Differential Expression of Murine Macrophage-mediated Tumor Cytotoxicity Induced by Interferons

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ABSTRACT

The requirements for interferon (IFN)-induced priming of murine peritoneal macrophages for cytolysis of tumor cell lines of distinct histological origin were investigated. Lysis of B16 melanoma targets required exposure of elicited macrophages to recombinant murine interferon plus lipopolysaccharide (LPS) together, while sequential treatment of macrophages with IFN-γ then LPS resulted in lysis of P815 mastocytoma targets. The kinetics of macrophage activation by IFN-γ and LPS for lysis of P815 and B16 melanoma targets varied considerably, 8 h being sufficient for P815 targets but 24 h being required for B16 targets. Pretreatment of the macrophages with the antibiotic polymyxin B was able to inhibit completely the induction of tumor lysis of B16 targets but not of P815 targets. In addition, IFN-α/β was able to prime macrophages for lysis of P815 targets but not of B16. Finally, the kinetics of priming macrophages with IFN-γ for lysis of B16 targets had a profound effect on the subsequent exposure time requirement for LPS. The results indicate that the induction of murine macrophage-mediated tumor cytotoxicity can vary considerably depending on the amount and type of interferon used, the presence of a second signal, and the type of tumor target used.

INTRODUCTION

Although macrophages are considered to be important effector cells in the host’s defense against neoplasia (1–4), little is presently known about the underlying cellular events involved in macrophage activation for tumor cytotoxicity (1). Recent evidence suggests that phenotypic alterations accompanying activation are acquired in a stepwise fashion after stimulation with a variety of agents and culminate in development of the tumoricidal state. The onset and loss of these specific phenotypic alterations depends on the nature and duration of agents involved (1, 5–10).

There is now definitive evidence that IFNs are an important class of naturally occurring cytokines that contribute to or participate in the acquisition of the tumoricidal state by macrophages (10–14). Although described originally as antiviral proteins, it is now well recognized that IFNs also have a wide spectrum of other regulatory effects including immunomodulatory as well as proliferative and antiproliferative effects (15–24). These observations have led to extensive Phases I and II clinical studies in cancer patients with a number of recombinant interferons. These studies have generally been disappointing, with results inferior to those obtained in a variety of animal models (25–28). This can be attributed, in part, to a general lack of knowledge regarding the mechanism of action, optimal dose, route, and sequence of IFN administration. Within the context of therapeutic manipulation of macrophage-mediated host defense by IFN, an important goal, therefore, is acquisition of a better understanding of how the process of macrophage activation for tumor cytotoxicity is regulated.

To this end, we have examined the steps required to activate murine macrophages optimally to express tumoricidal properties against tumor cell lines of distinct histological and histotypic origin. This study confirms and extends previous observations that macrophage activation for tumor cytotoxicity in vitro is dependent on an ordered treatment sequence; however, using r-MuIFN-γ and a highly purified preparation of MuIFN-α/β, the temporal sequence of events required for acquisition and expression of tumoricidal properties was found to differ significantly with the assay system and target cell used.

MATERIALS AND METHODS

Animals. Specific pathogen-free male C57BL/6N mice (H-2b) and C3H/HeN (H-2*), were obtained from Charles River, Inc., Portage, MI. Mice were matched for age, sex, and body weight within each experiment. Macrophages from HeN and C57BL mice were compared in the two assay systems utilized, and similar results were obtained regardless of the strain of mouse used (29).

Agents. DMEM and EMEM were obtained from Grand Island Biological Co., Grand Island, NY. FBS was obtained from Sterile Systems, Inc., Logan, UT. LPS from Escherichia coli (0127:B8 and 0111:B4) was obtained from Difco Laboratories, Detroit, MI. PMB and antibiotics were from Pfizer, Groton, CT. All of the reagents, media, and IFN at the concentrations tested contained less than 0.1 ng/ml of endotoxin as assessed by the Limulus lysate assay. Recombinant E. coli-derived murine IFN-γ, specific activity 6 x 10^8 units/mg protein was provided by Genentech, Inc., South San Francisco, CA. MuIFN-α/β, specific activity 1 x 10^8 units/mg, was prepared using a modification of procedures described previously (30). Briefly, L929 cells (5 x 10^5) were seeded in roller bottles containing basal medium Eagle supplemented with 10% FBS. Bottles were refed with growth media after incubation at 37°C for 4 days; 24 h later, Newcastle disease virus was added in serum free media at an input multiplicity of infection of 1. The IFN-α/β containing supernatant was collected 24 h later, clarified by centrifugation, concentrated by ultrafiltration, and acidified to pH 2 for 5 days to inactivate the Newcastle disease virus.

The IFN, which was stored lyophilized in 0.25% bovine plasma albumin, was reconstituted in 0.01 M phosphate buffer containing 0.3 NaCl, pH 7.4 and purified by antibody affinity chromatography on a Sepharose bound sheep anti-IFN-α/β globulin column as described (31), with the bound interferon eluting in pH 2.2, 0.1 M sodium citrate buffer. The purified IFN-α/β was dialyzed against 0.1 M sodium borate, pH 9.0, buffer containing 1 M NaCl and stored at −70°C. The protein content of this material was determined by the method of Lowry et al. (32), and the specific activity of the purified IFN was 1 x 10^7 units/mg protein. Sodium dodecyl sulfate-polyacryl-
amid gel analysis of purified IFN-α/β revealed approximately 10% IFN-α and 90% IFN-β.

IFN Assay. IFN titers are expressed in units of antiviral activity as measured by the inhibition of encephalomyocarditis virus CPE in mouse L929 cells. The assay was performed using duplicate 0.1-ml 2-fold serially diluted IFN samples in 96-well flat-bottomed microtiter plates, to which 30,000 L929 cells in 0.1 ml were added. After an overnight incubation at 37°C in 5% CO2, cultures were challenged with 0.05 ml of encephalomyocarditis virus (multiplicity of infection = 0.3). Twenty-four h later following complete CPE, the cells were viewed with an inverted microscope to determine the extent of viral CPE inhibition.

The IFN titer is the reciprocal of the dilution of the test sample that protects 50% of the cells from viral destruction. Titters are expressed as international reference units per ml based on the NIH Mouse Interferon Reference Standard G-002-904-511 distributed by the Resource Agents Branch of the National Institute of Allergy and Infectious Disease.

Tumor Cell Lines. The B16-F10 melanoma, syngeneic to the H-2k C57Bl/6N mouse and the P815 methylcholanthrene-induced mastocytoma, syngeneic to the DBA/2 mouse (H-2b) were used. The B16-F10 cell line was maintained as a monolayer in DMEM supplemented with 10% FBS. The P815 mastocytoma was maintained in EMEM supplemented with 10% FBS. Both cell lines were free of Mycoplasma and pathogenic murine viruses.

Assays for Macrophage-mediated Cytotoxicity. Macrophage-mediated cytotoxicity was assessed by in vitro 18- and 72-h radioactive release assays as described elsewhere (9, 33). Briefly, peritoneal exudate macrophages were obtained 4–6 days after i.p. injection with 1.0–2.0 ml of Brewer's thioglycollate medium and washed once with Hanks' balanced salt solution. Macrophage suspensions (104 cells) were plated into the flat-bottomed wells of a 96-well plate and incubated for 2 h, and nonadherent cells were removed. The monolayers were incubated with control medium or an activating agent for 2–24 h, both with and without FBS, after which the medium was removed, and the cultures were washed thoroughly with Hanks' balanced salt solution. Macrophage suspensions (106 cells) were plated into the monolayers and incubated with medium containing radiolabeled target cells. For the 72-h macrophage-mediated cytotoxicity assay, B16 melanoma target cells (104) labeled for 18 h with [5-125I]iodo-2'-deoxyuridine (2000 Ci/mmol; New England Nuclear), washed once by centrifugation, and incubated for 2 h, and nonadherent cells were removed. The monolayers were incubated with medium containing radiolabeled target cells. For the 72-h macrophage-mediated cytotoxicity assay, B16 melanoma target cells (104) labeled for 18 h with [5-125I]iodo-2'-deoxyuridine (2000 Ci/mmol; New England Nuclear) were added to each well in 0.2 ml of DMEM supplemented with 10% FBS. Seventy-two h after plating, adherent target cells were washed and then lysed with 0.1 ml of 0.2 N NaOH. The lysates were absorbed on cotton swabs, placed directly into 12- x 75-mm tubes, and radioactivity was measured in a gamma counter.

The percentage of cytotoxicity was computed by the mathematical relationship

\[
\text{% cytotoxicity} = \frac{\text{cpm in target cells cultured with normal macrophages} - \text{cpm in target cells cultured with test macrophages}}{\text{cpm in target cells cultured with normal macrophages}} \times 100
\]

For the 18-h assay, P815 mastocytoma cells were labeled for 1 h with 300 μCi 53Cr (sodium chromate, specific activity of 50–400 μCi/mg; New England Nuclear), washed once by centrifugation, and incubated for 1 h in EMEM supplemented with 10% FBS. The targets were then washed again with EMEM-FBS and 10⁴ labeled cells were added to each well. Eighteen h later the assay was terminated by removal of 100-μl aliquots from each well and assessment of released radioactive label in a gamma counter. Cytotoxicity was determined by the mathematical relationship

\[
\text{% net cytosis} = \frac{\text{cpm from wells with macrophages} - \text{cpm from wells without macrophages}}{\text{Total cpm} - \text{cpm from wells without macrophages}} \times 100\%
\]

Under these assay conditions, normal (untreated) macrophages were not cytotoxic to target cells.

RESULTS

Sequence of Activation by IFN-γ and LPS. In the first set of experiments, macrophage cultures were incubated with either control or test agents for 24 h before addition of target cells. The data presented in Fig. 1 demonstrate that treatment of macrophages for 2–24 h with 10 units/ml of IFN-γ followed by addition of LPS was insufficient for development of tumoricidal activity against the B16 melanoma. Macrophage-mediated cytotoxicity against the B16 melanoma required concomitant exposure to IFN-γ and LPS for 24 h. In contrast, treatment of replicate macrophage cultures with IFN-γ for 2 h followed by treatment with LPS alone resulted in development of significant macrophage-mediated cytotoxicity against the P815 mastocytoma (65% cytotoxicity). Macrophage-mediated cytotoxicity expressed against B16 melanoma or P815 mastocytoma cells could not be demonstrated when the macrophages were first treated with LPS followed by IFN-γ. Macrophages treated with IFN-γ (50 units/ml) or LPS (50 ng/ml) alone failed to express tumoricidal activity against B16 melanoma or P815 mastocytoma cells (Fig. 1).

Dose Requirement and Kinetics of Macrophage Activation by IFN-γ and LPS. Macrophages exposed to either 5 or 10 units/
ml of r-MuIFN-γ in the presence of exogenously supplied LPS required a treatment time of 24 h to develop tumoricidal properties against the B16 melanoma (40–50% cytotoxicity) (Fig. 2, top). In contrast, treatment of macrophages with identical doses of IFN-γ and LPS for 8 h was sufficient to develop maximal tumoricidal properties against P815 mastocytoma cells (65% cytotoxicity) (Fig. 2, bottom). Treatment of macrophages with increasing amounts of r-MuIFN-γ (5–50 units/ml) induced a dose-dependent increase in the kinetics of macrophage activation as well as the quantitative level of cytotoxicity expressed against the B16 melanoma. Similar changes in the kinetics of activation were obtained when the P815 mastocytoma was used as the target (Fig. 2).

Effect of Polymyxin B on Macrophage Activation by γ Interferon and LPS. Macrophage cultures treated with PMB (40 μg/ml) for 2 h prior to the addition of r-MuIFN-γ (10 U/ml) plus LPS (50 ng/ml) were inhibited completely from induction of tumoricidal activity against B16 melanoma cells (Table 1). In contrast, PMB failed to inhibit the induction of tumoricidal properties against P815 mastocytoma cells (Table 1).

Macrophage Activation Induced by IFN-α/β. Macrophages treated with IFN-α/β (100 units/ml) in the presence or absence of LPS (50 ng/ml) failed to express tumoricidal activity against B16 melanoma cells (Table 2). In contrast, exposure of replicate macrophages to IFN-α/β in the presence of LPS (50 ng/ml) induced a dose-dependent acquisition of macrophage-mediated cytotoxicity against P815 mastocytoma cells, but only when FBS was present during the priming step (Table 2).

Effect of Treatment Sequence on Macrophage Activation by IFN-γ and LPS. Macrophages treated initially with either LPS (50 ng/ml) or medium alone prior to the addition of r-MuIFN-γ (10 units/ml) plus LPS lost their capacity to become activated by 8 h (Fig. 3). In contrast, macrophages primed initially with r-MuIFN-γ and subsequently challenged with the addition of LPS retained their capacity to become activated and express tumoricidal activity against B16 melanoma (Fig. 3). To evaluate if the kinetics of priming influences the exposure time required for LPS to render macrophages tumoricidal, cultures were treated with IFN-γ and challenged with LPS for different periods of time (Fig. 4). Macrophages treated for 8 h with IFN-γ (10 units/ml) and challenged with LPS (50 ng/ml) for 2–4 h before removal of both agents failed to acquire significant macrophage-mediated cytotoxicity against B16 melanoma (0; 13% cytotoxicity). Macrophages treated with r-MuIFN-γ for 20 h and challenged with LPS 4 h before the addition of B16 melanoma cells, however, resulted in acquisition of significant tumoricidal properties (76% cytotoxicity) (Fig. 4).

**DISCUSSION**

The underlying cellular events associated with or essential for acquisition and expression of tumoricidal properties remain obscure. Numerous studies have emphasized that activation of murine peritoneal macrophages in vitro to express tumoricidal properties results from a complex cascade of events induced in a stepwise progression following exposure to a series of immunomodulatory agents (reviewed in Ref. 1). These conclusions were derived from experiments that for the most part used impure reagents, a variety of different effector and target cell populations, and a diverse array of assay methodologies. The purpose of the present study was to define more precisely the temporal sequence of events required for induction of tumori-
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Fig. 3. Decay of ability of macrophages to be activated to express tumoricidal properties against B16 melanoma targets. Macrophage monolayers were exposed to either medium alone, 50 ng/ml of LPS, or 10 units/ml of IFN-γ for various periods of time. At the end of each time point, the macrophages were washed and 10 units/ml IFN-γ plus 50 ng/ml LPS were added for the remaining time to total 24 h. Cytotoxic activity against the B16 melanoma was then assessed. D, medium; •, IFN-γ plus LPS; ■, IFN-γ; □, LPS.

Fig. 4. Kinetics of priming of macrophages for development of tumoricidal activity. Macrophage monolayers were treated with UN- (10 units/ml) and then challenged with LPS (50 ng/ml) for different periods of time. Medium was then added after washing for a total exposure time of 24 h for all reagents. Cytotoxic activity against B16 melanoma cells was then determined. D, medium; •, UN- plus LPS; ■, UN-; □, LPS.

The macrophages treated with highly purified preparations of IFN either alone or in combination with LPS. Concomitant exposure of macrophages to r-MuIFN-γ plus LPS was an absolute requirement for induction of tumoricidal properties against B16 melanoma targets. Macrophage monolayers were exposed to either medium alone, 50 ng/ml of LPS, or 10 units/ml of IFN-γ for various periods of time. At the end of each time point, the macrophages were washed and 10 units/ml IFN-γ plus 50 ng/ml LPS were added for the remaining time to total 24 h. Cytotoxic activity against the B16 melanoma was then assessed.

To accomplish this task we systematically compared two commonly used assay methodologies that utilize identical effector but different target cell populations for monitoring the acquisition and expression of tumoricidal properties. The results suggest that cellular events regulating expression of the tumoricidal phenotype may be different depending on the assay system utilized. This observation is supported by four lines of evidence obtained in the present study as described below.

Concomitant exposure of macrophages to r-MuIFN-γ plus LPS was an absolute requirement for induction of tumoricidal properties against B16 melanoma cells. In contrast, macrophages treated for as little as 2 h with IFN-γ followed by the addition of LPS for the remaining activation period resulted in significant macrophage-mediated cytotoxicity against P815 mastocytoma cells.

To determine if the duration of exposure and dose of IFN could influence the kinetics of activation, we examined the effect that different concentrations of r-MuIFN-γ induced following concomitant exposure with LPS. The kinetics for induction of significant tumoricidal properties against P815 mastocytoma cells following exposure to r-MuIFN-γ (10 units/ml) plus LPS (50 ng/ml) was essentially complete after 8 h. In contrast, macrophages treated in an identical fashion required a treatment time of 24 h to express tumoricidal properties against B16 melanoma cells. Increasing the concentration of r-MuIFN-γ to 50 units/ml decreased the time necessary for induction of macrophage-mediated cytotoxicity against both types of target cells.

Treatment of macrophage cultures with the polypeptide antibiotic, PMB, at a concentration (40 μg/ml) sufficient to inhibit the effect of high levels of exogenously supplied LPS failed to inhibit the induction of tumoricidal properties against P815 mastocytoma cells following treatment with r-MuIFN-γ (10 units/ml) plus LPS (50 ng/ml). Conversely, PMB inhibited completely the induction of tumoricidal properties against B16 melanoma cells.

To investigate if other interferons fulfilled similar or different activation criteria, we evaluated the effect MuIFN-α/β induced in the two assay systems. A highly purified preparation of IFN-α/β (10⁸ units/ml) in the presence of LPS (50 ng/ml) was insufficient to activate macrophages for cytotoxicity against B16 melanoma cells. In contrast, a dose-dependent induction of tumoricidal properties was obtained against P815 mastocytoma cells as has been reported recently (10). Unlike results obtained with IFN-γ, however, activation with IFN-α/β was only achieved in the presence of serum.

Since the induction of tumoricidal properties expressed against B16 melanoma required concomitant exposure to two agents, and we are unlikely to achieve synchronous delivery of macrophage activating agents in vivo, we wished to establish whether the presence of one activation signal influenced the subsequent development of tumoricidal activity when challenged with a second activating agent. The results demonstrate that the initial duration of exposure to lymphokine influences the time required for treatment with LPS for development of tumoricidal activity against B16 melanoma, suggesting that the goal of achieving successful therapeutic immunomodulation in vivo will require sophisticated rather than random delivery protocols.

Two aspects of these results are worth noting: (a) the serum dependence of macrophage priming by IFN-α/β is apparently

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systems as described, IFN-γ was effective either in the presence or absence of serum (data not shown). The basis for the dependence on serum for priming by IFN-α/β is not known but may relate simply to a dependence on a cofactor component present in serum; (b) the ability of PMB to inhibit priming by IFN-γ for lysis of B16 but not P815 targets may be due to multiple effects of the antibiotic, not just neutralization of endotoxin. If endotoxin neutralization were the sole effect of PMB pretreatment, then lysis of P815 targets would be expected to be affected as well. Since this was not the case (Table 1), alternative explanations must be explored. One possibility is that PMB might be affecting macrophage activation for lysis of B16 targets by interfering with protein kinase C activation. Two lines of evidence support this contention: (a) PMB has been demonstrated to inhibit protein kinase C activity, probably by interacting with phosphatidylserine (34); (b) IFN-γ has been shown to activate protein kinase C in murine macrophages, while IFN-α/β had no effect (35). Taken together, these published observations in conjunction with our data support the possibility that lysis of B16 targets by IFN-γ activated murine macrophages proceeds via a mechanism dependent on protein kinase C. Lysis of P815 might not be dependent on protein kinase C since PMB did not block IFN-γ mediated macrophage activation for P815 lysis (Table 1), and IFN-α/β also activated the macrophages for lysis of P815 (Table 2) (10).

The results reported here support the view that the biochemical events regulating the process of macrophage activation are dependent on sequence of addition, duration of exposure, and concentration and type of IFN. In addition, expression of macrophage-mediated cytotoxicity induced by immunomodulatory agents can vary substantially with respect to the nature of the cells used to monitor the expression of this phenotype.

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