IDENTIFICATION OF INTERFERON-γ AS THE LYMPHOKINE THAT ACTIVATES HUMAN MACROPHAGE OXIDATIVE METABOLISM AND ANTIMICROBIAL ACTIVITY*

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In the later stages of infection or after the host recovers, lymphocytes encountering antigens of the infecting organism confer upon macrophages an enhanced capacity to kill the same or unrelated pathogens (1). This process is termed macrophage activation (2). Over a decade ago, antigen-stimulated lymphocytes were found to release a glycoprotein(s) (3) that enhanced the activity of the hexose monosphosphate shunt in macrophages (4). Soon thereafter, supernatants from antigen- or mitogen-stimulated lymphocytes were shown to augment macrophage antimicrobial activity (5–9). More recently, it was demonstrated that lymphoid supernatants increase the capacity of both murine (10–13) and human (14, 15) macrophages to secrete chemically reactive, incompletely reduced metabolites of molecular oxygen, including hydrogen peroxide. The capacities of macrophages to secrete hydrogen peroxide and to kill various microorganisms are closely correlated (10, 11). In fact, reactive oxygen intermediates appear to mediate much (though not all [15, 16]) of the antimicrobial function of activated macrophages against such intracellular pathogens as *Toxoplasma gondii, Trypanosoma cruzi, Leishmania, Candida* sp., and mycobacteria (reviewed in reference 17). Thus, cell-mediated immunity to intracellular pathogens appears to depend in large part on the secretion by lymphocytes of a factor(s) that activates macrophage oxidative metabolism and antimicrobial activity. In this paper, the lymphokine (LK)[1] meeting this description is called macrophage-activating factor (MAF).

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[1] *Abbreviations used in this paper*: IFN, interferon; IFNα, formerly leukocyte IFN; IFNβ, formerly fibroblast IFN; IFNγ, formerly immune IFN; LK, lymphokine(s); LPS, bacterial lipopolysaccharide; MAF, macrophage-activating factor; MLL-IFNγ, partially purified IFNγ from human leukocytes exposed to mezerein and lentil lectin; MNL, peripheral blood mononuclear leukocytes; PMA, phorbol myristate acetate; R-HuS, Roswell Park Memorial Institute medium 1640 with penicillin, streptomycin, and human serum; SEA-IFNγ, partially purified IFNγ from human leukocytes exposed to staphylococcal enterotoxin A; SEM, standard error of the mean.

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The synthesis of MAF by lymphocytes in trace amounts, and its secretion even by cloned lymphoid populations in admixture with other LK, have frustrated attempts to identify it physicochemically. One hypothesis is that MAF is interferon gamma (IFNγ). There is strong evidence that IFNγ is the LK that induces the expression of Ia or DR antigens on Macrophages (18, 19) and primes them for antitumor activity (20, 21). Various IFNs stimulate monocyte plasminogen activator release (22, 23) and Fc receptor expression (24, 25). However, none of these effects is known to be linked directly with enhanced antimicrobial activity. Partially purified, leukocyte-derived IFNs may contain other LK. Polyclonal antibodies may neutralize not only IFN but the contaminants as well. Even proof that cloned, recombinant IFN can activate macrophages would not establish that lymphocytes do so by means of IFN. For all these reasons, the relationship between MAF and IFN has remained undefined.

In this report, we made use of unpurified antigen- and mitogen-induced LK, partially purified lymphocyte-derived IFNγ, pure IFNγ produced by bacteria containing the cloned human gene (26), and a monoclonal antibody that neutralizes IFNγ. This combination of reagents has permitted the identification of IFNγ as the LK that enhances both the production of hydrogen peroxide by human macrophages and their ability to kill an intracellular microbial pathogen.

Materials and Methods

Culture of Monocytes. Mononuclear leukocytes (MNL) were isolated from the venous blood of normal adult donors as described (14, 27). 13-mm diam glass coverslips were pretreated for 1 wk with 50% HNO_3 before they were cleaned in ethanol as detailed (27). For experiments with H_2O_2 release, 1×10^6 MNL suspended in 0.1 ml RPMI-1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin and 25% fresh-frozen human serum (R-HuS) were plated per coverslip. After 2 h, the coverslips were rinsed three times in warm Eagle's minimum essential medium and transferred to 16-mm diam wells in 24-well trays (Costar Data Packaging, Cambridge, MA) in 0.3 ml R-HuS. The medium was replaced the following day (day 1), on day 3, and every 2–3 d thereafter. For experiments with *T. gondii,* 12-mm diam coverslips received 1.5×10^6 MNL in R-HuS containing 20% heat-inactivated human serum (Sabin-Feldman dye test-negative), and were cultured in groups of three coverslips per 35-mm diam plastic petri dish. Cultures were rinsed after 2 h, and given fresh medium on day 1 and every third day thereafter.

Hydrogen Peroxide Secretion. Secretion of H_2O_2 in response to 100 ng/ml phorbol myristate acetate (PMA) (Consolidated Midlands Co., Brewster, NY) was measured by the fluorescent scopoletin assay as described (27). The initial concentration of scopoletin was selected so that <50% was oxidized. Six coverslips were used per data point: three for peroxide secretion and three to measure adherent cell protein by the method of Lowry et al. (28), with bovine serum albumin as the standard. Results were expressed as nmol H_2O_2 per mg adherent cell protein.

Antitoxoplasma Activity. One million RH strain *T. gondii* trophozoites obtained from infected mouse peritoneal exudates (29) were added to each 35-mm dish for 30 min. Uningested organisms were removed by washing, and coverslips were then cultured in standard medium (without added lymphocyte products). At 4 h and 18–20 h after infection, replicate coverslips were fixed, stained, and scored microscopically for number of toxoplasmas per vacuole and per 100 macrophages (16, 29).

LK and IFN Preparations. Buffy coats from 87 U of blood were pooled at the New York Blood Center, and incubated for 48 h in serum-free RPMI-1640 containing 1 mg/ml human albumin, 5 ng/ml mezerein, and 30 μg/ml lentil lectin. At the concentrations added to monocytes, mezerein and lentil lectin or supernatants containing them did not themselves trigger H_2O_2 release. Some of these supernatants were enriched for IFNγ by

sequential affinity chromatography to a specific activity of 1×10^6 antiviral U/ml; these are designated MLL-IFNγ. Details of the purification will be furnished elsewhere (M. Wiebe, M. Chang et al., manuscript in preparation). Another preparation, termed SEA-IFNγ, was obtained by stimulating buffy coat cells with staphylococcal enterotoxin A and purifying the supernatant by sequential column chromatography to a specific activity of 1×10^7 U/mg protein, as described (references 30, 31, and B. Y. Rubin et al., manuscript in preparation). In addition, supernatants were collected from MNL cultures stimulated for 48 h with concanavalin A or toxoplasma lysate as reported previously (15). Control LK were obtained by adding concanavalin A to MNL cultures at the end of the 48-h incubation or by adding toxoplasma antigen at the outset of culture of MNL from Sabin-Feldman dye test-negative donors (15). Recombinant IFNγ synthesized by *E. coli* (26) was provided by Genentech, Inc., South San Francisco, CA. The lot used, which gave a single band on analytical polyacrylamide gel electrophoresis (personal communication, Dr. Sang-He Lee), had a specific activity of 6×10^5 U/mg. For consistency, we have arbitrarily expressed all antiviral titers in this paper according to unitage of assays performed by one of us (B. R.) in comparison to a laboratory standard, using a cytopathic effect inhibition assay with vesicular stomatitis virus in WISH (HeLa) cells (32). It should be noted that different antiviral activities were recorded for some of these preparations in other laboratories.

Antibodies to IFN. Monoclonal IgG_1 antibody GIF-1 neutralizes human IFNγ but not IFNα or IFNβ (31). The GIF-1 hybridoma supernatant used here had a neutralizing activity of 5,000 U/ml. Sheep globulins to human IFNα and to human IFNβ were National Institutes of Health (NIH) reference preparations GO 26-502-568 and GO 28-501-568, respectively. Test media were preincubated with antibody for 30–90 min before use. Percent inhibition of peroxide-releasing capacity was calculated as $100(1-[P_{LK+A}-P_M]/[P_{LK}-P_M])$, where P = nmol H_2O_2/mg cell protein after treatment of monocytes as indicated by the subscripts (LK = lymphokine or IFNγ, A = antibody, M = medium control).

Results

Elimination by Monoclonal Anti-IFNγ Antibody of the Ability of Unfractionated Lymphoid Supernatants to Enhance Peroxide Release from Human Macrophages. The 15 experiments summarized in Table I are in accord with previous observations on the capacity of lymphoid supernatants generated with concanavalin A (14, 15) or toxoplasma antigen (15) to enhance the peroxide-releasing capacity of human macrophages. Similar results were seen with supernatants of buffy coat cells pooled from multiple blood donors and exposed to lentil lectin and mezerein. As expected (33–35), these supernatants all contained substantial titers of IFNγ (Table I). IFNα and IFNβ could not be detected. To determine whether the stimulatory activity in these unfractionated supernatants could be ascribed to their content of IFNγ, we made use of a monoclonal antibody (GIF-1) that neutralizes IFNγ but has no detectable effect on IFNα or IFNβ (30). In 8 of 12 experiments, GIF-1 antibody removed all stimulatory activity from the supernatants. In the remaining four experiments it was partially effective. Complete inhibition was seen with six of the seven LK preparations studied.

To rule out the possibility that GIF-1 was toxic, we added the antibody to monocytes for 3 d beginning on day 1 of culture. When tested on days 2, 3, and 4, these monocytes released 107%, 119%, and 113% as much peroxide as those cultured in medium alone (the latter released 529 ± 13 [day 2], 420 ± 7 [day 3], and 630 ± 70 [day 4] nmol/mg cell protein). This demonstrated the lack of direct suppressive effects of antibody GIF-1, and further suggested that the

TABLE I

Stimulation of Human Macrophage Peroxide-releasing Capacity by Unfractionated Lymphoid Supernatants: Prevention by Monoclonal Antibody to IFNγ

Stimuli for LK production*	IFNγ titer	Concentration IFNγ used	H_2O_2 release by LK-treated cells‡	H_2O_2 release, experimental‡/control§ cells	Inhibition by monoclonal antibody‖
	U/ml	*U/ml*	*nmol/mg protein/60 min*		%
Lentil lectin + mezerein					
Prep. 1	100	1–10	321, 520, 529, 657, 696¶	1.5, 1.8, 2.9, 2.9, 12.0¶	141, 311¶
Prep. 2	1,000	100	1094	1.7	170
Concanavalin A					
Prep. 1	1,000	67–100	339, 1052	2.7, 3.1	85, 89
Prep. 2	3,000	100–300	368, 912	2.3, 5.1	49, 104
Prep. 3	1,500	100	511	4.7	105
Control**	0	0	172, 305, 394	0.8, 0.9, 1.2	ND‡‡
Toxoplasma antigen					
Prep. 1	6,000	400–600	514, 919, 1022	2.3, 4.5, 4.8	78, 113, 146
Prep. 2	2,000	134	529	4.9	95
Control§§	0	0	157, 189	1.2, 1.2	ND
Overall means ± SEM (N):			666 ± 67 (15)	3.8 ± 0.6 (15)	124 ± 19 (12)

* MNL from individual donors (for concanavalin A or toxoplasma antigen preparations) or buffy coat cells pooled from 87 donors (lentil lectin + mezerein) were incubated with the indicated stimuli and the supernatants collected as described in Materials and Methods. *Prep.*, preparations made with different cells on different days. Different batches of toxoplasma lysate were used for the two antigen-stimulated supernatants.

‡ Human macrophages were exposed to the indicated concentration of LK for 3 d beginning on the 3rd to 12th d of culture, washed, and stimulated with PMA to measure H_2O_2 release.

§ Control cells were tested in parallel with those in note ‡, except that LK was not added.

‖ Antibody GIF-1 used at 60–600 U/ml of neutralizing capacity for antiviral activity. Inhibition calculated as described in Materials and Methods.

¶ Values for individual experiments performed on different days or with different donors' macrophages, each in triplicate for both H_2O_2 release and adherent cell protein. SEM for H_2O_2 release by triplicates averaged 8.6% of the mean for individual experiments.

** MNL received concanavalin A just before harvesting the supernatant, which was added to macrophages at a 10-fold dilution.

‡‡ Not tested.

§§ Supernatants from MNL of Sabin-Feldman dye test-negative donors incubated with toxoplasma antigen were used at a 10% concentration on macrophages.

elevated H_2O_2-releasing capacity typically observed over the first 3–4 d of culture of adherent MNL may not be sustained by IFNγ from contaminating T cells.

In three experiments, incubation of macrophages in supernatant plus anti-IFNγ antibody resulted in H_2O_2-releasing capacity substantially lower than that of macrophages incubated in medium alone (inhibition >140%, Table I). This suggested that lymphoid supernatants may contain factors suppressing macrophage oxidative metabolism. This possibility was also raised by the dose-response profile shown in Fig. 1, in which 30% by volume of unfractionated LK was much less stimulatory than 1%.

On the average, adherent cell protein was 1.7 ± 0.3 times greater after 3 d

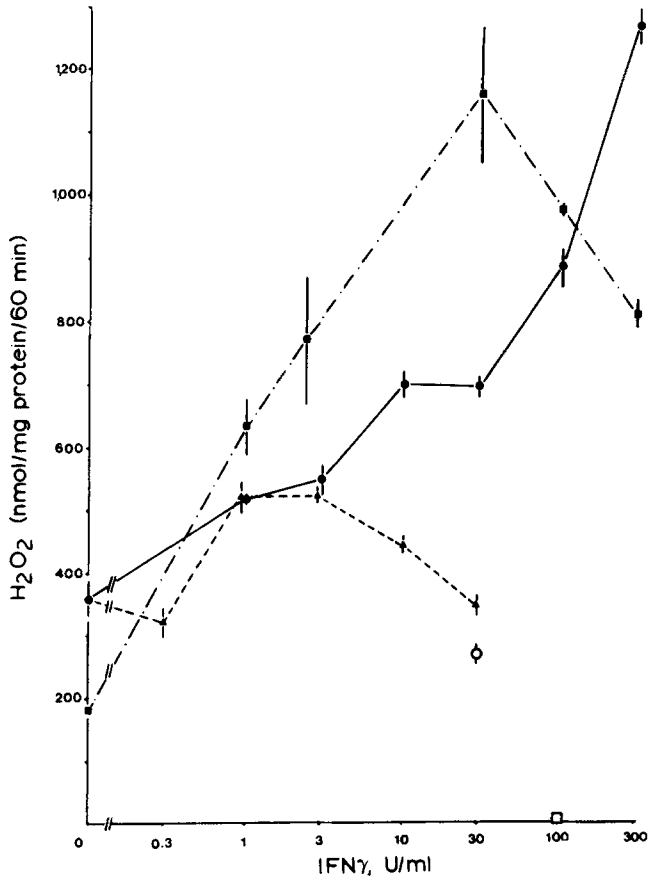

FIGURE 1. Enhancement of macrophage peroxide-releasing capacity by three IFNγ-containing preparations and its prevention by monoclonal anti-IFNγ antibody. Human macrophages were exposed to the indicated concentrations of IFNγ on days 4–7 of culture, washed, and stimulated with PMA to measure H_2O_2 release. An unpurified supernatant from buffy coat cells stimulated with mezerein and lentil lectin (solid triangles) had 10^2 U/ml antiviral activity. A fraction obtained from this supernatant (solid circles) had an antiviral activity of 10^4 U/ml or 1×10^6 U/mg protein. An independently purified preparation from buffy coat cells stimulated with staphylococcal enterotoxin A (solid squares) had an activity of 6×10^3 U/ml or 1×10^7 U/mg protein. The open symbols are results with the corresponding preparations exposed to 120–350 neutralizing U/ml of monoclonal anti-IFNγ antibody. An equivalent amount of monoclonal antibody to a mouse H2 antigen (the kind gift of Dr. R. Steinman, The Rockefeller University) had no effect (not shown). Means ± SEM for triplicates.

incubation of macrophages in the lymphoid supernatants than in control media (n = 15). Neither the elevations nor the reductions in adherent cell protein seen in individual experiments were consistently affected by monoclonal anti-IFNγ antibody (data not shown).

Effects of Partially Purified IFNγ. The foregoing experiments implied that IFNγ was the sole factor in the mitogen- or antigen-induced lymphoid supernatants tested that could enhance human macrophage peroxide-releasing capacity. If so, then native IFNγ should have MAF activity. The results of 13 experiments with two independently derived preparations enriched in IFNγ are summarized

in Table II. On the average, IFNγ-rich fractions enhanced macrophage peroxide-releasing capacity 8.8-fold. Peroxide released by IFNγ-treated macrophages on days 7–9 sometimes exceeded that secreted by samples of the same monocytes on day 0. Monoclonal anti-IFNγ antibody inhibited IFNγ-mediated enhancement of macrophage peroxide-releasing capacity by an average of 97% in seven experiments (Table II). In two titrations of preparation SEA-IFNγ (Fig. 1), 50% of maximal stimulation of macrophage peroxide-releasing capacity followed incubation in 0.4 and 1.3 antiviral U/ml, and peak effects were seen at 30 and 100 U/ml.

Peroxide-releasing capacity usually (but not invariably) peaked on the third day of exposure to partially purified IFNγ, and thereafter declined somewhat, despite replenishment with fresh IFNγ. However, peroxide releasing-capacity of cells continuously exposed to IFNγ remained markedly elevated for at least 5 d, the longest period tested. For example, macrophages treated with IFNγ from days 5–8 released 1,479 ± 124 nmol/mg protein/60 min, compared to 393 ± 20 for controls. After 2 more days in IFNγ, peroxide secretory capacity fell to 652 ± 53, compared to 101 ± 25 for the controls. However, when IFNγ was removed on day 8, secretion on day 11 fell to 23 ± 4, compared with 12 ± 2 in the controls. Thus, the enhancing effect of partially purified IFNγ on macrophage peroxide release was reversible. These kinetics closely parallel those reported earlier for unfractionated lymphoid supernatants (14).

On the average, cultures incubated in IFNγ-rich fractions contained 1.5 ± 0.3 times as much adherent cell protein as those in medium alone (n = 13). This was unaffected by the antibody (not shown).

Effects of Recombinant IFNγ. To establish conclusively that IFNγ could activate macrophages for enhanced hydrogen peroxide release, and to evaluate the possible contribution of other lymphoid cell products that might copurify with

TABLE II

Stimulation of Human Macrophage Peroxide-releasing Capacity by Partially Purified Native IFNγ: Prevention by Monoclonal Antibody to IFNγ

Preparation	Specific activity (U/mg protein)	Concentration IFNγ used	H_2O_2 release by IFN-treated cells‡ (nmol/mg protein/60 min)	H_2O_2 release experimental‡ control§ cells	Inhibition by monoclonal antibody¶
		U/ml			%
MLL-IFNγ**	1×10^6	10–242	392, 482, 706, 1226¶	2.0, 6.8, 7.1, 12.7¶	102, 124¶
SEA-IFNγ‡‡	1×10^7	30–300	211, 367, 381, 652, 1168, 1236, 1479, 1549, 2102	2.0, 3.5, 5.3, 6.5, 8.9, 11.4, 15.1, 24.5, 435	41, 80, 103, 108, 122
Overall means ± SEM (n):			919 ± 155 (13)	8.8 ± 1.8 (12)§§	97 ± 10 (7)

‡§¶ See Table I. SEM for H_2O_2 release by triplicate cultures averaged 9.5% of the mean in individual experiments.

** LK induced with mezerein + lentil lectin and fractionated as described in Materials and Methods.

‡‡ LK induced with staphylococcal enterotoxin A and fractionated as described in Materials and Methods.

§§ Excludes the highest value (435).

IFNγ, we next tested the effects of pure IFNγ produced by bacteria transformed with the cloned human gene for this LK. The potent MAF activity of recombinant IFNγ is illustrated for three independent titrations in Fig. 2. The positions of the three dose-response curves varied considerably. Half-maximal stimulation of macrophage peroxide-releasing capacity followed 3 d of exposure to 0.009, 0.14, and 1.3 U/ml of IFNγ, with peak responses at 0.3, 10, and 1000 U/ml. The variability in the dose-response curves might be due in part to the use of different donors for the macrophages and the serum in each experiment. Another possible contributory factor is the fact that the time to peak response varied from 2 d of exposure (not shown) to as long as 4 d (Fig. 3). With continuous exposure to IFNγ, peroxide-releasing capacity remained elevated for at least 6 d, but fell toward baseline within 3 d of removing the recombinant IFNγ (Fig. 3), as already noted for native IFNγ. In the experiment illustrated in Fig. 3, some of the macrophages were exposed to 100 U/ml of IFNγ for 10 min on day 6 of culture, extensively washed to remove IFNγ, and then cultured in standard medium for 3 more days. Their peroxide-releasing capacity was at least as great as that of

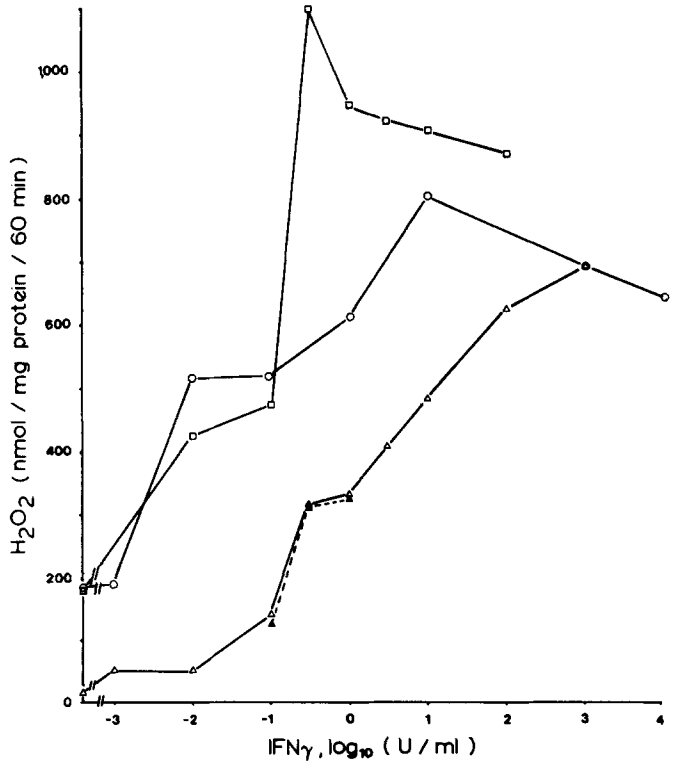

FIGURE 2. Enhancement of macrophage peroxide-releasing capacity by pure, recombinant IFNγ. Two experiments are shown in which macrophages were exposed to the indicated concentrations of IFNγ on days 6–9 (open circles and triangles) and one experiment in which exposure was from days 5–8 of culture (open squares). The dashed line (solid triangles) indicates results in the presence of monoclonal antibody to native IFNγ added at (from left to right) 3, 10, or 100 times the neutralizing activity for the indicated concentration of native IFNγ. Means of triplicates are indicated. SEMs averaged 13% of the mean.

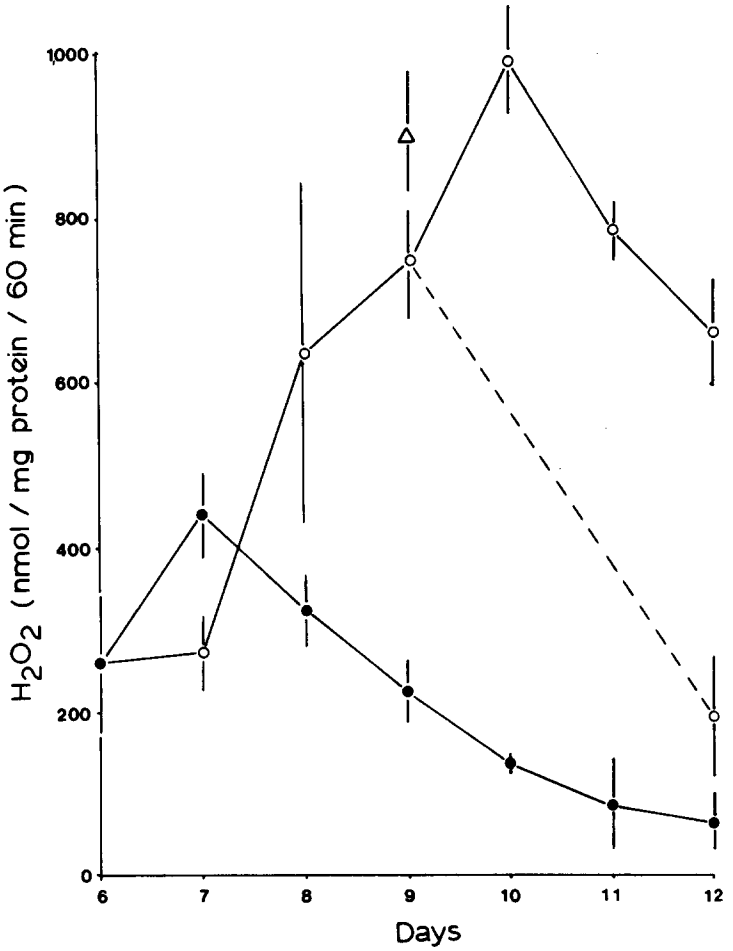

FIGURE 3. Kinetics of response to recombinant IFNγ. Macrophages on day 6 of culture were incubated in standard medium (solid circles) or 100 U/ml of IFNγ (open circles) for the indicated times before assay of H_2O_2 release. Fresh medium of the same type was added on day 8 or 9 for cells assayed on days 11 or 12, respectively. The dashed line indicates results for cells which were removed from IFNγ on day 9, washed four times in standard medium, then incubated in the latter for 3 d before assay. The open triangle indicates cells which were pulsed with 100 U/ml IFNγ for 10 min on day 6, washed four times, and incubated in standard medium until assay on day 9. Means ± SEM of triplicates.

cells exposed continuously to 100 U/ml IFNγ for the entire 3-d period. Results were similar in a second experiment using a 2-h pulse.

In contrast to the results with native IFNγ, monoclonal antibody GIF-1 had no inhibitory effect on the MAF activity of recombinant IFNγ. This is illustrated in Fig. 2 with antibody concentrations ranging from 3 to 100 times the amount required to neutralize an equivalent antiviral activity of native IFNγ. Antibody GIF-1 also failed to inhibit the antiviral activity of recombinant IFNγ (data not shown).

The remarkable potency of recombinant IFNγ compared with native IFNγ in some experiments raised the possibility that the former might contain a co-

stimulator or the latter might contain a suppressor of MAF activity. To test these possibilities, mixing experiments were carried out as shown in Table III. An effective but suboptimal dose of recombinant IFNγ did not give greater than additive effects when combined with a suboptimal but effective dose of the native product. An optimal dose of native IFNγ, when neutralized by monoclonal antibody and added to recombinant IFNγ, did not give less than additive effects. Thus, neither costimulator nor suppressor factors could be detected by mixing.

Secretion of hydrogen peroxide by macrophages was undetectable or barely detectable after incubation in either native or recombinant IFNγ, unless a secretagogue such as PMA was added (data not shown).

Induction of Antitoxoplasma Activity. The above experiments established that IFNγ enhances the oxidative metabolism of human macrophages. We next tested whether IFNγ also augments the antimicrobial activity of macrophages against an intracellular pathogen—the cardinal criterion for macrophage activation. As a test organism, we used the protozoan, *T. gondii.* The killing of this parasite by murine macrophages is closely related to their capacity to secrete hydrogen peroxide (11) and is mediated largely by oxidative mechanisms (11, 29), but to some extent by oxygen-independent processes as well (16, 36).

As shown in Fig. 4, unstimulated human macrophages cultured for 13–23 d before infection killed >5% of ingested toxoplasmas in the first 4 h, and supported intracellular replication (5.5 toxoplasmas/vacuole at 20 h). In contrast, when the cells were preincubated for 3 d with unfractionated mitogen- or

TABLE III

*Evidence Against a Suppressor Factor in Partially Purified IFNγ or a Costimulator in Recombinant IFNγ**

SEA-IFNγ‡	Recom.§	Aby‖	H_2O_2, nmol/mg protein/60 min	Predicted value for additive effects¶
U/ml	*U/ml*	*U/ml*		
0	0	0	15 ± 11**	
6	0	0	178 ± 16	
30	0	0	367 ± 16	
30	0	100	85 ± 8	
0	0.1	0	139 ± 28	
0	0.3	0	313 ± 11	
0	0.3	3	313 ± 11	
0	1.0	0	332 ± 8	
0	1.0	100	320 ± 9	
6	0.1	0	199 ± 13	302
30	0.3	100	448 ± 12	383
30	1.0	100	478 ± 8	390

* Macrophages were treated with IFNγ from days 6–9 of culture.

‡ Partially purified, native IFNγ elicited with staphylococcal enterotoxin A (see Table II).

§ Recombinant IFNγ.

‖ Monoclonal antibody GIF-1 (does not neutralize recombinant IFNγ).

¶ ([SEA-IFNγ ± Aby] − 15) + (Recom. − 15) + 15, where 15 is the value in medium alone.

** Means ± SEM for triplicates.

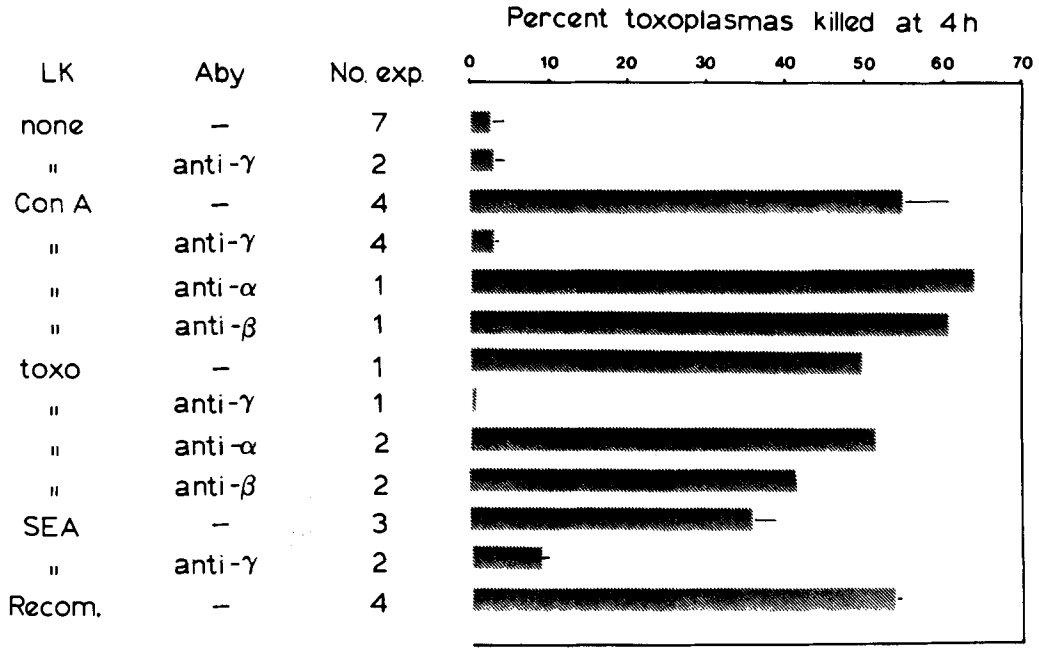

FIGURE 4. Toxoplasmacidal activity of macrophages incubated in lymphokine (LK) preparations with or without antibody to the indicated types of IFN. Incubations were in 100–300 U/ml IFNγ for 2–3 d beginning on days 13–23 of culture. Monoclonal anti-IFNγ antibody was used at 300–600 neutralizing U/ml. Anti-IFNα and anti-IFNβ antibodies were used at 600 neutralizing U/ml. Unpurified LK were induced with concanavalin A (*Con A*) or toxoplasma antigen (*toxo*). Partially purified native IFNγ preparation SEA-IFNγ had an activity of 10^7 U/mg protein. *Recom.*, recombinant IFNγ. Means ± SEM for the indicated number of experiments.

antigen-induced LK or partially purified native IFNγ, they killed 38–60% of the initially ingested parasites within 4 h, and inhibited the replication of surviving organisms (1.8–1.9 parasites/vacuole at 20 h). The induction of antiparasitic activity was abrogated by treatment of the unfractionated supernatants or the partially purified IFNγ with monoclonal anti-IFNγ antibody, but not with polyclonal sheep antibodies against human IFNα or IFNβ (Fig. 4). Direct exposure to 10% concanavalin A-induced LK or to 300 U/ml of partially purified native IFNγ for 1 h at 37° C did not affect the ability of toxoplasmas to survive and replicate when subsequently ingested by unstimulated macrophages (data not shown).

Pure, recombinant IFNγ also markedly enhanced macrophage antitoxoplasma activity (Fig. 4) in a dose-dependent manner (Fig. 5). In the experiment illustrated in Fig. 5, macrophages that had been in culture for 13 d were exposed to IFNγ for 3 more days before challenge with the parasite. Under these conditions, toxoplasmacidal activity was evident after treatment with as little as 1 U/ml IFNγ. Addition of 300 U/ml of recombinant IFNγ had no effect when it was added after infection of the macrophages (not shown).

As with enhancement of peroxide-releasing capacity, there was variability in the optimal period of preincubation in recombinant IFNγ to activate macrophage

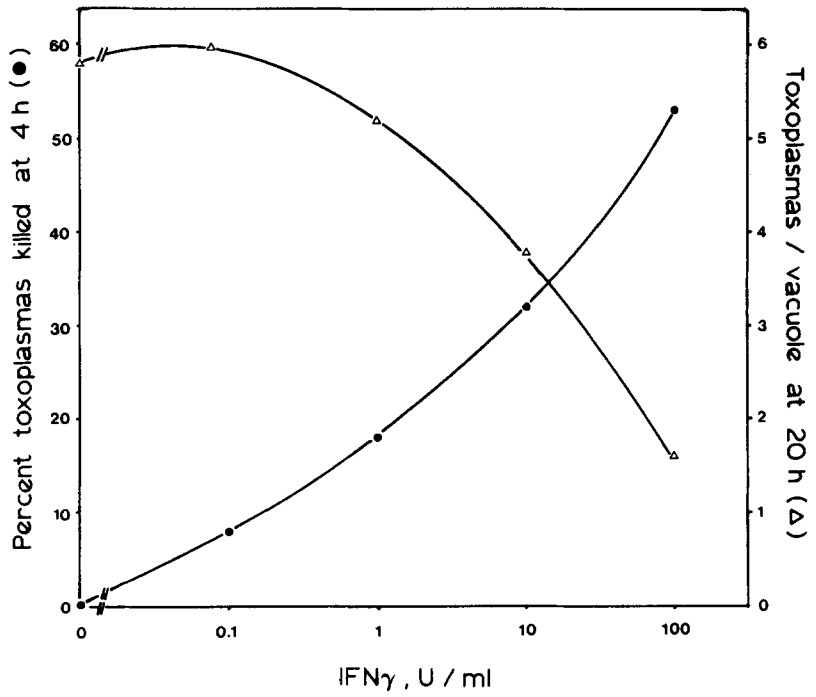

FIGURE 5. Effect of the concentration of recombinant IFNγ on the toxoplasmacidal (solid circles) and toxoplasmastatic (open triangles) activity of human macrophages after 3 d exposure to IFNγ beginning on day 13 of culture.

toxoplasmacidal activity. Peak activity was seen from 2–3 d after adding IFNγ. Fig. 6 summarizes three such experiments.

The effects of recombinant IFNγ were less dramatic when IFNγ was added from the outset of culture (day 0) to monocytes that were challenged with toxoplasmas on day 2. In this case, the untreated controls, which could release copious H_2O_2, killed 23% of ingested organisms by 4 h and limited the replication of the remainder to 2.0 per vacuole by 20 h. Monocytes treated from days 0 to 2 with 100 U/ml IFNγ killed 42% of the organisms at 4 h and displayed 1.4 toxoplasmas/vacuole at 20 h.

Effects of IFNγ on Cell Shape. Decreased spreading of human monocytes incubated in IFNα or IFNβ has been attributed to inhibition of their maturation into macrophages (37, 38). We examined the effects of applying IFNγ-containing media to cells that had already differentiated into macrophages. Macrophages incubated in control medium were usually bipolar, multipolar, or fan-shaped, with flat nuclear regions that were dark by phase-contrast microscopy (Fig. 7 *a*). In unfractionated lymphoid supernatants or in partially purified native IFNγ, the cells tended to spread in a more disklike fashion and often had highly rounded, refractile nuclear regions (Fig. 7 *b*). Addition of monoclonal anti-IFNγ forestalled these shape changes (Fig. 7 *c*). The same shape changes were seen after prolonged exposure to recombinant IFNγ (Fig. 7 *d*), but were not always evident by the time peroxide-releasing capacity was elevated. Thus, the morpho-

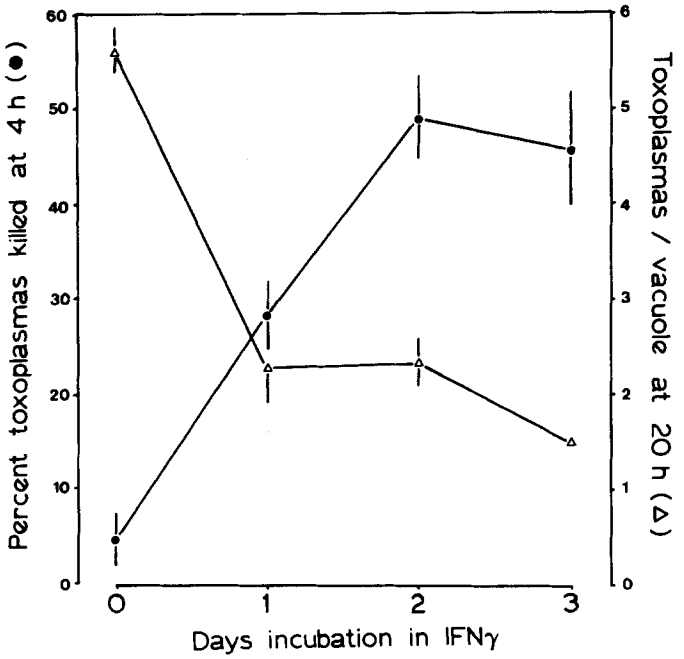

FIGURE 6. Time course of induction of toxoplasmacidal (solid circles) and toxoplasmastatic (open triangles) activity of macrophages exposed to 300 U/ml recombinant IFNγ beginning on days 10, 16, or 23 of culture. Means ± SEM for three experiments at each time point except two experiments at 3 d.

logic alterations did not appear to be necessary for enhanced peroxide-releasing capacity.

Discussion

We conclude that IFNγ is a potent activator of human macrophage oxidative metabolism and antimicrobial activity. Under the conditions tested, the only such activator consistently detected in the medium of antigen- or mitogen-stimulated human leukocytes was IFNγ. IFNγ is the first secretory product of T lymphocytes (33, 34, 39, 40) of known structure (41, 42). Thus it is of special interest that it capacitates macrophages to release a microbicidal product (H_2O_2) whose chemical composition is also known.

IFNγ is the LK for which the most sensitive bioassay is available, namely, induction of antiviral resistance. With recombinant IFNγ, activation of macrophage peroxide-releasing capacity was stimulated to 50% of the maximal value with a geometric mean concentration of 0.1 antiviral U/ml. Assuming that recombinant IFNγ is a nonglycosylated dimer of 34,292 daltons, then the 50% maximally effective concentration of the preparation used here can be estimated at 0.4–63 picomolar (geometric mean, 6 pM). The remarkable sensitivity of the oxidative metabolism of human macrophages to enhancement by IFNγ suggests that macrophage activation may be one of the primary physiologic functions of this LK.

It is now unarguable that more than one LK activity (as defined by effects on

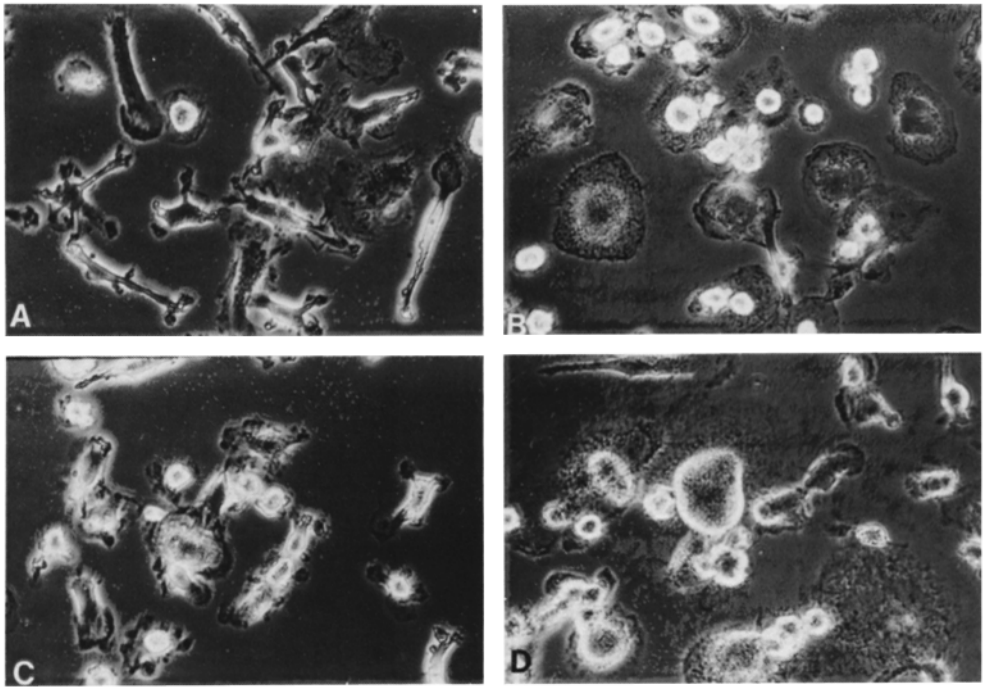

FIGURE 7. Morphology of IFNγ-treated macrophages by phase contrast microscopy. A, control cells in standard medium on day 8. B, macrophages exposed from days 5–8 to partially purified native IFNγ. C, same as B but also exposed to monoclonal antibody against IFNγ. D, treated from days 6–12 with 100 U/ml recombinant IFNγ.

different target cells) can be ascribed to the same molecule. Thus, IFNγ induces antiviral activity in a variety of cell types and enhances the capacity of macrophages to secrete H_2O_2 and to kill toxoplasmas. In addition, it is now clear that a single LK can cause pleiotropic effects in the same cell population. For example, the recombinant IFNγ used in our studies has also been reported to enhance the expression of DR antigens (19) and Fc receptors (25) on monocytes, and to activate monocytes to kill tumor cells (43).

Four qualifications require emphasis. First, the term "MAF" has been applied to factors that induce a variety of physiologic changes in macrophages. We have used the term in a restricted sense, and our results do not bear on the issue whether MAF as defined in other ways or in other species is IFNγ (reviewed in reference 21). Second, our studies are fully compatible with the possibility that there may be other factors besides IFNγ that can induce the same changes in macrophages as described here. IFNα, a product of non-T leukocytes, is of special interest in this regard (C. Nathan, H. Murray, B. Rubin, and M. Wiebe, unpublished observations). Our experiments do suggest that other MAFs were either not consistently present in active amounts in the lymphoid supernatants we studied, or if present, were masked by inhibitors or dependent on IFNγ in order to activate macrophages. Third, as already noted, the biochemical and functional changes we measured in macrophages in response to IFNγ are only a few of an unknown number of alterations induced in the same cells by the same

molecule. Experiments are in progress to determine whether the lymphokine that enhances oxygen-independent antimicrobial activity in human macrophages (15, 16) may also be IFNγ. Finally, we have not formally excluded the possibility that the effects of IFNγ on macrophages could have been mediated by another type of cell contaminating the cultures. However, results were similar whether IFNγ was added starting on the 3rd through the 23rd day of culture. In most of these cultures, no cells other than macrophages could be identified by morphologic criteria.

Neutralization of MAF activity in crude lymphoid supernatants by the monoclonal anti-IFNγ antibody was strong evidence that MAF was IFNγ. However, the epitopes recognized by monoclonal antibodies may sometimes be shared by seemingly unrelated proteins (44). Accordingly, it was necessary to determine directly whether native, leukocyte-derived IFNγ had MAF activity. Using two independent protocols for induction and partial purification of IFNγ, MAF activity was readily demonstrated, was enriched to at least the same degree as antiviral activity, and was again neutralized by monoclonal anti-IFNγ antibody. Nonetheless, these preparations of IFNγ were not pure, and the possibility remained that a contaminant contributed to MAF activity. For a definitive answer, we turned to a preparation in which IFNγ was the only human protein and probably the only protein, namely, IFNγ purified to apparent homogeneity from bacteria transformed with the cloned gene for human IFNγ. This preparation displayed especially potent MAF activity. The time required for macrophages to display an optimal response to recombinant IFNγ varied from 2–4 d. Exposures to IFNγ as brief as 10–120 min led to substantial activation when macrophages were tested 3 d later. After 3 d of exposure to IFNγ, peroxide secretory capacity fell to barely detectable levels when IFNγ was removed, but was elevated for at least 6 d when IFNγ remained.

Monoclonal antibody GIF-1 neutralized both the antiviral and macrophage-activating effects of native IFNγ, yet neutralized neither of these effects of the recombinant bacterial product. There are believed to be two carbohydrate chains on native IFNγ (26, 41, 42) and none on the recombinant product. It is possible that the carbohydrate groups contribute to the epitope seen by the antibody. These carbohydrates are not necessary for the expression of at least some of the antiviral (45) or MAF activity of IFNγ. However, the carbohydrate moieties may border a domain necessary for IFNγ activity, such as a receptor-binding site. The antibody might interfere with the function of this domain by steric hindrance.

Recombinant IFNγ was usually considerably more potent than the equivalent concentration of native IFNγ, based on antiviral activity. There are at least three possible explanations for this apparent discrepancy. First, the partially purified native IFNγ could have been contaminated with a factor suppressing macrophage oxidative metabolism. Such a suppressive factor has been observed in the culture medium of murine lymphoid cells (46), and inhibitors of IFN antiviral activity are reportedly produced by stimulated lymphocytes (47). The ability of monoclonal antibody GIF-1 to neutralize native IFNγ without neutralizing recombinant IFNγ made it possible to perform mixing experiments, in which native IFNγ preparations plus antibody GIF-1 were added to recombinant IFNγ. No

suppression of the macrophage-activating effect of the latter was observed. However, a suppressive factor seemed to be present in the unpurified LK preparations when they were used at high concentrations. The nature of such a suppressive factor and its possible role in anergic states warrant further study.

A second possible explanation for the relative potency of recombinant IFNγ is that there could be a costimulator contaminating it, such as bacterial LPS (48). However, in mixing experiments, no costimulator activity could be demonstrated. Furthermore, we have not been able to enhance human macrophage H_2O_2 release with LPS, using the same culture conditions as in the present work (C. Nathan, unpublished observations). Finally, no LPS was detectable in the purified recombinant IFNγ by the limulus amoebocyte lysate test (personal communication, Dr. C. Sevastopoulos) (limit of detection, 0.05 ng/ml). This preparation was effective after a dilution of more than 10^7-fold. Effectiveness after such dilution virtually rules out a contribution by traces of LPS in the IFNγ, or by any other minor contaminant.

Thus, a third possibility must be considered, that one molecule of recombinant IFNγ may either have more potent MAF and/or less potent antiviral activity than one molecule of native IFNγ, perhaps because of differences in posttranslational modifications. In fact, on a protein basis, SEA-IFNγ and recombinant IFNγ had similar MAF activity. There is precedent for variations in ratios of different IFN effects when comparing natural and recombinant IFNs (49). The reasons for these variations are not well understood.

There have been almost no previous reports on the effects of IFN on macrophage oxidative metabolism. Boraschi et al. (50) reported that IFNβ inhibited the superoxide-releasing capacity of mouse peritoneal macrophages. However, pure IFNβ was not used. It will be of interest to determine the relation between the inhibitory activity in that study and the factor suppressing macrophage oxidative metabolism that was detected in medium conditioned by a variety of cell types, including fibroblasts (46). In contrast, numerous reports have suggested that IFN may enhance antimicrobial activity of a variety of cells against pathogens other than viruses, including *T. gondii* (51–54), malaria (55), *Rickettsia* (56), shigellae (57), salmonellae (58), staphylococci (59), and mycobacteria (60).

The capacity of host lymphocytes to secrete IFNγ mounts in parallel with other manifestations of delayed-type hypersensitivity and cell-mediated immunity (34, 39, 61, 62). This is consistent with the evidence presented here in support of the hypothesis that IFNγ mediates macrophage activation during the latter responses. Thus it seems appropriate to ask whether provision of IFNγ might favorably affect the course of diseases in which persistent parasitization of macrophages is a prominent feature. These include some of the most prevalent chronic infections, such as tuberculosis, lepromatous leprosy, and leishmaniasis.

Summary

Human blood mononuclear leukocytes stimulated with toxoplasma antigen, concanavalin A, mezerein plus lentil lectin, or staphylococcal enterotoxin A secreted a factor (macrophage-activating factor, or MAF) that enhanced the capacity of human macrophages to release H_2O_2 and to kill toxoplasmas. The same lymphoid supernatants contained IFNγ but not IFNα or IFNβ. The MAF

activity of six of seven unfractionated supernatants was completely eliminated by a monoclonal antibody that neutralizes IFNγ, and MAF in the remaining supernatant was almost completely neutralized. Native IFNγ partially purified by two independent protocols to specific activities of 1×10^6 and 10^7 U/mg protein was enriched in MAF activity at least as much as in antiviral activity. The capacity of macrophages to secrete H_2O_2 after incubation in partially purified native IFNγ (mean peak stimulation, 8.8-fold) was greater than with unpurified lymphokines (3.8-fold) and sometimes equaled or exceeded the capacity of freshly harvested monocytes. The MAF activity of the partially purified native IFNγ preparations was abolished by monoclonal anti-IFNγ. Finally, IFNγ of >99% estimated purity was isolated (at Genentech, Inc.) from bacteria transformed with the cloned human gene for this lymphokine. Recombinant IFNγ had potent MAF activity, stimulating the peroxide-releasing capacity of macrophages an average of 19.8-fold at peak response and enhancing their ability to kill toxoplasmas from 2.6 ± 1.3% for untreated cells to 54 ± 0.4% for treated cells. Attainment of 50% of the maximal elevation in peroxide-releasing capacity required a geometric mean concentration of 0.1 antiviral U/ml of recombinant IFNγ, which is estimated to be ~6 picomolar for this preparation. Peroxide secretory capacity and toxoplasmacidal activity of macrophages peaked 2–4 d after exposure to IFNγ. Peroxide-secretory capacity remained elevated during at least 6 d of continuous exposure, but the effect of IFNγ was reversed within about 3 d of its removal. Activation was usually but not invariably accompanied by characteristic changes in cell morphology. Thus, IFNγ activates human macrophage oxidative metabolism and antimicrobial activity, and appeared to be the only factor consistently capable of doing so in the diverse LK preparations tested.

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