Brief Definitive Report

STIMULATING EFFECT OF MDP AND ITS ADJUVANT-ACTIVE ANALOGUES ON GUINEA PIG FIBROBLASTS FOR THE PRODUCTION OF THYMOCYTE-ACTIVATING FACTOR*

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A synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine, or muramyl dipeptide (MDP), is known to be a minimal effective structure for adjuvant activity of cell wall peptidoglycans (1–3). MDP-stimulated macrophages release monokines or interleukin 1 (IL-1) that are mainly concerned with the activation of T cells (4–8).

Recently, Luger et al. (9, 10) described a cytokine, epidermal cell-derived thymocyte-activating factor that is biologically and biochemically similar to IL-1. More recently, Fontana et al. (11) also demonstrated a IL-1-like factor derived from astrocytes and glioma cells stimulated with lipopolysaccharide. Okai et al. (12) showed that murine 3T3 fibroblasts stimulated with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) produced soluble factors that augment the proliferative response of thymocytes to concanavalin A. These findings suggest that epidermal cells, astrocytes, and fibroblasts interact with the immune system by elaborating nonspecific factors.

In the present study, we show that MDP could stimulate skin fibroblasts of the guinea pig to produce thymocyte-activating factor. Furthermore, using four MDP analogues that were shown to be adjuvant-active or -inactive (3), we found a striking parallelism between adjuvant activity of these compounds and the stimulating effect on the production of thymocyte-activating factor from fibroblasts. The fibroblast-derived factor was also shown to be distinct from T cell growth factor (TCGF) or T cell-activating monokines.

Materials and Methods

Animals. Inbred strain 2 and JY-1 guinea pigs that were 1–2 mo old were used. Strain 2 guinea pigs were obtained from Nisseiken Co., Ltd., Tokyo, Japan. JY-1 guinea pigs were kindly supplied from the National Institute of Health (Japan). These guinea pigs were bred in this laboratory.

Fibroblast Cultures. Skin fibroblasts were prepared as described by Wahl et al. (13). Skin explants from strain 2 or JY-1 guinea pigs were placed in 21-cm^2 tissue culture dishes (Falcon Labware, Div. Becton, Dickinson and Co., Oxnard, CA) and the fibroblasts grown to confluency in Dulbecco's modified Eagle's medium (DME) (Gibco Laboratories, Grand Island, NY)

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supplemented with 10% heat-inactivated fetal calf serum (FCS), 7 mM Hepes (Doujin Yakukagaku Institute, Japan), 50 U/ml of penicillin, and 50 μg/ml of streptomycin. These primary fibroblasts were maintained in culture at 37°C in 10% CO_2 and subcultured by treatment of *Bacillus polymixa* protease, 1,000 U/ml of Dispase (Godoshusei Co., Ltd., Japan). The fibroblasts between two and four passages were used in the following experiments. These fibroblasts consisted of >99% fibroblasts, as morphologically determined by a phase contrast microscope, and ≦0.5% nonspecific esterase-positive cells.

Culture Supernatants of Fibroblasts Stimulated with MDP or its Analogues. The confluent cultured fibroblasts (0.8–1 × 10^6 cells in 5 ml/dish) were washed once with Hank's balanced salt solution (HBSS) and refilled with fresh DME. The culture were added with MDP or its analogues at various concentrations and cultured for 72 h. MDP and its analogues were synthesized as described previously (14). The synthetic MDP was kindly provided by Dr. Atsuro Inoue (Daiichi Pharmaceutical Co., Tokyo) or purchased from Protein Research Foundation, Osaka. The culture supernatants were collected by centrifugation (200 *g*, 10 min and 10,000 *g*, 30 min). The culture supernatants of fibroblasts incubated without MDP or its analogues were supplemented with these compounds at the end of the culture and served as the control supernatants. These culture supernatants were dialyzed three times against HBSS, and finally against tissue culture medium 199 (Chiba-ken Serum Inst., Chiba, Japan) and filtered through a membrane filter (pore size, 0.45 μm; Sartorius, Göttingen, West Germany).

Proliferative Response of Thymocytes to PHA. Thymocytes of the guinea pig were suspended at 5 × 10^6 cells/ml in tissue culture medium 199 supplemented with 5% FCS, 7 mM Hepes, 5 × 10^{-5} M 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin. Thymocytes (1 × 10^6 cells/0.25 ml) were added with phytohemagglutinin (PHA-P, Difco Laboratories, Detroit, MI) (2 μg/ml) and cultured in flat-bottomed Falcon Microtest II tissue culture plates in 10% CO_2 in air for 72 h. The culture supernatants of fibroblasts were added to the cultures at 20% (vol/vol) concentration. The cultures were pulsed with 0.2 μCi tritiated thymidine ([^{3}H]TdR, 5 Ci/nmol, RCC, Amersham, England) for the final 18 h of incubation. The [^{3}H]TdR incorporation was measured as described previously (7). The data are expressed as the arithmetic mean of cpm ± standard error of triplicate culture.

Fractionation of the Culture Supernatant on a Sephacryl S-200 Column. The culture supernatant (200 ml) of fibroblasts stimulated with MDP at 5 μg/ml was concentrated and fractionated on a Sephacryl S-200 column (2.6 × 89 cm) in 0.5 M NaCl, 0.1 M Tris-HCl buffer at pH 8.0. Each fraction was dialyzed against HBSS and finally against medium 199, and filtered through a membrane filter.

TCGF Activity. TCGF activity of the culture supernatants was measured as described previously (15). Briefly, PHA-activated lymphoblasts (5 × 10^4 cells/0.25 ml/well) were cultured with or without the culture supernatants in the presence of 0.2 μCi of [^{3}H]TdR for 24 h. The cells were harvested and assayed for the incorporation of [^{3}H]TdR as described previously (7).

Results

The confluent-cultured fibroblasts were stimulated with MDP at varying concentrations (0.001–50 μg/ml) and the culture supernatants were tested for an augmenting effect on the proliferative response of thymocytes to PHA. As shown in Table I, the culture supernatants of fibroblasts incubated without MDP slightly enhanced the proliferation of thymocytes to PHA. The culture supernatants of fibroblasts stimulated with MDP at concentrations of 1–50 μg/ml exhibited marked thymocyte-activating activity. The control culture supernatant of fibroblasts supplemented with MDP at the end of culture exhibited, after dialysis, as low activity as the supernatant of fibroblasts cultured without MDP (data not shown). The culture supernatant of fibroblasts stimulated with MDP did not induce the proliferation of thymocytes in the absence of PHA (see footnote to Table I).

The culture supernatants of fibroblasts stimulated with MDP analogues were tested for thymocyte-activating activity. As shown in Table II, adjuvant-active analogues

TABLE I

Production of Thymocyte-activating Factor from Fibroblasts Stimulated with MDP

Culture supernatants added to the culture	Proliferative response of thymocytes to PHA*
	cpm ± SE
None	8,320 ± 201
Fibroblasts	11,860 ± 966
Fibroblasts stimulated with MDP at	
0.001 μg/ml	12,503 ± 232
0.01	13,027 ± 1,792
0.1	16,945 ± 884
1.0	24,650 ± 233
10.0	24,969 ± 1,748
50.0	23,522 ± 433

* Thymocytes cultured without PHA incorporated 283 ± 73 cpm of [^{3}H]TdR. Thymocytes cultured with only culture supernatant of fibroblasts stimulated with MDP at 50 μg/ml incorporated 339 ± 66 cpm of [^{3}H]TdR.

TABLE II

Thymocyte-activating Activity of the Culture Supernatants of Fibroblasts Stimulated with MDP and its Analogues

Stimulants	Adjuvant activity	Concentration (μg/ml)	[^{3}H]TdR incorporation (Δcpm ± SE)*
Mur-NAc-L-Ala-D-isoGln (MDP)	+	2	15,479 ± 1,713
		20	16,223 ± 6,218
		100	14,759 ± 1,850
Mur-NAc-L-Val-D-isoGln	+	2	15,304 ± 2,093
		20	14,429 ± 4,113
Mur-NAc-L-Ser-D-isoGln	+	2	20,787 ± 1,277
		20	20,247 ± 2,825
Mur-NAc-L-Ala-L-isoGln	−	2	481 ± 1,696
		20	2,007 ± 1,163
Mur-NAc-L-Ala-D-isoAsn	−	2	5,458 ± 3,951
		20	2,155 ± 1,347

* Δcpm = (cpm of culture added with the culture supernatant of MDP-stimulated fibroblasts) − (cpm of culture added with the culture supernatant of nonstimulated fibroblasts to which MDP or its analogues were added at the end of culture).

(Mur-NAc-L-Val-D-isoGln, Mur-NAc-L-Ser-D-isoGln) as well as the MDP effectively stimulated fibroblasts to produce thymocyte-activating factor, whereas adjuvant-inactive analogues (Mur-NAc-L-Ala-L-isoGln, Mur-NAc-L-Ala-D-isoAsn) could not stimulate fibroblasts. Thus, a striking parallelism was found between the adjuvant activity of these compounds and their ability to stimulate fibroblasts to produce thymocyte-activating factor.

Enhanced production of thymocyte-activating factor by MDP-stimulated fibroblasts was seen when they were cultured in the medium supplemented with FCS at concentrations of 1–10%, but not when cultured without FCS (data not shown). The culture supernatant of MDP-stimulated fibroblasts incubated in the presence of 2% FCS was concentrated and fractionated on a Sephacryl S-200 column. As shown in Fig. 1, the thymocyte-activating factor mainly appeared in the molecular weight range of 30,000–60,000 with a major peak activity at ~38,000. Three lower peaks with activity were found in the molecular weight range of >60,000 and 10,000–30,000.

The culture supernatants of fibroblasts were tested for the TCGF activity that

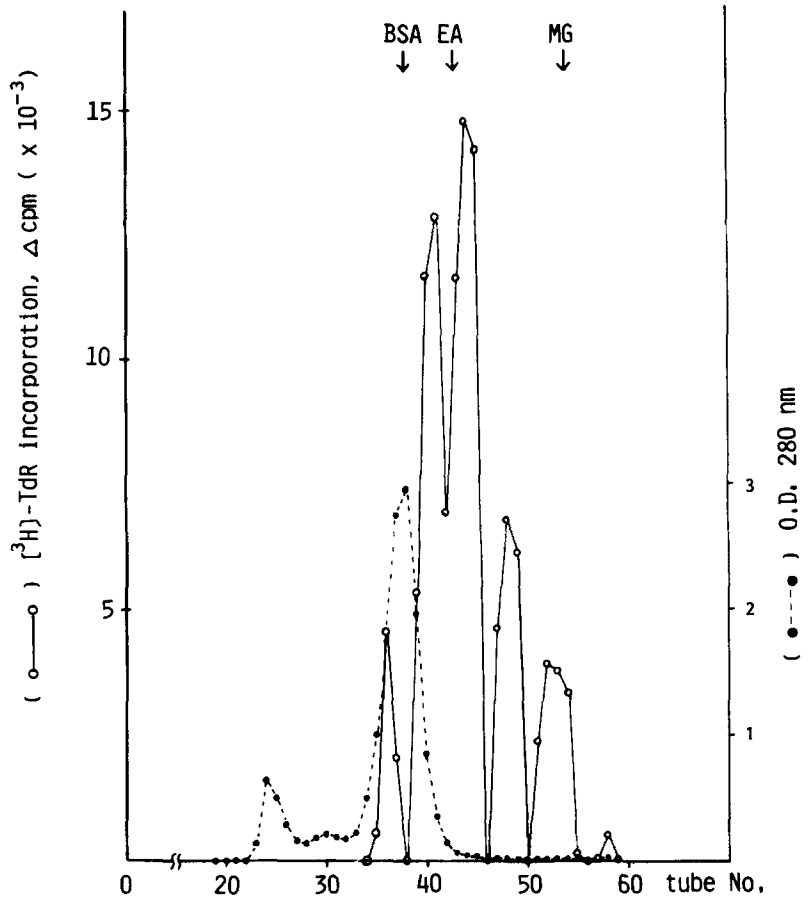

FIG. 1. Fractionation of the culture supernatant of MDP-stimulated fibroblasts on a Sephacryl S-200 column. The supernatant (200 ml) of fibroblasts cultured with MDP at 5 μg/ml were concentrated and fractionated on a Sephacryl S-200 column in 0.5 M NaCl, 0.1 M Tris-HCl buffer at pH 8.0. Each fraction of the eluate was dialyzed and assayed for thymocyte-activating activity. Samples were tested at 20% (vol/vol) concentration. Thymocyte-activating activity of the samples was expressed as Δcpm [^{3}H]TdR incorporation: Δcpm = cpm (thymocytes + PHA + sample) − cpm (thymocytes + PHA). Arrows indicate the position where reference proteins were eluted from the same column: *BSA*, bovine serum albumin; *EA*, egg albumin; *MG*, whale myoglobin.

stimulates the proliferation of PHA-induced lymphoblasts. The culture supernatants of fibroblasts stimulated with or without MDP did not exhibit TCGF activity (data not shown), suggesting that thymocyte-activating factor from MDP-stimulated fibroblasts is distinct from TCGF of the guinea pig that we described previously (15).

Discussion

A synthetic adjuvant, MDP, was shown to stimulate the guinea pig fibroblasts to produce thymocyte-activating factor for, to our knowledge, the first time. The effect of MDP that may be carried over to the culture supernatant of MDP-stimulated fibroblasts would be negligible, since the control culture supernatant of fibroblasts supplemented with MDP at the end of culture exhibited, after dialysis, activity only

as low as the supernatant of fibroblasts cultured without MDP.

Macrophage-derived IL-1 has been shown to exhibit thymocyte-activating activity (16). The confluent culture of fibroblasts used in our study consisted of >99% fibroblasts and ≦0.5% nonspecific esterase-positive cells. If all of the nonspecific esterase-positive cells are macrophages, their concentration is estimated to be $\sim 10^3$ cells/ml. At such low concentration the stimulating effect of MDP on the production of thymocyte-activating factor from macrophages would not be detected, because enhanced production of thymocyte-activating factor from macrophages by MDP stimulation was shown when they were cultured at $>10^5$ cells/ml (H. Iribe and T. Koga, unpublished data). Furthermore, thymocyte-activating factor from MDP-stimulated fibroblasts was mainly found in the molecular weight range of 30,000–60,000 by gel filtration, whereas T cell-activating monokines from guinea pig macrophages were found in two major fractions, one in the 50,000–90,000 mol wt fraction and the other in the 10,000–30,000 mol wt fraction (7, 8). These findings suggest that thymocyte-activating factor detected in the supernatant of MDP-stimulated fibroblasts is not macrophage-derived, but fibroblast-derived.

Recently, it was shown that 3T3 fibroblasts stimulated with TPA released thymocyte-activating factors (12). These factors were found in two major fractions with peak activity at mol wt 30,000 and 10,000, respectively, by gel filtration (12). On the other hand, thymocyte-activating factors from MDP-stimulated fibroblasts were mainly found in the fraction of mol wt 30,000–60,000. It is not known whether this difference is due to the difference of stimulants used or the species difference.

In this study, we showed that adjuvant-active analogues of MDP as well as MDP could stimulate fibroblasts to produce thymocyte-activating factor, whereas adjuvant-inactive analogues could not. Thus a parallelism was found between the adjuvant activity of these compounds and the ability to stimulate fibroblasts to produce thymocyte-activating factor. Previously we also showed a striking parallelism between adjuvant activity of these compounds and the macrophage-stimulating effects on the production of T cell-activating monokines (7). These findings suggest that in addition to the T cell-activating monokine, fibroblast-derived factor contributes to the immunopotentiating effect of the MDP. Furthermore, fibroblast-derived factor may play an important role in the immune response at the sites of chronic inflammation.

Summary

Synthetic muramyl dipeptide (MDP) could stimulate skin fibroblasts of the guinea pig to produce thymocyte-activating factor, which augments the proliferative response of thymocytes to phytohemagglutinin (PHA). Adjuvant-active analogues of MDP also stimulated fibroblasts to produce the factor, whereas adjuvant-inactive analogues failed to do so. Thus a marked parallelism was found between adjuvant activity of these compounds and the stimulating effect on fibroblasts. Thymocyte-activating factor derived from MDP-stimulated fibroblasts was found in the fraction of mol wt 30,000–60,000 by gel filtration on a Sephacryl S-200 column. Furthermore, this factor did not exhibit T cell growth factor activity.

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