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Host Reaction against Alginate-polylysine Microcapsules Containing Living Cells

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Abstract—Syngeneic and nude mice were injected intraperitoneally with saline, empty microcapsules, aggregates of tumourogenic MO_4 cells, encapsulated non-tumourogenic MO cells and encapsulated MO_4 cells. The host reaction 4 days after injection, was evaluated by counting the leucocytes in the peritoneal cavity and the cells sticking to recovered microcapsules. A significantly lower number of peritoneal leucocytes was found in mice injected with empty microcapsules or encapsulated MO cells as compared with encapsulated MO_4 cells. Histological evaluation showed a significantly higher number of cells sticking to microcapsules containing cells as compared with empty microcapsules. These observations showed that the cellular host reaction against intraperitoneal implants can be evaluated by counting the leucocytes in the peritoneal lavage and histology of recovered capsules.

Spontaneous and experimental tumours form a complex ecosystem with the host (Mareel et al 1991). Two types of interactions can be recognized: an immediate cellular interaction and an interaction by humoral mediation. It is difficult to study those interactions separately during experiments in-vivo. Cellular contact between the tumour and the host cells can be excluded by separating the cells by a membrane. Implantation of cells, isolated in membranes with pore sizes that are permissive or not (cut-off) for humoral factors implies a molecular barrier.

Islets of Langerhans have been protected against immunorejection in diffusion chambers (Theodorou et al 1980; Klomp et al 1983), microcapsules (Sun et al 1982, 1984, 1987; O'Shea et al 1984; Darquy & Reach 1985; Sun & O'Shea 1985a, b; O'Shea & Sun 1986; Goosen 1987) or hollow fibres (Tze et al 1976; Chick et al 1977; Aebischer et al 1986; Altman et al 1986a, b). Microcapsules have been used to produce monoclonal antibodies in-vitro (Jarvis & Grdina 1983; Duff 1985; Posillico 1986), to test antitumour drugs in-vivo (Gorelik et al 1986, 1987) and to study the influence of cellular and humoral interactions with the host on differentiation (Taketo-Hosotani 1987). Microcapsules have been successfully used with different cell types (Lim 1984a, b; Jarvis et al 1986; Sun et al 1986; Goosen 1987) and were described to have variable pore sizes (Goosen et al 1985; Goosen 1987; King et al 1987).

The proposed encapsulation technique, originally described for the encapsulation of islets of Langerhans by Lim & Sun (1980), is based on the formation of a polyionic membrane around a calcium alginate bead containing living cells. We adapted this technique for the encapsulation of tumour cells. The feasibility of the use of microcapsules to investigate the cellular host reaction against encapsulated cells was studied. The host reaction against an intraperitoneal injection of saline, empty microcapsules, non-encapsulated aggregates of MO_4 cells, encapsulated MO or MO_4 cells was evaluated by counts of peritoneal leucocytes and histological examinations of the recovered microcapsules.

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Materials and Methods

Preparation of the microcapsules

Three types of microcapsules were prepared: empty microcapsules, microcapsules containing non-tumourogenic MO cells or tumourogenic MO_4 cells. A low passage of MO cells (fibroblastic cells derived from a foetal C_3H mouse) did not form tumours after intraperitoneal injection into syngeneic C_3H mice (Mareel et al 1975), whereas the MO_4 cell line, derived from MO cells after transformation with Kirsten murine sarcoma virus (Billiau et al 1973), reproducibly formed tumours (Mareel et al 1975). The cells were cultured in 75 cm^2 tissue culture flasks (Falcon, Becton Dickinson, Sumnter, SC) in Eagle minimum essential medium (modified) with Earle's salts and non-essential amino acids (EMEM, Flow Laboratories, Irvine, Scotland) supplemented with 10% (v/v) heat-inactivated foetal bovine serum, 0·05% (w/v) L-glutamine and 250 int. units mL^{-1} penicillin.

Confluent cultures of MO cells (40th passage) or MO_4 cells were treated with Moscona's solution (950 mL distilled water, 8 g NaCl, 0·3 g KCl, 0·05 g NaH_2PO_4, 0·025 g $KH_2PO_4.H_2O$, 1·0 g $NaHCO_3$, 2·0 g dextrose, 1 M HCl to pH 7·2) and with 0·025% (w/v) trypsin 1:250 (Difco, Detroit, MI) in order to detach the cells. Next, the cells were suspended in 5 mL of the Moscona's solution. Sodium alginate (Manucol DH, Kelco, London, UK) and Moscona's solution were added to 4×10^5 cells in order to obtain 8·0 mL of a 1·0% (w/v) dispersion of alginate. The alginate-cell-suspension was dropped in 30 mL of a 1·3% (w/v) $CaCl_2$ solution for jellification. Using a droplet-forming device (Lim 1984b), droplets with a diameter of approximately 750 μm were produced. Calcium alginate beads, prepared from 1·0 mL sodium alginate, were kept for 5 min more in the $CaCl_2$ solution. After 2 washes with 30 mL saline (0·9% (w/v) NaCl), 30 mL of a 0·08% (w/v) solution of poly-L-lysine HCl (mol. wt 39 000 Da, Sigma, St Louis, MO) in saline, was

poured over the calcium alginate beads. After 2 washes with 30 mL saline, a dispersion of 0·05% (w/v) sodium alginate in saline was added. Next the microcapsules were washed 5 times at 4°C with 100 mL Ringer solution in order to remove non-encapsulated cells. The microcapsules were transferred to the culture medium for incubation at 37°C. Free cells and broken microcapsules, sticking to the culture flask, were removed by transferring the supernatant and the free-floating microcapsules to a new culture flask after the 1st and 3rd day. The growth of the encapsulated cells was followed through the transparent capsule wall. Microcapsules were prepared for transmission electron microscopy as described previously (Vandenbossche et al 1993).

Empty microcapsules were prepared according to the same protocol without cells and were kept in the culture medium. The host reaction against empty microcapsules, kept in saline, is described elsewhere (Vandenbossche et al 1993).

Preparation of the aggregates

Aggregates of MO_4 cells were prepared, as described by Mareel et al (1979). The cells were detached with Moscona's solution and trypsin as described above. Cell aggregates were prepared by incubation, at 37°C and in an atmosphere of 5% CO_2–95% air with 7 mL medium with 7×10^5 cells in 50 mL shakers on a Gyrotory shaker (model G10, New Brunswick Scientific, USA) at 70 rev min^{-1}.

Injection of mice

Five days after microencapsulation, the microcapsules were washed twice with Moscona's solution and selected for a smooth surface and an intact wall. Aggregates of MO_4 cells with a diameter of about 0·1 mm were selected with a Macroscope (×60, Wild, Heerbrugg, Switzerland). One hundred microcapsules or aggregates, suspended in 0·5 mL Moscona's solution, were transferred to 3 mL syringes (Luer-Lock, Becton Dickinson, Rutherford, NJ) and were injected intraperitoneally in mice by means of a 16 G × 2″ catheter needle (Terumo, Tokyo, Japan).

We used female 4-week old nude (Swiss-nu, Iffa Credo, Lyon, France) and male 10-week old syngeneic mice (C_3H/OuJIco, Iffa Credo). Groups of 6 C_3H mice were injected intraperitoneally with saline, empty microcapsules, aggregates of non-encapsulated MO_4 cells or encapsulated MO or MO_4 cells. Groups of 6 nude mice were injected intraperitoneally with saline or encapsulated MO_4 cells.

Evaluation of the host-reaction

The mice were killed by cervical dislocation 4 days after intraperitoneal injection. The abdominal skin was removed and the abdominal muscle wall was incised. Next, 1·0 mL Ringer's solution (at 4°C) was injected into the peritoneal cavity and repeatedly sucked with a pasteur pipette. This peritoneal lavage was repeated once.

The peritoneal lavage was analysed as previously described (Vandenbossche et al 1993), using an automated Haematology Analyzer H*1 (Technicon, Tarrytown, NY) and May-Grünwald stained smears.

Free-floating microcapsules were recovered from the peritoneal cavity, fixed in Bouin-Hollande's solution, embedded in paraffin and consecutive, 7 μm thick sections were stained with haematoxylin and eosin. Equatorial sections through 2 microcapsules per mouse were photographed. The nuclei of the focused cells sticking to a zone of 250 μm along the capsule wall were counted. The values obtained by cell counts in the peritoneal lavages ($n = 6$) or on the histological sections ($n = 12$) were statistically evaluated using the two-tailed Mann-Whitney U test. If $P \leqq 0{\cdot}05$, the values were concluded to be significantly different.

As described previously (Vandenbossche et al 1993), the cells sticking to the capsule wall were morphologically identified on haematoxylin-eosin stained sections, and on sections stained for cytokeratin, vimentin or acid phosphatase.

Results

Culture in-vitro

MO_4 cells formed aggregates whereas MO cells remained solitary inside the microcapsules during preculturing (Fig. 1). An electron microscopic picture of an aggregate of MO_4 cells is shown in Fig. 2. The inspected membranes were intact and no intercellular contact via pseudopodia was possible.

Cells recovered from the peritoneal cavity

As shown in Table 1, there was no significant difference in the number of leucocytes recovered from the peritoneal cavity of the C_3H mice after an injection with empty microcapsules compared with saline ($P > 0{\cdot}1$). A significant rise in perito-

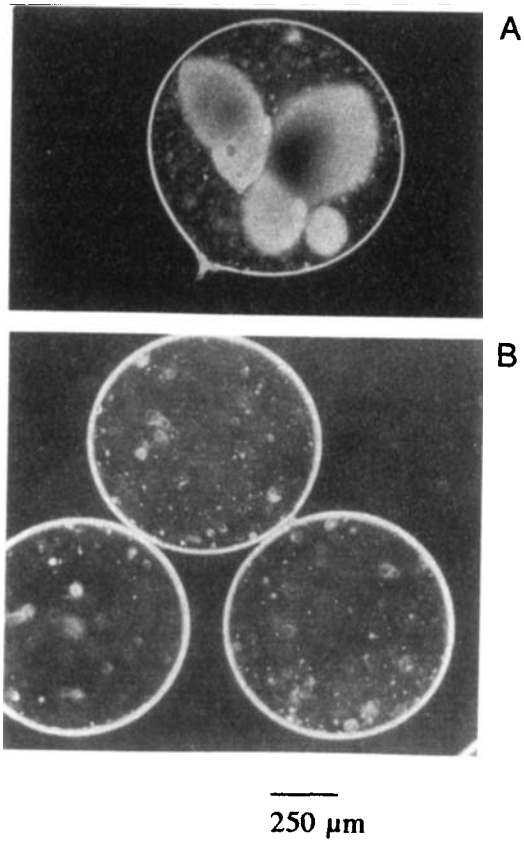

FIG. 1. Phase contrast micrograph of encapsulated individual MO cells (A) and aggregates of (B) MO_4 cells 5 days after encapsulation.

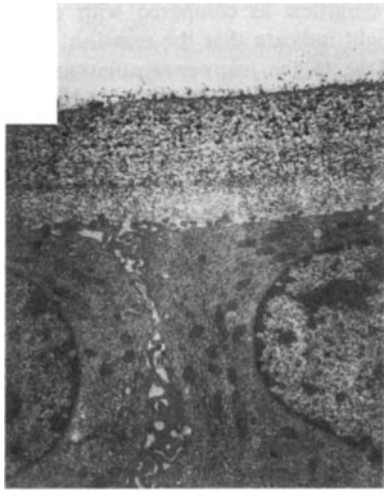

FIG. 2. Transmission electron micrograph of a microcapsule containing MO_4 cells (M). No pseudopodia, crossing the capsule membrane (C) were found after two weeks of culture in-vitro.

Table 1. Mean number of leucocytes (±s.d.) recovered from the peritoneal cavity of 6 syngeneic or nude mice, 4 days after intraperitoneal injection of aggregates of MO_4 cells, empty microcapsules, encapsulated MO or MO_4 cells. The controls were injected with saline.

	Number of cells	
	Syngeneic mice	Nude mice
Empty microcapsules	3·2±2·7	—
Encapsulated MO cells	5·4±3·2	—
Encapsulated MO_4 cells	11·3±2·9[a,b]	13·6±4·6[a]
MO_4 aggregates	5·7±1·5[a]	—
Saline controls	3·6±1·7	2·7±2·2

[a] Significantly higher than for saline. [b] Significantly higher than for empty microcapsules, aggregates of MO_4 cells or encapsulated MO cells.

neal leucocytes was noticed after injection of non-encapsulated aggregates of MO_4 cells ($P<0·05$). The reaction against encapsulated MO_4 cells was significantly higher than against empty microcapsules, encapsulated MO cells and non-encapsulated MO_4 cells ($P<0·002$, $P=0·004$ and $P=0·004$, respectively).

No significant difference in the number of leucocytes recovered from the peritoneal cavity of C_3H compared with nude mice was noticed after a saline injection ($P>0·1$) or after injection of encapsulated MO_4 cells ($P>0·1$).

Cells sticking to the capsule wall

On histological sections of encapsulated MO or MO_4 cells, 3 groups of cells were observed (Fig. 3). The cells close to the capsule wall were morphologically recognized as leucocytes. Most of them had an indented nucleus and were neutrophils. A minority of macrophages, staining for acid phosphatase, was found. Eosinophils and lymphocytes were present in some sections. Further away from the surface, a band of spindle-shaped fibroblastic cells, orientated parallel to the capsule wall, was found. Immunostaining with antivimentin but not with anticytokeratin confirmed the fibroblastic origin of the cells. At the outside of the section activated,

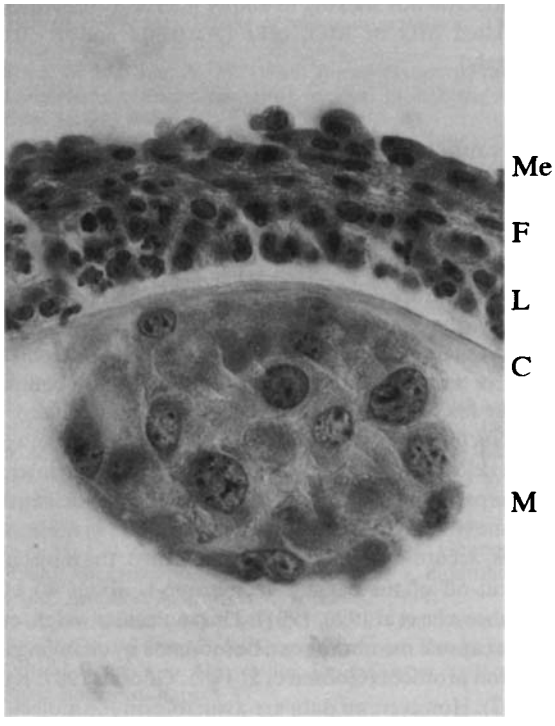

FIG. 3. A micrograph of a histological section through encapsulated MO_4 cells (M) recovered from a C_3H mouse 4 days after intraperitoneal injection. Three different types of host cells sticking to the capsule wall (C) can be distinguished: leucocytes (L), fibroblastic cells (F) and mesothelial cells (Me).

cuboidal mesothelial cells with a prominent nucleus and abundant, sharply demarcated, granular cytoplasm were present. Those cells showed an immunostaining for both vimentin and cytokeratin.

After 4 days the host reaction against empty microcapsules consisted mainly of macrophages. This was indicated by morphology and by a staining for acid phosphatase. Some macrophages were fused and formed multinuculeate giant cells.

As indicated in Table 2, a significant difference in the number of cells sticking to the capsule wall was observed between encapsulated MO_4 cells injected in C_3H mice as compared with nude mice ($P<0·05$). A significantly lower

Table 2. The mean number of cells (±s.d.) counted on histological sections of empty microcapsules, encapsulated MO or MO_4 cells recovered, 4 days after injection, from the peritoneal cavity of 6 syngeneic or nude mice.

	Number of cells	
	Syngeneic mice	Nude mice
Empty microcapsules	43±8	—
Encapsulated MO cells	197±54[a]	—
Encapsulated MO_4 cells	121±79[a]	121±57[a,b]

[a] Significantly higher than for empty microcapsules. [b] Significantly higher than for encapsulated MO_4 cells injected in syngeneic mice.

number of cells was sticking to empty microcapsules than to encapsulated MO or MO_4 cells ($P<0{\cdot}002$ and $P<0{\cdot}02$, respectively).

Discussion

Microencapsulation offers the possibility of evaluating the host reaction against different implants in different hosts. Besides a lower number of peritoneal leucocytes and cells sticking to empty microcapsules as compared with microcapsules containing cells, different types of cells were observed after 4 days intraperitoneal residence. This difference is not caused by the presence of the culture medium, as the reactions against empty capsules, pretreated in culture medium or saline (Vandenbossche et al 1993) are comparable. The increase in host reaction was thus caused by released factors or by direct intercellular interaction between the host cells and the implanted cells. Intercellular interaction via pseudopodia is prevented by an intact capsule membrane (Fig. 2). Secreted factors are limited in molecular weight as, according to data obtained in-vitro, the molecular weight cut-off of the capsule membrane is about 40 kDa (Vandenbossche et al 1990, 1991). This molecular weight cut-off of the capsule membrane can be modified by changing the production protocol (Goosen et al 1985; Goosen 1987; King et al 1987). However, no data are available on the molecular weight cut-off of the capsule membrane when kept in-vivo: lytic enzymes might increase the molecular weight cut-off, whereas cells surrounding the microcapsules might inhibit the diffusion of biological molecules. Although not observed in the present experiments, we cannot exclude rupture of some capsules during injection. This might allow direct intercellular contact between the host cells and the implanted cells (Taketo-Hosotani 1987). The significantly lower reaction against non-encapsulated aggregates of MO_4 cells as compared with encapsulated MO_4 cells provides an argument against a predominant influence of direct cellular interaction. The higher reaction against the encapsulated cells is not caused by the microcapsules alone since no significant difference in the number of peritoneal leucocytes was found between empty capsules and saline. Presumably, a synergistic effect between the tumour cells and the capsule wall led to a higher host reaction.

The higher number of peritoneal leucocytes found after intraperitoneal injection of encapsulated MO_4 cells as compared with encapsulated MO cells, might be caused by the release of molecules from the tumourogenic MO_4 cells. Baumann et al (1984) and Yarden & Weinberg (1989) showed that transformed malignant cells produce cytokines and other signals. Another explanation for the higher response of encapsulated MO_4 cells is the higher number of cells at the moment of injection. In this study equal numbers of cells have been encapsulated, but the MO cells did not proliferate inside the microcapsules due to their inability to grow in calcium alginate. The capability of cells to grow in calcium alginate is related to their tumourogenic phenotype (Kupchik et al 1983). This deficiency could be overcome by the addition of substrates for anchorage-dependent cells, as suggested by Lim (1982). One has to consider that excessive growth of encapsulated tumour cells led to rupture of the capsule membrane after one month of culture in-vitro.

The observation that only a quantitative difference in reaction in syngeneic as compared with nude mice was observed, might indicate that the reaction was not T-cell-dependent. Due to the microencapsulation technique the host is not limited to syngeneic or nude mice. Because of immunoprotection, xenogenic transplantations are possible (Darquy & Reach 1985). It should, however, be noted that the type of host influences the host reaction. Waterfall et al (1990) showed differences in host reaction after injection of similar microcapsules into different strains of rats. Christenson et al (1989) observed differences in host reaction after implantation of polymer graft in mice vs rats. Successful transplantation of islets of Langerhans in streptozotocin-induced diabetic rats has been reported (O'Shea et al 1984; Sun et al 1984; Sun & O'Shea 1985a; O'Shea & Sun 1986). Other papers, using non-obese diabetic rats, reported a failure of the transplant to respond to glucose stimulation after about 2 weeks, probably because of the overgrowth of the capsule membrane (Ricker et al 1986; Gin et al 1990; Mazaheri et al 1991). The latter phenomenon was also described for implant systems consisting of macroencapsulated cells in hollow fibres (Archer et al 1980; Christenson et al 1989) or in diffusion chambers (Theodorou et al 1980; Klomp et al 1983).

In conclusion, we assume that the greater host reaction, as compared with empty capsules, has to be ascribed to soluble factors released by encapsulated cells.

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