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*Research Article*

# Protein Precoating of Polylactide Microspheres Containing a Lipophilic Immunopotentiator for Enhancement of Macrophage Phagocytosis and Activation

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Biodegradable microspheres containing a lipophilic muramyl dipeptide, MDP-B30, were prepared from a L-lactic acid–glycolic acid copolymer. The effect of precoating the microspheres with water-soluble polymers including proteins on the antitumor activity of mouse peritoneal macrophages (Mϕ) was investigated. Macrophages activated by phagocytosis of the microspheres exhibited growth inhibitory activity toward Meth-A tumor cells. The activity correlated with the extent of Mϕ phagocytosis of the microspheres. Mϕ phagocytosis was greatly augmented by gelatin precoating of the microspheres, resulting in a significant increase of *in vitro* antitumor activity of Mϕ by the microspheres. However, potentiation of Mϕ activity by gelatin precoating was minimal after intraperitoneal injection of the microspheres, but cross-linking of the coated gelatin with glutaraldehyde afforded potentiation of the antitumor activity *in vivo*.

KEY WORDS: polylactide microspheres; macrophage; phagocytosis; antitumor activation; gelatin; immunopotentiator.

## INTRODUCTION

Macrophages (Mϕ) activated by various immunopotentiators are able to recognize and destroy neoplastic cells, while leaving nonneoplastic cells unharmed (1,2). However, one of the major problems associated with therapeutic trials using immunopotentiators is serious side effects of the high-dosage regimens required for significant therapeutic efficacy. Drug delivery systems of the immunopotentiators for targeting to the site of action may overcome this limitation. We have been studying biodegradable polylactide microspheres as a sustained-release vesicle for delivering a lipophilic immunopotentiator, MDP-B30, to Mϕ. After being phagocytosed by Mϕ, the microspheres are degraded in the Mϕ interior, resulting in the slow release of MDP-B30 in the cells. MDP-B30 incorporated in the microspheres is more efficient in enhancing the inhibitory activity of Mϕ against tumor-cell growth than free MDP-B30 under *in vitro* and *in vivo* conditions (3).

A preliminary study has revealed that Mϕ phagocytosis of polymer microspheres is enhanced by surface precoating with some opsonic proteins such as immunoglobulin, fibronectin, and gelatin, in contrast to the reduction of phagocytosis by surface coating with various water-soluble polymers without opsonic ability (4).

The present work was undertaken to study in more detail the effect of surface precoating of microspheres with various polymers on Mϕ phagocytosis and the resulting tumor growth inhibitory activity of Mϕ. We also describe the *in situ* Mϕ activation by intraperitoneal injection of the precoated microspheres.

## MATERIALS AND METHODS

### Culture Media and Reagents

Culture medium (RPMI-FCS) was prepared by supplementing RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Japan) with 10% fetal calf serum (FCS; M.A. Bioproducts, Walkersville, Md.), 5 m*M* L-glutamine, and penicillin (100 units/ml) and buffered with 5 m*M* 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid and $NaHCO_3$ at pH 7.0. Phosphate-buffered saline (PBS) solution was obtained from Nissui Seiyaku Co., Ltd., Tokyo. Lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) was obtained from Difco Laboratories, Detroit, Mich. The immunopotentiator used here, 6-*o*-(2-tetradecyl-hexadecanoly)-MDP (MDP-B30), was kindly supplied by Daiichi Seiyaku Co., Ltd., Tokyo, and the preparations were free of endotoxins as determined by the *Limulus* amoebocyte lysate assay. Proteins employed here were bovine serum albumin (BSA) (Seikagaku Kogyo Co., Ltd., Tokyo), bovine immunoglobulin (IgG) (Cohn faction II, Sigma Chemical Co., St. Louis, Mo.), tuftsin (Cambridge Research Biochemicals, Ltd., Harston, England), gelatin (Nitta Gelatine Co., Ltd., Osaka, Japan), and human plasma fibronectin (FN) isolated from frozen human plasma by affinity chromatography with a gelatin–Sepharose column (5).

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Water-soluble polymers are poly(vinyl alcohol) (PVA; degree of polymerization, 1000; saponification value, 87.69%; Unitika Kasei Ltd., Osaka, Japan), carboxymethyl cellulose (CMC; F-SH, 1%; viscosity, 3500 cps; degree of saponification, 0.6; Daiichi Kogyo Seiyaku Co., Ltd., Kyoto, Japan), poly(acrylamide) (PAAm; Daiichi Kogyo Seiyaku Co., Ltd., Kyoto, Japan), dextran (weight-average molecular weight, 200,000; Nakarai Chemicals, Ltd., Kyoto, Japan), and poly(vinylpyrrolidone) (PVP; weight-average molecular weight, 24,500; Nakarai Chemicals, Ltd., Kyoto, Japan). L-lactic acid (90% aqueous solution) and glycolic acid (70% aqueous solution) were purchased from C.V. Chemie Combinatie, Amsterdam, The Netherlands, and Dupont Chemical Co., Ltd., respectively, and used as obtained. Other chemical reagents of guaranteed grade were purchased from Nakarai Chemicals, Ltd., Kyoto, Japan.

### Preparation of Microspheres Containing MDP-B30

The L-lactic acid and glycolic acid copolymer (PGLA) used here was synthesized by copolycondensation of L-lactic acid and glycolic acid at an equivalent weight ratio for 5 hr at 180°C under reduced pressure from 760 to 100 mm Hg. No catalyst and diluent were employed for copolymerization. The polymerization product was purified by repeated precipitation into methanol from the chloroform solution for removal of the monomers. The monomer conversion to PGLA copolymer was 50%. The weight-average molecular weight and the molar composition of the copolymer were 2900 and 43/57 (L-lactic acid/glycolic acid), respectively (3). Reproducibility of the polymer synthesis was satisfactory.

Microspheres were prepared by the solvent evaporation method (3). One milliliter of methylene chloride solution containing 20 mg of PGLA and 0.4 mg of MDP-B30 was added to 10 ml of 2% PVA aqueous solution, followed by ultrasonic emulsification at 64 W for 2 min. The resulting emulsion was agitated continuously at 30°C until the complete evaporation of methylene chloride. The microspheres were then washed with cold distilled water four times by centrifugation at 5000 rpm for 5 min. No appreciable dissolution of the copolymer in water was observed during washing of the microspheres with water. The washed microspheres were lyophilized and stored at 4°C until use. The yield of the microspheres was 23%, and the amount of MDP-B30 included was $1.8 \pm 0.2$ μg/mg microsphere as determined by the modified Levvy and McAllan method (6). The low content of the drug may be due to partial solution of the drug into the PVA solution during emulsification of the microspheres.

The surface of microspheres containing MDP-B30 was modified by physical adsorption of proteins or other water-soluble polymers, such as BSA, IgG, tuftsin, gelatin, and FN, or PVA, CMC, PAAm, dextran, and PVP. Five-tenths milligram of the microspheres was placed in 1 ml of PBS solution, containing the polymers to be coated, for 1 hr at 37°C, and then 0.1 ml of the resulting suspension was added to 0.9 ml of culture medium for *in vitro* assays. The concentrations of polymer solutions were in the range of $1.5 \times 10^{-10}$ to 1.5 mg/ml. Cross-linking of gelatin adsorbed onto the microsphere surface to reduce the water solubility was conducted with 0.25% glutaraldehyde in PBS for several minutes up to 60 min at 4°C.

### Phagocytosis Assay

Mϕ preparation and phagocytosis assay were performed according to the method reported previously (4). The adherent cells collected from the peritoneal cavity of BALB/c mice 4 days after intraperitoneal injection of thioglycollate broth were used as mouse peritoneal Mϕ. More than 98% of the cells had morphologic and phagocytic properties of Mϕ.

For Mϕ phagocytosis, 1 ml of RPMI-FCS containing 50 μg of microspheres precoated with 1.5 mg/ml of polymer solutions was added to each 16-mm dish of 24-well multidish culture plates (A/S Nunc, Kampstrup, Rosklide, Denmark) with a round cover-glass slip on which $2 \times 10^5$ Mϕ had been adhered. After incubation for 6 hr, the slips were washed with RPMI-1640 medium, fixed with 2.5% glutaraldehyde in the medium, and embedded with glycerin jelly. The average number of the microspheres taken up by one Mϕ was estimated by phase-contrast microscopy for 400 cells. Experiments were independently performed three times for each microsphere.

### *In vitro* Mϕ Activation by PGLA Microspheres

*In vitro* Mϕ activation was estimated according to the method reported previously (3). Mϕ ($2 \times 10^5$) in 1 ml of RPMI-FCS per dish were pretreated for 24 hr at 37°C with PGLA microspheres precoated with 1.5 mg/ml of various proteins or nonprecoated. In all cases, the dose of the microspheres was 50 μg/$2 \times 10^5$ cells, which was below the toxic level to Mϕ. Mϕ cultures were rinsed thoroughly with RPMI-1640 medium to remove nonphagocytosed microspheres before the addition of tumor cells.

### Inhibitory Activity of Mϕ on Tumor-Cell Growth

Meth-A cells, methylcholanthrene-induced fibrosarcoma cells of BALB/c mice, originally established by Dr. Uno (7) were used for assessing the effect of Mϕ on tumor-cell growth. Assays were always performed on cells in the exponential growth phase. Tumor cells ($1 \times 10^4$) in 1 ml of RPMI-FCS were added to the Mϕ monolayers prepared as described above. Under these conditions, untreated Mϕ exerted no inhibitory effect on tumor growth. The number of viable tumor cells was counted after culture for 48 hr at 37°C in a 5% $CO_2$–95% air atmosphere. The growth inhibitory activity of Mϕ toward Meth-A cells was evaluated according to the following formula (7): percentage growth inhibition = [(No. of tumor cells cultured with untreated Mϕ) − (No. of tumor cells cultured with activated Mϕ)]/(No. of tumor cells cultured with untreated Mϕ) × 100.

### *In Situ* Mϕ Activation by PGLA Microspheres

Mice were intraperitoneally injected with 0.5 ml of PBS containing 200 μg of free MDP-B30, 1.4 mg of empty microspheres, or 1.4 mg of microspheres containing 2.5 μg of MDP-B30. Gelatin-coated microspheres with or without cross-linking were also injected to estimate the effect of gelatin coating on *in situ* Mϕ activation. Six, 24, and 48 hr after injection, Mϕ were harvested from peritoneal cavities to assess the inhibition activity on tumor-cell growth as described above.

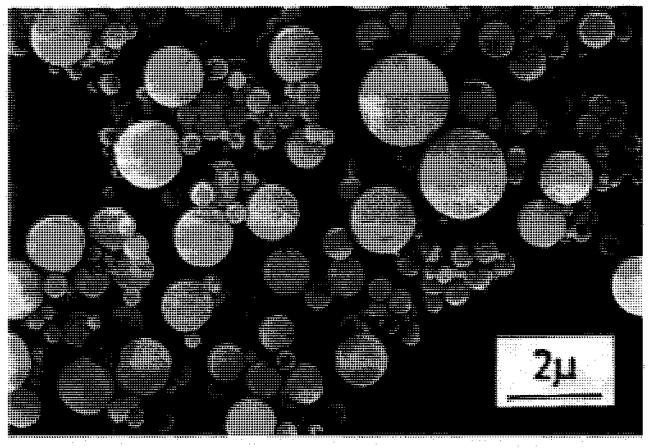


Fig. 1. An SEM photograph of PGLA microspheres containing MDP-B30.

## RESULTS

### Characterization of PGLA Microspheres

PGLA microspheres containing MDP-B30 were heterogeneous in size, with diameters of 2 μm or less, as shown in Fig. 1. The microspheres were degraded, and the consequent release of MDP-B30 from the microspheres was observed in PBS for 6 days at 37°C. The results suggested that the molecular weight of the copolymer (MW 2900) gave suitable release rates of MDP-B30. The microsphere surface became more rough upon degradation, as usually observed for PGLA (8). No influence of polymer precoating on the release profiles was observed (9).

### Mϕ Phagocytosis of PGLA Microspheres

Phase-contrast microscopy occasionally fails to distinguish clearly the microspheres merely attached to the macrophage surface from those internalized into the cells. Thus, experiments were carried out at 4°C for PGLA microspheres containing MDP-B30 precoated with various polymers, because of suppression of Mϕ phagocytosis at low temperatures (10). As shown in Table I, regardless of the precoating polymers and the presence of FCS, the number of microspheres associated with each macrophage is always below 0.2 and quite small compared with that for phagocytosis at 37°C, indicating the validity of this method for evaluating the number of microspheres actually internalized into the cells.

Results on phagocytosis of microspheres containing MDP-B30 at 37°C show that microsphere phagocytosis is suppressed by precoating with BSA, PVA, dextran, CMC, PVP, and PAAm. On the contrary, phagocytosis is remarkably enhanced by precoating with gelatin, FN, tuftsin, and IgG, irrespective of the presence of FCS. It is noteworthy that the presence of FCS increases the uptake of gelatin-precoated microspheres to a remarkable extent. The effect of protein concentration of precoating solutions on phagocytosis of the microspheres in the presence of FCS is illustrated in Fig. 2. It is seen that no appreciable effect of protein precoating on phagocytosis is observed when the protein concentration is below about $10^{-9}$ mg/ml, whereas a significant influence of the precoating appears at concentrations above $10^{-8}$ mg/ml. Microsphere phagocytosis is reduced by BSA precoating, whereas precoating of the microspheres with gelatin remarkably promotes the phagocytosis, compared with IgG and FN. Figure 3 shows the effect of FCS on phagocytosis of microspheres precoated with polymers at 1.5 mg/ml. FCS addition apparently increases the phagocytosis of gelatin-precoated microspheres, in remarkable contrast with that of other protein-precoated and nonprecoated microspheres. In addition, little change in the phagocytosis of gelatin-coated microspheres was found by glutaraldehyde cross-linking of the precoated gelatin.

Table I. Phagocytosis at 4 and 37°C by Macrophages for PGLA Microspheres Precoated with Various Polymers

| Polymer | No. microspheres/cell, 4°C[a] | | No. microspheres/cell, 37°C[a] | | |
|---|---|---|---|---|---|
| | FCS(−)[b] | FCS(+)[c] | FCS(−)[b] | FCS(+)[c] | FCS(+)/FCS(−)[d] |
| None | 0.15 | 0.10 | 3.80 | 2.64 | 0.69 |
| BSA | 0.08 | 0.09 | 1.70 | 1.60 | 0.94 |
| IgG | 0.10 | 0.13 | 6.36 | 6.38 | 1.00 |
| FN | 0.12 | 0.10 | 4.61 | 4.86 | 1.05 |
| Tuftsin | 0.09 | 0.07 | 3.66 | 3.73 | 1.02 |
| Gelatin | 0.20 | 0.14 | 3.99 | 9.33 | 2.34 |
| Cross-linked gelatin[e] | 0.12 | 0.10 | 4.20 | 9.42 | 2.24 |
| PVA | 0.02 | 0.06 | 0.60 | 0.53 | 0.88 |
| Dextran | 0.06 | 0.07 | 2.20 | 2.00 | 0.91 |
| PAAm | 0.10 | 0.12 | 1.41 | 1.30 | 0.92 |
| CMC | 0.18 | 0.15 | 2.43 | 2.09 | 0.86 |
| PVP | 0.16 | 0.14 | 2.91 | 2.47 | 0.85 |

[a] The values denote the number of microspheres phagocytosed by one macrophage.
[b] In the absence of FCS.
[c] In the presence of FCS.
[d] Ratio of the number of microspheres phagocytosed in the presence of FCS to that in the absence of FCS.
[e] Cross-linked with 0.25% glutaraldehyde in PBS for 15 min at 4°C.

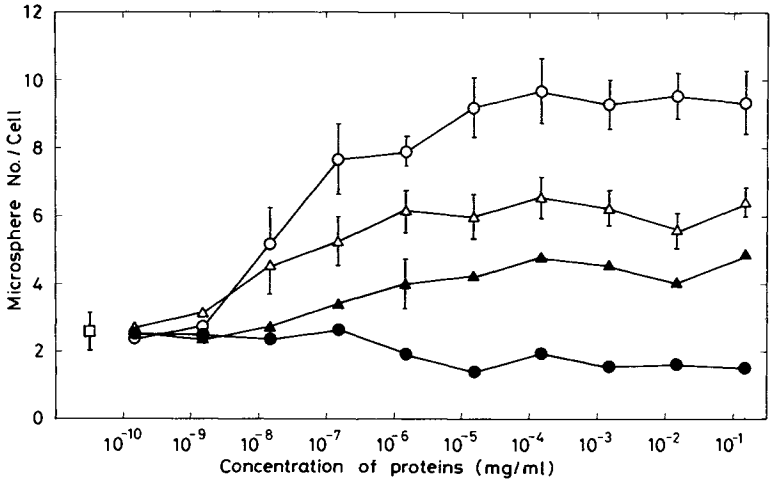


Fig. 2. Influence of protein precoating on Mϕ phagocytosis of PGLA microspheres containing MDP-B30 in RPMI-1640 culture medium with 10% FCS: (○) gelatin, (△) IgG, (▲) FN, (●) BSA, and (□) none.

### *In Vitro* Mϕ Activation by PGLA Microspheres

The tumor-cell growth inhibitory activity of Mϕ pretreated with the PGLA microspheres, uncoated, or precoated with various polymers at 1.5 mg/ml is plotted in Fig. 4 against the number of microspheres phagocytosed by one Mϕ. Each microsphere contains 45 ng of MDP-B30 on the average. The activity increases with an enhancement in the phagocytosis, approaching 55%. The effect of the protein concentration of precoating solutions on the activity is illustrated in Fig. 5. No precoating effect on the activity is observed, if the concentration is below about $10^{-9}$ mg/ml. Interestingly, a notable influence appears at concentrations above $10^{-8}$ mg/ml. This is in accordance with the trend of Mϕ phagocytosis of the precoated microspheres shown in Fig. 2. Therefore precoating of the microspheres with gelatin increases phagocytosis along with inhibitory activity against tumor cells. No influence on the activity enhancement of gelatin was observed by cross-linking, as shown in Fig. 4.

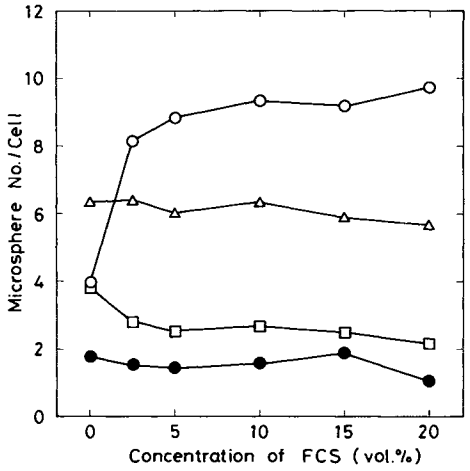


Fig. 3. Influence of FCS concentration on Mϕ phagocytosis of protein-precoated PGLA microspheres containing MDP-B30: (○) gelatin, (△) IgG, (●) BSA, and (□) none.

### *In Situ* Mϕ Activation by Gelatin-Precoated Microspheres

Results on *in situ* Mϕ activation by intraperitoneal injection of nonprecoated and gelatin-precoated microspheres containing MDP-B30 are given in Table II. The result obtained by cross-linking of gelatin following precoating the microspheres with gelatin is also give in Table II. Six, 24, and 48 hr after injection, Mϕ were collected to evaluate the inhibitory effect against tumor-cell growth. The Mϕ of mice did not exhibit any activity, when injected with PBS, free MDP-B30, empty microspheres, or a mixture of empty microspheres and free MDP-B30. On the contrary, the inoculation of microspheres containing MDP-B30 could stimulate Mϕ to exhibit a significant growth inhibitory activity, even

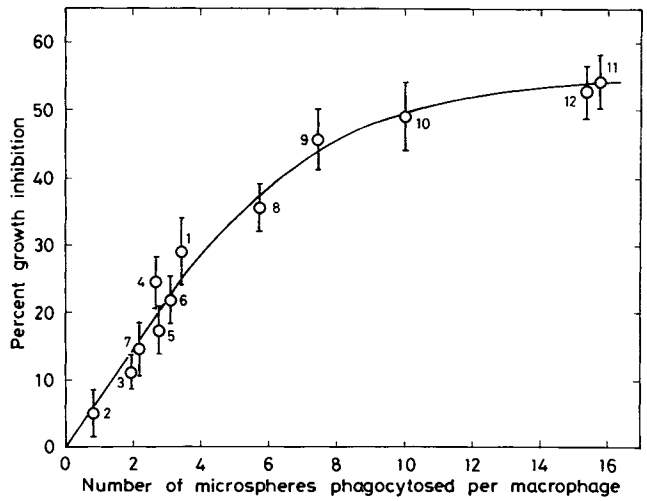


Fig. 4. Influence of polymer precoating on the tumor-cell growth inhibitory activity of Mϕ pretreated *in vitro* with PGLA microspheres containing 45 ng of MDP-B30. The microspheres were precoated with (1) nothing, (2) PVA, (3) PAAm, (4) dextran, (5) CMC, (6) PVP, (7) BSA, (8) tuftsin, (9) FN, (10) IgG, (11) gelatin, and (12) gelatin cross-linked with glutaraldehyde for 15 min at 4°C. Each point represents the mean of triplicate cultures, and the bar indicates the S.E.

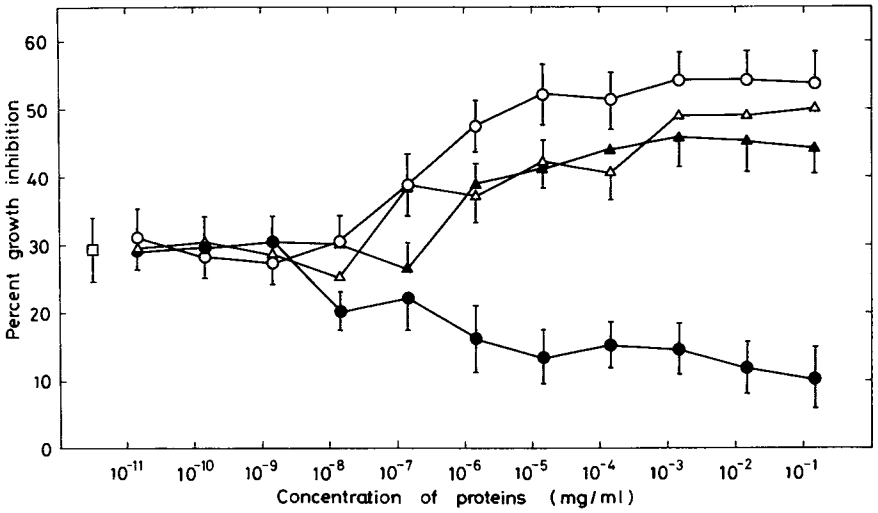


Fig. 5. Effect of the protein concentration of precoating solutions on the *in vitro* tumor-cell growth inhibitory activity of Mϕ pretreated with PGLA microspheres containing 45 ng of MDP-B30. The microspheres were precoated with (□) nothing, (○) gelatin, (△) IgG, (▲) FN, and (●) BSA. Each point represents the mean of triplicate cultures and the bar indicates the SE.

though a small amount of MDP-B30 was contained in the microspheres compared with that of free MDP-B30. The activity became maximal 24 hr after inoculation. Unexpectedly, no effect of gelatin precoating of microspheres was observed on *in situ* Mϕ activation, in contrast to *in vitro* activation. However, cross-linking of the precoated gelatin with glutaraldehyde led to its potentiation effect of Mϕ activation, similar to the *in vitro* system, as shown in Table II. It is suggested that the gelatin molecules precoated on the microspheres might have detached or hydrolyzed *in vivo*. The dependence of gelatin cross-linking on the antitumor activity of Mϕ is given in Fig. 6. The activity increases with the cross-linking time up to 15 min, but a slight decrease in the activity was observed thereafter. This may be interpreted in terms of enhanced fixation or resistance against hydrolysis of the cross-linked gelatin in the initial stage of cross-linking and in terms of structural change of the gelatin by excessive cross-linking in the later stage of the reaction, leading to reduced opsonization.

## DISCUSSION

Particle uptake by phagocytic cells is largely affected by physicochemical properties of the particle surface, particularly by its hydrophobicity (11–14). In general, an increase in hydrophobicity of a particle surface leads to an enhanced uptake. For instance, van Oss and Gillman (12) have shown that bacteria can be readily phagocytosed by neutrophils, if the bacteria surface is more hydrophobic than that of neutrophils. Further, some opsonic proteins remarkably enhance phagocytosis of particles (15,16). Our data can be interpreted on the basis of these facts. The hydrophobic surface of PGLA microspheres can absorb proteins (17),

Table II. *In Situ* Tumor-Cell Growth Inhibitory Activity of Macrophages by Intraperitoneal Injection of PGLA Microspheres Containing MDP-B30

| Treatment of macrophage donors | Percentage growth inhibition | | |
|---|---|---|---|
| | 6 hr | 24 hr | 48 hr |
| PBS | 0 | 0.5 | 0.4 |
| Free MDP-B30 (200 μg) | 2.2 | 2.0 | 1.6 |
| PGLA microsphere (1.4 mg) | 0.5 | 0.6 | 1.2 |
| PGLA microsphere (1.4 mg) + free MDP-B30 (2.5 μg) | 1.0 | 0.5 | 0.5 |
| MDP-B30 (2.5 μg) in PGLA microsphere (1.4 mg) | 25 | 30 | 17 |
| MDP-B30 (2.5 μg) in gelatin-precoated PGLA microsphere (1.4 mg) | 29 | 31 | 16 |
| MDP-B30 (2.5 μg) in cross-linked-gelatin-precoated PGLA microsphere (1.4 mg) | 40 | 41 | 18 |

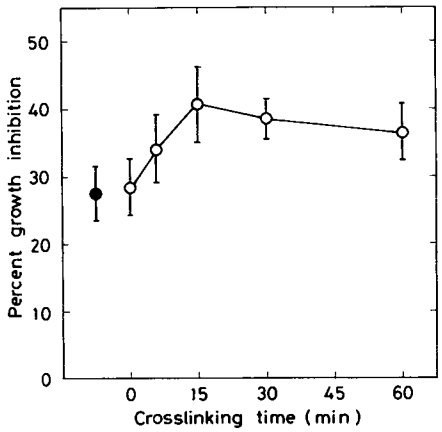


Fig. 6. Effect of the cross-linking time on the *in situ* tumor-cell growth inhibitory activity of Mϕ by intraperitoneal injection of cross-linked-gelatin-precoated PGLA microspheres containing 2.5 μg of MDP-B30: (●) nonprecoated microspheres and (○) microspheres precoated with cross-linked gelatin. Each point represents the mean of triplicate cultures and the bar indicates the SE.

resulting in the lowering of the hydrophobicity of the microsphere surface. Some of the proteins present in FCS, such as albumin, and other nonproteinaceous polymers do not have the opsonizing ability and, hence, may simply alter the hydrophobicity of the microsphere surface to reduce phagocytosis. On the contrary, the addition of FN, IgG, and gelatin enhances phagocytosis of the microsphere, most probably through its opsonizing ability (4,13,18). Fibronectin or other cell-adhesive proteins found in FCS may be bound predominantly to the gelatin-precoated microsphere surface, because of its biospecific affinity to gelatin. As a result, the gelatin-precoated microspheres undergo increased opsonization in the presence of FCS (4,19). Hence, an important function of phagocytic Mϕ seems to be the uptake of gelatin, which being denatured collagen, is a catabolic product of connective tissues. However, so far, nobody has taken advantage of this propensity of Mϕ for gelatin to target immunopotentiators.

The inhibitory activity of Mϕ on tumor-cell growth by PGLA microspheres containing MDP-B30 is directly related to the extent of Mϕ phagocytosis of the microspheres (Fig. 4), which can be widely regulated through precoating with various proteins. The dependence of protein concentrations of precoating solutions on the tumor growth inhibitory activity is entirely consistent with that on phagocytosis of the microspheres, as seen from comparison of Figs. 5 and 2. It appears that, upon phagocytosis of the microspheres containing MDP-B30, Mϕ are stimulated to acquire the antitumor activity, which is remarkably potentiated through the enhancement of microsphere phagocytosis by gelatin precoating.

Cross-linking of gelatin precoated to the microspheres enhanced the inhibitory activity of Mϕ against tumor-cell growth under *in vivo* conditions, presumably because of longer retention of gelatin on the microspheres. In conclusion, gelatin is a promising protein for the enhancement of microsphere phagocytosis by Mϕ, resulting in potentiation of Mϕ activation for *in vivo* as well as *in vitro* systems.

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