ENHANCED PRODUCTION OF MURINE INTERFERON γ BY T CELLS GENERATED IN RESPONSE TO BACTERIAL INFECTION*

By EDWARD A. HAVELL,‡ GEORGE L. SPITALNY, and PARSOTTAM J. PATEL

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

It is generally accepted that T cells produce lymphokines during the generation and expression of cell-mediated immunity (reviewed in 1). Lymphokine preparations often exhibit antiviral activity that is known to be mediated by interferon (IFN).[1] This IFN, because of its distinct antigenic and physicochemical properties, has been designated IFNγ to differentiate it from the IFNα and IFNβ classes (2). In addition to its distinct molecular nature, IFNγ has been reported to be relatively more active than other classes of IFN in its ability to directly inhibit the growth of neoplastic cells (3, 4) as well as in its ability to function as an immunomodulator (5, 6). Because of its T cell origin and pronounced actions on immune responses, it is conceivable that the primary role of the lymphokine IFNγ might be one other than its antiviral action.

IFNγ has been detected in sera of immunized mice after injection of sensitizing antigen (7) or in vitro after exposure of lymphoid cells of immune animals to antigen (8, 9). In place of specific antigenic stimulation, polyclonal T cell mitogens such as phytohemagglutinin (PHA) or concanavalin A (Con A) have been used in vitro to induce the synthesis of low levels of IFNγ from lymphoid cells of nonimmune animals (10, 11). Because polyclonal mitogens stimulate a far greater number of T cells than does specific antigen, it was considered of interest to determine whether lymphocytes from animals actively expressing T cell-mediated immunity might produce higher IFNγ levels in response to PHA or Con A. The studies presented in this paper show that spleen cells from animals given an immunizing infection with the bacterium *Listeria monocytogenes* consistently produced 10- to 20-fold more IFNγ than cells from normal mice after exposure to PHA or Con A. This finding has enabled the development of a reproducible method for the production of high-titered murine IFNγ (MuIFNγ). These studies also reveal a striking parallel between the development of T cell-mediated anti-*Listeria* immunity (12, 13) and an enhanced ability of spleen cells from the responding mice to produce IFNγ in response to PHA or Con A stimulation. Some physicochemical properties of mitogen-induced MuIFNγ are also presented.

* Supported by grants CA-30517, AI-15348, CA-21360, AG-01234, and RR-05705 from the U. S. Public Health Service, the Division of Research Resources, National Institutes of Health, Bethesda, Maryland.

‡ To whom reprint requests should be made.

[1] *Abbreviations used in this paper:* C, complement; Con A, concanavalin A; FBS, fetal bovine serum; HuIFNγ, human interferon γ; IFN, interferon; LDH, lactic dehydrogenase; MuIFNγ, murine interferon γ; NDV, Newcastle disease virus; PHA, phytohemagglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEA, staphylococcal enterotoxin A; TPA, diterpene ester 12-*O*-tetradecanoylphorbol-13-acetate.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/82/07/0112/16 $1.00
Volume 156 July 1982 112–127

Materials and Methods

Animals. Male and female AB6F$_1$ mice (A/Tru × C57BL/6 Tru) 8–12 wk of age were used in these studies. Animals were supplied by the Trudeau Institute Animal Breeding facility, Saranac Lake, NY, and were shown to be free of 11 common murine viruses according to tests by Microbiological Associates Viral Testing Service, Cockeysville, MD. Also, mice were found free of lactic dehydrogenase (LDH) virus as determined by the failure of an injection of AB6F$_1$ sera to raise the serum level of LDH enzyme in germ-free mice (14). Serum levels of LDH were measured with an LDH diagnostic kit (500) from Sigma Chemical Co., St. Louis, MO.

New Zealand white rabbits were originally purchased from Dutchland Laboratories, Denver, PA and have been randomly bred at the Trudeau Institute for a generation. Rabbit sera was used as a source of complement (C).

Bacteria. L. monocytogenes (strain EGD, serotype 3b) was grown to log phase in Trypticase soy broth (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, MD), dispensed in 1.0-ml aliquots, and frozen at −70°C. An inoculum of bacteria was prepared for intravenous injection by diluting bacteria in saline and injecting 0.2 ml of the appropriate dilution. The standard inoculum for intravenous immunization was 2×10^3, and 10^5 was used for challenge in the studies on the passive transfer of immunity.

Listeria Antigen. The soluble *Listeria* antigen used in these studies was prepared according to published procedures (15). Briefly, *L. monocytogenes* was grown in 1,000 ml of low molecular weight Trypticase soy broth at 37°C with agitation for 72 h. The culture supernatant containing antigen was concentrated 10-fold by ultrafiltration on an Amicon XM-50 membrane (Amicon Corp., Scientific Sys. Div., Lexington, MA). The antigen was then precipitated from the concentrate by the addition of solid $(NH_4)_2SO_4$ to a final concentration of 75% saturation. The precipitate was resuspended in sterile H_2O and dialyzed overnight against running tap H_2O. The antigen was sterilized by 0.45-μM millipore filtration, and protein concentration determined by the method of Lowry (16), and lyophilized. The resulting antigen preparation was assessed as to its relative potency by its ability to elicit delayed-type hypersensitivity in the footpads of 6-d *Listeria*-immune mice and its failure to cause an increase in footpad size of nonimmune animals.

MuIFNγ Induction in Spleen Cell Cultures. Spleens were removed aseptically from groups of mice and placed in serum-free RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10 μg/ml gentamycin. Cells were separated from the spleen capsule by holding the spleen stationary and gently pressing out the cords of cells from the cut ends with a glass rod. The cell cords were dispersed into a single cell suspension by several cycles of aspiration with a pasteur pipette. The cells were washed two times with medium and counted in a hemacytometer. Cell viability was always >95% as determined by exclusion of trypan blue. Spleen cells were suspended at a concentration of 10^7 cells/ml and 2 ml of this suspension was placed into 35 × 22-mm tissue culture dishes (Costar, Data Packaging, Cambridge, MA), and IFN synthesis was induced by incubating the cultures with the designated concentration of mitogen for 24 h unless specified otherwise.

Passive Transfer of Anti-Listeria Immunity with Spleen Cells. Passive transfer of anti-*Listeria* immunity was measured by removing spleens from control or *Listeria*-injected mice and preparing spleen cells as described in the preceding section, except that cells were suspended in RPMI 1640 supplemented with 1% fetal bovine sera (FBS) (Gibco Laboratories, Grand Island Biological Co.). The cell suspension was filtered through gauze, washed two times with medium, and then one spleen equivalent was injected intravenously into recipient mice as previously reported (17). 30 min before cell infusion, the recipients were injected intravenously with 10^5 *L. monocytogenes*. The level of immunity transferred is expressed as the $\log_{10}$ protection, which was determined by subtracting the number of bacteria in the spleens of immune cell recipients from the number present in the spleens of normal cell recipients 48 h after injection of bacteria.

Rabbit Anti-Mouse IgG Serum. Rabbits were immunized by administering two sequential intramuscular injections, 2 wk apart, of 200 μg of purified mouse IgG emulsified in complete Freund's adjuvant. The mouse IgG was purified by protein A-Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) affinity chromatography (18). 3 wk after the last injection, the rabbits were bled, and the specificity of the antisera was determined

against whole mouse serum and purified IgG by immunoelectrophoresis. The rabbit anti-mouse IgG (1:10) destroyed 50% of murine spleen cells in C-mediated lysis.

Identification of MuIFNγ-producing Cells. The MuIFNγ-producing cells were tested for their susceptibility to C-mediated lysis after treatment with monoclonal antibodies directed against Thy-1.2, Lyt-1.2, Lyt-2.2, and I-A^k. The monoclonal anti-Thy-1.2 was originally produced by Marshak-Rothstein et al. (19) and obtained from the Salk Institute, La Jolla, CA. The anti-Lyt-1.2 and anti-Lyt-2.2 was purchased from New England Nuclear, Boston, MA. Anti-I-A^k antibody was produced from a hybridoma derived by Oi et al. (20) and obtained from the Salk Institute. Rabbit serum C was prescreened on thymocytes to determine lytic activity and was found at all dilutions tested to give <10% spontaneous lysis. Hybridomas secreting anti-Thy-1.2 antibody or anti-I-A^k were propagated in RPMI 1640 supplemented with 10% FBS. Supernatants from these cultures were used as a source of antibody at a 1:10 dilution. The anti-Lyt antibodies were used at a two- to fivefold excess of that required for lysis of the specific cell type in the preparation.

Spleen cells from animals immunized 6 d earlier with *L. monocytogenes* were suspended at 5 × 10^6 cells/ml in 1.0 ml of RPMI 1640 containing 1% FBS and 10 μg/ml gentamycin. The desired concentration of antibody was added to the appropriate tubes and the suspension incubated in a 37°C water bath. 10 min later, C was added to a final dilution of 1:10. After a 1-h incubation, cells were washed free of antibody, and cell death was quantitated by exclusion of ethidium bromide (21).

For the C-mediated cytolytic studies, a modification of the procedure outlined above for induction of MuIFNγ synthesis was used. The antibody-treated cells were distributed in 0.2-ml quantities to wells of a 96-well microtiter plate (Costar, Data Packaging). PHA was added to a final concentration of 5 μg/ml to each experimental group consisting of triplicate wells. After 24 h incubation, the supernatants from each well of the respective experimental group were pooled and assayed for IFN activity.

Chemicals and Reagents. Purified Con A (type IV) was purchased from Sigma Chemical Co., and purified PHA was obtained from Burroughs Wellcome, Research Triangle Park, NC. Staphylococcal enterotoxin A was produced by the Microbiology Biochemistry Branch, Division of Microbiology, Food and Drug Administration, Cincinnati, OH. Stock solutions (1 mg/ml) of the mitogens were prepared in sterile distilled H_2O.

Cultures of L cells (strain Lpa) and pure bone marrow-derived macrophages were induced with either Newcastle disease virus (NDV) or polyinosinic-polycytidylic acid (poly [I]·poly [C]) according to published procedures (22–24) to provide preparations of MuIFNα and MuIFNβ for use in these studies.

IFN and Antiserum Neutralization Assays. IFN assays were carried out as previously reported (25) using the L929B strain of L cells and vesicular stomatitis virus as the challenge virus. Because no MuIFNγ standard is available, included in each assay was the MuIFN international standard (G-002-904-511), and all titers were corrected against this international standard.

The antiserum neutralization assays of antiviral activities were done as described by Havell et al. (26) also using L929B cells and vesicular stomatitis virus. The anti-L cell MuIFN serum used was the kind gift of Dr. Erwin Braude and Dr. W. E. Stewart II (Memorial Sloan-Kettering Cancer Center, NY). This antiserum, which neutralizes both MuIFNα and MuIFNβ, was raised in a goat by immunization with NDV-induced L cell IFN (specific activity, 10^6 U/mg) known to possess both MuIFNα and MuIFNβ activity (27, 28). The rabbit anti-MuIFNγ neutralizing serum was the kind gift of Dr. Juana Wietzerbin (Curie Institute, Paris, France). This antiserum was raised according to published procedures (29).

Molecular Weight and Isoelectric Point Determinations. Molecular weight determinations were carried out by means of gel filtration column chromatography using Bio-Gel P-60, 100–200 mesh (Bio-Rad Laboratories, Richmond, CA) as the column matrix. The column buffer was 0.05 M Na citrate with 1 M NaCl, pH 6.0. The column (73 × 2.5 cm) was run at 4°C and calibrated with the following molecular weight markers: rabbit IgG, 150,000; ovalbumin, 43,000; myoglobin, 18,500; and cytochrome C, 12,000. 1-ml fractions were collected at a column flow rate of 15 ml/h.

Chromatofocusing, a technique originally described by Sluyterman et al. (30, 31), was used to determine the isoelectric points of the IFN in various preparations. A chromatofocusing kit

containing the ion exchanger PBE 94 as well as the polybuffer exchangers, polybuffers 96 and polybuffer 74, were purchased from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc. Samples and the ion exchanger PBE 94 were equilibrated in starting buffer (0.025 M Tris-acetate, pH 8.4), and the column was packed (14 × 0.5 cm) with PBE 94. The sample to be chromatofocused was then run into the column bed, and the generation of a pH gradient (5.0–8.0) was achieved by elution with the polybuffer system (3% polybuffer 96 and 7% polybuffer 74, adjusted to pH 5.0 with acetic acid). The column was run at room temperature with a flow rate of 15 ml/h. 1-ml fractions were collected and assayed for IFN activity, and the pH of each sample measured at room temperature.

Results

Enhanced IFN Production by Spleen Cells from Listeria-immune Mice. Because T cell mitogens are inducers of IFNγ in nonimmune animals (10, 11), it was considered of interest to determine the relative ability of spleen cells obtained from mice actively expressing T cell-mediated antibacterial immunity to produce IFN in response to polyclonal T cell activators. Spleen cells from both control and mice infected intravenously 6 d earlier with *Listeria* were incubated for 24 h with the designated concentrations of the mitogenic agents (Table I). The results of these studies show that spleen cells from *Listeria*-immune animals produced levels of IFN at least 16-fold higher than similarly treated cultures from nonimmune mice. It was repeatedly observed in 20 separate experiments that spleen cell cultures from *Listeria*-immune mice produced 10–20 times more antiviral activity than normal cells induced with Con A or PHA, although the absolute IFN units fluctuated two- to fourfold between experiments. Specific antigenic stimulation of spleen cells from *Listeria*-immune mice with a preparation of soluble *Listeria* antigen(s) resulted in the synthesis of low levels of IFN, but the antigen failed to elicit IFN in nonimmune spleen cell cultures. Marginal levels of IFN activity (≤2) were detected in supernatants from *Listeria*-immune spleen cell cultures not incubated with the mitogens.

Characterization of Immune Spleen Cell IFN. The γ class of interferons is characterized as being inactivated at low pH (2.0) and by the failure of antisera raised against IFNα and IFNβ to neutralize its antiviral activity (32, 33). In the following studies,

TABLE I

MuIFNγ Production after Mitogenic or Antigenic Stimulation of Spleen Cells from Normal or Listeria-immune Mice

Spleen cell source	Mitogenic agent*	24-h IFN titer
		U/ml
Control mice	PHA (5 μg/ml)	256
	Con A (1 μg/ml)	64
	Listeria antigen (5 μg/ml)	<2
	None	<2
+6-D *Listeria*-immune mice	PHA (5 μg/ml)	4,096
	Con A (1 μg/ml)	3,072
	Listeria antigen (5 μg/ml)	32
	None	≤2

* 2-ml cultures of spleen cells (10^7 cells/ml) were incubated for 24 h with the designated final concentrations of mitogenic agents.

the IFN produced by *Listeria*-immune spleen cell cultures stimulated with Con A or PHA were analyzed as to their stabilities at pH 2.0 and to their antigenic properties. Murine IFN preparations containing both MuIFNα and MuIFNβ were included as controls in these experiments. These MuIFN were produced by L cells and by pure cultures of macrophages induced with NDV or poly (I)·poly (C) (22–24). All samples were dialyzed at 4°C against a pH 2.0 buffer for 5 d and returned to pH 7.4 by dialysis against PBS. The results in Table II show that acidification of the L cell and macrophage IFN preparations resulted in no more than a twofold decrease in antiviral activities, whereas the mitogen-induced spleen cell IFN were found to be relatively more acid labile. In repeated pH 2.0 lability studies, it was observed that each MuIFNγ preparation produced in vitro possesses a fraction (3–15% of the original activity) that was not inactivated by the 5-d acid treatment.

The antigenic classification of the IFN preparations used in the pH stability studies was done through the use of specific anti-MuIFN sera. A goat anti-MuIFN serum possessing both anti-MuIFNα and anti-MuIFNβ activity and a rabbit anti-MuIFNγ serum were used to determine the degrees to which the antiviral activities of each test IFN could be neutralized (Table II). The antiserum possessing specific populations of antibodies directed against MuIFNα and MuIFNβ exhibited very high neutralizing titers for the macrophage and L cell IFN but failed to inhibit the antiviral activities of either the PHA or Con A-induced spleen IFN preparations. Conversely, the rabbit anti-MuIFNγ serum neutralized only the mitogen-induced spleen cell IFN. Similar neutralization studies performed with the acid-stable portion of the PHA-induced spleen cell IFN showed that it also was IFNγ by its antigenic properties (data not shown). Thus, based on the relative acid lability and the serological results, the major IFN activity produced by the spleen cell cultures was MuIFNγ. However, it should be mentioned that, whereas the major antiviral activity in these preparations is MuIFNγ, we cannot exclusively eliminate the possible presence of low levels of other antigenic

TABLE II
Properties of Different MuIFN Preparations

Source of MuIFN	Inducing agent	pH 2.0 treatment*		Neutralizing titer of‡	
		Before	After	Anti-MuIFN (α + β)§	Anti-Mu-IFNγ
Lpa cells	NDV	12,288	8,192	14,746	<12
	poly(I)·poly(C)	3,072	1,536	52,428	<12
Bone marrow macrophages	NDV	4,096	2,048	6,554	<12
	poly(I)·poly(C)	512	512	39,322	<12
Listeria-immune spleen cell cultures	PHA	1,024	32	<32	50
	Con A	384	64	<32	50

* Samples dialyzed at 4°C for 5 d against Clark Lubs pH 2.0 KCl-HCl buffer, followed by dialysis for 1 d against PBS, pH 7.4.

‡ The antiserum neutralizing titer is expressed as the reciprocal of the highest dilution of antiserum that when mixed with an equal volume of test IFN (20 U/ml) neutralizes 50% of the antiviral activity of the IFN.

§ Antiserum possessing anti-MuIFNγ and anti-MuIFNβ neutralizing antibodies.

classes of MuIFN. In the antibody neutralization studies, a final concentration of 10 U/ml of antiviral activity is reacted, and if <10% of the activity (<1 unit) is mediated by an IFN molecule not neutralized by the antibody, its antiviral activity would not be detected in this neutralization assay (34).

Temporal Relationship between the Development of Anti-Listeria Immunity and Enhanced Capacity of Spleen Cells to Produce MuIFNγ. The following experiments were designed to determine whether the enhanced production of MuIFNγ by spleen cells from *Listeria*-immune mice coincided with the peak of the antibacterial-immune response. This was tested by measuring the capacity of spleen cells harvested against time of *Listeria* infection to produce PHA-induced MuIFNγ and to passively transfer antibacterial immunity to normal recipients. The results illustrated in Fig. 1 clearly demonstrate a parallel between the development of antibacterial immunity and the ability of spleen cells to produce high-titered MuIFNγ. Thus, the ability of spleen cells to produce MuIFNγ and to transfer protective immunity developed, peaked, and declined at the same times.

Cellular Origin of MuIFNγ. Evidence indicates that T cells are the major producers of lymphokines, although B cells have also been implicated as potential contributors of these soluble mediators (35, 36). In the following studies, monoclonal antibodies and C were used to determine the nature of the spleen cells producing MuIFNγ. The findings presented in Table III reveal that treatment of cells with anti-Thy-1.2 plus C virtually abolished production of MuIFNγ. It should be noted that the low level of IFN activity that was produced after anti-Thy-1.2 plus C treatment was identified as MuIFNγ by its lability at pH 2.0 and by its antigenic properties (results not shown). The synthesis of this residual MuIFNγ could conceivably by attributed to small numbers of T cells possessing little or no Thy-1 antigen (37). In contrast to the almost total elimination of MuIFNγ production by anti-Thy-1.2, destruction of B lympho-

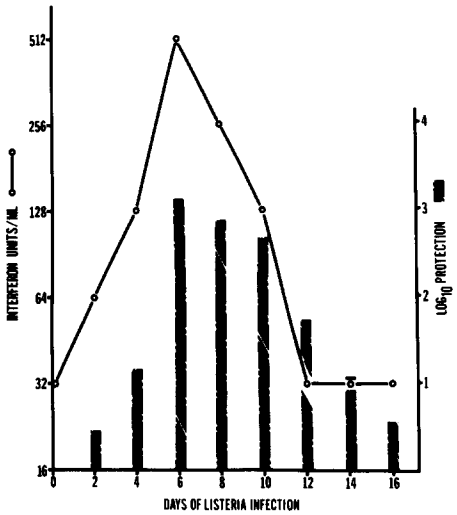

FIG. 1. Development of anti-*Listeria* immunity and the capacity for enhanced MuIFNγ synthesis by spleen cells from *Listeria*-infected mice. At the indicated day after intravenous injection of 2×10^3 *L. monocytogenes*, pooled spleen cells from groups of five mice were either induced with 5 μg/ml of PHA for 24 h to determine their ability to produce MuIFNγ (line graph) or tested for their ability to adoptively transfer anti-*Listeria* immunity (bar graph).

TABLE III

T Cell Dependence of MuIFNγ Synthesis in Spleen Cultures from Mice Immunized with Listeria Monocytogenes

Spleen source	Spleen cell treatment*	24-h IFN yield
		U/ml
Nonimmune control mice	None	16
6-d *Listeria*-immune mice	None	2,048
6-d *Listeria*-immune mice	Antibodies − C (separate groups treated with either anti-Thy-1.2, anti-I-A^k, or anti-IgG)	1,024
6-d *Listeria*-immune mice	C	1,024
6-d *Listeria*-immune mice	Anti-Thy-1.2 + C	32
6-d *Listeria*-immune mice	Anti-I-A^k + C	2,048
6-d *Listeria*-immune mice	Anti-IgG + C	2,048
6-d *Listeria*-immune mice	Anti-Thy-1.2 + I-A^k + C	16

* Spleen cells were suspended at 5×10^6 cells/ml in 1.0 ml of RPMI 1640 supplemented with 1% FBS and 10 μg/ml gentamycin. Antibody and C were added to the designated experimental groups. After antibody treatment, all cells were induced with PHA (5 μg/ml).

TABLE IV

Lyt Phenotype of MuIFNγ-producing T Cells

Spleen source	Spleen cell treatment*	24-h IFN yield
		U/ml
Nonimmune control mice	None	16
6-d *Listeria*-immune mice	None	2,048
6-d *Listeria*-immune mice	Antibody − C (separate groups treated with either anti-Thy-1.2, anti-Lyt-1.2, or anti-Lyt-2.2)	2,048
6-d *Listeria*-immune mice	C	1,024
6-d *Listeria*-immune mice	Anti-Thy-1.2 + C	32
6-d *Listeria*-immune mice	Anti-Lyt-1.2 + C	64
6-d *Listeria*-immune mice	Anti-Lyt-2.2 + C	384
6-d *Listeria*-immune mice	Anti-Lyt-1.2 + Lyt-2.2 + C	16
6-d *Listeria*-immune mice	Anti-Lyt-1.2 + anti-Lyt-1.2 + C‡	96
6-d *Listeria*-immune mice	Anti-Lyt-2.2 + anti-Lyt-2.2 + C‡	512
6-d *Listeria*-immune mice	Recombination of anti-Lyt-1.2 + C-treated cells with anti-Lyt-2.2 + C-treated cells.	1,024

* Spleen cells suspended at 5×10^6 cells/ml in 1.0 ml of RPMI 1640 supplemented with 1.0% FBS and 10 μg/ml gentamycin. Antibody plus C was added to the appropriate groups as described in Materials and Methods. After antibody treatment, all groups were induced with PHA (5 μg/ml).

‡ After antibody plus C treatment, viable cells were determined by exlusion of ethidium bromide, and the number of viable cells was then adjusted to 5×10^6 cells/ml before the addition of PHA.

cytes with anti-IgG plus C or anti-I-A plus C had no detectable effect on the titer of PHA-induced MuIFNγ.

Similar studies were performed to identify the T cell subpopulation(s) producing MuIFNγ through the use of specific cytolytic monoclonal antibodies directed against Lyt differentiation antigens. In Table IV, it is shown that the T cells producing MuIFNγ were more susceptible to destruction by anti-Lyt-1.2 plus C than with anti-

Lyt-2.2 and C. The combined effects of the two anti-Lyt antibodies reduced by MuIFNγ titer to slightly below that of the anti-Thy-1.2 plus C-treated cells.

Reduced MuIFNγ titers were also observed when cells treated with anti-Lyt-1.2 plus C were restored to the original number per culture (5×10^6 cells/ml). Based on this observation, it is reasonable to conclude that the T cell subpopulations not destroyed by anti-Lyt-1.2 plus C are not the contributors of the major portion of the MuIFNγ activity. This finding does not rule out the possibility that for maximum MuIFNγ production, Lyt-1^+2^- cells might have to interact with other cell population(s). This is based on the additional findings presented in Table IV, which show that almost maximum MuIFNγ titers were obtained by (*a*) restoring the cells remaining after anti-Lyt-2.2 plus C treatment to the original number of cells per culture (5×10^6 cells/ml) or (*b*) by combining cells surviving after treatment with anti-Lyt-1.2 with those surviving after treatment with anti-Lyt-2.2 plus C. However, the increase in titers in each of the above cases might be on the borderline of significance because the MuIFNγ titer after anti-Lyt-2.2 plus C treatment was only threefold (384) lower than C-treated controls (1,024). Thus, this difference falls close to the inherent twofold error of the interferon assay.

Kinetics of MuIFNγ Release. The kinetics of MuIFNγ release from 6-d *Listeria*-immune spleen cell cultures stimulated with either 5 μg/ml of PHA or 1 μg/ml of Con A are presented in Fig. 2. Similar patterns of IFN release were exhibited by cultures induced with either T cell mitogen. IFN was first detected in the culture

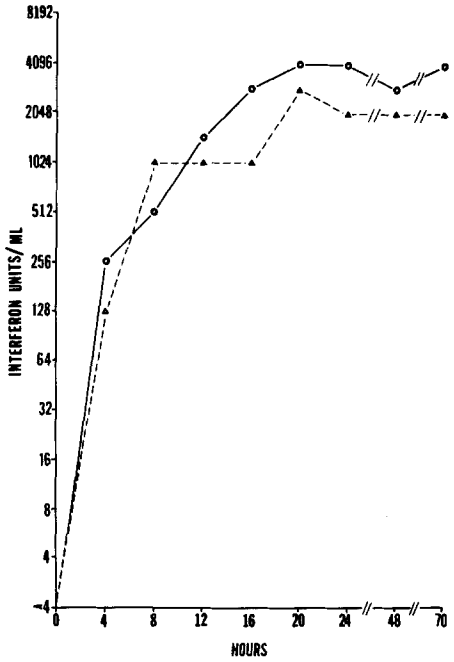

FIG. 2. Kinetics of MuIFNγ release from spleen cell cultures. Replicate cultures of 6-d *Listeria*-immune spleen cells (10^7 cells/ml) were incubated with PHA (5 μg/ml) or Con A (1 μg/ml). Supernatants of duplicate cultures from each mitogen-treated group were collected at the times indicated, pooled, and centrifuged. The supernatants were stored at 4°C until they were assayed for antiviral activity. Δ, Con A; O, PHA.

supernants as early as 4 h after the addition of the mitogens, with peak levels occurring at 18 h. After this time, MuIFNγ levels remained constant for an additional 48 h. From the slopes of the production curves and the levels of IFN released, it is concluded that the rate of synthesis and quantities of MuIFNγ produced by cultures of *Listeria*-immune spleen cells was the same when PHA or Con A were used as inducers. The kinetics of MuIFNγ release are similar to those reported for alloantigen-induced MuIFNγ (38).

MuIFNγ Induction by Different Concentrations of T Cell Mitogens. A series of studies was carried out to establish the optimum concentrations of T cell mitogens required to induce the maximum synthesis of MuIFNγ in spleen cell cultures from *Listeria*-immune and control mice. In addition to Con A and PHA, staphylococcal enterotoxin A (SEA) was included in these studies because it has been reported to be a potent inducer of IFNγ in both murine and human cell systems (11, 39). The findings presented in Table V reveal that the MuIFNγ levels from the *Listeria*-immune spleen cells were at least 10-fold greater than the corresponding yields from similarly treated nonimmune control cells. MuIFNγ production plateaued in immune spleen cell cultures incubated with PHA concentrations in the range of 0.5–10 μg/ml, whereas Con A elicited the highest titers of MuIFNγ activity in both control and immune cultures at a concentration of 1 μg/ml. The SEA failed to induce MuIFNγ in nonimmune control cultures with any of the concentrations tested. In fact, SEA was only effective in inducing MuIFNγ in immune spleen cell cultures, with the highest level of antiviral activity being detected in cultures incubated with the highest concentration (10 μg/ml) of SEA tested.

The diterpene ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the structurally related compound-mezerein have been reported (40) to enhance the levels of human IFNγ (HuIFNγ) produced by primary cultures of human leukocytes stimulated by PHA or several other T cell mitogens. Therefore, experiments were performed to determine whether these esters might also further augment the yield of MuIFNγ produced by *Listeria*-immune spleen cells. In these studies, cultures of spleen cells from immune mice were incubated with different concentrations of TPA or mezerein 3 h before the addition of PHA (5 μg/ml). Little or no enhancement in interferon yields

TABLE V

MuIFNγ Production by Normal and Listeria-immune Spleen Cell Cultures Induced with Different Concentrations of T Cell Mitogens

Source of spleen cells	Mitogen	24-h IFN yield (U/ml) induced by mitogen concentrations (μg/ml)					
		0.05	0.1	0.5	1	5	10
Control	PHA	ND‡	<4	16	32	128	128
Listeria immune*	PHA	ND	80	1,024	1,024	1,024	1,536
Control	Con A	<4	<4	8	32	<4	<4
Listeria immune*	Con A	<4	4	256	768	384	32
Control	SEA	<8	<8	<8	<8	<8	<8
Listeria immune*	SEA	<8	24	48	32	128	192

* Spleen cell cultures obtained from mice injected intravenously 6 d before with 2×10^3 *L. monocytogenes*.
‡ Not done.

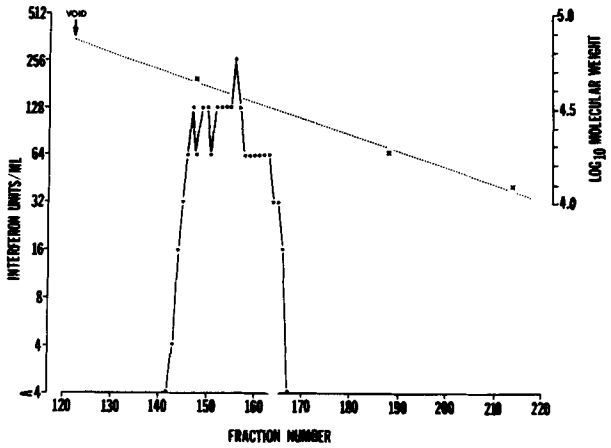

FIG. 3. Molecular weight estimation of MuIFNγ by Bio-gel P-60 filtration chromatography. Column buffer was 0.05 M Na citrate with 1 M NaCl, pH 6.0. Column dimensions and conditions for elution are given in Materials and Methods. Interferon activities (●) were determined on each 1-ml fraction. Column void volume was determined by exclusion of rabbit IgG, and calibration was done with the following markers (X): ovalbumin, 43,000; myoglobin, 18,500; and cytochrome C, 12,000.

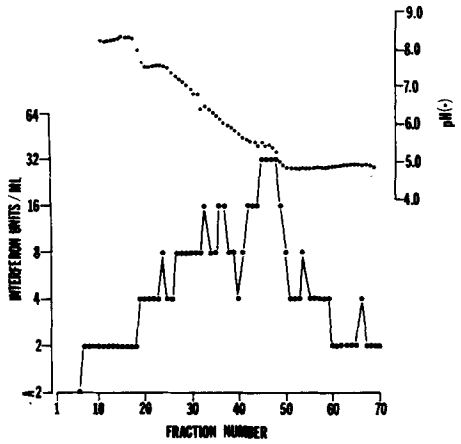

FIG. 4. Isoelectric point determination of MuIFNγ by chromatofocusing. 1 ml of MuIFNγ, equilibrated in starting column buffer (0.025 M Tris-acetate, pH 8.4), was applied to the chromatofocusing column. Conditions of elution and generation of the pH gradient are as presented in Materials and Methods. 1-ml fractions were assayed for antiviral activity, and the pH of each fraction was determined at room temperature. The MuIFNγ unitage applied was 1,536 units, and the total units recovered was 484.

was observed in the cultures pretreated with either agent in the concentration ranges (1–50 ηg/ml) reported by others to enhance HuIFNγ levels (results not shown).

Molecular Weight of MuIFNγ. The availability of high-titered MuIFNγ preparations produced by PHA-induced *Listeria*-immune spleen cells has enabled the analysis of certain of its physicochemical properties. Molecular weight determinations were made by gel filtration chromatography using Bio-Gel P-60 under nondenaturing conditions. It would have been preferable to estimate the molecular weight of MuIFNγ by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), however, treatment

of MuIFNγ with SDS (0.1 or 1%) under both reducing and nonreducing conditions performed at a number of temperatures consistently resulted in substantial losses of antiviral activity. Treatment of HuIFNγ with this anionic detergent has also been reported to cause a ≥90% loss in HuIFNγ antiviral activity (41). The elution profile of MuIFNγ from the Bio-Gel P-60 column is presented in Fig. 3. The MuIFNγ activity has an estimated 38,000 ± 4,000 mol wt, and the recovery of antiviral activity was in the order of 50% of the applied unitage.

Isoelectric Point of MuIFNγ. The isoelectric point of MuIFNγ was determined by chromatofocusing (30, 31). Preliminary isoelectric focusing studies using ampholytes to generate a pH gradient in acrylamide gels revealed that, unlike MuIFNα and MuIFNβ (42), the MuIFNγ focused in the acidic region of the gradient. Therefore, the polybuffer system used to develop a pH gradient during chromatofocusing was adjusted to generate a linear pH gradient between pH 5.0–8.0. The resulting elution profile of MuIFNγ activity from the chromatofocusing column is presented in Fig. 4. Greater than 90% of the recovered MuIFNγ focused between pH 5.0–7.0, and the MuIFNγ is shown to exhibit considerable molecular charge heterogeneity. A major peak of MuIFNγ activity focused between pH 5.0–5.6, with a second extremely disperse region of activity from pH 5.8–7.0.

Discussion

The studies presented in this paper show that T lymphocytes from the spleens of animals with an ongoing T cell-mediated immune response to *Listeria* infection acquire the ability to produce significantly more MuIFNγ than splenic T cells from uninfected mice. In addition, it was revealed that the ability of spleen cells from immune mice to produce high-titered MuIFNγ developed and declined concordantly with the production and loss of T cells capable of passively transferring anti-*Listeria* immunity. Prior studies have shown that a number of other parameters of cellular activity associated with an onging cell-mediated anti-*Listeria* response display similar kinetics of generation and decay. These include spleen cell proliferation, spleen cellularity, delayed sensitivity to *Listeria* antigens (17), and macrophage activation (43). Our results indicate that during the generation of anti-*Listeria* immunity, there is an enlargement of a T cell subpopulation capable of producing MuIFNγ. An alternative explanation is that the T cells in the normal spleen that are capable of producing low titers of MuIFNγ are individually activated to produce more IFN as a result of infection. Although we have only quantitated the enhancement of MuIFNγ synthesis by spleen cells stimulated with T cell mitogens during the generation of antibacterial immunity, it would be of interest to determine whether the production of other mitogen-induced lymphokines is also enhanced.

The T cell origin of MuIFNγ was established by showing that treatment with anti-Thy-1.2 plus C greatly dimishishes the production of this lymphokine. In contrast, treatment of cells with anti-I-A or anti-IgG to destroy B cells did not inhibit MuIFNγ synthesis. The MuIFNγ-secreting T cells were relatively susceptible to C-mediated lysis with anti-Lyt-1.2, which reduced the MuIFNγ titer to nearly the same level as treatment with anti-Thy-1.2. This is not surprising because published studies have shown that virtually all T cells outside the thymus display Lyt-1 antigens on their surface, though not all are susceptible to C-mediated lysis (37). Treatment of spleen cells with anti-Lyt-2.2 + C, on the other hand, only slightly reduced the titer of

MuIFNγ. If the MuIFNγ-secreting lymphocytes displayed Lyt-1^+2^+, then they should be equally susceptible to destruction by either anti-Lyt-1 or anti-Lyt-2. Therefore, there can be little doubt that the Lyt-1^+2^- T lymphocyte is the main producer of MuIFNγ in this system. A small contribution by Lyt-1^-2^+ T cells must be considered, however, even though the reduced production caused by anti-Lyt-1^-2^+ antibody is close to the limitations of the inherent twofold error of the IFN assay. These findings are in contrast to an earlier publication (44) that reported that an Lyt-1^-2^+ T cell was the major source of MuIFNγ in animals immunized with Calmette-Guerin bacillus and challenged with specific antigen. These authors also reported the MuIFNγ-producing cell was destroyed by anti-Ia and anti-IgG. It might be significant that T cells within the Lyt-1^+2^- population are known to secrete interleukin 2 and that interleukin 2 is generated without participation of other T cells (45).

The criterion most widely used to differentiate IFNγ from other IFN is its loss of antiviral activity after acidification. It has been repeatedly observed that MuIFNγ is relatively more labile at pH 2.0 than other MuIFN. However, we have always detected a variable fraction (3–15% of the total pre-acidified unitage) within MuIFNγ preparations that remains active after 5 d pH 2.0 treatment. Similar findings were reported by Wietzerbin et al. (46). A potent rabbit antiserum raised against a partially purified MuIFNγ (5×10^5 U/mg protein) neutralized the acid-stable MuIFNγ component, whereas a neutralizing antiserum possessing specificity for both MuIFNα and MuIFNβ did not neutralize this activity. Thus, based on this serological analysis, it is possible that not all MuIFNγ molecules are acid labile. The heterogeneity of the MuIFNγ molecules in the PHA-induced preparations is evidenced by additional findings in this paper. For example, isoelectric point determinations by means of chromatofocusing revealed that the MuIFNγ molecules are primarily acidic proteins focusing in a broad pH range from 5.0–7.0. Addtional studies (E. A. Havell and G. L. Spitalny, manuscript submitted for publication) have shown that MuIFNγ antiviral activity focusing between pH 5.0 and 5.6 binds specifically to a Con A affinity column, which indicates its glycoprotein nature. Molecular weight determinations by means of gel filtration chromatography on Bio-gel P-60 revealed the molecular weight of MuIFNγ to be 38,000 ± 4,000. Studies by others have established the molecular weight of other murine lymphokines to be within a similar size range (47, 48). However, because our molecular weight studies were carried out under nondenaturing conditions, we cannot rule out the possibility of complexes of low molecular weight MuIFNγ yielding an apparent 38,000 mol wt under the conditions used for the molecular weight estimations. With regard to this last point, Yip et al. (48) originally reported that the molecular weight of HuIFNγ estimated by gel filtration chromatography to be 58,000, but later found (41) that the remaining activity after SDS treatment could be resolved by SDS-PAGE into 25,000- and 20,000-mol wt components. This suggests the molecular aggregates cause the apparent higher molecular weight forms observed under nondenaturing conditions. The molecular diversity of MuIFNγ could conceivably result from a variety of factors that have been shown to be responsible for the molecular heterogeneity within other IFN preparations. These include (*a*) distinct IFN gene classes and their differential induction in different cell types (50, 51), (*b*) multigene IFN families within antigenically distinct IFN classes (52), and (*c*) microheterogeneity caused by differing degrees of post-translational processing, such as in the glycosylation of IFN proteins (53, 54).

The exact role, if any, played by MuIFNγ in the regulation of immunity is still a matter of conjecture. Although there is no direct evidence for a function of MuIFNγ in anti-*Listeria* immunity, the parallel development of T cells that exhibit enhanced production of MuIFNγ and of T cells that mediate immunity is perhaps suggestive of an important role for MuIFNγ in cell-mediated immunity in general. The elucidation of the possible functional role(s) of MuIFNγ in immunity will be made more difficult in view of the fact that distinct molecular populations of MuIFNγ are produced by T cells.

Summary

Spleen cell cultures derived from animals infected 6 d earlier with *Listeria monocytogenes* produced 10–20-fold more murine interferon γ (MuIFNγ) than spleen cells from nonimmune mice in response to stimulation with T cell mitogens. A striking temporal association was found between the enhanced synthesis of MuIFNγ and the development of anti-*Listeria* immunity in that both the potential for increased MuIFNγ production and the generation of *Listeria*-protective T cells developed and then decayed in unison. Treatment of spleen cells with monoclonal anti-Thy-1.2 plus complement virtually abolished the ability of cells from *Listeria*-immune mice to synthesize MuIFNγ. The T cells producing MuIFNγ were found to be more susceptible to complement-mediated lysis with monoclonal anti-Lyt-1.2 than with monoclonal anti-Lyt-2.2. The production of MuIFNγ was not affected by treating spleen cells with anti-IgG antisera or with a monoclonal antibody directed against I-A specificities. MuIFNγ was detected 4 h after the beginning of mitogenic stimulation of spleen cell cultures, and peak levels of MuIFNγ were reached by 18 h. The IFN synthesized by mitogen-induced spleen cells derived from *Listeria*-immune mice were relatively labile at pH 2.0 and neutralized by a rabbit anti-MuIFNγ serum but not by an antiserum having specificities for MuIFNα and MuIFNβ. The apparent molecular weight of the MuIFNγ, as estimated by molecular sieving on a Bio-gel P-60 column, was estimated to be 38,000, and the isoelectric point as determined by chromatofocusing was extremely heterogeneous, ranging between pH 5.0 and pH 7.0.

The authors would like to acknowledge the excellent technical assistance of Donald Auclair and Bruce Rankin.

Received for publication 9 March 1982.

References

1. North, R. J. 1981. An introduction to macrophage activation. *In* Lymphokines 3. Lymphokines in Macrophage Activation. E. Pick and M. Landy, editors. Academic Press, Inc., New York. 1.
2. Stewart, W. E. II, J. E. Blalock, D. C. Burke, C. Chany, J. K. Dunnick, E. Falcoff, R. M. Friedman, G. J. Galasso, W. K. Joklik, J. T. Vilcek, J. S. Younger, and K. C. Zoon. 1980. Interferon nomenclature. *Nature (Lond.)*. **286:**110.
3. Glasgow, L. A., J. L. Crane, E. R. Kern, and J. Youngner. 1978. Antitumor activity of interferon against murine osteogenic sarcoma *in vitro* and *in vivo*. *Cancer Treat. Rep.* **62:**1881.
4. Blalock, J. E., J. A. Georgiades, M. P. Langford, and H. M. Johnson. 1980. Purified human immune interferon has more potent anticellular activity than fibroblast or leukocyte interferon. *Cell. Immunol.* **49:**390.

5. Sonnenfeld, G., A. D. Mandel, and T. C. Merigan. 1977. The immunosuppressive effect of Type II mouse interferon preparations on antibody production. *Cell. Immunol.* **34:**193.
6. Virelizier, J. C., E. L. Chan, and A. C. Allison. 1977. Immunosuppressive effects of lymphocyte (type II) and leucocyte (Type I) interferon on primary antibody responses *in vivo* and *in vitro*. *Clin. Exp. Immunol.* **30:**299.
7. Salvin, S. B., J. S. Younger, and W. H. Lederer. 1973. Migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity. *Infect. Immun.* **7:**68.
8. Gifford, G. E., A. Tibor, and D. L. Peavy. 1971. Interferon production in mixed lymphocyte cell cultures. *Infect. Immun.* **3:**164.
9. Kirchner, H., R. Zavotsky, and V. Schirrmacher. 1979. Interferon production in the murine mixed lymphocyte culture. I. Interferon production caused by differences in the H-2K and H-2D region but not by differences in the I or the M locus. *Eur. J. Immunol.* **9:**97.
10. Wheelock, E. F. 1965. Interferon-like virus inhibitor induced in human leukocytes by phytohemagglutinin. *Science* (*Wash. D. C.*). **149:**310.
11. Johnson, H. M., G. J. Stanton, and S. Baron. 1977. Relative ability of mitogens to stimulate production of interferon by lymphoid cells and to induce suppression of the *in vitro* immune response. *Proc. Soc. Exp. Biol. Med.* **154:**138.
12. North, R. J. 1973. Importance of thymus-derived lymphocytes in cell-mediated immunity to infection. *Cell. Immunol.* **7:**166.
13. North, R. J., and G. L. Spitalny. 1974. Inflammatory lymphocyte in cell-mediated antibacterial immunity: factors governing the accumulation of mediator T-cells in peritoneal exudates. *Infect. Immun.* **10:**489.
14. Snodgrass, M. J., D. S. Lowrey, and M. G. Hanna, Jr. 1972. Changes induced by lactic dehydrogenase virus in thymus and thymus dependent areas of lymphatic tissue. *J. Immunol.* **108:**877.
15. Mackaness, G. B. 1969. The influence of immunologically committed lymphoid cells on macrophage activity *in vivo*. *J. Exp. Med.* **129:**973.
16. Lowry, D. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the folin phenol reagent. *J. Biol. Chem.* **193:**265.
17. North, R. J. 1973. Cellular mediators of anti-*Listeria* immunity as an enlarged population of short-lived, replicating T cells. *J. Exp. Med.* **138:**342.
18. Ey, P. L., S. J. Prowse, and C. R. Jenkin. 1978. Isolation of pure IgG, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using Protein-A-sepharose. *Immunochemistry.* **15:**429.
19. Marshak-Rothstein, A., P. Fink, T. Gridley, D. H. Roulet, M. J. Bevan, and M. L. Gefter. Properties and applications of monoclonal antibodies directed against determinants of the Thy-1 locus. *J. Immunol.* **122:**2491.
20. Oi, V., P. P. Jones, J. W. Goding, L. A. Herzenberg, and L. A. Herzenberg. 1978. Properties of monoclonal antibodies to mouse Ig antigens, H-2 and Ia antigens. *Curr. Top. Microbiol. Immunol.* **81:**115.
21. Parks, D. R., V. M. Bryan, V. M. Oi, V. T. Oi, and L. A. Herzenberg. 1979. Antigen-specific identification and cloning of hybridomas with a fluorescence-activated cell sorter (FACS). *Proc. Natl. Acad. Sci. U. S. A.* **76:**1962.
22. Havell, E. A. 1979. Isolation of a subspecies of murine interferon antigenically related to human leukocyte interferon. *Virology.* **92:**324.
23. Havell, E. A., and W. A. Carter. 1981. Effects of tunicamycin on the physical properties and antiviral activities of murine L cell interferon. *Virology.* **108:**80.
24. Havell, E. A., and G. L. Spitalny. 1980. The induction and characterization of interferon from pure cultures of murine macrophages. *Ann. N. Y. Acad. Sci.* **350:**413.
25. Havell, E. A., and J. Vilcek. 1972. Production of high-titered interferon in cultures of human diploid cells. *Antimicrob. Agents Chemother.* **2:**476.

26. Havell, E. A., J. Vilcek, E. Falcoff, and B. Berman. 1975. Suppression of human interferon production by inhibitors of glycosylation. *Virology.* **63:**475.
27. Yamamoto, Y., and Y. Kawade. 1980. Antigenicity of mouse interferons: distinct antigenicity of the two L cell interferon species. *Virology.* **103:**80.
28. Fugisawa, J., and Y. Kawade. 1981. Properties of nonglycosylated and glycosidase-treated mouse L cell interferon species. *Virology.* **112:**480.
29. Stefanos, S., L. Catinot, J. Wietzerbin, and E. Falcoff. 1980. Production of antibodies against mouse immune T (Type II) inteferon and their neutralizing activities. *J. Gen. Virol.* **50:**225.
30. Sluyterman, L. A. E., and O. Elgersma. 1978. Chromatofocusing: isoelectric focusing on ion exchange columns. I. General principles. *J. Chromatogr.* **150:**17.
31. Sluyterman, L. A. E., and J. Wijdenes. 1978. Chromatofocusing: isoelectric focusing on ion exchange columns. II. Experimental verification. *J. Chromatogr.* **150:**31.
32. Valle, M. J., G. W. Jordan, S. Haahr, and T. C. Merigan. 1975. Characteristics of immune interferon produced by human lymphocyte cultures compared to other human interferons. *J. Immunol.* **115:**230.
33. Youngner, J., and S. B. Salvin. 1973. Production and properties of migration inhibitory factor and interferon in the circulation of mice with delayed hypersensitivity. *J. Immunol.* **111:**1914.
34. Havell, E. A. 1981. Qualitative and quantitative virus-yield assay for antibody neutralization of interferons. *In* Interferons. A Volume of Methods in Enzymology 79 (part B). S. Pestka, editor. Academic Press, Inc., New York. 575.
35. Yoshida, T., H. Sonozaki, and S. Cohen. 1973. The production of migration inhibition factor by B and T cells of the guinea pig. *J. Exp. Med.* **138:**784.
36. Bloom, B. R., G. Stoner, J. Gaffney, E. M. Shevach, and I. Green. 1975. Production of MIF and LT by non-T-cells. *Eur. J. Immunol.* **5:**220.
37. Ledbetter, J. A., R. V. Rouse, H. S. Micklem, and L. A. Herzenberg. 1980. T cell subsets defined by expression of Lyt-1,2,3 and Thy-1 antigens. Two parameters immunofluorescence and cytotoxicity analysis with monoclonal antibodies modifies current views. *J. Exp. Med.* **152:**280.
38. Pevissia, G., L. Mangoni, H. D. Engers, and G. Trichneri. 1980. Interferon production by human and murine lymphocytes in response to alloantigens. *J. Immunol.* **125:**1589.
39. Dianzani, F., L. Salter, W. R. Fleischman, and M. Zucca. 1978. Immune interferon activates cells more slowly than does virus-induced interferon. *Proc. Soc. Exp. Biol. Med.* **159:**94.
40. Yip, Y. K., R. H. L. Pang, J. D. Oppenheim, M. S. Nachbar, D. Henriksen, I. Zerebeckj-Eckhardt, and J. Vilcek. 1981. Stimulation of human gamma interferon production by diterpene esters. *Infect. Immun.* **34:**131.
41. Yip, Y. K., B. S. Barrowclough, C. Urban, and J. Vilcek. 1982. Molecular weight of human gamma interferon is similar to that of other human interferons. *Science (Wash. D. C.).* **215:**411.
42. Stancek, D., M. Gressnerova, and K. Paucker. 1970. Isoelectric components of mouse, human, and rabbit interferons. *Virology.* **41:**740.
43. North, R. J. 1974. Cell-mediated immunity and the response to infection. *In* Mechanisms of Cell-Mediated Immunity. R. T. McCluskey and S. Cohen, editors. Wiley and Sons, New York. 185.
44. Sonnenfeld, G., A. D. Mandel, and T. C. Merigan. 1979. *In vitro* production and cellular origin of murine type II interferon. *Immunology.* **36:**883.
45. Pickel, K., V. Hammerling, and M. K. Hoffmann. 1976. Ly phenotype of T-cells releasing T-cell replacing factor. *Nature (Lond.).* **264:**72.

46. Wietzerbin, J., S. Stefanos, M. Lucero, and E. Falcoff. 1979. Physicochemical characterization and partial purification of mouse immune interferon. *J. Gen. Virol.* **44:**773.
47. Leonard, E. J., L. P. Ruco, and M. S. Meltzer. 1978. Characterization of macrophage activation factor, a lymphokine that causes macrophages to become cytotoxic for tumor cells. *Cell. Immunol.* **41:**347.
48. Watson, J., S. Gillis, J. Harbrook, D. Mackizuki, and K. A. Smith. 1979. Biochemical and biological characterization of lymphocyte regulatory molecules. I. Purification of a class of murine lymphokines. *J. Exp. Med.* **150:**849.
49. Yip, Y. K., H. L. Pang, C. Urban, and J. Vilcek. 1981. Partial purification and characterization of human gamma (immune) interferon. *Proc. Natl. Acad. Sci. U. S. A.* **78:**1601.
50. Havell, E. A., T. G. Hayes, and J. Vilcek. 1978. Synthesis of two distinct interferons by human fibroblasts. *Virology.* **89:**330.
51. Erickson, J. S., and K. Paucker. 1979. Molecular species of interferon induced in mouse L cells by Newcastle disease virus and polyinosinic-polycytidylic acid. *J. Gen. Virol.* **43:**521.
52. Nagata, S., C. Brack, K. Henco, A. Schambock, and C. Weissmann. 1981. Partial mapping of ten genes of the human interferon $-\alpha$ family. *J. Interferon Res.* **1:**333.
53. Dorner, F., M. Scriba, and R. Weil. 1973. Interferon: evidence for its glycoprotein nature. *Proc. Natl. Acad. Sci. U. S. A.* **70:**1981.
54. Havell, E. A., S. Yamazaki, and J. Vilcek. 1977. Altered molecular species of human interferon produced in the presence of inhibitors of glycosylation. *J. Biol. Chem.* **252:**4425.