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Synergistic induction of cytotoxicity in macrophages by murine interferon-γ and biological response modifiers derived from microorganisms

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Summary. The ability of recombinant murine interferon-gamma (rMuIFN-γ) to activate murine macrophages with or without several biological response modifiers (BRM), including synthetic muramyl dipeptide derivatives (MDPs), was investigated. Mouse peritoneal macrophages were activated by rMuIFN-γ alone to the cytostatic state, but not the cytolytic state. Other BRM as well as bacterial lipopolysaccharide (LPS), including a lyophilized preparation of an attenuated strain of *Streptococcus hemolyticus*, a cell wall skeleton of bacillus Calmette-Guerin and synthetic MDPs, were highly active in generating the synergism with rMuIFN-γ. Macrophages were endowed with the cytolytic activities by combinations of rMuIFN-γ and MDP-Lys(L18); the combination of 100 U/ml of rMuIFN-γ with 10 ng/ml of MDP-Lys(L18) was sufficient to induce cytolytic activities in macrophages. The synergism was observed when the macrophages primed with rMuIFN-γ were treated with LPS or MDP-Lys(L18), but not when the sequence of treatment was reversed. The cytotoxicity of macrophages induced by rMuIFN-γ with MDP-Lys(L18) was suppressed by priming with MDP-Lys(L18). The suppressive effect was also observed by priming with LPS in combinations of rMUIFN-γ and LPS. The reason for the suppression of macrophage activation by priming with LPS and MDP-Lys(L18) is at present unknown.

Introduction

It is known that certain lymphokines activate macrophages to the tumoricidal state, and are thus designated as macrophage activating factors (MAF) [3, 12]. Interferon-gamma (IFN-γ) is said to be a MAF in view of its biological properties, including the stimulation of nonspecific tumoricidal activities of macrophages [29], and the induction or production of Ia antigen expression [33, 34], of plasminogen activator [11], and of hydrogen peroxide [17]. Biosynthetic and biochemical similarities of MAF and IFN-γ have also been demonstrated [9, 24, 28, 35]. However, induction by IFN-γ alone of the tumoricidal activity of macrophages is as yet unclear [13, 25, 30, 35, 37]. On the other hand, it is unquestionable that IFN-γ, in combination with lipopolysaccharide (LPS), stimulates macrophages to kill tumor cells [2, 25, 30, 35, 37]. Recently, Saiki and Fidler [26] reported that the combination of IFN-γ and muramyl dipeptide derivative (MDP) was effective in inducing the cytotoxicity of macrophages. We also have studied partners for IFN-γ other than LPS that are applicable to clinical use. The biological response modifiers (BRM), including LPS [8, 32], whole bacteria [1, 18, 38], bacterial cell wall skeleton [31], microorganic products [16], and MDPs [36] are well known to activate macrophages to the tumoricidal state in vitro and/or in vivo. Accordingly, this paper deals with the effect of combinations of whole bacteria, bacterial cell wall skeleton, or synthetic MDPs with recombinant murine IFN-γ (rMuIFN-γ) on macrophage activation to kill tumor cells.

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Materials and methods

Animals. Inbred (specific pathogen-free) 6 to 12-week old male mice of strains DBA/2NCrj and C57BL/6NCrj (Charles River Japan Inc., Kanagawa, Japan) were used. Animals were quarantined for at least 7 days before use, and given standard rodent chow and drinking water ad libitum. They were maintained under semibarrier conditions.

Tumor cell lines. P815 mastocytoma was donated by T. Tokunaga, Department of Cellular Immunology, National Institute of Health, Tokyo, Japan. Lewis lung adenocarcinoma (3LL) was obtained from Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo. P815 cells and 3LL cells were maintained in the peritoneal cavity of DBA/2NCrj mice and intramuscularly in C57BL/6NCrj mice in accordance with the passage protocol of National Cancer Institute, USA, respectively. Primary cultures of these cells were used for the testing.

IFN and other agents. The rMuIFN-γ (Genentech Inc., San Francisco, Calif., USA) [10] was diluted with RPMI 1640 medium containing 10% v/v fetal calf serum (FCS, Mitsubishi Chemical Ind., Ltd., Tokyo) and 100 μg/ml sodium kanamycin (KM, Banyu Pharmaceutical Co., Ltd., Tokyo) just before use. The medium was ascertained to be free of endotoxin (<0.05 ng/ml) by the Limulus amebocyte assay using the Limulus Test Wako (Wako Pure Chemical Ind., Ltd., Osaka, Japan), and designated as the culture medium. The antiviral activity of each preparation of IFN was measured by the plaque assay using Ly cells infected with vesicular stomatitis virus, and expressed in

laboratory units (U) with reference to that of the natural type of murine IFN-α/β. The active concentrations were measured in comparison with those of the reference reagents provided by the National Institute of Health, USA. The specific activity of rMuIFN-γ was 5.2×10^6 U/mg of protein. As for the other BRM, LPS of *Escherichia coli* 0111:B4 (Westphal method, Difco Lab., Detroit, Mich., USA), cell wall skeleton of bacillus Calmette-Guerin (BCG-CWS, Ribi Immunochem Research, Inc., Mont., USA), a lyophilized preparation of an attenuated strain of *Streptococcus hemolyticus* (OK432, Chugai Pharmaceutical Co., Ltd., Tokyo), a periodate-modified mannoglucan from the culture filtrate of *Microellobosporia grisea* (MGA), staphylococcal enterotoxin A (SEA), and synthetic MDPs [5, 14, 19–23], MDP-Lys(L18), L18-MDP, and MDP(MeAla)-Lys(L18) (Daiichi Seiyaku Co., Ltd., Tokyo) were used. These agents were also dissolved or suspended in the culture medium just before use.

Macrophages. The peritoneal resident cells were harvested by washing out the abdominal cavities of normal mice of each strain with RPMI 1640 medium containing 2% v/v FCS, which is hereafter termed washing medium. Then 100-μl quantities of the cell suspension (2.5×10^6 cells/ml) were inoculated into each well of 96-well microtiter plates (Flat bottom, Nunc Inc., Roskilde, Denmark). Following a 2-h incubation of the plates, they were rinsed with warm washing medium three times, and the cells adhering to the bottom of each well were designated peritoneal macrophages. More than 90% of the cells thus prepared were identified as macrophages by Wright's staining.

Cytostasis and cytolysis assay. The monolayers of macrophages purified using the above procedure were incubated for 20 h with culture medium containing rMuIFN-γ with or without other agents, and then rinsed with warm washing medium. For cytostasis assay, 200 μl of the suspension of target cells was poured into each well (1×10^4 cells/well) and the plates were incubated for a further 20 h. Then 20 μl of 25 μCi/ml of ^{3}H-thymidine (^{3}H-TdR, 20.0 Ci/mmol, New England Nuclear, Boston, Mass., USA) was added to each well, and 3 h later, the radioactivity of the tumor cells was measured using an Aloka liquid scintillation spectrometer LSC-672 (Aloka Co., Ltd., Tokyo). The cytostasis ratio (R) was calculated according to the following formula: $R = (TAM - AM)/(TM - M) \times 100$ where TAM is the cpm of the mixed culture of tumor cells and activated macrophages; AM, the cpm of activated macrophages; TM, the cpm of mixed culture of tumor cells and nonactivated macrophages; and M is the cpm of nonactivated macrophages. The terms activated and nonactivated macrophages mean the macrophage preparations cultivated with the medium containing rMuIFN-γ or other agents, and those cultivated with medium free of these agents, respectively.

The cytolytic activities of macrophages were assayed by incubation for 20 h with target cells labeled with 150 μCi of ^{51}Cr ($Na_2{}^{51}CrO_4$, New England Nuclear) for 1 or 2 h. The radioactivity (cpm) of 100 μl of culture supernatant was measured using an Aloka Auto Well Gamma System, Model JDC-751 (Aloka Co., Ltd.). The percent release of ^{51}Cr (R) was calculated by the following formula: $R = (T - S)/(Max - S) \times 100$ where T is the cpm of the test sample; S, the cpm of spontaneous release from the labeled cells; and Max is the cpm of samples treated with a detergent, sodium dodecyl sulfate.

In addition to the above simultaneous treatment of macrophages with rMuIFN-γ and other BRM, sequential treatment with these agents was carried out. The macrophage preparations mentioned above were exposed for 12 h to 100 U/ml of rMuIFN-γ, followed by the 12-h exposure to 25 ng/ml of LPS or to 100 ng/ml of MDP-Lys(L18). Exposure to these agents was also carried out in reverse sequence. The incubation time was selected on the basis of the results of preliminary examinations that showed that macrophages were sufficiently activated by simultaneous treatment with rMuIFN-γ and LPS or MDP-Lys(L18) for 12 h. After incubation at 37 °C for the indicated durations, these macrophage preparations were rinsed thoroughly with warm washing medium, and subjected to cytostasis or cytolysis assay. These experiments were repeated at least twice and a representative experiment is given.

Statistical analysis. Statistical significance was determined by Student's *t*-test.

Results

Enhancement of macrophage activation by BRM

Macrophages were treated with combinations of rMuIFN-γ with several kinds of BRM, OK432, BCG-CWS, and

Table 1. Synergistic macrophage activation by combinations of rMuIFN-γ and other BRMs against P815 cells

Doses of[a]		^{3}H-TdR uptake by P815 cells with macrophages exposed to rMuIFN-γ and another BRM (%)				
rMuIFN-γ (U/ml)	BRM (ng/ml)	LPS	BCG-CWS	OK432	MGA	SEA
0	100	NT[b]	69.2± 9.0[c]	NT	NT	137.7±16.3
0	1,000	81.7±10.8	110.7±19.3	118.3±24.7	100.4±22.2	162.3±28.5
0	10,000	NT	286.8±51.0	136.5± 2.2	125.9± 4.6	156.7±50.1
100	100	NT	11.4± 3.6	NT	NT	168.7±25.8
100	1,000	0.8± 0.6	1.2± 0.2	155.6±26.1	135.9±10.4	152.9±27.0
100	10,000	NT	0.9± 0.5	30.5± 7.3	147.3±23.8	96.4±18.2

[a] Macrophage preparations from DBA/2N mice were exposed for 20 h to 100 U/ml of rMuIFN-γ and dilutions (100 to 10,000 ng/ml) of BRM

[b] Not tested

[c] Mean ± SD of three wells (rMuIFN-γ alone, 119.7±13.6)

Table 2. Synergistic macrophage activation by combinations of rMuIFN-γ and synthetic MDPs against P815 cells

Doses of[a]		^{3}H-TdR uptake by P815 cells with macrophages exposed to rMuIFN-γ and synthetic MDPs (%)		
rMuIFN-γ (U/ml)	MDP (ng/ml)	MDP-Lys(L18)	L18-MDP	MDP(MeAla)-Lys(L18)
0	100	82.3 ± 34.4[b]	85.1 ± 27.4	129.3 ± 45.3
0	1,000	87.0 ± 24.8	89.8 ± 9.4	127.1 ± 11.3
0	10,000	99.8 ± 16.4	105.2 ± 32.0	100.8 ± 15.4
100	100	1.7 ± 1.9	2.6 ± 1.8	142.0 ± 42.4
100	1,000	0.8 ± 0.2	1.3 ± 0.4	33.2 ± 22.5
100	10,000	1.1 ± 0.4	3.6 ± 5.1	19.2 ± 5.0

[a] Macrophage preparations from DBA/2N mice were exposed for 20 h to 100 U/ml of rMuIFN-γ and dilutions (100 to 10,000 ng/ml) of synthetic MDP

[b] Mean ± SD of three wells (rMuIFN-γ alone, 118.7 ± 29.3)

MGA and SEA. Small doses of OK432 and BCG-CWS, at which no effect was detectable, activated macrophages in combination with rMuIFN-γ, while MGA and SEA did not (Table 1). Some synthetic MDP derivatives also stimulated highly cytostatic activities of macrophages in combination with rMuIFN-γ; the simultaneous exposure of macrophages to 100 ng/ml of MDP-Lys(L18) and L18-MDP was sufficient to exert synergism with 100 U/ml of rMuIFN-γ (Table 2). Since the efficacy of these two MDPs on synergistic macrophage activation with rMuIFN-γ was almost the same and since clinical trials of antimicrobial activities of MDP-Lys(L18) are now proceeding in Japan, we chose MDP-Lys(L18) as the partner for rMuIFN-γ. Synergism between rMuIFN-γ and MDP-Lys(L18) was observed in the syngeneic test system with 3LL cells (C57BL/6NCrj mice) as well as in that with P815 cells (DBA/2NCrj mice) (Table 3). Contact with macrophages exposed to 10 to 10,000 ng/ml of MDP-Lys(L18) alone or to 100 U/ml of rMuIFN-γ alone yielded little release of ^{51}Cr from the labeled tumor cells (cytolysis). Nevertheless, the release of ^{51}Cr from the tumor cells was detectable when they came in contact with macrophages exposed to such doses of both agents: the percent release of ^{51}Cr was between 14.8% and 16.4%. The TdR uptake by tumor cells was suppressed by macrophages exposed to rMuIFN-γ alone, but not by those exposed to MDP-Lys(L18) alone. This suppression was strengthened further by exposure of macrophages to both agents (Table 3). These BRM preparations were ascertained to be free of endotoxin (< 0.05 ng/ml) by the Limulus amebocyte lysate assay, and polymyxin B did not affect the synergism between rMuIFN-γ and MDP-Lys(L18).

Table 3. Stimulation of cytolytic and cytostatic activities in macrophages by combinations of rMuIFN-γ and MDP-Lys(L18) against 3LL cells

Doses of[a]		Macrophage activation[b]		
rMuIFN-γ (U/ml)	MDP (ng/ml)	% ^{51}Cr release		% ^{3}H-TdR uptake
0	10	0	(−343 ± 491)	83.4 ± 16.7
0	100	0	(−87 ± 242)	77.6 ± 3.3
0	1,000	0	(−246 ± 292)	66.1 ± 6.3
0	10,000	0	(−234 ± 460)	77.1 ± 24.9
0.1	0	0	(−580 ± 358)	NT[d]
1	0	0	(−674 ± 509)	NT
10	0	1.9 ± 1.1	(644 ± 366)	NT
100	0	0.9 ± 1.2	(308 ± 387)	9.1 ± 3.4
0.1	100	8.3 ± 3.1	(2766 ± 1033)*[c]	NT
1	100	15.6 ± 3.0	(5215 ± 1021)*	NT
10	100	15.8 ± 2.6	(5272 ± 867)**	NT
100	10	14.8 ± 1.1	(4948 ± 340)***	0.4 ± 0.1
100	100	15.2 ± 1.5	(5083 ± 493)***	0.3 ± 0.1
100	1,000	16.4 ± 0.5	(5468 ± 156)***	0.4 ± 0.1
100	10,000	15.5 ± 0.9	(5186 ± 303)***	0.4 ± 0.3
0	0	0	(−0.3 ± 421)	100.0 ± 14.7

[a] Macrophage preparations from C57BL/6N mice were exposed for 20 h to 100 U/ml of rMuIFN-γ and dilutions of MDP-Lys(L18)

[b] Radioactivities of culture supernatant are given in parentheses

[c] * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

[d] Not tested

Priming effect of rMuIFN-γ on macrophage activation

Having demonstrated that LPS and MDP-Lys(L18) enhanced the activation, by rMuIFN-γ, of macrophages to kill tumor cells, we studied the effect of the duration of priming with rMuIFN-γ. The macrophage preparations were incubated with medium containing 100 U/ml of rMuIFN-γ for 2, 4, 6, 8, and 10 h at 37 °C. After each interval of time, preparations were rinsed thoroughly with washing medium and then incubated with the medium containing 100 ng/ml of MDP-Lys(L18) for 12 h. The priming effect of macrophages with rMuIFN-γ, followed by treatment with MDP-Lys(L18), appeared at and after the 4th h. In the absence of the second stimulation with MDP-Lys(L18), on the other hand, the inhibitory effect on the TdR uptake by tumor cells appeared at 8 and 10 h. Tumoricidal activities of macrophages (^{51}Cr release from tumor cells) also appeared at and after the 4th h (Table 4).

To clarify the priming effect of rMuIFN-γ on macrophage activation, previous treatment with LPS or with MDP-Lys(L18) was carried out under the same experimental conditions as above. The TdR uptake by tumor cells in contact with macrophages that had been primed with rMuIFN-γ, followed by exposure to LPS or to MDP-Lys(L18), was suppressed significantly: the percentage uptake of TdR in both cases was 1.2%. On the other hand, when macrophages were primed with LPS and MDP-Lys(L18), followed by rMuIFN-γ, the percentage uptakes of TdR by target cells in contact with these macrophages were 100.6% ± 10.1 and 26.6% ± 7.8, respectively. Further-

Table 4. Time course of the priming effect of rMuIFN-γ on macrophage activation by MDP-Lys(L18)

Exposure (time)[a]		Macrophage activation[b]	
1st	2nd	% ^{3}H-TdR uptake	% ^{51}Cr release
IFN (2 h)	MDP	100.9± 7.8	0 (–48± 88)
IFN (4 h)	MDP	12.3± 1.3	5.8±2.6 (493±219)*[c]
IFN (6 h)	MDP	7.6± 1.7	7.3±2.6 (615±221)**
IFN (8 h)	MDP	4.4± 0.4	7.1±2.3 (596±193)**
IFN (10 h)	MDP	3.1± 0.7	5.9±0.8 (498± 69)***
IFN (2 h)	–	128.9±27.2	NT[d]
IFN (4 h)	–	91.7±30.2	NT
IFN (6 h)	–	88.5±27.8	NT
IFN (8 h)	–	61.7± 5.7	1.2±2.4 (93±203)
IFN (10 h)	–	42.8± 7.4	0.1±1.2 (9± 99)
–	MDP	113.8±24.0	1.7±0.6 (62± 53)
–	–	100.0± 9.2	0 (0± 55)

[a] Macrophage preparations were exposed to 100 U/ml of rMuIFN-γ for 2 to 10 h, followed by incubation with 25 ng/ml of MDP-Lys(L18) for 12 h

[b] Target cells were 3LL cells for macrophages from C57BL/6N mice. Radioactivities of culture supernatant are given in parentheses. Mean ± SD of three wells

[c] * $P<0.05$, ** $P<0.01$, *** $P<0.001$

[d] Not tested

Table 5. Sequential macrophage activation by rMuIFN-γ and LPS or MDP-Lys(L18) against 3LL cells

Exposure[a]		^{3}H-TdR uptake by 3LL cells (%)	
1st	2nd		
IFN	LPS	1.2± 0.1[b]	–
IFN	MDP	–	1.2±0.3
LPS	IFN	100.6±10.1	–
LPS	IFN plus LPS	37.3±11.8	–
–	IFN plus LPS	1.8± 0.3	–
MDP	IFN	–	26.6±7.8
MDP	IFN plus MDP	–	13.6±5.6
–	IFN plus MDP	–	2.4±1.7

[a] Macrophage preparations were exposed to 100 U/ml of rMuIFN-γ, to 25 ng/ml of LPS, to 100 ng/ml of MDP-Lys(L18), or to rMuIFN-γ with LPS of MDP-Lys(L18) for 12 h

[b] Mean ± SD of three wells

more, the macrophages which had been exposed to LPS or MDP-Lys(L18) exhibited lower cytostatic activities on further stimulation with combinations of rMuIFN-γ with LPS or with MDP-Lys(L18) than unprimed macrophages: the uptakes of TdR in cases with rMuIFN-γ plus LPS and rMuIFN-γ plus MDP-Lys(L18) were 37.3%±11.8 and 13.6±5.6, respectively, as against 1.8%±0.3 and 2.4%±1.7, respectively, in cases that were not primed with LPS and MDP-Lys(L18) (Table 5). These results were reproduced in four separate experiments, indicating that in the macrophages stimulated by rMuIFN-γ, a mechanism must exist to induce in them the tumoricidal state, and that the second stimulation, by LPS or MDP-Lys(L18), enhances the activation processes; at least 4 h is required for the macrophages to be able to receive the second stimulation. These are in good agreement with the findings of Saiki et al. [27] with human rIFN-γ and MDP.

Discussion

It has been reported that IFN-γ activates human monocytes [13] and murine macrophages [35, 37] to become cytotoxic for tumor cells. As Varesio et al. [37] emphasized, the susceptibilities of tumor cells, including P815 mastocytoma, Meth A fibrosarcoma, 3LL lung adenocarcinoma, B16 melanoma, MM46 mammary carcinoma, and MH134 hepatoma, to the cytostatic activities of macrophages stimulated by rMuIFN-γ differed from cell to cell. All of them, on the other hand, were highly susceptible to macrophages that had been primed with both rMuIFN-γ and LPS, though they were resistant to macrophages primed with LPS alone (data not shown). Fogler and Fidler [7] have also reported that syngeneic tumoricidal macrophages nonselectively destroyed murine tumor cells. Furthermore, we [15] have observed that the growth in the lung of 3LL cells was suppressed by combination therapy with these two agents. Fidler [6] stated that tumoricidal macrophages can be used to kill the few tumor cells that have survived destruction by conventional therapies. Nevertheless, LPS is highly toxic to man. Therefore, we were interested in the potency of any less toxic substances that might exist.

OK432 and BCG-CWS significantly induced cytostatic activities in macrophages by combination with rMuIFN-γ, while MGA and SEA did not. Furthermore, Saiki and Fidler [26] recently reported the synergism between rMuIFN-γ and MDP in the induction of the cytotoxicity of macrophages. In this study, each of the synthetic derivatives of MDP was also effective in inducing synergistically with rMuIFN-γ the cytostatic activities of macrophages against P815 cells: MDP-Lys(L18) and L18-MDP had almost the same efficacy and were more effective than MDP-(MeAla)-Lys(L18). A small amount (10 ng/ml) of MDP-Lys(L18) was sufficient, in combination with 100 U/ml of rMuIFN-γ, to activate macrophages to both the cytostatic and cytolytic states against 3LL cells, suggesting that MDP-Lys(L18) can be substituted for LPS.

Potent tumoricidal activities of macrophages were elicited by treatment with LPS or MDP-Lys(L18) after that with rMuIFN-γ and treatment in the reverse order was not at all effective, or only slightly so. These results are in good agreement with published reports [25, 27]. Once exposed to LPS or to MDP-Lys(L18), macrophages seem to become tolerant to subsequent stimulation by combination of rMuIFN-γ with LPS or MDP-Lys(L18) (Table 5). Thus, the immune IFN should play a preferential role in the activation of macrophages to kill tumor cells. Celada et al. [4] found the cell surface receptors for rMuIFN-γ on murine macrophages. The binding of rMuIFN-γ to the receptors may be involved in the triggering of macrophage activation. This preactivation of macrophages was induced by incubation for at least 4 h at 37 °C. No detailed biochemical or cytobiological process involving such a short time has previously been evidenced. When exposed to rMuIFN-γ, macrophages may provide a larger number of binding sites for LPS [2] or MDP-Lys(L18). However, as Saiki et al. [26, 27] stated, liposome-encapsulated MDP derivatives were effective in the synergism with rMuIFN-γ (data not shown), indicating that macrophage activation may not always require the interaction of these activators with the macrophage cell surface. The question of why, in the reversed treatment sequence, MDP-Lys(L18) or LPS interfered with subsequent stimulation by rMuIFN-γ with MDP-Lys(L18) or LPS has still to be answered.

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