Brief Definitive Report

MONOCLONAL ANTIBODY TO MURINE GAMMA INTERFERON INHIBITS LYMPHOKINE-INDUCED ANTIVIRAL AND MACROPHAGE TUMORICIDAL ACTIVITIES

BY GEORGE L. SPITALNY AND EDWARD A. HAVELL

From The Trudeau Institute, Inc., Saranac Lake, New York 12983

Lymphokines capable of mediating a variety of biological activities are secreted by T cells after exposure to polyclonal T cell mitogens, such as phytohemagglutinin (PHA) or concanavalin A (Con A), or after specific antigenic stimulation during the development of cell-mediated immunity (reviewed in 1). One of these lymphokines, gamma interferon (IFN-γ), possesses antiviral activity and is able to induce macrophage tumoricidal activity in vitro (2). Still unresolved is whether IFN-γ is the only lymphokine capable of activating macrophages to destroy tumor cells. One approach for resolving this issue would be to produce a monoclonal antibody able to neutralize the activities of IFN-γ. Here we report the isolation of a hybridoma that secretes a monoclonal rat IgG1 antibody (MAb) which neutralizes both the antiviral activity of murine IFN-γ (MuIFN-γ) and the ability of lymphokine preparations to induce macrophage tumoricidal activity.

Materials and Methods

Animals. Male (A × C57BL/6)F_1 (AB6F_1) mice, 8–12 wk of age, and female DA strain rats, 8–10 wk of age, were used throughout the studies. All animals were provided by the Trudeau Institute Animal Breeding Facility.

Production and Partial Purification of MuIFN-γ. High-titered MuIFN-γ was produced by PHA-stimulated spleen cells derived from AB6F_1 mice infected intravenously 6 d earlier with a sublethal dose of 2×10^3 *Listeria monocytogenes* (3). From the unfractionated spleen cell supernatant, MuIFN-γ was partially purified 50-fold to a specific activity (sp act) of 5×10^5 U/mg protein by Con A affinity chromatography (4, 5). This partially purified MuIFN-γ was used for the immunization of rats.

Immunization of Rats, Cell Fusion Procedure, and Cloning of Hybridomas. Rats of the DA strain were injected each time subcutaneously with 32,000 antiviral units of the partially purified MuIFN-γ emulsified in Freund's adjuvant. The schedule of injections, timing of bleedings, and specificity of antisera were detailed previously (5). 19 wk after the last injection, one rat was injected intravenously with 32,000 U of MuIFN-γ. 4 d later, spleen cells from the rat were mixed at a 5:1 ratio with cells of the mouse P3U-1 myeloma line and hybridomas were allowed to form in the presence of polyethylene glycol 1450 (J. T. Baker Chemical Co., Phillipsburg, NJ) using standard procedures (6). Cells were suspended in Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS) and seeded into 16-mm diam wells. The following day, all wells received an

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additional equal volume of medium containing 2× hypoxanthine, aminopterin, and thymidine (HAT). 1 wk later, this medium was removed and the cultures replenished with medium containing only hypoxanthine and thymidine. After 7–10 d, the hybridoma supernatants were screened for antibodies capable of neutralizing the antiviral activity of MuIFN-γ.

1 well out of 240 was positive for neutralization activity and hybridomas were expanded and screened again for anti-MuIFN-γ activity. Positive hybridomas were subcloned twice by limiting dilution in microtiter plates with irradiated thymocyte feeder layers in DME containing 20% FBS (7). The cloned hybridoma (R4-6A2) was expanded and the Ig in the culture supernatant was precipitated by 45% saturation with $(NH_4)_2SO_4$. The precipitate was resuspended in phosphate-buffered saline (PBS), pH 7.4, to 1/30 of the original volume, dialyzed exhaustively in PBS, and then sterile filtered.

IFN and Antibody Neutralization Assays. IFN assays were carried out as previously described (8) using L929B cells and vesicular stomatitis virus (VSV) as the challenge virus. Since no MuIFN-γ international standard is available, included in each assay was the MuIFN international standard G-002-904-511.

The antiviral activity neutralization assay for the detection and quantitation of anti-MuIFN-γ activity in hybridoma supernatants was performed as described (5). In brief, an equal volume (0.05 ml) of hybridoma culture supernatant and the partially purified MuIFN-γ (final concentration 10 U/ml) were mixed together in 96-well microtiter plates and incubated at 37°C for 1 h. After this time, 0.1 ml of L929B cells (1.8×10^5/ml) were added to each well and incubated for 18 h. Each of the wells was then challenged with VSV at a multiplicity of infection of 0.1 and examined at 48 h for neutralization of the antiviral activity of the test MuIFN-γ.

Macrophage Tumoricidal Assay. The ability of lymphokine preparations to induce macrophage tumoricidal activity in vitro was assayed by established procedures (9). Macrophage-rich exudates were harvested from the peritoneal cavities of $AB6F_1$ mice injected 3 d earlier with 1.0 ml of a 10% solution of proteose-peptone. The cells were washed and resuspended at 10^6 cells/ml in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with sodium pyruvate and 5% FBS (Sterile Systems, Logan, UT). 0.2 ml of cells were allowed to adhere to the wells of a microtiter plate (Costar, Data Packaging, Cambridge, MA). 2 h later, nonadherent cells were washed off and 0.2 ml of test lymphokine preparations were added to groups of triplicate wells. Control macrophages without lymphokines were included in every experiment as a measure of spontaneous cytotoxicity and this activity was subtracted as background (spontaneous release) from the data. To each of the wells, 10 ng of endotoxin (*Escherichia coli* 0111:B4; Sigma Chemical Co., St. Louis, MO) was also added. Each of the lymphokine preparations was incubated for 1 h at 37°C with different concentrations of the MAb against MuIFN-γ, before the addition to the macrophages. 5 h later, after the addition of lymphokines, 0.01 ml of P815 mastocytoma cells (provided by Dr. R. Schreiber), which had been labeled with ^{51}Cr (50–400 mCi/mg; Amersham Corp., Arlington Heights, IL) according to published methods (9), was added directly to each of the wells at an effector to target ratio of 5:1. Sixteen hours later, the uppermost 0.1 ml of fluid from each well was collected and counted in an LKB Rack Gamma II gamma counter (LKB Instruments, Inc., Gaithersburg, MD). Tumoricidal activity was assessed by the following formula: Percent specific ^{51}Cr release = [(experimental release − spontaneous release)/(total release − spontaneous release)] × 100.

Immunoadsorption of Lymphokine Preparations. The concentrated MAb culture supernatant was coupled to CNBr-activated Sepharose 4B beads according to manufacturer's instructions (Pharmacia, Inc., Piscataway, NJ). Mock Sepharose beads were generated by treating CNBr-activated Sepharose 4B, in the absence of protein, under identical conditions. Lymphokines were diluted to 128 IFN U/ml, mixed either with MAb coupled to Sepharose beads or mock Sepharose beads, and rotated for 15 h at room temperature. The beads were then pelleted at 200 *g* for 5 min and the supernatant tested for antiviral and tumoricidal inducing activities.

Biological Reagents. The isotype of the MAb was determined by double diffusion in agar using anti–heavy chain antisera purchased from Miles Laboratories, Inc., Kankakee,

TABLE I

Specificity of Rat Monoclonal Anti-MuIFN-γ

Interferon	Production mode		Fraction	Neutralization titer* for antiviral activity
	System	Induction		
MuIFN-γ	Spleen cells	Con A‡	—	14,000
MuIFN-γ	Spleen cells	PHA§	—	13,000
MuIFN-γ	Spleen cells	PHA‖	Con A-bound‖	13,000
MuIFN-γ	Spleen cells	PHA‖	Con A-unbound‖	13,000
MuIFN-γ	Recombinant DNA¶	—	—	13,000
MuIFN-γ	Serum	BCG/OT**	—	13,000
MuIFN-γ	MLR	Allogeneic cells‡‡	—	26,000
MuIFN α + β§§	C-243	Virus	—	<20
Rat IFN-γ	Spleen cells	PHA‖‖	—	<20
Human IFN-γ	Blood leukocytes	PHA¶¶	—	<20

* Neutralization titer is defined as the reciprocal of the highest dilution of antibody that, when mixed with an equal volume of IFN-γ (final IFN-γ concentration, 10 U/ml), neutralizes 50% of the antiviral activity as judged by the development of viral cytopathic effect (5).

‡ Spleen cells from *Listeria*-immune AB6F$_1$ mice were stimulated in vitro with 2 μg/ml Con A as described.

§ Same as in ‡, except spleen cells were stimulated with 5 μg/ml PHA.

‖ A PHA-induced MuIFN-γ was fractionated by Con A affinity chromatography into Con A–bound and Con A–unbound fractions (4, 5).

¶ Highly purified IFN-γ (>10^7 U/mg) produced by cloned MuIFN-γ genes transfected to *E. coli* and kindly supplied by Genentech, Inc.

** (A × C57BL/6)F$_1$ mice were injected with 5 × 10^6 *Mycobacterium bovis* (BCG) and 21 d later inoculated with old tuberculin (OT) (10 mg total protein). Serum was collected 2 h after injection of old tuberculin.

‡‡ Mixed lymphocyte reaction (MLR)-induced IFN-γ was produced as described previously (5).

§§ Pure IFN α + β were purchased from Enzo Biochem Inc., New York.

‖‖ Spleen cells from DA rats injected 6 d earlier with 5 × 10^6 *Listeria monocytogenes* subcutaneously were suspended at 10^7 cells/ml in RPMI 1640 with 10 μg/ml gentamycin and stimulated with 5 μg/ml PHA for 24 h.

¶¶ Human IFN-γ induced by PHA induction of blood leukocytes and generously supplied by Flow Laboratories, Inc.

IL. By this procedure the MAb was determined to be in the IgG1 subclass. With a radial immunodiffusion kit (Miles Laboratories, Inc.), the concentration of MAb protein in the concentrated culture supernatant was determined to be 1.55 mg/ml. To neutralize the antiviral activity of 10 U of MuIFN-γ, 30.0 ng of MAb was required.

A variety of mouse, rat, and human IFN were included in studies to test the specificity of the MAb. The source and mode of production for each of these IFN are included in the footnotes to Table I.

Results and Discussion

The specificity of the neutralizing activity of the concentrated MAb, R4-6A2, was tested against a variety of MuIFN-γ preparations. The results in Table I show that regardless of the source of MuIFN-γ, the antiviral activity of each of the samples was neutralized to an approximately similar degree as the original immunogen. It was also determined that the MAb neutralized equally the antiviral activity of both the native 41,000 mol wt form (3) and the 20,500 mol wt species generated under denaturing conditions (10) (results not shown). In contrast, the antiviral activity of MuIFN-α plus MuIFN-β, human IFN-γ, and rat IFN-γ was not neutralized by the MAb. Our finding that the antiviral activities of glycosylated and nonglycosylated (recombinant MuIFN-γ produced by *E. coli*) MuIFN-γ were neutralized equally indicates that the epitope recognized by the MAb is a constituent of the polypeptide structure of the MuIFN-γ molecule.

Experiments next investigated whether the MAb could neutralize the ability of lymphokine preparations to induce macrophage tumoricidal activity in vitro. The amount of MuIFN-γ used for activation of macrophages was based on the lowest quantity of antiviral activity (U/ml) that would consistently induce the

TABLE II

Inhibition of Lymphokine-induced Macrophage Tumoricidal Activity by MAb to MuIFN-γ

IFN preparation	Percent specific ^{51}Cr release*				IFN titer‡		
	Relative excess of MAb§				No MAb	Plus excess MAb	
	No MAb	2×	5×	10×		2×	5×
					U/ml		
Con A–induced MuIFN-γ‖	30.8 ± 1.4	21.4 ± 3.4	14.2 ± 0.5	10.5 ± 1.2	8	<4	<4
PHA-induced MuIFN-γ‖	31.3 ± 1.4	23.0 ± 1.4	13.8 ± 1.0	11.1 ± 2.3	8	<4	<4
PHA-induced MuIFN-γ‖ (Con A–bound fraction)	29.1 ± 3.3	17.5 ± 2.9	11.3 ± 2.3	10.5 ± 1.7	16	<4	<4
PHA-induced MuIFN-γ‖ (Con A–unbound fraction)	33.4 ± 2.8	26.7 ± 1.2	20.0 ± 3.2	13.6 ± 3.1	8	<4	<4
Recombinant DNA MuIFN-γ‖	32.6 ± 2.0	23.8 ± 1.8	14.7 ± 2.1	10.6 ± 1.7	16	<4	<4
MuIFN-α + MuIFN-β¶	6.3 ± 1.2	4.0 ± 2.0	—	—	128	128	—
Con A–induced MuIFN-γ**	32.6 ± 1.7	—	—	35.3 ± 2.7	—	—	—

* Total cell-associated ^{51}Cr determined by lysing P815 target cells with Triton X-100. Mean of triplicate samples for three separate experiments ± SEM.

‡ The IFN titer is defined as the reciprocal of the highest dilution of the sample that protects 50% of the assay cells (L929 B) from viral (vesicular stomatitis virus) cytopathic effect.

§ The quantity of MAb reacted was based on neutralization of the antiviral activity of each preparation. Since the antibody titer was determined by the inhibition of 50% of the activity (see legend to Table I), it was necessary to add at least twofold more antibody to abolish all antiviral activity.

‖ Lymphokines prepared according to methods outlined in legend to Table I.

¶ Pure IFN α + β were produced by Newcastle disease virus-induced mouse C-243 cells.

** Macrophages were incubated with lymphokines for 5 h before the addition of MAb.

maximum expression of tumoricidal activity. Each sample tested for its ability to activate macrophages was simultaneously assayed for antiviral activity. In addition, individual control wells consisting of macrophages or ^{51}Cr-labeled tumor cells were incubated with each of the concentrations of lymphokines, antibody, or combinations of both, and these were consistently found not to be toxic to the cells as determined by either trypan blue dye exclusion or measurement of ^{51}Cr release. The results presented in Table II show that unfractionated lymphokines (sp act, 10^4 U/mg) and partially pure (Con A bound: sp act, 5×10^5 U/mg) and highly purified (recombinant: sp act, 10^7 U/mg) MuIFN-γ preparations all induced macrophages to become tumoricidal. In contrast, a preparation consisting of a mixture of MuIFN-α plus MuIFN-β failed to induce tumoricidal activity, even when present at 10 times the quantity of antiviral activity of MuIFN-γ. When test lymphokines were preincubated for 1 h with twice the amount of MAb required to neutralize 50% of the stated MuIFN-γ concentration before the addition to macrophages, antiviral activity was completely abolished while tumoricidal activity was inhibited 25–40%. To reduce the tumoricidal-inducing activity of all lymphokines to <50% of maximum activity, it was necessary to add 5–10-fold more of the MAb than required for the abolishment of antiviral activity. With 50–100-fold excess, antibody tumoricidal activity was inhibited by 75–90% (not shown). The addition of MAb to macrophages after an initial 5 h period of exposure to lymphokines did not inhibit tumoricidal activity. The reason that more MAb was required to neutralize tumoricidal activity than antiviral activity is unknown but may be related to the greater sensitivity of the tumoricidal assay.

Immunoadsorption was also used to demonstrate that MuIFN-γ present in lymphokine preparations was the active mediator required for induction of macrophage tumoricidal activity. The MAb was coupled to CNBr-activated

TABLE III

Specific Binding of Lymphokine-induced Antiviral and Macrophage Tumoricidal Activities to Immobilized Anti-IFN-γ MAb

MuIFN-γ preparation‡	Activities of lymphokines before and after incubation with MAb coupled to Sepharose beads*			
	Antiviral (IFN titer)		Tumoricidal (Percent specific ^{51}Cr release)	
	Before	After	Before	After
Con A–induced	128	<4	34.6 ± 1.8	1.3 ± 1.1
PHA-induced	128	<4	34.4 ± 2.0	1.5 ± 2.3
PHA-induced (Con A–bound fraction)	128	<4	33.8 ± 1.5	0.7 ± 1.3
PHA-induced (Con A–unbound fraction)	128	<4	34.4 ± 2.0	1.5 ± 0.7
Recombinant DNA	128	<4	35.0 ± 2.1	4.9 ± 3.1
	Mock Sepharose beads without coupled MAb*			
PHA-induced	128	128	33.2 ± 1.5	34.1 ± 2.2
Recombinant DNA	128	96	32.9 ± 4.4	31.6 ± 3.4

* Lymphokines were diluted to 128 IFN U/ml, mixed either with MAb coupled to Sepharose beads or mock Sepharose beads and processed as described in Materials and Methods.

‡ Lymphokines prepared according to methods outlined in legend to Table I.

Sepharose 4B beads, which were then incubated with each of the lymphokine preparations. It was found that this procedure removed all of the antiviral activity and macrophage tumoricidal activity from each of the lymphokine preparations (Table III). Sepharose beads made without MAb failed to remove the antiviral and tumoricidal-inducing properties of lymphokines.

These studies demonstrate that a rat MAb against MuIFN-γ, initially screened for neutralization of antiviral activity, can also neutralize the ability of lymphokine preparations to activate macrophage tumoricidal activity in vitro. However, to achieve this effect, relatively more MAb was required than was needed to neutralize antiviral activity. Similar results were obtained whether unfractionated lymphokines or partially or highly purified MuIFN-γ preparations were used. In addition, published studies (11, 12) have shown that an MAb against human IFN-γ was capable of completely inhibiting not only antiviral activity but also the ability of these preparations to activate the secretory and microbicidal functions of peripheral blood monocytes. The results of the combined studies with MAb to mouse and human IFN-γ, plus our findings that immobilized MAb specifically removed all soluble factors capable of activating macrophage tumoricidal activity from lymphokine preparations, indicates that the induction of macrophage activation and the expression of antiviral activity are mediated by the same molecule or by molecules sharing similar epitopes.

Summary

Fusion of rat immune spleen cells with mouse myeloma cells resulted in the formation of a stable hybridoma that secretes monoclonal antibody (MAb) directed against murine gamma interferon (MuIFN-γ). This MAb specifically neutralized the antiviral activity of a variety of MuIFN-γ preparations, including a sample produced by recombinant DNA technologies. In contrast, the antiviral activities of a mixture of MuIFN-α plus MuIFN-β, as well as those of rat or human IFN-γ, were not neutralized by this antibody. The ability of the MAb to

inhibit lymphokine-induced macrophage activation was also tested. It was found that in relation to the quantity of antibody needed to completely neutralize antiviral activity, much higher concentrations of MAb were required to abolish the capacity of lymphokine preparations to induce macrophage tumoricidal activity in vitro. The MAb was also coupled to cyanogen bromide-activated Sepharose beads and used as an immunoadsorbent. By reacting lymphokines with MAb coupled to an insoluble matrix, it was possible to show that this immobilized antibody completely and specifically removed from the lymphokine preparations the ability both to invoke macrophage tumoricidal activity and to mediate antiviral activity.

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