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# The use of albumin microspheres in the treatment of carrageenan-induced inflammation in the rat

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**Abstract**—Free hydrocortisone, hydrocortisone incorporated into microspheres and empty microspheres have been administered orally to rats with carrageenan-induced hindpaw inflammation. Hydrocortisone administered in particles was effective at a lower dose than free steroid in reducing inflammation. Inflammatory exudates were able to release steroid from the microspheres by proteolytic degradation.

Targeting drugs employed in the treatment of inflammatory disease to local sites, e.g. inflamed joints in rheumatoid arthritis, would result in a reduction in the amount of drug necessary to control the disease, with possible additional benefits in decreasing or even eliminating adverse side-effects. Recent work (Alpar et al 1989b) has shown that colloidal particles introduced into the circulation can concentrate in inflammatory exudates. Earlier reports (Volkheimer & Schultz 1968a, b) gave evidence for the transfer of small microparticles from the gastrointestinal tract to the circulation, a process called persorption. In order to test the possibility of a passive targeting system where orally administered particles would reach inflammatory exudates and tissues, we recently (Alpar et al 1989a, b) administered latex UV fluorescent microspheres (1–3 μm diam.) to rats with carrageenan-induced inflammatory air-pouches. We found that particles were rapidly transferred from the gastrointestinal tract to the circulation and that about 0·2% of the particles administered were detected in the exudate and tissues of the air-pouches. The transfer of orally administered latex particles from the gastrointestinal tract to various organs and to the circulation has recently been demonstrated (Jani et al 1990). In this communication we report on the use of orally administered biodegradable microspheres, prepared from bovine serum albumin and hydrocortisone, in the treatment of carrageenan-induced inflammation in the rat.

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## Materials and methods

*Preparation of plain microspheres.* The method employed was based on that of Gallo et al (1984) except that olive oil was substituted for cotton-seed oil. A water in oil emulsion (w/o) was prepared from a mixture of bovine serum albumin (BSA) (250 mg dissolved in 0·5 mL of water) and olive oil (30 mL). The mixture was homogenized for 1 min at 14000 rev $min^{-1}$ in an Omi homogenizer. The emulsion was then added at 40 drops $min^{-1}$ to 100 mL of rapidly stirred olive oil at 125°C. This denatured the albumin which remained in suspension as microspheres. The temperature of the oil bath was maintained with stirring at 125°C for 10 min and then allowed to cool to room temperature (22°C). The olive oil was diluted with ether (60 mL) and the microspheres obtained by centrifugation at 3000 rev $min^{-1}$ for 10 min. The particles were washed with ether and freeze-dried.

*Preparation of microspheres labelled with fluorescein.* The particles were prepared as described above but were suspended in a saturated solution of fluorescein isothiocyanate (FITC) in 1% sodium bicarbonate solution for 1 h. Excess FITC was removed by washing with water and the particles were centrifuged and dried by freeze-drying.

*Preparation of microspheres loaded with steroid.* Two methods were employed. Initially, plain particles, prepared as described above, were allowed to soak for 1 h in a saturated solution of hydrocortisone in methanol. After centrifugation, and washing with water to remove excess steroid, the particles were freeze-dried. However, higher and more uniform loading was obtained by employing a modification of the procedure for preparing microspheres previously described. In this modification the composition of the original emulsion was altered by adding 100 mg of hydrocortisone sodium phosphate and 5 mg of alkaline phosphatase to the original mixture which was pre-cooled to 4°C

to avoid premature enzyme action. After homogenization the mixture was allowed to warm to 30°C for 1 h before microspheres were formed by heat denaturation at 125°C as described previously.

*Examination of microspheres by electron microscopy.* The particles were sized using scanning electron microscopy. In addition, some particles were sectioned after embedding in resin followed by examination using a JEOL 100 electron microscope.

*Determination of hydrocortisone levels.* Hydrocortisone concentrations were determined in particles by refluxing 10 mg of particles with 5 mL of methanol. After centrifugation the methanol supernatant was diluted to 25 mL with methanol and an extra 25 mL of water added. The steroid content was determined by HPLC using a Waters 600 system and a reversed-phase stainless steel column (3·9 mm × 30 cm) packed with Bondapak C18 (Millipore UK Ltd; Waters Chromatography Division, Harrow, Middlesex, UK). The column was eluted with a solution consisting of 0·01% trifluoroacetic acid dissolved in 50% v/v aqueous acetonitrile. Detection was at 254 nm.

*Release of steroid from albumin microspheres in-vitro.* The test fluids consisted of rat plasma, human rheumatoid synovial fluid and a 1% trypsin solution in phosphate buffer (pH 7·4). Ten mg of steroid-containing particles was incubated at 37°C in 5 mL of each test fluid diluted with phosphate buffer to 1:1 v/v. The length of incubation was determined by preliminary experiments which determined the time necessary for complete disintegration of the particles. This was 1 h in the trypsin solution but intact particles were still present in the synovial fluid and plasma suspensions after 24 h. The incubation times were therefore fixed at 1 h for trypsin incubation and 24 h for plasma and synovial fluid. Incubations were performed in triplicate. After incubation the suspensions were diluted with methanol (1:1 v/v) and centrifuged to remove particles and precipitated protein. The clear supernatants were assayed for hydrocortisone by HPLC.

*The oral dosing of particles.* FITC-microspheres were used for tracing the passage of particles from the gastrointestinal tract to other tissues and fluids in the rat. In some experiments, particles ($3·5 \times 10^{10}$) were suspended in water, 10% α-1-proteinase inhibitor (to inhibit trypsin in-vivo) or 1% poloxamer 908 solution, and each suspension (1 mL) dosed separately to the animals. At various times blood samples were taken from the tail vein and counts of FITC-labelled particles were made either by using a haemocytometer, under both UV and visible light, on a Zeiss universal microscope fitted with both deuterium and tungsten lamps or by using the fluorescent activated cell sorter (FACS) facility at the University of Birmingham Medical School. Fluid and tissue samples were also examined under the microscope for the presence of particles.

*Carrageenan-induced inflammation.* The method employed was based on that of Winter et al (1962). Lambda carrageenan in aqueous solution (Sigma Chemical Co, Poole, Dorset, UK) (0·5% m/v; 50 μL) was injected into the left hindpaw of a Wistar strain male rat (90–110 g). The paw volume was measured by an electronic modification (Lewis 1989) of the plethysmographic method of Harris & Spencer (1962) immediately before and 3 h after injection.

Rats were dosed by gavage needle 1 h before the injection of carrageenan. There were five rats in each group. One group (control) was dosed with 1 mL water, another group was dosed with 1 mL of plain microspheres in 1 mL of a hydrocortisone suspension (free steroid group) and a further group was dosed with microspheres containing hydrocortisone 5% suspended in 1 mL of water. The mass of particles administered was adjusted to supply the total steroid load under test. In one experiment plain microspheres were tested for possible anti-inflammatory action via a counter-irritant (Laden et al 1958) action and in another experiment the free steroid was administered i.p.

## Results

*Preparation of microspheres.* The method employed gave 80–95 mg of particles (32–38% yield). When steroid was incorporated 12·1–14·7% was found to be present when using the phosphate ester method and 6–9% when the plain microspheres were incubated in methanol/hydrocortisone solutions. When the amount of phosphate ester incorporated into the emulsion was reduced the amount of steroid found in the microspheres was also reduced (Table 1). The control solutions using hydrocortisone solutions in 50% aqueous methanol over the range 0–1 mg/100 mL gave a linear response when peak heights were measured against concentration. The retention time of hydrocortisone on the column using the experimental conditions described was 3·4 min.

Table 1. Hydrocortisone concentrations in microspheres.

| Amount of hydrocortisone sodium phosphate added to emulsion (mg) | Concn of hydrocortisone in microspheres (mg/10 mg particles) |
|---|---|
| 100 | 1·47 |
| 100 | 1·21 |
| 40 | 0·55 |
| 40 | 0·56 |
| 20 | 0·36 |
| 15 | 0·20 |

*Examination of microspheres by electron microscopy.* The size range of particles formed was 0·5–10 μm with a mean ± s.d. diameter of 3·1 (± 1·7) (Fig. 1A). The particles were spherical but with some surface irregularities (Fig. 1B). The interiors of particles loaded with steroid by enzyme action showed no evidence of a matrix (Fig. 1C).

*Release of hydrocortisone from the microspheres.* When the particles were incubated with trypsin solution the complete disintegration of the particles led to the complete release of steroid (100 ± 5%). After 24 h incubation in rheumatoid synovial fluid 41 ± 6% of the steroid was released and in plasma 0·05 ± 0·03% of the steroid was released. Examination of the particles by electron microscopy after incubation with plasma or rheumatoid synovial fluid showed morphological differences. The particles recovered from synovial fluid appeared spongy in nature. This suggests that the attack of proteolytic enzymes on the exposed surface of the particle is irregular rather than a smooth erosion of the particle into a smaller diameter spheroid. The particles from the plasma solution showed no change in shape or size after incubation.

*The distribution of particles after oral dosing.* The appearance of FITC-labelled microspheres is reported in Table 2. The results show that uptake from particles placed in the stomach by gavage was rapid with particles appearing in rat tail vein samples five min after dosing. Considerable variation between individual animals was found (Table 2). In other experiments (not reported) co-administration with α-1-proteinase inhibitor or poloxamer 908 did not significantly increase either the rate of uptake or the number of particles in circulation over a 60 min period after administration. However, in the absence of polox-

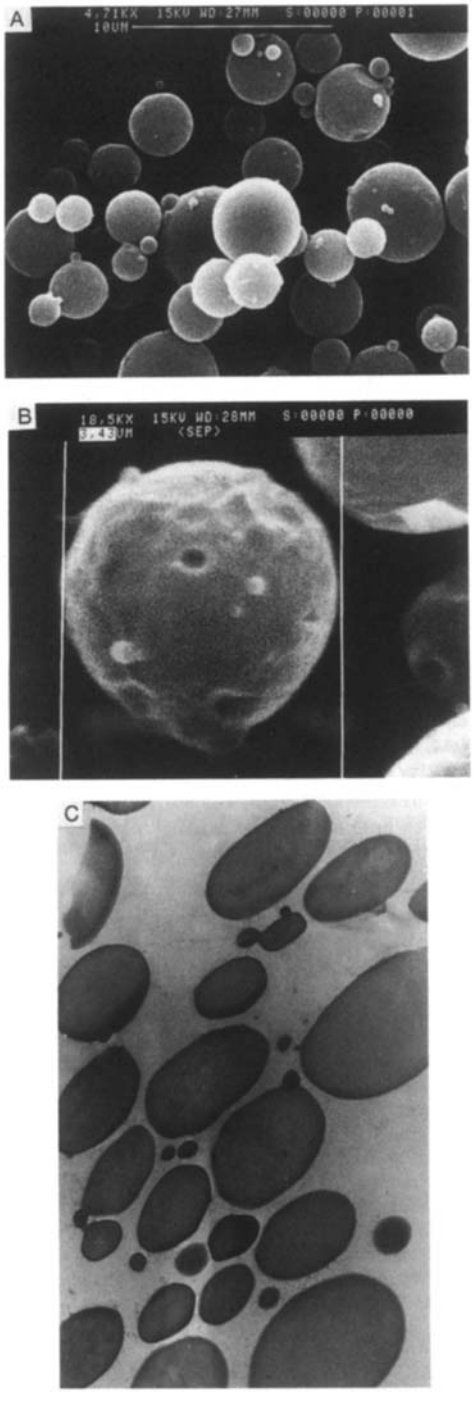


FIG. 1. The plate consists of three photographs of microspheres. A is a general view of microspheres prepared by the heat denaturation method described in the text. B shows surface detail of an individual microsphere. C shows the absence of a matrix in microspheres loaded with steroid by the enzyme hydrolysis of water-soluble steroid phosphates, described in the text.

amer pretreatment no particles could be detected in 24 h samples. Poloxamer pretreatment where 0·5% was added to the microsphere suspension 1 h before dosing resulted in 2% of the particles administered being detected in tail vein samples 6 days after dosing. Rats administered with FITC-labelled particles were killed at various times after the administration of carrageenan and the fluid and tissues from amputated paws examined by UV microscopy. Particles were found to be present in fluids and tissues from 2 h after administration of carrageenan. It was not possible to quantify the number of particles by this method as some particles may have been broken down by local proteolytic action and others embedded too deeply in the tissues to be observed under the microscope. The presence of particles did, however, indicate that the albumin microspheres were able to reach the site of local inflammation from the circulation.

Table 2. Appearance of FITC-labelled albumin microspheres in the circulation (rat tail vein samples) after oral dosing. Particle numbers calculated from FACS data.

| Rat | No. of particles administered ($\times 10^{-10}$) | Body weight (g) | % particles in circulation (min) | | | |
|---|---|---|---|---|---|---|
| | | | 5 | 15 | 60 | 120 |
| 1 | 4·2 | 131 | 7·5 | 7·9 | 6·9 | 5·2 |
| 2 | 3·9 | 121 | 9·8 | 12·2 | 9·4 | 8·0 |
| 3 | 3·8 | 117 | 28·4 | 16·6 | 19·0 | 5·8 |
| 4 | 3·5 | 108 | 16·6 | 14·2 | 12·1 | 12·6 |
| 5 | 3·2 | 100 | 13·0 | 12·3 | 5·9 | — |
| Mean ±s.e.m. | | | 15·0 | 12·6 | 10·7 | 7·9 |
| | | | 3·7 | 1·4 | 2·3 | 1·7 |

*The action of steroid-loaded microspheres on carrageenan-induced inflammation.* In the experiments listed in Table 3 the particles were administered in a water suspension (1 mL). The results show that the oral administration of steroid-loaded microspheres was more effective than free steroid in inhibiting carrageenan-induced paw oedema in the rat. Plain microspheres alone did not have an anti-inflammatory action, which eliminates the possibility of a counter-irritant effect (Laden et al 1958) from the particles.

Table 3. Effect of hydrocortisone-loaded microspheres on carrageenan induced inflammation in the rat (5 rats in each group) $*P < 0·01$ (Student's *t*-test). Plain hydrocortisone administered i.p. 17·5 mg $kg^{-1}$ produced an inhibition of 67 ± 6·2% oedema whereas the same dose incorporated into microspheres and doses orally produced an inhibition of 72·1 ± 25·6% (results mean ± s.e.m.).

| Dose (mg $kg^{-1}$) | Inhibition of inflammation % of control (adjusted to 100%) | | |
|---|---|---|---|
| | Particles alone | Hydrocortisone in particles | Free oral steroid |
| 0·0 | 107 ± 6 | — | — |
| 1·0 | — | 61·0 ± 22·5 | 44·2 ± 10·3 |
| 0·1 | — | 50·0 ± 6·0* | 25·7 ± 6·0 |
| 0·075 | — | 49·5 ± 15·8* | 16·2 ± 16·6 |

## Discussion

The results are similar to those obtained with latex microspheres (Alpar et al 1989a, b) in that the albumin microspheres were rapidly absorbed from the gastrointestinal tract and transported to the circulation. The mode of entry into the circulation is unproven. Particles have also been found in blood by other workers (Florence et al 1990; Jani et al 1990), but these were 50 and 100 nm in diameter. Larger particles are taken up via gut-associated lymphoid tissue (Peyer's patches) (Jani et al 1989a, b; Florence et al 1990) and the distribution of particles of various sizes in body organs after oral administration has recently been reported (Jani et al 1990). We administered particles with a size

range of 500 nm to 10 μm (mean diam. 3 μm) to the rats. An important difference between our experiments and those of Jani et al (1989a, b, 1990) and Florence et al (1990) was that we administered the particles in 1 mL of water. We employed this water overload since the persorption theory concerning the oral administration of particles (Volkheimer & Schultz 1968a,b) suggests that the transient trauma of food or water overload allows the passage of relatively large particles into the circulation. Our previous work (Alpar et al 1989b) using latex particles and an identical dosing technique showed a possible cut-off of particles of about 3 μm diameter appearing in the circulation.

Examination of stomachs removed from animals after water overload showed no macroscopic evidence of damage. In our experiments it is possible that two mechanisms of uptake may be operating: uptake via Peyers patches (Jani et al 1989a, b, 1990; Lefevre et al 1989) and persorption, as suggested by Volkheimer & Schultz (1968a,b). Also the administration of a large volume of water (1 mL) to the stomach might increase the clearance of particles from the stomach to the intestines where uptake occurs. This may explain the rapid appearance of particles in the circulation.

The structure of the particles is also of interest. When the particles were sectioned the interior structure appeared homogeneous which suggested that the steroid was evenly distributed throughout. This may have been due to the novel method of loading via the enzymic hydrolysis of the soluble steroid phosphate ester. The loading of particles by immersing them in organic solutions of the steroids results in crystals of the steroid being deposited in the microsphere when the solvent is removed. Soaking of the particles in solutions of the drugs may result in much of the drug being attached to, or located near, the surface of the particle. Particles loaded by this method show biphasic release of drug; initially about 60% of the drug is released rapidly and the remaining 40% of the drug is released more slowly. The mode of release of drugs from particles has been extensively discussed elsewhere (Widder et al 1979; Willmot et al 1985; Gupta et al 1986). Under high magnification our particles were found to possess irregular surfaces and this increase in surface area could well affect the rate of release of drug and the speed of proteolysis. Preliminary experiments described in the results where particles were incubated with inflammatory proteolytic enzymes suggest that the partially digested particles took on a sponge-like structure which would allow penetration of the enzymes into the interior of the microsphere. Albumin microspheres swell to various degrees depending on the amount of denaturation in aqueous media and swelling would allow easier access of proteases to the interior. Smaller fragments found by electron microscopy suggest that the large sponge-like structures break down into smaller fragments before final disintegration.

Denatured albumin is not highly immunogenic (Lee et al 1981) which probably accounts for the fact that the particles were able to remain in the circulation long enough to enter into fluids and tissues of the rat paw. In addition, plasma is rich in antiproteases which would inhibit any proteolytic breakdown. Poloxamer 908 prolonged the period that particles were in the circulation by inhibiting their uptake by the reticulo-endothelial system (RES).

In conclusion steroid administered in microspheres is more effective than free steroid against carrageenan-induced hindpaw oedema in the rat. What is not clear is the mode of release. It was observed that particles were able to accumulate in inflammatory exudates and tissues. However, based on our previous report where 0·2% of the particles administered orally were located in inflammatory air pouches (Alpar et al 1989a, b) it appears likely that the percentage of dosed particles that reached the site of inflammation would also be around that figure. The majority of the particles in circulation would be removed by the RES and by the Kupffer cells of the liver. This would result in the release of systemic steroid which would be maintained at a steady level in the blood. Therefore we have two possible anti-inflammatory mechanisms, the first being the local release of steroid by local proteolytic action and the second being the steady release of steroid from particles degraded by the RES.

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