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Antitumor Effect of Mycoplasmas

II. Effects of Mf-B (Macrophage-activating Factor from Mycoplasmas) on Macrophage Functions

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Abstract Mycoplasmas (Myco), in concert with IFN-γ, were effective in activating macrophages (Mϕ) to be tumoricidal. Mϕ-activating factor (Mf-B) was extracted from Myco with chloroform-methanol and water (Mf-B). Mf-B was also effective in activating Mϕ in the presence of IFN-γ. Mf-B induced a tumor necrosis factor (TNF) and IL-1 in Mϕ. these effects of Mf-B to Mϕ may be mediated by a Gi-like receptor, because intracellular Ca^{++} levels of Mϕ was increased transiently by Mf-B and this reaction was blocked by pre-treatment of IAP.

The cytotoxicity of Mϕ activated by Mf-B or LPS in the presence of IFN-γ, was not affected by the addition of anti-TNF antiserum. On the other hand, depletion of L-arginine or addition of D-arginine in the medium at effector phase blocked the cytotoxicity. The cytotoxicity of Mϕ activated by Mf-B plus IFN-γ was dependent on L-arginine in the culture, suggesting that L-arginine metabolites are involved in Mϕ cytotoxicity.

INTRODUCTION

Macrophages (Mϕ) play important roles in self-defense systems. It is well known that mouse exudate Mϕ can be activated to cytotoxic under *in vitro* conditions by being treated with two-step stimuli (Meltzer et al.,1982), for example, IFN first and bacterial lipopolysaccharide (LPS) second. These activated Mϕ can kill a wide variety of tumor target cells but cannot kill normal cells (Fidler,1985). There has been much discussion on the killing mechanisms of activated Mϕ. Thus, TNF (Decker et al.,1987; Ichinose et al.,1988), IL-1 (Ichinose et al.,1988), hydrogen peroxide (Mavier & Edgington,1984) and cytolytic protease (Adams,1980a; Adams et al.,1980b) have been envisaged to play roles in Mϕ cytotoxicity.

Recently, we have reported that mycoplasmas (Myco) replace the role of LPS to activate Mϕ in concert with IFNs and activate Mϕ even from an LPS-hyporesponsive strain (C3H/HeJ) of mice (Takema et al.,1990). Furthermore, we obtained the Mϕ-activating factor preparation (Mf-B) from a pure cultured organisms of *M. gallisepticum* (Takema et al.,1990). The Mϕ-activating principle in Mf-B was apparently

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different from LPS according to the following reasons: 1) Myco species we used was reported not to contain LPS (Smith et al. 1976), 2) LPS was undetectable in Mf-B by endotoxin-specific assay, 3) Mf-B was effective for C3H/HeJ Mϕ, and 4) polymyxin B did not neutralize the Mf-B activity.

Independent of our work, Gallily et al (1989) and Sher et al.(1990) have reported that Myco by themselves activate Mϕ to be cytotoxic, and that whole organisms or their membrane components were strong inducer of TNF. They speculate that the TNF may be a main cytotoxic factor of Mϕ activated by Myco (Sher et al.,1990).

Recent studies have suggested that the cytotoxicity of activated Mϕ is dependent on L-arginine metabolites, such as reactive radical nitric oxide (Hibbs et al.,1987a; Hibbs et al.,1987b; Stuehr & Nathan,1989). Then, in this paper, we examined the cytotoxic mechanism of Mϕ activated by Mf-B and LPS in relation to arginine metabolism.

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.

Agents and culture media

Recombinant (r) murine IFN-γ ($2x10^7$ IU/mg protein) was provided by Shionogi Research Laboratories (Osaka). r-Human tumor necrosis factor-α (TNF-α) and r-human IL-1α were provided by the Research Institute of Dainippon Pharmaceutical Co. (Osaka). Rabbit anti-mouse tumor necrosis factor (anti-TNF antiserum, $6x10^6$ U/ml) was a product of Genzyme (Boston, MA). Lipopolysaccharide B (LPS from Escherichia coli 0111:B4) and Brewer's thioglycollate medium (TGC) were purchased from Difco Laboratories (Detroit, MI). Actinomycin D, L-arginine, and D-arginine were products of Sigma Chemical Co.(St. Louis, MO). (Methyl-^{3}H) thymidine (^{3}H-TdR: 60 Ci/mmole) was obtained from ICN Radiochemicals (Irvine, CA).

PPLO (pleuropneumonia-like organisms) 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). Eagle's MEM (Nissui Seiyaku Co., Tokyo), which contains 126 μg/ml L-arginine and 60 μg/ml kanamycin, was supplemented with 0.2 mM L-glutamine, 5 mM hydroxyethylpiperazine ethanesulfonic acid (HEPES) and 1.6 mg/ml sodium bicarbonate. L-arginine-free MEM was prepared by supplementing a four amino acids-free MEM (MEM-5: Nissui) with 52 μg/ml L-leucine, 15 μg/ml methionine, and 32 μg/ml phenylalanine. RPMI-1640 medium (Nissui) was supplemented with 5 mM HEPES, 1.6 mg/ml sodium bicarbonate, and 100 U/ml penicillin and 100 μg/ml streptomycin. Endotoxin-free fetal bovine serum (FBS), which contains about 60 μM of L-arginine, 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 Mϕ-activating factor from Myco

Chicken mycoplasmas (*Mycoplasma gallisepticum*) were used as a representative of

Myco because of their inability to infect humans or mice. They 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 x g, 30 min), and lyophilized. We obtained about 1 g of Myco in dry weight from 6 liters of culture medium. 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 chloroform, 3 ml 2 M KCl and 60 μl 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).

Mϕ activation in vitro

Mice were injected i.p. 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 x 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 x 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 x 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 x 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 putative IL-1 and TNF

IL-1 activity of Mϕ culture supernatant was determined by the co-mitogenicity for thymocytes responding to PHA (phytohemagglutinin P, Difco). C3H/HeJ thymocytes (5 x 10^5) were cultured with the Mϕ supernatant in RPMI-1640 medium containing 5 x 10^{-5}M 2-ME, 10% FBS and 5 μg/ml PHA in 96-well tissue culture plates for 72 hr. ^{3}H-TdR uptake was proceeded for the last 6 hr of culture.

TNF activity was determined by the cytotoxicity for L-M cells according to by the method of Nakano et al.(1986) with minor modifications. Thus, 5 x 10^4 L-M cells (a strain of L929 cells, CCL-1.2, ATCC)/well were incubated in 96-well plates in the presence of test samples and 1 μg/ml actinomycin D at 37°C for 24 hr in a CO_2 incubator. After incubation, the remaining L-M cells were fixed with glutaraldehyde, stained with 0.05% methylene blue, and washed. The cell-bound dye was extracted with 0.33 N HCl, and absorbance of the extract was measured at 665 nm with Multiscan MCC (Flow Laboratories, Mclean, VA). The TNF activity (U/ml) of test samples was evaluated by reference to that of the simultaneously run r-human TNF-α, the titer of which had been determined as the reciprocal of the dilution which caused 50% inhibition of cell growth.

Estimation of intracellular calcium levels in macrophages

Suspensions of TGC Mϕ (2 x 10^6/ml) were loaded with Fura-II/AM (Sigma Chemical Co.) and changes in fluorescence were monitored by fluorescence spectrophotometer (RF-5000, Shimadzu,Co., Kyoto) at an excitation wavelength of 339 nm and emission wavelength of 500 nm.

RESULTS

Change of intracellular calcium levels in Mϕ

Adams and Hamilton (1987) reported that lipid A, which is second messenger of Mϕ activation, raised the level of intracellular calcium level in Mϕ from a basal level of 80 nM to almost 600 nM within 30 sec. We examined the change of intracellular calcium level of Mϕ after being treated with 100 μg/ml Mf-B. As shown in Fig.1A, Mf-B caused rapid and marked rise of intracellular level of calcium. This change was blocked entirely by the

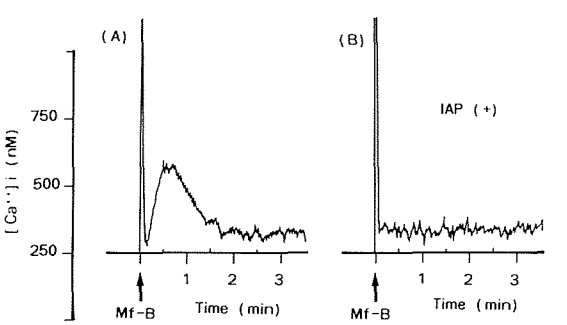

Fig. 1. Effect of Mf-B on intracellular Ca^{++} level in Fura II-loaded Mϕ.Suppressions of normal (A) or IAP pretreated (B) TGC Mϕ (2x10^6/ml) were loaded with Fura II/AM and the changes in fluorescence was monitored. (A) Cells were stimulated with 100 μg/ml Mf-B. (B) Cells were pretreated with 50 ng/ml IAP for 3 hr at 37°C before stimulation with Mf-B.

Table 1. IL-1 activity in culture medium of MØ stimulated with Mf-B or LPS.[a]

Agents	IL-1 activity in supernatant of MØ culture (U/ml) Concentrations (μg/ml) of agents						
	0	10^{-3}	10^{-2}	10^{-1}	1.0	10	10^{2}
Mf-B			ND[b]	ND	ND	2.7	2.8
LPS	ND	ND	2.4	4.8	2.9	5.6	

[a] TGC MØ (1×10^{6}/1.0 ml/well) incubated with graded doses of Mf-B or LPS for 24 hr at 37°C. IL-1 activity in the culture medium was determined as the comitogenic activity in PHA response of C3H/HeJ thymocytes. IL-1 activity (U/ml) of test samples was evaluated by reference to that of the simultaneously run r-human IL-1α.

[b] Not detectable: <0.5 U/ml.

Table 2. TNF activity in culture medium of MØ stimulated with Mf-B or LPS in the presence or absence of IFN-γ.[a]

Agents	IFN-γ	TNF activity (U/ml) Concentrations (μg/ml) of agents						
		10^{-3}	10^{-2}	10^{-1}	1.0	10.0	10^{2}	(anti TNF)[b]
Mf-B	−			ND[c]	ND	3.4	2.0	(ND)
Mf-B	+			ND	2.0	23.6	27.3	(ND)
LPS	−	1.5	8.4	23.8	18.2	46.3	16.4	(ND)
LPS	+	2.2	17.0	39.1	32.7	147.9	120.0	(ND)

[a] TGC MØ (1×10^{5}/0.2 ml/well) were incubated with graded concentrations of Mf-B or LPS in the presence or absence of IFN-γ (20 IU/ml) for 6 hr at 37°C. TNF activity in the culture medium was measured as the cytotoxicity for L-M cells. Results represent the mean of triplicate cultures, individual values of which were included within $\pm 10\%$ of the mean.

[b] In parentheses, results of experiments are shown in which anti-TNF antiserum (0.5% final concentration) was added to the culture medium of MØ treated with 10^{2} μg/ml of agents.

[c] Not detectable (<0.5 U/ml).

pretreatment of Mϕ with 25 ng/ml islet activate protein (IAP, Sigma Chemical Co.,) which is an inhibitor of guanine nucleotide regulatory protein (Fig. 1B). Furthermore, IAP pretreatment tended to blocked the activation of TGC Mϕ by Mf-B plus IFN-γ (data not shown). These results suggest that Mf-B stimulates Mϕ through some receptor on the membrane.

Induction of IL-1 in Mϕ stimulated with Mf-B

TGC Mϕ from C3H/He mice were cultured with graded doses of Mf-B or LPS in 24 well multi dish plate at the density of 1 x 10^{6}/1.0 ml/well for 24 hr at 37°C. Table 1 shows that LPS was a moderately strong inducer and Mf-B was a weak inducer of IL-1. Although, more than 10 μg/ml of Mf-B was needed to induce IL-1, only 10^{-2} μg/ml LPS induced IL-1.

Induction of TNF secretion

Mϕ from C3H/He mice were cultured with LPS or Mf-B in the presence or absence of IFN-γ for 6 hr, and the culture supernatant was titrated for cytotoxicity of L-M cells. Table 2 shows that LPS was a strong inducer and Mf-B a weak inducer of the cytotoxic

Table 3. Ineffectiveness of anti-TNF antiserum in reducing cytotoxicity of MØ activated with IFN-γ plus Mf-B or LPS.[a]

IFN-γ plus agents	Anti-TNF	Cytotoxicity of MØ (% specific ^{3}H-release)[b] Concentrations (μg/ml) of agents					
		0	10^{-2}	10^{-1}	1.0	10.0	10^2
Mf-B	−		−5.9	−1.2	1.8	72.9	65.9
Mf-B	+		−6.2	−7.5	−1.9	67.0	62.8
LPS	−	−1.0	54.5	58.5	59.5	56.6	64.2
LPS	+	−6.2	63.0	67.2	51.6	65.9	54.6

[a] MØ (2×10^5/0.2 ml/well) were incubated with rIFN-γ (20 IU/ml) plus graded doses of Mf-B or LPS for 8 hr, washed, and cocultured for 36 hr with ^{3}H-TdR-labeled P815 cells in the presence or absence of anti-TNF antiserum (0.5% final concentration).

[b] Radioactivity released to the culture medium was measured to quantify MØ cytotoxicity. Results represent the mean of triplicate cultures, individual values of which were included within ±10% of the mean.

factor. The amount of the factor secreted by Mφ was augmented by the further addition of IFN-γ. This factor seems to most probably be TNF, because the cytotoxic activity of culture supernatant of Mφ treated with 10^2 μg/ml of Mf-B or LPS in the presence or absence of IFN-γ, was entirely abolished by the addition of anti-TNF antiserum at a final concentration of 0.5% in the L-M cell culture medium.

Effect of anti-TNF antiserum on the cytotoxicity of activated Mφ

Gallily et al.(1989) reported that the cytotoxicity of Mφ in the presence of Myco was partially abolished by the addition of anti-TNF. Thus, we tested the effect of anti-TNF antiserum (final concentration 0.5%) on the cytotoxicity of activated Mφ by IFN-γ plus Mf-B or LPS (Table 3). In contrast to the result in Table 2, the cytotoxicity of activated Mφ against P815 cells was not affected by anti-TNF antiserum. Coinciding with this, the culture supernatant of activated Mφ was not cytotoxic for P815 cells (data not shown).

Requirement of L-arginine for effector function of activated Mφ, but not for activation of Mφ

We examined the effect of L-arginine content in the medium on the cytotoxicity of Mf-B-or LPS-activated Mφ in the activation phase (Fig. 2A) and in the cytotoxic effector phase (Fig. 2B). TGC Mφ were incubated with graded doses of Mf-B or LPS in the presence of IFN-γ (20 IU/ml) in the medium containing different concentrations (0.2-0.02 mM) of L-arginine. After 8 hr, Mφ were cocultured with ^{3}H-TdR-labeled P815 tumor cells for 36 hr in normal MEM medium supplemented with 10% FBS to measure the cytotoxic activity. As shown in Fig. 2A, Mφ were activated to be cytotoxic by Mf-B plus IFN-γ or LPS plus IFN-γ irrespective of the concentration of L-arginine. In both cases of Mf-B and LPS, the Mφ activity induced in L-arginine-deficient MEM medium, which actually contained 0.006 mM L-arginine derived from 10% FBS, was almost equal to that in MEM containing 0.2 mM L-arginine.

The effect of L-arginine on the cytotoxicity of activated Mφ was shown in Fig. 2B. TGC Mφ (2×10^5/well) were activated by graded doses of Mf-B or LPS in the presence of 20 IU/ml IFN-γ for 8 hr in normal MEM medium. Then, the cytotoxicity of activated Mφ against P815 target cells was measured in MEM medium containing varying concentrations

of L-arginine. The cytotoxicity of Mϕ, either Mf-B-activated or LPS-activated, tended to decrease as the concentration of L-arginine was reduced (Fig. 2B). Actually, Mϕ cytotoxic activity was not measurable in media containing 0.05 mM or less of L-arginine, in contrast with the relatively high cytotoxicity in the presence of 0.2 mM or 0.1 mM L-arginine. This indicated that the presence of L-arginine in the medium was required for the manifestation of cytotoxic activity against tumors.

Addition of D-arginine in the medium block the effector function of activated Mϕ, but not affect activation

In order to confirm the L-arginine dependence of cytotoxic activity of Mϕ, D-arginine as an antagonist of L-arginine was added to the culture. As shown in Fig. 3A, the addition of D-arginine to the MEM containing 0.2 mM L-arginine, gave no effect on the activation of Mϕ by Mf-B or LPS.

On the other hand, the tumoricidal activity of Mϕ activated by Mf-B or LPS decreased as the concentration of D-arginine increased. The addition of 20 mM

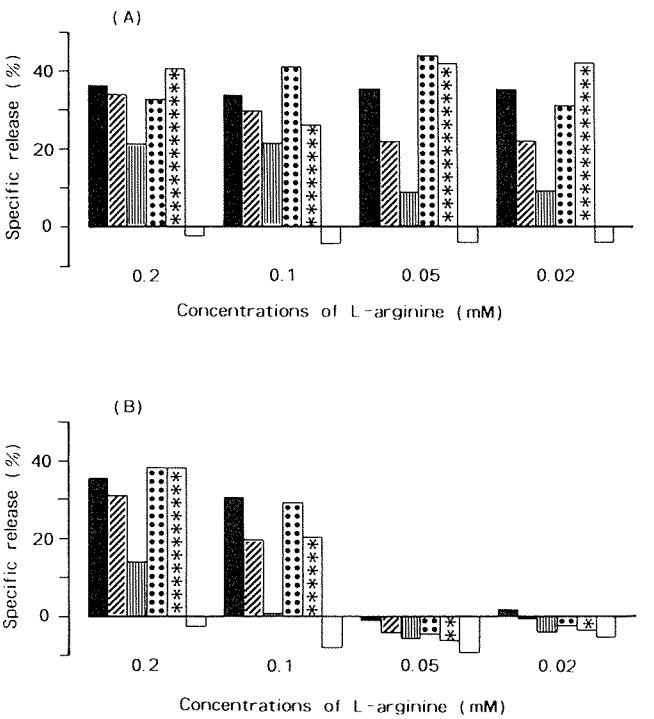

Fig. 2. Effect of depletion of L-arginine from the medium on Mϕ cytotoxicity. TGC Mϕ ($2x10^5$ /well) were activated by LPS(■, 1 μg/ml; ▨, 0.1 μg/ml; ▥, 0.01 μg/ml) or Mf-B (⊡, 100 μg/ml; ⊡, 10 μg/ml; □, 0 μg/ml) in the presence of 20 IU/ml IFN-γ for 8 hr, and cultured with $2x10^4$ ^{3}H-TdR-labeled P815 cells for 36 hr. Tumoricidal activity was determined by measuring the radioactivity released into culture medium. (A) Mϕ were activated in MEM containing indicated concentrations of L-arginine and measured the tumoricidal activity in normal MEM medium (containing 0.6 mM L-arginine). (B) Mϕ were activated in normal MEM medium and measured the tumoricidal activity in MEM containing indicated concentrations of L-arginine. The results are the means of triplicate assays.

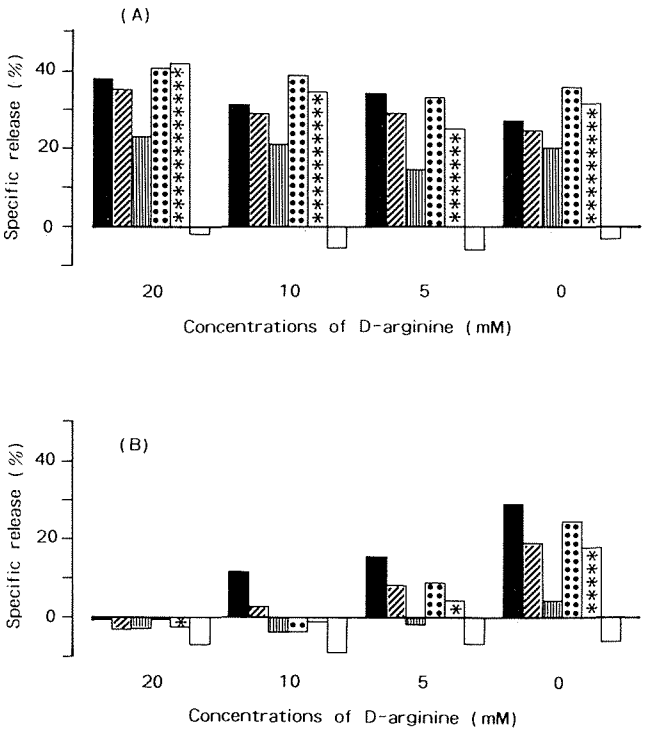

Fig.3. Effect of addition of D-arginine to the medium containing 0.2 mM L-arginine on Mϕ cytotoxicity. TGC Mϕ ($2x10^5$/well) were activated by LPS (■, 1 μg/ml; ▨, 0.1 μg/ml; ▥, 0.01 μg/ml) or Mf-B (⊡, 100 μg/ml; ⊡, 10 μg/ml; □, 0 μg/ml)in the presence of 20 IU/ml IFN-γ for 8 hr, and cultured with $2x10^4$ ^{3}H-TdR-labeled P815 cells for 36 hr. Tumoricidal activity was determined by measuring the radioactivity released into culture medium. (A) Mϕ were activated in MEM containing 0.2 mM L-arginine and indicated concentrations of D-arginine, and their tumoricidal activity in normal MEM medium (containing 0.6 mM L-arginine) was determined. (B) Mϕ were activated in normal MEM and measured their tumoricidal activity in MEM containing 0.2 mM L-arginine and indicated concentrations of D-arginine was determined. Results are the means of triplicate assays.

D-arginine to MEM (containing 0.2 mM L-arginine) perfectly blocked the Mϕ function and 10 mM D-arginine blocked nearly completely (Fig. 3B). These results seem to indicate that Mϕ activated by Mf-B plus IFN-γ, as well as those activated by LPS plus IFN-γ, metabolize L-arginine to manifest cytotoxic activity.

Affect of L-arginine depletion on the TNF production of Mϕ

The cytotoxicity of activated Mϕ was abolished by the depletion of L-arginine in the medium. The reduction of tumoricidal activity of activated Mϕ in the L-arginine depleted medium, may be associated with the reduction of TNF production. Thus, we examined whether depletion of L-arginine from the medium decrease the TNF production of Mϕ in the presence or absence of IFN-γ. As shown in Table 4, however, TNF production of activated Mϕ did not affected by depletion of L-arginine. TNF production was augmented by the addition of IFN-γ irrespective of the presence or absence of L-arginine.

Table 4. Effect of L-arginine depletion on the TNF production of MØ.[a]

IFN-γ	Agents	Medium[b]	TNF activity (U/ml) Concentrations (μg/ml) of agents				
			10^{-3}	10^{-2}	10^{-1}	1.0	10
−	Mf-B	MEM(+)			ND[c]	ND	ND
		MEM(−)			ND	ND	ND
	LPS	MEM(+)	ND	6.0	6.7	9.5	
		MEM(−)	ND	4.4	4.7	3.9	
+	Mf-B	MEM(+)			ND	6.2	10.4
		MEM(−)			ND	10.9	27.7
	LPS	MEM(+)	ND	61.4	21.9	18.6	
		MEM(−)	ND	37.6	20.1	17.3	

[a] TGC MØ (1×10^5/0.2 ml/well) were incubated with graded concentrations of Mf-B or LPS in MEM(+) or MEM(−) medium in the presence or absence of IFN-γ (20 IU/ml) for 6 hr at 37°C. TNF activity in the culture medium was measured as the cytotoxicity for L-M cells. Results represent the mean of triplicate cultures, individual values of which were included within ±10% of the mean.

[b] MEM(+): normal Eagle's MEM medium (containing 0.6 mM L-arginine) supplemented with 10% FBS. MEM(−): L-arginine deficient Eagle's MEM medium supplemented with 10% FBS.

[c] Not detectedable (<0.5 U/ml).

DISCUSSION

As reported in a previous paper, chicken Myco, as well as LPS, activate Mϕ in concert with IFN-γ, and Mϕ-activating factor (Mf-B) can be extracted from Myco cultured in PPLO broth. Furthermore, it was demonstrated that Mf-B was not LPS and Mf-B was not contaminated with LPS (Takema et al. 1990).

In this paper, effects of Mf-B on various Mϕ functions of Mϕ activated by Mf-B or LPS are documented. Prpic et al. (1987) reported that both LPS and lipid A in combination with IFN-γ could induce Mϕ to become tumoricidal and bactericidal, and only lipid A, but not LPS, increased intracellular Ca^{++} levels in Fura II-loaded Mϕ. As shown in Fig. 1A, Mf-B stimulated Mϕ to raise the intracellular Ca^{++} level. This phenomenon seem to give an evidence that Mf-B could be distinguished from LPS in addition to the augment described previously (Takema et al. 1990). Jakway and Defranco (1986) reported that pertussis toxin, IAP, inhibit the B cell and Mϕ responses to bacterial LPS. In the present study, pretreatment of Mϕ with IAP prohibited the increase of intracellular Ca^{++} level. These results strongly argues that activation of Mϕ by Mf-B is mediated by a Gi-like receptor-effector coupling protein. In addition, Mf-B induced Mϕ to secrete immunoregulatory substance, IL-1 (Table 1). But the ability of Mf-B to stimulate IL-1 secretion from Mϕ was lower than LPS.

Mf-B stimulate Mϕ, though weakly, to deliver a cytotoxic factor against L-M cells, most probably TNF. This finding is compatible with that Gallily et al.(1989) who reported that heat-killed Myco strongly induced TNF in Mϕ. Recently, Sher and Gallily (1990) documented that the membrane preparation of Myco is also a strong autonomous inducer of TNF in Mϕ. In our experiment, however, the ability of Mf-B to induce TNF was not as strong as that of their membrane preparation. The induction of TNF by Mf-B was

augmented by the addition of IFN-γ (Table 2), in agreement with the results of Boutler et al. (1986) who studied TNF induction by LPS. Taking these results together, we surmise that Myco membranes contain, in addition to a direct TNF inducer, the inducer of some co-factor which lacks in Mf-B.

As mentioned above, Mϕ activated by Mf-B with IFN-γ deliver TNF, but this does not seem to cause the cytotoxicity for P815 cells, which are generally known as TNF-α-resistant cells. Decker et al.(1987) reported that cell-associated TNF plays an important role in the killing of TNF-sensitive tumor cells by activated Mϕ and, furthermore, Peck et al.(1989) reported that cell surface TNF can kill TNF-resistant tumor cells. However, the results in Table 3 shows that the cytotoxicity of Mϕ activated with IFN-γ plus Mf-B or LPS was not reduced by the addition of anti-TNF antiserum at the concentration enough to neutralize the plausible TNF delivered from activated Mϕ. Therefore, we should consider another mechanism concerning the cytotoxic mechanism of activated Mϕ for P815 cells.

Hibbs and his colleagues (1987a & 1987b) have documented that L-arginine metabolites are potent effector molecules of cytotoxic Mϕ preactivated with IFN-γ plus LPS. As shown in Fig. 2, the cytotoxicity of Mϕ preactivated with IFN-γ plus either Mf-B or LPS is diminished by reduced concentrations of L-arginine in the medium at the effector phase. Similarly, D-arginine which is a specific substrate analog, inhibited the tumoricidal action of Mϕ activated by Mf-B or LPS (Fig. 3). On the other hand, L-arginine depletion had no effect on the activation of Mϕ by Mf-B or LPS (Fig. 2 and Fig. 3). These results strongly suggest that Mϕ activated by IFN plus LPS or Mf-B deliver L-arginine metabolites with cytotoxicity (L-arginine dependent effector mechanism) against target tumor cells.

In addition, L-arginine depletion in the medium is not accompanied with the decrease of TNF secretion from activated Mϕ (Table 4). Therefore, it may be envisaged that activated Mϕ can manifest at least two cytolytic mechanisms independently of each other. Higuchi et al.(1990) reported that TNF and L-arginine dependent mechanisms of activated Mϕ act synergistically to destroy the TNF-resistant cell line.

Thus, it can be surmised that L-arginine dependent effector mechanism is important in the Mϕ-cytotoxicity, and that TNF is directly effective in deteriorating TNF-sensitive tumor cells and indirectly as a co-factor of L-arginine metabolites.

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