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Interferon-γ Is Required in Activation of Macrophages for Tumor Cytotoxicity

DANIELA N. MÄNNEL[1] AND WERNER FALK

Institut für Immunologie und Genetik, Deutsches Krebsforschungszentrum, D-6900 Heidelberg, West Germany

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The participation of interferon-γ in activation of murine macrophages for tumor cell lysis was investigated. Biochemically macrophage activation factor and interferon-γ have not been separated. Antiviral titers correlated closely with macrophage activation in antigen- or mitogen-induced spleen cell supernatants. A monoclonal rat antibody that neutralized virus-induced interferon was also found to neutralize interferon-γ in such supernatants. These monoclonal antibodies were coupled to CH-Sepharose 4B and used for absorption of antiviral activity from mitogen-induced spleen cell supernatants. Absorption of the interferon was paralleled by the reduction of the macrophage-activating capacity of the supernatants. Data from control absorptions supported the specificity of the absorption effect. These results indicate that interferon-γ is required for activation of macrophages for tumor cell lysis. These results can be interpreted in two ways: (a) the monoclonal antibodies cross-react with interferon-γ and with a mediator that is required for activation of macrophages for tumor cell lysis or (b) interferon-γ itself is an essential cofactor for macrophage activation.

INTRODUCTION

Evidence for a prominent role of interferons in modulating macrophage antitumor function has been extensively described (reviewed in (1)). Nonspecific tumoricidal activity was induced in macrophages utilizing semipurified fibroblast interferon (IFN) preparations (2, 3). Of these IFN (α and β) preparations 10^3 units/ml of culture medium were necessary for significant activation of macrophages. The mechanism of activation of IFN (α and β) seems to be qualitatively and quantitatively different from that of macrophage-activating factor (MAF) obtained from antigen- or mitogen-induced spleen cell supernatants (3, 4). On the other hand, striking similarities between MAF and another type of interferon, IFN-γ, in physicochemical characteristics and coordinate production have been observed (5). Physicochemical separation of MAF and IFN-γ has not yet been achieved. Also, cloned T cells and T-cell hybridomas were reported to be producer cells for both IFN-γ and MAF activity (6–8).

In this report a different approach for evaluation of the function of IFN-γ in macrophage activation is described. Monoclonal rat antibodies with inhibitory capacity for antiviral activity were tested for their effects on macrophage activation by MAF-containing lymphokines.

[1] To whom correspondence should be addressed.

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MATERIALS AND METHODS

Mice. Male C_3H/Tif, 6 to 12 weeks of age, were purchased from Bomholtgard, Ry, Denmark. Female LOU rats were obtained from R. Kemler, F. Miescher Institute, Tübingen, West Germany.

Lymphokine preparations. Concanavalin A (ConA) supernatants were prepared by culturing 10^7 spleen cells/ml culture medium (RPMI 1640, GIBCO) + 10% heat-inactivated FCS + 50 μg gentamicin (Sigma)/ml for 24 hr in the presence of 5 μg ConA (Pharmacia)/ml at 37°C in 5% CO_2 in moist air. *α-Methyl*-D-mannoside (Carl Roth KG, Karlsruhe, West Germany) was added to the cell-free supernatants to a concentration of 1 m*M*. For antigen-stimulated spleen cell supernatants mice were immunized intraperitoneally (ip) with 5×10^6 viable *Mycobacterium bovis,* strain BCG (Phipps substrain, TMC 1029, Trudeau Mycobacterial Collection, Saranac Lake, N.Y.) and 3 weeks after immunization 5×10^6 spleen cells/ml culture medium were cultured with 50 to 100 μg of purified protein derivative/ml (PPD, Connought Medical Research Laboratories, Toronto, Canada) for 48 hr at 37°C in 5% CO_2 in moist air. Cell free supernatants were stored at −20°C until used.

Assay for interferon activity. Interferon titrations were performed by using a microtiter assay with L929 cells (0.2 ml of medium (RPMI 1640 + 5% FCS) per well) and vesicular stomatitis virus (Indiana strain) as challenge virus. One unit corresponds to the minimal amount of interferon capable of conferring protection to 50% of the cells. One microtiter unit/0.2 ml corresponds to 2 NIH reference units/ml. All titers are expressed in laboratory units.

Assay for interleukin 2 activity. The amount of interleukin 2 (IL-2) in supernatants was measured by their ability to support the growth of the IL-2 dependent cell line W-2 (9). Thymidine incorporation after a 20-hr incubation and a 4-hr pulse with 0.5 μCi of [*methyl*-^{3}H]thymidine ([^{3}H]TdR, sp act 50 Ci/mmol, Amersham International Ltd, Amersham, U.K.) was determined. Results are expressed as laboratory units as described by Gillis *et al.* (10).

Assay for macrophage cytotoxic activity. The detailed protocol of the cytotoxicity assay was described earlier (11). Mice were treated ip 1 day previously with 1 ml of phosphate-buffered salaine (PBS). Peritoneal exudate cells (PEC) were collected after ip injection of 10 ml of Dulbecco's minimal essential medium (DMEM) containing 10% heat-inactivated FCS and 50 μg of gentamicin/ml. Peritoneal fluid was withdrawn through the anterior wall with a 19-gauge needle. Fluids were pooled and a sample was taken for differential and total cell counts, and the remainder was centrifuged in polypropylene tubes (2074, Falcon Plastics) at 250*g* for 10 min. Differential counts were made on Wright-stained cell smears prepared by cytocentrifugation (Cytospin centrifuge, Shandon Southern Instruments, Camberley, U.K.). Washed PEC suspensions were adjusted to 12×10^5 macrophages/ml DMEM (10% FCS, gentamicin) and 0.1 ml of the cell suspension per flat-bottom well of a 96-well tissue culture cluster (Costar 3596) was incubated at 37°C in 5% CO_2 in moist air. Macrophages were exposed to dilutions of lymphokine supernatants as adherent PEC for 5 hr at 37°C in 5% CO_2 in moist air or continuously during the 48-hr culture period. [^{3}H]TdR-prelabeled tumor target cells (1023) (3-methylcholanthrene-induced fibrosarcoma of C3H/HeN origin) were obtained from washed and trypsin-digested cell monolayers. Two to three million adherent cells in 20 ml of DMEM (10% FCS, gentamicin) were labeled with 1 μCi of [^{3}H]TdR/ml (sp act 5 Ci/mmol, Amersham International Ltd)

for 20 hr at 37°C in 5% CO_2 in moist air. Six thousand tumor cells were added to the PEC in a total volume of 0.2 ml of DMEM (10% FCS, gentamicin). After 48 hr, labeled tumor cell monolayers lysed with 0.5% SDS in water were used to estimate total incorporated counts. Cytotoxicity was estimated by measurement of [^{3}H]TdR release from prelabeled tumor cells in triplicate cultures and expressed as percentage of SDS total counts ± SD or as specific cytotoxicity according to the formula:

$$100 \times \left(\frac{\text{experimental cpm} - \text{background cpm}}{\text{total incorporated cpm} - \text{background cpm}} \right).$$

IgG preparation from rat hybridomas. For mass production of antibodies 5×10^6 rat hybridoma cells secreting monoclonal IgG directed against murine IFN (12) were injected ip into adult female rats (LOU) that had received 2 ml of Pristan mineral oil (Carl Roth KG, Karlsruhe, West Germany) ip 1 week previously. One to two weeks later, ascites fluid was recovered from the peritoneal cavity and IgG purified by ammonium sulfate precipitation (45% saturated) followed by gel filtration on Sephacryl-S300.

Coupling of IgG to activated CH-Sepharose 4B. Per 4 g activated CH-Sepharose 4B (Pharmacia) 20 mg of commercial rat IgG or monoclonal IgG from 20 ml ascites fluid was mixed in bicarbonate buffer 0.1 *M*, pH 8, containing 0.5 *M* NaCl, for 1 hr at room temperature. Free binding sites were saturated with ethanolamine 1 *M*, pH 9, and the immunosorbent was washed with five cycles of 0.1 *M*, formate buffer, pH 4, containing NaCl (0.5 *M*) and Tris buffer 0.1 *M*, pH 8, also containing NaCl (0.5 *M*).

RESULTS

Correlation of Antiviral Titer and MAF Activity

Spleen cell suspensions from individual mice infected with BCG were challenged *in vitro* with PPD. The supernatants of these cultures were tested for both their antiviral and macrophage-activating capacities. In all tested samples of over 40 lymphokines from individual mice the activating capacity correlated very well with the IFN titer of the preparation. Figure 1 shows the correlation of antiviral titers and macrophage cytotoxicity obtained from one experiment in which 10 of these lymphokines were tested. From these results one can calculate that a final concentration of less than 10 international units of immune IFN was sufficient for significant macrophage activation. In addition, earlier reports on pH 2 lability of macrophage-activating factor (MAF) (13) and IFN-γ (14, 15) were confirmed with these lymphokines (data not shown).

Neutralization of IFN-γ Activity by Monoclonal Antibodies

A monoclonal rat antibody that neutralized virus-induced interferon was described earlier (12). IgG of this clone was purified from ascites fluid by ammonium sulfate precipitation followed by gel filtration on Sephacryl-S300. Table 1 shows that the monoclonal antibodies not only reduced the antiviral titer of virus-induced interferon, but also inhibited two different IFN-γ preparations in a dose-dependent manner. Commercial rat IgG at the same concentration had no inhibitory effect. IFN-γ activity was destroyed at pH 2 and not neutralized by a sheep antivirus-induced IFN antiserum

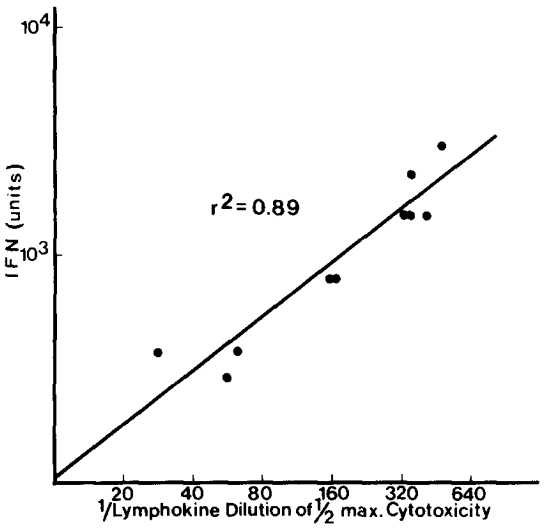

FIG. 1. Correlation of antiviral and macrophage-activating activities in lymphokines. The antiviral titers of 10 mitogen-induced lymphokine preparations were plotted versus the concentration of these lymphokines that resulted in 1/2 maximal tumor cytotoxicity in one experiment.

that completely neutralized the virus-induced IFN (data not shown). Thus, the monoclonal rat IgG besides reacting with all molecular weight species of mouse virus-induced interferon cross-reacts with the antiviral activity of murine IFN-γ.

Absorption of Antiviral and MAF Activities with Monoclonal Antibodies

Since rat IgG interfered with the assay system for macrophage activation, monoclonal antibodies were coupled to activated CH-Sepharose 4B and the immobilized antibodies were used for absorption of interferon activity from ConA supernatants. The antiviral activity was reduced by 37% of the original sample after 2 hr of end-over-end mixing at 37°C (Table 2). Antigen specificity of the absorption was tested by measuring the interleukin 2 activity of the fractions in parallel. IL-2 activity was almost completely recovered indicating the specificity of the absorption for interferon. No reduction of interferon activity was measured when the incubation was performed at room temperature or in the cold (4°C). In contrast to the virus-induced IFN (12) it was not possible to recover antiviral IFN-γ activity from the immunosorbent because

TABLE 1

Neutralization of Antiviral Activity by Monoclonal Antibodies Directed against Interferon

	IFN titer in presence of anti-IFN antibodies[a]		
	0	0.2 μg	2 μg
ConA lymphokine	384	192	72
BCG-PPD lymphokine	144	144	36
Virus-induced IFN	96	48	24

[a] Purified anti-IFN antibodies were added to the regular interferon assay.

TABLE 2

Estimation of Antiviral and IL-2 Activities after Absorption on Monoclonal Anti-IFN Antibodies Fixed on CH-Sepharose 4B

	IFN (units)	IL-2 (units)
ConA lymphokine applied	3840	100
Eluted fraction 1	960	62
Eluted fraction 2	360	28
Eluted fraction 3	90	4
Eluted fraction 4	0	0
Recovery	37%	94%

IFN-γ is labile at pH 2 and eluting the immunosorbent after absorptions at 37°C with buffers containing high salt concentrations did not result in desorption of antiviral activity. These lymphokine preparations were tested before and after absorption on coupled antibodies for their capacity to activate macrophages for tumor cytotoxicity. Figure 2 shows the activation of macrophages after continuous culture with the lymphokines (Panel A) or the activation by pulsing the cells for just 5 hr (panel B). The macrophages were highly cytotoxic when kept at a 1:32 or 1:64 dilution of the untreated lymphokine for 48 hr or when they were pulsed with a 1:10 dilution of the ConA supernatant. The ability for activation was markedly reduced with the absorbed lymphokine preparation. The dose–response curve was shifted to higher concentrations and the continuous presence of a 1:16 dilution of lymphokine was necessary to induce the same degree of cytotoxicity (panel A). No cytotoxic response was induced in this experiment by pulsing the macrophages for 5 hr with the absorbed ConA supernatant (panel B).

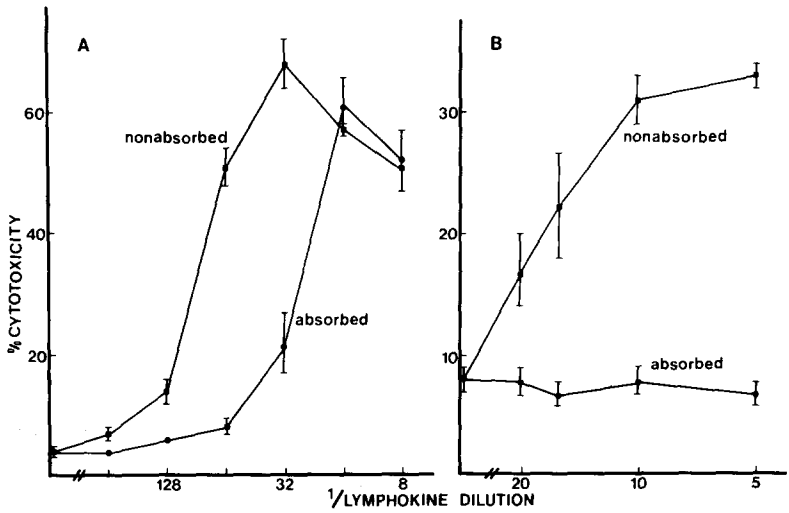

FIG. 2. Activation of macrophages for tumor cytotoxicity for (A) 48 hr or (B) 5 hr with ConA-induced spleen cell supernatants before or after absorption on monoclonal anti-IFN antibodies coupled to CH-Sepharose 4B. Cytotoxicity (released [^{3}H]TdR) was estimated at 48 hr and expressed as percentage of total incorporated counts.

Correlation of Absorption of Antiviral and MAF Activities

Nonspecific absorption or destruction of interferon activity during the 2-hr mixing period could not be excluded, even though another lymphokine, IL-2, was almost completely recovered (Table 2). Commercial rat IgG was coupled to CH-Sepharose 4B to estimate the possible effect of nonspecific absorption. ConA supernatant was absorbed on this material and in a second absorption step (a) on monoclonal anti-IFN antibodies or (b) for a second time on rat IgG. Antiviral titers were reduced by 50% after the first absorption (1950 to 950 units) and again by half after the second treatment with coupled rat IgG (400 units). The second absorption on anti-IFN antibodies, however, reduced the antiviral titer to the detection limit. The ability of these preparations to induce macrophage cytotoxicity is shown in Table 3. The lymphokine absorbed on rat IgG had lost some macrophage activation capacity so that a 1:10 dilution of the once- and a 1:5 dilution of the twice-absorbed material was necessary to reach cytotoxicity values comparable to induction with a 1:80 dilution of the original material. The lymphokine that was absorbed on anti-IFN antibodies after the nonspecific absorption step showed little activating effect even at the highest dilution tested (1:5).

DISCUSSION

In a previous report it was published that monoclonal rat antibodies raised against a mixture of highly purified mouse IFN-α and IFN-β neutralized and bound fibroblast-derived antiviral activities of all molecular weight species (12). In this report it is shown that antiviral activities from other sources like mitogen- or antigen-induced lymphokine preparations were inhibited by these antibodies as well. The specificity of the monoclonal antibodies seems to be directed against a rather common structure of all interferons. Carbohydrates could be the binding site for the antibodies since no peptide sequences common for all IFN species were described.

Our results show a very close relationship of antiviral titers with MAF activity when lymphokines from individual inbred mice were prepared following an identical protocol and when they were tested in parallel for the two activities (Fig. 1, $r^2 = 0.89$). This is in contrast to recent findings that describe the segregation of macrophage-stimulating factors from antiviral activity in ConA-induced supernatants of T-cell hybridomas (6). However, the source of these supernatants, tumor cells, was entirely

TABLE 3

Activation of Macrophages for Tumor Cytotoxicity with Lymphokines before and after Absorption

Lymphokine[a]	1:10	1:5
Nonabsorbed	43.8[b]	48.2
Absorbed on rat IgG	40.6	44.7
Absorbed twice on rat IgG	13.1	41.2
Absorbed on rat IgG and anti-IFN	2.4	16.5

[a] Macrophages were activated for 5 hr with ConA-induced spleen cell supernatants nonabsorbed or absorbed on anti-IFN antibodies and/or rat IgG coupled to CH-Sepharose 4B.

[b] Specific cytotoxicity was estimated after 48 hr and expressed as the percentage of total incorporated counts.

different from the one described in this paper. The possibility exists that there is a number of different activating factors depending on the macrophage function tested. Also, in the activation of macrophages for tumor cytotoxicity, IFN-γ could be an essential cofactor which is not required for induction of other macrophage functions.

The monoclonal rat antibodies used in this study are rather unusual in so far as they do not differentiate between different forms of mouse IFNs. However, they are unable to bind or neutralize IL-2. From this finding and from control absorptions on rat IgG coupled to CH-Sepharose 4B it was demonstrated that besides the nonspecific loss of antiviral and MAF activity during treatment with the immunosorbent, specific absorption of IFN and macrophage-activating factor occurred. The concurrent absorption of the two activities indicates two possibilities. (a) Both mediators share at least an antigenic site even if they are not identical. This means that MAF in antigen- or mitogen-induced lymphokine preparations belongs to the IFN family and is closely related to IFN-γ. (b) IFN-γ is a cofactor necessary to activate macrophages for tumor cell lysis in combination with another activating factor and both are contained in antigen- or mitogen-induced lymphokines. Which of the two possibilities is true for the activating mechanism can only be revealed by utilizing purified and defined substances as they might become available by recombinant DNA techniques.

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