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# Macrophage activation through phagocytosis of muramyl dipeptide encapsulated in gelatin microspheres

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Gelatin microspheres containing muramyl dipeptide (MDP) were prepared by crosslinking with glutaraldehyde. They were added to mouse peritoneal macrophages (PMs) to potentiate the tumour growth inhibitory activity. The PMs which had internalized the microspheres exhibited growth inhibitory activity to syngeneic, allogeneic, and xenogeneic tumour cells. A similar effect was observed for PMs incubated with free MDP, but the MDP encapsulated in the microspheres was more efficient in enhancing the PM activity than the free MDP. In addition, PMs were activated in much shorter periods upon incubation with the microsphere-encapsulated MDP. The duration of activity could be controlled for up to 7 days by changing the extent of crosslinking of microspheres. Dose-response experiments established that microsphere-encapsulated MDP is able to activate PMs to inhibit growth of tumour cells at concentrations approximately 2000 times lower than the free MDP present in media. The activity of PMs was also acquired on intraperitoneal injection of the microspheres, in contrast to PMs with the free MDP.

Muramyl dipeptide (MDP) and its derivatives have been reported to function as immunopotentiators in host defence systems. They have the ability to activate macrophages but do not exhibit any toxicity and antigenicity (Elouz et al 1974; Candid et al 1979; Mater 1979). However, their duration of activity in-vivo is too short, probably because parenterally administered MDP is rapidly excreted in urine (Mater 1979; Parant et al 1979; Ledever 1980). Therefore, it is of prime importance to develop new dosage forms of MDP to lengthen its immune activity. Fidler et al (1981, 1982, 1983) have demonstrated that encapsulation of MDP or its derivative in liposomes makes possible the prolonged tumouricidal activation of macrophages, even in-vivo. Recently, we have found that a lipophilic derivative of MDP is capable of activating mouse peritoneal macrophages at extremely low concentrations in-vitro as well as in-vivo, when given to the macrophages encapsulated in a biodegradable microsphere comprising glycolic acid and L-lactic acid copolymer. The minimum concentration necessary for macrophage activation in-vitro was about one thousand times lower for the encapsulated immunopotentiator than for the free, unencapsulated one. Such a high efficacy of the drug may be explained in terms of the phagocytic propensity of the macrophages.

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As is well known, the phagocytosis of macrophages is greatly dependent on the size and the surface properties of the microspheres to be phagocytosed. Opsonization also enhances phagocytosis by macrophages. In this connection we have previously studied the effects of precoating microspheres with a variety of synthetic and biological macromolecules on macrophage phagocytosis. One of the interesting findings of the work was that gelatin was the most effective precoating substance to accelerate the phagocytosis whereas precoating with serum albumin conspicuously retarded it (Ikada & Tabata 1986). This suggests that gelatin is a promising promoter for the uptake of a drug by macrophages. Gelatin, a biodegradable polymer, has been used for drug delivery systems (Nixon et al 1968; Hashida et al 1979; Yoshioka et al 1981), but there have been no reports of gelatin microspheres delivering immunomodulating substances to macrophages.

The aim of the present work has been to encapsulate water-soluble MDP in microspheres of gelatin crosslinked to different extents and to study the macrophage activation caused by phagocytosis of the microspheres containing MDP.

## MATERIALS AND METHODS

### *Animals*

Specific pathogen-free inbred male and female BALB/cCrSlc mice, 5–7 weeks old, were obtained

from the Shizuoka Animal Facility Center, Shizuoka Animal Center, Shizuoka, Japan.

*Cells*

A subline of Meth-A cells (Meth-A-R1) employed here (methylcholanthrene-induced fibrosarcoma of BALB/c mice) was originally established as interferon-resistant and non-adherent (Uno et al 1985). Allogeneic tumour P815 is a chemically induced mastocytoma in DBA/2 mice. Xenogeneic tumour AH130 is an ascites hepatoma induced in Donryu rats by dimethylaminoazobenzene. All tumour cells have been adapted to grow in-vitro. Cultures of syngeneic mouse embryo cells were prepared by trypsinization of 14- to 17-day-old BALB/c mouse foetuses (Fidler 1975). The embryo cell cultures used in the assays were between the second and fifth passage in-vitro, and assays were always performed on cells in their exponential growth phase.

*Media and buffers*

Culture medium (RPMI-FCS) was prepared by supplementing RPMI-1640 medium (Nissui Seiyaku Co., Ltd, Tokyo, Japan) with 10% foetal calf serum (FCS; M.A. Bioproducts, Walkersville, MD), 5 mM L-glutamine, and penicillin (100 units $mL^{-1}$), and buffered with 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid and $NaHCO_3$ at pH 7·4. Hanks' balanced salt solution (HBSS) and phosphate-buffered saline were obtained from Nissui Seiyaku Co., Ltd, Tokyo, Japan. Lipopolysaccharide (LPS; *E. coli* 0111:B4) was obtained from Difco Laboratories, Detroit, MI. The immunopotentiator used, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), was supplied by Daiichi Seiyaku Co., Ltd, Tokyo, Japan, and the preparations were free of endotoxins as determined by the limulus amoebocyte lysate assay. Gelatin (Alkaline type, pI 4·9) was supplied by Nitta Gelatine Co., Ltd, Osaka, Japan. Other chemical reagents were of special grade and purchased from Nakarai Chemicals, Ltd, Japan and used without further purification.

*Mouse peritoneal macrophage*

To obtain homogeneous mouse peritoneal macrophages (PMs) (Fidler 1975), 5–7 week-old mice were injected aseptically with 2 mL thioglycollate broth (Brewer's Medium, Difco Laboratories, Detroit, MI) intraperitoneally (i.p.) 4 days before harvest. On the day of harvest, mice were exsanguinated by decapitation and injected i.p. with cold HBSS, and the peritoneal exudate cells were collected by syringe aspiration. After centrifugation (1000 rev $min^{-1}$, 5 min, 0°C), pelleted peritoneal exudate cells were resuspended in RPMI-FCS, counted on a haemocytometer, and brought to a cell density of $2 \times 10^5$ macrophages $mL^{-1}$ RPMI-FCS. 1 mL of cell suspension was seeded into 16 mm wells on tissue culture plates (A/S. Nunc, Kamstrup, Roskilde), or into 16 mm wells into each of which a round, glass cover-slip had been placed for the phagocytosis assays. After being incubated at 37 °C in a 5% $CO_2$, 95% air atmosphere for 2 h, non-adherent cells were removed by washing three times with RPMI without the FCS. More than 98% of the adherent cells had morphologic and phagocytic properties as macrophages. These cells were used for in-vitro assays.

*Gelatin microspheres containing MDP*

0·5 g of Span 80 dissolved in a mixture of 5 mL of toluene and 5 mL of chloroform was added to a 30 mL sampling tube. 1 mL of aqueous solution containing 100 mg of gelatin and 1 mg of MDP was poured into the organic mixture containing Span 80 and the whole emulsified by sonification. The resulting emulsion was poured rapidly into 80 mL of a precooled organic solution of 25% chloroform in toluene containing 4 g of Span 80. Subsequently, gelatin in the emulsion was crosslinked by glutaraldehyde in toluene as follows: 10 mL of 25% aqueous glutaraldehyde solution and 10 mL of toluene were added to a 30 mL sampling tube and mixed to give a dispersion. When two-phase separation was completed, the toluene phase saturated with glutaraldehyde was pipetted out and 1, 2, 4, and 8 mL of the toluene phase was added to the gelatin emulsion. The glutaraldehyde concentration in the toluene phase was determined to be 5·6 mg $mL^{-1}$ with 3-methyl-2-benzothiazolinone hydrazone (Sawicki et al 1961). The emulsion, mixed with the glutaraldehyde-saturated toluene, was continuously agitated at 0 °C for 6 h for crosslinking. After that, the emulsion was centrifuged at 4000 rev $min^{-1}$ for 5 min and the supernatant discarded. The resulting microspheres were washed four times each with 25% chloroform in toluene, isopropanol, and distilled water. Between each washing, the microspheres were centrifuged at 4000 rev $min^{-1}$ for 5 min, the supernatant discarded, and the pellets resuspended. Finally, the washed microspheres were suspended in PBS. The empty microspheres were also prepared in the same manner as mentioned above. The characteristics of the gelatin microspheres encapsulating MDP are summarized in Table 1. A typical SEM photograph is shown in Fig. 1. The microspheres are spherical with diameters less than 2 μm. Micro-

Table 1. Characteristics of gelatin microspheres encapsulating MDP.

| Microspheres code | Glutaraldehyde-saturated toluene | | MDP loaded in 1 mg microspheres (μg) |
|---|---|---|---|
| | Volume added (mL) | Moles of glutaraldehyde | |
| Gel-1 | 1 | $0{\cdot}6 \times 10^{-4}$ | $5{\cdot}9 \pm 0{\cdot}2$ |
| Gel-2 | 2 | $1{\cdot}2 \times 10^{-4}$ | $5{\cdot}8 \pm 0{\cdot}2$ |
| Gel-3 | 4 | $2{\cdot}4 \times 10^{-4}$ | $5{\cdot}9 \pm 0{\cdot}2$ |
| Gel-4 | 8 | $4{\cdot}8 \times 10^{-4}$ | $5{\cdot}3 \pm 0{\cdot}2$ |

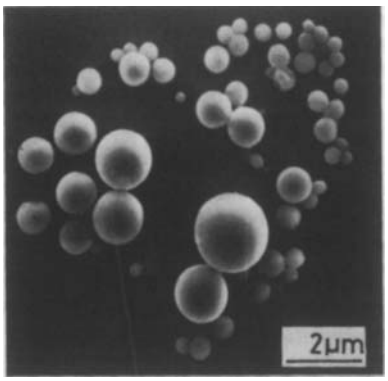


FIG. 1. Scanning electron micrograph of Gel-4 microspheres.

spheres of different sizes, that is, <2 μm, from 2 to 10 μm, and >10 μm diameters, were also prepared by changing the input power at emulsification from 24 to 64 W.

The in-vitro release profile of MDP from the microspheres was determined as follows: 1 mg of gelatin microspheres containing about 12 μg of MDP was suspended in 1 mL of PBS and shaken at 37 °C. At predetermined time intervals, the supernatants were collected by centrifugation (9000 rev $min^{-1}$, 5 min) and the amount of MDP released was determined by the Levvy & McAllan (1959) method. The overall amount of MDP in microspheres was determined after alkaline hydrolysis of the microsphere matrix.

*Phagocytosis assay*

1 mL of RPMI-FCS containing 10 μg of microspheres was added to each of the wells with a round glass cover-slip (14 mm diameter) to which macrophages had adhered and had been incubated for 6 h. After incubation, the glass cover-slips were washed several times with PBS and shaken and then fixed with 2·5% glutaraldehyde in RPMI-1640 medium. After washing with distilled water, the glass cover-slips were inverted and embedded in glycerol jelly on slides. The average number of the microspheres phagocytosed by one macrophage was counted by phase-contrast microscopy. In each test at least 400 macrophages were examined and counting was repeated at least three times for each of the microspheres.

*In-vitro activation of mouse PMs by free MDP or microsphere-encapsulated MDP*

PMs were incubated for 24 h in RPMI-FCS or RPMI-FCS containing 5 μg $mL^{-1}$ LPS as reported by Sone & Fidler (1980). PMs were also incubated with free or microsphere-encapsulated MDP for various times up to 30 h at 37 °C. In additional control experiments, macrophages were incubated with the microspheres containing no MDP together with the free MDP. In all cases the microspheres were added to the macrophage culture at a density of 10 μg/$2 \times 10^5$ PMs. The level of free MDP and the dose of microspheres applied in the present work were not so high as to be toxic to target cells. The PM cultures were rinsed thoroughly with RPMI-FCS before the addition of target cells.

*In-situ activation of mouse PMs*

Mice were injected i.p. with PBS, 200 μg of free MDP, 425 μg of empty microspheres, or 425 μg of microspheres encapsulating 2·5 μg of MDP. The microspheres were suspended in PBS and 0·5 mL of the suspension was used. 425 μg of empty microspheres was also injected together with 2·5 μg of free MDP. Mice were killed 6, 24, and 48 h after injection. PMs were harvested to prepare PM monolayer cultures as described above.

*Assay of PM-mediated tumour growth inhibitory activity*

The target cells used were syngeneic, allogeneic, or xenogeneic tumour cells and syngeneic mouse embryo cells. $1 \times 10^4$ target cells were plated in a well containing $2 \times 10^5$ PMs in 1 mL of RPMI-FCS to obtain an initial PM to target cell ratio of 20:1. At this population density, untreated PMs did not exhibit growth inhibitory activity to the neoplastic cells, in contrast with activated PMs. The target cells were incubated at 37 °C in a 5% $CO_2$, 95% air atmosphere and the assay was terminated 48 h later for syngeneic cells and 18 h later for allogeneic or xenogeneic cells. The tumour cells used here were all non-adherent cells. The cell suspension was counted on a haemocytometer. The number of embryo cells

grown was counted after they had been detached by trypsinization. Assays were in triplicate.

Tumour growth-inhibitory activity mediated by the activated PMs was calculated according to the following formula (Uno et al 1985) and the data were treated statistically with Student's $t$-test ($P < 0{\cdot}01$).

$$\text{Growth inhibition \%} = \frac{\text{No. of target cells cultured with normal PMs} - \text{No. of target cells cultured with activated PMs}}{\text{(No. of target cells cultured with normal PMs)}} \times 100$$

## RESULTS

### *In-vitro release of MDP from microspheres*

The in-vitro release profiles of MDP from microspheres are shown in Fig. 2. It is obvious that its release rate can be controlled by the extent of crosslinking of the gelatin microspheres. As expected, MDP is released more slowly from the microspheres prepared with the higher concentration of glutaraldehyde. Addition of FCS had no significant effect on release profiles (data not shown).

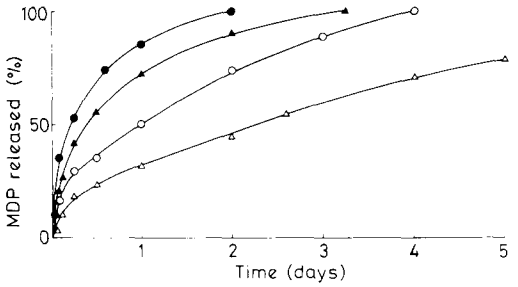


FIG. 2. The in-vitro release profiles of MDP from the different microspheres: (●) Gel-1, (▲) Gel-2, (○) Gel-4, and (△) Gel-8 microspheres.

### *In-vitro cytostatic activation of mouse PMs by microsphere-encapsulated MDP*

In the first set of experiments, the mouse PMs were treated in culture wells for 24 h with a medium containing free MDP (50 μg), empty Gel-4 microspheres (10 μg), empty microspheres (10 μg) plus free MDP (1 μg), LPS (5 μg), or microspheres (10 μg) containing MDP (59 ng). After careful washing of PM monolayers, the target cells were placed into the wells and incubation was continued for given times. The results of a representative experiment are in Table 2. The control PMs, treated with MDP-free medium, did not exhibit tumour growth inhibitory activity. However, a significant and reproducible enhancement of the activity was observed for PMs treated with free MDP, LPS, or microsphere-encapsulated MDP. It should be noted that the total amount of MDP in the microspheres was about $10^{-3}$ × the concentration of free MDP added to PMs. Moreover, the activated PMs exhibited growth-inhibitory activity against syngeneic Meth-A-R1, allogeneic P815, and xenogeneic AH130 tumour cells. Treatment with the empty microspheres did not induce any growth-inhibitory activity by the PMs, but when PMs were treated with empty microspheres plus free MDP there was detectable inhibition. This indicated that the microsphere matrix itself had no effect on the PM activity and did not interfere with the immunopotentiation of MDP. The PM-mediated growth-inhibitory activity was also examined using non-tumourigenic syngeneic embryo cells. These cells grew at a rate that doubled their count after 48 h incubation, regardless of the presence of PMs or the kind of PM treatment (data not shown).

Table 2. Tumour growth inhibitory activity of mouse PMs treated in-vitro with free and Gel-4 microsphere-encapsulated MDP against syngeneic, allogeneic, and xenogeneic target cells.

| PM preincubation with | PM-mediated growth inhibitory activity against: Syngeneic Meth-A-R1 fibrosarcoma | Allogeneic P815 mastocytoma | Xenogeneic AH130 hepatoma |
|---|---|---|---|
| No. PMs, tumour cells alone | 4·2 ± 0·1 × ($10^5$/mL) | 4·3 ± 0·1 | 4·3 ± 0·1 |
| Normal PMs (RPMI-FCS) | 4·2 ± 0·1 | 4·3 ± 0·1 | 4·3 ± 0·1 |
| Microsphere alone (10 μg) | 4·1 ± 0·1 | 4·3 ± 0·1 | 4·3 ± 0·1 |
| Free MDP (50 μg) | 2·9 ± 0·1 (30) | 2·6 ± 0·1 (39) | 3·1 ± 0·1 (28) |
| MDP (1 μg) + microsphere (10 μg) | 3·7 ± 0·1 (12) | 3·8 ± 0·1 (11) | 3·8 ± 0·1 (13) |
| MDP (59 ng) in microsphere (10 μg) | 2·8 ± 0·1 (34) | 2·7 ± 0·1 (37) | 3·0 ± 0·1 (30) |
| LPS (5 μg) | 1·8 ± 0·2 (56) | 2·0 ± 0·2 (54) | 2·3 ± 0·2 (48) |

The values denote the number of target cells (mean ± s.d. for triplicate cultures) and those in parentheses denote percent growth inhibition ($P < 0{\cdot}01$).

The effect of microsphere size on the tumour growth inhibition of PMs is shown in Table 3. The gelatin microspheres used were Gel-4 and the amount of MDP encapsulated was kept to 60 ng, irrespective of microsphere size. As can be seen, the phagocytosis of microspheres was reduced with increase in size, indicating that the activity of PMs treated with the microspheres has a definite relation to the microsphere size. When the size was larger than 10 μm, no uptake of the microspheres by PMs occurred, leading to insignificant PM activation.

Table 3. Tumour growth inhibitory activity of mouse PMs treated in-vitro with Gel-4 microspheres of different sizes.

| PM preincubation with | Microsphere number/cell | Tumour cell number ($\times 10^4$) | Percent growth inhibition |
|---|---|---|---|
| No. PMs, tumour cells alone | — | 4·2 ± 0·3 | — |
| Normal PMs (RPMI-FCS) | — | 4·1 ± 0·1 | — |
| MDP (60 ng) in gel M.S. diameter < 2 μm | 10·6 | 2·8 ± 0·1 | 34 |
| MDP (60 ng) in gel M.S. 2 μm < diameter < 10 μm | 6·3 | 3·6 ± 0·1 | 23 |
| MDP (60 ng) in gel M.S. diameter > 10 μm | 0·0 | 3·8 ± 0·2 | 9 |

To study the kinetics of activation of PMs preincubated for different times with the MDP encapsulated within Gel-4 microspheres, mouse PMs were incubated for 3, 6, 12, 18, 24, and 30 h with 50 μg of free MDP or with microspheres containing 59 ng of MDP, followed by washing and incubating with Meth-A-R1 cells in RPMI-FCS for 48 h. The results are in Fig. 3. It can be seen that PMs were activated

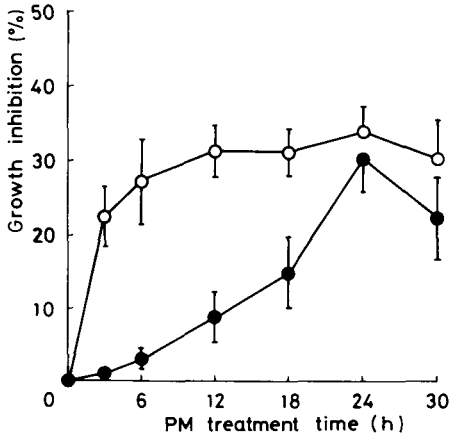


FIG. 3. Kinetics of PM activation by free or microsphere-encapsulated MDP; (●) 50 μg of free MDP, and (○) Gel-4 microspheres containing 59 ng of MDP. Each point represents the mean of triplicate cultures and bar is s.d. ($P < 0·01$).

more strongly with increasing preincubation time in both cases. However, only 3 h incubation with the microsphere-encapsulated MDP resulted in a significant increase of tumour growth-inhibitory activity of PM, compared with that for free MDP.

To study how long the activity of PMs can be maintained, an experiment was performed in which PMs were incubated for 24 h with free MDP or MDP encapsulated in microspheres crosslinked to different extents. After thorough washing and refeeding with RPMI-FCS, Meth-A-R1 cells were added 1 to 8 days later to determine the PM-mediated tumour growth-inhibitory activity. The results are illustrated in Fig. 4. As can be seen, immediately after

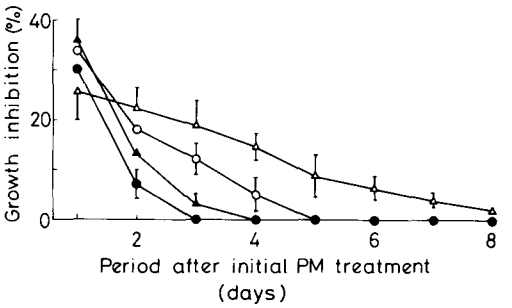


FIG. 4. Maintenance of tumour growth inhibitory activity of PMs treated in-vitro with free MDP or MDP encapsulated in different microspheres; (●) 50 μg of free MDP, (▲) 58 ng of MDP in Gel-2, (○) 59 ng of MDP in Gel-4, and (△) 53 ng of MDP in Gel-8 microspheres. Each point represents the mean of triplicate cultures and bar is s.d. ($P < 0·01$).

treatment with the microspheres containing MDP, a significant activity was observed, irrespective of the extent of crosslinking of the microspheres. Moreover, it is clear that the maintenance of the activity of PMs was greatly enhanced by encapsulation of MDP and influenced by the extent of crosslinking of gelatin. The activity of PMs treated with free MDP disappeared in 2 days, whereas PMs treated with the microsphere-encapsulated MDP maintained activity much longer than 2 days when the extent of crosslinking was high. The duration of the activity was in good accord with the in-vitro release profile of MDP shown in Fig. 2, indicating that the maintenance of activity is primarily dependent on the extent of crosslinking of the microspheres. The result for Gel-1 microspheres, though not shown in Fig. 4, was similar to that of Gel-2.

*In-situ activation of PMs by microsphere-encapsulated MDP*

Free MDP and Gel-4 microsphere-encapsulated MDP was injected intraperitoneally to examine the possibility of in-situ activation of PMs. PMs were collected from mice at 6, 24, and 48 h after injection, and the in-vitro evaluation of PM-mediated tumour growth inhibitory activity was made using Meth-A-R1 cells in a manner similar to that described for the in-vitro activation. As can be seen in Table 4, the

Table 4. Tumour growth inhibitory activity of mouse PMs activated in-situ by intraperitoneal injection of free and Gel-4 microsphere-encapsulated MDP

| Treatment of PM donors | Tumour cell number ($\times 10^4$) (percent growth inhibition) 6 h | 24 h | 48 h |
|---|---|---|---|
| None, tumour cell alone | 4·1 ± 0·2 | 4·2 ± 0·1 | 4·1 ± 0·2 |
| PBS | 4·1 ± 0·2 | 4·1 ± 0·1 | 4·1 ± 0·1 |
| Free MDP (200 μg) | 4·0 ± 0·1 | 4·1 ± 0·1 | 4·1 ± 0·1 |
| MDP (2·5 μg) in Gel-4 microsphere (425 μg) | 3·3 ± 0·1 (20) | 3·2 ± 0·2 (23) | 3·2 ± 0·1 (22) |
| Gel-4 microsphere (425 μg) | 4·0 ± 0·2 | 4·1 ± 0·2 | 4·1 ± 0·1 |
| Gel-4 microsphere (425 μg) + free MDP (2·5 μg) | 4·1 ± 0·2 | 4·1 ± 0·1 | 4·1 ± 0·1 |

The values denote the number of target cells (mean ± s.d. for triplicate cultures) and those in parentheses denote percent growth inhibition ($P < 0·01$).

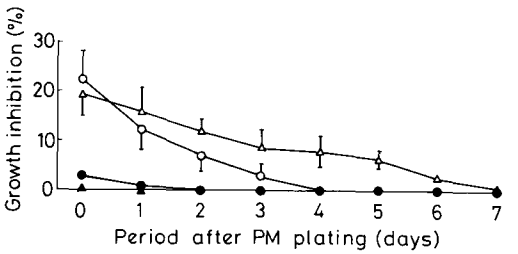


FIG. 5. Maintenance of tumour growth inhibitory activity of mouse PMs activated in-situ with free and microsphere-encapsulated MDP; (●) 200 μg of free MDP, (○) 2·5 μg of MDP encapsulated in 425 μg of Gel-4 microspheres, (△) 2·5 μg of MDP encapsulated in 425 μg of Gel-8 microspheres, and (▲) 2·5 μg of free MDP plus 425 μg of Gel-4 empty microspheres. Each point represents the mean of triplicate cultures and bar is s.d. ($P < 0·01$).

PMs do not exhibit activity when collected from mice after inoculation with PBS alone. Moreover, the PMs collected after inoculation with the free MDP, the empty microspheres, or the mixture of empty microspheres and free MDP, do not exhibit any activity. In the in-vitro system, the free MDP induced PMs to become active in growth inhibition of the tumour cells (Table 2), but no activity was observed when PMs were treated with free MDP in the in-situ system. This may be explained in terms of the rapid diffusion and excretion of free MDP from the peritoneal cavity preventing the activation of macrophages. On the contrary, the PMs from the mice inoculated with the microsphere-encapsulated MDP acquired a significant and reproducible tumour growth inhibitory activity, which became maximal 24 h after inoculation. In addition, the activity lasted longer than 3 days and the duration could be extended further when the extent of crosslinking of microspheres increased. As can be seen in Fig. 5, the effect of crosslinking is similar to that of the in-vitro system shown in Fig. 4.

## DISCUSSION

In this study we have defined PM activation as induced activity in the growth inhibition of tumourigenic target cells. In the present assays, mouse PMs have not exhibited any spontaneous activity and all assays were made under endotoxin-free conditions. The MDP used was entirely synthetic and no lipopolysaccharides were detected by limulus amoebocyte lysate assay. Moreover, the PMs were not activated by empty microspheres or the medium used. Mouse PMs treated with gelatin microspheres containing MDP exhibited growth inhibitory activity against phenotypically diverse tumour cells, but not against non-tumourigenic syngeneic normal cells.

The results clearly demonstrate that the MDP encapsulated in the gelatin microspheres is far more efficient in increasing growth inhibitory activity of mouse PM than free MDP. The amount of MDP within the microspheres needed to effect PM activation was approximately 1000 times lower than that of free MDP (Table 2). In addition, shorter exposure times were sufficient to induce activation of PMs where MDP encapsulated in microspheres was used than when free MDP was used (Fig. 3) and the PMs treated with the microspheres containing MDP maintained their activity for much longer than PMs treated with free MDP (Fig. 4). The times involved were in good accord with those for MDP release from the microspheres. These findings strongly indicate that the activation of PMs in this system is due to the MDP encapsulated in microspheres. In other words, the microspheres are first internalized into macrophages via phagocytosis and then the encapsulated MDP is released inside the cells, resulting in PM activation.

Thus, the gelatin microspheres encapsulating MDP are expected to have a therapeutic potential. The major problem associated with therapeutic trials of immunomodulators is the requirement of high-dosage which invokes toxicity and adverse effects. To answer this problem, several approaches (Fidler et al 1981, 1982, 1983; Lepoz-Berestein et al 1983; Kleinerman et al 1983; Phillips et al 1985) have been attempted using liposomes, but gelatin microspheres have not been studied for this purpose. Gelatin has been commonly used for microencapsulation and

various preparation methods (Tanaka et al 1963; Nixon et al 1968; Madan et al 1974; Hashida et al 1979; Yoshioka et al 1981) have been reported. According to our method, it is possible to obtain gelatin microspheres small enough to be phagocytosed by macrophages. Moreover, the immunomodulator can be encapsulated in the microspheres to a high extent without denaturing. Like liposomes, gelatin is biodegradable. However, the toxicity of the gelatin microsphere crosslinked with glutaraldehyde needs close study before this microsphere is applied in therapy, although gelatin has been used as a plasma expander and the content of glutaraldehyde in the microspheres seems extremely low. One of the advantages of gelatin is that it works as an opsonin even in the presence of FCS (Gudewicz et al 1980; Van de Water et al 1981), probably because of a high affinity to fibronectin or other cell-adhesive proteins in serum. As a result, gelatin must accelerate the phagocytosis of microspheres by PMs (Ikada & Tabata 1986). Thus, gelatin seems to be promising as a targeting and slow release matrix in the activation of macrophages in-vivo.

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