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Host Reaction against Empty Alginate-polylysine Microcapsules. Influence of Preparation Procedure

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Abstract—Microcapsules, prepared with alginate and polylysine, were injected intraperitoneally into mice and the number of peritoneal leucocytes as well as the cells sticking to the capsule wall were counted after 4–28 days. A significant increase in host reaction was observed when the microcapsules contained an outer layer of polylysine as compared with calcium alginate beads without polylysine or microcapsules coated with an outer layer of alginate. The alginate sources influenced the host reaction significantly. After an intraperitoneal residence of 4 days, the microcapsules were mainly surrounded by macrophages. After 28 days, several cell layers surrounded the microcapsules; macrophages, multinucleate giant cells, fibroblasts and mesothelial cells.

Microencapsulation might avoid immune rejection of allograft or xenograft transplants such as islets of Langerhans (Lim & Sun 1980; O'Shea et al 1984; Sun & O'Shea 1985; O'Shea & Sun 1986; Sun 1987), liver cells (Wong & Chang 1986; Sun et al 1986; Cai et al 1988), parathyroid cells (Darquy & Sun 1987), tumour cells (Gorelik et al 1987) or foetal mouse ovaries (Taketo-Hosotani 1987). Successful long-term transplantation in chemically induced diabetic animals, using alginate-polylysine microcapsules, was reported by Sun and coworkers (O'Shea et al 1984; Sun & O'Shea 1985; O'Shea & Sun 1986; Sun 1987). Other groups, using spontaneous diabetic animals as non-obese diabetic mice (NOD) or diabetic BB/W rats, did not observe a restoration of the normoglycaemic blood levels after more than two weeks. The failure of encapsulated transplants was ascribed to fibrosis against the capsule membrane (Ricker et al 1986; Darquy et al 1990; Gin et al 1990; Weber et al 1990; Mazaheri et al 1991). Mazaheri et al (1991) found a survival time in-vivo of encapsulated islets of about 2 weeks. The use of immunosuppressive drugs such as cyclosporin and dexamethasone extended the survival up to three months. A clear correlation between survival time of the encapsulated islets and fibrosis against the microcapsules was demonstrated (Mazaheri et al 1991). Calafiore et al (1987) used superoxide scavengers to prolong the survival of encapsulated islets.

In this study the host reaction against 8 different types of empty microcapsules was investigated after an intraperitoneal residence of 4 to 28 days. The reproducibility of the host reaction against one type of microcapsule was studied.

Materials and Methods

Characterization of the alginates

The molecular weight distribution of both alginates and the ratio guluronic/manuronic acid, were kindly determined by Protan (Drammen, Norway) by intrinsic viscosity and by nuclear magnetic resonance (Grasdalen et al 1981; Grasdalen 1983), respectively. The endotoxin content of both alginate dispersions was determined using the LAL test described in the USP XXII (1990).

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Preparation of the microcapsules

Microcapsules with a diameter of 750 μm were prepared using alginate from two different sources: Manucol DH (Batch 514 851, Kelco, London, UK) and Pronova MVG (Batch 902 282 02, Protan, Drammen, Norway). The purchased Pronova MVG was purified from proteins and was already filtered through a 0·22 μm filter, whereas a set of prefilters with pore sizes from 8 to 0·6 μm (Sartorius, Göttingen, Germany) was required for the Manucol DH. As membrane filtration did not significantly affect the physicochemical properties of the alginates (Vandenbossche & Remon 1993), both dispersions were filtered through a 0·45 μm membrane filter before use.

A flow chart of the production of the microcapsules is shown in Fig. 1. Samples were taken at different steps. Sodium alginate (Manucol DH) 1·0% (w/v) in saline (0·9% (w/v) NaCl) was dropped, via a droplet forming device (Lim 1984), into 30 mL of a 1·3% (w/v) $CaCl_2$ solution. The droplets jellified and formed calcium alginate beads. Calcium alginate beads, prepared from 1·0 mL sodium alginate, were kept for 5 min in the $CaCl_2$ solution. After 2 washes with 30 mL saline (A), 30 mL of a 0·08% (w/v) solution of poly-L-lysine hydrochloride (Sigma mol. wt 39×10^3, St Louis, MO) in saline was poured over the beads. After 2 washes with 30 mL saline (AL), 30 mL of a 0·05% (w/v) sodium alginate dispersion in saline was added. The microcapsules were washed with saline (ALA) and were, once again, placed in a 0·05% (w/v) sodium alginate dispersion. This procedure was repeated 3 (ALA3) and 6 (ALA6) times.

The same production procedure was followed to prepare microcapsules with the Pronova MVG. As indicated on Fig. 1, the microcapsules were finally soaked once (A'LA'), 3

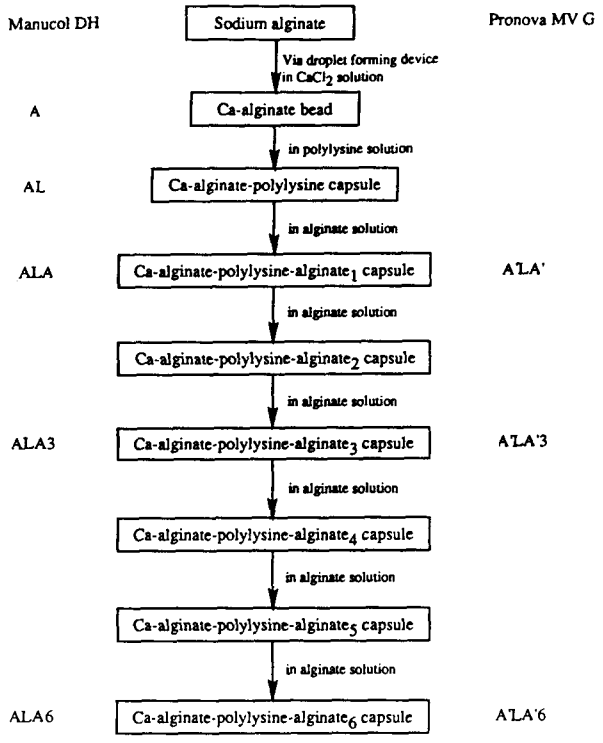

FIG. 1. Flow chart showing the production steps of the microcapsules. Sampling codes are indicated.

(A'LA'3) or 6 (A'LA'6) times in an 0·05% (w/v) alginate dispersion.

Injection of mice

Microcapsules with smooth surfaces and without visible rupture of the wall were selected with a Macroscope (×60, Wild, Heerbrugg, Switzerland). One hundred microcapsules in 0·5 mL saline were transferred to 3 mL syringes (Luer-Lock, Becton Dickinson, Rutherford, NJ).

Groups of 6 male adult mice (C_3H/OuJIco, Iffa Credo, Lyon, France) were injected intraperitoneally with saline or microcapsules by means of a 16 G×2″ catheter needle (Terumo, Tokyo, Japan).

The reproducibility of the host reaction was tested injecting 3 different batches of microcapsules soaked once in Pronova MVG (A'LA').

A group of 15 C_3H mice was injected with a fourth batch of the same (A'LA') microcapsules to evaluate the host reaction over 28 days.

Evaluation of the host reaction

In a first set of experiments, the mice were killed by cervical dislocation 4 days after injection. In a second set of experiments, mice were injected with one batch of capsules and were killed after 4, 7, 14, 21 and 28 days. The abdominal skin was removed and the muscle wall was incised. One millilitre Ringer solution (at 4°C) was injected into the peritoneal cavity and the solution was repeatedly sucked from the peritoneal cavity to obtain a homogenous sample. This procedure was repeated once and the peritoneal lavages were collected.

Cells recovered from the peritoneal cavity

The peritoneal lavages were analysed by means of an automated Haematology Analyzer H*1 (Technicon, Tarrytown, NY). Differential counts on the basis of the volume, nuclear shape, resistance to detergents and peroxidase content were performed. The number of peritoneal leucocytes was expressed per 2 mL peritoneal lavage. Further, the peritoneal lavage was centrifuged and smears were prepared for a May-Grünwald staining. The differential counts obtained by the Haematology Analyzer H*1 were checked by microscopy.

Cells sticking to the capsule wall

Recovered microcapsules were fixed in Bouin-Hollande's solution and embedded in paraffin. For each mouse, two microcapsules were sectioned. Seven-micrometre equatorial sections, stained with haematoxylin-eosin, were photographed and the nuclei of the focused cells sticking to a zone of 250 μm of the capsule wall were counted. Counts were performed on microcapsules with a smooth wall; collapsed capsules with a folded capsule wall were excluded because counting of the cells sticking to a known distance along the capsule wall was technically not possible.

The deparaffinized sections were incubated overnight at 4°C with a polyclonal antibody against cytokeratin (dilution 1:100, cat. PKE, Organon Technika, Turnhout, Belgium) or vimentin (dilution 1:200, cat. PVI, Organon Technika) and further processed in a Midas II (Harleco-Merck, Darmstadt, Germany) automated stainer with the TissuGnost (Uni-pak, Merck) reagent.

Five-micrometer cryosections were stained for acid phosphatase according to the method modified from Anderson & Song (1962).

For analysis by transmission electron microscopy (TEM), microcapsules were fixed for 1·5 h in 2·5% (v/v) glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·4), postfixed for 30 min in 1% (w/v) OsO_4 in cacodylate buffer, dehydrated in an ethanol gradient and embedded in LX-112 (Ladd Research Industries, Burlington, VT). Fifty-nanometre sections were stained for 30 min at 40°C with uranylacetate (LKB, Bromma, Sweden) and for 15 min at 20°C with lead citrate (LKB). The sections were examined with a Joel JEM 100B electron microscope at 80 kV.

Statistics

The values obtained from the cell counting of the peritoneal lavages and of the histological sections (n indicated on Fig. 2) were statistically evaluated using the two-tailed Mann-Whitney U-test (Siegel 1956). The values were considered to be significantly different for $P \leqq 0{\cdot}05$.

Results

Characterization of the alginates

The mol. wt distribution of both alginates, determined by intrinsic viscosity, showed a 180–350 kDa range for Pronova MVG and a 280–360 kDa range for Manucol DH. The ratio guluronic/manuronic acid was 63/37 for Pronova MVG and 29/71 for Manucol DH. The Manucol DH dispersion contained 630 endotoxin units mL^{-1} and the Pronova MVG dispersion contained 108 endotoxin units mL^{-1}.

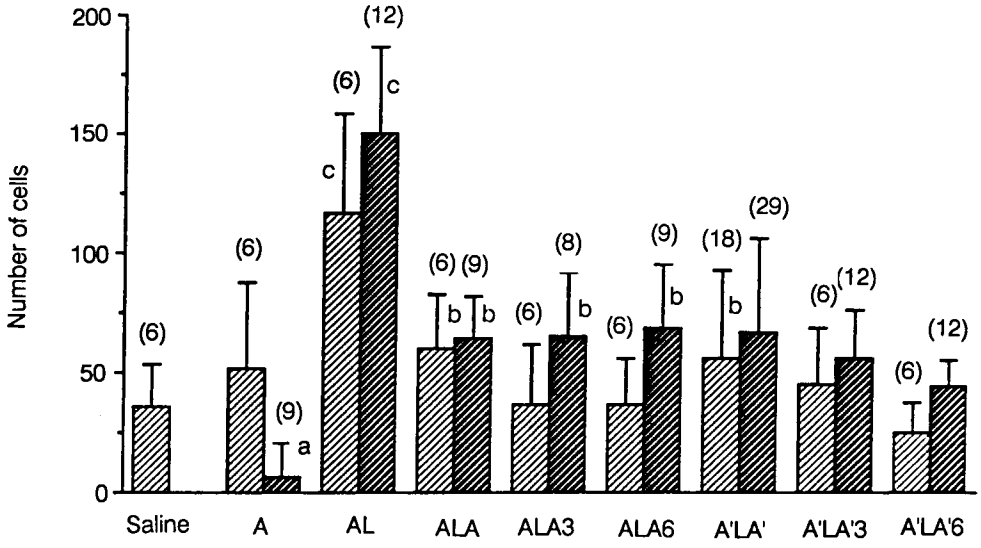

FIG. 2. Host reaction against different types of empty microcapsules or saline, 4 days after injection in C_3H mice. Lightly hatched bars, mean number ($\times 10^5$) of leucocytes (+s.d.) recovered from the peritoneal cavity. The number of mice is indicated in brackets. Darkly hatched bars, mean number of cells (+s.d.) counted on histological sections of recovered microcapsules. The number of microcapsules counted is indicated in brackets. [a]Significantly lower than all other microcapsules. [b]Significantly higher than A'LA'6 microcapsules. [c]Significantly higher than all other microcapsules.

Cells recovered from the peritoneal cavity

A significantly higher number of peritoneal leucocytes was raised against microcapsules with an outer polylysine layer than against calcium alginate beads ($P<0{\cdot}002$) (Fig. 2). Single or repeated incubation of the alginate-polylysine microcapsules in Manucol DH led to a significant decrease in the number of peritoneal cells ($P<0{\cdot}02$). Similar results were obtained also after soaking the alginate-polylysine capsules in Pronova MVG ($P<0{\cdot}04$). The number of peritoneal leucocytes raised against microcapsules incubated 6 times in Pronova MVG was significantly lower than against microcapsules incubated once in Manucol DH or in Pronova MVG ($P=0{\cdot}026$ or 0·05, respectively).

Injection of 3 different batches of microcapsules resulted in a significantly higher number of peritoneal leucocytes against the third batch than against the first and the second ($P=0{\cdot}002$ and 0·026, respectively).

No significant differences were found between the numbers of peritoneal leucocytes, recovered at different time intervals after injection of A'LA' microcapsules (Fig. 3).

The automated differential counts were useful for the discrimination between granulocytes and agranulocytes,

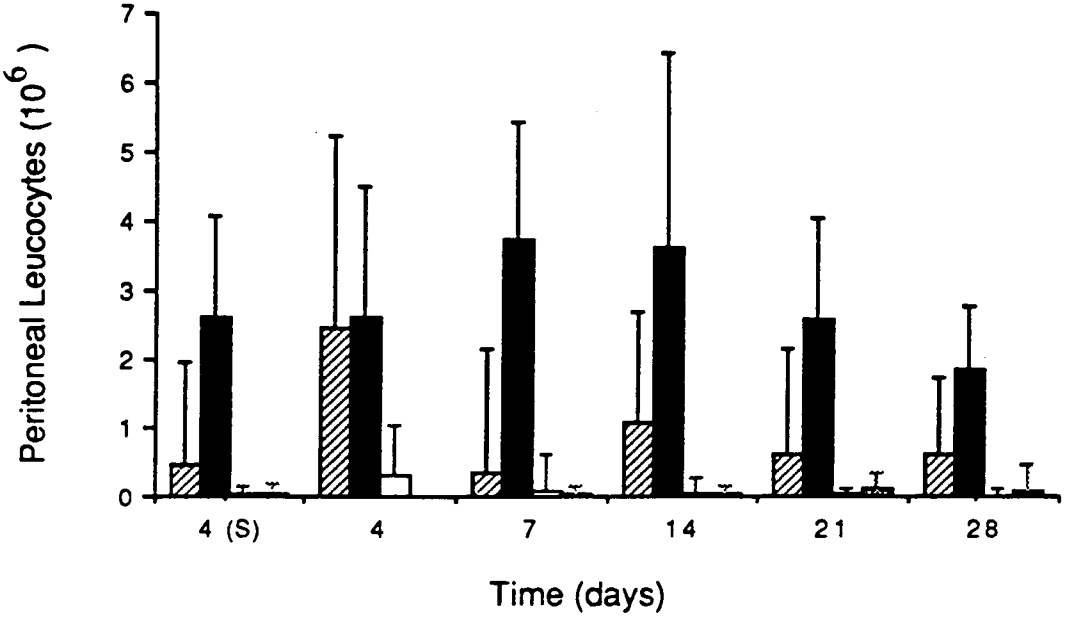

FIG. 3. Differential counts of peritoneal leucocytes, recovered at different time intervals after injection of A'LA' microcapsules or saline (S) in 3 C_3H mice. Hatched bars, lymphocytes; solid bars, macrophages; open bars, PMNs; dotted bars, mast cells.

whereas microscopic evaluation of the smears allowed discrimination between polymorphonuclear cells, mast cells, macrophages and lymphocytes.

Analysis, by the automated method, of the peritoneal lavages obtained after 4 days always showed a majority of agranulocytes. Differential counts of the smears, obtained after 4–28 days are shown in Fig. 3.

Cells sticking to the capsule wall

As for the peritoneal lavage, the number of cells sticking to calcium alginate beads significantly increased by coating with polylysine (AL) ($P<0{\cdot}002$). The number of cells sticking to AL microcapsules significantly decreased by single or repeated soaking in alginate. However, the reaction against alginate-polylysine microcapsules soaked in alginate remained significantly higher than against pure calcium alginate beads ($P<0{\cdot}002$).

A significantly lower number of cells was sticking to microcapsules incubated 6 times in Pronova MVG (A′LA′6) than to microcapsules prepared with Manucol DH ($P<0{\cdot}05$).

As for the peritoneal lavage, a significantly higher number of cells were sticking to the third batch of A′LA′ microcapsules as compared with the first and the second ($P<0{\cdot}002$).

Most of the cells sticking to microcapsules with an outer layer of alginate and recovered after 4 days were macrophages. This was indicated by acid phosphatase staining and ultrastructural characteristics (Rhodin 1963) (Figs 4, 5). Fibroblastic cells that stained for vimentin but not for cytokeratin, were found in foci, except for the AL sections that were surrounded with up to three layers of spindle-shaped fibroblasts. No granulocytes were found, except for sections through AL and the third batch of A′LA′ microcapsules.

Although no significant difference in the total number of cells sticking to the capsule wall was observed as a function of time (Table 1), a change in arrangement of the cells was observed. After 14 days, on top of the layer of macrophages and multinucleate giant cells, a single or double layer of fibroblastic cells surrounded the microcapsules. The fibroblastic cells became more flattened and collagen fibres with characteristic cross-banding were observed in the intercellular space (Fig. 5). The outer cell layer, surrounding most of the microcapsules, consisted of mesothelial cells, staining for vimentin and cytokeratin, showing short microvilli with a central core on TEM, and cell junctions.

Table 1. The mean number of cells (±s.d.) counted on histological sections of A′LA′ microcapsules (n = 6), recovered after intraperitoneal injection to three C_3H mice.

Time after injection (days)	Number of cells
4	57±27
7	99±50
14	77±39
21	46±9
28	61±37

Discussion

The increase in host reaction, observed after coating of calcium alginate beads with polylysine, was ascribed to positive charges of the polylysine. Steadman et al (1990) reported an increase in neutrophil degranulation in-vitro when a negatively charged *E. coli* strain was coated with polylysine. The increase in number of peritoneal leucocytes and cells sticking to AL microcapsules may be explained by degranulation of neutrophils, resulting in the attraction of various kinds of leucocytes (Marchant et al 1983; Anderson & Marchant 1984). Stronger adherence of cells to a positively charged surface may also explain the higher number of cells sticking to the capsule wall. The concommitant increase of cells in the peritoneal lavage makes the latter explanation unlikely.

The significant reduction in host reaction by soaking alginate-polylysine microcapsules in an alginate bath led to the conclusion that alginate restricts the host reaction by shielding the polylysine. This reduction is probably due to a negative surface charge. This is in agreement with observations of Steadman et al (1990), who described a decrease in neutrophil degranulation after treatment of polylysine-coated *E. coli* with heparin. The significantly lower host reaction, observed for microcapsules coated with Pronova MVG as compared with microcapsules prepared with Manucol DH, may be explained by differences in charge density. Because of its steric 1C conformation, guluronic acid has a higher charge density than manuronic acid. Not only the higher guluronic content of Pronova MVG, but also the lower endotoxin content as compared with Manucol DH, both induced a lower interleukin-1 and tumour necrosis factor-α release from the macrophages, and therefore stimu-

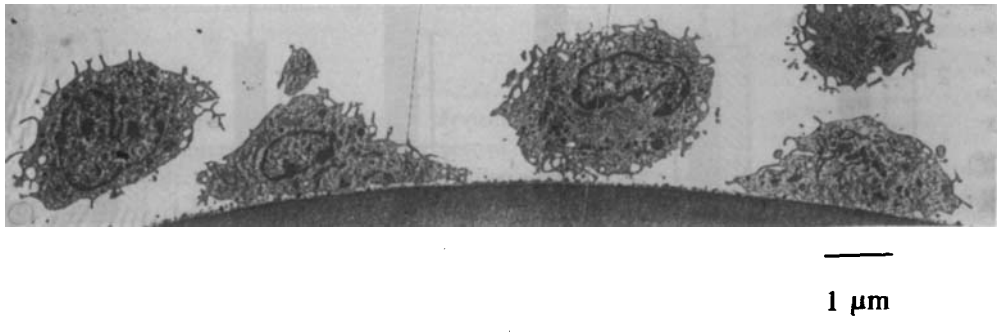

FIG. 4. Transmission electron micrograph of the cells adhering to A′LA′ capsules recovered from the peritoneal cavity of C_3H mice after 4 days, mainly macrophages (Ma) were sticking to the capsule wall (C).

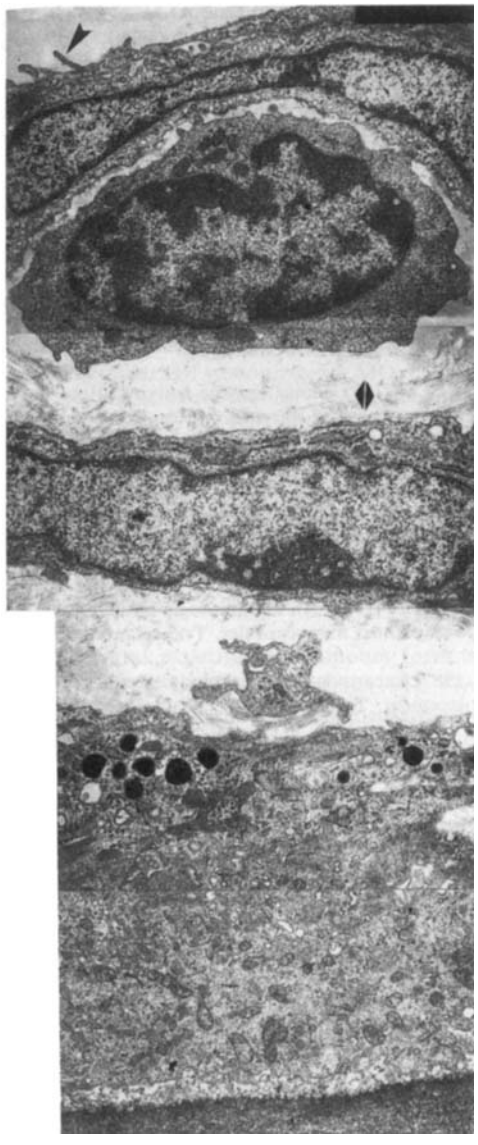

FIG. 5. Transmission electron micrographs of the cells adhering to A′LA′ microcapsules recovered from the peritoneal cavity of C_3H mouse after 28 days. Macrophages (Ma) were sticking to the capsule wall (C), followed by a layer of fibroblastic cells (F) which produced collagen fibres (◀▶) with characteristic cross-bandings. Only a few lymphocytes (L) were found in the sections. Mesothelial cells (Me) were found at the outer margin of the cell layer. The mesothelial cells showed short microvilli (◄), with a central core and were connected by cell junctions.

lated fibroblast proliferation in a less pronounced way (Soon-Shiong et al 1991). Differences in molecular weight of both alginates may also influence their ability to shield the polylysine.

The polylysine layer was probably not completely shielded after a single or repeated soaking in the alginate bath, as the host reaction remained significantly higher as compared with pure calcium alginate beads. Incomplete shielding may also explain variations in the host reaction observed after a single soaking in alginate. A majority of neutrophils was sticking to the AL microcapsules and one batch of A′LA′ microcapsules, whereas on all other microcapsules tested, mainly macrophages and practically no neutrophils were present.

A significantly lower number of peritoneal leucocytes, raised against microcapsules soaked 6 times as compared with once in Pronova MVG, suggested multiple soaking was favourable. However, this was not observed with Manucol DH.

The literature does not provide standard criteria for host reaction to implants (Ratner & Hoffman 1976). Some authors measure the activity of acid and alkaline phosphatase, β-glucuronidase or leucine-amino-peptidase (Salthouse 1976; Burpee et al 1978; Taylor & Gibbons 1983; Marchant et al 1983; Anderson & Marchant 1984) or count the leucocytes at the side of implantation (Taylor & Gibbons 1983; Marchant et al 1983; Anderson & Marchant 1984). Other authors use histology to score for the thickness of the fibrous tissue reaction against the implant (Taylor & Gibbons 1983; Marchant et al 1983; Anderson & Marchant 1984; Anderson et al 1974).

In this study, counts of peritoneal leucocytes and histological examination were used to evaluate the host reaction. There was a positive correlation between the methods. The type of cells counted via both techniques was, however, different. Free floating cells were observed by peritoneal lavage, whereas adherent cells were observed by histology. The presence of fibroblasts and mesothelial cells on the surface of free-floating microcapsules is in contradiction with their absence in the peritoneal lavage. However, the automated analysis of the peritoneal lavage did not allow discrimination between leucocytes and fibroblastic or mesothelial cells, released from the site of injection or from the peritoneal wall. The presence of fibroblasts and mesothelial cells might also result from temporary adherence of the microcapsules to the peritoneal wall.

The concentric delineation of the cell layers surrounding microcapsules, recovered after 28 days, indicate the chronology of the reaction. First leucocytes stick to the capsule wall, followed by fibroblastic cells and finally by mesothelial cells. Further research will be required to study the impact of the surrounding cell layers and collagen fibres on the diffusion of biological molecules through the capsule wall.

Acknowledgements

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