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Macrophage Stimulation In Vitro by an Inactive Muramyl Dipeptide Derivative After Conjugation to a Multi-Poly(DL-Alanyl)-Poly(L-Lysine) Carrier

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It has been previously reported that *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP), which represents the minimal structure that can substitute for mycobacteria in Freund complete adjuvant, activated macrophages in vitro and in vivo. In the present study we show that, in contrast to MDP, the nonadjuvant MDP(DD) stereoisomer has no effect on cytostatic activity of thioglycolate-induced macrophages as measured by uptake of [^{3}H]thymidine. However, surprisingly, after conjugation to an inert carrier, multi-poly(DL-alanyl)-poly(L-lysine), this compound activates macrophages in vitro and becomes at least as effective as MDP. It has also been shown in other studies that after conjugation MDP(DD) remained devoid of antigenicity and of adjuvant activity although such a conjugate could increase resistance to infection. It, therefore, appears that there exists no correlation between the structure required for adjuvant activity and the structure required for macrophage activation or for enhancement of nonspecific immunity.

After stimulation by lipopolysaccharides or by Freund complete adjuvant (FCA), macrophages can inhibit the growth of neoplastic or of transformed target cells in vitro (2, 8, 9). Recent studies have shown that *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (hereafter referred to as MDP for muramyl dipeptide) represents the minimal active structure that can substitute for mycobacteria in FCA and enhances both humoral and cell-mediated immune responses (3, 4, 7, 10, 12). It was also reported that MDP-activated macrophages could inhibit the in vitro and in vivo growth of tumor target cells (11, 15). Other investigators have shown that MDP derivatives can stimulate several activities of these phagocytic cells (1, 16, 19, 18, 20, 21) and increase nonspecific resistance to infection (6). In most cases it was observed that derivatives which were devoid of adjuvant activity in vivo were also inactive in the other test systems used. More recently, after conjugation to a synthetic carrier, multi-poly(DL-alanyl)-poly(L-lysine), hereafter referred to as A-L (17), several biological activities of MDP were shown to be enhanced or modified (L. Chedid, C. Carelli, and F. Audibert, RES J. Reticuloendothel. Soc., in press; L. Chedid, M. Parant, F. Parant, F. Audibert, P. Lefrancier, J. Choay, and M. Sela, Proc. Natl. Acad. Sci. U.S.A., in press). After coupling to the same chain, its inactive MDP(DD) stereoisomer acquired the capacity of protecting mice against a *Klebsiella* challenge, although it still lacked antigenicity and adjuvant activity (Chedid et al., Proc. Natl. Acad. Sci. U.S.A., in press). In the present study, MDP and MDP(DD) were conjugated to A-L, and the macrophage-activating ability of these conjugates, hereafter referred to as (MDP)-A-L and [MDP(DD)]-A-L, and of the unconjugated glycopeptides was compared. Macrophage activation was evaluated by measuring the growth inhibition of mastocytoma cells in vitro.

MATERIALS AND METHODS

Synthetic glycopeptides. The following glycopeptides were used: (i) AcMur-L-Ala-D-Glu-NH_2 = MDP; (ii) AcMur-D-Ala-D-Glu-NH_2 = MDP(DD); (iii) multi-poly(D,L-alanyl)-poly(L-lysine) = A-L; (iv) multi-poly[MDP]-poly(DL-alanyl)-poly(L-lysine) = [MDP]-A-L; (v) multi-poly[MDP(DD)]-poly(DL-alanyl)-poly(L-lysine) = [MDP(DD)]-A-L.

The A-L chain was kindly provided by M. Sela. MDP used in these experiments was MDP-Pasteur (Institut Pasteur Production). The synthetic analog MDP(DD) was prepared as previously described (14). These derivatives were conjugated to the multi-poly(DL-alanyl)-poly(L-lysine) (17) according to the following procedure described for the coupling of MDP.

To a solution of 675 mg (1.35 mmol) of AcMur-L-Ala-D-Glu-NH_2 in 12.5 ml of dimethylformamide, 188 mg (1.38 mmol) of *N*-hydroxybenzotriazole, and 575 mg (1.38 mmol) of *N*-ethyl-*N'*(dimethylaminopropyl)-carbodiimide hydrochloride were added. After 1 h, this organic solution was added to an aqueous one (25 ml), adjusted at pH 8.5 with M $NaHCO_3$ of 250 mg of

multi-poly(DL-alanyl)poly(L-lysine) (around 0.135 meq of free amino groups). The reaction mixture was stirred for 24 h at room temperature, diluted with 80 ml of water, and lyophilized. The solution of the resulting powder in 50 ml of water was ultra-filtered through an Amicon membrane PM 10, then lyophilized to give 226 mg of dry material containing 21% (wt/wt) MDP, as determined by amino acid analysis after acid hydrolysis.

Conjugates, referred to as (MDP)-A-L and [MDP(DD)]-A-L, contained, respectively, 21% MDP or MDP(DD) (wt/wt).

Cells. Six- to 8-week-old BDF_1 (C57BL/6xDBA/2) mice were obtained from the Centre National de la Recherche Scientifique (Orléans, France). Macrophages harvested from peritoneal exudates 4 days after an intraperitoneal injection of 1.5 ml of thioglycolate medium (Pasteur Institute) were used as effector cells. The mastocytoma P815 used as target cells were peritoneal ascitic tumor cells recovered from DBA/2.

Preparation of macrophage monolayers. After harvesting, macrophages were washed and seeded at a concentration of 5×10^5 cells in 0.25 ml of growth medium in the wells of Falcon Microtest II plates. The culture medium was 199 medium (Institut Pasteur Production) containing 10% heat-inactivated fetal calf serum (GIBCO Laboratories), 1% glutamine, and antibiotics. The cultures were incubated at 37°C under continuous flow of a mixture of 5% CO_2–95% air. After cells were allowed to adhere for 90 min at 37°C, the nonadhering cells were removed by intensive washing of monolayers with 199 medium.

Macrophage activation test. Various amounts of the different substances tested were incubated with macrophages during 24 h, after which they were removed by washing. 2.5×10^4 P815 mastocytoma cells were then added to the macrophage monolayers and incubated for 24 h. Four hours before the end of the assay, 0.1 μCi of [^{3}H]thymidine (1 Ci/mmol; Saclay, France) was added to each well. The labeled cells were collected on glass fiber disks in a multiple sample collector (Skatron; Flow Laboratories).

Estimation of target cell cytostasis. Results were expressed as growth inhibition percent by the following formula: Percent growth inhibition = $(CR - AR)/(CR \times 100)$, where CR represents radioactivity incorporated by mastocytoma cells cultured on control macrophage monolayers and AR represents radioactivity incorporated by mastocytoma cells cultured on activated macrophage monolayers. P values were calculated by Student's t test.

RESULTS

Influence of compounds on mastocytoma cell growth in the absence of macrophages. In view of detecting a possible direct effect on tumor cells, these substances were incubated with mastocytoma cells in the absence of macrophages for 24 h. Four hours before the end of the experiment, [^{3}H]thymidine was added to the cultures. None of these agents was found to inhibit the growth of mastocytoma cells (Table 1). Moreover, surprisingly, unconjugated MDP(DD) by itself significantly increased thymidine incorporation. The A-L carrier by itself had the same effect but to a lesser degree and only at the lower dosage. This experiment was repeated under the same conditions and gave the same results.

Influence of conjugation on macrophage activation by MDP or by its adjuvant inactive DD stereoisomer. In each experiment, macrophages were pooled from five mice and incubated with amounts varying between 0.3 and 30 μg/0.25 ml of either free MDP, MDP(DD), A-L, or of either of the two conjugates (each

TABLE 1. *Absence of direct cytotoxicity of MDP and analogs on mastocytoma cells*

Analog	[^{3}H]thymidine incorporated (cpm) with the following doses of analog (μg/0.25 ml) added to mastocytoma cell cultures:[a]			
	0.03	0.3	3	30
Expt 1				
MDP	6,897 ± 104	5,974 ± 853	6,096 ± 648	5,749 ± 438
MDP(DD)	10,307 ± 400[b]	12,538 ± 597[b]	10,965 ± 617[b]	9,009 ± 677[b]
(MDP)-A-L	6,309 ± 578	7,270 ± 590	6,338 ± 857	5,531 ± 439
[MDP(DD)]-A-L	6,302 ± 679	6,262 ± 825	6,173 ± 68	6,622 ± 502
A-L	8,963 ± 306[b]	7,813 ± 529[c]	6,441 ± 152	5,809 ± 104
Expt 2				
MDP	8,744 ± 684	9,505 ± 809	9,088 ± 567	8,465 ± 1,193
MDP(DD)	10,062 ± 916	12,627 ± 1,042[b]	12,462 ± 207[b]	13,084 ± 653[b]
(MDP)-A-L	8,711 ± 571	9,763 ± 324	8,443 ± 268	8,749 ± 356
[MDP(DD)]-A-L	9,117 ± 573	8,721 ± 258	9,228 ± 270	10,100 ± 499
A-L	11,582 ± 627[b]	8,992 ± 457	9,208 ± 597	8,779 ± 739

[a] [^{3}H]thymidine incorporated by mastocytoma cells incubated for 24 h with the compounds indicated. Values in unstimulated control culture were 6,626 ± 698 for experiment 1 and 9,546 ± 410 for experiment 2.

[b] $P < 0.001$.

[c] $P < 0.02$.

containing approximately 20% of either glycopeptide).

The data in Fig. 1 represent the average of seven experiments performed under the same experimental conditions. As previously shown (11, 15), MDP activates macrophages in vitro, and small structural changes influence biological activities (1, 16, 18–20); thus, after incubation with the inactive MDP(DD) stereoisomer, effector cells did not inhibit the growth of mastocytoma target cells. As in Table 1, increased incorporation of label was even observed in test samples incubated with MDP(DD) or with the random polymer A-L. However, surprisingly, when MDP(DD) was coupled to A-L it strongly activated macrophages in vitro and became more active than MDP, if one considers that the conjugate contains only 20% of the glycopeptide. The activity of MDP was also maintained (and even increased on a weight basis) when conjugated to the A-L polymer. Care was taken to confirm that, as previously shown with MDP (11), these effects could not be related to the cytolysis of tumor target cells by the activated macrophages. The same percentage of ^{51}Cr release (8%) was found in test samples containing tumor cells incubated with stimulated or control macrophages. In these experiments, the background value was very low, since the spontaneous release was also of the same order (7%).

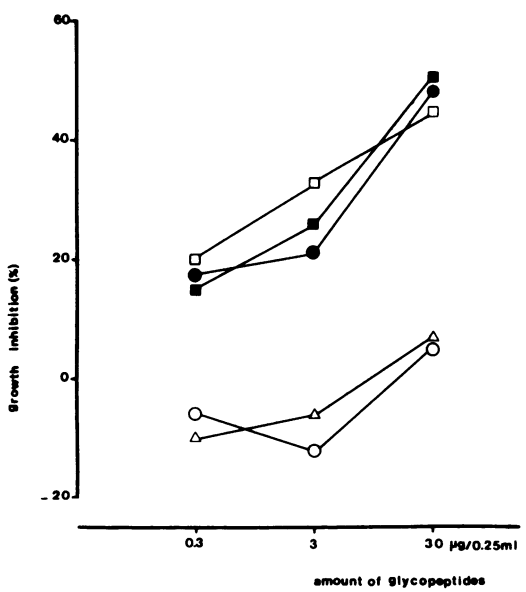

FIG. 1. *Growth inhibition of mastocytoma cells by in vitro-activated peritoneal macrophages. Macrophages were recovered from thioglycolate-treated mice as described in the text and then activated by MDP* (□); *MDP(DD)* (○); *(MDP)-A-L* (■); *[MDP-(DD)]-A-L* (●); *or A-L* (△).

Finally, the possibility that the effect observed could be due to the secretion of cold thymidine by activated macrophages was also ruled out, since the same incorporation of [^{3}H]thymidine by tumor cells was found when they were incubated with supernatants recovered from stimulated or control macrophage cultures.

DISCUSSION

MDP, which represents the minimal structure that can substitute for mycobacteria in Freund complete adjuvant (7), has been previously shown to activate macrophages in vitro and in vivo (11, 15), whereas an inactive derivate *N*-acetyl-muramyl-L-alanine had no effect (11). In these studies, activation was studied within the limited context of growth inhibition of tumor target cells as measured by uptake of [^{3}H]thymidine. Other investigators have confirmed and extended those findings, showing that MDP could stimulate various other macrophage responses. In most cases, strict stereospecificity was required and good correlations were found between chemical structure and biological activities (1, 16, 18–20). In the present study, the macrophage activation capacity of MDP was compared with that of its MDP(DD) stereoisomer which is devoid of adjuvant activity both in vitro and in vivo (5, 13). Results reported here also showed that, in contrast to MDP, the MDP(DD) stereoisomer did not activate macrophages in vitro. The copolymer A-L which can be used as an inert carrier has no detectable stimulatory effect on macrophages. Surprisingly, however, since both MDP(DD) and A-L were unable to stimulate effector cells, the [MDP(DD)]-A-L conjugate activated macrophages in vitro at least as effectively as MDP. When MDP was conjugated to the same carrier, its activity was maintained but less significantly increased.

The effects of these various agents on several biological responses are summarized in Table 2. As can be seen after conjugation, MDP becomes capable of eliciting a positive skin test response in sensitized guinea pigs. Moreover, its immunogenicity, pyrogenicity, and anti-infectious activity against several organisms (*Klebsiella*, *Pseudomonas*, or *Listeria*) are increased. It must be recalled that, in the same study (Chedid et al., Proc. Natl. Acad. Sci. U.S.A., in press), whereas MDP(DD) was unable to increase nonspecific immunity, [MDP(DD)]-A-L protected mice against an infectious challenge, although this conjugate remained devoid of immunogenicity, antigenicity, pyrogenicity and of adjuvant activity. It, therefore, appears that there exists no correlation between the structure required

TABLE 2. *Dissociation between various biological activities of two MDP derivatives and their conjugates*[a]

Compound	Adjuvant activity	Delayed-type hypersensitivity		Anti-infectious activity[d]	Pyrogenicity[e]	Macrophage activation
		Sensitization[b]	Elicitation[c]			
MDP	+	+	−	+	+	+
MDP(DD)	−	−	−	−	−	−
A-L	−	−	−	−	−	−
(MDP)-A-L	+	++	+	++	++	+
[MDP(DD)]-A-L	−	−	−	+	−	+

[a] See Chedid et al., RES J. Reticuloendothel., in press, and Chedid et al., Proc. Natl. Acad. Sci. U.S.A., in press.

[b] Sensitization to conjugated MDP when the free or conjugated derivative has been administered to guinea pigs in FCA.

[c] Elicitation of positive skin test by free or conjugated MDP derivatives administered in FCA to guinea pigs.

[d] Protective activity in mice infected with *K. pneumoniae*.

[e] Pyrogenicity in the rabbit.

for the latter biological activities and the structure required for macrophage activation or enhancement of nonspecific resistance against infection. However, whether these two activities are strictly related remains to be proven. In any case, since nonspecific resistance to infection is produced by a cascade of several complex immune responses, macrophage activation offers a unicellular, and therefore much simpler, system to study the influence of chemical configuration on a cell which participates very strongly in several immune responses. Various parameters such as chain length, degree of substitution, and degree of modification of glycopeptides will be evaluated on cells from good or low responders.

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