International Journal of Pharmaceutics, 52 (1989) 183–190
Elsevier

IJP 01762

Research Papers

Biochemical and cellular effects of degraded starch microspheres on macrophages

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(Received 10 October 1988)
(Accepted 13 November 1988)

Key words: Microsphere; Macrophage; Phagocytosis; Drug-carrier; Release of inflammatory mediator

Summary

Soluble fragments of starch microspheres, Spherex, having a molecular weight ranging from $< 4 \times 10^4$ to 1×10^6 were obtained by degradation with α-amylase. The effects of the starch fragments on mouse peritoneal macrophages were compared with those of small starch microspheres (mean diameter 1.4 μm). Toxicity, rate of phagocytosis, protein and RNA synthesis, release of arachidonic acid, LTC_4 and PGE_2 were studied after exposure to different doses of the soluble fragments or the microspheres. No negative effect was seen on the number of adherent cells. Microspheres were rapidly phagocytosed whereas the soluble fragments were taken up at a considerably slower rate indicating fluid phase pinocytosis. Protein and RNA synthesis was significantly increased. Arachidonic acid metabolites were released from cells exposed to microspheres whereas the soluble fragments had no effect on the release of these inflammatory mediators. Neither the starch microspheres nor the fragments stimulated the macrophages to express cytostatic or cytocidal activity against the L-929 cells. The results indicate that fragments of epichlorhydrin cross-linked spheres, Spherex, are weakly stimulatory but non-toxic to macrophages.

Introduction

Intra-arterial injection of large degradable starch microspheres with a mean diameter of 45 μm (Spherex), induces an arrest of the blood flow to the receiving organ. The embolization will last until the spheres are degraded by serum α-amylase (Forsberg, 1978). This transient arterial flow reduction in a localized area (organ) means that a co-administered drug could be retained in that area longer resulting in a lower peripheral concentration of the drug and thereby a reduction in possible side-effects. Thus, large starch microspheres and cytotoxic drugs have been utilized in liver cancer chemotherapy (Dakhil et al., 1982; Lindell et al., 1978). Further, arterial microembolization by degradable starch microspheres can be used to produce regional hyperthermia (Akuta et al., 1987).

In order to avoid ischemic damage, the blockage must be reversible and consequently, the spheres must be rapidly degraded in the blood stream. Before complete hydrolysis, the mean diameter of Spherex diminishes gradually (Lindberg et al., 1984). Complete degradation of Spherex by α-amylase in vitro results in soluble polymers or oligomers substituted with glycerol ether moieties (Lindberg et al., 1984). Thus, it is possible that

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different types of fragments, ranging from relatively large particles to low molecular weight metabolites are released in vivo. Fragments with a low molecular weight are excreted by renal filtration while larger fragments are likely to be cleared from the circulation by the macrophages of the reticuloendothelial system.

The effects of such fragments or "metabolites" of the microspheres, on macrophages have so far not been studied. In this study, the effects of starch microspheres with a mean diameter of 1.4 μm and small, soluble metabolites from α-amylase degraded microspheres on isolated macrophages were investigated. The microspheres were chosen as models for the larger fragments of spheres resulting immediately after ending of the embolization.

Materials and Methods

Starch microspheres and soluble fragments of starch spheres

The microspheres (Pharmacia AB, Sweden) consisted of epichlorhydrin-crosslinked starch with a mean diameter of 1.44 ± 0.37 μg (mean $\pm$ S.D.). The in vitro degradation time with α-amylase (240 IE) was 16 h following the method outlined by Lindberg et al. (1984).

The fragments were produced by degradation of Spherex (mean diameter 45 μm) with endotoxin-free α-amylase in vitro under aseptic conditions. The resulting solution was characterized chromatographically on Superose 6 (Pharmacia AB, Sweden). The molecular weight distribution was as follows: 50% of the fragments had a size of $< 10{,}000$, 30% had a molecular weight between 10^4 and 10^5, and the rest between 10^5 and 10^6.

Radiolabelled microspheres and fragments were produced by using ^{14}C-labelled epichlorhydrin. The specific activity was 11.5 kBq/mg and 30 kBq/mg, respectively.

The amount of endotoxin was determined by a *Limulus* Amoebo lysate assay (LAL) and found to be < 0.25 EU/ml.

Cells

Resident peritoneal macrophages were used in all experiments. The cells were obtained from male Balb/c mice (Bomholtsgård, Ry, Denmark; cytostasis and cytotoxicity experiments) and from male NMRI-mice (A-lab, Stockholm, Sweden; all other experiments). The peritoneal lavage from the 8–12-week-old mice generally yielded $1\text{–}2 \times 10^6$ (NMRI-mice) and $2\text{–}4 \times 10^6$ (Balb/c mice) macrophages from each animal (Artursson et al., 1987a). Occasionally, higher yields of macrophages were obtained. Such samples were always discarded since large numbers of macrophages in the peritoneal cavity indicate the presence of elicited or pre-stimulated cells. The macrophages were maintained in RPMI 1640 medium containing 10% heat-inactivated sterile-filtered fetal calf serum, 1% HEPES buffer (1.0 M, pH 7.4), benzylpenicillin (100 U/ml), and streptomycin (10 μg/ml). All tissue culture media were obtained from Gibco Biocult, Paisley, U.K. The mouse fibroblast tumor cell line L-929 (American Type Culture Collection, Rockville, MD, U.S.A.), previously shown to be sensitive to polysaccharide stimulated macrophages (Bögwald et al., 1982), was maintained in the same medium.

The macrophages were suspended to give an optimal concentration of $1\text{–}2 \times 10^6$ cells/ml and seeded in tissue culture wells; 3.5 cm diameter; Nunc, Roskilde, Denmark (phagocytosis and toxicity experiments) and 1.6 cm diameter; Flow laboratories, Irvine, U.K. (all other experiments). After 45 min the cultures were washed in order to remove non-adherent cells. The macrophage cultures were characterized and maintained as described previously (Artursson et al., 1987a). All culture media were free of endotoxin (< 0.2 ng/ml) as revealed with the *Limulus* Amoebo lysate assay.

Exposure of macrophages to fragments and microspheres

Twenty-four hours after seeding, the macrophages were exposed to 0.01–10.0 mg/ml (given in 100 μl PBS) of the test materials for 0.25–4 h (uptake experiments) or 2 h (all other experiments) at 37 °C. Non-ingested and unbound fragments were removed by washing the monolayers 8 times with medium (Artursson et al., 1987a).

Toxicity of fragments and microspheres

The toxicity of the fragments and the microspheres was studied according to a previously reported method (Edman et al., 1984). Briefly, the number of spread adherent cells per culture dish was estimated immediately after the exposure to the fragments or microspheres and then every other day for 5 days. The cells were counted at the center of 10 circles drawn on the bottom of each dish, using an inverted phase contrast microscope and a 32 × objective lens.

Non-degradable microparticles of 1.6-α-D-mannan (mean diameter 1–2 μm; 10 mg/ml) were used as positive controls since these particles have previously been shown to be toxic to macrophages when given in large doses (Artursson et al., 1987a).

Phagocytosis

Macrophages were exposed to 1.0 mg/ml of ^{14}C-labelled fragments or microspheres for up to 4 h. After washing, the monolayers were dissolved in 1% sodium dodecylsulphate solution (SDS; 37 °C; 2 h) and counted in a liquid scintillation counter.

Assays of protein and RNA biosynthesis

Protein and RNA synthesis by the macrophages were determined by following the uptake of [^{3}H]leucine (spec. act. 176.5 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) and [^{3}H]uridine (spec. act. 39.6 Ci/mmol; New England Nuclear, Boston MA, U.S.A.), respectively (Juliano et al., 1985). Twenty-four and 96 h after fragment exposure, the radiolabelled markers were added at a final concentration of 0.5 μCi/ml. After 90 min at 37 °C, the monolayers were washed and 1.0 ml ice-cold 10% trichloroacetic acid (TCA) was added. The cell layers were thoroughly scraped and the insoluble material was collected by centrifugation. The pellet was dissolved in 1% SDS and counted in a liquid scintillation counter. Initially, the precipitates were washed with 1–2 additional portions of TCA. However, since no difference could be observed between values obtained with 1, 2 or 3 washes, the additional washes were omitted. Cycloheximide (final concentrations 10^{-5}–10^{-9} μM) and actinomycin D (final concentrations 10^{-4}–10^{-8} μM) were used as positive controls for the inhibition of protein and RNA biosynthesis, respectively. All results were expressed as % of protein or RNA synthesis as compared with unstimulated control cultures.

Assays of arachidonic acid, PGE_2 and LTC_4

Macrophages were incubated with [^{3}H]arachidonic acid (final concentration 0.5 μCi/ml; spec. act. 240 mCi/mmol; New England Nuclear, Boston, MA, U.S.A.) for 16–24 h according to Scott et al. (1980). The uptake of the radioactive compound by the macrophages was estimated to be 65–80%. After rinsing, the monolayers were incubated with 0.01–1.0 mg/ml of the fragments for 2 h. 0.5 ml of the medium was then withdrawn and the release of radioactivity measured in a liquid scintillation counter. PGE_2 and LTC_4 were determined with commercial RIA-kits according to the manufacturers specifications (New England Nuclear, Boston, MA, U.S.A.). 1,3-β-glucan microparticles (mean diameter 1–2 μm; lichenan; Sigma St. Louis, MI, U.S.A.), previously known to induce the release of arachidonic acid metabolites from macrophages were used as positive controls (Artursson et al., 1987b; Czop and Austen, 1985).

Assay for cytostasis

The target cells (L-929), were added to the macrophage cultures 24 h after exposure to 0.01–1.0 mg/ml of the fragments. The ratio between tumour cells and macrophages was 1 : 20. After 24 or 96 h of co-culture, ^{3}H-labelled thymidine (final concentration 0.5 μCi/ml; spec. act. 25 mCi/mmol; New England Nuclear, Boston, MA, U.S.A.) was added to the cells. After 90 min at 37 °C, the monolayers were dissolved in 1% SDS and the radioactive DNA was collected on nitrocellulose filter papers (Whatman, Kebo AB, Sweden), washed and counted in the scintillation counter. The uptake by macrophages cultured in the absence of tumour cells was insignificant. The cytostatic activity was expressed as a percentage of the radioactivity obtained in non-stimulated control cultures.

Assay for cytolysis

L-929 cells were labelled with [^{3}H]thymidine (final concentration 0.5 μCi/ml) for 16–20 h,

washed and added to macrophage monolayers (macrophage-to-target cell ratio 20 : 1), which had been stimulated with 0.01–1.0 mg/ml of the fragments 24 or 96 h earlier. After 3 days of co-culture, the release of [^{3}H]thymidine in the culture medium was estimated using 0.5 ml of the culture supernatants.

Statistics

All figures are expressed as mean values ± S.D. ($n = 3$–5).

Results

Toxicity of fragments and microspheres

Macrophages were stimulated with 0.01–10.0 mg/ml of fragments or microspheres for 2 h and the number of adherent cells was estimated 5 days later. A decrease in adherence has previously been shown to correlate with macrophage toxicity (Czop and Austen, 1985). In general no decrease in adherence could be observed for exposed macrophages as compared with the unstimulated cells. However, cells exposed to the higher concentrations of fragments or microspheres appeared to be well-stretched and enlarged as compared with the control cells (phase-contrast microscope). When the cells were exposed to a toxic dose, of mannan particles (10.0 mg/ml), the amount of adherent cells was reduced to 4.7 ± 1.0% as compared with controls.

Uptake of microspheres and soluble fragments

The uptake of ^{14}C-labelled fragments and microspheres was studied for up to 4 h (Fig. 1A and B). The uptake of microspheres by the macrophages was greater than that of the soluble fragments. After 2 h when the uptake of the microspheres reached a plateau, 2520 ± 440 ng was taken up by 10^6 cells (Fig. 1B). The corresponding value for the soluble fragments was 40 ± 5 ng/10^6 cells, i.e. 63 times lower (Fig. 1A). The difference in uptake between soluble fragments and microspheres is comparable to the reported difference in uptake between polyacryl starch microspheres and soluble starch (Artursson et al., 1987a). This indicates that the soluble fragments are taken up by fluid phase pinocytosis while the microspheres are internalized via receptor-mediated, saturable phagocytosis.

Protein synthesis by exposed macrophages

Since the rates of protein and RNA synthesis have been used to monitor short-term cytotoxicity of particulate drug carriers on macrophages (Juliano et al., 1985), the effects of fragments and microspheres on these biosynthetic pathways were studied. A dose-dependent increase in protein biosynthesis, measured as incorporation of [^{3}H]leu-

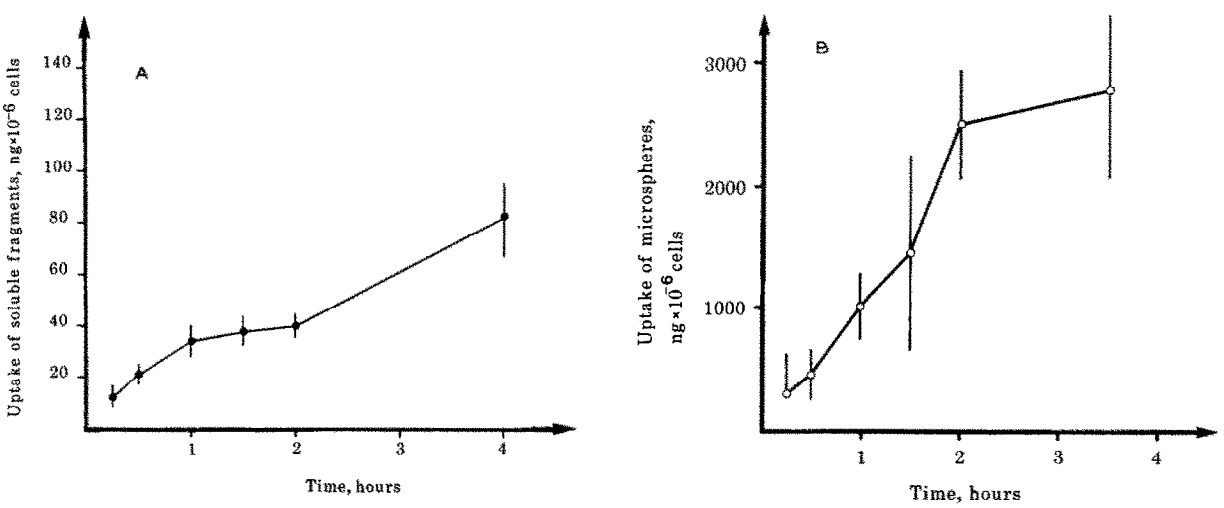

Fig. 1. Uptake of (A) soluble fragments, and (B) microspheres by macrophages.

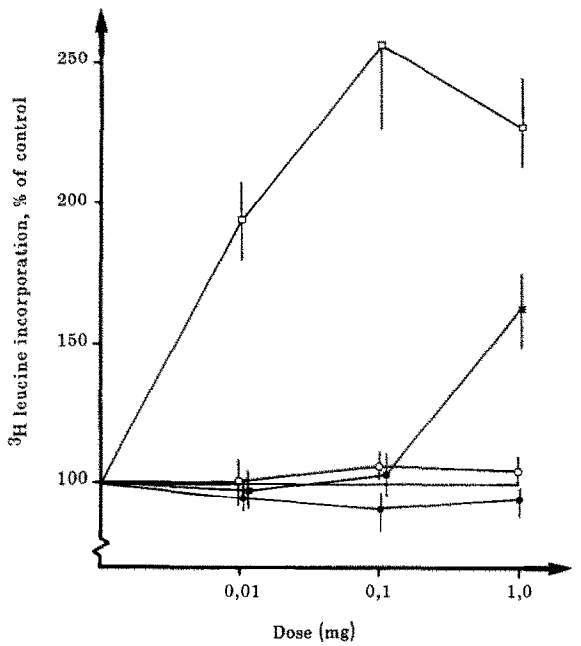

Fig. 2. Protein synthesis in macrophages stimulated with fragments (●, ■) or microspheres (○, □) 24 h (circles) or 96 h (squares) previously.

cine, was found for both fragments and microspheres (Fig. 2). However, the increased protein synthesis was not an immediate response to the uptake.

Thus, no significant difference in [^{3}H]leucine uptake was seen 24 h after the exposure to fragments and microspheres. 96 h after exposure, a significant increase in protein synthesis was observed for all doses of microspheres (0.01–1.0 mg/ml) and for the highest dose of the soluble fragments. The maximal increase in protein synthesis recorded was $257 \pm 31\%$ (0.1 mg microspheres/ml) and $163 \pm 13\%$ (1.0 mg soluble fragments/ml) (fig. 2).

RNA synthesis by exposed macrophages

The effect of the starch fragments and the microspheres on RNA synthesis, measured as the incorporation of ^{3}H-labelled uridine, was also investigated (Fig. 3). In general, the starch fragments and the microspheres were found to stimulate RNA biosynthesis by the macrophages. The results were similar to those found in the protein synthesis assay and demonstrated that: (1) the microspheres were more stimulatory than soluble fragments; and (2) a dose–response relationship existed for both microspheres and fragments. However, macrophages stimulated with microspheres showed an increase in [^{3}H]uridine after 24 h, i.e. at a time when no increase in protein synthesis was detected (Figs. 3A and 2). The stimulation of RNA synthesis became more pronounced 96 h after the exposure. At this time, the uptake was increased from $100 \pm 4\%$ in the control cultures to $191 \pm 13\%$ (1.0 mg/ml of microspheres) and $155 \pm 2\%$ (1.0 mg/ml of soluble fragments). The corresponding values at 24 h were $138 \pm 9\%$ for microspheres and $109 \pm 9\%$ for soluble fragments.

An exception from the overall stimulatory effect of fragments and microspheres on the RNA synthesis was observed when 0.1 mg/ml of soluble fragments were added (Fig. 3A and B). At this concentration, a slight decrease in [^{3}H]uridine uptake was observed.

Release of arachidonic acid, LTC_4 and PGE_2

In order to investigate whether the fragments would induce the release of potentially inflammatory mediators from the macrophages, the release of [^{3}H]arachidonic acid and its two major metabolites, PGE_2 and LTC_4, was assayed (Fig. 4 and Table 1). A clear dose–response relationship could be seen (Fig. 4). The soluble fragments induced a smaller release of arachidonic acid than the microspheres. The maximal release of arachidonic acid from the microsphere stimulated macrophages was $16.4 \pm 2.2\%$. This should be

TABLE 1

Release of PGE_2 and LTC_4 from resident peritoneal macrophages

Macrophages were exposed to 1.0 mg/ml of the various stimuli for 2 h.

Stimulus	PGE_2 ng/10^6 cells	LTC_4 ng/10^6 cells
None	3.9 ± 1.6	0.2 ± 0.2
Soluble fragments	2.5 ± 0.0	0.5 ± 0.2
Microspheres	53.0 ± 12.7	2.4 ± 0.6
1,3-β-glucan	32.0 ± 8.5	5.2 ± 0.4

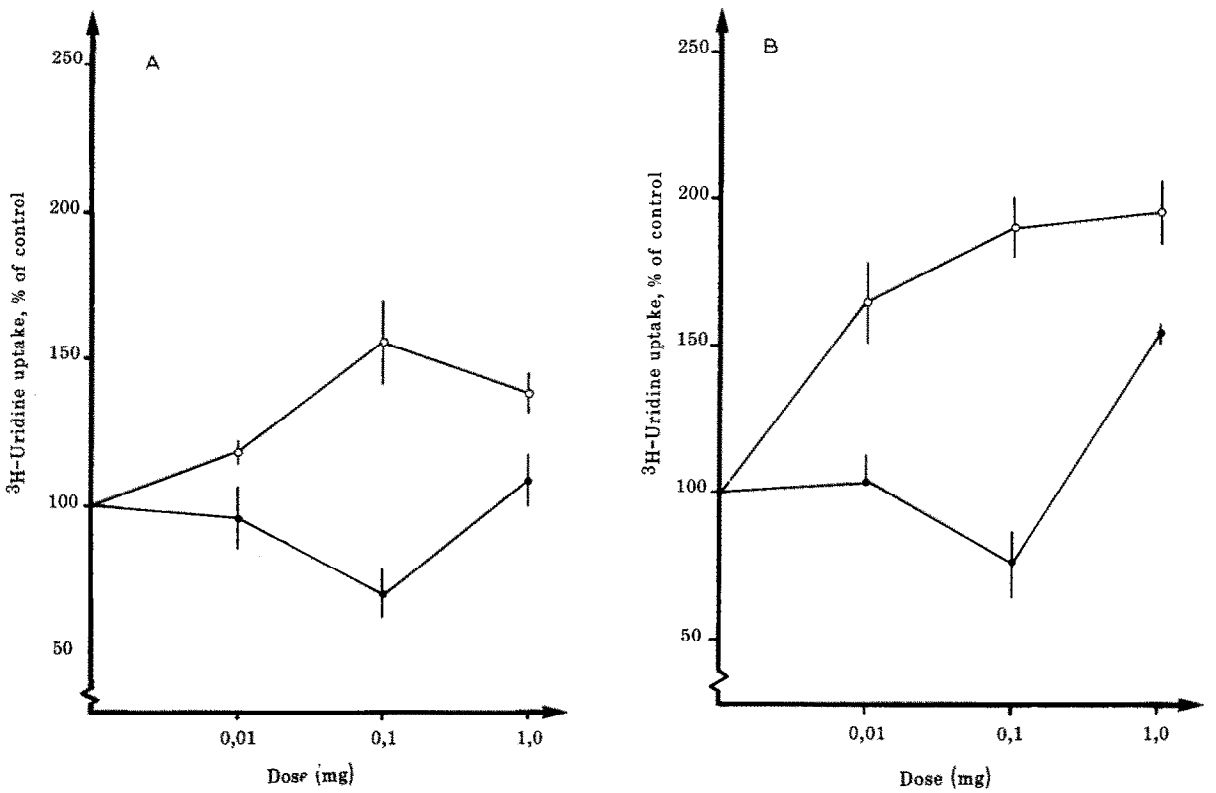

Fig. 3. RNA synthesis by macrophages stimulated with soluble fragments (●) or microspheres (○) (A) 24 h or (B) 96 h previously.

compared with a release of 30.4 ± 3.2% obtained with the inflammatory/ macrophage-activating agent, 1,3-β-glucan. As expected, macrophages stimulated with microspheres also released significant amounts of PGE_2 and LTC_4, whereas no release could be detected for the soluble fragments (Table 1).

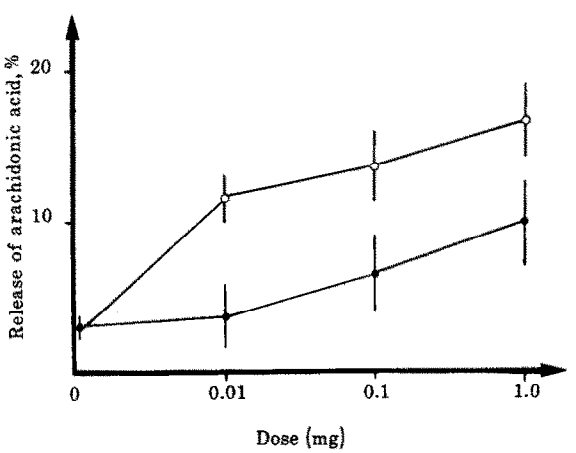

Fig. 4. Release of arachidonic acid from macrophages stimulated with various doses of soluble fragments (●) and microspheres (○).

Effect of stimulated macrophages on tumour cells

Since the increased synthesis of RNA and pro-

TABLE 2

Cytostatic activity of fragment stimulated macrophages

Macrophages were exposed to the fragments for 2 h. The tumor cells were added 24 or 96 h after the exposure to the fragments. The cytostatic activity is expressed as % of that in control cultures exposed to PBS (100 μl; 2 h). Mean ± S.E.M. are given ($n = 3$).

Stimulus (mg/ml)	Soluble fragments		Microspheres	
	24 h	96 h	24 h	96 h
None	100 ± 10	100 ± 14	100 ± 9.8	100 ± 14
0.01	85 ± 16	96 ± 6	99 ± 10	116 ± 21
0.1	77 ± 8	73 ± 6	89 ± 9	100 ± 18
1.0	113 ± 15	93 ± 4	83 ± 11	86 ± 11

teins by the stimulated macrophages, as well as their increased adherence to the culture plates implies macrophage activation, the effect of the macrophages on tumor cells was studied (Table 2). In general, the co-culturing of fragment stimulated macrophages with tumor cells had no effect on the growth kinetics of the latter, as monitored by [^{3}H]thymidine uptake (Table 2). Thus, only macrophages stimulated with 0.1 mg/ml of soluble fragments slightly inhibited the growth of the tumor cells. At 24 and 96 h, the [^{3}H]thymidine uptake was decreased to $77 \pm 8\%$ and $73 \pm 6\%$, respectively. It should be noted that the RNA synthesis was also inhibited at this concentration. No significant cytotoxicity towards L-929 cells was observed. The result indicates that macrophages exposed to soluble fragments of starch microspheres or small starch microspheres were not activated to cytotoxicity, only stimulated.

Discussion

The effects of soluble as well as particulate Spherex metabolites on macrophages were studied. Small cross-linked starch microspheres with a chemical composition identical to that of the large (Spherex) microspheres were used as model compounds for the particulate metabolites. A broad molecular weight range of soluble metabolites were obtained from α-amylase-degraded microspheres.

Both the soluble and the particulate fragments were non-toxic to the macrophages as judged from the number of adherent cells. In addition, no suppression of RNA or protein biosynthesis could be observed upon exposure to the fragments. Relatively small doses of biologically inert microspheres have previously been shown to suppress protein biosynthesis by macrophages (Massaro et al., 1970).

In contrast, the small starch microspheres generally stimulated the macrophages to increase their protein and RNA biosynthesis. This indicated that the intracellular metabolism of the cells was stimulated by the fragments. It was of interest to investigate if these metabolic changes were followed by altered macrophage functions. Therefore, the fragment-stimulated macrophages were assayed for cytostatic and cytocidal activity against tumor cells. The cytotoxic/cytocidal assays were chosen since the cytotoxicity towards tumor cells is considered as the most stringent assay for functional macrophage differentiation (Baggiolini, 1985). However, no significant effects on the tumor cells could be observed. From these results, it could be concluded that the macrophage was stimulated but not fully activated (as measured by the cytotoxic/cytostatic assays) by the fragments. These characteristics are typical for inflammatory macrophages, i.e. macrophages that have been recruited to inflammatory sites (Baggiolini, 1985).

Another characteristic of inflammatory macrophages is their ability to release a large number of secretory products. Among these are inflammatory mediators, such as arachidonic acid metabolites (Scott et al., 1980) and lysosomal enzymes (Baggiolini, 1985). Arachidonic acid metabolites were released when the macrophages were stimulated with the small microspheres. It is interesting to note that the release of prostaglandin (E_2) was relatively high since this compound is known to suppress certain macrophage responses such as tumoricidal activity (Artursson et al., 1987b; Taffet and Russel, 1980). The microspheres were generally more stimulatory than the soluble fragments at a given dose. A plausible explanation can be the relatively high uptake of the spheres as compared with soluble fragments.

The effects observed for the microspheres were similar to those reported previously for polyacryl starch microparticles (Artursson et al., 1987a). Thus, particulate polysaccharides such as starch or dextran, may have inherent macrophage stimulatory properties in vitro. Alternatively, certain structural requirements for stimulation may be fulfilled only in the particulate form (Artursson et al., 1987b).

The findings indicating the Spherex fragments are only weakly stimulatory on macrophages are encouraging. Thus, it should be possible to repeat the administration of Spherex and, in the future, to use Spherex itself as a carrier for covalently linked drugs which are released locally when the matrix is degraded by α-amylase.

Another opportunity is to use the small starch microspheres (mean diameter 1.4 μm), as carriers

for drugs for targeting to the reticuloendothelial system (Edman et al., 1987).

Acknowledgement

We thank Prof. Jan L.E. Ericsson, Department of Pathology University of Uppsala, for placing the cell culture facilities at our disposal.

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