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# Biodegradation Rate of Embolized Protein Microspheres in Lung, Liver and Kidney of Rats

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**Abstract**—The targeting and sustained release characteristics of cytotoxic drug-loaded protein microspheres may prove useful in the therapeutic chemoembolization of solid tumours. Because biodegradation rate of embolized particles will influence rate of incorporated drug release and duration of exposure, this parameter was studied for microspheres (10–30 μm mean diam.) prepared from the proteins albumin and casein, that we have previously used as carriers for doxorubicin. As a measure of microsphere loss in-vivo the radionuclide $^{125}I$ was chosen because it can be covalently bound to proteins and also homogeneously distributed throughout the matrix. Radiolabelled microspheres were administered to rats both intravenously (lung as target organ, 1·4–2·2 mg/100 g) and via the hepatic artery (liver as target organ, 0·4–0·8 mg/100 g). In both cases it was observed that the casein system biodegraded more slowly than the albumin in-vivo. Thus, time taken for loss of 50% of embolized microspheres from lung was: albumin 2·0 days; casein 3·5 days and from liver: albumin 3·6 days; casein 6·8 days. Microsphere "debris" did not markedly accumulate in other organs. In-vitro experiments showed that microspheres were stable in serum and that albumin microspheres were not innately more sensitive to enzymic digestion than casein. The results may be useful in estimating duration of exposure of target organs to drug-loaded microsphere systems prepared from these proteins.

Embolization, following intravascular injection of spherical particles of requisite size, has been used to treat a variety of conditions such as arteriovenous malformation and haemorrhage (Dion et al 1986; Horak et al 1986; Reidy 1987). When properly applied the technique is considered safe and effective. The advent of biodegradable microspheres (albumin or starch) has enabled workers to use embolization in other conditions; for example, to attempt to change haemodynamics of target organs harbouring tumour deposits, and consequently disposition of co-administered drug (Ensminger et al 1985; Goldberg et al 1988).

An elaborate pharmacokinetic rationale can be constructed to support the use of microspheres co-administered with cytotoxic agents via the hepatic artery; however, essentially it means" . . . for the time that the hepatic arterial blood column is held up by microsphere embolization, the regional exposure advantage of a hepatic arterial drug infusion should increase" (Wollner et al 1986). Thus, rate of biodegradation of embolized particles should be an important factor in drug efficacy.

A more elegant way of using degradable microspheres is to incorporate drug into the matrix and thereby take advantage of the capacity of the system for targeting (Willmott 1987; Goldberg 1987) and sustained release (Willmott et al 1988). We have succeeded in preparing albumin and casein microspheres containing the anti-cancer drug, doxorubicin, and found that it is incorporated in both native form and in a form tightly (probably covalently) bound to protein matrix (Chen et al 1988). The rate of biodegradation of the particles in the various embolized organs will clearly influence bioavailability of drug (especially the form bound to matrix protein) and subsequent anti-tumour effect. Therefore, we have examined biodegradability of embolized albumin and casein microspheres in lung, liver and kidney, these being potential target organs.

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## Materials and Methods

*Radiolabelled albumin microspheres*

1 mL of [$^{125}I$]human serum albumin (Amersham International) supplied at a concentration of 20 mg $mL^{-1}$ (sp. act = 71 kBq $mg^{-1}$) was mixed with a solution of 380 mg cold human albumin (Sigma Chemicals) dissolved in 800 μL water containing 2mg of sodium dodecylsulphate. This constituted the disperse phase of a water-in-oil emulsion prepared with a Silverson mixer. Water droplets were stabilized by the addition of 240 μL 15% glutaraldehyde solution and the resulting microspheres separated and made ready for in-vivo use as described elsewhere (Willmott et al 1985).

*Radiolabelled casein microspheres*

5 mg of casein (sodium salt, Sigma Chemicals) was radiolabelled with 74MBq of Na[$^{125}I$] (Amersham International) by the chloramine-T method, essentially as described by Willmott & Simpson (1983). Free radiolabel was separated from protein bound on a G-50 fine Sephadex column and the product concentrated to the desired volume by pressure dialysis. Finally, radiolabelled casein was dialysed against a solution of 0·9% NaCl and 0·9% benzyl alcohol to make it comparable with the [$^{125}I$]human serum albumin supplied by Amersham (see above). Denatured protein was separated by centrifugation, 1·2 mL of supernatant mixed with 1 mL of water containing 2mg of sodium dodecyl sulphate and the solution added to 400mg of cold casein wetted with 800 μL absolute ethanol. The whole was heated at 95°C for 1 min to

dissolve as much casein as possible. This constituted the disperse phase of a water-in-oil emulsion and microspheres were prepared as described for albumin, i.e. using 240 μL 15% glutaraldehyde.

*Rats*

Inbred Wistar rats were used for the intravenous injection of microspheres via a tail vein (injection volume 0·2–0·3 mL). Because of the small diameter of a rat artery intra-arterial injections (injection volume 0·2–0·4 mL) require a larger strain and Sprague-Dawley rats were used.

At intervals following injection of radiolabelled microspheres, rats were killed by ether inhalation and radioactivity in blood, lungs, liver, spleen and kidneys measured on a Packard Autogamma 500C.

*Microsphere size*

The size of microspheres was measured by Coulter Counter. In all cases the volume distribution was used to compute the 50% average size because this is a more accurate reflection of drug (or radioisotope) content than size distribution.

*Time course of microsphere degradation in-vitro*

Microspheres were suspended in a 0·4% solution of trypsin (Difco 1 : 200) in phosphate buffered saline and maintained at 37°C. Degradation and solubilization of particles as a function of time was assessed by laser diffraction (Malvern 2600C Particle Sizer) using changes in volume concentration. Volume concentration is computed from both amount of light transmitted and particle size distribution and we have found it to be a more sensitive measure of particle loss than changes in particle size alone.

*Stability of microspheres*

On storage at 4°C in phosphate buffered saline + 0·5% Tween 80, both albumin and casein microspheres were remarkably stable. Less than 2·5% of radiolabel was released into the supernatant over 3 months. To assess stability in rat serum, 2–5 mg of microspheres were incubated in 1 mL serum at 37°C and at the first time point particles were separated from supernatant and radioactivity in both measured. After recording counts $min^{-1}$ in both fractions they were remixed and reincubated at 37°C until the next time point, whereupon the procedure was repeated. The amount of $^{125}I$ released is expressed as % of total counts $min^{-1}$.

*Injection technique*

Intravenous injection of microspheres (1·4–2·2 mg/100 g) was via a tail vein in rats anaesthetized with halothane. Intra-arterial injections were made following laparotomy in rats anaesthetized with halothane or ether. Hepatic arterial injection (0·4–0·8 mg/100 g) was via a cannula positioned in the gastroduodenal artery (which feeds the hepatic artery) and renal arterial injection (0·2 mg/100 g) via an angled needle inserted into the renal artery. Intra-arterial injections were carried out as slowly as possible to minimize back pressure, and equipment was flushed through with saline. All radiolabelled microspheres for injection were suspended in phosphate-buffered saline + 0·5% Tween 80 and disaggregated if necessary by sonication.

*Statistics*

Although numbers in each experimental group are small, they appeared on inspection of raw data to be derived from a Poisson distribution as is usual for results generated from radioisotopes. Consequently, the equation $\bar{x} \pm 1{\cdot}96\ \sqrt{(\bar{x}/n)}$ (n = number of observations) was used to compute 95% confidence intervals (Wetherill 1982).

## Results

*Biodegradation of microspheres in lung*

Following intravenous injection of microspheres radiolabelled with $^{125}I$ prepared from albumin (12·7 μm and 25·4 μm diameter) and casein (27·6 μm diameter), rats were killed at intervals and blood (1 mL), lungs, kidney, spleen and liver taken for γ-counting. The results in Fig. 1A show the radioactivity remaining in the lung as percent of radioactivity injected.

It can be seen that casein microspheres biodegrade more slowly than albumin and that reducing particle size did not increase biodegradation rate. Thus, for the casein system, the time taken to reduce radioactivity in the lung by 50% (using the mean radioactivity in the lung immediately after injection as the 100% value) was 3·5 days. On a similar basis, 50% of lung radioactivity was lost in 2 days for both albumin systems. The radioactivity localizing in the lung immediately after injection approached 100% of injected dose.

It was anticipated that, following embolization in the lung and subsequent biodegradation, microsphere "debris" would appear in organs containing phagocytic cells, i.e. liver and spleen. In fact this prediction did not prove correct, radioactivity in these organs always being < 1% of injected activity. Activity in kidney was similarly low. In the albumin systems, radioactivity in total blood volume did occasionally rise to between 1–3% of injected dose (data not shown) when activity in lung was falling sharply, i.e. when biodegradation was taking place.

*Biodegradation of microspheres in liver and kidney*

Tumour deposits in liver and kidney of patients can be embolized with albumin microspheres (Willmott 1987; Goldberg et al 1987). Therefore, it is important to have information on the expected rate of biodegradation of embolized particles in these organs and also their subsequent distribution. Following intrahepatic arterial injection of $^{125}I$-labelled microspheres prepared from albumin and casein (25·4 and 27·6 μm diameter, respectively) rats were killed at intervals and radioactivity residing in the target organ (liver) measured. In addition radioactivity in lung (to assess the degree of shunting), thyroid and femur were recorded.

The efficiency of entrapment immediately following intra-arterial injection (i.e. liver and kidney as target organs) was only 39–55% (see Fig. 1B for liver. For albumin microspheres embolized in kidney: 1 min 39·3 ± 6·3%; 5 days 2·0 ± 1·4% n = 4) whereas following intravenous injection (i.e. lung as target organ) it approached 100% (Fig. 1A). This is because in the latter case the injection is into a low pressure system and back flow is not a problem. Following intra-arterial injection, "resistance" can build up in the target organ capillaries and back flow may occur. That this had occurred was confirmed by the detection, after intrahepatic

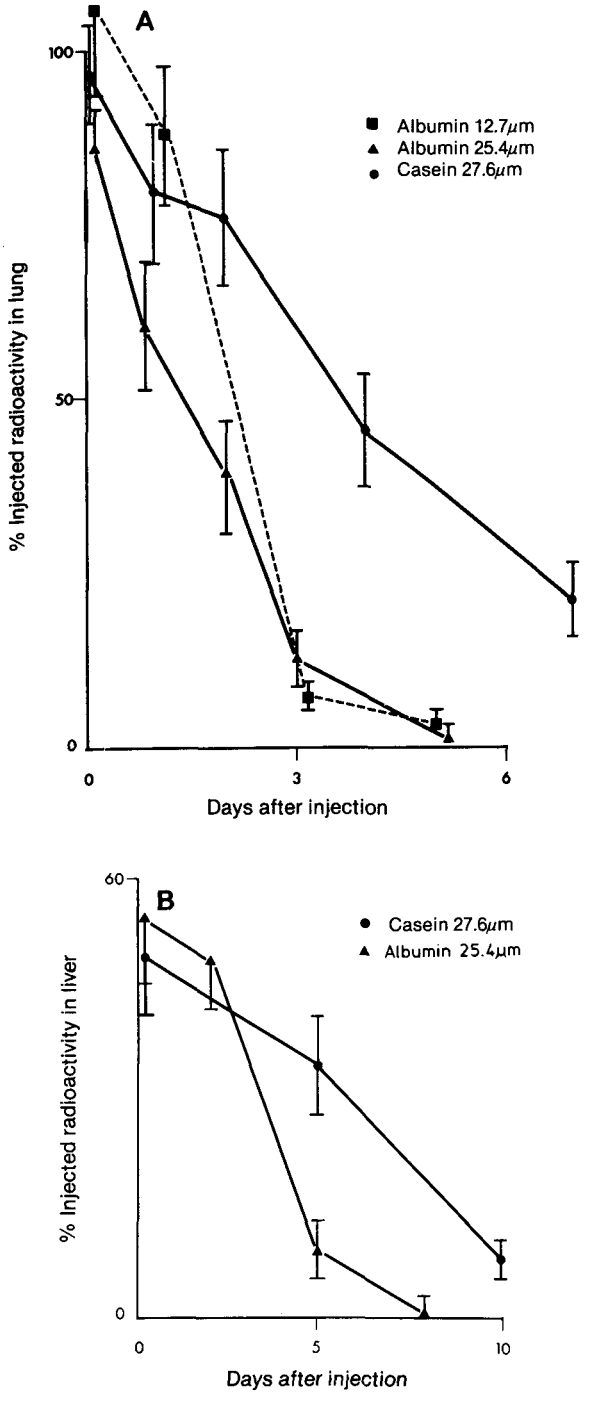


FIG. 1. In-vivo biodegradation rate of protein microspheres in A, rat lung and B, rat liver. Rats were injected intravenously via a tail vein (A) or via hepatic artery at laparotamy (B) with the different preparations of protein microspheres labelled with $^{125}I$. At intervals rats were killed and activity in lungs expressed as percent of activity administered. 3–4 rats per group. Error bars represent 95% confidence interval.

arterial injection, of radiolabelled particles in stomach and small bowel. Indeed, excluding the liver, these organs were the repository of the highest amount of microspheres (data not shown).

From Fig. 1B it can be seen that, for the casein system, the time taken to lose 50% of embolized particles (again using the mean radioactivity in the liver immediately after injection as the 100% value) was 6·8 days and for the albumin system 3·6 days. The process of biodegradation of casein microspheres embolized in the rat liver is shown in Fig. 2A, B. Clearly, by day 5 although particles are still present they are in the process of erosion, as evidenced by loss of sphericity and fragmented appearance, and by day 10 nothing resembling spherical particles could be seen.

Table 1 shows the degree of shunting of microspheres through the liver into the lungs after intrahepatic arterial injection (as assessed by radioactivity in lungs immediately after injection) and also the fate, after biodegradation, of microspheres embolized in the liver. The organs of particular interest were lung (because it receives venous drainage from the liver via the heart) and bone (because marrow depression is frequently dose-limiting in cancer chemotherapy). It can be seen that, in general, little shunting of microspheres takes place (0, 0 and 1·2% of injected dose for albumin and 1·5, 3 and 14% for casein). Moreover, during the time embolized microspheres are lost from the liver through biodegradation, little radioactivity accumulates in the lung. Radioactivity steadily accumulates in the thyroid, as expected for $^{125}I$. Importantly, the level of radioactivity in bone was never substantially more than background.

To examine whether albumin microspheres (25·4 μm

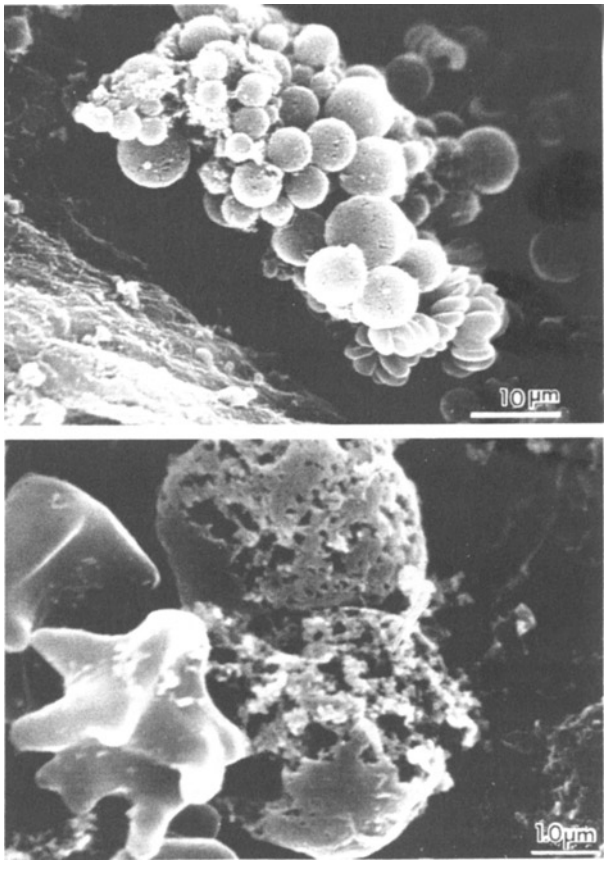


Fig. 2. Scanning electron photomicrographs of casein microspheres embolized in rat liver. (A) upper, immediately after injection, (B) lower, 5 days after injection. Note the loss of sphericity and fragmented appearance of microsphere after 5 days.

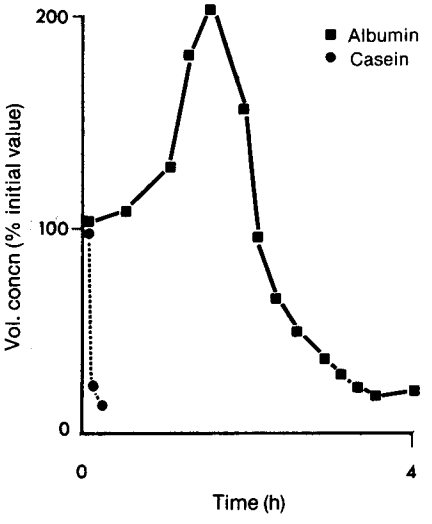


Fig. 3. In-vitro degradation rate of protein microspheres. The different microsphere preparations were suspended in 0·4% trypsin at 37°C by stirring and changes in volume concentration with time monitored by laser diffraction. Results are expressed as percent of initial value.

diameter) biodegraded in the kidney, radiolabelled particles were administered via the renal artery and groups of rats killed immediately after injection and at 5 days. It is clear from the results (1 min 39%, 5 days 2%) that at the latter time microspheres had virtually disappeared; moreover, histologically, the injected and contralateral kidney had the same appearance (unpublished).

*In-vitro degradation of microspheres*

From the foregoing it is clear that embolized albumin and casein microspheres degrade in-vivo. To investigate which in-vivo systems contribute to biodegradation, radiolabelled microspheres were incubated at 37°C in rat serum and digestion and solubilization of microspheres measured by

Table 1. Distribution of radioactivity following embolization and biodegradation of microspheres in rat liver (Activity in femur after administration of albumin microspheres was 0%).

| | % Injected activity in | | |
|---|---|---|---|
| | Lung | | Thyroid |
| Time after injection | Albumin | Casein | Albumin |
| 1 min | 0·4 [0–1·2] | 6·0 [1·5–14] | 0 |
| 2 days | 0·6 [0–1·7] | NT | 0·9 [0·4–1·6] |
| 5 days | 1·2 [0·2–1·9] | 0·7 [0·2–1·4] | 2·7 [1·5–3·5] |
| 8 days | 0·1 [0–0·1] | NT | 2·0 [1·2–2·5] |
| 10 days | NT | 0 | NT |

$^{125}$I-labelled microspheres were injected into the hepatic artery of rats. At the time points shown groups of 3–4 rats were killed and radioactivity in liver (Fig. 1B), lung, thyroid and femur (see above) measured. Because distribution of values in some groups was discontinuous (i.e. values of zero occurred), mean and [range] are quoted.

NT—not tested

Table 2. Stability of microspheres in serum.

| | % Release of radiolabel from[a] | |
|---|---|---|
| Day examined | Albumin microspheres | Casein Microspheres |
| 0 | 0·1 | 1·0 |
| 1 | 0·4 | 5·2 |
| 3 | 0·7 | 7·2 |
| 6 | 1·0 | 7·0 |
| 7 | 1·5 | 15·0 |
| 9 | 1·6 | 15·7 |

Protein microspheres (2–5 mg) were incubated in rat serum (1 mL) at 37°C.

At the times shown microspheres were separated from serum by centrifugation and radioactivity in supernatant (serum) and sediment (microspheres) measured. Serum and microspheres were remixed and reincubated at 37°C until the next time.

(a) % release =

$$\frac{\text{Counts min}^{-1}\text{ in supernatant}}{\text{Counts min}^{-1}\text{ in sediment} + \text{Counts min}^{-1}\text{ in supernatant}} \times 100\%$$

appearance of radiolabel in serum. Table 2 shows that after 9 days incubation 1·6% of radiolabel was released from albumin microspheres and 15·7% from casein.

A further in-vitro experiment was performed to assess the sensitivity of the albumin and casein systems to protease digestion. Microspheres were incubated in a 0·4% solution of trypsin at 37°C and degradation and solubilization of particles recorded as a function of time on a laser diffraction apparatus, using changes in volume concentration. The two different types of microsphere were prepared using identical protein/glutaraldehyde ratios. Fig. 3 shows representative data from a series of experiments and it is clear that the time course of degradation of the two systems is very different. With albumin microspheres a latent period was evident before the particles appeared to 'swell' and degrade. With casein microspheres the process was considerably faster.

## Discussion

In these studies with $^{125}$I-labelled microspheres it was essential that loss of radiolabel from the target organ should be an accurate and reliable measure of microsphere biodegradation and loss from that organ. Thus, we have used a radionuclide that can be covalently bound to protein and also incorporated the radionuclide during the emulsification process, rather than post-label performed microspheres, so that incorporated $^{125}$I should be homogeneously distributed throughout microsphere matrix. That loss of radiolabel from that target organ is indeed a good measure of albumin microsphere biodegradation and loss is supported by the finding of similar half lives using the technique described above and also by monitoring loss of actual microspheres in histological sections (Willmott et al 1985). In addition, for casein microspheres the presence of particles as seen by scanning electron microscopy (Fig. 2A, B) corresponded to biodegradation rate as measured by loss of radiolabel (Fig. 1B).

The salient points to emerge from this study are that (a) embolized protein microspheres biodegrade in lung, liver and kidney, (b) casein microspheres biodegrade in lung and

liver more slowly than albumin systems (half-life for albumin in lung was approximately 2 days and for casein 3·5 days; half-life for albumin in liver was 3·6 days and for casein 6·8 days), (c) albumin microspheres were not innately more sensitive to digestion by protease enzymes than casein.

A technical consideration that may influence the accuracy of these results is the problem of back flow when a volume of material is injected into a restricted space (i.e. artery of a small animal). This resulted in only 39–55% of particles being trapped by the target organ after intra-arterial injection, compared with approaching 100% after intravenous injection. Therefore, all in-vivo results are normalized by expressing them as a percent of the value immediately after injection (nominally set at 1 min). The problem of low target organ entrapment encountered in rats is not anticipated in humans because of the larger diameter of arteries.

Microspheres of the required size for embolization offer considerable scope for altering disposition of cytotoxic drugs and thereby the antitumour effect. In the two therapeutic strategies based on microspheres to be discussed, rate of biodegradation is an important consideration. The simplest approach is to mix particles and drug, and this has received attention for regional chemotherapy of liver metastases. In rats, co-administration of a high dose (100 mg $kg^{-1}$) of degradable starch microspheres via the hepatic artery with radiolabelled 5-fluorouracil resulted in significant, if modest, increases in liver drug concentrations and corresponding decreases in plasma concentrations compared with drug alone (Teder et al 1986). In similar animal systems, increases in doxorubicin and 5-fluorouracil concentrations in tumour deposits in liver were observed after co-administration with degradable starch microspheres (Sigurdson et al 1986; Flowerdew et al 1987). The dose of microspheres injected in these reports was not stated: this is unfortunate because the effect on drug disposition appears dependent on the amount of particles administered (Ensminger et al 1985).

Although microspheres co-administered with cytotoxic drugs can change the disposition of active agent, generally seen as reduced plasma concentrations, the reduction is modest (Teder et al 1986; Ensminger et al 1985) and, indeed, may not always be observed (Goldberg et al 1988). The approach of incorporating drugs within microsphere matrix, either in native form or by attachment to matrix protein, should reduce plasma concentrations of active agents further and is better suited to take advantage of the targeting potential of these systems (Burton et al 1985; Ackerman & Jacobs 1985; Willmott 1987; Goldberg et al 1987). We have shown that administration of doxorubicin in microspherical form reduced plasma drug concentrations, measured within 1h of administration, by 8- to 9-fold compared with drug in solution whilst maintaining concentrations in the target organ (Kerr et al 1988). An interesting feature of this drug delivery system is that a proportion of incorporated doxorubicin is apparently covalently bound to the protein matrix (Chen et al 1988) and therefore only available on biodegradation. So, in this system also, the rate at which particles degrade should be an important determinant of drug exposure and consequent anti-tumour effect.

A less obvious point is the degree and duration of hypoxia induced when microspheres embolize and interrupt blood supply. This point may be significant because a class of cytotoxic anticancer agents, exemplified by the quinone-containing antibiotics mitomycin C and doxorubicin possess the capacity for bioactivation under anaerobic conditions (Moore 1977). Indeed, we have recently shown that in-vivo metabolism by tumour tissue of doxorubicin incorporated into albumin microspheres is via anaerobic bioreduction (Willmott & Cummings 1987).

The sequence of events involved in particle embolization in rodent organs has been recorded (Willmott et al 1985; Fujimoto et al 1985; Schoen et al 1986). Even with inert particles a foreign body reaction occurs, reaching its maximum extent at 48 h. Overlaid on this is tissue damage due to any cytotoxic drug incorporated. With biodegradable systems, evidence for the restoration of homeostasis can be seen within 7 days. As regards toxicity of embolized particles in humans, studies by our group have shown that 300mg of empty albumin microspheres can be administered, to embolize in the liver of patients, without undue toxicity (Goldberg et al 1988) despite the long residence time (expected to be days on the basis of our results) compared with starch microspheres (reported to be minutes) (Wollner et al 1986).

Our in-vitro studies, showing resistance of microspheres to digestion by serum (Table 2) suggest that, unlike starch microspheres, protein microspheres are not degraded by blood components. The invariable occurrence of an inflammatory response to embolized particles suggests that enzymes involved in this event are responsible. Moreover, the differences in biodegradation rate between albumin and casein systems in-vivo (Fig. 1A, B) suggest a degree of enzyme specificity. This is supported by the reversed order of rate of degradation by trypsin in-vitro (Fig. 3).

It is of interest that protein microspheres, when biodegraded in the liver, did not appear to relocate to any marked extent to "downstream" organs such as lung and bone marrow (Table 1). Similarly, subsequent to biodegradation in lung, radiolabel did not relocate to liver or spleen. Thus, the original embolized particles are not eroded to particles of smaller diameter that would localize in organs of the reticuloendothelial systems e.g. liver, spleen and bone marrow. In view of the slightly raised blood radioactivity, corresponding to loss of microspheres from the lung, a more likely explanation is that particles are biodegraded into soluble protein/peptide fragments at the site of embolization.

In summary, we have examined the rates of biodegradation of albumin and casein microspheres in different organs. The casein system exhibited a slower rate of biodegradation than albumin in-vivo (but not in-vitro). The results have significance for efficacy (and toxicity) of drugs either co-administered with or incorporated into microspheres prepared from these proteins.

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