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Antitumor Effect of Mycoplasmas

I. Isolation and Purification of Macrophage-activating Factor from Mycoplasmas

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Abstract We previously reported that various species of mycoplasmas served as the second signal for the activation of murine macrophages (Mϕ) following IFN as the first signal. In the present study, chicken mycoplasmas (Myco: *Mycoplasma gallisepticum*) were used. Mϕ-activating capacity of Myco was stable to heating (120°C for 15 min), 1–2 N HCl, or trypsin. The relative molecular mass of the active factor extracted by 0.1 N NaOH was larger than 30 kDa. This factor was effective in activating C3H/HeJ mouse Mϕ, which are known to be very low responsive to LPS, indicating that the Mϕ-activating principle was most probably different from LPS. The activating factor was also extracted with chroloform-methanol and water by the method of Bligh and Dyer. This factor was then submitted to HPLC gel filtration. Active peak fraction which comprised almost solely nucleic acids was obtained. Treatment of the peak fraction with DNase and RNase, however, did not result in the reduction of activity. The principle of the factor is still uncertain and remain to be further analyzed.

INTRODUCTION

Macrophages (Mϕ) can be activated by a large variety of bacteria or bacterial components to lyse tumor cells (Weinberg et al. 1978, 1983, Nagao et al. 1988, Schafer et al. 1988). Recently it has been reported that *Mycoplasma orale* and *M. capricolum* stimulate Mϕ to induce tumor necrosis factor α (TNF-α) production which is, at least partially, responsible for killing of TNF-sensitive tumor cells (Loewenstein et al. 1983, Gallily et al. 1989, Sher et al. 1990). It is now believed, however, that Mϕ are activated under *in vitro* conditions to be unselectively cytotoxic by being treated with two-step stimuli (Pace et al. 1983, Hamilton and Adams, 1987, Hogan and Vogel 1987), for example, IFN first and bacterial lipopolysaccharide (LPS) second.

Independent of the work of Gallily and his colleagues, we documented that various species of mycoplasmas (Myco), whether tumor cell-bound or free form, play a role as a second signal, in the activation of Mϕ to acquire tumoricidal capacity(Uno et al. 1990). In the present study, chicken Myco (*M. gallisepticum*) was used as an representative of Myco, since they lack LPS (Smith et al. 1976), and do not infect humans and mice. The Myco were grown as a pure culture and examined to activate Mϕ in concert with IFN-γ. Then the

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extraction of Mϕ-activating factor from Myco organisms was attempted in order to convince that the effect of Myco on Mϕ was dependent on the specific factor different from LPS. In contrast with the toxicity of bacterial LPS to animals and humans, factor(s) of Myco, which appears nontoxic in nature, may be of therapeutic value in the treatment of cancer.

MATERIALS AND METHODS

Mice

Female C3H/He Slc and C3H/HeJ Jcl mice aged 8–12 wk were obtained from Japan SLC Inc. (Shizuoka) and CLEA Japan Inc. (Osaka), respectively. They were used as donors of peritoneal Mϕ.

Agents and culture media

Recombinant (r) murine IFN-γ ($2x10^7$ IU/mg protein) and r-human IFN-α A/D (IFN-α) were provided by Shionogi Research Laboratories (Osaka) and the Nippon Roche Research Center (Kamakura), respectively. Lipopolysaccharide B (LPS from Escherichia coli 0111:B4) and Brewer's thioglycollate medium (TGC) were purchased from Difco Laboratories (Detroit, MI). DNase-1, RNase-A, 3-[(3-chloramidopropyl) dimethylammonio]-1-propane sulfate (CHAPS), and concanavalin A (Con A) were products of Sigma Chemical Co.(St. Louis, MO). (Methyl-^{3}H) thymidine (^{3}H-TdR: 60 Ci/m mol) was obtained from ICN Radiochemicals (Irvine, CA).

PPLO broth for chicken Myco (bovine heart infusion, 100 g; peptone, 10 g; NaCl, 5 g; D-glucose, 1g; thallium acetate, 0.25 g; water, 1 liter, pH 7.8)was a product of Eiken Chemical Co. Ltd. (Tokyo). RPMI-1640 medium (Nissui) was supplemented with 5 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfomic acid (HEPES), 1.6 mg/ml sodium bicarbonate, and 100 U/ml penicillin and 100 μg/ml streptomycin. Endotoxin-free fetal bovine serum (FBS) was obtained from Bocknek Laboratories Inc. (Toronto, Canada). Endotoxin was undetectable in any of the tissue culture reagents tested by a chromogenic endotoxin-specific assay (Endospacy: Seikagaku Kogyo Co. Ltd., Tokyo).

Preparation of Myco

Chicken mycoplasmas (*Mycoplasma gallisepticum*) were grown in a standing culture in PPLO medium containing 12% horse serum (Flow Lab. Inc., Mclean, VA) for 7–10 days at 37°C. Myco were collected, washed twice with saline by centrifugation (5,000 × g, 30 min), lyophilized, and stored at 4°C until use. We obtained about 1 g of Myco in dry weight from 6 liters of culture medium.

Preparation of Mϕ-activating factor from Myco

Crude or partially purified preparations of Mϕ-activating factor were obtained by means of the following two different methods. 1) Myco were solubilized by being treated with 0.1 N NaOH for 1 hr at room temperature. The fluid was neutralized with HCl, and centrifuged at 105,000 × g for 2 hr. The supernatant was diluted with endotoxin-free distilled water, and a lower molecules of molecular weight 30,000 or less were cut off by filtration through YM-30 membrane (Amicon Corp. Danvers, MA). After repeating this procedure, the residue in a small volume of distilled water was lyophilized. We obtained

100 mg of lyophilizate from 1 g Myco. This preparation is referred to as Myco factor Takema (Mf-T). 2) Extraction of Myco factor was performed by the method of Bligh and Dyer (1959). To Myco (dry weight 1 g) suspended in 3 ml of 50 mM phosphate-buffer (pH 6.8), 12 ml chloroform-methanol (1:2) was added, and the suspension was stirred vigorously. To this, 3 ml of chloroform, 3 ml of 2 M KCl and 60 μl of 250 mM EDTA pH 8.0 were further added with stirring, and the mixture was centrifuged at 3,000 rpm for 10 min. Mϕ-activating activity resided in the water layer but not in the organic solvent layer. The water layer was dialyzed overnight against distilled water and lyophilized. Yield of the lyophilizate was 10-15 mg from 1 g Myco. This preparation was designated as Myco factor Bligh & Dyer (Mf-B).

Ion exchange chromatography was performed with a HPLC column (TSK gel, DEAE-3SW, 7.5 mm ID $\times$ 60 cm) at a flow rate of 1.0 ml/min. The salt concentration of eluent was changed with linear gradient from 5 min to 25 min until 50% of 150 mM NaCl solution containing 1mM CHAPS, and then that was changed to 100% of NaCl solution containing 1mM CHAPS.

Gel-fractionation was performed with a HPLC column (TSK gel, G4000SW, 7.5 mm ID $\times$ 60 cm) at a flow rate of 1.0 ml/min. The eluant was 50 mM tris-HCl buffer pH 7.4, containing 1 mM CHAPS.

Mϕ activation in vitro

Mice were injected ip. with 2.0 ml of TGC, and peritoneal exudate cells (PEC) were harvested 4 days later by lavage with Hanks' balanced salt solution (HBSS) containing 5 U/ml heparin. PEC were washed three times by centrifugation and resuspended in the culture medium. The cells were incubated in flat-bottomed 96-well plates (Coster, Cambridge, MA) at a density of 2×10^5 PEC/0.2 ml/well for 2 hr at 37°C in a CO_2 incubator with humidified 5% CO_2-95% air. Then nonadherent cells were removed by flushing with HBSS, and adherent cells were used as the Mϕ population. Mϕ activation was performed by incubating Mϕ in 0.2 ml of culture medium containing different Mϕ activators, usually for 8 hr. The incubated Mϕ were washed and submitted to cytolytic assay.

Mϕ-dependent cytolytic assay

Target tumor cells, P815 mastocytoma cells from DBA/2 mice, were radiolabeled by incubating 2×10^6 cells with 0.2 μCi/ml ^{3}H-TdR in 10 ml of the culture medium for 10 hr at 37°C in a CO_2 incubator, washed twice in HBSS, and resuspended in the culture medium. The ^{3}H-labeled cells were added (2×10^4 cells/0.2 ml/well) to the Mϕ monolayers, and cultured for 36 hr. At the end of culture, the plates were centrifuged at 200 $\times$ g for 10 min, and 100 μl of cell-free supernatant from each well was placed on a glass filter. After being airdried, the radioactivity in the filters was measured with a liquid scintillation counter, and the percentage of specific ^{3}H-TdR release was calculated by the following formula:

$$[cpm_{exp} - cpm_{spont} \,/\, cpm_{max} - cpm_{spont}] \times 100$$

in which cpm_{max} (maximum release) was the radioactivity of lysate supernatant of ^{3}H-P815 cells treated with 0.7% sodium dodecyl sulfate, cpm_{spont} (spontaneous release) was the

radioactivity of culture supernatant of ^{3}H-P815 cells incubated without Mϕ, and cpm_{exp} (experimental release) was that of those incubated with Mϕ. Target cells were checked regularly for contamination with Myco.

Assay for mitogenicity

Spleen cells were loosened from normal mouse spleens on stainless steel meshes in petri dishes containing cold HBSS, washed twice with HBSS, resuspended in RPMI-1640 medium, and served as a source of following cell populations. Dendritic cells were prepared by the method of Steinman et al. (1979). Nylon wool column-passed, monoclonal anti-Ia antibody (10.2.16) plus rabbit complement-treated cells were used as splenic T cells. Sephadex G-10 column-passed, anti-Thy 1 antibody (HO-13-4) plus rabbit complement-treated cells were used as splenic B cells.

For examining the mitogenicity for T cells, splenic T cells (3×10^5/well) were cultured with graded doses of Myco or Mf-T for 60 hr in the presence of 10^2 dendritic cells. For B cells, splenic B cells (2×10^5/well) were cultured with graded doses of Myco or Mf-T for 60 hr. The incorporation of ^{3}H-TdR into T or B cells proceeded for the last 12 hr of culture. Con A and LPS were used as positive control mitogens for T and B cells, respectively.

RESULTS

Mϕ activation by Myco in the presence of IFN-α

Mϕ were treated with graded doses of heat killed (120°C, 15 min) Myco (Fig. 1A) or LPS (Fig. 1B) in combination with IFN-α for 8 hr, and their antitumor activities were assessed against ^{3}H-P815 cells.

Maximum Mϕ activation in the presence of 10^4 IU/ml IFN-α was attained by the treatment with 10^7/ml Myco or 100 ng/ml LPS, or more, and that in the presence of 10^3 IU/ml IFN-α was observed by the addition of 10^8/ml Myco or 1 μg/ml LPS.

Mϕ activation by Myco in the presence of IFN-γ

Mϕ were treated with graded doses of chicken Myco (Fig. 2A) or LPS (Fig. 2B) in combination with IFN-γ for 8 hr, and their antitumor activity was assessed.

TGC Mϕ were only slightly activated by treatment with Myco or LPS alone; at concentrations of 10^3 μg/ml of Myco or 10^{-2} μg/ml or more of LPS. However, Mϕ were strongly activated when IFN-γ was added. Thus, 10^2 μg/ml Myco or 10^{-3} μg/ml LPS or more, were effective in activating Mϕ in the presence of 1 IU/ml or more of IFN-γ.

Rough estimation of characters of Mϕ-activating substance in Myco

Myco were suspended at 1 mg/ml in RPMI 1640 medium without FBS, and heated by autoclaving at 120°C for 15 min, or treated with NaOH or HCl at room temperature for 24 hr, or with 1 mg/ml trypsin at 37 °C for 24 hr. NaOH-treatment resulted in complete (at final concentration 1.0 N), nearly complete (0.1 N), and partial (0.01 N) solubilization of Myco. HCl at final concentration of 1 N did not solubilize Myco. Myco were partially degraded by being treated with trypsin. The Myco suspensions thus treated, or their supernatant prepared in the case of NaOH-treatment by centrifugation at 105,000 $\times$ g for 2 hr, were neutralized, if necessary, and submitted for Mϕ activation test at a final dilution

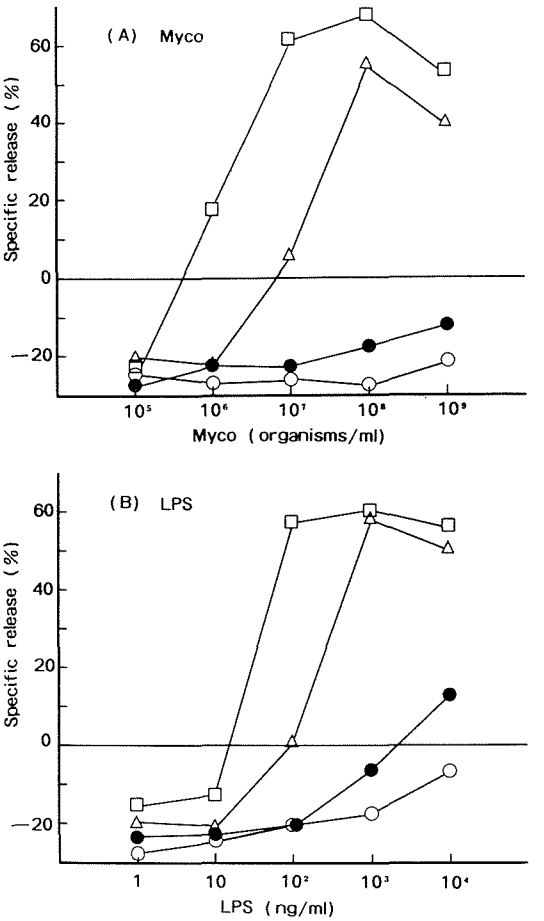

Fig. 1. Activation of Mϕ by Myco (A) or LPS (B) in concert with IFN-α. TGC-elicited Mϕ (2 x 10^5 /well) were treated with graded doses of Myco or LPS plus IFN-α at concentrations of 10^4 IU (□), 10^3 IU (△), 10^2 IU (○), or 0 IU (●)/ml for 8 hr, and cultured with 2 x 10^4 ^{3}H-P815 cells for 36 hr to measure the specific ^{3}H-release from P815 cells. Each symbol represents the mean of triplicate cultures. Standard errors are included within the vertical length of symbols.

of 1:100. Results are shown in Fig. 3A.

The Mϕ-activating capacity of Myco was resistant to heat, 0.01-0.1 N NaOH, 1 N HCl, and trypsin, but deteriorated by 1 N NaOH. In the case of NaOH-treatment, the capacity was found to release from Myco bodies to the medium partially (0.01 N) or almost completely (0.1 N). The supernatant of 0.1 N NaOH-treated Myco was filtered through membrane filters at different MW cutoffs (MWCO, Amicon Corp., Danvers, MA) at 1 kg/cm^2, and MW of the Mϕ-activating factor was estimated (Fig. 3B). The Mϕ-activating factor of Myco did not pass through Amicon YM30 (30 kDa-MWCO) filter. A small part of activity passed through UK10 (100 kDa-MWCO), but a large part remained in the residue. Thus, it seems likely that MW of the Mϕ-activating factor is around 100 kDa.

Activation of Mϕ by Myco factor

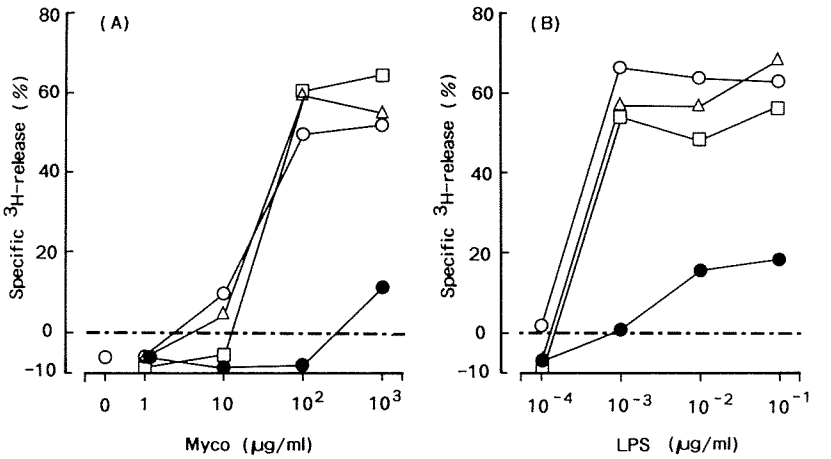

Fig. 2. Activation of Mϕ by Myco (A) or LPS (B) in concert with IFN-γ. TGC-elicited Mϕ (2 x 10^5 /well) were treated with graded doses of Myco or LPS plus IFN-γ at concentrations of 10^2 IU (○), 10 IU (△), 1.0 IU (□), or 0 IU(●)/ml for 8 hr, and cultured with 2 x 10^4 ^{3}H-P815 cells for 36 hr to measure the specific ^{3}H-release from P815 cells. Each symbol represents the mean of triplicate cultures. Standard errors are included within the vertical length of symbols.

Two kinds of crude extracts which activates Mϕ in concert with IFN-γ, were obtained from Myco by NaOH extraction (Mf-T) and by a method of Bligh and Dyer (Mf-B). In order to further purify the Mϕ-activating factor from Myco, we compared in the titer to activate Mϕ between Mf-T and Mf-B in the presence of 10 IU/ml IFN-γ. C3H/He (Fig. 4A) and C3H/HeJ (Fig. 4B) mice were employed, the latter of which are known to be low responsive to LPS (Ruco and Meltzer 1978, Boraschi and Meltzer 1979). As shown in Fig. 4A, the necessary minimum concentration of Mf-T, Mf-B and LPS for activating Mϕ was 10^1, 10^0 and 10^2 μg/ml respectively. Thus, the titer of Mf-B was 10 times higher than Mf-T, though more than 10^2 times lower than that of LPS on the basis of dry weight. The responsiveness to LPS of C3H/HeJ Mϕ was much lower than that of C3H/He Mϕ. However, no difference was found between these two strains of mice in the responsiveness of Mϕ to Mf-T and Mf-B (Fig. 4 A,B). In C3H/HeJ also, the titer of Mf-B was 10 times higher than that of Mf-T.

Mitogenic activity of Myco and Mf-T on T and B cells

Since some workers reported the mitogenic activity of certain species of Myco (*M. arthritidis* and *M. hyorhinis*) for T and B cells (Proust et al. 1985, Atkin et al. 1986, Cole et al. 1990), we examined whether Myco in this experiment and Myco factor (Mf-T) have similar activity. As shown in Table 1, Myco slightly stimulated the T cell proliferation, but the mitogenic activity was incomparably lower than that of Con A. Mf-T showed insignificant mitogenic effect on T cells at any doses under examination. A similar trend was also observed in the experiment for B cells. As shown in Table 2, LPS strongly stimulated B cell ploliferation, but Myco did slightly and Mf-T insignificantly.

Attempt to purify Mϕ-activating factor

As Mf-B is still a crude extract from Myco, we tried to further purify the

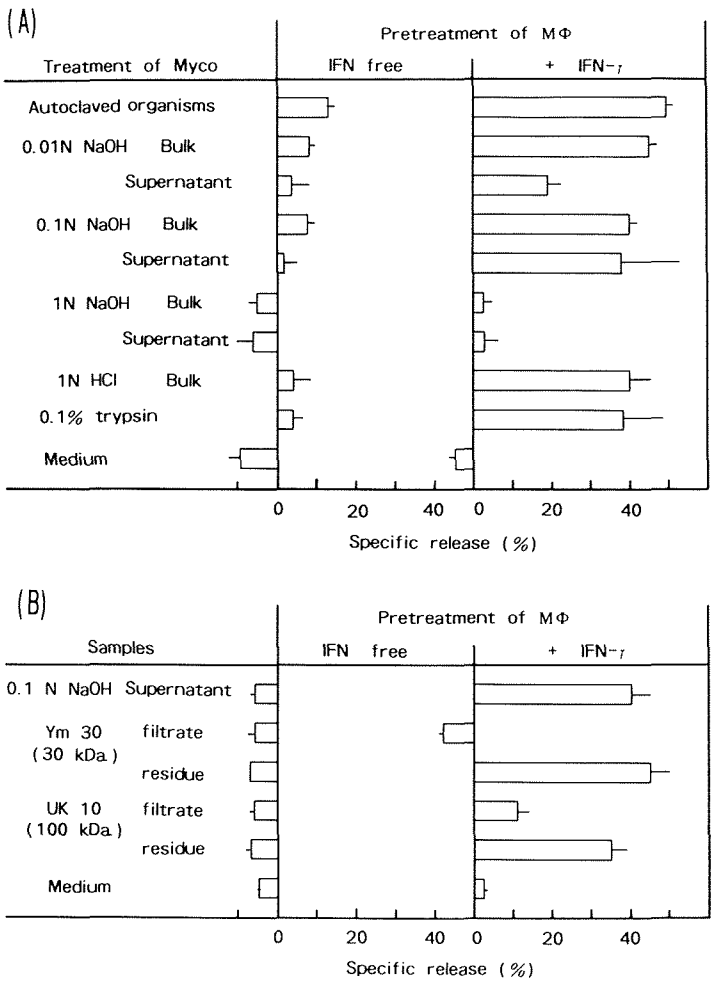

Fig. 3. Characterization of Mϕ-activating factor of Myco. A) Myco (1 mg/ml) were treated with NaOH, HCl, or trypsin. B) The supernatant of 0.1 N NaOH-treated Myco was filtered through Amicon ultrafiltration membranes as described in Materials and Methods. TGC-elicited Mϕ (2×10^5 /well) was pretreated with these Myco samples at 10% concentration with or without IFN-γ for 8 hr at 37°C, washed, and cocultured with ^{3}H-TdR-labeled P815 cells for 36 hr. The specific ^{3}H-release from P815 cells was quantified. Each horizontal column and bar represent the mean of triplicate cultures and standard errors, respectively.

Mϕ-activating factor. Lectin columns (Con A-agarose column, WGA-Ultrogel column and RCA-agarose column) and a hydroxylapatite HPLC column were ineffectual in the purification of Mϕ-activating principle from Mf-B, since the principle had no affinity to them. The Mf-B activity was trapped by ion exchange HPLC, but this was not useful for purification, because the active fraction was eluted by 150 mM NaCl solution with most of charged substances (Fig. 5). Therefore, Mf-B was fractionated with a HPLC gel filtration column. Each fraction was assessed for its ability to activate Mϕ at a final dilution of 10^{-3} in the presence of 20 IU/ml IFN-γ. As shown in Fig.6A, a peak of activity was recovered from fractions at around 19 min elution, which coincided with the first peak of 280 nm

absorbance. In this peak, Lowly's method and amino acid analysis showed no protein, gas chromatography no lipid, and orcin and resorcinol tests no sugar. Only nucleic acid was detected by gas chromatography. In order to digest the nucleic acid, 5 mg Mf–B was pretreated with 2 μg of DNase 1 and 2 μg of RNase A for 1 hr at 37°C, and gel–filtered (Fig. 6B). The 280 nm absorbance at around 19 min elution mostly disappeared without loss of Mϕ–activating capacity. This indicates that the Mϕ–activating principle forms a very small part of Mf–B. The chemical nature of the principle remains to be clarified.

DISCUSSION

We documented previously that various species of Myco were able to activate Mϕ primed by IFN–α to make them antitumor effector cells (Uno et al. 1990). In this study, we have shown that chicken Myco, as well as LPS, activate Mϕ in concert with either IFN–α or IFN–γ (Fig. 1 and Fig. 2). The Mϕ–activating capacity of Myco is quite resistant to heat, acid, or trypsin, and relatively labile in alkaline solutions. These results suggest that Mϕ activation by Myco is caused by some substance contained in Myco organisms, and that

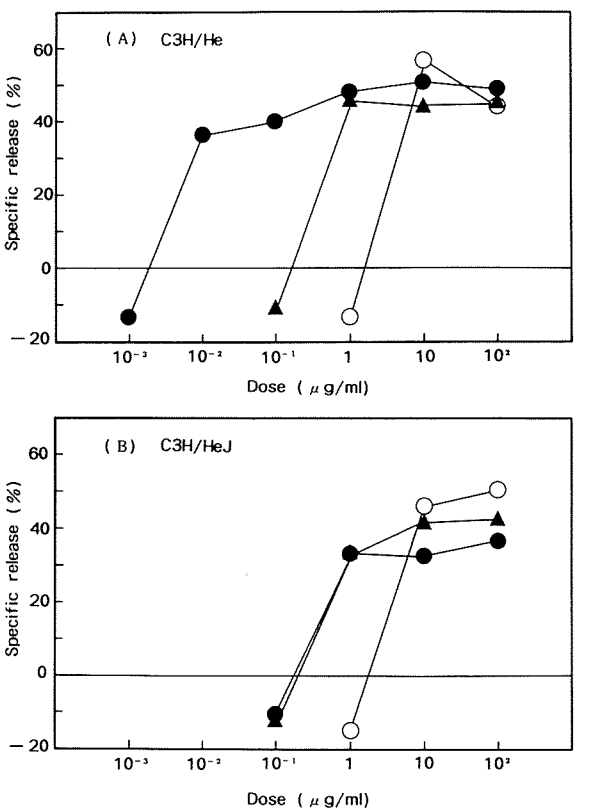

Fig. 4. Comparison between C3H/He and C3H/HeJ Mϕ in the responsiveness to LPS, Mf–T, and Mf–B. TGC–elicited Mϕ of C3H/He (A) or C3H/HeJ (B) mice were treated for 8 hr with LPS (●), Mf–T (○), or Mf–B (▲) in the presence of 10 IU/ml IFN–γ and their cytolytic activity of them was quantified. Each symbol represent the mean of triplicate cultures. Standard errors are included within the vertical length of symbols.

the Myco need not be alive.

The Mϕ-activating activity of Myco was found in the two preparations of Myco-extracts, Mf-T and Mf-B. The principle does not seem to be LPS on the basis of following reasons. 1) Both Mf-T and Mf-B can activate to similar extent between Mϕ of C3H/He and those of C3H/HeJ mice (Fig. 4), the latter known to be genetically low responders to LPS. 2) Polymyxin B, which can neutralize LPS did not affect the activity of them (data not shown). 3) LPS was undetectable in them even by a highly sensitive chromogenic endotoxin-specific assay (data not shown). 4) Smith et al. (1976) reported that all species of acholeplasmas and anaeloplasmas under examination and one species of mycoplasma contained LPS, but most species of mycoplasmas including *M. gallisepticum* were devoid of it. These results suggest that the Mϕ-activating principle in Mf-T and that in Mf-B may be identical with each other in spite of the titer of Mf-B is ten times high than that of Mf-T (Fig. 4). Thus we fractionated Mϕ-activating factors from Mf-B with an HPLC gel filtration column (Fig. 6).

In the fractions with high activity, a large amount of nucleic acid was found by gas

Table 1. Effect of Myco and Mf-T on spleen T cell proliferation.[a]

Dose (μg/ml)	^{3}H-TdR uptake ($\times 10^{-2}$ cpm)[b]	
	Myco	Mf-T
100	14.7	2.2
50	11.7	1.9
25	9.5	1.8
12	6.0	1.9
medium	1.2	
Con A (2 μg/ml)	852.6	

[a] Splenic T cells (3×10^5) were cultured for 60 hr with Myco, Mf-T, or Con A in the presence of 10^2 dendritic cells per dish of 96-well plates. ^{3}H-TdR uptake proceeded for the last 12 hr of culture.

[b] Values are means of triplicate cultures in which standard deviations were $<10\%$.

Table 2. Effect of LPS, Myco, and Mf-T on spleen B cell proliferation.[a]

Dose (μg/ml)	^{3}H-TdR uptake ($\times 10^{-3}$ cpm)[b]		
	Myco	Mf-T	LPS
30	13.6	0.6	65.8
10	5.7	0.9	58.0
3	3.1	1.0	48.9
1	2.0	0.9	39.2
medium	0.6		

[a] Splenic B cells (2×10^5) were cultured for 60 hr with Myco, Mf-T, or LPS in 96-well plates. ^{3}H-TdR uptake proceeded for the last 12 hr of culture.

[b] Values are means of triplicate cultures in which standard deviations were $<10\%$.

chromatography. This is reminiscent of the report by Alexander and Evans that synthetic double stranded RNA activates Mϕ to be tumoricidal under both *in vitro* and *in vivo* conditions (1971). However, the Mϕ-activating principle in Mf-B did not seem to be nucleic acid, since the enzymatic digestion of nucleic acid in Mf-B did not affect the activity of Mf-B . The possibility that the factor is protein, lipid, or sugar also seems to be quite low, as mentioned above. In addition, the fact that the activity of Myco remains after treatment with heat, 0.1 N NaOH, 1 N HCl, or trypsin rules out the possibility that the Mϕ-activating principle is a protein. Some workers have reported that the mitogen for T and B cells secreted by Myco is protein (Proust et al. 1985, Atkin et al. 1986, Cole et al. 1990). This and the fact that Mf-T has no mitogenic activity (Table 1 and 2) seem to indicate that the Mϕ-activating principle and the mitogen in Myco are different. Thus, we have not determined yet the identity of Mϕ-activating principle in Mf-B. Some other technical approaches to identify it will be required.

In the present study, we extracted Myco's Mϕ-activating factor from Myco themselves. Our preliminary experiment shows, however, that the factor can be prepared also from the supernatant of Myco-culture. The yield of the factor from the medium is much higher than from Myco themselves. It is necessary, however, to determine whether the factors from Myco themselves and from the culture supernatant are identical.

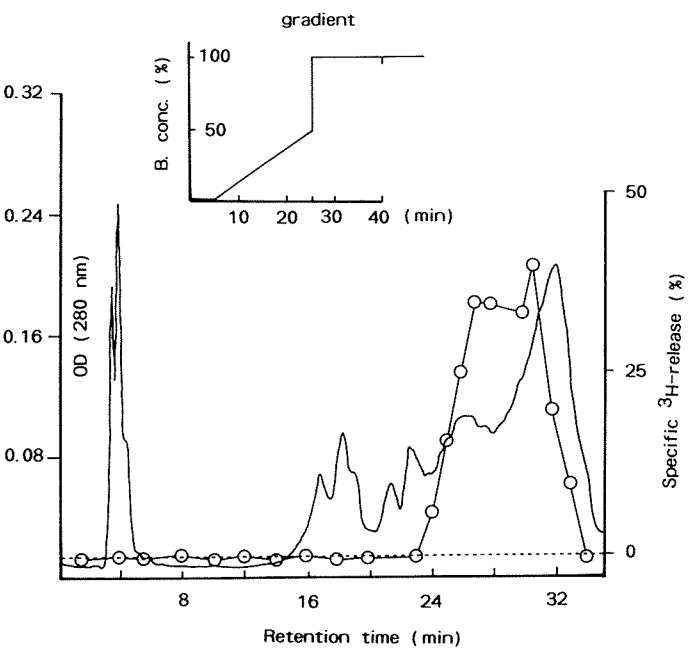

Fig. 5. Failure in the separation of Mϕ-activating factor from Mf-B by ion exchange HPLC (DEAE-3SW, 7.5 mm ID x 60 cm). Chromatography was performed at a flow rate of 1.0 ml/min. The salt concentration of eluent was linearly gradiented from 5 min to 25 min until 50% of 150 mM NaCl solution containing 1 mM CHAPS, and then that was changed to 100% of NaCl solution containing 1 mM CHAPS. Absorbance (–) was read at 280 nm, and the Mϕ-activating activity, expressed in terms of specific ^{3}H-release from ^{3}H-P815 cells (◯), of each fraction was assessed by the cytotoxicity of Mϕ treated with 10^{-3} diluted elutes plus 20 IU/ml IFN-γ. Each symbol represents the mean of triplicate cultures. Standard errors are included within the vertical length of symbols.

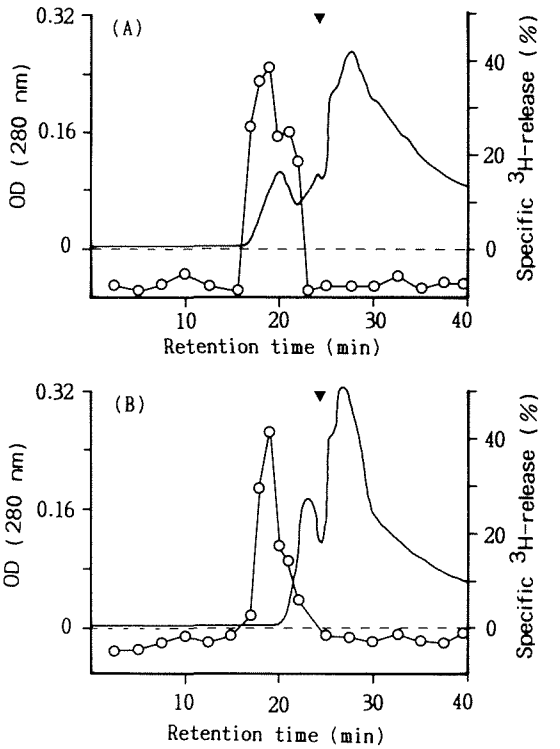

Fig. 6. Separation of Mϕ-activating factor from untreated (A) and nuclease-treated (B) Mf-B by gel filtration (G4000 SW, HPLC column, 7.5 mm ID x 60 cm) eluted with 50 mM tris-HCl buffer containing 1 mM CHAPS pH 7.4 at a flow rate of 1 ml/min. Absorbance (–) was read at 280 nm, and the Mϕ-activating activity, expressed in terms of specific ^{3}H-release from ^{3}H-P815 cells (○), of each fraction was assessed by the cytotoxicity of Mϕ pre-treated with 10^3-fold diluted elutes plus 20 IU/ml IFN-γ. Each symbol represents the mean of triplicate cultures. Standard errors are included within the vertical length of symbols. ▼: Elution point of a 100 kDa standard protein.

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