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Potentiation of direct antitumor cytotoxicity and production of tumor cytolytic factors in human blood monocytes by human recombinant interferon-gamma and muramyl dipeptide derivatives

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Summary. We investigated whether human peripheral blood monocytes isolated by centrifugal elutriation from healthy donors could be acitivated to become tumoricidal and release tumor cytolytic factor (TCF) subsequent to incubation with recombinant human interferon-gamma (r-IFN-γ) or a derivative of muramyl dipeptide (nor-MDP), or both. Blood monocytes incubated in endotoxin-free medium containing up to 1000 U/ml of r-IFN-γ or in medium containing less than 1 μg/ml of nor MDP were not activated to lyse radiolabeled allogeneic human tumor cells. In contrast, the incubation of monocytes with various dose combinations of r-IFN-γ and nor-MDP generated significant direct cytotoxic activity as well as production of TCF. Preincubation of the r-IFN-γ and nor-MDP mixture with polymyxin B did not inhibit the synergism, thus ruling out the possibility that the process was due to endotoxin contamination. TCF harvested from monocyte culture supernatants was cytolytic against five allogeneic tumor targets, but not against a nontumorigenic cell line. Collectively, the data demonstrate that r-IFN-γ can prime human blood monocytes to allow their activation by synthetic nor-MDP.

Introduction

Previous reports from our laboratories and many others have demonstrated that human monocytes-macrophages can be activated by various agents such as lymphokines (macrophage activating factor, or MAF) [5, 8, 9, 20, 21, 26, 28], muramyl dipeptide (MDP) [9, 19, 26, 44, 46, 47, 48ß], lipophilic derivaties of MDP [19, 26, 47], lipopolysaccharide (LPS) [14, 46], or interferon-gamma (IFN-γ) [7, 17, 18, 24, 25, 27, 32, 35-38, 41, 42]. These agents render macrophages able to distinguish between tumorigenic targets, which they lyse, and nontumorigenic cells, which they do not harm [8]. In rodent systems, the lymphokine MAF and bacterial products such as LPS [39, 40] or MDP [10, 43] have been shown to synergistically activate tumoricidal properties of macrophages. Moreover, the encapsulation of MAF and MDP within liposomes has been shown to produce synergistic activation of macrophages both in vitro [10, 43] and in vivo [10].

Lymphokines released by antigen- or mitogen-stimulated lymphocytes contain significant IFN-γ activity [21, 22, 37, 38]. Recent attention has focused on the immunoregulating role of IFN-γ with respect to modulating the activity of natural killer (NK) cells [6, 14, 16, 17] and macrophages [7, 11, 21, 22, 25, 27, 32, 35-38, 41, 42]. In various systems IFN-γ has been shown to influence macrophage functions such as expression of HLA-DR antigen [1], increased expression of Fc receptor [12], activation of oxidative metabolism and antimicrobial activity [32], and potentiation of monocyte-mediated antitumor cytotoxicity and antiviral activity [23, 24].

Macrophages activated by bacterial products such as LPS and MDP can also become tumoricidal and produce a factor that is cytolytic to tumor cells and not to normal cells. This tumor cytotoxic factor (TCF) can be responsible for at least one mechanism by which activated macrophages destroy tumor targets [45, 49]. In the present study, we wished to determine wheter recombinant IFN-γ (r-IFN-γ) and MDP can act synergistically to activate tumoricidal properties in monocytes, and whether such monocytes also release TCF into the culture supernatant fluids.

Materials and methods

Cell cultures. A375, derived from a human melanoma, HT-29, a line derived from a human colon carcinoma, PC-3, a line derived from a human prostatic carcinoma, and the Natusch line, derived from a human glioblastoma, were adapted to growth in culture as described in detail previously [8, 9]. Nontumorigenic human cell line Flow-2000, derived from an embryonic lung, was purchased from Flow Laboratories, Rockville Md. MDA-MB 468 and MDA-MB 435 S are lines derived from different metastatic human breast adenocarcinomas [3]. All cultures were free of *Mycoplasma* and were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and gentamicin (CRPMI-1640), at 37 °C in a humidified atmosphere containing 5% CO_2 and air.

Reagents. RPMI-1640, human AB serum, FBS, and Hanks' balanced salt solution (HBSS) were purchased from M. A. Bioproducts, Walkersville, Md. Hydrophilic nor-MDP (desmethyl-N-acetyl-muramyl-L-alanyl-D-isoglutamine) was the kind gift of Ciba-Geigy, Ltd., Basel,

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Switzerland. LPS (*Escherichia coli* 055:B 5) was purchased from Difco Laboratories, Detroit, Mich. Human r-IFN-γ was the kind gift of Genentech, Inc., South San Francisco, Calif. Polymyxin B was purchased from Sigma Chemical Company, St. Louis, Mo.

Isolation and culture of human peripheral blood monocytes. Mononuclear cells were obtained from incidental samples derived from collections of platelet concentrates using an IBM 2997 blood-cell separator [15]. Monocytes were isolated from the mononuclear cell sample by centrifugal elutriation using a JE-6B Beckman elutriation rotor as described previously [26]. Briefly, a fraction containing greater than 90% of the total monocyte population was obtained at a speed of 3000 rpm and a flow rate of 41 ml/min. These cells were >95% monocytes as determined by nonspecific esterase staining and morphological examination, and were >97% viable by a trypan blue dye exclusion. The fraction was pooled, washed twice with Ca^{2+}- and Mg^{2+}-free HBSS, and resuspended in RPMI-1640 supplemented with 5% human AB serum to a concentration of 5×10^5 monocytes/ml. These cells were then plated in a 96-well Microtest plate (Linbro, Flow Laboratories, McLean, Va). After a 2-h incubation, the nonadherent cells were removed by aspiration of the supernatant and washing of the monolayer with medium. At this point the purity of moncytes was >99% as assessed by examination of cell morphology, phagocytosis, and nonspecific esterase staining.

In vitro activation of monocytes. Monocytes were incubated at 37 °C in medium alone, or in medium containing various amounts of r-IFN-γ, or nor-MDP, or both suspended in RPMI-1640 supplemented with 5% human AB serum. Unless otherwise described, after 24 h the monocytes were washed thoroughly with medium prior to the addition of radiolabeled target cells.

Monocyte-mediated cytotoxicity. Cytotoxicity was assessed by measuring release of radioactivity as described in detail previously [8, 44]. Target cells in their exponential growth phase were incubated for 24 h in the appropriate medium containing ^{125}I IUrd (0.3 μCi/ml; sp. act. 200 mCi/μmol; New England Nuclear, Boston, Mass.). The cells were then washed twice to remove unbound radioiodine, harvested by a 1-min trypsinization (0.25% trypsin-0.02% EDTA), and washed. The labeled cells were resuspended in CRPMI-1640, and 1×10^4 cells were plated into culture wells to obtain an initial target-to-effector cell ratio of 1:10. Radiolabeled cells were plated alone as a control group. After 16 h, the cultures were washed to remove the nonadherent target cells, refed with fresh medium and then cultured for an additional 2 days. Because we used an adherent cell assay in which cell-to-cell contact between effector and target cells is required to achieve killing [8, 20], washing after 24 h removed the error introduced by cells that did not adhere, but were not necessarily killed in the 3-day assay. In this assay, the initial plating efficiency of the target cells is >85% when plated alone or with either control or activated monocytes. Time course studies have shown that when target cells are cocultivated with activated monocytes, loss of radioactivity begins after 24 h and reaches a maximum at 72 h [8, 20]. Therefore, 72 h after the addition of tumor cells, the cultures were washed twice with HBSS, and the adherent viable cells were lysed with 0.1 ml 0.5 N NaOH. The radioactivity of the lysate was measured in a gamma counter, and the cytotoxic activity of the monocytes was calculated as follows:

$$\text{Percentage of specific cytotoxicity mediated by activated monocytes} = \frac{A - B}{A} \times 100,$$

where A represents cpm in cultures of untreated monocytes and target cells and B represents cpm in cultures of test monocytes and targets cells.

Production of TCF by human monocytes. Monocytes freshly isolated by centrifugal elutriation were plated into culture wells and later all nonadherent cells were removed by being washed with fresh medium. The resulting monocyte monolayers were incubated in CRPMI-1640 with r-IFN-γ, or nor-MDP, or both, or neither. After 24 h, the cell-free supernatants were harvested, filtered through 0.22 μm Millipore membranes, and stored at −20 °C until use [44].

TCF-mediated cytotoxicity in vitro. Tumor lysis mediated by supernatants with TCF activity was measured by a radioactive release assay described previously [48, 49]. Radiolabeled target cells (0.5×10^4 or 1×10^4) were plated into 38-mm² wells (Microtest plate), and 14 h later the nonadherent target cells were removed and the cultures were refed with fresh CRPMI-1640. Cell-free supernatants (with or without TCF activity) were then added to the target cell monolayers. As an additional control group, radiolabeled target cells were incubated in medium alone. After 72 h the cultures were gently washed twice with HBSS, and adherent, viable cells were lysed with 0.1 ml of 0.5 N NaOH. The lysate was monitored for radioactivity in a gamma counter. The percentage of TCF activity was calculated from the formula:

$$\text{\% of TCF-mediated cytotoxicity} = 100 \times \frac{A - B}{A},$$

where A represents cpm in target cells cultured in supernatant from cultures of untreated monocytes, and B represents cpm in target cells cultured in supernatant from test monocytes.

Statistical analysis. The statistical significance of differences between test groups was analyzed by Student's t-test (2-tailed).

Results

Synergism for generating tumoricidal properties in human blood monocytes by recombinant human IFN-γ and nor-MDP

In the present studies as well as in previously published results [26, 49], highly purified human monocytes (>98%) that were isolated by centrifugal elutriation from the blood of healthy donors were not spontaneously cytotoxic against allogeneic tumor cells. Human monocytes incubated for 24 h in medium alone or medium containing r-IFN-γ (0.1 U/ml to 1000 U/ml) or with medium containing 0.001 μg/ml to 0.1 μg/ml nor-MDP were not rendered cytotoxic against the allogeneic A375 melanoma targets (Table I). Monocytes incubated with medium containing 1, 10, or 50 μg/ml nor-MDP were rendered tumoricidal.

Table 1. Synergistic activation of human blood monocytes by r-IFN-γ and nor-MDP

Dose of nor-MDP (μg/ml)	Radioactivity remaining in viable target cells cultured with monocytes incubated with[a]: Concentration of r-IFN-γ (U/ml)					
	0	0.1	1	10	100	1000
0	1046 ± 37[b]	1004 ± 60	1059 ± 18	1069 ± 35	1051 ± 60	1068 ± 66
0.001	1109 ± 32	1055 ± 40	1050 ± 15	1089 ± 14	1040 ± 38	1053 ± 18
0.01	1098 ± 22	1019 ± 41	1063 ± 33	1072 ± 25	1038 ± 51	1028 ± 47
0.1	1080 ± 20	1019 ± 32	839 ± 85 (20%)[c]	728 ± 37 (30%)[d]	737 ± 42 (30%)[c]	585 ± 20 (44%)[d]
1	860 ± 81 (18%)[c]	757 ± 42 (28%)[c]	666 ± 92 (36%)[d]	696 ± 31 (33%)[d]	537 ± 44 (49%)[d]	495 ± 56 (53%)[d]
10	763 ± 75 (27%)[c]	675 ± 25 (35%)[c]	681 ± 43 (35%)[c]	564 ± 29 (46%)[d]	511 ± 57 (51%)[d]	530 ± 191 (49%)[d]
50	798 ± 38 (24%)[c]	816 ± 65 (22%)[c]	671 ± 77 (36%)[c]	523 ± 45 (50%)[d]	550 ± 33 (47%)[d]	545 ± 26 (49%)[d]

[a] Human monocytes (10^5) were treated for 24 h in endotoxin-free medium alone or in medium containing nor-MDP and/or r-IFN-γ at the indicated doses. ^{125}IIUdR-labeled A375 melanoma cells (10^4) were added to the monocyte monolayers. The assays were terminated 72 h after cocultivation

[b] cpm ± SD of triplicate cultures. These are representative data of three separate experiments. Number in parentheses is percentage cytotoxicity as compared to untreated monocytes and tumor cells. The remaining activity in tumor cells cultured alone was 1123 ± 69

[c] $p < 0.05$

[d] $p < 0.01$

Monocytes incubated with endotoxin-free medium containing >0.1 μg/ml nor-MDP and >0.1 U/ml r-IFN-γ were highly cytotoxic against the A375 melanoma cells (Table 1).

Synergistic activation of monocyte antitumor activities was produced by their incubation with combinations of r-IFN-γ and nor-MDP. The combination of r-IFN-γ at subthreshold doses for monocyte activation (up to 1000 U/ml) and nor-MDP at subthreshold doses for monocyte activation (0.1 μg/ml) resulted in synergistic activation of monocyte-mediated cytotoxicity. Moreover, as the nor-MDP dose was increased (1 μg or 10 μg/ml) with the dose of r-IFN-γ held constant, higher levels of antitumor activities were found in the monocytes (Table 1). This synergism of monocyte antitumor activity is clearly illustrated in Fig. 1. Because r-IFN-γ alone, even at doses up to 1000 U/ml, did not activate monocyte cytotoxic properties, these data suggest that the combination of r-IFN-γ and nor-MDP had synergistic effects on the activation of monocytes.

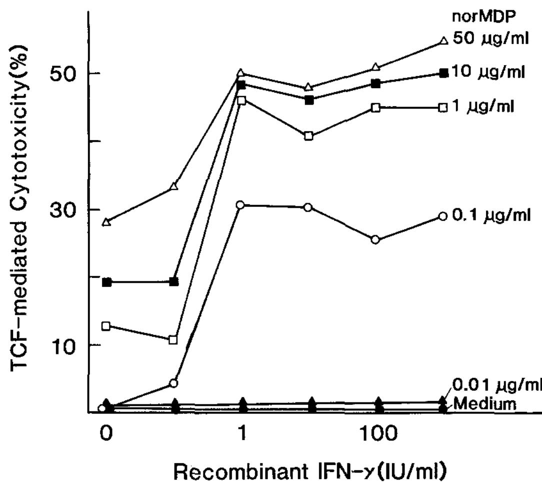

Fig. 1. Synergistic activation of human blood monocytes by r-IFN-γ and nor-MDP. Human monocytes (10^5) were treated for 24 h in endotoxin-free medium alone or in medium containing nor-MDP and/or r-IFN-γ at the indicated doses. ^{125}IIUrd-labeled A 375 melanoma cells (10^4) were added to the monocyte monolayers. The assays were terminated after 72 h of cocultivation. Percentage cytotoxicity was calculated by comparison with target cells incubated with control monocytes. The results are representative of those obtained in three independent experiments. nor-MDP alone (o); r-IFN-γ alone (◇); nor-MDP + r-IFN-γ, 0.1 U/ml (△); nor-MDP + r-IFN-γ, 1.0 U/ml (●); nor-MDP + r-INF-γ, 10 U/ml (□); nor-MDP + r-IFN-γ, 100 U/ml (◆): nor-MDP + r-IFN-γ, 1000 U/ml (▲)

TCF production by monocytes incubated with mixtures of r-IFN-γ and nor-MDP

We have recently shown that the activation of human macrophages to the tumoricidal state is associated with the production of diffusible molecules that lyse tumor cells but not normal cells [48, 49]. These molecules have been referred to as TCF. Partial characterization of TCF revealed that the material is resistant at pH 2, sensitive to boiling, and resistant to treatment with various protease inhibitors [49].

In the next set of experiments, we wished to determine wheter the synergistic activation of tumoricidal properties in monocytes by r-IFN-γ and nor-MDP correlated with release of TCF into the culture supernatant fluids. To do so, human blood monocytes were incubated with various dose combinations of r-IFN-γ and nor-MDP, and culture supernatant fluids were harvested 24 h later. The antitumor activity of these supernatants was assessed by incubation for 72 h with ^{125}IIUrd-labeled A375 cells as described in detail previously [48]. The data shown in Fig. 2 demonstrate that culture supernatants of monocytes cultured with endotoxin-free medium containing only r-IFN-γ (0.01 – 100 U/ml) or with endotoxin-free medium containing 0.01 μg/ml nor-MDP were devoid of TCF activity. The incubation of monocytes with endotoxin-free medium containing >0.1 μg/ml nor-MDP and >0.1 U/ml r-IFN-γ resulted in significant production of TCF ($p<0.01$). Indeed, as little as 1 U/ml r-IFN-γ admixed with 0.1 μg/ml nor-MDP produced significant levels of TCF activity in the culture supernatants.

In a parallel set of experiments, we determined the sequence for monocyte interaction with r-IFN-γ and nor-MDP required for the synergistic activation of tumoricidal

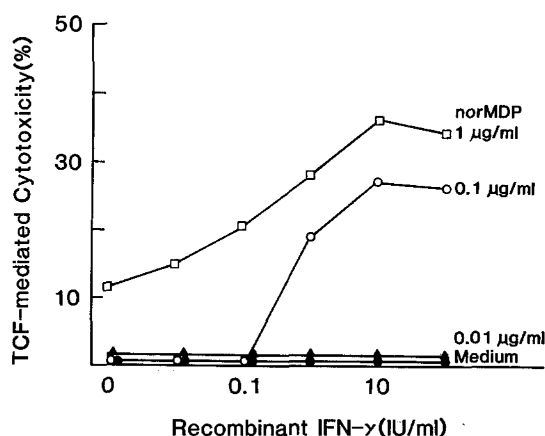

Fig. 2. Synergistic activation by r-IFN-γ and nor-MDP of human blood monocytes for TCF production. Freshly isolated human blood monocytes were incubated with different doses of r-IFN-γ or with r-IFN-γ and different amounts of of nor-MDR, then 24 h later, the culture supernatants were harvested and diluted 1:10 in fresh medium. TCF activity was determined against [125]IIUrd-labeld A 375 melanoma. Percentage cytotoxicity was calculated by comparison of target cells incubated with culture supernatant fluid of control (untreated) monocytes. Results are mean values from triplicate samples. Standard deviation from the mean did not exceed 10%. This is a representative experiment of three

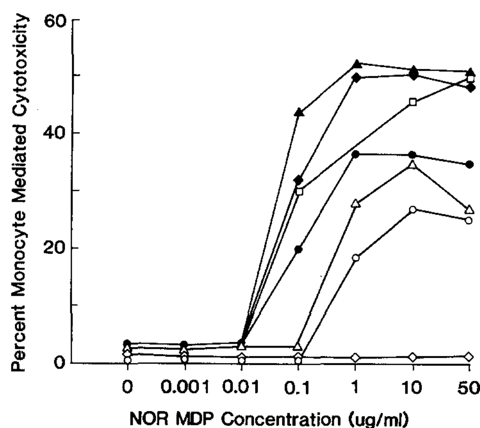

Fig. 3. Correct sequence of monocyte treatment by r-IFN-γ and nor-MDP required for synergistic activation toward TCF production. Monocytes were first treated with r-IFN-γ for 4 h. After washing, the monocytes were incubated for an additional 20 h with nor-MDP. Culture supernatant fluids were then harvested and diluted 1:10 with fresh medium. TCF activity was measured against [125]IIUrd-labeled A 375 melanoma. Percentage cytotoxicity was calculated by comparison with culture supernatant fluids of untreated monocytes. Results are a mean value from triplicate samples. Standard deviation from the mean did not exceed 10%. This is a representative experiment of four

properties in monocytes (Table 2) and for the production of TCF (Fig. 3). In these experiments monocytes were incubated for 4 h with various doses of r-IFN-γ alone or with various doses of nor-MDP alone. The monocytes were washed and refed with medium containing the second activator, i.e., r-INF-γ, then nor-MDP, or nor-MDP, then r-IFN-γ. After 20 h of incubation with the second signal the monocyte cultures were washed, and tumoricidal properties or TCF release were then determined. To achieve synergistic activation of monocytes for both tumoricidal properties (Table 2) and TCF production (Fig. 2), monocytes had to be first incubated with r-IFN-γ (10 U or 100 U/ml) and then with 0.1 µg/ml nor-MDP. The treatment of monocytes first with nor-MDP and then with r-IFN-γ led to little to no measurable TCF activity in the culture supernatants.

Spectrum of antitumor activity of TCF released from monocytes incubated with r-IFN-γ and nor-MDP

In these studies blood monocytes were incubated with medium alone, nor-MDP alone (0.1 or 50 µg/ml), r-IFN-γ alone (100 U/ml), or LPS (0.1 µg/ml) and a mixture of nor-MDP (0.1 µg/ml) and r-IFN-γ (100 U). Culture supernatant fluids were harvested and tested for TCF activity against five different tumorigenic cells and one nontumorigenic normal cell line. The data are shown in Table 3. Culture supernatant fluids of monocytes incubated in medium alone, in medium containing 0.1 µg/ml nor-MDP, or in medium containing 100 U/ml r-IFN-γ had no TCF activity. In contrast, culture supernatant fluids of monocytes incubated with LPS (0.1 µg/ml), a high dose of nor-MDP (50 µg/ml), or a mixture of r-IFN-γ (100 U/ml) and low

Table 2. The sequence of monocyte interaction with r-IFN-γ and nor-MDP and its influence on synergistic activation

Sequence of monocyte treatment: r-IFN-γ 4 h (U/ml)	then nor-MDP 20h (µg/ml)	% of monocyte mediated cytotoxicity[a]	Sequence of monocyte treatment: nor-MDP 4 h (µg/ml)	then r-IFN-γ 20h (U/ml)	% of monocyte mediated cytotoxicity[a]
0	0.01	0	0	10	0
0	0.1	0	0	100	0
0	1	0	0.01	0	0
10	0	0	0.01	10	0
10	0.01	12	0.01	100	0
10	0.1	18[b]	0.1	0	0
10	1	43[b]	0.1	10	0
100	0	0	0.1	100	7
100	0.01	16[b]	1	0	0
100	0.1	20[b]	1	10	10
100	1	35[b]	1	100	10

[a] Percentage monocyte-mediated cytotoxicity was determined by comparison with untreated monocytes and tumor cells
[b] Same as [a], but $P < 0.05$

Table 3. Spectrum of cytotoxicity mediated by TCF produced by human blood monocytes incubated with nor-MDP and r-IFN-γ

Treatment of monocytes[a]	Percentage cytotoxicity mediated by TCF-rich culture supernatant fluids against[b]:					
	Human tumorigenic target cells					Normal cells
	A375	HT-29	PC-3	MDA-MB468	MDA-MB435S	Flow-2000
Medium	0[b]	0	0	0	2	0
Nor-MDP (50 μg/ml)	65[c]	42[c]	0	32[c]	10	0
Nor-MDP (0.1 μg/ml)	0	4	4	1	0	0
r-IFN-γ (100 U/ml)	0	2	0	1	2	1
Nor-MDP (0.1 μg/ml) and r-IFN-γ (100 U/ml)	64[c]	36[c]	27[d]	44[c]	21[d]	0
LPS (0.1 μg/ml)	70[c]	48[c]	27[d]	30[c]	27[d]	0

[a] Monocytes were incubated for 24 h in medium with the indicated agents. Culture supernatant fluids were harvested and diluted 1 : 10 with fresh medium. TCF activity was then assayed as described in *Materials and methods*
[b] Percentage cytotoxicity as compared to tumor cells incubated in medium
[c] Sames as [b], but $P < 0.01$
[d] Sames as [b], but $P < 0.05$

dose nor-MDP (0.1 μg/ml) contained significant ($P < 0.001$) levels of TCF activity against the A375 melanoma, HT-29 colon carcinoma, PC-3 prostatic carcinoma, and MDA-MB468 and MDA-MB435S breast adenocarcinomas. No lytic activity was found against nontumorigenic fibroblasts.

Control studies with polymyxin B

Since small amounts of LPS have been shown to have synergistic effects on the activation of human monocytes by r-IFN-γ [21, 22], throughout our studies, we took great care to ascertain that all reagents used were endotoxin free. Nonetheless, we wished to directly rule out the possibility that the observed synergism could have been attributed to low levels of endotoxin contamination in the medium. In the next set of experiments, we incubated 20 μg/ml polymyxin B with 0.01 or 0.1 μg/ml LPS, with 1 μg/ml nor-MDP, with 100 U/ml r-IFN-γ, or with a mixture of 100 U/ml r-IFN-γ and 1 μg/ml nor-MDP. After 30 min of incubation, the agents (and polymyxin B) were added to the monocyte monolayers for 24 h. Culture supernatant fluids were harvested and TCF activity was assessed. The data shown in Table 4 clearly demonstrate that whereas incubation of LPS with polymyxin B completely abolished the LPS biological activity on monocytes, treatment of r-IFN-γ and nor-MDP with polymixin B did not diminish their synergistic activation of monocytes.

Table 4. Effects of polymyxin B on r-IFN-γ, nor-MDP, or LPS induction of TCF production in human monocytes

Treatment of monocytes	TCF activity in culture supernatants of monocytes stimulated with agents pretreated with[a]	
	Medium	20 μg/ml polymyxin B
Medium	1406 ± 38[b]	1386 ± 72
Nor-MDP (1 μg/ml)	927 ± 55 (34%)[c]	706 ± 63 (50%)[c]
r-IFN-γ (100 U/ml)	1364 ± 19	1393 ± 33
Nor-MDP (1 μg/ml) and r-IFN-γ (100 U/ml)	381 ± 20 (73%)[c]	323 ± 26 (77%)[c]
LPS (0.1 μg/ml)	321 ± 27 (77%)[c]	1391 ± 60

[a] Polymyxin B (20 μg/ml) was incubated for 30 min with r-IFN-γ, nor-MDP, or LPS. The mixtures were added to monocytes for 24 h. Culture supernatant fluids were diluted 1 : 10 with fresh medium and tested for TCF activity on ^{125}IIUrd-labeled A375 melanoma cells. Culture growth was terminated after 72 h of cocultivation
[b] Remaining radioactivity in viable cells. Mean cpm ± SD of triplicate cultures. This is one experiment of three
[c] Number in parentheses is percentage TCF mediated cytotoxicity as compared with tumor cells cultured with medium. $P < 0.01$

Discussion

Our present results demonstrate that human blood monocytes obtained by centrifugal elutriation from healthy donors can be rendered tumoricidal by incubation with subthreshold amounts of nor-MDP and r-IFN-γ. This synergistic activation of monocytes is well correlated with the production of TCF and requires a precise sequence of monocyte activation, i.e., priming by r-IFN-γ and triggering by nor-MDP. This sequence agrees with that reported for activation of human monocytes by r-IFN-γ and endotoxins [22, 36].

Our demonstration that human monocytes can respond to activation stimuli such as MDP and r-IFN-γ and release a cytolytic factor to tumor cells is consistent with obersvations of others [4, 5, 13, 29, 34, 48, 49]. Previous studies from our laboratories have shown that the TCF activity was not abolished by the presence of serum in the medium or by treatment with protease inhibitors [49]. The TCF produced by monocytes incubated with MDP and r-IFN-γ lysed cells in five different human tumorigenic lines, but not those in two nontumorigenic lines. These observations are consistent with previous results [8, 9] that activated human macrophages destroy tumorigenic but not nontumorigenic targets.

Previous reports have clearly shown that LPS and r-IFN-γ can produce synergistic activation of mouse [22, 30, 35, 36] and human [21] macrophages. The synergistic activation of blood monocytes was due to interaction with r-IFN-γ and nor-MDP and not to endotoxins. First, all reagents used in our assay were screened for endotoxins (<0.25 ng/ml) as determined with the standard *Limulus* amebocyte lysate assay. Second, incubation of r-IFN-γ

and nor-MDP with polymyxin B, a potent inhibitor of LPS [2, 31], did not diminish the synergistic activation of the monocytes.

Recently, it has been suggested that small numbers of NK cells contaminating monocyte cultures can respond to stimulation by r-IFN-γ and be responsible for the observed antitumor activity [6, 17, 18, 28]. The cytotoxicity we measured was mediated by activated monocytes. We used only preparations of highly purified blood monocytes (>98%), which we have observed to be devoid of NK cell activity against K562 (unpublished observation). Furthermore, the cytotoxic effector cells in the assay we routinely used have been recently shown to be monocytes and not NK cells [18].

We have previously reported that human blood monocytes can be rendered tumoricidal following interaction with a crude lymphokine preparation (MAF) released from mitogen-stimulated lymphocytes [8, 21]. Since the crude preparation could also contain IFN-γ, the relationship of IFN-γ to MAF has been controversial. Several investigators have suggested that in mice IFN-γ and MAF are similar molecules [22, 25, 32, 35, 41, 42, 50]. Other investigators have presented just as compelling but contradictory data to show that murine IFN and murine MAF are separate molecules [7, 21, 33, 37, 38], and studies from our laboratory have also shown that a human T-cell leukemia line (HTLV positive) can release MAF activity that is distinct from IFN-γ [21]. The present finding that under endotoxin-controlled conditions r-IFN-γ failed to activate tumoricidal properties in monocytes or TCF production also suggest that r-IFN-γ alone cannot account for all MAF activity in lymphokine preparations.

Our data agree with others [35, 36] that r-IFN-γ must prime monocyte-macrophages, and that either LPS or MDP (bacterial products) trigger the activation of these effector cells. The priming of monocytes with IFN-γ is also significant for its antiviral effects, since recently it has been shown that monocytes activated with free- or liposome-encapsulated IFN-γ can discriminate between virus-infected and uninfected cells [23, 24].

The demonstration of synergistic activation of monocytes by r-IFN-γ and nor-MDP for both direct tumoricidal activity and indirect TCF production has several important ramifications. First, both agents are produced in a pure and standardized form. Second, the low levels of MDP used for synergistic activation are unlikely to be toxic in vivo [9] and thus have a tremendous advantage over endotoxins.

In summary, these data indicate that human r-IFN-γ can prime freshly isolated human blood monocytes to respond to a second signal such as synthetic nor-MDP. Activation of monocytes to the tumoricidal state and to TCF production by r-IFN-γ and nor-MDP may have therapeutic implications as potent biological response modifiers for the treatment of malignant diseases. Indeed, the encapsulation of r-IFN-γ and MDP within liposomes could provide a powerful mechanism for generating macrophage activity in vivo, in a manner similar to that of MAF and MDP [10]. Studies of this possibility are now under way.

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