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In-vitro cytotoxic activity of cross-linked protein microcapsules

B. DESOIZE*, J. C. JARDILLIER, K. KANOUN, D. GUERIN†, M C. LEVY†

Laboratoire de Biochimie, Faculté de Pharmacie et Institut Jean Godinot, GIBSA (Groupe d'etude des Interactions Biologiques des Substances Anticancéreuses), Reims, and †Laboratoire de Pharmacotechnie UA/CNRS 492, Faculté de Pharmacie, Reims, France

Microcapsules (5–100 μm) were prepared through interfacial cross-linking of various proteins (human serum albumin, lysozyme, haemoglobin, casein, pepsin) with glutaraldehyde or terephthaloylchloride. Surprisingly they all showed an inhibitory effect on cultured cells in a concentration range of 100 μg ml^{-1} to 10 mg ml^{-1}. This effect seemed non-specific, reversible and to depend on contact with the cell plasma membrane. The electric charges of microcapsules could be involved in the inhibition phenomenon.

In previous studies, we reported the preparation of microcapsules through interfacial cross-linking of various proteins or polysaccharides (Rambourg et al 1982; Levy et al 1982; Guerin & Levy 1983; Gourdier et al 1983; Hamard et al 1983).

These microcapsules seemed to be suitable for preparing immobilized derivatives of anticancer drugs through covalent linkage with functional free groups of the wall. Such derivatives have been prepared by several workers to demonstrate a direct cytotoxicity of adriamycin at the membrane surface. Tritton performed covalent attachment to a polymeric material (agarose or cross-linked polyvinylalcohol (Tritton & Yee 1982; Tritton et al 1983); Tökés et al (1984) used polyglutaraldehyde microspheres as a carrier for covalently coupled adriamycin.

Before using cross-linked protein microcapsules as carriers, we studied their own effect on several cell lines in cultures. The first experiments were with lysozyme, cross-linked with terephthaloylchloride or glutaraldehyde. The microcapsules showed a cytotoxic effect in-vitro. As their size (5–100 μm) did not allow internalization, an effect of capsules on the cell membrane was supposed. Some biological properties of lysozyme have been actually related to the multiplication and membrane properties of the mammalian cells (Osserman et al 1973; Asdourian et al 1975; Gordon et al 1979; Keusch & Jacewicz 1977). Furthermore, lysozyme has shown an in-vivo antitumor effect (Gambetti 1967; Choné & Müller 1968; Amato & Ciano 1977) and Osserman et al (1974) considered the enzyme as a natural anticancerous factor.

To our surprise, cross-linking of lysozyme appeared to enhance its in-vitro cytotoxicity more than one hundred times, while it suppressed enzymatic activity.

Assays were then performed with microcapsules prepared from various other proteins: serum-albumin, haemoglobin, casein, pepsin. A cytotoxicity was observed in all cases. These results are now presented.

* Correspondence.

MATERIALS AND METHODS

Materials

Lysozyme from hen egg white (generously provided by Boehringer Ingelheim) and casein (from bovine milk, Cooperation Pharmaceutique Française) were used as solutions in 0·6 M NaOH. The other proteins were dissolved in a buffer (0·45 M Na_2CO_3; HCl to pH 9·8): lyophilized human serum-albumin and haemoglobin (Centre Régional de Transfusion Sanguine, Reims), pepsin (from porcine stomach mucosa, Sigma). The organic solvent was a chloroform–cyclohexane mixture (1 : 4 v/v); it was used to prepare a saturated solution of terephthaloylchloride (Aldrich). The surfactants (Seppic Montanoir) were sorbitan trioleate as a 5% solution v/v in the organic solvent and polysorbate as a 5% solution v/v in 95% ethanol. A 12% etheral solution of glutaraldehyde (v/v) was prepared by partition with the commercial (Aldrich) 25% aqueous solution after saturation with NaCl.

Microencapsulation procedure. (Levy et al 1982; Rambourg et al 1982)

In a 100 ml cylindrical tube, 600 mg of the proteins were dissolved in 3 ml of alkaline aqueous phase, (0·6 M NaOH for lysozyme and casein, buffer for human serum albumin, haemoglobin and pepsin). The organic solution of sorbitan oleate (15 ml) was added and emulsification was obtained after a 3 min

stirring (2000 rev min^{-1}) with a glass anchor (Heidolph Stirrer, stirring motor type RZRII, adaptation system type RK6).

When glutaraldehyde was used as the cross-linking reagent, an etheral solution (1 ml) and 15 ml of the organic solvent were successively added. After a 15 min agitation, the suspension was diluted with 20 ml 95% ethanol, stirred for 5 min and centrifuged (350*g*, 30 s). The sediment was first washed with the ethanolic solution of polysorbate and then with ethanol (twice) and finally with water. After the last centrifugation, microcapsules were resuspended in distilled water and lyophilized. When terephthaloylchloride was used, the saturated solution of acylchloride in the organic solvent (20 ml) was added and stirred for 10 min; 30 ml more solvent was then mixed with the suspension. After a 5 min stirring, the capsules were centrifuged and the sediment was washed as before.

Cell culture

A human erythroleukaemic cell line K562 was cultured in Eagle Dulbecco medium (Gibco) supplemented with 4% artificial serum (Ultroser, IBF, France), antibiotics and sodium bicarbonate. Except where otherwise specified, cells were seeded at a concentration of 50 000 ml^{-1}, and harvested after three days of culture in an incubator at 37 °C, containing 5% CO_2. L1210 cells (a murine lymphoid leukaemia cell line) and human fibroblasts were seeded at lower concentrations, 5000 ml^{-1} and 40 000 ml^{-1}, respectively, in the same medium containing 10% of foetal calf serum instead of artificial serum. The culture volumes varied from 0·2 to 10 ml. At the harvest, the cell concentration was measured and lethality evaluated using a phase contrast microscope, except for fibroblasts which were fixed with glutaraldehyde after harvesting with trypsin and EDTA.

Addition of microcapsules to the cultured cells

Lyophilized microcapsules were weighed, and added to the cultures as sterile suspensions in the culture medium. Control experiments were performed in the same way with Biosilon microcarriers (Nunc; bead diameter = 160–300 μm) and cationic exchange resins (Dowex 50w-8, Biorad, bead diameter 63–150 μm). Anionic exchange resins were used as fibres (DE 52, Whatman fibre diameter = 10–30 μm).

Reversibility assay

After counting, both control and treated cells were centrifuged on 2 ml of a solution of Ficoll-Paque (Pharmacia) for 10 min at 1000*g*. Microcapsules sedimented to the bottom and cells remained at the interface medium/Ficoll-Paque. Cells were removed, centrifuged, and reset in culture at 50 000 ml^{-1} for another three days. For a further reset of culture, the cells were centrifuged once at 400*g* for 10 min, and reset at 50 000 cells ml^{-1} for another three days. In a series of experiments, cells were reset as before for a third period of three days.

Agitated cultures

In a series of experiments cultures were set in 5 ml tubes and bound at the periphery of a slightly tilted rotating disc (6 rev min^{-1}).

K562 differentiation test

Under the influence of an inducer of differentiation, K562 cells synthesize haemoglobin which may be detected by the benzidine test, under the conditions of Jeannesson et al (1984).

Enzymatic evaluation of lysozyme

Enzymatic activity was measured according to Litwack (1955) on a lysate of *Micrococcus lysodeiticus*, with a Kit from Eurobio (Paris, France).

Except where otherwise specified, all experimental cultures were set in tri- or quadruplicate and control cultures in quadruplicate. For statistical study, the one- or two-way analysis of variance was used.

RESULTS

Microcapsules aspect

Lyophilization of the batches yielded 380 to 500 mg dry microcapsules. The powder was white when terephthaloylchloride was used, and coloured for the batches prepared with glutaraldehyde (yellow or orange).

After rehydration in water, all the microcapsules were spherical. They had a thin membrane and their sizes ranged from 5 to 100 μm. Under a phase contrast microscope capsules looked darker than cells (Fig. 1).

Cytotoxic activity of glutaraldehyde cross-linked lysozyme (LG) microscapsules

(1) *Dose related effects*

The cytotoxicity of LG microcapsules was perceptible at 100 μg ml^{-1}; at 1 mg ml^{-1} the number of cell divisions was 0·04/day, which corresponded to a 96% inhibition compared with control cultures where the cell division rate was 1·01/day (Fig. 2). The IC50 (concentration which inhibits 50% of the cell

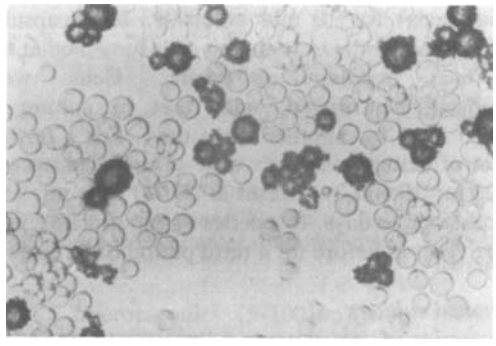

FIG. 1. K562 cells in culture with LG microcapsules. Cells are clear and regular spheres, their mean diameter is 18 μm, microcapsules are darker spheres.

divisions), evaluated with the help of the dose response curve, was 520 μg ml^{-1}.

Lysozyme in solution (non cross-linked) from the same batch was more than 100 times less toxic; at 10 mg ml^{-1} it inhibited cell division by 12% (statistically non significant).

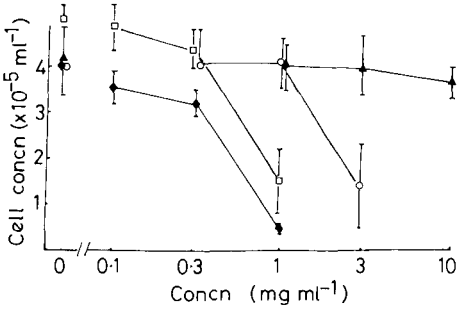

FIG. 2. Dose–response curve of lysozyme (▲), and three different sorts of microcapsules: lysozyme cross-linked with glutaraldehyde (□) or with terephthaloylchloride (◆) and serum-albumin cross-linked with glutaraldehyde (□). Each point is the mean (±s.d.) of 2 to 4 different experiments in triplicate; the LG curve is obtained with 4 different preparations of LG microcapsules.

(2) *Search for a toxic compound released from microcapsules*

Extensive washing of microcapsules was performed 5 times each with 95% ethanol and distilled water. These supplementary washes did not modify the cytotoxicity of LG microcapsules. In another series of experiments LG microcapsules were incubated for three days at 37 °C in a regular culture medium. The supernatant was used to culture K562 cells for three days; it did not affect the cell growth compared with control cultures. This also means that capsules did not adsorb, from the culture medium, molecule(s) essential for cell growth.

(3) *Reversibility*

After a three day culture, the cells were separated from microcapsules, and reset in culture; after another three days incubation, the cells recovered the greater part—if not all—of their growth potential depending on the LG microcapsules concentration during the first period of incubation. Six days after washing, the cells completely recovered their growth potential (Table 1).

Table 1. Inhibition of cell division before and after separation from LG microspheres. Cell mortality is always <10%.

	% of inhibition at:			
		J3 + 3*		
Expt no.	J3*	One wash	Three washes	J3 + 6*
1	70	8		0
2	65	10		
3	75	16	16	
4	23	4	0	

* J3 = day three; cell numerations are done before wash. J3 + 3, J3 + 6; three or six days after wash.

(4) *Time related cytotoxic activity*

LG microcapsules cytotoxicity was uniform during the whole culture time, and the delay seemed short, probably less than 24 h (Fig. 3).

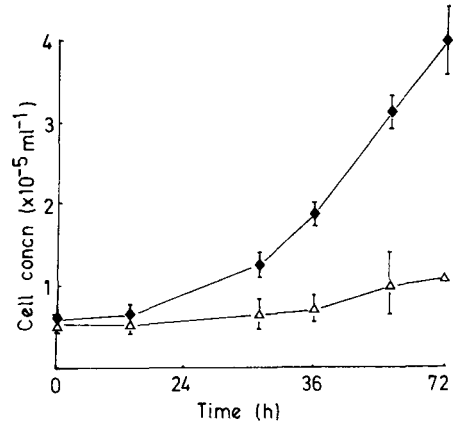

FIG. 3. Cell concentration during three days in control culture (◆) and in experimental cultures containing 1 mg ml^{-1} of LG microcapsules (△). Each point is the mean (±s.d.) of triplicates.

Influence of agitation. Under a gentle agitation LG microcapsules kept their cytotoxic activity; in three different experiments set up in duplicate or triplicate, the mean inhibition rate was 87, 65 and 70%, which corresponds to the toxicity of these LG batches in non-agitated cultures.

Differentiation test

Haemoglobin synthesis by K562 cells was evaluated using the benzidine test after three days of culture with different concentrations of LG. No synthesis of haemoglobin was perceptible (Table 2).

Table 2. Study of a differentiating effect of LG microcapsules with the benzidine test done after three days of culture (triplicate).

Microcapsule concn	Cell concn (±s.d.)	% Dead cells	Benzidine positive cells (%)
0	572 000 ±38 000	2·6	3·2
300 μg ml^{-1}	453 000 ±50 000	6·2	2·9
1 mg ml^{-1}	200 000 ±37 000	4·3	2·0

Cytotoxicity on other cell lines

LG microcapsules were tested on two other cell lines: a non-human leukaemic cell line (L1210 cells), and a human non-cancerous cell line (fibroblasts). The activity of the microcapsules was in the same range of magnitude for L1210 cells and fibroblasts (Table 3).

Table 3. LG microcapsules cytotoxicity on murine L 1210 cells and fibroblasts after three days of culture.

Microcapsules concn	Cell concentration ± s.d. (% dead cells)
L 1210	Cells ml^{-1}
0	872 000 ± 112 000 (5·9)
300 μg ml^{-1}	760 000 ± 39 000 (4·2)
1 mg ml^{-1}	64 000 ± 37 000 (7·2)
Fibroblasts	Cells/culture
0	63 000 ± 8200
300 μg ml^{-1} [a]	16 000 ± 3400
1 mg ml^{-1} [a]	6 750 ± 3300
300 μg ml^{-1} [b]	20 000 ± 3200
1 mg ml^{-1} [b]	11 500 ± 1000

[a] Microcapsules are added 6 h after culture onset.
[b] Microcapsules are added at the culture onset.

Enzymatic activity

No enzymatic activity was found for the experimental microcapsules. A slight lysozymic activity was detected for LG microcapsules prepared from a neutral aqueous solution.

Influence of cross-linking reagent

Cytotoxicity of terephthalolylchloride cross-linked lysozyme microcapsules (LT). They showed an almost identical activity to LG: at 300 μg ml^{-1} the inhibition was 5%, and at 1 mg ml^{-1} 72%; the calculated IC50 was 630 μg ml^{-1} (Fig. 2).

Activity of microcapsules prepared by cross-linking of various proteins

Serum-albumin microcapsules were more toxic when cross-linked with terephthaloylchloride (AT) than with glutaraldehyde (AG); at 1 mg ml^{-1} the inhibition was close to 100% with AT microcapsules, and close to 0% with AG. Their IC50 values were 420 μg ml^{-1} and 2·5 mg ml^{-1}, respectively (Fig. 2). Solutions of serum-albumin were also tested in our cultures; at concentrations up to 10 mg ml^{-1} they did not alter the cell growth (Fig. 2).

Microcapsules were prepared through cross-linking of various proteins, haemoglobin (HT), casein (CT) and pepsin (PT), with terephthaloylchloride and were tested on K562 cells. They were less toxic than LT and AT; their IC50 values were 850 μg ml^{-1} for HT, 2·4 mg ml^{-1} for CT and 10·7 mg ml^{-1} for PT microcapsules.

The cytotoxicity of microcapsules seemed to be related to the isoelectric pH of their constituting proteins (Fig. 4). The correlation coefficient, r, was −0·7705 and $P < 0·05$. If we discarded AT microcapsules which may behave differently, r = −0·9549 and $P < 0·001$. So the higher the isoelectric point, the higher the cytotoxicity.

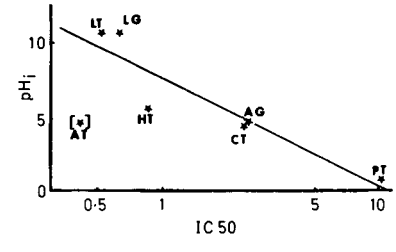

FIG. 4. Correlation between the isoelectric pH (pH_i) of the microcapsules constituting proteins, and the logarithm of the IC50. r: −0·9549 and $P < 0·001$ when AT are excluded, and r: −0·770, $P < 0·05$ when AT are included. Abbreviations are those given in the text above.

In our control experiments, the microcarrier Biosilon had no significant activity on K562 cells even at 50 mg ml^{-1}. At the highest concentration tested, 5 mg ml^{-1}, cationic exchange resins slightly stimulate cell cultures, and anionic exchange resins inhibit 30% of the cell growth.

In all these experiments cell lethality did not exceed 5%, except when the inhibition was higher than 80%.

DISCUSSION

The results show that microcapsules prepared by cross-linking of various proteins exhibit a cytotoxic activity in-vitro both towards leukaemic cells (human or not) and towards non-cancerous human cells. This effect appears to be proportional to the concentration of microcapsules involved. It is not a lethal activity (for an inhibition percentage < 80) but it appears as an inhibitory effect on cell division; this is confirmed by a preliminary experiment which shows an accumulation of the cell cycle at the G2 phase.

Inhibition occurs after a short contact (within 24 h); it is reversible and does not involve differentiation. It is observed even in agitated cultures.

To our knowledge, such a property has never been previously described. However, Oppenheim et al (1984) reported on a slowing of the onset of division of cultured cells (B16 and MMTV cell lines) when adding drug-free serum albumin nanoparticles, although growth, when started, was normal. These submicron particles, prepared by desolvating and glutaraldehyde hardening of the protein, were internalized into tumour cells. The mechanism may then be different.

When larger spheres were used in cell cultures by different workers, in order to study an effect on cell membranes, they were usually made of various non-proteinaceous material, so that the above results apparently are unprecedented.

Many authors have reported on the influence of electric charges of liposomes on the in-vitro effect on cell cultures. Drug-free stearylamine-containing positive liposomes produced an inhibition of in-vitro cell growth of mouse peritoneal macrophages cultures as used by Boots et al (1979) and EMT-6 cells by Dunnick et al (1976), while neutral or negative liposomes were well tolerated by the cells. Moreover, cationic liposomes were reported by Magee & Miller (1972) to attach to cells (ML cells) almost instantaneously. A similar observation was made by Schwendener et al (1984) when studying the liposome-cell association with rat peritoneal macrophages. This particular effect of cationic liposomes is also observed when they are drug-loaded and results then in an enhancement of cytotoxicity. The ID50 of cytosine arabinoside on L1210 cells was 30–40% lower than that of the free drug, while no change in ID50 was observed for negatively charged vesicles (Mayhew et al 1978). Similar conclusions were reported by Hennick et al (1983) with doxorubicin-loaded liposomes. Positive liposomes were the most effective in increasing the life span of CDF1 mice implanted i.p. with P388 leukaemia cells.

While microcapsules cannot be compared with liposomes, since their larger size prevents internalization and their wall cannot be incorporated in cell plasma membrane, the influence of the electric charges cannot be excluded as shown by the results of our control experiments with ion-exchange resins, used in the same concentration range. Positively charged anionic exchange resins inhibited the cell growth by 30%. Replacement of resin fibres by resin beads might have given a different result. On the contrary, cationic exchange resin beads exhibited a stimulating effect on cell cultures. The electric charge of our microcapsules was not measured but, when dispersed in the buffered culture medium (pH 7·4), all microcapsules prepared from a protein with an isoelectric pH $< 7{\cdot}4$ (serum-albumin, haemoglobin, casein, pepsin) were obviously negatively charged. Blockade of the amino groups by cross-linking results in lowering the isoelectric pH and thereby increases the negative charges of the capsules. Tomlinson et al (1984) have demonstrated that microspheres prepared by glutaraldehyde cross-linking of serum-albumin are negatively charged.

In the case of lysozyme, the effect of cross-linking on the electric charge of microcapsules is less predictable, due to the very high isoelectric pH of the protein. Most of the microcapsules discussed here, especially AT capsules, which are the most toxic, are negatively charged. Their cytotoxic effect then appears not to be consistent with the results obtained from the cationic exchange resin beads at the same concentrations. These beads, which are supposed to bear numerous negative charges, exhibit a stimulating effect on cell cultures. Determination of zeta potential of the microcapsules will be necessary for better understanding. It would perhaps afford an explanation for the apparent correlation (Fig. 4) existing between the isoelectric pH of the microcapsule constitutive protein and logarithm of the IC50 (with the exception of albumin).

Because of the high sensitivity of cell cultures, other factors may be also involved in the toxicity. Some of them could be excluded.

The activity does not seem to be related to a problem of space requirement for cell division on the bottom of the culture flasks. Biosilon microcarriers do not inhibit cell growth. Moreover, the cytotoxicity is not modified by agitation.

Microcapsules did not adsorb molecules essential for cell growth from the culture medium. After incubation of the microcapsules in the culture medium, the supernatant allowed normal cell growth.

Release of toxic substances in the culture medium also seems to be excluded by this experiment. Moreover extensive washing of microcapsules did not decrease their activity.

In any case, the phenomenom obviously proceeds through a non-specific mechanism as it is equally observed with various proteins and cross-linking agents. It could result in discrete cell membrane alterations, which would then induce cytotoxicity.

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