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Effect of uptake of albumin microspheres on cellular activity of mouse peritoneal macrophages

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Summary

Empty and drug-containing albumin microspheres (2–5 μm) produced by an emulsion cross-linking process were presented to mouse peritoneal macrophages. Empty microspheres caused a 36.7 and 43.3% release of β-galactosidase and β-glucuronidase, respectively. The inclusion of prednisolone (19 μg/mg), prednisolone succinate (26 μg/mg) and methotrexate (50 μg/mg) caused a reduction in lysosomal release. Similarly the presence of incorporated anti-inflammatory drugs caused a depression in glycolytic activity, illustrated by monitoring levels of $^{14}CO_2$. Both effects were found to be dose dependent. This report demonstrates that the possible toxicity of the albumin matrix can be suppressed by inclusion of an anti-inflammatory drug into the microsphere. Potentially, this system could be administered by the intra-articular route to deliver steroidal drugs to the synovial joint in order to treat rheumatoid arthritis.

Introduction

Albumin microspheres have been proposed as a carrier of anti-inflammatory agents to treat rheumatoid arthritis by the intra-articular route (Ratcliffe et al., 1987). It has been established that the administration of corticosteroids to the synovial joint results in a rapid biexponential clearance from the site of inflammation (Winter et al., 1967) and hence the drug does not exert its full therapeutic effect (Hollander, 1972). Wallis and Simkin (1983) suggested that the initial phase related to intra-articular distribution and the second slower phase reflected true trans-synovial exchange. Thus the incorporation of drugs into a suitable delivery system, such as a particulate carrier, could therefore delay clearance and lead to a beneficial therapeutic effect.

Histopathological studies of the synovium in rheumatoid arthritis have demonstrated the localisation of macrophages in areas of abundant plasma and lymphoid cells within the synovial layers (Zvaifler, 1973). Lysosomal enzyme release by macrophages and their production of mediators (e.g. arachidonic acid metabolites and growth factors), which cause or facilitate tissue degradation, are thought to be important in the pathogenesis of rheumatoid arthritis (Allison, et al., 1975). It has been demonstrated that microspheres composed of starch (Artursson et al., 1989), polylactic

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acid (Smith and Hunneyball, 1986) are phagocytosed in vitro by mouse peritoneal macrophages which exhibit similar phagocytic capacities to those of macrophages in the synovium (Hauser and Vaes, 1978). Hence, mouse peritoneal macrophages have been used as a model in the present studies.

The objective was to investigate the effect of empty and drug-laden particles on non-stimulated macrophage cells by observing lysosomal enzyme release and glycolytic activity.

Materials and Methods

Materials

Bovine serum albumin (BSA) fraction V, purified olive oil, prednisolone, prednisolone 21-succinate sodium and methotrexate were all obtained from Sigma Chemicals, U.K. Glutaraldehyde solution (50% v/v) used as the cross-linking agent was obtained from BDH Chemicals. The substrates for the lysosomal assays – (*p*-nitrophenyl-β-D-glucopyranosiduronic acid and *p*-nitrophenyl-β-D-galactopyranoside) were obtained from BDH Chemicals and Sigma Chemicals, respectively.

Experimental procedure

Resident peritoneal macrophages were obtained from untreated Olac MFI mice (25 g) killed by carbon dioxide asphyxiation. A sample was taken from the pooled cell suspension and diluted in order to obtain a concentration of 1×10^6 cells/ml. The final dilution was checked by counting on a Coulter counter (model TAII). Then 250 μl aliquots of the cells were plated out on Nunclon multiwell plates and incubated at 37°C in 5% carbon dioxide/95% air gas phase for 3 h to allow the formation of homogeneous monolayers. Next, nonadherent cells were removed by washing with phosphate-buffered saline (PBS) and then replaced with fresh medium. The cells were incubated at 37°C for 24 h, the medium removed and the cells washed with PBS as described above and used immediately for further investigation. All cell culture operations were carried out under sterile conditions in a laminar flow cabinet at room temperature. Cultured cells were monitored continuously under the phase-contrast microscope to check viability. Biochemical analysis involving estimations of lactate dehydrogenase levels (Davies et al., 1974) showed no loss in cell viability.

Preparation of albumin microspheres

Albumin microspheres were prepared by the water-in-oil emulsion technique as described by Tomlinson et al. (1982). Glutaraldehyde solution (0.2 ml, 12.5% v/v) was added to crosslink the spheres. The hardened spheres were collected by centrifugation and washed with petroleum ether and ethanol to remove any traces of oil. Particles collected by sieving were sized using a laser diffraction technique (Malvern Particle Sizer 2600, Malvern Instruments, U.K.). The particles had a mean particle size of 2–5 μm.

Incorporation of drug substances

Microspheres were prepared containing prednisolone, prednisolone succinate and methotrexate. The drug substances were incorporated into the albumin solution before dispersion in the external oil phase. The procedure for microsphere formation was then followed as described above. The maxiumum incorporation level achieved for methotrexate, prednisolone, prednisolone succinate were 50, 19 and 26 μg/mg albumin, respectively.

Presentation of microspheres to macrophages

Cells were harvested and cultured in the manner described above. Samples of unloaded particles were suspended in freshly prepared nutrient medium supplemented with crystamycin (1.0 μl/ml), glutamine (100 μg/ml) and 10% heat-inactivated swine serum to obtain concentrations of between 12.5 and 200 μg/ml. Drug loaded particles were suspended in medium to give a concentration of 200 μg/ml. A sample of microsphere suspension (250 μl) was transferred onto sterile Nunclon multiwell plates already containing harvested macrophages (1×10^6 cells/ml). The samples were left in contact with the cells for 24 h before the cell medium and cell lysates were assayed for the presence of lysosomal enzymes. Non-opsonised zymosan (1.5×10^7 cells/ml) was used as a positive control.

Opsonisation of particles

The coating of particles with proteins capable of interacting with macrophage surface receptors is defined as opsonisation (Howard and Wardlow, 1958). A microsphere suspension (2% w/v) was incubated with heat-inactivated swine serum at 37°C for 30 min to allow adsorption of serum proteins. A similar suspension was incubated with mouse anti-rabbit serum antibody to enable specific opsonisation of the microspheres by the anti-rabbit serum antibody. Again the plates were subjected to the enzyme assay described below.

Assays

Lysosomal enzyme assay The medium and cell lysates were assayed for the presence of β-galactosidase (β-GAL) and β-glucuronidase (β-GLU) enzymes. The medium from each well was removed and transferred to the the wells of Dynatech 96-multiwell plates, 24 h after the addition of the appropriate test particulate system. The adherent cells were lysed with 200 μl of a 1% Triton solution (BDH). Media and cell lysates were stored at 0–5°C prior to the assay procedure.

p-Nitrophenyl-β-D-glucopyranosiduronic acid solution (30 μl, 530 μg/ml) was added to the wells of a non-sterile Nunclon plate followed by 20 μl of the appropriate media or cell lysate sample. This was repeated using a solution of *p*-nitrophenyl-β-D-galactopyranoside (30 μl, 800 μg/ml). The plates were incubated in a humid atmosphere (75% relative humidity) at 37°C for 24 h and the reaction terminated by the addition of glycine buffer (200 μl), thereby producing an intense colour. The absorbances of both media and cell lysate incubations were determined at 405 nm using a Multiskan spectrophotometer (Flow Laboratories, U.K.) and used to calculate the percentage enzyme released. The percentage enzyme released in the medium was calculated from $(A_{medium}/(A_{medium} + A_{cell})) \times 100$. A_{cell} values were obtained from cell lysates.

The effect of particles on cell glucose metabolism Mouse peritoneal macrophages were harvested and cultured as described above. Samples of loaded and unloaded microspheres were prepared and 1.0 ml of each sample applied to a well of a sterile multiwell plate containing cultured macrophages. Each well was labelled with D-[1-^{14}C]glucose (25 μl, specific activity 0.5 μCi/ml). The plates were incubated in 5% carbon dioxide/95% air for 3 h. Glass microfibre filters previously soaked in 10% potassium hydroxide solution (75 μl), were placed over the centre of each well of plates removed from the incubator. 1 M sulphuric acid (10 μl) was injected through the filter into the well below. The plates were covered with sealant to prevent loss of $^{14}CO_2$ and incubated for 30 min after which the filters were removed and transferred to scintillation vials. Radioactive carbon dioxide was detected by counting on a scintillation counter (Minimaxi Tricarb 4000, Packard).

Statistical analysis

A statistical *t*-test was carried out on the results obtained using 95% confidence limits with a degree of freedom of 10. A value of $p < 0.05$ indicated a significant difference.

Results and Discussion

The release of both β-galactosidase (β-GAL) and β-glucuronidase (β-GLU) was found to be proportional to the concentration of particles presented to the macrophages (Fig. 1). Zymosan, used as a positive control, caused the release of 66.7 and 74.2% of total available β-GAL and β-GLU, respectively, into the medium. The selective release of acid hydrolases may be due to the glucan

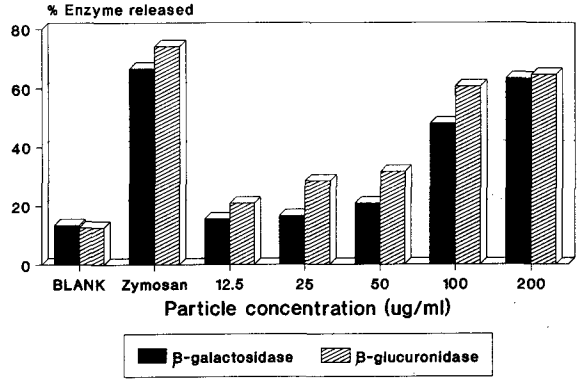

Fig. 1. Enzyme release as a function of microsphere concentration.

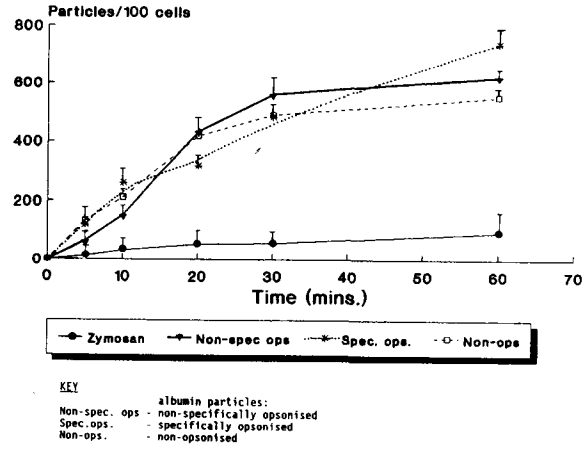

Fig. 2. Phagocytosis of albumin microspheres by macrophages.

composition of zymosan or alternatively due to the generation of cleavage products of complement components (Goldstein et al., 1973)

Phagocytosis of non-opsonised particles by mouse peritoneal macrophages were studied over a 1 h period (Fig. 2). The process of phagocytosis was interpreted as adherence to the cell followed by ingestion and subsequent engulfment as defined by Van Oss (1978). Non-opsonised particles were readily phagocytosed and this was enhanced further by coating with non-specific opsonins from serum and still further by adsorption of specific anti-rabbit serum albumin antibody. The maximum capacity for uptake appeared to be 10–12 particles per cell. In each case the particles rapidly adhered to the cell membrane with actual engulfment occurring within 10 min. The incorporation of drug substances into albumin microspheres did not alter the uptake profile. The initial rate of phagocytosis was maintained for 20 min with a second phase being considerably slower. The uptake of non-opsonised zymosan increased slightly from 20 to 90 particles/100 cells after 60 min yet approx. 550 non-opsonised albumin particles/100 cells were injested during the same time period.

TABLE 1

Release of lysosomal enzymes upon stimulation of macrophages by drug-associated microspheres

Sample	(percentage released into media)	
	β-GAL	β-GLU
Control: Zymosan Particles	72.8 (2.11)	74.2 (4.13)
Drug-free containing: methotrexate (50 μg/mg)	36.7 (4.74)	43.3 (4.83)
	28.6 (5.62)	26.6 (8.58)
Prednisolone succinate (26 μg/mg)	25.9 (5.27)	21.5 (2.19)
Prednisolone (19 μg/mg)	26.3 (3.12)	23.5 (4.02)

Particle concentration = 100 μg/ml. $n = 6$, mean with standard error in parentheses.

Table 1 shows the percentage of total available enzyme release into the media obtained when different samples were presented to non-stimulated peritoneal macrophages. The use of zymosan particles indicated that the cells were sensitive to stimulation with subsequent release of β-galactosidase (72.8 ± 2.11%) and β-glucuronidase (74.2 ± 4.13%). Unloaded microspheres caused a 36.7 and 43.3% release of β-GAL and β-GLU, respectively. It was established that there was a significant difference between enzyme levels from cells stimulated by zymosan and unloaded microspheres (t-test, $p < 0.05$). The incorporation of anti-inflammatory agents into the microspheres caused similar reductions in the amount of β-GAL and β-GLU released into the medium e.g. entrapped methotrexate resulted in release of 28.6 ± 5.62% β-GAL and 26.6 ± 8.58% β-GLU. The levels of enzyme released from the empty microspheres was significantly higher than that released from drug associated spheres (t-test, $p < 0.05$).

The phagocytic process can be divided into two stages:
(i) attachment of a particle to the cell surface and (ii) ingestion of the particle. Ingestion is highly temperature dependent and requires active cellular metabolism (Rabinovitch, 1969). After a phagocytic stimulus, macrophages experience a 'respiratory burst' which is characterised by an increase in oxygen consumption and an increase in glucose metabolism via the hexose monophosphate (HMP) shunt. The HMP shunt involves the activation of a plasma-membrane linked nicotinamide adenine dinucleotide phosphate (Briggs et al., 1975) or NADPH oxidase (Rossi et al., 1975) which con-

verts oxygen to hydrogen peroxide (Johnston and Lehmeyer, 1977). The production of hydrogen peroxide is thought to drive the HMP shunt (Fantone and Ward, 1982). It is the production of toxic oxygen-derived free radicals caused by activators such as opsonised zymosan (Root and Metcalf, 1977), or bacteria (Johnston et al., 1976) that is known to play a crucial role in tissue destruction (Burkhardht et al., 1986). The concurrent release of carbon dioxide is thought to be dependent on the cell and the nature of the stimulus (Artursson et al., 1987), hence the secretion of products of metabolism provides a useful tool in the determination of phagocytic uptake by macrophage cells (Artursson et al., 1986). In the present study, the ingestion of albumin microspheres was followed by monitoring the production of $^{14}CO_2$ from the oxidation of [^{14}C]glucose by macrophage cells. Zymosan, used as a control, induced a significant release of $^{14}CO_2$ from macrophages i.e. 1322.8 ± 147.1 cpm/ml cells. The use of unloaded particles resulted in the production of substantially lower carbon dioxide levels 408.7 ± 31.2 cpm/ml cells (Fig. 3). In comparison, the production of $^{14}CO_2$ by particles containing methotrexate (50 μg/mg), or prednisolone succinate (26 μg/mg) was not significantly different from the blank plates (cells only) (t-test, $p > 0.05$). Examination under the light microscope confirmed that the microspheres were readily ingested and incorporated into the lysosomal apparatus. Therefore, these particles were phagocytosed to the same extent as unloaded microspheres but did not lead to the production of significant levels of carbon dioxide released extracellularly. This implies that the incorporation of these drug substances served to overcome the potentially toxic nature of the microspheres. The results are consistent with those obtained from the lysosomal enzyme assays, where the inclusion of the drug substances caused a substantial reduction in β-GAL and β-GLU release. It was initially suggested that corticosteroids possess the ability to stabilise lysosomal membranes in vivo (Thomas et al., 1973). However, the synthesis and secretion of plasminogen activator (Vassalli et al., 1976), elastase and collagenase (Werb, 1977) and prostaglandins (Flower and Blackwell, 1979) have been shown to be inhibited by glucocorticoids due to the presence of specific surface receptors. Methotrexate possesses both immunosuppressant activity, which is cell-cycle specific, as well as anti-inflammatory properties; yet the actual mechanism is not fully understood (Marhaugh et al., 1980).

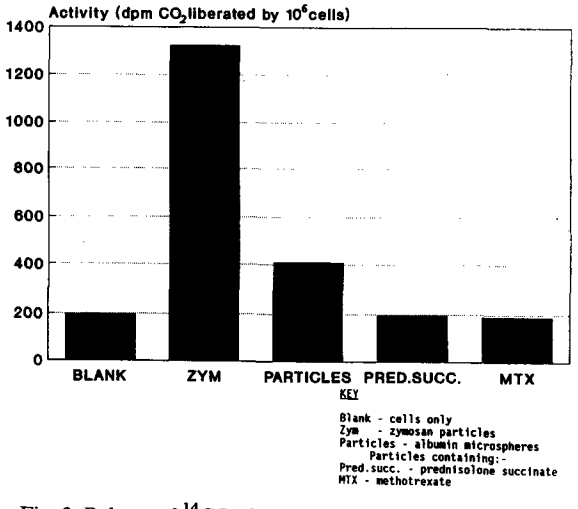

Fig. 3. Release of $^{14}CO_2$ from stimulated macrophage cells.

Conclusion

These experiments demonstrate the uptake of albumin microspheres by mouse peritoneal macrophages. The possible toxic nature of the spheres as evidenced by the observation of lysosomal enzyme release and glycolytic activity could be overcome by the incorporation of anti-inflammatory agents. This system could be used potentially as a means of delivering and retaining anti-inflammatory drugs within the synovial joint.

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