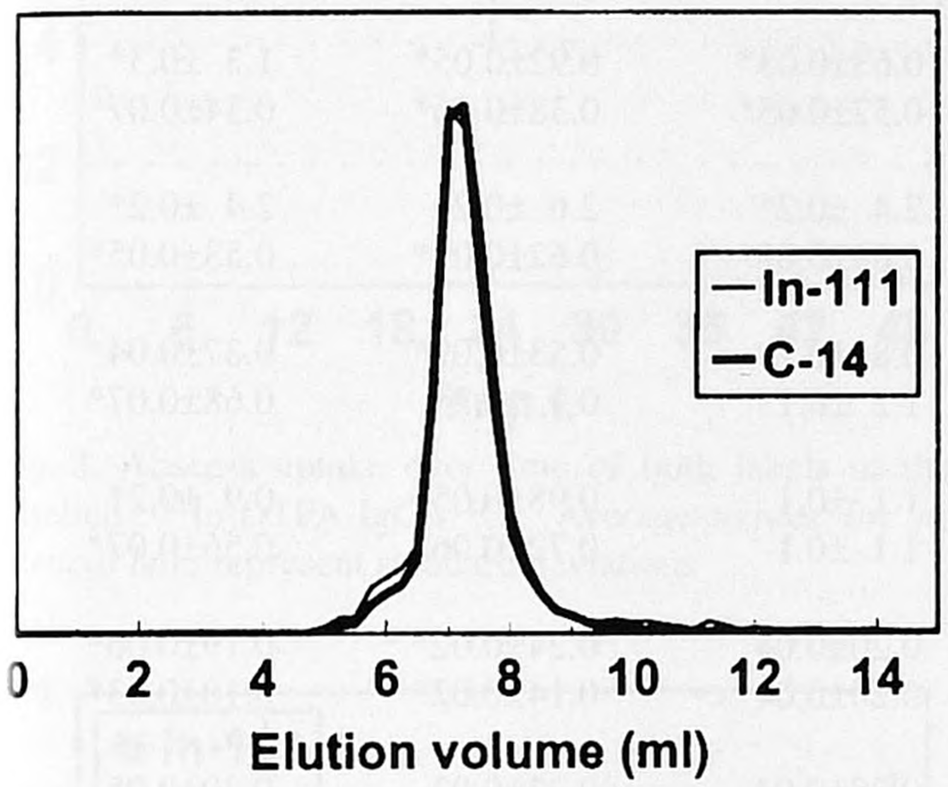
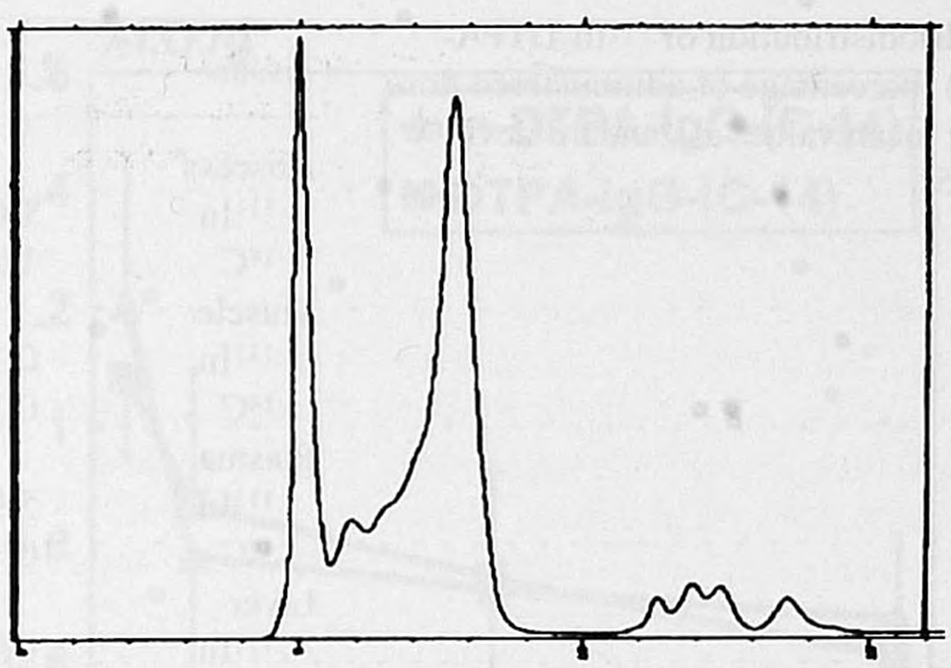


Fig. 1. HPLC analysis of double-labelled ^{111}In-DTPA-IgG-(^{14}C) after incubation in PBS buffer for 48 h at room temperature. The *upper curve* represents the OD_{280} profile. The *lower curves* represent the activity profiles of ^{111}In and ^{14}C



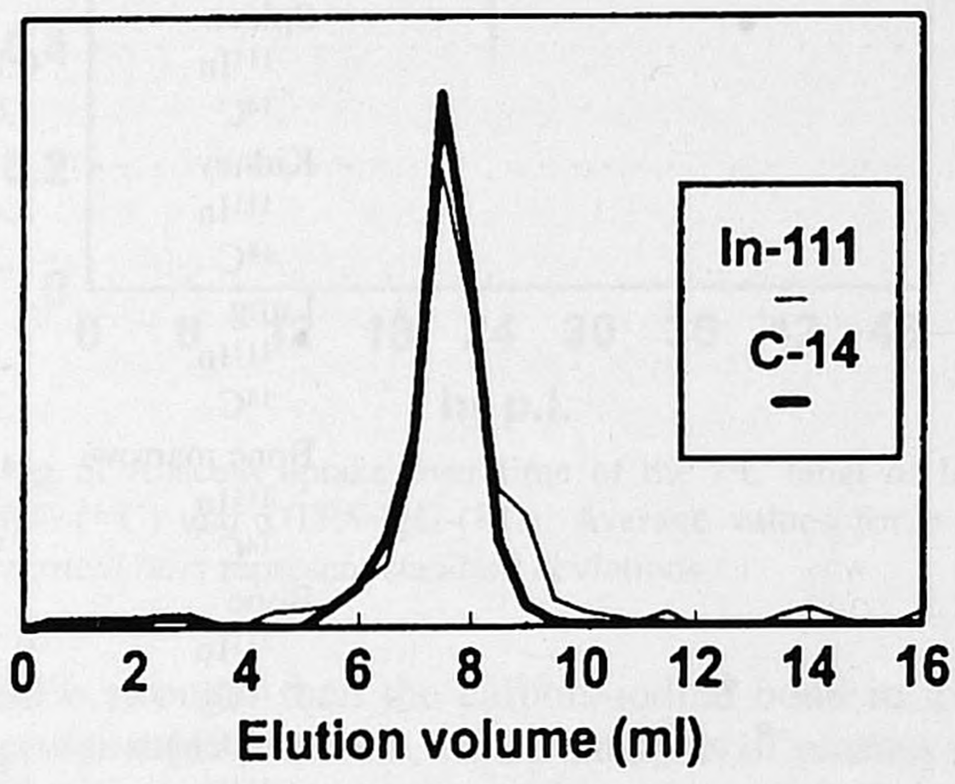


Fig. 2. HPLC analysis of a plasma sample taken at 24 h after administration of double-labelled ^{111}In-DTPA-IgG-(^{14}C). The *upper curve* represents the OD_{280} profile. The *lower curves* represent the activity profiles of ^{111}In and ^{14}C

of ^{111}In-transferrin [12]. The ^{111}In activity in this transchelation product is approximately 6% of the plasma activity at 6 h p.i., 9% at 24 h p.i. and 6% at 48 h p.i. Most of the urinary activity of ^{111}In had an elution volume on HPLC similar to that of ^{111}In-DTPA (not shown here). In urine HPLC demonstrated various low molecular peaks of ^{14}C activity.

Tissue distributions

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

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

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

At 48 h after administration of double-labelled ^{111}In-DTPA-IgG-(^{14}C), the abscess uptake of the ^{111}In label is significantly higher than the ^{14}C-abscess uptake (cf. Table 1). From 24 h after injection onwards, there is a significant difference between the ^{111}In and the ^{14}C concentration in plasma, liver, bone marrow and bone. In plasma from 24 h onwards, the ^{14}C 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; $n = 5$)

Tissue	2 h	6 h	24 h	48 h
Abscess				
^{111}In	1.0 ±0.1	1.0 ±0.1	1.0 ±0.1	1.0 ±0.2*
^{14}C	1.2 ±0.3	0.8 ±0.3	0.7 ±0.4	0.7 ±0.1*
Muscle				
^{111}In	0.10±0.02	0.11±0.02	0.16±0.03	0.15±0.04
^{14}C	0.12±0.03	0.15±0.05	0.23±0.03	0.16±0.09
Plasma				
^{111}In	5.9 ±0.5	3.8 ±0.3	1.8 ±0.2*	1.2 ±0.2*
^{14}C	10 ±1	3.7 ±0.7	3.5 ±0.3*	3.1 ±0.8*
Liver				
^{111}In	0.91±0.07	0.70±0.08	0.76±0.1*	1.1 ±0.3*
^{14}C	1.1 ±0.2	0.6 ±0.2	0.38±0.06*	0.35±0.03*
Spleen				
^{111}In	0.8 ±0.1*	0.63±0.03*	0.92±0.05*	1.3 ±0.3*
^{14}C	1.0 ±0.2*	0.52±0.05*	0.38±0.06*	0.34±0.07*
Kidney				
^{111}In	2.4 ±0.3*	2.4 ±0.2*	2.6 ±0.2*	2.4 ±0.2*
^{14}C	1.3 ±0.1*	0.69±0.08*	0.62±0.09*	0.53±0.05*
Lung				
^{111}In	1.1 ±0.1*	0.8 ±0.1*	0.53±0.09*	0.37±0.04*
^{14}C	1.7 ±0.2*	1.2 ±0.1*	0.9 ±0.2*	0.68±0.07*
Bone marrow				
^{111}In	1.5 ±0.3	1.1 ±0.1	0.98±0.05*	0.9 ±0.2*
^{14}C	1.9 ±0.5	1.1 ±0.1	0.72±0.06*	0.56±0.07*
Bone				
^{111}In	0.20±0.04	0.20±0.04	0.24±0.02*	0.19±0.06*
^{14}C	0.25±0.05	0.20±0.04	0.14±0.02*	0.10±0.03*
Duodenum				
^{111}In	0.36±0.05	0.28±0.04	0.23±0.02	0.20±0.05
^{14}C	0.57±0.08	0.43±0.09	0.34±0.03	0.25±0.13
Urine				
^{111}In	10 ±4	0.6 ±0.3	0.26±0.09	0.21±0.05
^{14}C	4 ±1	1.1 ±0.4	0.14±0.08	0.4 ±0.5

* Significant difference between ^{111}In and ^{14}C; paired t test; $P<0.05$

Table 2. Biodistribution of DTPA-IgG-(^{14}C) (percentage of administered dose per gram; mean values ± standard deviation; $n = 5$)

Tissue	2 h	6 h	24 h	48 h
Abscess	1.1 ±0.1	0.9 ±0.2	0.7 ±0.2	0.61±0.05
Muscle	0.08±0.02	0.11±0.02	0.18±0.02	0.19±0.03
Plasma	8 ±1	5.6 ±0.7	3.6 ±0.5	2.5 ±0.2
Liver	0.9 ±0.2	0.56±0.05	0.39±0.06	0.33±0.05
Spleen	0.7 ±0.1	0.6 ±0.2	0.39±0.06	0.34±0.04
Kidney	1.3 ±0.2	0.9 ±0.1	0.5 ±0.1	0.54±0.07
Lung	1.4 ±0.3	1.2 ±0.1	0.8 ±0.1	0.53±0.06
Bone marrow	1.6 ±0.3	0.6 ±0.6	0.8 ±0.1	0.6 ±0.1
Bone	0.19±0.04	0.16±0.06	0.14±0.05	0.11±0.02
Duodenum	0.51±0.05	0.40±0.04	0.31±0.06	0.26±0.03
Urine	3 ±2	0.8 ±0.2	0.21±0.03	0.14±0.03

cant difference is observed between the ^{111}In and the ^{14}C uptake. In spleen and kidney the ^{111}In uptake is higher; in lung the ^{14}C uptake is higher. In muscle, duodenum and urine no significant difference is observed between the uptake of the ^{111}In and the ^{14}C label (cf. Table 1).

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

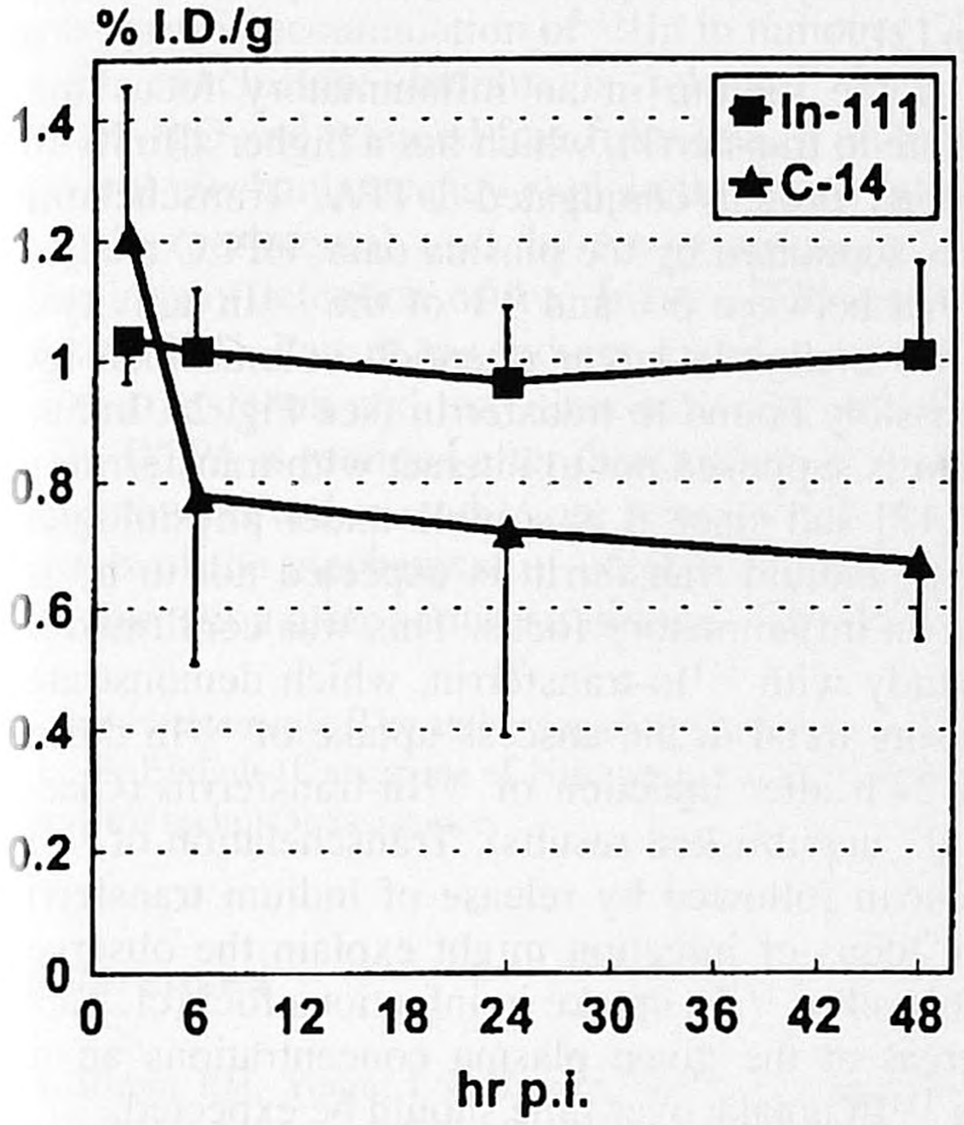


Fig. 3. Abscess uptake over time of both labels of the double-labelled ^{111}In-DTPA-IgG-(^{14}C). Average values for $n = 5$. The *vertical bars* represent standard deviations

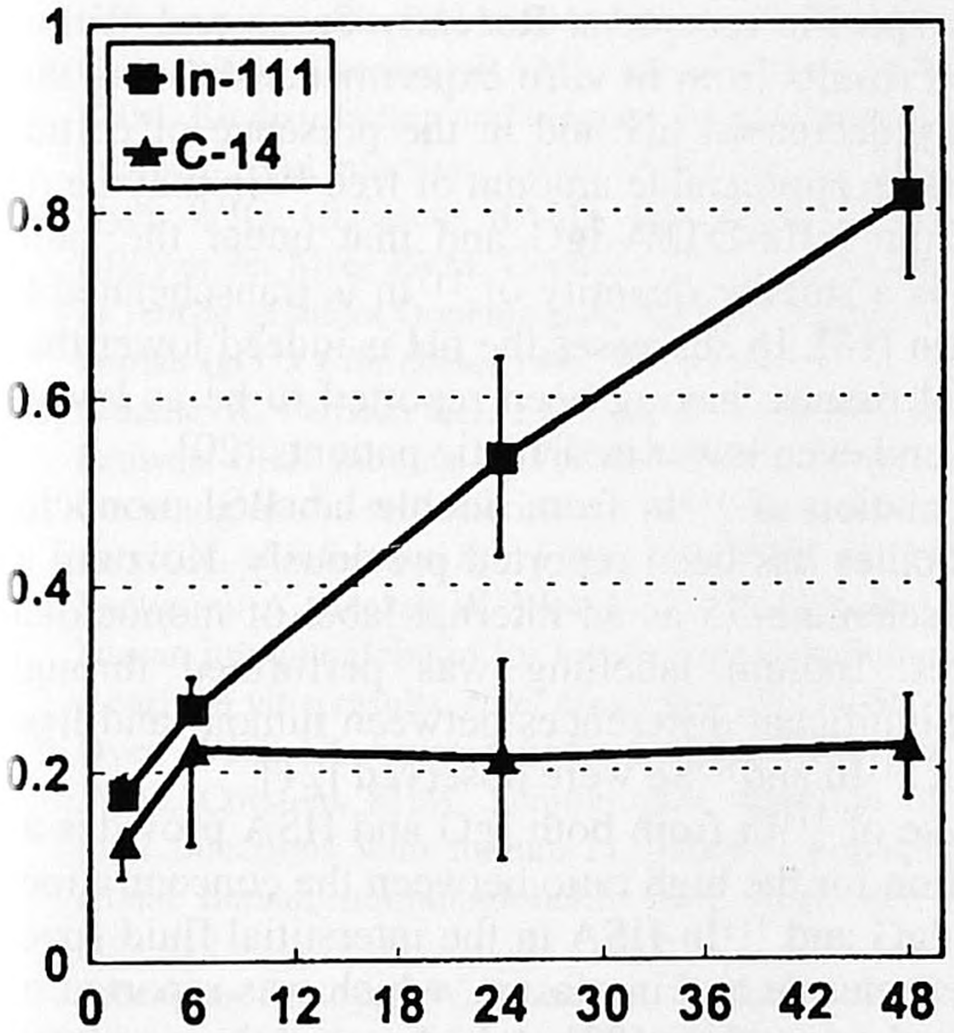


Fig. 4. Ratio of abscess activity uptake over plasma concentration of both labels of the double-labelled ^{111}In-DTPA-IgG-(^{14}C) over time. Average values for $n = 5$. The *vertical bars* represent standard deviations

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

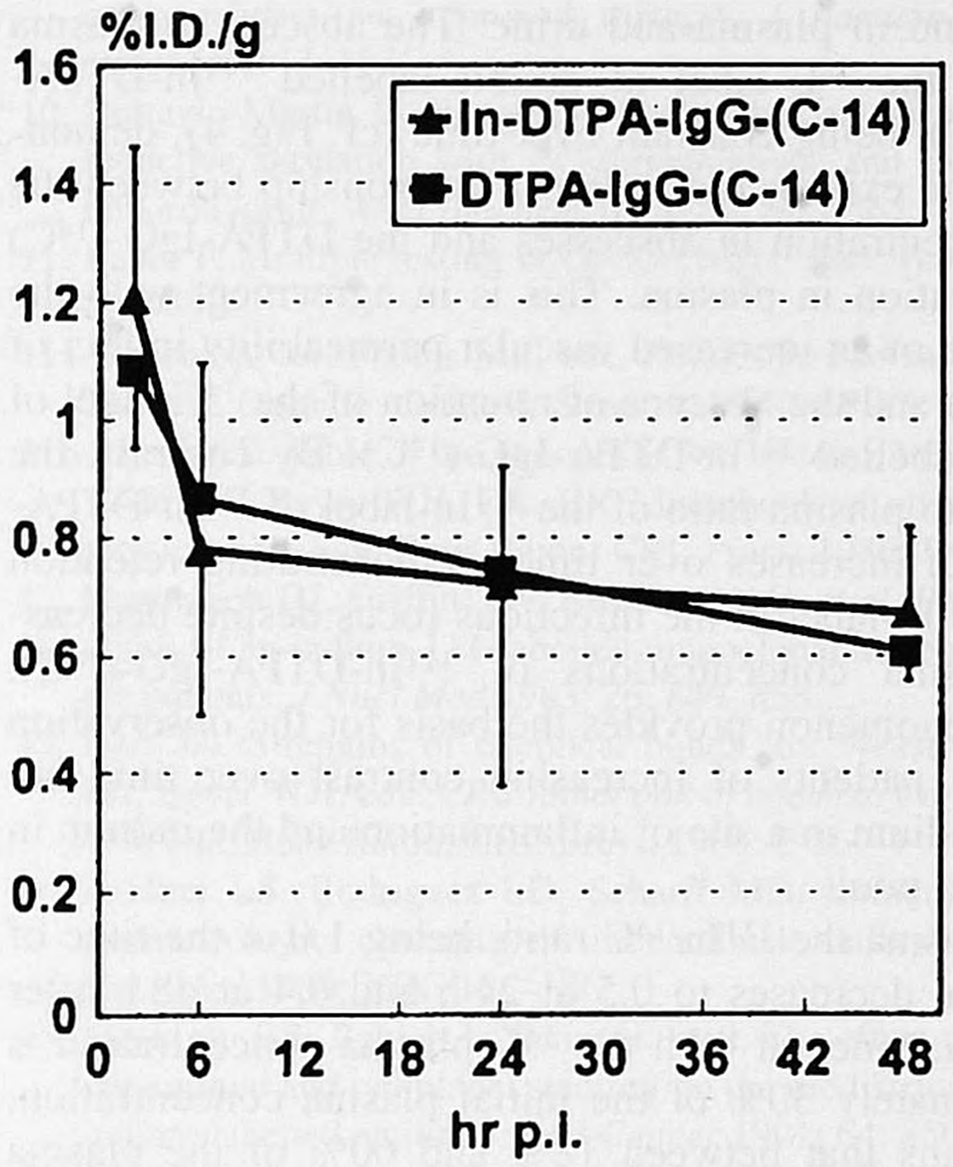


Fig. 5. Abscess uptake over time of the ^{14}C label of In-DTPA-IgG-(^{14}C) and DTPA-IgG-(^{14}C). Average values for $n = 5$. The *vertical bars* represent standard deviations

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 [^{111}In-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, indium 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 la-

belled antibody may provide an adequate explanation for non-specific accumulation of ^{111}In 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 ^{111}In-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 ^{111}In-DTPA-IgG.

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