Biodistributions of air-filled albumin microspheres in rats and pigs

Per WALDAY,* Helge TOLLESHAUG,*†‡ Tor GJØEN,† Grete M. KINDBERG,* Trond BERG,† Tore SKOTLAND* and Eckart HOLTZ*
*NYCOMED Imaging AS, P.O. Box 4220, Torshov, N-0401 Oslo, Norway,
and †Department of Biology, Division of Molecular Cell Biology, University of Oslo, P.O. Box 1050 Blindern, N-0316 Oslo, Norway

The air-filled microspheres of the ultrasound-contrast agent Albunex are unique in that the walls consist of human serum albumin molecules which have been made insoluble by sonication of the albumin solution. The microspheres were isolated by flotation, and the washed microspheres were labelled with ^{125}I. The labelled material was cleared from the circulation mainly as particles, not as soluble albumin molecules. In rats, 80 % of intravenously injected microspheres were cleared from the blood within 2 min. Nearly 60 % of the dose was recovered in the liver, only 5 % in the lungs, 9 % in the spleen, and negligible quantities in kidneys, heart and brain. Of the radioactivity in the liver, more than 90 % was taken up by Kupffer cells (liver macrophages). The protein in the liver was degraded apparently with first-order kinetics (half-life 40 min). In pigs, over 90 % of the intravenously injected dose was recovered in the lungs. The vastly increased recovery in pig lungs, compared with that in rats, is probably due to the pulmonary intravascular macrophages of the pig; macrophages are not normally found in this location in rats (or humans). In a separate series of experiments in rats, the biodistribution of shell material from the microspheres was examined. The microspheres were made to collapse by applying external pressure on the suspension, leaving sedimentable protein material consisting of layers of insoluble albumin from the 'shells' surrounding the air bubble. The 'shells' and the microspheres were cleared from the circulation and taken up by the liver with the same kinetics. In the lungs, a higher proportion (15 %) of shells than of microspheres was recovered.

INTRODUCTION

The ultrasound-contrast agent Albunex consists of air-filled microspheres. The air is enclosed within a thin layer of heat-aggregated human serum albumin (HSA). The microspheres are made by sonication of a 5 % albumin solution (Feinstein, 1989). They have a number-average diameter of 4 μm, with a range from approx. 1 to 15 μm. After intravenous injection, the product gives an increased contrast effect in ultrasound examination of the left ventricle, the echogenic properties being due to the air in the microspheres. The function of the protein layer, which constitutes about 2 % of the total protein in the product, is to stabilize the air bubbles.

The aim of this study was to examine the pharmacokinetics and biodistribution of the microspheres, with special attention being paid to the lungs, as a part of the safety evaluation of a new contrast agent.

After intravenous injection, the microspheres must pass the pulmonary capillaries in order to reach the left ventricle of the heart. Pulmonary intravascular macrophages are found in many familiar mammals of the order *Artiodactyla* (Winkler, 1989), which includes cattle, sheep, goats and pigs. These macrophages line the capillaries of the lung. In pigs, the injection of Albunex causes a powerful pulmonary vasoconstriction, apparently mediated by thromboxane (Östensen et al., 1992). This reaction is absent from rats, rabbits or humans (none of these species have pulmonary intravascular macrophages). One of the main purposes of the present study was to establish whether the species-specific differences in the physiological response to Albunex is paralleled by differences in the biodistribution of the microspheres.

If the microspheres are subjected to pressure above 0.3–0.4 MPa (3–4 standard atm), they collapse. About three-quarters of the microsphere protein, or 'shells', remains insoluble long enough to be isolated by centrifugation. The microsphere shell protein consists of intact albumin molecules with properties very similar to those of soluble albumin (Barnhart et al., 1990; Hellebust et al., 1993). The microsphere shell is stabilized mainly by non-covalent forces. Although the intact microspheres are stable when stored in the original suspension, the shell protein of pressure-collapsed microspheres becomes soluble when the shells are stored overnight in PBS (Hellebust et al., 1993).

EXPERIMENTAL

Albunex microspheres

Microspheres were produced from a 5 % HSA solution at NYCOMED Imaging AS, Oslo, Norway. One batch of Albunex was used in all of the labelling experiments.

^{125}I-labelling of Albunex microspheres

The microspheres were washed free of soluble albumin, labelled with ^{125}I by using sodium oxychloride as the oxidizing agent (Redshaw and Lynch, 1974), resuspended, and non-microsphere-associated radioactivity was removed by washing. The washing was performed by floating the microspheres by gently centrifuging the microsphere suspension.

The details of the procedure are as follows. The albumin carrier solution was removed from a vial containing 5 ml of Albunex (by pushing the needle of a syringe all the way to the bottom of the vial and aspirating the albumin solution underneath the microsphere layer). Then 3 ml of PBS–EDTA was introduced into the vial. The microspheres were suspended and subsequently floated by centrifuging for 5 min at 150 *g*. This procedure was repeated once. Before labelling, 0.5 ml of

Abbreviations used: HSA, human serum albumin; PBS, phosphate-buffered saline (0.9 % NaCl containing 10 mM sodium phosphate buffer, pH 7.4); PBS–EDTA, PBS containing 2 mM EDTA; TCA, trichloroacetic acid.

‡ To whom correspondence should be addressed, at NYCOMED.

PBS–EDTA was added, and the microspheres were resuspended. Then 2–5 mCi (148–370 MBq) of carrier-free $Na^{125}I$ was added, and 5 portions each of 10 μl of 1 mM NaOCl in 50 mM sodium phosphate buffer, pH 7.4, were added at 30 s intervals (Redshaw and Lynch, 1974; Tolleshaug, 1981). The vial was shaken after each addition. The reaction was terminated by addition of 10 μl each of 0.1 M Na_2SO_3 and 0.05 M KI, followed by 3 ml of PBS–EDTA containing 3 % HSA.

The iodine-labelled microspheres were separated from ^{125}I that had not reacted and soluble ^{125}I-HSA by centrifugation at 150 g for 5 min. The albumin solution underneath the microsphere layer was removed, and the microspheres were re-suspended in 3 ml of a 3 % solution of HSA in PBS–EDTA; this was repeated four times. After each washing cycle, total and trichloroacetic acid (TCA)-precipitable radioactivity were determined in the total suspension and in the albumin carrier solution (Table 1): portions (10 μl) were diluted to 0.5 ml with 1 % HSA in PBS, and 0.5 ml of 10 % TCA was added. The microsphere suspension tended to adhere to pipette tips, producing significant differences between parallel samples. If accurate determination of the amount of radioactivity was required, a larger volume was diluted into 0.1 % Triton X-100 (which causes the microspheres to disintegrate within seconds), and samples were counted for radioactivity.

Size distribution of the microspheres

The number and size distribution of the microspheres were checked in a Coulter Multisizer Mk II (Coulter Electronics, Luton, Beds., U.K.). In the size range between 1 and 30 μm, the Albunex batch used for radioactive labelling contained 8.0×10^8 microspheres/ml. The mean number diameter of the microspheres in this batch was 3.8 μm. After labelling, the different preparations contained from 5.0×10^8 to 6.3×10^8 microspheres/ml, with mean diameters in the range 4.4–4.6 μm; this variation in mean diameter between different batches of labelled Albunex was negligible. The small increase in mean diameter during labelling was mainly due to loss of some small microspheres during the washing process, as the small microspheres floated more slowly than the larger ones.

The pressure stability of the labelled batches was tested by exposing them to 100 mmHg for 1 min and determining the decrease in concentration of microspheres. The maximal fall was 4 %, which was regarded as acceptable. During intravenous injection, care was taken to keep the pressure in the syringe below 100 mmHg.

Microsphere shells were prepared by exerting mechanical pressure on the suspension of microspheres, causing them to collapse. The suspensions produced in this way were not echogenic in ultrasound tests *in vitro*. The shells could be sedimented by centrifuging at 8500 g for 10 min. Iodine-labelled microsphere shells were produced by introducing a small volume of ^{125}I-labelled Albunex into a 1 ml syringe, which was closed by a stopcock, and pressure was exerted on the piston until the milkiness almost disappeared, at which point only the empty shells remained. They were injected into rats immediately afterwards.

^{125}I-labelling of native HSA

A 20 μl portion of albumin carrier solution from the flotation of Albunex microspheres (containing 5 % HSA) was mixed with 5 μl of 0.5 M sodium phosphate buffer, pH 7.4, and 1 mCi (74 MBq) of $Na^{125}I$ in a volume of 10 μl. At 30 s intervals, four portions each of 5 μl of 1 mM NaOCl in PBS were added, with careful shaking after each addition. Then 10 min after the last addition, 5 μl each of 0.1 M Na_2SO_3 and 0.05 M KI were added. The reaction mixture was diluted to 0.5 ml with PBS and run on a PD-10 column (Pharmacia, Uppsala, Sweden); 1 ml fractions were collected. More than 97 % of the radioactivity in the peak fractions was precipitable in 5 % (w/v) TCA. About 70 % of the added radioactivity was recovered as TCA-precipitable radioactivity in the void volume. The two peak fractions were mixed with equal volumes of 3 % HSA in PBS–EDTA and stored in the freezer.

Blood clearance of ^{125}I-labelled microspheres in rats

Male Wistar rats (Møllegaard, Vejby, Denmark) weighing 220–270 g were anaesthetized by intraperitoneal injection of 1 ml of sodium pentobarbital solution (50 mg/ml)/kg body wt. After resuspension of the labelled microspheres in fresh buffer, 0.1 ml of the suspension was injected into the tail vein. On a body-weight basis, the microsphere dose was approximately twice the highest recommended clinical dose. The solution was injected at a rate of 1.2 ml/min, via a 23-gauge needle. Injection under these conditions were shown to produce a peak pressure of less than 75 mmHg, which would cause less than 4 % decrease in the concentration of intact microspheres, as shown by pressure-stability testing of the labelled batches.

Blood samples (50 μl) were drawn from the tail vein after the injection of ^{125}I-labelled microspheres (5 rats) or ^{125}I-HSA (3 rats). The samples were diluted 4–5-fold with distilled water, and 10 % TCA was added to give a final concentration of 5 %. After standing for 30 min at 4 °C, the samples were centrifuged at 1000 g for 10 min, and the supernatant was drawn off and counted for radioactivity along with the precipitates. After 30 min, the animals were killed, and the organ distribution of radioactivity was determined as described in the following subsection. For the purpose of calculating the radioactivity in the blood, the blood volume was taken as 6 % of the body weight (Ringler and Dabich, 1979).

Biodistribution of ^{125}I-labelled microspheres in rats

Rats were injected with ^{125}I-labelled microspheres or ^{125}I-HSA as above. They were killed after pre-determined intervals (5 rats in each group). Additional groups were placed in metabolic cages for the collection of urine and faeces. After laparotomy, a blood sample was drawn from the vena cava; acid-soluble and acid-precipitable radioactivity were determined as described above. Liver, lungs, spleen, kidneys, heart and brain were excised, and the total amounts of radioactivity were determined in each organ. A sample of bone marrow was taken from the femoral bone. For the purpose of calculating the radioactivity in the bone marrow, the total weight of bone marrow was taken as 3 % of the body weight (Ringler and Dabich, 1979).

Biodistribution of ^{125}I-labelled microspheres in pigs

The experimental study in pigs was performed at Scantox, Lille Skensved, Denmark. Pigs (cross-bred, weight about 25 kg) were given a pre-medication of azaperone (6 mg/kg) intramuscularly and anaesthetized by intraperitoneal administration of metomedati-HCl (2.5 mg/kg). The animals were kept anaesthetized throughout the experiment. In order to block the powerful pulmonary vasoconstrictory effect of Albunex in pigs, indomethacin (10 mg/kg) was administered intravenously and

sustained at a rate of 5 mg/kg per h. A dose of 0.12 ml of ^{125}I-labelled Albunex microspheres/kg was administered at 0.5 ml/s through a catheter in the ear vein. Three pigs were killed 3 min after the injection, and three pigs were killed after 90 min. Lungs, liver, spleen, kidneys, heart and brain were excised and weighed. The amounts of radioactivity in weighed samples were determined. A sample of bone marrow was taken from the sternal bone; the weight of the bone marrow was taken as 3 % of the body weight.

Blood clearance and biodistribution of ^{125}I-labelled microsphere 'shells' in rats

By applying pressure in a closed syringe, the labelled microspheres were collapsed to yield ^{125}I-labelled 'shells' (as described above), and 0.1 ml was injected into the tail vein of anaesthesized rats. In other respects, the procedure was substantially as described for the biodistribution of intact microspheres. Blood samples (50 μl) were drawn from the tail vein (5 rats) after the injection of ^{125}I-labelled microspheres (5 rats); TCA-soluble and TCA-precipitable radioactivities were determined as described for blood clearance of intact microspheres. After 30 min, the animals were killed, and the organ distribution of radioactivity was determined as described.

Biodistribution of 'shells' was also determined in a separate series of experiments. The animals were killed after 3, 10, 30 or 90 min (5 rats per group). After laparotomy, a blood sample was drawn from the vena cava into a heparinized syringe. Radioactivity in the blood, liver, lungs, spleen, kidneys and bone marrow were determined as described above for intact microspheres in rats.

Distribution of radioactivity from labelled microspheres between the main types of liver cells

Rats were injected with ^{125}I-labelled microspheres. Dissection of the animal was initiated so as to allow perfusion of the liver to start *in situ* 10 min after the injection (Eskild et al., 1989). The liver cells were separated and the three main types of liver cells were isolated by a two-step procedure involving differential centrifugation and centrifugal elutriation (Blomhoff et al., 1984a) in a Beckman JE 5.0 rotor at 4 °C. Contaminating hepatocytes and Kupffer cells in each fraction were identified by visual microscopy and peroxidase staining respectively (Blomhoff et al., 1984a) and corrected for contaminating cells. The recovery of injected radioactivity in the initial cell suspension was close to 50 %.

RESULTS

Labelling of Albunex microspheres

In the choice of labelling method, a prime consideration was to generate a small, diffusible, iodinating species which might penetrate the shells of the microspheres and be active near neutral pH. This was achieved by the use of oxychloride, which generates molecular iodine. After the introduction of radioactive iodine, the microspheres were washed several times by flotation, followed by a change of suspension medium, in order to remove labelled soluble albumin molecules. No attempt was made to determine the radioactivity in the actual microsphere layer; instead, it was assumed that the radioactivity in the microspheres could be calculated as the total radioactivity minus the radioactivity in the albumin carrier solution immediately after separation of the albumin solution underneath the microsphere layer by centrifuging.

A typical experiment is summarized in Table 1. During the first two changes of the suspension medium, there was a large decline in the amount of radioactivity in the suspension. The fraction of TCA-precipitable radioactivity increased to over 95 %, indicating that ^{125}I that had not reacted had been removed. During the last steps of the washing procedure, the percentage of radioactivity in the albumin carrier solution was nearly constant; neither was there any change in the percentage of TCA-precipitable radioactivity in either the total suspension or the albumin carrier solution. This shows that the composition of the system was nearly constant; in other words, the labelled microspheres had been separated from labelled soluble albumin.

In the final suspension, 12 % of the initially added radioactivity was recovered in the microspheres, and only 0.3 % in the albumin carrier solution. More than 96 % of the microsphere-associated radioactivity was TCA-precipitable. This shows that the microspheres had indeed been labelled, and that nearly all of the soluble labelled albumin had been removed.

After storage overnight, the percentage of non-microsphere-associated radioactivity increased 2–3-fold; TCA-precipitable radioactivity accounted for most of the increase. Consequently, labelled microspheres were used in experiments within a few hours after removal of the infranatant (the liquid phase underneath the microsphere layer) and resuspension in fresh albumin solution.

Clearance of labelled microspheres from the circulation of rats

After 3 min, less than 20 % of the injected radioactivity from ^{125}I-labelled microspheres remained in the circulation (Figure 1). The level of radioactivity was constant (within experimental error) from 5 min after the injection and for at least 30 min. It was much too high to be due to non-microsphere-associated labelled protein in the injected preparation. The amount of TCA-soluble radioactivity in the blood increased, beginning about 5 min after the injection. Part of the increase was due to the release of acid-soluble degradation products, which are filtered by the kidneys

Table 1 Radioactivity in the total suspension and soluble albumin phase during purification of ^{125}I-labelled Albunex microspheres

Step 1 refers to the iodination mixture (after the oxidizing agent was destroyed). First, total and TCA-precipitable radioactivities were determined in samples from the total suspension, then the microspheres were flotated by gentle centrifuging, and corresponding measurements were made on the infranatant before it was discarded. Finally, albumin solution was added to the microspheres to give the initial total volume. Step 2 refers to measurements on the next suspension and the corresponding infranatant. The microspheres were made up to the original volume before Step 3, and so forth. The infranatant is the soluble albumin phase underneath the microsphere layer, which was separated following gentle centrifuging (see the Experimental section). The 'Sum' values are in millions of c.p.m. Triplicate samples were taken from each total suspension and infranatant; median values are shown.

	Total suspension		Infranatant	
Step	Sum	TCA ppt. (%)	Sum	TCA ppt. (%)
1	8180	64.7	6118	35.3
2	2408	87.0	344	55.7
3	1192	95.1	109	79.3
4	1167	97.1	60	92.4
5	1009	96.1	24	90.6

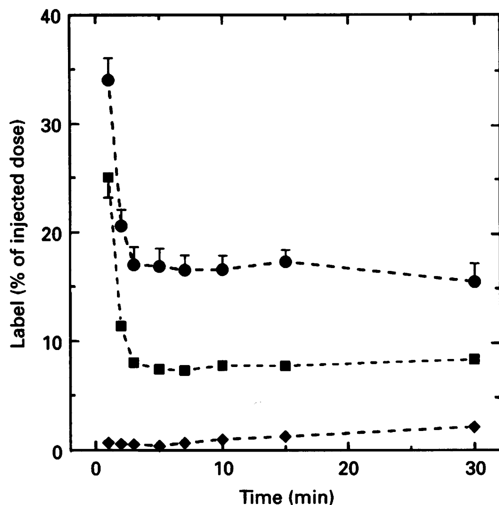

Figure 1 Clearance of ^{125}I-labelled air-filled microspheres and microsphere 'shells' from the circulation of rats

Microspheres and 'shells' were labelled and injected into rats as described in the Experimental section. Key: ●, intact microspheres; ■, 'shells'; ◆, TCA-soluble radioactivity from intact microspheres. Mean values ± S.E.M. are shown, from 5 rats per time point.

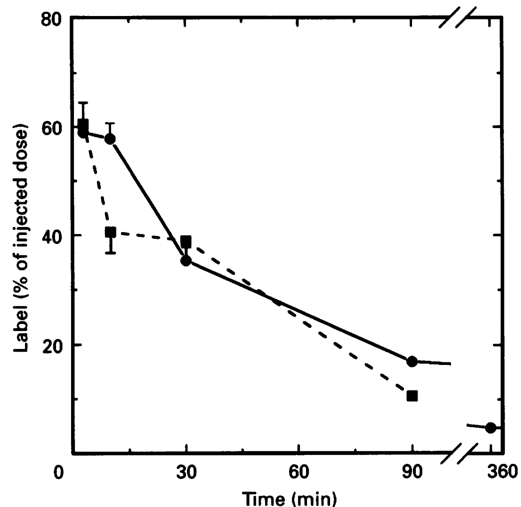

Figure 2 Kinetics of hepatic elimination of radioactivity from air-filled microspheres and 'shells' in rats

Key: ●, intact microspheres; ■, 'shells'. Values shown are means ± S.E.M., from 5 rats per time point.

to be recovered in the urine, preventing any appreciable build-up in the blood.

After injection of ^{125}I-HSA, about 85 % of the injected dose remained in the blood after 10 min (results not shown). Albumin molecules migrating into the extravascular space may account for most of the decrease from 100 %. The fraction of acid-soluble radioactivity in the ^{125}I-HSA-injected animals stayed below 1 % (results not shown). Thus this protein is handled in an entirely different manner from either microspheres or denatured albumin (Misquith et al., 1988).

Biodistribution of labelled microspheres in rats

At 3 min after the injection, 60 % of the injected radioactivity from ^{125}I-labelled microspheres was recovered in the liver (Figure 2). Minor sites of uptake were the lungs (3 %) and the spleen (7 %) (Figure 3). The amount in the bone marrow increased after injection up to a maximum of 3.3 % after 30 min, declining only slowly after that. Less than 0.3 % was found in the brain or in the heart. At any time point, less than 1.2 % was recovered in the kidneys (results not shown); the microspheres are not filtered in the glomeruli, and the degradation products are not re-absorbed from the ultrafiltrate. The sum of the recoveries in all organs studied, including the blood, was over 90 % after 10 min.

The radioactivity in the lungs and spleen declined steadily (Figure 3). Reckoned as a fraction of the load 10 min after the injection, radioactivity decreased fastest in the spleen and the lungs and more slowly from the liver. The degradation in the liver apparently followed first-order kinetics, the half-life being about 40 min. After 24 h, only trace amounts were recovered in the internal organs, but 75 ± 10 % of the original dose was found in the urine and 1.3 ± 0.5 % in the faeces.

Biodistribution of labelled microspheres in pigs

In pigs, the labelled microspheres were cleared from the circulation within 1 min (results not shown). At 3 min after the injection, over 90 % of the dose was recovered in the lungs (Table 2); 7 % remained in the blood; less than 1 % was found in the other organs which were examined (results not shown). The high uptake in the lungs may be due to the presence of pulmonary intravascular macrophages in pigs; in rats or humans, macrophages are not normally found in this particular location. In rats, the major part of the injected microspheres is recovered in the hepatic macrophages (Table 2, and below).

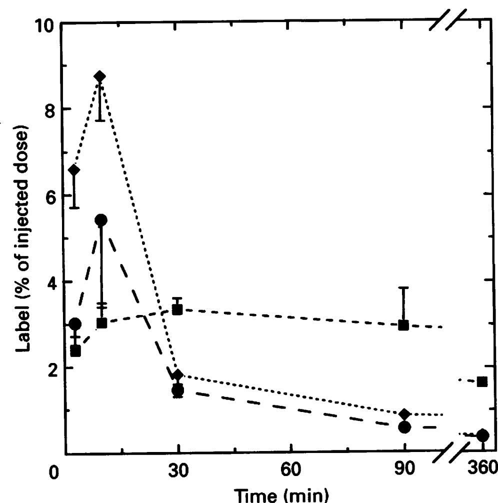

Figure 3 Time course of the biodistribution of ^{125}I-labelled microspheres in rats

Key: ●, lungs; ■, bone marrow; ◆, spleen. Mean values ± S.E.M. from 5 rats are shown.

At 90 min after the injection of labelled microspheres into pigs, the amount of radioactivity in the lungs had declined to about one-half of the original dose. The recovery in the bone marrow was 2.4 % of the dose, an increase from 0.36 % after 3 min

Table 2 Comparison of the biodistributions of radioactivity from ^{125}I-labelled microspheres in pigs and rats

Values shown are percentages of injected dose. Median values (pigs, 3 animals per group; rats, 5 animals per group) are shown. The average coefficient of variation was 28%.

	3 min post-injection		90 min post-injection	
Organ	Pigs*	Rats	Pigs	Rats
Lungs	103.4	3.13	50.8	0.51
Liver	0.29	59.8	0.93	16.5
Blood	6.76	14.3	8.39	12.3

* The sum of the values in this column exceeds 100%, due to the error inherent in calculating the total radioactivity in the organ from the radioactivity in weighed pieces.

(results not shown). In all of the other organs examined, increases in the amounts of radioactivity were observed, but the recoveries ranged downwards from less than 1% of the dose.

Blood clearance and biodistribution of labelled microsphere 'shells' in rats

At 3 min after the injection, only 8% of the ^{125}I-labelled microsphere 'shells' were left in the circulation (Figure 1). The clearance was even more complete than that of intact microspheres. From 5 min after the injection and for at least 30 min, the level of TCA-precipitable radioactivity was constant. The time course of the release of TCA-soluble radioactivity to the circulation (results not shown) was indistinguishable from the release from intact microspheres. The level of TCA-precipitable radioactivity in the circulation after injection of 'shells' is much lower than after injection of intact microspheres (Figure 1). There is no obvious explanation why this should be so. Possibly the disintegration of microspheres and 'shells' occurs by a different mechanism *in vivo*.

The liver accumulated 60% of the ^{125}I-labelled 'shells' within 3 min of the injection (Figure 2). During the next 90 min, over 80% of this radioactivity left the liver. This rate of loss indicates a rate of degradation of 'shells' similar to that of intact microspheres, with a half-time of about 40 min.

An even higher proportion of the 'shells' than of intact microspheres was retained in the lungs (Figure 4); the percentage was close to 15% after 10 min. In these organs also, the amount of radioactivity from 'shells' declined with the same kinetics as the radioactivity from intact microspheres.

The uptake in the spleen was approximately the same as for labelled microspheres. In the kidneys, less than 1% of the dose was recovered at any time point (results not shown). The total recovery of radioactivity in all of the organs examined was 74±9% after 10 min. In a different series of experiments, the biodistribution of 'shells' was examined after 24 h: 79±5% of the dose was found in the urine, 2.2±0.3% in the faeces, 1.3±0.1% in the liver, and only insignificant traces in the other organs studied (results not shown).

Uptake of microspheres in various rat liver cells

The data in Table 3 show that over 90% of the microspheres were taken up by the liver macrophages (Kupffer cells) in rats; these cells normally remove particles from the circulation. The values in Table 3 do not include degradation products which leave the cells after uptake *in vivo* and during separation of the cells. Degradation of endocytosed proteins during the separation procedure is retarded considerably by cooling of the cells (Duncan and Lloyd, 1978; Tolleshaug et al., 1980). The total hepatic recovery in the present experiments is close to that in the biodistribution experiments.

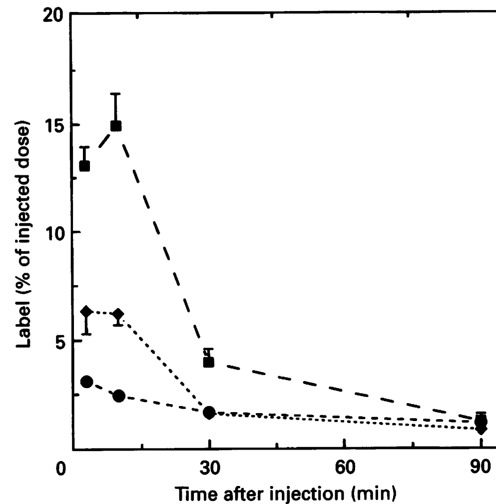

Figure 4 Time course of the biodistribution of ^{125}I-labelled microsphere 'shells' in rats

Key: ◆, spleen; ●, bone marrow; ■, lungs. Mean values±S.E.M. from 5 rats are shown.

Table 3 Distribution of radioactivity from labelled microspheres among the major types of rat liver cells

	Cells		
Radioactivity	Parenchymal	Endothelial	Kupffer
c.p.m./10^6 cells*	472	203	41328
c.p.m. in 1 g of liver†	56520	7870	684524
% of total	7.6	1.2	91.2‡

* Not corrected for contaminating cells.

† Corrected for contaminating cells; the number of cells of each type in 1 g of liver was taken from Blomhoff et al. (1984a).

‡ The SEM for the percentage in the Kupffer cells was 5.5 (3 experiments); the relative errors of the percentages in the other cells types were higher, due to much lower radioactivity in the parenchymal and endothelial cells.

DISCUSSION

The microsphere protein of collapsed microspheres may be sedimented by centrifugation, showing that the 'shell' structures are sufficiently stable to be used in experiments. After labelling, no significant loss of radioactivity was observed on repeated washing of the iodine-labelled microspheres, indicating that the radioactivity was covalently bound to the microspheres.

The most striking observation from the biodistribution studies is the extremely high recovery of radioactivity in the lungs of pigs, compared with rats. In the context of previous work, we feel that the difference may be best explained by the presence of pulmonary intravascular macrophages (Winkler, 1989); these cells are normally not found in rats or humans. The pulmonary intravascular macrophages are efficient scavengers; e.g.,

liposomes were taken up by these cells after injection in sheep (Miyamoto et al., 1988). In the present study, nearly all of the labelled material was recovered in the lungs. The complete pulmonary clearance explains the lack of ultrasound contrast in the carotid artery after intravenous injection of Albunex in pigs (Östensen et al., 1992). The high uptake in pig lungs parallels the powerful pulmonary vasoconstrictory effect of Albunex in pigs; this effect is not observed in rats, rabbits or humans, which lack pulmonary intravascular macrophages (Östensen et al., 1992).

In rats, the clearance rate of the labelled Albunex microspheres is comparable with that of solid microspheres (Morimoto and Fujimoto, 1985; Davis and Illum, 1988), at least as high as the clearance of asialo-orosomucoid by rat hepatocytes *in vivo* (Charlwood et al., 1979) and faster than the clearance of formaldehyde-denatured serum albumin (Misquith et al., 1988).

The protein in the microspheres may be removed from the circulation by trapping of intact microspheres in capillaries, by uptake of intact microspheres of 'shells' in capillary endothelium, by uptake of intact microspheres of 'shells' in phagocytes, or by uptake of soluble albumin molecules liberated from microspheres of 'shells'. An alternative explanation for the high recovery in pig lungs is trapping of microspheres in the lung microvasculature, which may trigger platelet-mediated vasoconstriction. Purely mechanical trapping probably occurs only to a very small extent, as over 90% of the microspheres are small enough to pass the pulmonary capillaries (Chilton and Witcofski, 1986).

Quantitative release of native HSA to the circulation does not occur, as the biodistribution of radioactivity from labelled HSA is entirely different from that of microsphere radioactivity; e.g., the recovery of native HSA in the kidneys is 3 times that of the microspheres. HSA molecules might be liberated as altered or denatured molecules. However, formaldehyde-denatured serum albumin is cleared more slowly from the circulation (Misquith et al., 1988) than is radioactivity from the microspheres. Radioactivity from Albunex microspheres is recovered in the Kupffer cells, whereas several different types of altered serum albumin (Eskild and Berg, 1984) are mainly taken up by liver endothelial cells *in vivo* (Blomhoff et al., 1984b; Eskild et al., 1989). Thus the radioactivity of the microspheres is taken up by an entirely different route from that for the usual preparations of denatured albumin.

Clearance of albumin particles from the lung occurs by breakdown into smaller particles, which are subsequently phagocytosed (Chilton and Witcofski, 1986). The fast decline of microsphere radioactivity in rat lungs may be explained by temporary trapping of microspheres in the pulmonary capillaries (rather than phagocytosis), followed by release of shell material or soluble albumin molecules.

On the other hand, solid albumin particles (Willmott et al., 1989) which remain trapped in the microvasculature are degraded much more slowly than phagocytosed particles; half-lives are measured in days rather than in hours. In the present study, hepatic uptake of the microsphere protein by endocytosis (presumably phagocytosis), followed by rapid degradation, is indicated by the high rate of elimination of radioactivity from the liver. Most of this radioactivity is eventually excreted as TCA-soluble material in the urine.

We conclude that the major uptake of radioactivity occurs as intact microspheres and microsphere shells. In the circulation, a stable level of TCA-precipitable radioactivity is measured 5–30 min after the injection of either intact or collapsed microspheres, indicating that release of material which is slowly endocytosed also occurs; this material could simply be soluble albumin molecules.

Observations on the ultrasound-contrast effects indicate that many microspheres are trapped or destroyed during the passage from the right to the left ventricle. In rats and humans, a substantial fraction of the microspheres reach the left ventricle. The material that is actually taken up by the Kupffer cells of rat liver may be largely 'shells', even if microspheres were injected originally. Uptake of microspheres versus shells cannot be distinguished kinetically, as microspheres as well as shells are cleared from the blood within 3 min.

The weight of the injected microsphere protein is less than one-thousandth of the weight of the Kupffer cells; accordingly, no overloading of the Kupffer cells with protein occurs. The rate of release of label from the liver mainly reflects the rate of degradation, as label from conventionally ^{125}I-labelled protein is quickly released from cells which degrade the protein (Strobel et al., 1985). The observed rate of hepatic degradation in the present study is faster than the degradation of asialoglycoproteins in isolated hepatocytes (Tolleshaug et al., 1980).

In rat lungs, the recovery of label from 'shells' is markedly higher than from microspheres. The more flexible 'shells' may be able to flatten themselves against the vessel walls, aided by contact over a larger fraction of their surface. Albumin-binding proteins on vascular endothelial cells have been identified (Dobrila et al., 1992); they bind altered or oligomeric albumin more strongly than the native protein (Schnitzer et al., 1992).

In pigs, the increase in the amounts of radioactivity in liver, kidneys and heart from 3 to 90 min after the injection may be explained by diffusion of labelled albumin molecules or labelled degradation products into the tissues. The striking increase in the bone marrow can only be caused by a scavenging function which is peculiar to this tissue.

In conclusion: Albunex microspheres are mainly cleared from the circulation as particles. In rats, the major uptake occurs in the Kupffer cells (liver macrophages), but in pigs nearly all of the injected microspheres are recovered in the lungs. They are very likely cleared by the pulmonary intravascular macrophages. As all of the blood passes through the pulmonary circulation, these macrophages are in a position to clear particles from the blood even faster than the Kupffer cells.

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