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Macrophage Activation for Antitumour Function by Muramyl Dipeptide-protein Conjugates

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Abstract—A muramyl dipeptide (MDP) has been conjugated directly with various proteins by means of a water-soluble carbodiimide. The enhancement of the antitumour activity of mouse peritoneal macrophages by the MDP-protein conjugates has been investigated to assess the ability of the proteins for targeting MDP to the macrophages. These were activated to inhibit the in-vitro growth of tumour cells much more effectively, when immunoglobulin (IgG), fibronectin (FN), and gelatin conjugates were used than when MDP was used alone. The minimum amount of MDP in both the MDP-gelatin and the MDP-IgG conjugates necessary for macrophage activation was approximately 2000 times lower than the amount of free MDP needed. The macrophages activated by the conjugates exhibited growth inhibitory activity against phenotypically diverse tumour cells. The activity induced by the MDP-gelatin conjugate was higher than that of the MDP-IgG conjugate over the range of MDP concentrations, regardless of the isoelectric point of the gelatin used. When MDP was conjugated with bovine serum albumin (BSA), the antitumour activity of macrophages was reduced as the amount of BSA conjugated increased. With both free MDP and MDP-protein conjugates, the macrophages were more strongly activated, the longer they were pretreated. However, less pretreatment time was needed to potentiate macrophage activation by the MDP-gelatin conjugate than by free MDP. Also, the macrophages pretreated with the MDP-gelatin conjugate could maintain their activated state for longer than those pretreated with free MDP. It is concluded that gelatin is an effective carrier protein for the targeting MDP to macrophages, resulting in their activation.

Macrophages are activated by a variety of immunomodulatory agents to exert cytocidal activity for phenotypically diverse tumour cells (Alexander & Evans 1971; Hibbs 1974; Piessens et al 1975; Doe & Henson 1978; Fidler 1978; Key 1983). Among immunomodulatory agents, muramyl dipeptide (MDP, *N*-acetylmuramyl-L-alanyl-D-isoglutamine), which is the smallest mycobacterial structure able to induce immunomodulatory activity (Adam et al 1981), does not exhibit toxicity and antigenicity, and activates macrophages for tumouricidal function (Ellouz et al 1974; Candid et al 1979; Mater 1979). However, MDP is rapidly cleared from the body (Parant et al 1979), and has a low in-vivo efficiency in inhibiting tumour growth (Chedid et al 1982). MDP was shown to inhibit the growth of hepatocarcinoma transdermally implanted in guinea-pigs (McLaughlin et al 1980), on injection with a suspension of trehalose-dimycolate, a glycolipid derived from mycobacterial cell wall. When MDP is carried by liposomes (Fidler et al 1981; Sone & Fidler 1981), neoglycoproteins (Monsigny et al 1984), or antibodies (Roche et al 1984), it is selectively recognized and endocytosed by macrophages, leading to an improvement in the efficacy of their activation. We have previously developed biodegradable gelatin microspheres containing MDP to activate macrophages, by taking advantage of the inherent opsonic ability of gelatin for macrophages (Ikada & Tabata 1986). The high effectiveness of the microspheres in macrophage activation was demonstrated in-vivo as well as in-vitro (Tabata & Ikada 1987).

In the present study, the active targeting of MDP to macrophages was attempted by chemical modification with gelatin. The enhancement of in-vitro inhibitory activity of macrophages on tumour cell growth by the gelatin conjugate with MDP was investigated to evaluate the ability of gelatin to target the macrophages, compared with that of other protein conjugates with MDP.

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Materials and Methods

Animals

Specific pathogen free inbred male and female BALB/cCrSlc mice, 5–7 weeks old, were from Shizuoka Laboratory Animal Center, Shizuoka, Japan.

Tumour cells

The Meth-A-R1 (R1) cell line, originally established as interferon-resistant and non-adherent cells (Uno et al 1985) from the Meth-A cell line (methylcholanthrene-induced fibrosarcoma of BALB/c mice) was used as a syngeneic tumour cell. Allogeneic and xenogeneic tumour cells are P815 mastocytoma cells induced chemically in DBA/2 mice and AH130 ascitic hepatoma cells induced in Donryu rats by methylaminoazobenzene, respectively. All tumour cells had been adapted to grow in-vitro and assays were always conducted on cells in their exponential growth phase.

Media and reagents

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 7·2. Hanks balanced salt solution (HBSS) and phosphate-buffered saline solution (PBS) were obtained from Nissui Seiyaku Co. Ltd, Tokyo, Japan. Lipopolysaccharide (LPS;

E. coli 0111: B4) was purchased from Difco Laboratories, Detroit, MI. The immunomodulatory agent 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 Limulus amoebocyte lysate assay. Proteins used were bovine serum albumin (BSA) (Seikagaku Kogyo Co. Ltd, Tokyo, Japan), bovine immunoglobulin (IgG) (Cohn fraction II, Sigma Chemical Co., St. Louis, MD), human plasma fibronectin (FN) isolated from frozen human plasma by affinity chromatography with a gelatin-sepharose column (Vuent & Veheri 1979), and various gelatins with different isoelectric points (Nitta Gelatine Co. Ltd, Osaka, Japan). $Na^{125}I$ (approx. 100 mCi mL^{-1}) was obtained from NEN Research Products, Dupont, USA. Other chemical reagents of guaranteed grade were purchased from Nakarai Tesque Inc., Kyoto, Japan and used as obtained.

Conjugation reaction of MDP to proteins

Proteins conjugating MDP (MDP-protein conjugates) were prepared with a water-soluble carbodiimide (EDCI; 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl) according to the Sheehan & Hess (1955) method. EDCI powder (30 mg) was added to phosphate buffer (2 mL, 0·05 M) at pH 4·7 containing different amounts of MDP, and left for 1 h at 4°C to activate the γ-glutamyl-carboxyl group of MDP. Phosphate buffer, pH 4·7, (2 mL, 0·05 M) containing 10 mg of protein was added to the activated MDP with agitation for times up to 40 h at 4°C. At desired times, MDP-protein conjugates were purified by gel filtration on a PD-10 column (Sephadex G-25, Pharmacia Fine Chemicals AB, Uppsala, Sweden) in PBS, sterilized by filtration through a 0·22 μm Millex-GS millipore filter (type: SLGS025OS, Japan Millipore Ltd, Tokyo, Japan), and stored at 4°C until used. The content of MDP in the conjugates was determined by the Levvy & McAllan (1959) method, and the amount of protein was evaluated by measuring the absorbance at 280 nm. Radioiodination of the conjugates was according to the Greenwood & Hunter (1963) method.

Macrophage preparation

Macrophages were prepared as described by Ikada & Tabata (1986). The adherent cells from the peritoneal cavity of BALB/c mice 4 days after intraperitoneal injection of thioglycollate broth were used as macrophages. More than 98% of the cells had morphologic and phagocytic properties of macrophages.

Pinocytosis assay

Pinocytosis assays were according to Barbara & Cohn (1967). RPMI-FCS (1 mL) containing different amounts of ^{125}I-labelled MDP-protein conjugates was added to each of the 16 mm dishes of 24-well multidish culture plates (A/S Nunc, Kamstrup, Roskilde, Denmark) on which 1×10^6 macrophages had been adhered. The plates were incubated for various times up to 24 h at 37°C, then the cell monolayers were washed four times with RPMI-1640 medium and the cells lysed with 2% sodium lauryl sulphate in PBS. The radioactivity of the lysates was measured to estimate the amount of conjugates pinocytosed by the macrophages. The composition of the conjugates was (μg (mg protein)$^{-1}$) MDP-gelatin 9·78 μg, MDP-IgG 8·03 μg, MDP-FN 8·26 μg and MDP-BSA 7·00 μg.

In-vitro activation of macrophages by MDP-protein conjugates

Macrophage activation was estimated according to Tabata & Ikada (1987). 2×10^5 cells in 1 mL of RPMI-FCS were pretreated for various times up to 30 h at 37°C with MDP-protein conjugates or with free MDP. The MDP conjugates were those used in the pinocytosis assay, unless mentioned otherwise. Additional control experiments were done in which macrophages were pretreated with MDP-free proteins with or without free MDP. In all cases, the amount of conjugate was in the range from 10·4 to 14·4 μg per 2×10^5 cells and was below the level toxic to them. The cultures were rinsed thoroughly with RPMI-1640 medium to remove non-pinocytosed conjugates before the addition of tumour cells.

Inhibitory activity of macrophages on tumour cell growth

Tumour cells (1×10^4) in 1 mL of RPMI-FCS were added to the macrophage monolayers prepared as described above. Under these conditions, untreated macrophages exerted no inhibitory effect on tumour growth. The number of viable tumour cells was counted after culture for 48 h at 37°C in 5% CO_2–95% air. The growth inhibitory activity of the macrophages towards the tumour cells was evaluated according to the formula of Tabata & Ikada (1987) and the data were treated statistically with Student's *t*-test ($P < 0·01$).

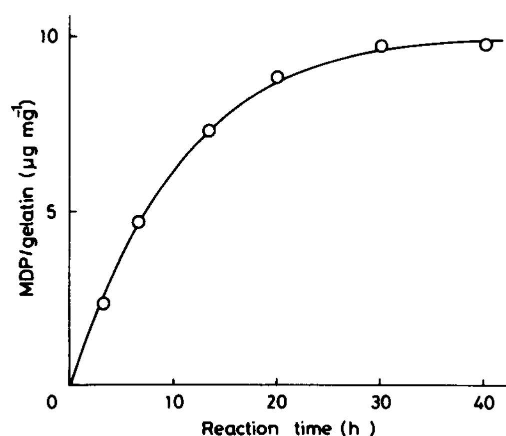

FIG. 1. Time course of the conjugation reaction of MDP with gelatin by the water-soluble carbodiimide method.

Table 1. Characteristics of MDP-gelatin conjugates.

In feed				
Gelatin (mg)	MDP (mg)	Yield (%)	MDP (mg protein)$^{-1}$ (μg)	MDP protein (mol mol^{-1})
10	0·5	78·0	1·92	0·39
10	0·75	82·8	3·40	0·69
10	1·0	84·6	5·99	1·22
10	1·25	91·6	8·33	1·69
10	1·5	83·8	9·51	1·98
10	1·75	78·6	9·78	1·99
10	2·0	80·7	14·48	2·94

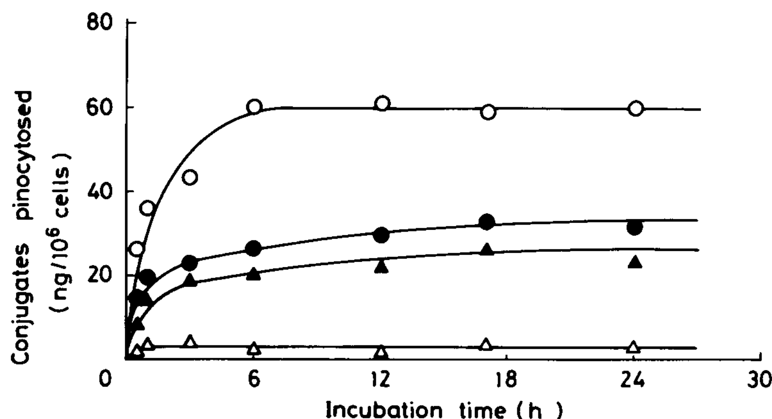

FIG. 2. Time course of the pinocytosis of MDP-protein conjugates by macrophages; (○) MDP-gelatin, (●) MDP-IgG, (▲) MDP-FN, and (△) MDP-BSA conjugates.

Percent growth inhibition = [(No. of tumour cells cultured with untreated macrophages) − (No. of tumour cells cultured with activated macrophages)]/(No. of tumour cells cultured with untreated macrophages) × 100.

Results

Characterization of MDP-protein conjugates

The amount of MDP conjugated to proteins is governed by the reaction time and the ratio of MDP to protein. Fig. 1 shows the effect of the reaction time on the amount of MDP coupled to gelatin. As reaction periods longer than 30 h did not increase the amount of MDP coupled, the time was fixed at 30 h for the present work. The yield of MDP-gelatin conjugates, the amount of MDP coupled in the conjugates, and the molecular number of MDP per gelatin molecule are summarized in Table 1, with the amount of gelatin and MDP added in conjugation. It is apparent that the MDP content in the conjugates can be regulated by changing the MDP/gelatin ratio. A similar trend was obtained with the other MDP-protein conjugates. The amounts of MDP conjugated varied from 2·18 to 18·4 μg mg^{-1} IgG and from 1·95 to 23·0 μg mg^{-1} BSA for MDP-IgG and MDP-BSA conjugates, respectively, by the same procedures. No cloudy appearance of solution from intermolecular crosslinking of the proteins was observed during the reaction.

Macrophage pinocytosis of MDP-protein conjugates

The results of macrophage pinocytosis of MDP-protein conjugates are shown in Figs 2 and 3, where the dependence of the incubation time and the amount of conjugates added to the cell cultures on the pinocytosis are shown. The amount of conjugates taken up by macrophages increased with incubation time up to 12 h. Among the conjugates, the saturation amount was highest for the MDP-gelatin conjugate. The amount of conjugates pinocytosed by the macrophages increased with increase of conjugates added, the largest amount pinocytosed being the MDP-gelatin conjugate (Fig. 3).

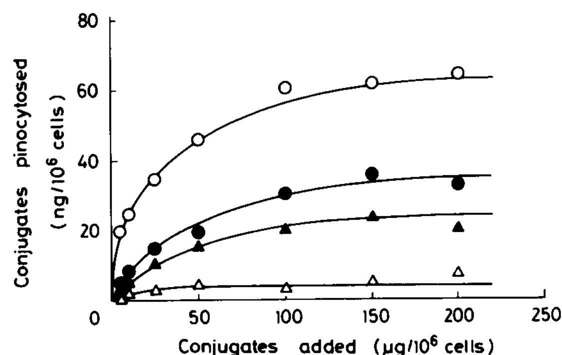

FIG. 3. Effect of the initial amount of MDP-protein conjugates on pinocytosis; (○) MDP-gelatin, (●) MDP-IgG, (▲) MDP-FN, and (△) MDP-BSA conjugates.

Effect of MDP-protein conjugates on inhibition of tumour growth by macrophages

Macrophages were pretreated for 24 h with RPMI-FCS containing 0·1 or 100 μg of free MDP, MDP-protein conjugates containing 0·1 μg of MDP, and MDP-free proteins with or without 0·1 μg of free MDP. The cell monolayers were washed thoroughly, and inoculated with syngeneic, allogeneic, or xenogeneic tumour cells, and incubated further for 48 h to assess the in-vitro inhibitory effect of macrophages on tumour cell growth. Control macrophages pretreated with culture medium free of MDP did not exhibit growth, in contrast to those pretreated with LPS used as a positive control (Table 2). However, a significant and reproducible enhancement of the activity was observed when the cells were pretreated with free MDP, or MDP-gelatin, MDP-IgG, or MDP-FN conjugates. The macrophage activation was in the ranking of gelatin > IgG > FN. Moreover, the activated cells exerted a growth inhibitory effect on syngeneic R1, allogeneic P815, and xenogeneic AH130 tumour cells. For the MDP-gelatin conjugate, the amount of MDP in the conjugate required for macrophage activation was approximately 2000 times less than for free MDP. No enhancement of the activity was observed with the MDP-BSA conjugate. Pretreatment of macrophages with the MDP-free proteins did not increase macrophage activity, and the antitumour activity induced by free MDP was not affected by the addition of the proteins, indicating that the protein had no effect on the cell activation and did not interfere with the immunopotentiation by MDP.

Table 2. In-vitro growth inhibitory activity of macrophages pretreated with various MDP-protein conjugates against syngeneic, allogeneic, and xenogeneic tumour cells.

	Macrophage mediated growth inhibitory activity (%) against		
Pretreatment with	Syngeneic R1 fibrosarcoma	Allogeneic P815 mastocytoma	Xenogeneic AH130 hepatoma
Tumour cells alone (no macrophages)	0	0	0
Medium free of MDP	0	0	0
Free MDP (100 μg)	28	30	23
Free MDP (0·1 μg)	5	4	6
MDP-free BSA (14·3 μg)	0	0	0
MDP-free FN (12·1 μg)	0	0	0
MDP-free IgG (12·5 μg)	0	0	0
MDP-free gelatin (10·3 μg)	0	0	0
Free MDP (0·1 μg)+MDP-free BSA (14·3 μg)	5	2	5
Free MDP (0·1 μg)+MDP-free FN (12·1 μg)	4	3	4
Free MDP (0·1 μg)+MDP-free IgG (12·5 μg)	2	2	2
Free MDP (0·1 μg)+MDP-free gelatin (10·3 μg)	3	4	2
MDP (0·1 μg)−BSA (14·3 μg) conjugate	3	2	1
MDP (0·1 μg)−FN (12·1 μg) conjugate	14	12	10
MDP (0·1 μg)−IgG (12·5 μg) conjugate	18	19	20
MDP (0·1 μg)−gelatin (10·3 μg) conjugate	24	25	23
LPS (5 μg)	52	50	44

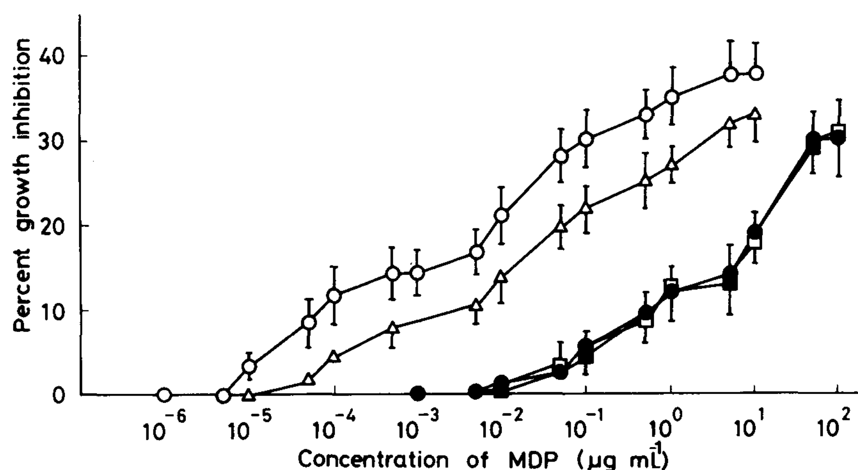

FIG. 4. In-vitro tumour growth inhibitory activity of macrophages pretreated with MDP-protein conjugates with different amounts of MDP; (●) free MDP, (○) MDP-gelatin conjugate, (△) MDP-IgG conjugate, (■) MDP-free gelatin plus free MDP, and (□) MDP-free IgG plus free MDP.

The results of the dose-response study of the MDP-protein conjugates for macrophage activation are given in Figs 4 and 5, which illustrate the antitumour activity of MDP-gelatin or MDP-IgG and MDP-BSA conjugates, respectively. The tumour cells used were R1 cells. As is apparent from Fig. 4, for both free MDP and MDP-protein conjugates, the in-vitro inhibition of tumour growth mediated by the activated macrophages increased with increase in MDP added to the macrophages. The amount of MDP, when conjugated with gelatin or IgG, was 2000 times less than the amount of free MDP needed to induce the activation of the macrophages. The activity induced by the MDP-gelatin conjugate was higher than that induced by the MDP-IgG conjugate over the range of MDP concentrations studied. The percentage growth inhibition appeared to level off when the dose was increased to an amount higher than 10^1 μg per well for the MDP conjugates or 10^2 μg per well for free MDP. Conjuga-

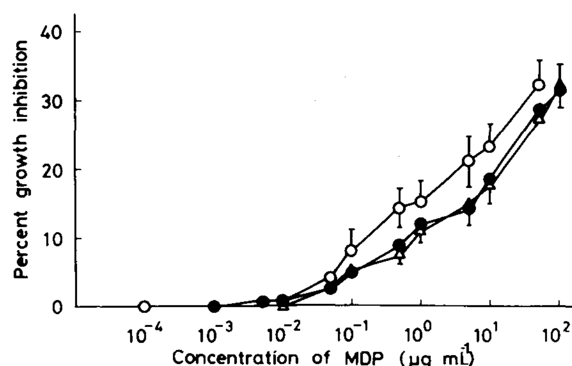

FIG. 5. In-vitro tumour growth inhibitory activity of macrophages pretreated with MDP-BSA conjugates with different amounts of MDP; (●) free MDP, (○) MDP-BSA conjugate, and (△) MDP-free BSA plus free MDP.

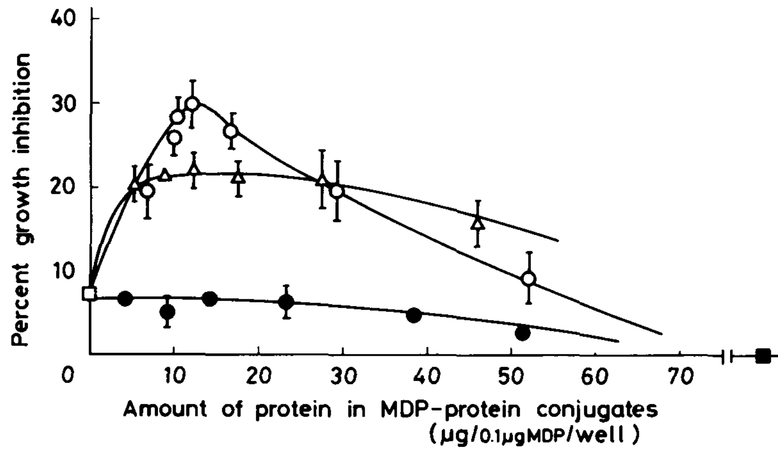

FIG. 6. Influence of the amount of protein on the in-vitro tumour growth inhibitory activity of macrophages pretreated with MDP-protein conjugates; (□) 0·1 μg of free MDP, (■) MDP-free proteins, (○) MDP-gelatin, (△) MDP-IgG, (●) MDP-BSA conjugate containing 0·1 μg of MDP.

Table 3. Influence of the isoelectric point of gelatin on tumour growth inhibitory activity of macrophages pretreated with MDP-gelatin conjugates.

Isoelectric point (pI)	Types	MDP (mg protein)$^{-1}$ (μg)	Percent growth inhibition (%)
4·9	Alkali (bone)	9·60	21
5·4	Acid (bone)	9·55	22
6·4	Acid (skin)	9·78	24
7·0	Acid (bone)	9·62	21
8·5	Acid (bone)	9·70	24
9·0	Acid (skin)	9·59	23

tion of MDP with BSA had little effect in enhancing the antitumour activity of macrophages (Fig. 5).

The effect of the amount of protein conjugated on the in-vitro inhibitory activity of macrophages towards R1 cell growth is shown in Fig. 6. When the protein content in the conjugates was varied with a fixed content of MDP, cell activation became maximal at a protein content of 12 μg for the MDP-gelatin conjugate, indicating that a balanced structure of the conjugate is required for macrophage activation. For the MDP-IgG conjugate, no significant influence was seen on cell activation, while the activity of macrophages pretreated with the MDP-BSA conjugate was decreased with increase in amount of BSA conjugated. The cells were not induced to exhibit any antitumour effect by any MDP-free protein.

Table 3 shows the effect of the isoelectric point of gelatin on macrophage activation by MDP-gelatin conjugates. No difference in the growth inhibitory activity was observed, irrespective of the isoelectric point and type of gelatin.

Time course of macrophage activation

Macrophages were pretreated for different times of 3 to 30 h with 0·1 μg of free MDP or MDP-protein conjugates containing 0·1 μg of MDP. The results (Fig. 7) show that the macrophages were progressively activated with increasing pretreatment time for any MDP conjugates. After 3 h incubation with the MDP-gelatin or MDP-IgG conjugate,

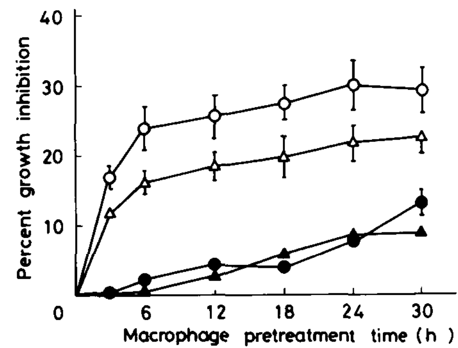

FIG. 7. Time course of macrophage activation by free MDP or MDP-protein conjugates; (●) 0·1 μg of free MDP, (○) MDP-gelatin, (△) MDP-IgG, and (▲) MDP-BSA conjugate containing 0·1 μg of MDP.

there was significant enhancement of the tumour growth inhibitory activity of the cells, whereas free MDP induced no activity. The macrophages were markedly activated by the pretreatment for 8 h with the two conjugates and the activity tended to increase in the subsequent 22 h, the activity of the MDP-gelatin conjugate being higher than that of the MDP-IgG conjugate. However, the BSA conjugate had no effect and the profile was similar to that for free MDP.

Durability of activated state of macrophages

To examine the durability of the activation of macrophages, they were pretreated for 24 h with 0·1 μg or 100 μg of free MDP and MDP-protein conjugates containing 0·1 μg of MDP, then thoroughly washed, and cultured in fresh RPMI-FCS. R1 cells were added 1 to 6 days later to estimate the in-vitro inhibitory effect of macrophages. The activated state was maintained for longer periods by macrophages pretreated with MDP-gelatin or the MDP-IgG conjugates than by those pretreated with free MDP. With the MDP-BSA conjugate, the activated state disappeared within two days, which was similar to that for free MDP.

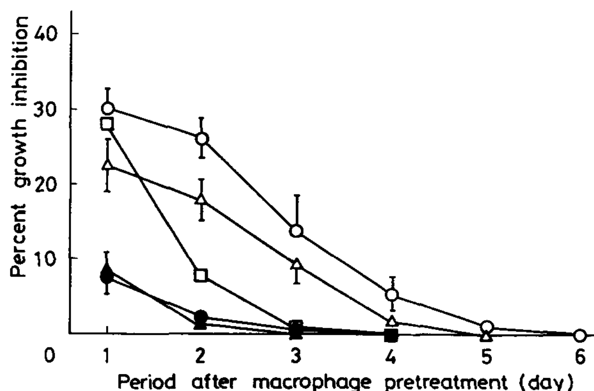

FIG. 8. Decrement patterns of the activated state of macrophages pretreated with free MDP or MDP-protein conjugates; (●) 0·1 μg of free MDP, (□) 100 μg of free MDP, (○) MDP-gelatin, (△) MDP-IgG, and (▲) MDP-BSA conjugate containing 0·1 μg of MDP.

Discussion

A variety of crosslinking agents for the conjugation between drugs and carrier molecules have been reported. Carbodiimides were selected to crosslink the carboxy group of MDP with the amino groups of proteins, because of the mild reaction conditions. The amount of MDP conjugated is controllable by changing the ratio of MDP to protein. With gelatin, the molecular number of MDP bound to one gelatin molecule could be varied from 0·39 to 2·94 by this reaction method (Table 1). Assuming the amino groups of lysine residues present in gelatin molecules are those involved in the coupling to MDP, it may be estimated that 10·6% of the lysine residues are coupled by MDP for the MDP-gelatin conjugate with 2·94 MDP molecules per gelatin molecule.

In the present study, the effectiveness of gelatin as a carrier for the targeting of MDP to macrophages has been shown by the in-vitro antitumour activity of macrophages pretreated with the MDP-gelatin conjugate. Gelatin has the ability to enhance phagocytosis of microspheres by macrophages (Ikada & Tabata 1986) and the inherent propensity to be taken up by macrophages. The opsonic ability of gelatin on macrophage endocytosis may be mediated by FN present in serum. Cultured peritoneal macrophages bind and ingest increased numbers of latex particles, if they are coated with gelatin, in the presence of FN (Doran et al 1980; Gudewicz et al 1980). Moreover, an increase in the pinocytosis of gelatin by macrophages had been observed in the presence of FN (unpublished data). Considering that gelatin is denatured collagen, a catabolic product of connective tissues, it is understandable that it is readily pinocytosed by reticuloendothelial macrophages. We have already reported that microspheres composed of gelatin are phagocytosed by macrophages and that the microspheres activated the antitumour function of macrophages, when the microspheres included MDP (Tabata & Ikada 1987) or interferon (Tabata et al 1988). The water-soluble MDP conjugate with gelatin is an intravenously injectable form preferable to microspheres, the size of which is limited for intravenous administration.

The present results clearly demonstrate that the conjugation with gelatin enables MDP to potentiate the in-vitro inhibitory activity of macrophages for tumour cell growth. Briefly, the MDP-gelatin conjugate was much more efficient than free MDP in respect of the MDP amount (Fig. 4) and time (Fig. 7) required for macrophage activation. The activated state of macrophages pretreated with the conjugate lasted longer than with free MDP (Fig. 8). A similar trend in macrophage activation was found with IgG and FN conjugates, although the activation was less. On the other hand, MDP-BSA conjugate did not lead to the enhancement of macrophage activation, which was similar to that induced with free MDP, when the amount of BSA conjugated was 14·3 μg per 0·1 μg MDP (Figs 7, 8). However, in high amounts the BSA conjugate reduced the antitumour activity, compared with free MDP (Fig. 6). These results may be explained by the difference in the affinity of the proteins for the macrophages. The order of conjugate pinocytosis by the cells was gelatin > > IgG > FN > > BSA (Figs 2, 3) and was in good accordance with that of cell activation. Macrophages are reported not to have MDP receptors on their surface, rather the take-up is by a fluid non-specific pinocytic process (Tenu et al 1982). Gelatin is thus most effective in increasing the amount of MDP internalized by macrophages, while BSA conjugation reduces the activity as a result of suppression of pinocytosis of MDP.

The dependence of the gelatin content in the conjugate on the growth inhibitory activity of macrophages may be interpreted in terms of the protein's affinity for the cells. When the amount of MDP conjugated is low, this is so high as to internalize MDP, resulting in cell activation. However, a higher amount of MDP conjugated to gelatin will hinder the protein's opsonic nature thereby reducing macrophage activation, suggesting that lysine residues present in the gelatin molecules may be necessary for the affinity to the cells. With the MDP-IgG conjugate, little change in the in-vitro inhibitory activity of macrophages for tumour cell growth was observed even when the amount of IgG conjugated was widely altered. It could be that the Fc portions of the IgG molecules, which accelerate the cell's pinocytosis of IgG, are not hindered over the conjugate range studied herein. A decrease in the inhibitory activity of the macrophages with increase in the BSA amount in the MDP-BSA conjugate may be explained in terms of BSA not being susceptible to pinocytosis by the macrophages. The MDP conjugate further suppresses the pinocytosis, resulting in reduced macrophage activation.

These results demonstrate that the efficacy of the MDP-gelatin conjugates for macrophage activation must be due to the high biospecific affinity of gelatin for the cells. It is concluded that the gelatin conjugate is a promising delivery form for targeting MDP to macrophages.

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