

AGONIST AND ANTAGONIST EFFECTS OF
INTERFERON α AND β ON ACTIVATION OF
HUMAN MACROPHAGES

Two Classes of Interferon γ Receptors and Blockade of the
High-Affinity Sites by Interferon α or β

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Although IFN- α , - β , and - γ differ in cell origin, inducing agents, molecular structure, and cell surface receptors, they share a number of properties, including the induction of antiviral and antiproliferative states and the ability to stimulate macrophages to develop tumoricidal activity and to downregulate mannosyl/fucosyl receptors (1, 2). Simultaneous exposure of fibroblasts, tumor cells, or macrophages to IFN- γ on the one hand, and IFN- α or - β on the other hand, frequently has a synergistic effect on antiviral and antiproliferative activity (3, 4) and induction of Fc receptors (5). However, with the neuroblastoma cell line T98G, IFN- γ antagonizes IFN- α -stimulated antiviral and antiproliferative activities (6). Conversely, with macrophages, IFN- α and/or - β interfere with regulation by IFN- γ of the expression of Ia antigens and mannosyl/fucosyl receptors, release of H₂O₂, and killing of tumor cells (7-11). The mechanisms underlying these inhibitory interactions are unknown.

In a survey of cytokines, IFN- γ was far more effective than IFN- α or - β for activation of human macrophage oxidative metabolism and antiprotozoal activity (12). Study of the latter IFNs was complicated by their apparent toxicity, as evidenced by sphering of the macrophages and decreased adherence to glass (12). Modifications in our method of cell preparation (13) designed to reduce platelet contamination have now permitted us to establish more robust macrophage monolayers. These cultures retain a higher proportion of adherent cells over 1-2 wk and resist the toxic effects of IFN- α and - β . Thus, we wished to reinvestigate the effects of the IFNs, alone and in combination, on macrophage H₂O₂ secretory capacity and antiprotozoal activity. Two novel findings emerged (14): on the one hand, activation of macrophages, but not monocytes, by fairly high concentrations of IFN- α and - β ; and on the other hand, antagonism of IFN- γ 's macrophage-activating effects by far lower concentrations of IFN- α or - β . Examination of the latter interaction at the receptor level led to two further observations. While monocytes and early macrophages displayed one class of

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IFN- γ -R (15–17), the IFN- γ -R on mature macrophages appeared to diverge into two classes of differing affinity. Moreover, IFN- α or - β seemed selectively and competitively to blockade the high-affinity IFN- γ -R. Differentiation of the human monocyte *in vitro* thus appears to be accompanied by complex changes in binding and signaling characteristics of receptors for each of the IFNs.

Materials and Methods

Interferons. Purified human rIFN- γ was generously provided by Genentech, Inc. (South San Francisco, CA). The specific activity was 3.4×10^7 U/mg protein, as assayed by inhibition of encephalomyelocarditis virus replication in human lung carcinoma A549 cells in comparison to the National Institutes of Health IFN- γ standard. Natural (n)¹ IFN- α , affinity purified with an mAb (18), was kindly donated by Dr. M. Wiebe of the New York Blood Center. The specific activity was 2×10^8 U/mg protein. Purified rIFN- α A, rIFN- α D, and rIFN- β (sp act 2×10^8 U/mg protein) were generous gifts from Drs. P. Trown, M. Brunda, and M. Sherman, Hoffman-La Roche, Inc., Nutley, NJ.

Antibodies. Sheep anti-human IFN- α IgG was purchased from Miles Laboratories Inc., Naperville, IL. 1 ml of this antibody neutralized 50,000 U of nIFN- α , as assayed by induction of H₂O₂-releasing capacity. Anti-rIFN- β ₁ rabbit serum was kindly provided by Dr. L. S. Lin of Cetus Corp., Emeryville, CA. The neutralization titer was 10,000 U/ml. Test media were preincubated with antibodies for 30–40 min at room temperature before use.

Culture of Monocytes. Healthy adult volunteers of both sexes were phlebotomized at the New York Blood Center. 450 ml of blood was collected with citrate/dextrose/phosphate/adenine (CDPA-1) as an anticoagulant. The buffy coat was obtained 2–3 h after venipuncture and mononuclear leukocytes (MNLs) were isolated by Ficoll-Hypaque ($\rho = 1.070$; Pharmacia Fine Chemicals, Piscataway, NJ) gradient centrifugation as described previously (13, 19) with some modifications. Buffy coat (~20 ml/tube) was centrifuged three times (500 g for 15, 10, and 10 min at 4°C, respectively) with 40 ml of cold PBS containing 5.5 mM glucose (PDG). After Ficoll-Hypaque gradient centrifugation at 1,050 g for 25 min at 25°C, the upper cell layer was suspended in 40 ml of cold PDG, and the suspension was thoroughly mixed in a 10-ml plastic pipette. After centrifugation at 200 g for 10 min at 4°C, the cell pellet was washed with cold PDG. After two further centrifugations (150 g for 10 min at 4°C), the final pellet was suspended in culture medium, consisting of RPMI 1640 containing 2 mM fresh glutamine (KC Biological, Inc., Lenexa, KS), 25% freshly frozen type A human serum prepared as described (13), 100 U/ml penicillin, and 100 μ g/ml streptomycin (complete medium). MNL number was determined by hemocytometer counting. Cell viability, determined by exclusion of 0.2% trypan blue, was >95%. For experiments with H₂O₂ release and for binding studies with rIFN- γ involving macrophages, 1–2 $\times 10^6$ MNL containing ~20–30% cells with monocyte morphology were dispensed in 0.08 ml of culture medium per 13-mm diam glass coverslip. For binding experiments using monocytes, 2–3 $\times 10^6$ MNLs were plated per coverslip. The coverslips were pretreated for 1 wk with 36% HNO₃ before they were cleaned in ethanol as described (13). After incubation for 2 h at 37°C in 5% CO₂/95% humidified air, the coverslips were rinsed three times in warm Eagle's MEM and transferred to 16-mm-diam plastic wells in 24-well trays (Costar, Data Packaging Corp., Cambridge, MA) in 0.3 ml of culture medium. The day of cell preparation was designated day 0. The medium was replaced on day 1 and every third day thereafter. Results are presented for cultures in which the cells adherent on day 8 represented $\geq 60\%$ of the monocytes initially plated. During the 2-yr course of this study, such cultures were consistently obtained for periods of up to 7 mo (22 consecutive experiments). However, there were two intervals, lasting several months, in which $\leq 15\%$ of the monocytes remained adherent by day 8. Stimulatory effects of IFN- α and - β on macrophage H₂O₂-releasing capacity were not uniformly seen in such cultures; these IFNs appeared instead to be toxic.

¹ Abbreviations used in this paper: MNL, mononuclear leukocyte; n, natural; PDG, PBS containing 5.5 mM glucose.

H₂O₂ Release. Secretion of H₂O₂ in response to 100 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) was measured in triplicate coverslips by the fluorescent scopoletin assay as described (13) except that the total volume of reaction mixture was reduced to 0.3 ml and the same coverslips were used for H₂O₂ and protein assays without removing the H₂O₂ assay solution. Coverslips were washed thoroughly by swirling in four successive beakers (200 ml each) of 0.9% NaCl at room temperature, drained on absorbent paper, and placed in 16-mm-diam wells containing 0.3 ml of Krebs-Ringer PBS with 5.5 mM glucose, from 5 to 35 nmol scopoletin, 0.3 purpurogallin unit of horseradish peroxidase, and 30 ng PMA in the presence (0–5 d after MNL preparation) or absence (6 d or later) of 1 mM NaN₃ to inhibit myeloperoxidase (13). As controls, cell-free coverslips exposed to complete medium were rinsed in the same manner and carried through the assay. The Costar trays were placed in a 37°C water bath for 60 min, the supernatant fluids transferred to a small glass culture tube (6 × 50 mm; Kimble Div., Owens-Illinois, Inc., Toledo, OH), and residual scopoletin fluorescence determined in a Perkin-Elmer-Hitachi MPF-4A or Spex Fluorolog F112A fluorometer at room temperature. The supernatant fluids were returned to the original wells and 30 μl of 1 N NaOH added to each well. After incubation for 30 min at 37°C, protein was measured by the method of Lowry et al. (20). Specific release (nmol H₂O₂/mg cell protein per 60 min) was calculated by microcomputer, with corrections for the protein content and fluorescence changes in the cell-free wells, as described (21).

Antitoxoplasma Activity. 1,000,000 RH strain *Toxoplasma gondii* trophozoites obtained from infected mouse peritoneal exudates (22) were added to each 35-mm dish for 30 min. Uningested organisms were removed by washing (time 0) and coverslips were then cultured in complete medium. At time 0 and at 20 h after infection, replicate coverslips were fixed, stained, and scored microscopically for number of toxoplasmas per vacuole and per 100 macrophages (22).

Radioiodination of rIFN-γ. rIFN-γ was labeled with ¹²⁵I as described (23) with modifications. The pH of rIFN-γ solution (25 μl, 1 mg/ml) was adjusted to 7.4 with 1 μl of 0.5 M potassium phosphate buffer, pH 7.4. The solution was mixed with 2 mCi of Na¹²⁵I (17 Ci/mmol, carrier-free; Amersham/Searle, Oakville, Ontario), transferred to an Iodogen (Pierce Chemical Co., Rockford, IL) -coated tube on ice, and mixed gently every 10 s. After 5 min at 4°C, the reaction was terminated by the addition of 20 μl of 1 M KI. The reaction mixture was layered on a Sephadex G-25 (fine) column (0.9 × 16 cm) in PBS containing 1% BSA, and ¹²⁵I-rIFN-γ was separated from free iodide. After chromatography and pooling of peak fractions (usually fraction 8–10, 250 μl/fraction), ~88% of the original protein was recovered. H₂O₂ release assay of all column fractions indicated there was no inactivation of rIFN-γ during the iodination. For nine ¹²⁵I-rIFN-γ preparations, specific activity ranged from 0.4–5.1 × 10⁷ cpm/μg protein (mean ± SE = [2.2 ± 0.5] × 10⁷ cpm/μg of protein). Approximately 10 molecules of I⁻ were incorporated per monomer of rIFN-γ (the rIFN-γ monomer contains four tyrosine residues). Iodinated rIFN-γ was stored at 4°C and could be used for at least 3 wk without significant loss of binding activity.

Assay for Binding of ¹²⁵I-rIFN-γ to Macrophages. All binding assays were performed using cells on glass coverslips in 24-well plastic plates at 4°C. Before assay, culture medium was replaced by binding buffer, consisting of RPMI 1640 medium, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mg/ml of BSA, and 50 mM HEPES, pH 7.2. Cells were incubated with or without competing ligand for 30 min at 4°C. Iodinated rIFN-γ was then added and incubation continued for 2 h at 4°C. Binding was maximal within this time. Coverslips were then washed in four successive beakers (200 ml each) of 0.9% NaCl at room temperature, drained on absorbent paper, and placed in the wells of a new 24-well tray containing 0.5 ml of PBS. The cells were scraped from the coverslips with a rubber policeman. Duplicate or triplicate suspensions were pooled, carefully layered over a cushion of PBS (3 ml) containing 5% sucrose, and centrifuged at 1,500 g for 10 min. The sucrose cushion was removed and the cell pellet was suspended in 200 μl of nuclear counting solution (0.1 M citric acid, 0.05% naphthol blue black [Buffalo black NBR] and 1% cetavlon, prepared as described [24]). After incubation for 30 min at room tempera-

TABLE I
Stimulation of H₂O₂ Release from Human Monocytes/Macrophages by IFN- α , - β , and - γ

Time of exposure to IFNs	IFN added	H ₂ O ₂ released at IFN concentrations (U/ml) of:		
		0	10	10 ⁵
<i>nmol/h/mg protein[‡]</i>				
Days 1-4*	None	364 ± 15 (6) [‡]		
	rIFN- γ		596 ± 26 (9)	699 ± 21 (9)
	rIFN- α A		361 ± 19 (9)	330 ± 55 (9)
	rIFN- α D		357 ± 15 (6)	325 ± 37 (6)
	nIFN- α		401 ± 29 (6)	330 ± 44 (6)
	rIFN- β		314 ± 24 (9)	319 ± 58 (9)
Days 8-11*	None	15 ± 3 (9)		
	rIFN- γ		50 ± 8 (9)	142 ± 31 (9)
	rIFN- α A		34 ± 7 (9)	82 ± 8 (9)
	rIFN- α D		19 ± 1 (6)	55 ± 5 (6)
	nIFN- α		16 ± 3 (6)	56 ± 3 (6)
	rIFN- β		19 ± 2 (9)	73 ± 10 (9)

* MNLs ($1-2 \times 10^6$ cells/cover slip) were plated and the adherent cells cultured as described in Materials and Methods. After 1 or 8 d, cells were exposed to the indicated concentrations of IFNs for an additional 3 d, washed, and stimulated with PMA to measure H₂O₂ release.

[‡] Mean ± SEM for the number of experiments in parentheses. Milligrams protein/cell averaged fourfold higher on day 11 than on day 4. Thus, H₂O₂ release by cells incubated with 10⁵ U/ml rIFN- γ was almost the same on days 4 and 11 when expressed on a per cell basis.

ture, the suspension was mixed in a pipette tip and transferred to a new plastic tube. Aliquots (7 μ l) were used for the determination of the number of nuclei and the remainder was counted in a gamma spectrometer. Specific binding of radiolabeled rIFN- γ was determined by subtracting the number of counts bound to cells in the presence of an 80-fold excess of nonlabeled rIFN- γ from the number of counts in the presence of iodinated rIFN- γ alone. Nonspecific binding of ¹²⁵I-rIFN- γ was <20% of total binding for either cell-bearing or cell-free coverslips. The percentage of multinucleated giant cells was <2% by day 8, the latest day in culture at which these assays were performed. Therefore, we used the number of nuclei per coverslip as the number of cells per coverslip. Binding activity was expressed as ng per 10⁶ cells.

Results

Stimulation of H₂O₂ Release from Human Macrophages by IFN- α or - β . With the modified methods of cell preparation described in Materials and Methods, macrophages resisted the dose-dependent rounding and detachment observed previously during incubation in IFN- α or - β (12). This permitted a reexamination of the ability of IFN- α or - β to activate mononuclear phagocytes at various stages of maturation. We shall refer to these stages as monocytes (≤ 4 d culture) and macrophages (≥ 5 d culture).

Natural IFN- α , rIFN- α A, - α D, and - β each stimulated H₂O₂-releasing capacity of macrophages in a dose-dependent manner at concentrations >10 U/ml (3 pM) (Table I and detailed dose-response curves not shown). However, even at 10³ U/ml (300 pM), the stimulated capacity (4.4 ± 1.6 -fold increase over medium

TABLE II
Induction of Antitoxoplasma Activity in Human Macrophages by rIFN- γ , - α A, and - β

IFN [‡]	U/ml	Fold increase* in toxoplasma				H ₂ O ₂ [§]
		Exp. 1	Exp. 2	Exp. 3	Mean \pm SD	
None	0	8.9	11.2	5.4	8.5 \pm 2.4	nmol/mg/h 7 \pm 5
rIFN- α A	10 ⁵	4.3	8.0	4.9	5.7 \pm 1.6	41 \pm 30
rIFN- β	10 ⁵	6.4	5.5	2.5	4.8 \pm 1.7	32 \pm 10
rIFN- γ	100	2.8	—	—	—	—
rIFN- γ	500	—	3.8	2.8	3.1 \pm 0.5	84 \pm 27

* Number of toxoplasma per 100 cells at 20 h divided by the number of toxoplasma per 100 cells immediately after infection (time 0).

[‡] Beginning on day 7 of culture, macrophages were incubated with the indicated cytokines for 3 d before being washed and challenged with toxoplasmas. The course of infection was followed in the absence of cytokines for the next 20 h.

[§] Mean \pm SEM for three experiments, each in triplicate.

control) was less than that (8.4 \pm 4.8-fold increase, mean \pm SD for three experiments) obtained with rIFN- γ (100 U/ml, 100 pM). When macrophages were exposed to 10⁵ U/ml of IFN- α or - β for 30 min on day 8 of culture, extensively washed to remove IFN, and then cultured in standard medium for an additional 3 d, their peroxide-releasing capacity was as great as that of cells exposed continuously to 10⁵ U/ml of IFN- α or - β for the entire 3-d period (data not shown). This suggested that the stimulatory effects of IFN- α or - β on mature macrophages were receptor mediated.

rIFN- α A (10⁵ U/ml) and rIFN- β (10⁵ U/ml) induced detectable but modest antitoxoplasma activity in mature macrophages. rIFN- γ (100–500 U/ml) afforded more pronounced macrophage activation by the same criterion. There was a rough correlation between the increase in H₂O₂-releasing capacity and induction of antitoxoplasma activity by these agents (Table II).

In contrast, at the stage of monocytes, 10⁵ U/ml of IFN- α or - β failed to increase the specific activity of H₂O₂-releasing capacity (Table I), and instead decreased by 20–40% the amount of protein adherent to coverslips (not shown), suggesting toxicity, as previously noted (12).

The fivefold lower value of H₂O₂/mg protein seen in Table I for rIFN- γ -activated macrophages compared to rIFN- γ -activated monocytes reflected the approximately fourfold increase in protein/cell which accompanied maturation of monocytes into 11-d macrophages (13).

Suppressive Effects of IFN- α or - β on rIFN- γ Stimulated H₂O₂-releasing Capacity. For all subsequent studies, we compared monocytes (exposed to cytokines from days 1–4 of culture) to macrophages (treated from days 8–11). We first tested the effects of simultaneous exposure of the cells to rIFN- γ plus IFN- α or - β . As shown in Fig. 1, cells in the two stages of differentiation gave contrasting results. With monocytes (Fig. 1A), IFN- α or - β had almost no effect on rIFN- γ 's action, while with macrophages (Fig. 1B), low doses of IFN- α or - β (3 fM–3 pM) blocked induction of H₂O₂-releasing capacity by far higher concentrations of rIFN- γ (10 pM). The maximum percent inhibitions were 83.3 \pm 8.5 (mean \pm SD for seven experiments), 68.0 \pm 8.0 (two experiments), 71.0 \pm 3.0 (two

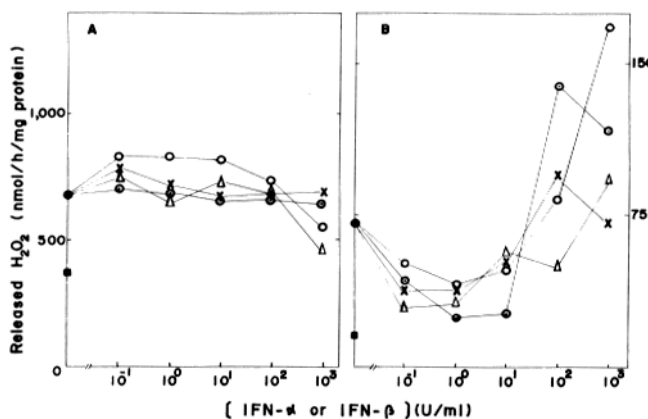


FIGURE 1. H_2O_2 release from human monocytes and macrophages exposed simultaneously to rIFN- γ and IFN- α or IFN- β . (A) Monocytes (1-d culture) or (B) macrophages (8-d culture) were exposed to various concentrations of IFN- α or - β with 10 U/ml of rIFN- γ . 3 d later, the cells were washed and stimulated with PMA to measure H_2O_2 release. Each point represents the mean value for six experiments. (■), Medium control; (●) rIFN- γ (10 U/ml) alone; (○) rIFN- γ (10 U/ml) + rIFN- α A; (△) rIFN- γ (10 U/ml) +

nIFN- α ; (×) rIFN- γ (10 U/ml) + rIFN- α D; (○) rIFN- γ (10 U/ml) + rIFN- β .

experiments), and 63.0 ± 11.0 (three experiments) with 0.18 pM of rIFN- α A, 0.17 pM of nIFN- α , 0.30 pM of rIFN- α D, and 0.12 pM of rIFN- β , respectively. At >3 pM (~ 10 U/ml), IFN- α or - β were no longer suppressive. Rather, H_2O_2 -releasing capacity appeared to equal or exceed the level obtained with rIFN- γ alone. Moreover, IFN- α or - β (3 fM–300 pM) could not inhibit the stimulatory effect of 1 nM (10^3 U/ml) rIFN- γ (not shown).

Antibodies against IFN- α or - β inhibited the nIFN- α - or rIFN- β -stimulated H_2O_2 -releasing capacity of macrophages by 78 and 85%, respectively, while these antibodies had almost no effect (-11.8 and 5.7% differences, respectively) on rIFN- γ 's action. Moreover, antibody specific for IFN- α markedly reduced the ability of low-dose (3 fM–3 pM, 0.01–10 U/ml) nIFN- α to inhibit the action of rIFN- γ , as well as the ability of high dose (≥ 3 pM) nIFN- α to augment the action of rIFN- γ . Similar results were seen with rIFN- β . Thus, both the antagonist and agonist actions of these IFN preparations appeared to be due to the IFNs themselves and not to contaminants (data not shown).

Time Course of IFN- α - or - β -mediated Inhibition of rIFN- γ -stimulated H_2O_2 -releasing Capacity. As a first step in determining the mechanisms of IFN- α - or - β -mediated suppression of rIFN- γ 's action, time course experiments were carried out. As shown in Fig. 2A, macrophages that were treated simultaneously with rIFN- α A (3 fM–3 pM) and rIFN- γ (10 pM) from days 8–11 of culture failed to show the full increase in H_2O_2 release induced by rIFN- γ alone. In contrast, the addition of rIFN- α A 6 h after the addition of rIFN- γ did not reduce rIFN- γ 's action (Fig. 2B). When macrophages had a 6-h preexposure to rIFN- α A and an additional 66-h exposure to rIFN- γ , rIFN- γ -stimulated H_2O_2 release was suppressed (Fig. 2C). Results were similar with rIFN- β (not shown). These results suggested that the inhibitory effect of IFN- α or - β on rIFN- γ -stimulated H_2O_2 -releasing capacity might involve interference with binding of rIFN- γ to its plasma membrane receptors.

Characterization of IFN- γ Receptors. To characterize rIFN- γ -Rs, we prepared 125 I-rIFN- γ with high specific activity and full bioactivity (see Materials and Methods). Mature macrophages (day 8) incubated at 4°C for 2 h in the presence of increasing concentrations of 125 I-rIFN- γ yielded specific saturation isotherms,

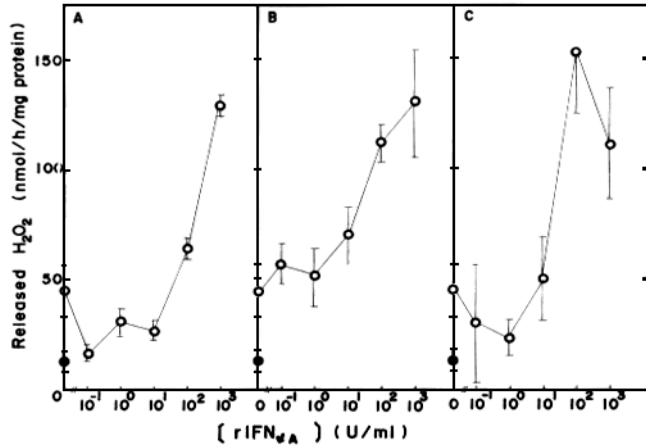


FIGURE 2. Time course of IFN- α -mediated inhibition of rIFN- γ -stimulated H_2O_2 -releasing capacity. (A) Macrophages (8-d culture) were incubated with rIFN- γ (10 U/ml) and various concentrations of rIFN- α . (B) Cells on day 8 were exposed to 10 U/ml of rIFN- γ . 6 h later, various concentrations of rIFN- α were added. (C) Cells were incubated on day 8 with various concentrations of rIFN- α for 6 h, and then 10 U/ml of rIFN- γ was added. In each case, H_2O_2 release was measured 3 d later. Each value represents the mean \pm SD of triplicates. (●) Medium control (no IFNs).

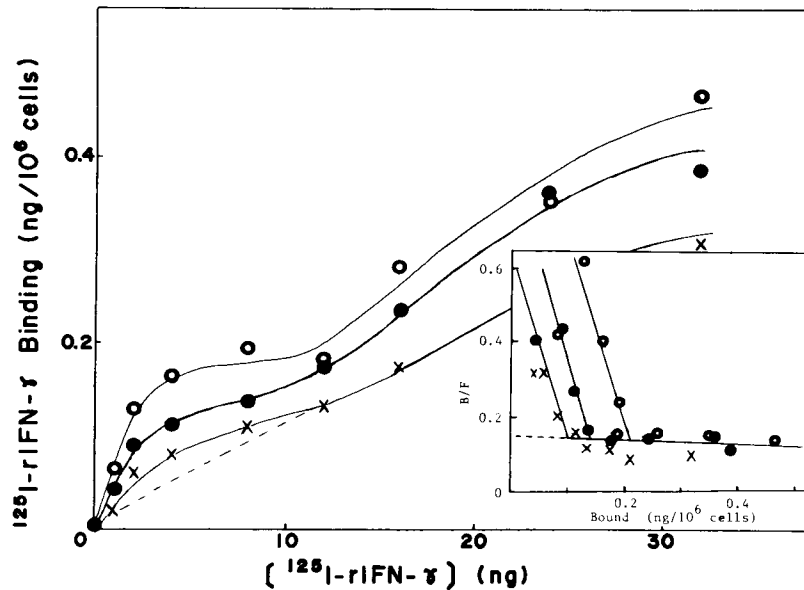


FIGURE 3. Binding of ^{125}I -rIFN- γ to human macrophages. Macrophages ($2-5 \times 10^5$ cells/coverlip) were incubated with increasing concentrations of ^{125}I -rIFN- γ at $4^\circ C$ for 2 h. Free radioactivity was separated from cell-bound radioactivity as described in Materials and Methods. Each point represents the mean of duplicate determinations from one or two experiments. (Inset) Scatchard plots of the binding data.

illustrated for one experiment in Fig. 3. Scatchard analysis gave biphasic curves (Fig. 3, inset). In three such experiments, K_d values of the two classes of rIFN- γ -Rs averaged $(4.33 \pm 0.33) \times 10^{-10}$ M and $(6.40 \pm 1.13) \times 10^{-9}$ M (mean \pm SD), and binding sites per cell averaged $3,859 \pm 391$ and $25,110 \pm 1,513$, respectively. In contrast, monocytes (day 1) and early macrophages (day 5 or 6) displayed a single class of rIFN- γ -Rs (Table III).

TABLE III
 Characteristics of Receptors for rIFN- γ on Human Monocytes and Macrophages

Parameter	Monocytes	Macrophages
K_d	1.0-1.1 $\times 10^{-9}$ M*	4.3 \pm 0.3 $\times 10^{-10}$ M (class 1) [†] 6.4 \pm 1.1 $\times 10^{-9}$ M (class 2) [†]
Sites/cell	1.3-2.5 $\times 10^{-10}$ M (reference 15) 6.8 $\times 10^{-9}$ M (reference 16) 2.0 \pm 0.5 $\times 10^{-9}$ M (reference 17) 1,324-1,850* 4,000 (reference 15) 2,400 (reference 16) 7,900 \pm 400 (reference 17)	2.0 \pm 0.3 $\times 10^{-9}$ M (reference 17) ~2,000 (class 1) [†] ~23,000 (class 2) [†] 7,800 \pm 300 (reference 17)

* Our preliminary data.
 † This paper.

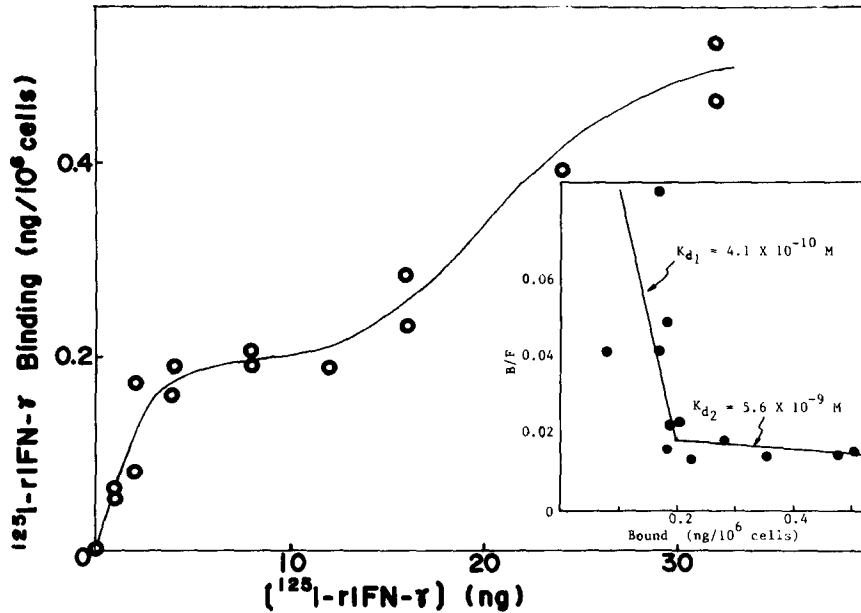


FIGURE 4. Displacement of ^{125}I -rIFN- γ from high-affinity receptors by nIFN- α . 1 U or 10^3 U/0.25 ml of nIFN- α was added together with various concentrations of ^{125}I -rIFN- γ . The cell-associated radioactivity was measured after a 2-h incubation at 4°C. Each point represents the mean of duplicate determinations. (Inset) Scatchard plots of the binding data. (O) Without nIFN- α ; (●) with 1 U of nIFN- α ; (x) with 10^3 U of nIFN- α ; (---) predicted results if all high-affinity binding sites for rIFN- γ were blocked.

Competitive Displacement of ^{125}I -rIFN- γ by Recombinant and Natural IFN- α or - β . In an attempt to examine whether IFN- α or - β could interfere with binding of rIFN- γ to one or both types of IFN- γ -Rs on macrophages, the effect of nIFN- α (1 or 10^3 U) on ^{125}I -rIFN- γ binding was determined as a function of ^{125}I -rIFN- γ concentration. As shown in Fig. 4 (inset), simultaneous addition of nIFN- α with ^{125}I -rIFN- γ caused a dose-dependent reduction in the apparent number of high-affinity receptor sites. The K_d value for high-affinity sites did not change. The approximate number (~2,200 sites/cell) of high affinity receptor sites for rIFN- γ , which were dissociable by nIFN- α , was obtained by calculating the number of

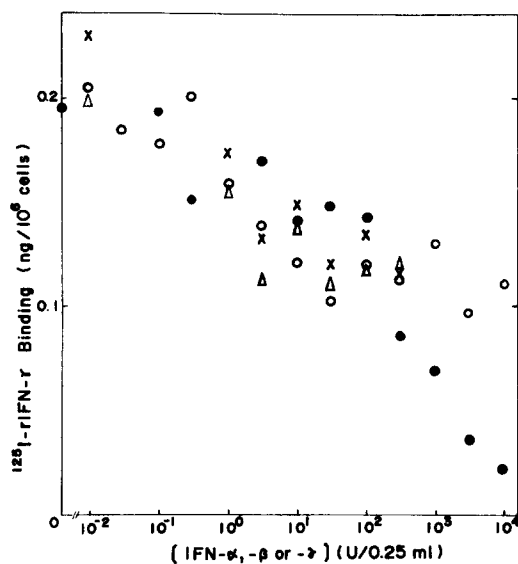


FIGURE 5. Binding site competition between ^{125}I -rIFN- γ and unlabeled nIFN- α , rIFN- α A, rIFN- β , or rIFN- γ . Macrophages ($\sim 3.5 \times 10^5$ cells/cover slip) were incubated with ^{125}I -rIFN- γ (4 ng, ~ 120 U) and various concentrations of unlabeled IFNs for 2 h at 4°C . Data points are means of triplicates for specific binding. (●) ^{125}I -rIFN- γ + rIFN- γ ; (○) ^{125}I -rIFN- γ + nIFN- α ; (Δ) ^{125}I -rIFN- γ + rIFN- α A; (x) ^{125}I -rIFN- γ + rIFN- β .

binding sites in the absence of nIFN- α minus the number of binding sites in the presence of nIFN- α (10^3 U).

In contrast, nIFN- α was all but incapable of competing with ^{125}I -rIFN- γ for binding to the low-affinity receptors (Fig. 4). These results are in line with the data (Fig. 1B) regarding the effect of IFN- α or - β on rIFN- γ -stimulated H_2O_2 release; the stimulatory effect of 10 pM rIFN- γ was inhibited by simultaneous addition of IFN- α or - β (3 fM–300 pM), while IFN- α or - β (3 fM–300 pM) could not inhibit the enhancement of H_2O_2 -releasing capacity stimulated by 1 nM rIFN- γ .

The ^{125}I -rIFN- γ specifically bound in the presence or absence of competitor was plotted as a function of concentration for each competing IFN (Fig. 5). In these experiments, 4 ng (~ 0.5 nM, ~ 120 U/0.25 ml) of ^{125}I -rIFN- γ was used, since at this concentration, binding to its high-affinity receptors reached a plateau (Fig. 4). In a dose-dependent manner, unlabeled rIFN- γ inhibited the binding of ^{125}I -rIFN- γ , and at 333 ng (~ 40 nM, $\sim 10^4$ U/0.25 ml), binding was almost completely suppressed. In contrast, although nIFN- α , rIFN- α A, and rIFN- β inhibited the binding of ^{125}I -rIFN- γ (4 ng, ~ 0.5 nM) in a dose-dependent manner, the maximum inhibition ($\sim 50\%$) was obtained with ~ 100 pg (~ 20 pM) of IFN- α or - β . Even at 50 ng (10 nM), IFN- α or - β suppressed ^{125}I -rIFN- γ binding by only 40–50%. These findings are consistent with the concept that IFN- α or - β inhibited the binding of rIFN- γ to its high-affinity sites but not to its low-affinity sites, while rIFN- γ competed with ^{125}I -rIFN- γ for both high- and low-affinity sites.

The above results indicated that IFN- α or - β interferes with binding of rIFN- γ to its high-affinity receptor on mature macrophages. This could be due to binding of IFN- α or - β in such a way as to sterically hinder binding of rIFN- γ , for example, by occupying the same receptor. Alternatively, IFN- α or - β might exert a metabolic effect on macrophages that leads to a decrease in the number of high-affinity rIFN- γ binding sites displayed on the cell surface, such as by

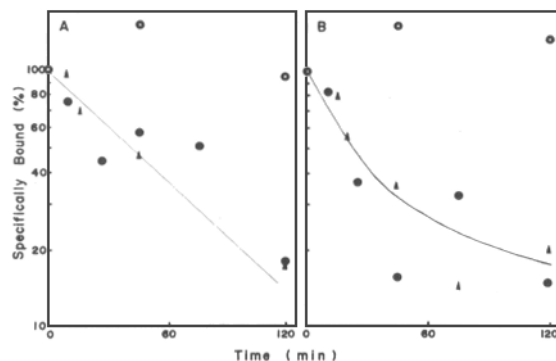


FIGURE 6. Effects of nIFN- α and rIFN- γ on ^{125}I -rIFN- γ binding to human macrophages. Macrophages ($\sim 3.5 \times 10^5$ cells/coverslip) were incubated with ^{125}I -rIFN- γ (4 ng) for 90 min at 4°C. The cells were then further incubated at 4°C in the presence (● and Δ [two different experiments]) or absence (○) of (A) nIFN- α (50 ng) and (B) rIFN- γ (300 ng). After the time shown, the amounts of ^{125}I -rIFN- γ specifically remaining bound were determined. Each point represents the mean of duplicate determinations.

promoting internalization or shedding. The latter possibility seemed remote, since the antagonism of IFN- α or - β on rIFN- γ binding was manifest rapidly and at 4°C. Nevertheless, to distinguish between a direct and indirect effect of the competing IFNs, we tested whether they could displace ^{125}I -rIFN- γ that had already been bound. The binding reaction was allowed to reach equilibrium for 90 min at 4°C. 10^3 U of nIFN- α or 10^4 U of rIFN- γ were then added without significant dilution of the incubation mixture, and the amount of ^{125}I -rIFN- γ specifically remaining bound was determined as a function of time (Fig. 6). In the case of nIFN- α , dissociation of ^{125}I -rIFN- γ from macrophages followed simple monoexponential first order kinetics with a $t_{1/2} \sim 40$ min and a dissociation rate constant (k_{-1}) of $\sim 0.017 \text{ min}^{-1}$. The association rate constant (k_1) was $\sim 3.7 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$ at 4°C, when k_{-1} was used for the calculation, as described (25). The $K_d (= k_{-1}/k_1)$ was calculated to be $\sim 4.7 \times 10^{-10} \text{ M}$. In contrast, dissociation of ^{125}I -rIFN- γ from macrophages after the addition of unlabeled rIFN- γ showed a deviation from linearity on a semilogarithmic plot. Approximately 50% of the bound ^{125}I -rIFN- γ dissociated within 30 min. The rest of the binding required >120 min for complete dissociation. Upon fitting two lines to the data, a fast rate with a $t_{1/2}$ of ~ 25 min and k'_{-1} of $\sim 0.028 \text{ min}^{-1}$, and a slow rate with a $t_{1/2}$ of ~ 180 min and k''_{-1} of 0.0038 , were observed. The association rate constants (k'_1 and k''_1) were $\sim 4.3 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ and $\sim 5.5 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$, respectively. The K_d values were calculated to be $\sim 6.4 \times 10^{-9} \text{ M}$ for k'_{-1} and $\sim 7.1 \times 10^{-10} \text{ M}$ for k''_{-1} , respectively. These K_d values were close to those ($4.3 \times 10^{-10} \text{ M}$ for the high-affinity receptor and $\sim 6.4 \times 10^{-9} \text{ M}$ for the low-affinity receptor) obtained from the Scatchard analysis (Fig. 3).

Discussion

These findings make four new points. First, in contrast to their toxic effects on monocytes, IFN- α or - β in high (submicromolar) concentrations enhanced the H_2O_2 -releasing capacity and antiprotozoal activity of human macrophages. Second, the ability of low (picomolar) concentrations of rIFN- γ to enhance macrophage H_2O_2 -releasing capacity was inhibited by even lower (femtomolar) concentrations of IFN- α or - β . Third, two classes of receptors for rIFN- γ emerged after monocytes differentiated into macrophages in vitro. Fourth, and most surprising, the suppressive effect of IFN- α or - β on rIFN- γ -enhanced H_2O_2 -releasing

capacity could be attributed to displacement of rIFN- γ from its high-affinity receptors by nonstimulatory binding of IFN- α or - β to the same sites. Thus, the differentiated human macrophage displays a biphasic response to IFN- α or - β : antagonism of IFN- γ -mediated activation at low doses, and an agonist action at higher doses.

It has been repeatedly demonstrated that IFN- γ binds to a receptor different from that for IFN- α and - β (25–27), although several groups reported some crossreactivity between IFN- β and the binding site for IFN- γ (28–32). The presence of two kinds of receptors for human IFN- α (29) or - β (28) has been suggested. Recently, Aiyer et al. (33) observed a curvilinear Scatchard plot for binding of ^{125}I -murine IFN- γ to WEHI-3 cells. Although binding was nonsaturable, the authors inferred the existence of two classes of IFN- γ receptors on this cell line. Finbloom et al. (15) reported binding curves of ^{125}I -human rIFN- γ to U937 and HL60 cells similar to those of Aiyer et al., but interpreted the results as showing negative cooperative interaction between receptors of a single class. To our knowledge, there has heretofore been no clearcut evidence for two classes of receptors for IFN- γ , nor any such evidence with primary macrophages.

Table III summarizes published binding studies using rIFN- γ and human mononuclear phagocytes. Methodologic differences may account for the 52-fold range in K_d s and sixfold span in the number of binding sites reported on monocytes. Nonetheless, four laboratories concur in describing a single class of IFN- γ -Rs at this stage in the cell's maturation; our results for the K_d fall in the middle of the reported range. Our limited results for early macrophages (days 4 and 6) agree closely with the one other report using cells at this stage (17). To our knowledge, data with more mature macrophages have not previously been provided. Our findings suggest that a dramatic change occurs between days 6 and 8 of culture, resulting in the emergence of a new receptor, a high-affinity site shared by all three classes of IFNs. Competition binding experiments suggest that the high-affinity site has a higher affinity for IFN- α and - β than for IFN- γ .

The results described above imply that two classes of binding sites for IFN- α should also be detectable on mature human macrophages. Preliminary experiments using radioiodinated rIFN- α A are consistent with this prediction; binding sites for rIFN- α A on day-11 macrophages appeared to have K_{d1} $\sim 6.7 \times 10^{-12}$ M and K_{d2} $\sim 3.5 \times 10^{-10}$ M. Moreover, binding of ^{125}I -rIFN α A (0–0.5 ng/0.25 ml) to the high-affinity sites was almost completely suppressed by addition of unlabeled rIFN- γ (200 ng/0.25 ml) (data not shown).

When the antagonism between IFNs demonstrated in this study is described in terms of molecules per macrophage, the remarkable efficiency of IFN- α and - β at blocking the binding of rIFN- γ to its high-affinity receptors becomes apparent. 50% inhibition of binding of rIFN- γ to $\sim 2,000$ high-affinity sites per macrophage required an input of ~ 900 molecules per cell (~ 10 pg per coverslip) of IFN- α or - β (Fig. 5). Given that one term in these calculations is the specific activity of the IFN preparations, which has an accuracy of a factor of 2, it can be estimated that 50–100% of the added IFN- α or - β was bound, while in the absence of IFN- α or - β , only $\sim 0.8\%$ of input rIFN- γ was bound under the same conditions. Therefore, the high-affinity sites on mature human macrophages appear to have an ~ 100 -fold higher affinity for IFN- α or - β than for rIFN- γ .

Yet, binding of IFN- α or - β to these sites elicited no response by the criteria used in this study. Of course, such binding may have other biologic effects. The basis of the crossreactive binding may lie in the structural similarities between IFN- α , - β , and - γ described by DeGrado et al. (34).

The capacity of low-affinity IFN- α and - β Rs to signal an agonist response, and the emergence of high-affinity binding sites for IFN- α or - β mediating an antagonist response to simultaneously added IFN- γ , should be considered in conjunction with the possible production of small amounts of IFN- α or - β by unstimulated macrophages (35, 36) and large amounts by stimulated cells (37). Thus, depending on how much IFN- α or - β they make, macrophages could either interfere with or promote their own activation. For example, there is a gradual rightward shift in the rIFN- γ dose-response curve for induction of H₂O₂-releasing capacity as macrophages progress in culture to day 8; this shift can be forestalled by addition of neutralizing antibody for IFN- α (data not shown).

Speculation on the possible physiologic import of these findings must keep in mind the resistance of monocytes and young macrophages to the IFN- γ -antagonizing action of low-dose IFN- α and - β , the ability of moderate concentrations of rIFN- γ ($\sim 10^3$ U/ml, ~ 1 nM) to overcome this antagonism, and the agonist rather than antagonist effects of higher concentrations of IFN- α or - β . Thus, the phenomenon of antagonism is unlikely to impinge on immigrant monocytes or inflammatory macrophages (which are young cells), or on resident tissue macrophages at the site of an immune response (where the concentration of IFNs is likely to be high). However, trace amounts of IFN- α or - β produced by stromal cells, parenchymal cells, and resident macrophages, perhaps constitutively, could be expected to damp the response of mature tissue macrophages to 1–10 U/ml IFN- γ . This may serve to prevent oxidant- or protease-mediated tissue damage by resident macrophages when low levels of IFN- γ circulate or diffuse into uninvolved tissues from sites of an immune response. Thus, the interaction of cytokines described here may function in the regional containment of macrophage activation.

Summary

H₂O₂-releasing capacity and limited antitoxoplasma activity could be induced in human macrophages (derived from monocytes cultured ≥ 5 d) but not in monocytes themselves (cells cultured ≤ 4 d) by a further 3-d incubation with pure natural or rIFN- α or - β . More than 3 pM (10 U/ml) of these IFNs was required, with greatest effects at ~ 300 pM (10^3 U/ml). At 300 pM, H₂O₂-releasing capacity was enhanced 4.4 ± 1.6 -fold over medium control (mean \pm SD for natural IFN- α , rIFN- α A, rIFN- α D, and rIFN- β) compared to an 8.4 ± 4.8 -fold increase with rIFN- γ (100 pM, 100 U/ml) in the same experiments. Unexpectedly, low concentrations of IFN- α or - β (3 fM–300 pM) blocked induction of H₂O₂-releasing capacity by rIFN- γ (10 pM), with a 50% inhibitory dose of ~ 80 fM. However, IFN- α or - β (3 fM–300 pM) could not inhibit the effect of higher concentrations of rIFN- γ (1 nM). In contrast to results with monocytes or young macrophages, Scatchard plots of binding of ¹²⁵I-rIFN- γ to mature macrophages (day 8 of culture) indicated two classes of binding sites: $\sim 2,000$ high-affinity sites

($K_d \sim 0.43$ nM) and $\sim 23,000$ low-affinity sites ($K_d \sim 6.4$ nM) per cell. Binding of ^{125}I -rIFN- γ to the high- but not the low-affinity sites was blocked by simultaneously added IFN- α or - β , with a 50% inhibitory dose of ~ 2 U/0.25 ml (~ 2 pM), or reversed by subsequently added IFN- α or - β .

Thus, differentiation of human mononuclear phagocytes in vitro is accompanied by the emergence of (a) an agonist response to submicromolar concentrations of IFN- α or - β , (b) antagonism of the effect of picomolar IFN- γ by femtomolar IFN- α or - β , (c) two classes of IFN- γ -Rs, and (d) nonstimulatory binding of IFN- α or - β to the high- but not the low-affinity IFN- γ -Rs, with higher affinity than rIFN- γ itself. We speculate that traces of IFN- α or - β derived from stromal cells, parenchymal cells, or resident macrophages may dampen the activation of mature tissue macrophages by the small amounts of IFN- γ that diffuse from inflammatory sites into normal tissues. Such a mechanism could constrain the potentially destructive phenomenon of macrophage activation to areas where monocytes have recently immigrated and/or the concentration of IFNs is high.

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