

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 monophosphate 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 (L.K.)¹ meeting this description is called macrophage-activating factor (MAF).

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¹ *Abbreviations used in this paper:* IFN, interferon; IFN α , formerly leukocyte IFN; IFN β , formerly fibroblast IFN; IFN γ , formerly immune IFN; LK, lymphokine(s); LPS, bacterial lipopolysaccharide; MAF, macrophage-activating factor; MLL-IFN γ , partially purified IFN γ 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-IFN γ , partially purified IFN γ from human leukocytes exposed to staphylococcal enterotoxin A; SEM, standard error of the mean.

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 ($\text{IFN}\gamma$). There is strong evidence that $\text{IFN}\gamma$ 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 $\text{IFN}\gamma$, pure $\text{IFN}\gamma$ produced by bacteria containing the cloned human gene (26), and a monoclonal antibody that neutralizes $\text{IFN}\gamma$. This combination of reagents has permitted the identification of $\text{IFN}\gamma$ 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 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$ 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 $\text{IFN}\gamma$ by

sequential affinity chromatography to a specific activity of 1×10^6 antiviral U/ml; these are designated MLL-IFN γ . Details of the purification will be furnished elsewhere (M. Wiebe, M. Chang et al., manuscript in preparation). Another preparation, termed SEA-IFN γ , 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 IFN γ 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-Hee 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 IgG₁ antibody GIF-1 neutralizes human IFN γ but not IFN α or IFN β (31). The GIF-1 hybridoma supernatant used here had a neutralizing activity of 5,000 U/ml. Sheep globulins to human IFN α and to human IFN β 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 H₂O₂/mg cell protein after treatment of monocytes as indicated by the subscripts (LK = lymphokine or IFN γ , A = antibody, M = medium control).

Results

Elimination by Monoclonal Anti-IFN γ 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 mezerin. As expected (33–35), these supernatants all contained substantial titers of IFN γ (Table I). IFN α and IFN β could not be detected. To determine whether the stimulatory activity in these unfractionated supernatants could be ascribed to their content of IFN γ , we made use of a monoclonal antibody (GIF-1) that neutralizes IFN γ but has no detectable effect on IFN α or IFN β (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 IFN γ

Stimuli for LK production*	IFN γ titer	Concentration IFN γ used	H ₂ O ₂ release by LK-treated cells [‡]	H ₂ O 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 \pm SEM (N):			666 \pm 67 (15)	3.8 \pm 0.6 (15)	124 \pm 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 H₂O₂ 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 H₂O₂ release and adherent cell protein. SEM for H₂O₂ 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 H₂O₂-releasing capacity typically observed over the first 3-4 d of culture of adherent MNL may not be sustained by IFN γ from contaminating T cells.

In three experiments, incubation of macrophages in supernatant plus anti-IFN γ antibody resulted in H₂O₂-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 \pm 0.3 times greater after 3 d

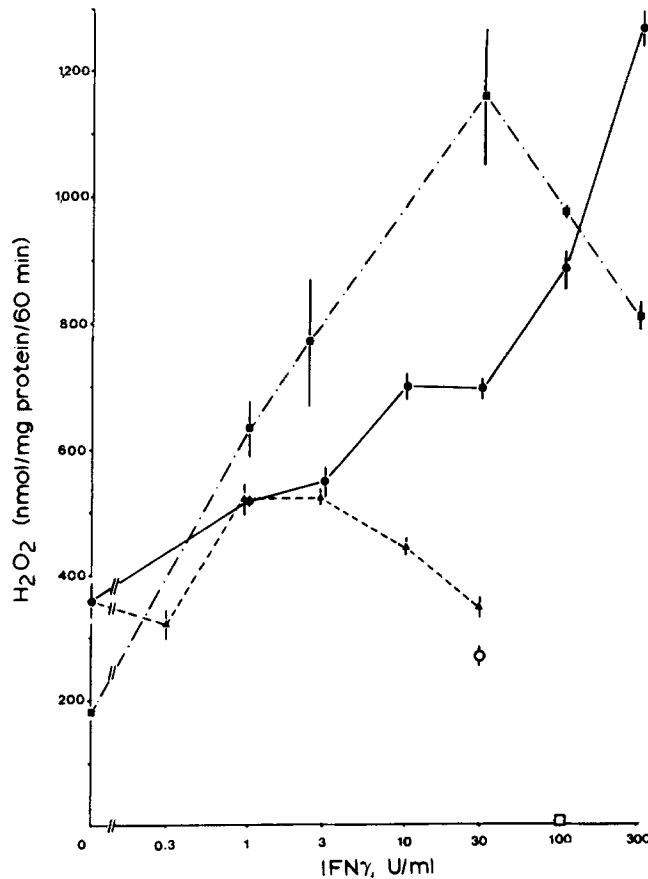


FIGURE 1. Enhancement of macrophage peroxide-releasing capacity by three IFN γ -containing preparations and its prevention by monoclonal anti-IFN γ antibody. Human macrophages were exposed to the indicated concentrations of IFN γ on days 4–7 of culture, washed, and stimulated with PMA to measure H₂O₂ release. An unpurified supernatant from buffy coat cells stimulated with mezerein and lentil lectin (solid triangles) had 10² U/ml antiviral activity. A fraction obtained from this supernatant (solid circles) had an antiviral activity of 10⁴ U/ml or 1 \times 10⁶ U/mg protein. An independently purified preparation from buffy coat cells stimulated with staphylococcal enterotoxin A (solid squares) had an activity of 6 \times 10³ U/ml or 1 \times 10⁷ U/mg protein. The open symbols are results with the corresponding preparations exposed to 120–350 neutralizing U/ml of monoclonal anti-IFN γ 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 \pm 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-IFN γ antibody (data not shown).

Effects of Partially Purified IFN γ . The foregoing experiments implied that IFN γ 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 IFN γ should have MAF activity. The results of 13 experiments with two independently derived preparations enriched in IFN γ are summarized

in Table II. On the average, IFN γ -rich fractions enhanced macrophage peroxide-releasing capacity 8.8-fold. Peroxide released by IFN γ -treated macrophages on days 7–9 sometimes exceeded that secreted by samples of the same monocytes on day 0. Monoclonal anti-IFN γ antibody inhibited IFN γ -mediated enhancement of macrophage peroxide-releasing capacity by an average of 97% in seven experiments (Table II). In two titrations of preparation SEA-IFN γ (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 IFN γ , and thereafter declined somewhat, despite replenishment with fresh IFN γ . However, peroxide releasing-capacity of cells continuously exposed to IFN γ remained markedly elevated for at least 5 d, the longest period tested. For example, macrophages treated with IFN γ from days 5–8 released $1,479 \pm 124$ nmol/mg protein/60 min, compared to 393 ± 20 for controls. After 2 more days in IFN γ , peroxide secretory capacity fell to 652 ± 53 , compared to 101 ± 25 for the controls. However, when IFN γ 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 IFN γ on macrophage peroxide release was reversible. These kinetics closely parallel those reported earlier for unfractionated lymphoid supernatants (14).

On the average, cultures incubated in IFN γ -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 IFN γ . To establish conclusively that IFN γ 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 IFN γ : Prevention by Monoclonal Antibody to IFN γ

Preparation	Specific activity (U/mg protein)	Concentration IFN γ used	H ₂ O ₂ release by IFN-treated cells [†] (nmol/mg protein/60 min)	H ₂ O ₂ release experimental [‡] control [§] cells	Inhibition by monoclonal antibody [¶] %
		U/ml			
MLL-IFN γ **	1×10^6	10–242	392, 482, 706, 1226 [†]	2.0, 6.8, 7.1, 12.7 [†]	102, 124 [†]
SEA-IFN γ **	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 \pm SEM (n):			919 ± 155 (13)	8.8 ± 1.8 (12) ^{**}	97 ± 10 (7)

^{†††} See Table I. SEM for H₂O₂ 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).

IFN γ , we next tested the effects of pure IFN γ produced by bacteria transformed with the cloned human gene for this LK. The potent MAF activity of recombinant IFN γ 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 IFN γ , 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 IFN γ , peroxide-releasing capacity remained elevated for at least 6 d, but fell toward baseline within 3 d of removing the recombinant IFN γ (Fig. 3), as already noted for native IFN γ . In the experiment illustrated in Fig. 3, some of the macrophages were exposed to 100 U/ml of IFN γ for 10 min on day 6 of culture, extensively washed to remove IFN γ , and then cultured in standard medium for 3 more days. Their peroxide-releasing capacity was at least as great as that of

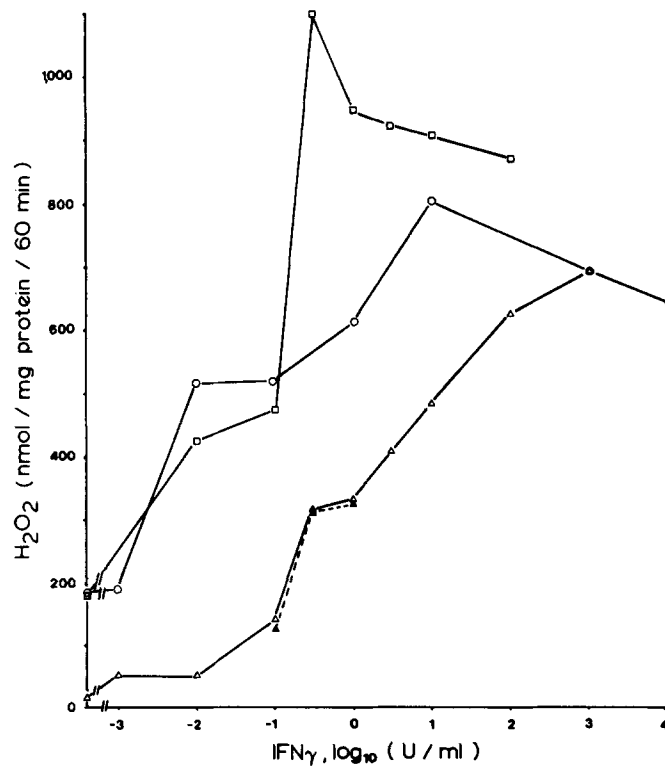


FIGURE 2. Enhancement of macrophage peroxide-releasing capacity by pure, recombinant IFN γ . Two experiments are shown in which macrophages were exposed to the indicated concentrations of IFN γ on days 6–9 (open circles and triangles) and one experiment in which exposure was from days 5–8 of culture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

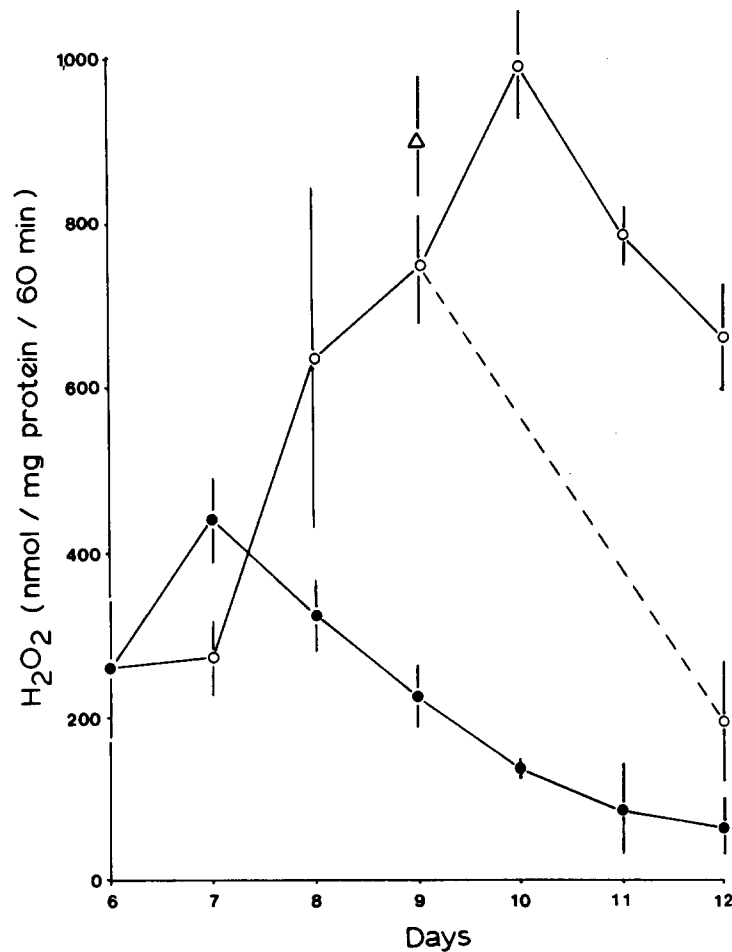


FIGURE 3. Kinetics of response to recombinant IFN γ . Macrophages on day 6 of culture were incubated in standard medium (solid circles) or 100 U/ml of IFN γ (open circles) for the indicated times before assay of H₂O₂ release. Fresh medium of the same type was added on day 8 or 9 for cells assayed on days 11 or 12, respectively. The dashed line indicates results for cells which were removed from IFN γ 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 IFN γ for 10 min on day 6, washed four times, and incubated in standard medium until assay on day 9. Means \pm SEM of triplicates.

cells exposed continuously to 100 U/ml IFN γ 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 IFN γ , monoclonal antibody GIF-1 had no inhibitory effect on the MAF activity of recombinant IFN γ . 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 IFN γ . Antibody GIF-1 also failed to inhibit the antiviral activity of recombinant IFN γ (data not shown).

The remarkable potency of recombinant IFN γ compared with native IFN γ 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 IFN γ did not give greater than additive effects when combined with a suboptimal but effective dose of the native product. An optimal dose of native IFN γ , when neutralized by monoclonal antibody and added to recombinant IFN γ , 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 IFN γ , unless a secretagogue such as PMA was added (data not shown).

Induction of Antitoxoplasma Activity. The above experiments established that IFN γ enhances the oxidative metabolism of human macrophages. We next tested whether IFN γ 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 IFN γ or a Costimulator in Recombinant IFN γ *

SEA-IFN γ [‡]	Recom. [§]	Aby [¶]	H ₂ O 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 IFN γ from days 6–9 of culture.

[‡] Partially purified, native IFN γ elicited with staphylococcal enterotoxin A (see Table II).

[§] Recombinant IFN γ .

[¶] Monoclonal antibody GIF-1 (does not neutralize recombinant IFN γ).

[†] [(SEA-IFN γ ± Aby) – 15] + (Recom. – 15) + 15, where 15 is the value in medium alone.

** Means ± SEM for triplicates.

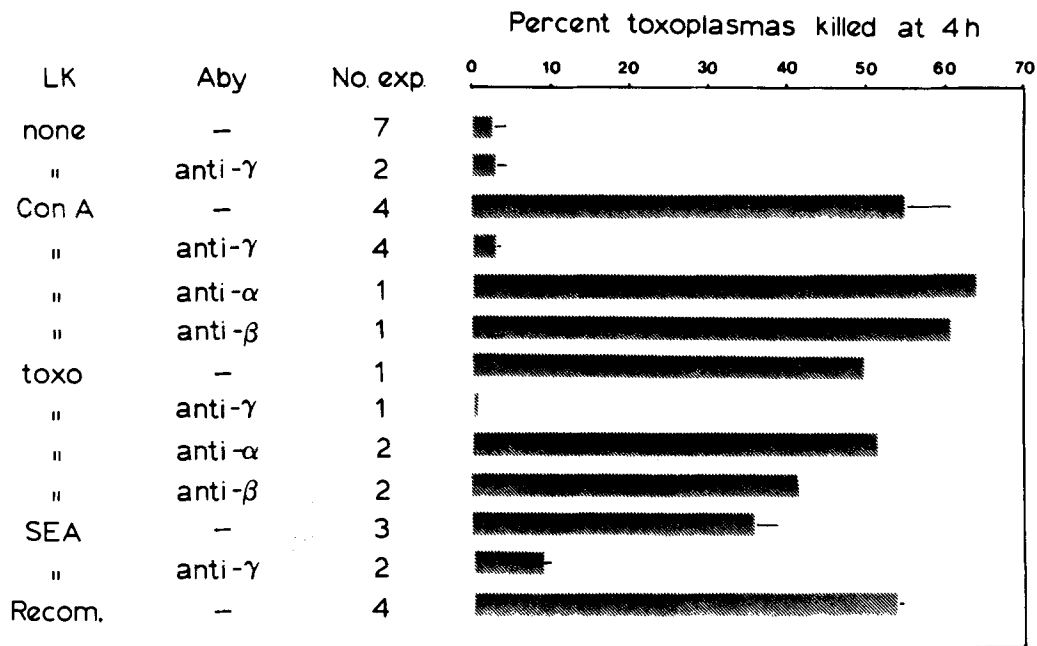


FIGURE 4. Toxoplasmodicidal activity of macrophages incubated in lymphokine (LK) preparations with or without antibody to the indicated types of IFN. Incubations were in 100–300 U/ml IFN γ for 2–3 d beginning on days 13–23 of culture. Monoclonal anti-IFN γ antibody was used at 300–600 neutralizing U/ml.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

antigen-induced LK or partially purified native IFN γ , 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 IFN γ with monoclonal anti-IFN γ antibody, but not with polyclonal sheep antibodies against human IFN α or IFN β (Fig. 4). Direct exposure to 10% concanavalin A-induced LK or to 300 U/ml of partially purified native IFN γ 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 IFN γ 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 IFN γ for 3 more days before challenge with the parasite. Under these conditions, toxoplasmodicidal activity was evident after treatment with as little as 1 U/ml IFN γ . Addition of 300 U/ml of recombinant IFN γ 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 IFN γ to activate macrophage

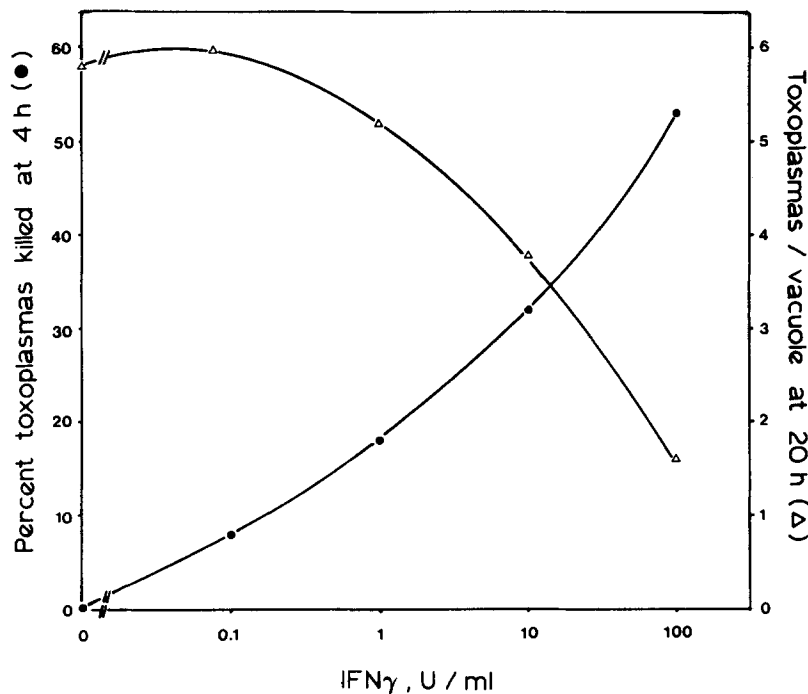


FIGURE 5. Effect of the concentration of recombinant IFN γ on the toxoplasma-killing (solid circles) and toxoplasma-replication (open triangles) activity of human macrophages after 3 d exposure to IFN γ beginning on day 13 of culture.

toxoplasma-killing activity. Peak activity was seen from 2–3 d after adding IFN γ . Fig. 6 summarizes three such experiments.

The effects of recombinant IFN γ were less dramatic when IFN γ 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 H₂O₂, 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 IFN γ killed 42% of the organisms at 4 h and displayed 1.4 toxoplasmas/vacuole at 20 h.

Effects of IFN γ on Cell Shape. Decreased spreading of human monocytes incubated in IFN α or IFN β has been attributed to inhibition of their maturation into macrophages (37, 38). We examined the effects of applying IFN γ -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 IFN γ , 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-IFN γ forestalled these shape changes (Fig. 7 c). The same shape changes were seen after prolonged exposure to recombinant IFN γ (Fig. 7 d), but were not always evident by the time peroxide-releasing capacity was elevated. Thus, the morpho-

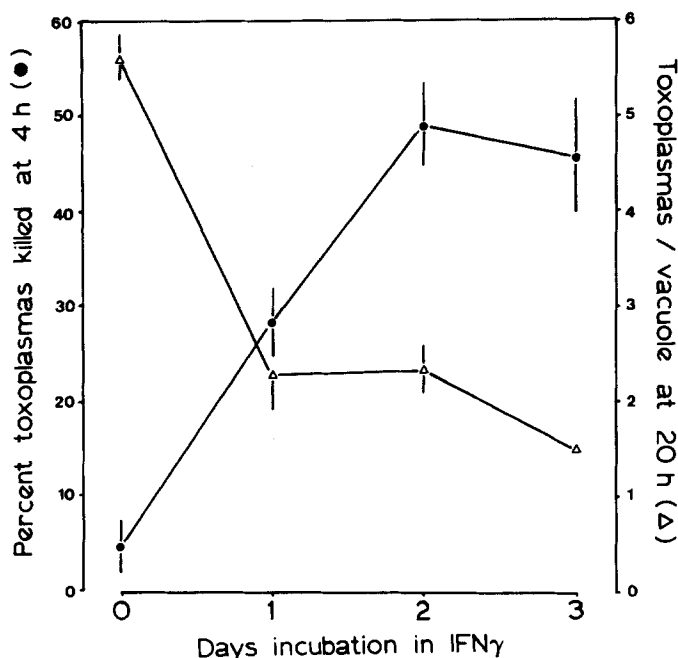


FIGURE 6. Time course of induction of toxoplasma-killing (solid circles) and toxoplasma-vacuole formation (open triangles) activity of macrophages exposed to 300 U/ml recombinant IFN γ beginning on days 10, 16, or 23 of culture. Means \pm SEM for three experiments at each time point except two experiments at 3 d.

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

Discussion

We conclude that IFN γ 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 IFN γ . IFN γ 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.

IFN γ is the LK for which the most sensitive bioassay is available, namely, induction of antiviral resistance. With recombinant IFN γ , 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 IFN γ 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 IFN γ 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

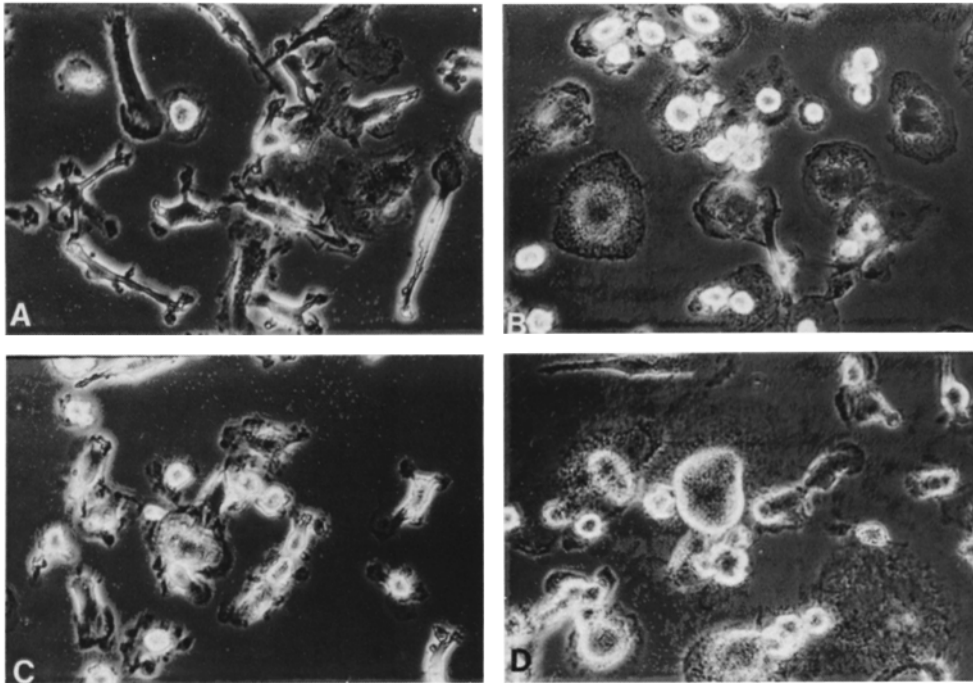


FIGURE 7. Morphology of IFN γ -treated macrophages by phase contrast microscopy. A, control cells in standard medium on day 8. B, macrophages exposed from days 5-8 to partially purified native IFN γ . C, same as B but also exposed to monoclonal antibody against IFN γ . D, treated from days 6-12 with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

different target cells) can be ascribed to the same molecule. Thus, IFN γ induces antiviral activity in a variety of cell types and enhances the capacity of macrophages to secrete H₂O₂ 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 IFN γ 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 IFN γ (reviewed in reference 21). Second, our studies are fully compatible with the possibility that there may be other factors besides IFN γ that can induce the same changes in macrophages as described here. IFN α , 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 IFN γ in order to activate macrophages. Third, as already noted, the biochemical and functional changes we measured in macrophages in response to IFN γ 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 IFN γ . Finally, we have not formally excluded the possibility that the effects of IFN γ on macrophages could have been mediated by another type of cell contaminating the cultures. However, results were similar whether IFN γ 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-IFN γ antibody was strong evidence that MAF was IFN γ . 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 IFN γ had MAF activity. Using two independent protocols for induction and partial purification of IFN γ , MAF activity was readily demonstrated, was enriched to at least the same degree as antiviral activity, and was again neutralized by monoclonal anti-IFN γ antibody. Nonetheless, these preparations of IFN γ 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 IFN γ was the only human protein and probably the only protein, namely, IFN γ purified to apparent homogeneity from bacteria transformed with the cloned gene for human IFN γ . This preparation displayed especially potent MAF activity. The time required for macrophages to display an optimal response to recombinant IFN γ varied from 2–4 d. Exposures to IFN γ as brief as 10–120 min led to substantial activation when macrophages were tested 3 d later. After 3 d of exposure to IFN γ , peroxide secretory capacity fell to barely detectable levels when IFN γ was removed, but was elevated for at least 6 d when IFN γ remained.

Monoclonal antibody GIF-1 neutralized both the antiviral and macrophage-activating effects of native IFN γ , yet neutralized neither of these effects of the recombinant bacterial product. There are believed to be two carbohydrate chains on native IFN γ (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 IFN γ . However, the carbohydrate moieties may border a domain necessary for IFN γ activity, such as a receptor-binding site. The antibody might interfere with the function of this domain by steric hindrance.

Recombinant IFN γ was usually considerably more potent than the equivalent concentration of native IFN γ , based on antiviral activity. There are at least three possible explanations for this apparent discrepancy. First, the partially purified native IFN γ 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 IFN γ without neutralizing recombinant IFN γ made it possible to perform mixing experiments, in which native IFN γ preparations plus antibody GIF-1 were added to recombinant IFN γ . 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 IFN γ 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 H₂O₂ 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 IFN γ 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 10⁷-fold. Effectiveness after such dilution virtually rules out a contribution by traces of LPS in the IFN γ , or by any other minor contaminant.

Thus, a third possibility must be considered, that one molecule of recombinant IFN γ may either have more potent MAF and/or less potent antiviral activity than one molecule of native IFN γ , perhaps because of differences in posttranslational modifications. In fact, on a protein basis, SEA-IFN γ and recombinant IFN γ 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 IFN β inhibited the superoxide-releasing capacity of mouse peritoneal macrophages. However, pure IFN β 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 IFN γ 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 IFN γ mediates macrophage activation during the latter responses. Thus it seems appropriate to ask whether provision of IFN γ 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 H₂O₂ and to kill toxoplasmas. The same lymphoid supernatants contained IFN γ but not IFN α or IFN β . The MAF

activity of six of seven unfractionated supernatants was completely eliminated by a monoclonal antibody that neutralizes IFN γ , and MAF in the remaining supernatant was almost completely neutralized. Native IFN γ 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 H₂O₂ after incubation in partially purified native IFN γ (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 IFN γ preparations was abolished by monoclonal anti-IFN γ . Finally, IFN γ of >99% estimated purity was isolated (at Genentech, Inc.) from bacteria transformed with the cloned human gene for this lymphokine. Recombinant IFN γ 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 $2.6 \pm 1.3\%$ for untreated cells to $54 \pm 0.4\%$ 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 IFN γ , which is estimated to be ~ 6 picomolar for this preparation. Peroxide secretory capacity and toxoplasmodicidal activity of macrophages peaked 2–4 d after exposure to IFN γ . Peroxide-secretory capacity remained elevated during at least 6 d of continuous exposure, but the effect of IFN γ was reversed within about 3 d of its removal. Activation was usually but not invariably accompanied by characteristic changes in cell morphology. Thus, IFN γ 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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