JOURNAL OF MATERIAL SCIENCE: MATERIALS IN MEDICINE 3 (1992) 10–13

Cytotoxic effects of some inflammatory mediators on microencapsulated cells*

Ch. CADIC, B. DUPUY
INSERM U. 306 Université de Bordeaux II, 146, rue Léo-Saignat, 33076 Bordeaux Cedex, France

S. VITIELLO
INSERM U. 259 Domaine de Carreire 1, rue Camille Saint Saëns, 33077 Bordeaux Cedex, France

One of the implantation problems in immunoprotected living cells is the appearance of local inflammatory phenomena around microcapsules. Some of the mediators released in such pathophysiological conditions were tested. A toxic action of compounds such as elastase, collagenase, free radicals and lysozyme was evidenced. Interleukins 1 and 2 revealed no cytotoxicity within the test limits on the experimental cellular model chosen. These results underline the importance of inflammatory mediators released by adjacent, or even resident, cells in the implant.

1. Introduction

The implantation of living and functional cells in microcapsules has in recent years represented an attractive alternative in the treatment of some human diseases. The bioartificial pancreas has been proposed for the treatment of insulin-dependent diabetes. Microcapsules have been developed with various methods and materials, including biopolymers [1–4]. Hybrid functional units have been tested *in vitro* with varying success. The fundamental properties of these entities are based on two characteristics: the absence of material toxicity and a controlled porosity of the capsular membrane. The aim here is to avoid the fatal contact between the antibody and the implanted cells. Nevertheless, whatever capsular structure is used and despite immunoprotection, many *in vivo* studies have shown that grafted cells do not survive, or that they lose some of their functional characteristics after a period of time [5, 6]. These observations led us to examine some immunological phenomena, which might intervene in this cell destruction, in particular inflammatory local phenomena, which are of great importance regarding the dual functions of immune surveillance and responsiveness in the direct vicinity of such implants.

These experiments were designed to test some well-known soluble mediators which are permeable to the capsule wall. Our results indicate that embedded natural killer sensitive cultured tumour YAC-1 cell line are effectively protected from cell–cell mediated lysis. Proteolytic enzymes killed these embedded cells when pathophysiological concentrations were used. Moreover, the different soluble cell products tested, which are released and are present in the fluid isolated from an inflammatory site and which are endowed with a potential cytotoxic effect upon encapsulated cells, provided mixed results.

2. Materials and methods

2.1. Reagents

Natural killer (NK) cells were obtained from rat spleen according to Heberman's technique [7].

Supernatant of spleen cell culture was obtained by stimulating 1.5×10^6 cells ml^{-1} of RPMI 1640 (Gibco) with 2 $\mu g\ ml^{-1}$ Concanavalin A for 24 h and Il-2 level in such supernatant was determined according to the technique of Gillis *et al.* [8].

Human recombinant Il-1-β was obtained from Genzyme. One nanogram protein correspond to 100 U Il-1 according to the manufacturer's specifications.

Collagenase from Clostridium histolyticum, lysozyme and riboflavin were supplied by Boehringer, Gibco, and Sigma, respectively.

Human pancreatic elastase was used. The enzyme was purified in our laboratory by the procedure of Largman *et al.* [9] and used after 30 min incubation with trypsin.

2.2. Cell lines, agarose embedding and cytotoxicity assays

The different inflammatory products studied were assayed by using a ^{51}Cr-release test with the following procedure. Target cells were U 937 (a human myelomonocyte cell line) and YAC-1 (a Moloney leukemia virus-induced-murine-lymphoma) cell lines. They were labelled by incubating 6×10^6 cells with 200 μCi

* This paper was presented at the BIOMAT 90 Conference.

0022–2461/92 $03.00 + .12

Sodium-^{51}Cr-chromate overnight at 37 °C in humidified 5% CO_2 atmosphere. After washing the labelled cells three times, the cells were divided in two parts and centrifuged. One part acted as a reference and was tested without any modification; the second half of the cells was embedded in agarose beads according to a technique previously described [10]. Target-labelled cells were dispersed in a 1.5% agarose solution (type IX, gelification $t < 15$ °C, Sigma). The agarose microspheres were obtained by extrusion of these mixtures through a needle into the inner tube of a coaxial polyethylene capillary, into which paraffin oil (Isopar M, Exxon Chemical) was pumped through the outer tube. Lowering the temperature produced perfectly spherical and homogeneous microbeads, which were collected in the bottom of a cool receiving flask. Then, the cells, either embedded or not in agarose, were placed in plastic tubes (about 1.5×10^5 cells in each tube). Cell culture supernatant, cytokine or other reagents were then added in different concentrations to each tube and incubated for 4 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. All the samples were performed in triplicate. The tubes were then centrifuged and the amount of ^{51}Cr released into the supernatant was measured. Spontaneous release (target cells in medium alone) was less than 30% of maximum release in all experiments. Total release was obtained by incubating labelled target cells with HCl 1 M. All reagent activities were expressed as the percentage cytotoxicity as follows:

$$\% \text{ cytotoxicity} = \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100.$$

3. Results and discusssion

Natural killer cells: Target (T) YAC-1 cells were mixed with splenic effector (E) cells at E/T ratios of 200:1, 100:1, 50:1, 25:1. The NK cells, which require a direct contact with tumour target cells to be lysed, showed no cytolytic action on agarose-embedded YAC-1 labelled cells (Fig. 1). The percentage of specific ^{51}Cr-release, i.e. the number of cells killed during the 4 h test, was equal to spontaneous ^{51}Cr-release, whatever the E/T. The polymer obstructed cell penetration. This classical NK cell-mediated cytotoxicity was not MHC-restricted. The cells had a cytotoxic action irrespective of the presence of the antibody, and seemed to secrete no substance capable of lysing these tumour target cells.

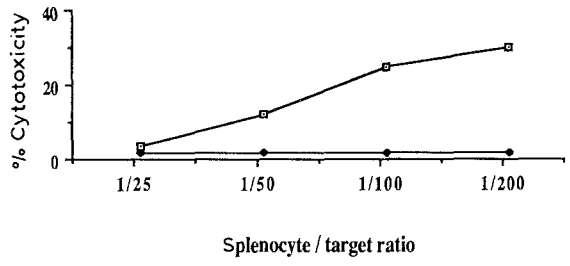

Figure 1 Four-hour natural killer cell activity on cells embedded in agarose microbeads (–●–), or on free cells (–□–).

Culture supernatants from mitogen-stimulated rat spleen cells mainly contain molecules synthesized and released by the T-helper lymphocytes. When such cultures are stimulated by mitogen Concanavalin A (Con A), supernatants are tested, routinely used and titrated as a typical growth factor assay in the proliferation of an Interleukin 2-dependent cytotoxic T-cell line [8]. This medium obtained with Con A was used at different concentrations, the first being similar to those found when lymphocytes are stimulated *in vivo* during a pathological state. These supernatants (pure or diluted) did not show any toxic effect on the cell lines tested either on the embedded cell line or on the free cell suspension (data not shown).

Interleukin-1β (Il-1β) is a cytokine which plays an important role in many physiological responses. It is produced by many cell types, particularly by macrophages and monocytes. Il-1 affects a wide range of target tissues and is thought to be an important mediator of the inflammatory response by acting as a growth factor for fibroblasts, by inducing the release of prostaglandin E_2, by stimulating the secretion of proteases and new cytokines, by inhibiting some metabolic functions and by generating free radicals [11]. Such are a few of the direct or indirect multiple actions of this polypeptide. Il-1 was tested at concentrations varying between 20 ng ml^{-1} and 2 μg ml^{-1} of medium. Some authors have clearly shown that Il-1 concentrations greater than 1 ng ml^{-1} inhibit insulin secretion, and even account for severe cell injuries within pancreatic islets [12, 13]. On our cell model no significant direct cytotoxicity was observed when the Il-1 was used at such concentrations (data not shown).

The lysozyme is a βglucosidase detected early at an abnormal rate in response to a non-specific inflammatory stimulus, i.e. non-antigen specific [14]. *In vivo* assays are performed during the inflammatory phase and correspond to the minimal concentrations tested in our experiment (5 μg ml^{-1}). This enzyme is released by degranulation of various cells (polynuclears, macrophages, etc.). We found only a limited toxicity on the cells used, and only at relatively high lysozyme concentrations. At 5 mg of lysozyme per ml, the cytotoxicity rate was about 5% (data not shown).

Elastase and collagenase are two proteases in the set of proteolytic enzymes which intervene systematically in the presence of an inflammatory phenomenon. Concentrations of 500 μg ml^{-1} to 100 mg ml^{-1} for collagenase and of 1.89 μg ml^{-1} to 70 μg ml^{-1} for elastase were used in this study. Minimal values of these concentrations took into account the usually enzymatic content of secreting cells and the specific activity of our proteases (especially collagenase) [15]. These enzymes can kill cells swiftly and at a high rate (Figs 2 and 3). Agarose in this case has a slight protective action towards elastase (Fig. 3).

Our latest test is an assessment of the toxic power of free radicals. These chemical compounds are widely encountered mediators in the multiple stages and reactions of cellular life [16]. The cells which intervene in the inflammatory process are sensitized: they produce superoxide anions and other oxidizing species. These species are the consequences of respiratory

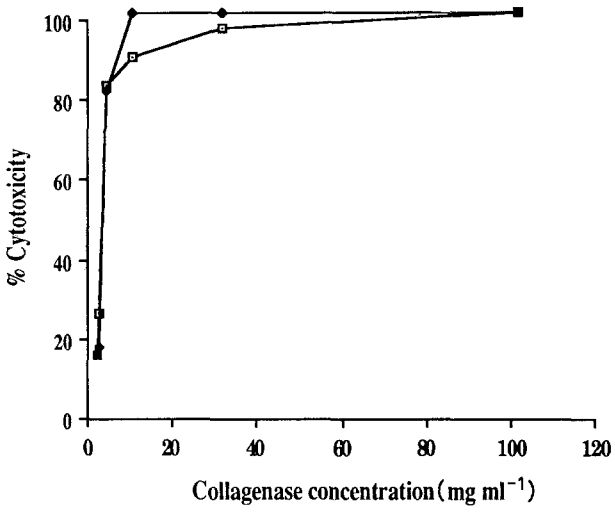

Figure 2 Four-hour collagenase activity on cells embedded in agarose microbeads (–●–), or on free cells (–□–).

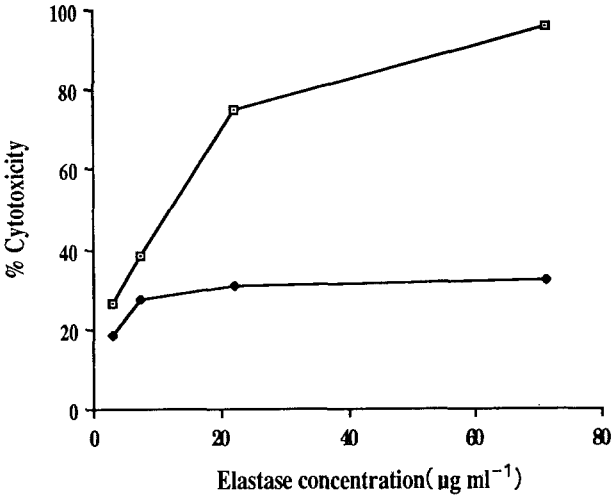

Figure 3 Four-hour elastase activity on cells embedded in agarose microbeads (–●–), or on free cells (–□–).

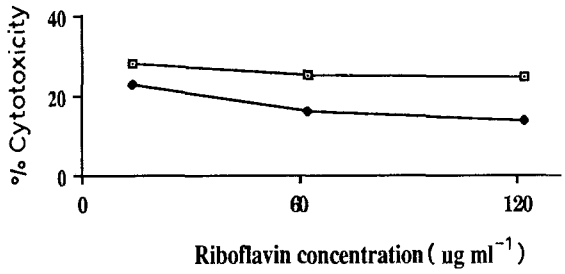

Figure 4 Four-hour free radicals activity on cells embedded in agarose microbeads (–●–), or on free cells (–□–). Free radicals were generated by incorporating riboflavine to culture medium.

burst, but may also be the result of more specific reactions such as those of the arachidonic acid metabolic cascade. Free radicals have strong effects but are short-lived. For this reason, to test their action on embedded or free cells, we mimicked their action by introducing riboflavin into the culture medium. We used riboflavin concentrations of 12–120 μg ml^{-1} of medium. The mixture was then exposed to light. The riboflavin became oxidized and therefore produced free radicals. Results showed that both on embedded and free cells the toxic action of these compounds was exerted during the 4 h test (Fig. 4).

4. Conclusion

This initial study investigates whether some molecular products known to form during host defence could pass through microcapsule-protected cells. These molecules are of low molecular mass (less than 150 kDa). Some are secreted proteins such as cytokines (Il-1 and Il-2) involved in immunity and inflammation, where they regulate the amplitude and duration of response. They are usually produced transiently and locally, acting in a paracrine or autocrine manner. Others are enzyme molecules also produced by stimulated immunocompetent cells such as elastase. These results confirm the cytotoxicity of compounds such as elastase, collagenase, free radicals, and lysozyme at high concentrations. No direct toxic effect of Il-1 and Il-2 was found in our target cells.

However, the initial context is important, i.e. the action of such mediators toward grafted cells such as Langerhans islets. These are groups composed of various cell types. It may be that some of these cells react to cytokines. When passing through the encapsulation membrane, the Il-1 (for example) could bind to various cells of the islets (resident macrophage receptors) and therefore trigger the immunological cascade. A real attack on the implanted cells results in a generalized reaction such as degranulation, protease synthesis and free radical genesis. In time, a sort of slow-acting cytokine-mediated autodestruction causes definitive cell necrosis.

We have demonstrated the toxic action of some of the inflammatory mediators which could act without the direct intervention of immune complexes such as antibody–antigens. This list is, of course, not exhaustive but underlines the complexity of the problem. With a direct action, they could kill cells if their synthesis were not correctly regulated. Although their function is only indirect, it is nevertheless fundamental insofar as these compounds could thus maintain and amplify the previous inflammatory response. Despite the presence of a semi-permeable membrane, it seems possible that in living cell implantation, initiation of an inflammatory response against these foreign bodies mediates in a more or less complex way the production of such molecules; cell survival is then seriously compromised. These problems will probably be solved by technical improvements in the production of better non-inflammatory materials and polymers.

This preliminary study indicates that beyond the strictly immunological problem and the fact that the cells of the immune system in particular depend on regulated interactions with other cells to activate and direct the response to infection, the implantation of hybrid functional units must take into account the various biological molecules synthesized and secreted by the cells which are activated after an inflammatory stimulus.

References

1. H. OHGAWARA, N. MOCHIZUKI, T. TAIRA, S. NISHIJIMA, N. IWASAKI, R. YUI, Y. HIRATA and T. HAYAKAWA, *In vitro Cell. Dev. Biol.* **26** (1990) 348–352.
2. H. IWATA, H. AMEMIJA, T. MATSUDA, H. TAKANO, T. AKUTSU, *J. Bioact. Comp. Polymers* **3** (1988) 356–369.

3. M. E. SUGAMORI and M. V. SEFTON, *Trans. Amer. Soc. Artif. Intern. Organs* **35** (1989) 791–799.
4. S. DARQUY and G. REACH, *Diabetologia* **28** (1985) 776–780.
5. R. L. BROUGHTON and M. V. SEFTON, *Biomaterials* **10** (1989) 462–465.
6. S. R. WINN, P. AEBISCHER and P. M. GALLETTI, *J. Biomed. Mater. Res.* **23** (1989) 31–44.
7. R. B. HEBERMAN, in "Natural cell-mediated immunity against tumors" (Academic Press, New York, 1980).
8. S. GILLIS, M. H. FERM, W. OU and K. A. SMITH, *J. Immunol.* **120** (1978) 2027–2034.
9. C. LARGMAN, J. W. BRODRICK and M. C. GEOKAS, *Biochem.* **15** (1976) 2491–2500.
10. B. DUPUY, H. GIN, Ch. BAQUEY and D. DUCASSOU, *Innov. Tech. Biol. Med.* **7** (1986) 627–632.
11. L. B. LACHMAN, in "Mediators in cell growth and differentiation", edited by R. J. Ford and A. L. Maizel (Raven Press, New York, 1985) pp. 171–183.
12. U. SIEBERS, T. ZEKORN, R. G. BRETZEL, M. REMARDY, P. ZSCHOCKE, H. PLANCK and U. FEDERLIN, *Transplant. Proc.* **22** (1990) 2035–2036.
13. S. SANDLER, K. BENDTZEN, L. A. H. BORG, D. L. EIZIRIK, E. STRANDELL and N. WELSH, *Endocrinol.* **124** (1989) 1492–1501.
14. M. ERRASFA and F. RUSSO-MARIE, *Agents Actions* **24** (1988) 123–129.
15. R. F. REST, in "Methods in enzymology" (Academic Press, San Diego, California, 1988) vol. 163, pp. 309–328.
16. D. R. BLAKE, R. E. ALLEN and J. LUNEC, *Brit. Med. Bull.* **43** (1987) 371–385.

Received 6 January
and accepted 8 April 1991