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Research Article

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Demonstration and Partial Characterization of the Interferon-Gamma Receptor on Human Mononuclear Phagocytes

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Abstract

Radioiodinated recombinant human interferon-gamma (IFNγ) bound to human monocytes, U937, and HL60 cells in a specific, saturable, and reversible manner. At 4°C, the different cell types bound 3,000–7,000 molecules of IFNγ, and binding was of comparable affinity ($K_a = 4$–12×10^8 M^{-1}). No change in the receptor was observed after monocytes differentiated to macrophages or when the cell lines were pharmacologically induced to differentiate. The functional relevance of the receptor was validated by the demonstration that receptor occupancy correlated with induction of Fc receptors on U937. Binding studies using U937 permeabilized with digitonin showed that only 46% of the total receptor pool was expressed at the cell surface. The receptor appears to be a protein, since treatment of U937 with trypsin or pronase reduced ^{125}I-IFNγ binding by 87 and 95%, respectively. At 37°C, ligand was internalized, since 32% of the cell-associated IFNγ became resistant to trypsin stripping. Monocytes degraded ^{125}I-IFNγ into trichloroacetic acid-soluble counts at 37°C but not at 4°C, at an approximate rate of 5,000 molecules/cell per h. The receptor was partially characterized by SDS-polyacrylamide gel electrophoresis analysis of purified U937 membranes that had been incubated with ^{125}I-IFNγ. After cross-linking, the receptor-ligand complex migrated as a broad band that displayed an M_r of 104,000±18,000 at the top and 84,000±6,000 at the bottom. These results thereby define and partially characterize the IFNγ receptor of human mononuclear phagocytes.

Introduction

Macrophage activation has been defined as a series of functional and biochemical modifications in macrophage populations (1–4) that are induced by a T cell-derived lymphokine known as macrophage activating factor (MAF).[1] Previous reports from

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1. *Abbreviations used in this paper:* DMEM, modified Eagle's medium; DMS, dimethyl suberimidate 2HCl; DMSO, dimethylsulfoxide; DSS, disuccinimydyl suberate; EGS, ethylene glycol-*bis* (succinimidyl succinate); FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HPLC, high performance liquid chromatography; HSA, human serum albumin; IFN, interferon; IRU, international reference units; K_a, association equilibrium constant; KIU, kallikrein inhibitory units; KPBS, DMEM with

several laboratories, including our own, have shown that gamma-interferon (IFNγ) can activate mononuclear phagocytes and may well represent the major type of MAF produced by physiological T cell populations (reviewed in references 5 and 6).

These observations have prompted attempts to elucidate the biochemical basis for IFNγ-dependent cellular responses. Recently, we demonstrated the presence of a specific receptor for IFNγ on murine macrophages by its ability to bind ligand in a saturable, reversible, and specific manner (7). We have also shown that receptor occupation is a prerequisite for induction of macrophage tumoricidal activity, Ia antigen expression, or enhancement of intracellular microbicidal activity (7, 8), biological activities that are all characteristic of activated macrophages.

In the current communication, we wish to extend these observations to the IFNγ receptor of human mononuclear phagocytes. In addition, we report the partial physicochemical characterization of the human monocyte IFNγ receptor and describe the ultimate fate of the ligand after receptor binding.

Methods

Chemicals, media, and reagents. Media, supplements, and buffers used in these experiments were purchased or prepared as previously described (7, 9), and were determined to contain <10 pg/ml of endotoxin using the *Limulus amebocyte* lysate assay (Sigma Chemical Co., St. Louis, MO). Reagents used specifically for this study were purchased from the following sources: phenylmethylsulfonyl fluoride (PMSF), leupeptin, 4β-phorbol-12β myristate-13α acetate (PMA), dimethylsulfoxide (DMSO), ATP, and aprotinin were obtained from Sigma Chemical Co.; disuccinimydyl suberate (DSS), ethylene glycol-*bis* (succinimidyl succinate) (EGS) and dimethyl suberimidate 2 HCl (DMS) were purchased from Pierce Chemical Co., Rockford, IL.

Interferons. Natural human IFNγ was induced from partially purified human peripheral blood lymphocytes essentially as described by Blalock et al. (10). Lymphocytes at 6×10^6 cells/ml in RPMI medium containing 1% fetal calf serum (FCS) were stimulated with 1 μg/ml staphylococcal enterotoxin B (SEB, Sigma Chemical Co.) for 24 h. The resulting culture supernatants were cleared of cell debris by centrifugation and filtration and stored at −20°C. This preparation had a specific activity of 1.1 × 10⁴ international reference units (IRU)/mg.

Escherichia coli-derived recombinant human IFNγ was produced as previously described (11) and generously provided by Genentech, Inc. (San Francisco, CA). Two preparations (lots D0002SF and E9026A) were used that had been purified to specific antiviral activity of 6.8 and 2 × 10⁷ IRU/mg, respectively. IFNγ was stored at 4°C in phosphate-buffered saline (PBS), pH 6.5.

Human alpha and beta interferons were purchased from Lee Biomolecular Laboratories, San Diego, CA. The unpurified preparations had

potassium-PBS; MAF, macrophage activating factor; NP40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonyl fluoride; SEB, staphylococcal enterotoxin B; WGA, wheat germ agglutinin.

specific activities of 2.1×10^5 IRU/mg for alpha interferon and 1×10^5 IRU/mg for beta interferon.

Preparation of radioiodinated IFNγ. Purified recombinant human IFNγ (57 µg) was labeled with ^{125}I Bolton-Hunter reagent (1 mCi) (ICN Chemicals, Radioisotope Division, Irvine, CA) for 2 h at 4°C as described (7). Protein associated and free ^{125}I were separated by centrifugation through Biogel P6. Typical preparations displayed specific activities of 8.3 µCi/µg (13 µCi/10^6 IRU). ^{125}I-IFNγ was further purified by gel filtration on a high performance liquid chromatography (HPLC) system using a 7.5×300-mm Biosil TSK 125 column (Bio-Rad Laboratories, Richmond, CA) (7). Fractions were collected in tubes containing $^1/_{10}$ vol of heat-inactivated FCS. IFNγ-specific activities were determined either with a cytopathic effect assay, which used purified vesicular stomatitis virus, human WISH cells, and the National Institutes of Health human IFNγ standard, or by IFNγ-specific radioimmunoassay (Centocor, Inc., Malvern, PA). Values obtained with the two assays were in close agreement. Radioiodinated IFNγ was stored at 4°C and retained biological activity for at least 2 wk.

Monoclonal antibodies. Monoclonal antibodies to recombinant human IFNγ (HGI/M6 and HGI/M125) were produced by fusion of immune murine splenocytes with the hypoxanthine, aminoplerin, and thymidine (HAT)-sensitive murine myeloma cell line P3 × 63-Ag8.653 at a splenocyte/myeloma ratio of 10:1 using a previously described fusion protocol (8). The resulting hybridomas were cloned three times by limiting dilution. Specificity was confirmed by enzyme-linked immunosorbent assay and radioimmunoassays that used recombinant human IFNγ as antigen. In functional assays, the antibodies were found to neutralize the antiviral activity of natural human IFNγ. Purified M6 and M125 were prepared by protein-A chromatography of clone culture supernatants. M6 and M125 are IgG$_1$, κ, type antibodies. Monoclonal antibody 543 is specific for the human C3b receptor and was produced as described elsewhere (12). Monoclonal anti-CR3 α-chain (OKM 10) and β-chain (60.3) were obtained from Ortho Diagnostics, (Raritan, NJ) and from Dr. Patrick Beatty (Fred Hutchinson Cancer Research Center, Seattle, WA), respectively. Hamster monoclonal antibodies against murine IFNγ (H22) were prepared as described previously (8).

Monocytes. Human monocytes were purified by Ficoll-Hypaque centrifugation and centrifugal elutriation. As starting material, mononuclear cell-enriched leukocyte concentrates were used that were obtained from normal donors undergoing lymphocytapheresis on a continuous flow centrifugation machine (model 2297; IBM, Princeton, NJ). 80 ml of concentrate (containing $\sim 10^8$ leukocytes) was diluted 1:1 with sterile 0.9% sodium chloride and 4 ml layered into tubes containing 10 ml of Lymphopaque (Nyegaard and Co., Oslo, Norway). After centrifugation for 20 min at 23°C at 800 g, the mononuclear cells were collected, washed, and resuspended in 10 ml of elutriation medium (DMEM) with potassium-PBS (KPBS) containing 0.1 M NaCl, 0.002 M KCl, 0.01 M KH$_2$PO$_4$, 0.008 M Na$_2$HPO$_4$, and 250 mg/ml U. S. Pharmacopeia sterile human serum albumin (HSA) (Armour Pharmaceutical Co., Kankakel, IL) (DMEM-KPBS-HSA). The cells were injected into the elutriation chamber of an elutriation system (JE-6B Elutriation System, Beckman Instruments, Inc., Palo Alto, CA) and introduced into the separation chamber with 125 ml DMEM-KPBS-HSA buffer at a flow rate of 5 ml/min and a rotor speed of 2,000 rpm. Thereafter, two 100-ml fractions were collected at flow rates of 13 and 15 ml/min, and 11 50-ml fractions were collected at flow rates of 16.0, 16.5, 17.0, 17.5, 18.0, 18.5, 19.0, 19.5, 20.0, 21.0, and 22.0 ml/min. Monocyte-containing fractions (6-13) were combined and collected by centrifugation at 400 g for 10 min. The resulting cell preparation contained >95% monocytes as assessed by cytocentrifugation (Cytospin 2, Shandon Instruments, Sewickley, PA) and Giemsa-peroxidase staining (13) and displayed 99% viability.

Cultured cell lines. The human monocytic cell line U937 (14) and the promyelocytic cell line HL60 (15) were maintained in RPMI supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, 1 mM sodium pyruvate, 0.075% (wt/vol) sodium bicarbonate, and 10% heat-inactivated FCS.

Membrane preparations. U937 cells were lysed by nitrogen cavitation and the plasma membranes purified in sucrose gradients using a modi-

fication of the method described by Jesaitis et al. (16, 17). Briefly, U937 cells (2×10^9) were washed and then suspended in 20 ml of homogenization buffer (10 mM Hepes, 0.34 M sucrose, 0.1 mM MgCl$_2$, 10 mM EDTA, 1 mM dithiothreitol, 250 mM ATP, and 23 kallikrein inhibitory units (KIU)/ml aprotinin) in a 50-ml polyethylene tube containing a cylindrical stir bar. The cell mixture was placed in a Parr cell disruption bomb (Parr Instruments, Moline, IL) that had been precooled to 0°C. The stirred cell mixture was equilibrated for 15 min at 600 psi N$_2$ at 0°C. After cavitation, 90% of the starting volume was recovered and the lysate was immediately centrifuged at 1,000 g for 5 min. The foam residue, which floated on top of the supernatant from this centrifugation, was collected and homogenized with buffer in a Dounce homogenizer and combined with the first low-speed supernatant. The mixture was centrifuged again for 5 min at 1,000 g. The supernatant of the second centrifugation was collected and layered into tubes containing sucrose gradients prepared with Hepes buffer. The gradients consisted of a 3-ml cushion of 60% sucrose and a 22-ml linear gradient ranging from 55 to 20% sucrose. The samples were centrifuged in a Sorval TV 850 vertical rotor (DuPont Co., Newton, CT) at 45,000 rpm (163,000 g) for 1 h at 4°C, and 1.5-ml fractions were collected. Fractions were frozen in liquid nitrogen and stored at −80°C. In some experiments, U937 were surface labeled using ^{125}I-wheat germ agglutinin (WGA) according to the procedure described by Jesaitis et al. (17).

Binding studies with ^{125}I-recombinant IFNγ. The method used to quantitate cellular binding of IFNγ has been described in detail elsewhere (7). In brief, cells were incubated at 4°C for 2 h with varying amounts of radiolabeled human IFNγ. Free and cell-associated ^{125}I-IFNγ were separated by centrifugation through pthalate oil. Specific binding was defined as the difference between total binding and the binding that occurred in the presence of a 100 to 1000-fold excess of unlabeled IFNγ (nonspecific binding).

To study binding of IFNγ to isolated cell membranes, 200 µg of purified U937 membranes were incubated for 1 h with varying amounts of ^{125}I-IFNγ in a total volume of 100 µl in RPMI-1640 containing 5% FCS and 1% Nonidet P-40 (NP40) (Sigma Chemical Co.). Free and membrane-bound IFNγ were separated by ultracentrifugation at 4°C in a L8-70 ultracentrifuge (Beckman Instruments, Inc.) for 45 min at 40,000 rpm (154,000 g) using a LP42Ti rotor. Specific binding was quantitated as above.

Binding assays in the presence of digitonin or saponin. Five-million U937 cells were preincubated in 1 ml of assay medium (Hanks' balanced salt solution) containing 10 mM Hepes, 10 mM N-Tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES), and 10 mg/ml bovine serum albumin (BSA) (U. S. Biochemical Corp., Cleveland, OH), 0.1% glucose, 1 mM PMSF, and 5 µg/ml of leupeptin, pH 7.4) at 4°C for 15 min in the presence or absence of 0.055% digitonin (Matheson, Coleman and Bell, Norwood, OH) (18) or 0.5% saponin (Sigma Chemical Co.) (19). Digitonin was added from a stock solution (14 mg/ml) in absolute ethanol. Control cells for the digitonin experiments received an equal volume of absolute ethanol. Treated cells were then incubated with radiolabeled IFNγ either alone or in the presence of excess unlabeled IFNγ for 2 h at 4°C. Binding was assessed by the centrifugation through oil method.

Preparation of fluorescenated human IgG$_1$. Fluorescein isothiocyanate (FITC) labeling of a human myeloma IgG1 (generously provided by Dr. H. Spiegelberg, Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA) was accomplished by incubation of 1 mg/ml protein with 2 mM FITC (Molecular Probes Inc., Plano, TX) in 10 mM borate buffer, pH 9.7, for 30 min at 37°C. Bound and free FITC were separated by gel filtration on a 10-ml Biogel P-6 column (Bio-Rad Laboratories). Protein concentration was determined by the method of Lowry (20).

Quantitation of IFNγ-dependent induction of Fc receptors. Induction of Fcγ receptors on U937 was measured with a direct immunofluorescence assay. Three-million U937 in 10 ml of IFNγ-containing medium were incubated for 48 h at 37°C in T25 tissue culture flasks (3013, Falcon Labware, Oxnard, CA). Cells were harvested and incubated (1 × 10^6) at 4°C for 45 min with 10^{-7} M FITC-labeled human IgG$_1$ in a total volume of 50 µl. To determine nonspecific binding, cells were preincubated with

50 μl of 10^{-5} IgG$_1$ before addition of fluorescenated ligand. After incubation, cells were washed two times with PBS containing 0.5% HSA and 10 mM sodium azide. Cellular fluorescence was quantitated by flow cytometry with a FACS IV instrument (Becton-Dickinson & Co., FACS Systems, Mountain View, CA) using linear amplification. The data are presented as the difference between the fluorescence intensity (mean fluorescence channel number) of cells incubated either with IFNγ or medium.

Induction of cellular differentiation. Purified monocytes from normal donors were allowed to mature into macrophages by suspension culture in 50-ml teflon beakers (Nagle Co., Rochester, NY) at 37°C in a 5% CO$_2$ atmosphere (21). Each beaker contained 20 × 10^6 monocytes in 40 ml of RPMI-1640 supplemented with 5 × 10^{-2} M 2-mercaptoethanol, 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% autologous serum. Cultures were fed every 3 d. Maturation of U937 cells was induced by 5 ng/ml PMA and incubation for 5 d (22). HL60 were differentiated to macrophages by culture with PMA (5 ng/ml) for 5 d (23) or to granulocytes by culture with DMSO (1.25% vol/vol) for 5 d (24).

Monocyte maturation was assessed morphologically by light microscopy and cytochemically by loss of peroxidase positivity. Differentiation in U937 or HL60 was confirmed by induction of C3bi (CR3) and Fc receptors and conversion of the cells to an adherent population.

Uptake and degradation of IFNγ. These studies were performed in 6-well tissue culture plates (Costar, No. 3506, Cambridge, MA) that had been preincubated overnight with medium containing 20% FCS. The wells contained 5 × 10^6 purified human monocytes and 200 ng ^{125}I-IFNγ in 1 ml of medium. After various lengths of time at 37°C, the culture supernatants containing unbound or degraded ^{125}I-IFNγ were collected. The supernatants were mixed with 500 μl of FCS and 1 ml 20% TCA at 4°C and TCA soluble and insoluble material separated by centrifugation at 4°C for 20 min at 2,000 g. Monocytes were washed at 4°C, solubilized with 1% NP40, and counted for the determination of total cell-associated radioactivity. As a control, ^{125}I-IFNγ was incubated at 37°C in empty wells, then processed as above. The control levels of TCA soluble counts were subtracted as background from all data points. An increase in TCA-soluble ^{125}I-IFNγ in the absence of cells was never observed.

Cross-linking of membrane components to ^{125}I-IFNγ and SDS-polyacrylamide gel electrophoresis (PAGE) analysis. 200 μg of membrane protein corresponding to ~5 × 10^7 U937 were incubated with 160 ng ^{125}I-IFNγ for 2 h at 4°C. In parallel, a control tube was incubated with a 100-fold excess of unlabeled IFNγ. Membranes were washed by centrifugation in an airfuge (Beckman Instruments Inc.) for 30 min at 25 psi (120,000 g). Pelleted membranes were resuspended in 45 μl of PBS, pH 7.3, and 5 μl was added of a 10-mM solution of selected cross-linking agents (DMS, DSS, or EGS) dissolved in DMSO. After incubation for 20 min at 4°C, 100 μl of 20 mM Tris HCl, pH 7.3, was added and the membranes were washed again and resuspended in 50 μl of 1% NP40. After 30 min at 4°C, 50 μl SDS sample buffer was added and membranes were fractionated by SDS-PAGE using 4–12% polyacrylamide gradient slab gels as described by Laemmli (25). The SDS sample buffer contained 100 mM dithiothreitol. Gels were dried and autoradiography performed at −70°C with XAR-5 film (Eastman Kodak Co., Rochester, NY) and a Cronex Lightning Plus intensifying screen (DuPont Co.). The following proteins were used as molecular weight markers: myosin (M_r = 20,000), β-Galactosidase (M_r = 116,250), phosphorylase B (M_r = 92,500), BSA (M_r = 66,200), and ovalbumin (M_r = 45,000) (Bio-Rad Laboratories, Chemical Division).

In some cases, whole cells were used as the receptor source. U937 (2 × 10^8 cells) were incubated in 5 ml of medium (PBS with 0.20 KIU/ml of aprotonin and 1 mM PMSF, pH 7.3) containing 200 ng ^{125}I-IFNγ for 2 h at 4°C. Cells were washed twice with ice-cold medium and cross-linking was initiated by resuspension of the cells in 5 ml of 1 mM cross-linking reagent. After incubation for 20 min at 4°C, 10 ml of 20 mM Tris HCl, pH 7.3, was added and the cells were washed again by centrifugation. Cells were lysed in a Dounce homogenizer and the lysates were centrifuged at 500 g for 10 min. The supernatants were centrifuged

again at 40,000 g for 30 min at 4°C. The high speed pellets were extracted with 100 μl of 1% NP40 for 30 min at 4°C, treated with 100 μl of SDS sample buffer, and subjected to SDS-PAGE analysis as above.

Results

Demonstration of a specific IFNγ receptor on Monocytes, U937, and HL60. The IFNγ receptor of human mononuclear phagocytes was defined in ligand binding experiments. These studies used radioiodinated recombinant human IFNγ that had been subjected to HPLC gel filtration to remove any degraded material that may have formed during storage. As detected by ultraviolet absorption at 280 nm and radioactivity, IFNγ eluted from the column at an M_r of 35,000 (data not shown). 62% of the recombinant IFNγ was recovered after iodination and column purification. The ^{125}I-IFNγ displayed antigenic reactivity with monoclonal anti-IFNγ and retained 80% of its starting specific antiviral activity and 90% of the capacity to induce Fc receptors on U937.

Fig. 1 shows that ^{125}I-recombinant human IFNγ binds to peripheral blood human monocytes or U937 in a specific and saturable manner at 4°C. In these experiments, 96–98% of the total binding was specific, since it was blocked in the presence of a 100- and 1,000-fold excess of unlabeled IFNγ, respectively. Binding was homogeneous, noncooperative, and of moderately high affinity. Binding did not require the presence of calcium or magnesium ions. Specific binding of ^{125}I-IFNγ to HL60, the human promyelocytic cell line, was also demonstrable. Mean values for the receptors on the different cell types are listed in Table I. Whereas the receptors on all three cell types displayed virtually identical association equilibrium constant (K_a) values, receptor expression on U937 and HL60 was 40–55% lower than on monocytes. Two different preparations of radiolabeled recombinant IFNγ produced similar values.
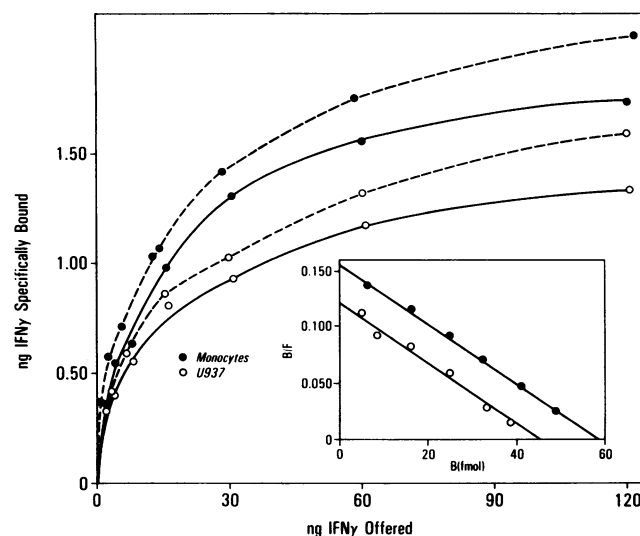

Figure 1. Quantitation of IFNγ binding to monocytes and U937. 5 × 10^6 cells were incubated at 4°C for 2 h with different amounts of radiolabeled, HPLC-purified, recombinant IFNγ. Cell associated and free ^{125}I-IFNγ were separated by centrifugation over pthalate oil as outlined in Methods. Specific binding (solid lines) was defined as the difference between total binding (dotted lines) and the nonspecific binding occurring in the presence of a 1,000-fold excess of unlabeled IFNγ. The inset represents Scatchard analysis of the data. B, bound IFNγ; F, free IFNγ.

Table I. Number of IFNγ Receptors and K_a on Human Monocytes U937 and HL60

Cell type	IFNγ preparation	Number of experiments	Receptors/cell	$K_a \times 10^{-8}$ (M^{-1})
Monocytes	D 0002 SF	5	6,900±1,000*	4.0±2.3
U937	D 0002 SF	20	4,200±1,000	5.0±3.4
HL60	D 0002 SF	3	3,200±700	4.1±1.8
U937	E 9026 A	15	5,500±1,600	12.1±4.7

* Mean±SD. Monocytes were obtained from five different normal individuals. The quantitation of IFNγ binding was determined as described in Fig. 1.

The specificity of the binding reaction is demonstrated in Fig. 2. Crude preparations of natural human IFNγ (SEB-stimulated lymphocyte culture supernatants) efficiently competed with ^{125}I-recombinant IFNγ for binding to U937 (67% inhibition was achieved at inputs of 6,800 IRU natural and 2,040 IRU ^{125}I-rIFNγ). Crude preparations of natural human IFNβ displayed only a weak ability to inhibit binding of recombinant IFNγ when added in large excess (100,000 IRU), while natural human IFNα displayed no inhibitory activity. Lower doses of IFNα or IFNβ were not inhibitory at all. Monoclonal antibodies (M6 and M125) specific for human IFNγ, which neutralized IFNγ biological activity, abrogated specific IFNγ binding to U937. Other monoclonal antibodies that did not react with human IFNγ (H22, 543, OKM10) were without effect on the binding reaction.

Fig. 3 demonstrates that the binding of IFNγ to U937 was reversible, since bound ligand dissociated from the cells in a homogeneous manner. The $t_{1/2}$ for the dissociation was 68 min at 4°C and the reaction could be described by a single dissociation rate constant (k_{off}) of 9×10^{-5} s^{-1}.
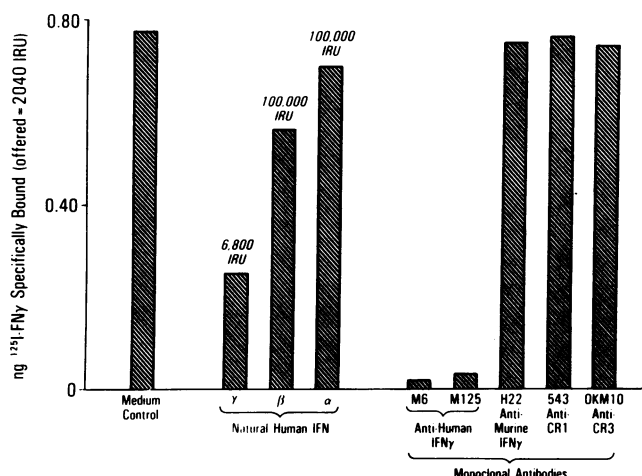

Figure 2. Specificity experiments for recombinant ^{125}I-IFNγ binding to macrophages. U937 cells were preincubated for 30 min at 4°C with designated amounts of competitor in a total reaction volume of 100 μl. 20 μl of ^{125}I-IFNγ (30 ng) was then added to each tube and incubation continued for 2 h at 4°C. Specific binding and separation of bound and free ^{125}I-IFNγ as in Fig. 1. For these studies, the natural IFNγ was concentrated 10-fold before use by ultrafiltration. A sham supernatant concentrated 10-fold did not have any inhibitory effect on the ^{125}I-IFNγ binding to the cells.
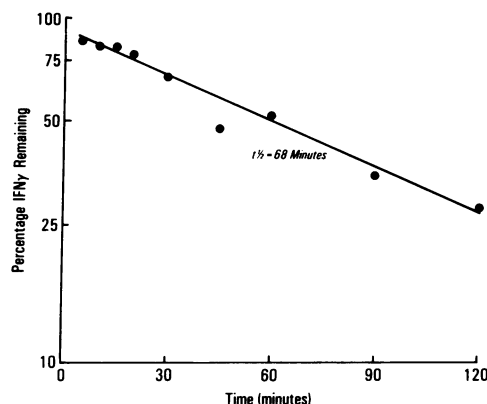

Figure 3. Dissociation of bound IFNγ from U937. Sixty-million U937 were incubated in 1 ml of medium containing 800 ng of ^{125}I-IFNγ for 2 h at 4°C. Cells were washed and resuspended to the original volume in the presence of a 100-fold excess of unlabeled IFNγ. At designated times, aliquots of 5×10^6 cells were centrifuged through phthalate oil and the radioactivity in the cell pellet measured. The data are presented as the log of the percentage of IFNγ remaining bound to the cells. Zero time is taken as 100%. The specific IFNγ binding at zero time was 0.90 ng/5×10^6 cells.

Demonstration that occupation of the IFNγ receptor induces a cellular response. The biological relevance of the receptor was validated by assessing the relationship between receptor occupancy and stimulation of a functional response in mononuclear phagocytes. For this purpose we measured the IFNγ-dependent induction of Fc receptors on U937 (26, 27). Fig. 4 depicts the dose-response profile for Fc receptor induction by purified human recombinant IFNγ. 50% maximal induction was produced by 3 ng of IFNγ (60 IRU) and maximal stimulation occurred at 50 ng input (1,000 IRU). When the data from the above experiment and the receptor binding experiments were normalized to similar cell densities (Fig. 4, inset), the concentration of IFNγ required for cell binding was found to correlate with that needed to induce the cellular response.

Ontogeny of the IFNγ receptor on mononuclear phagocytes. The ability to differentiate U937, HL60, and peripheral blood monocytes in vitro provided a mechanism to study the ontogeny of the IFNγ receptor. Table II demonstrates that receptor expression and affinity was not altered when normal peripheral blood monocytes matured to macrophages by 6–8 d culture in teflon beakers or when U937 and HL60 were differentiated into monocytes/macrophages by 5 d culture with PMA. No changes in the receptor were noted even when HL60 was induced to become granulocytes by treatment with DMSO.

In all experiments, differentiation was confirmed by morphological, cytochemical, and immunochemical analysis (see Methods). After 5 d induction with PMA, >90% of the U937 and 86% of HL60 expressed monocyte/macrophage markers and morphology. After 5 d incubation with DMSO, the HL60 culture consisted of 6.1% promyelocytes, 65.3% myelocytes, 24.6% metamyelocytes, 1.6% band cells, and 2.4% mature polymorphonuclear leukocytes.

Protease sensitivity of the IFNγ receptor. Pretreatment of U937 with 1 mg/ml trypsin or pronase for 5 min at 37°C abrogated subsequent binding of ^{125}I-IFNγ at 4°C (Fig. 5). Whereas 5×10^6 untreated cells bound 1.4 ng of IFNγ at saturation, the same number of trypsin-treated cells bound only 0.18 ng and
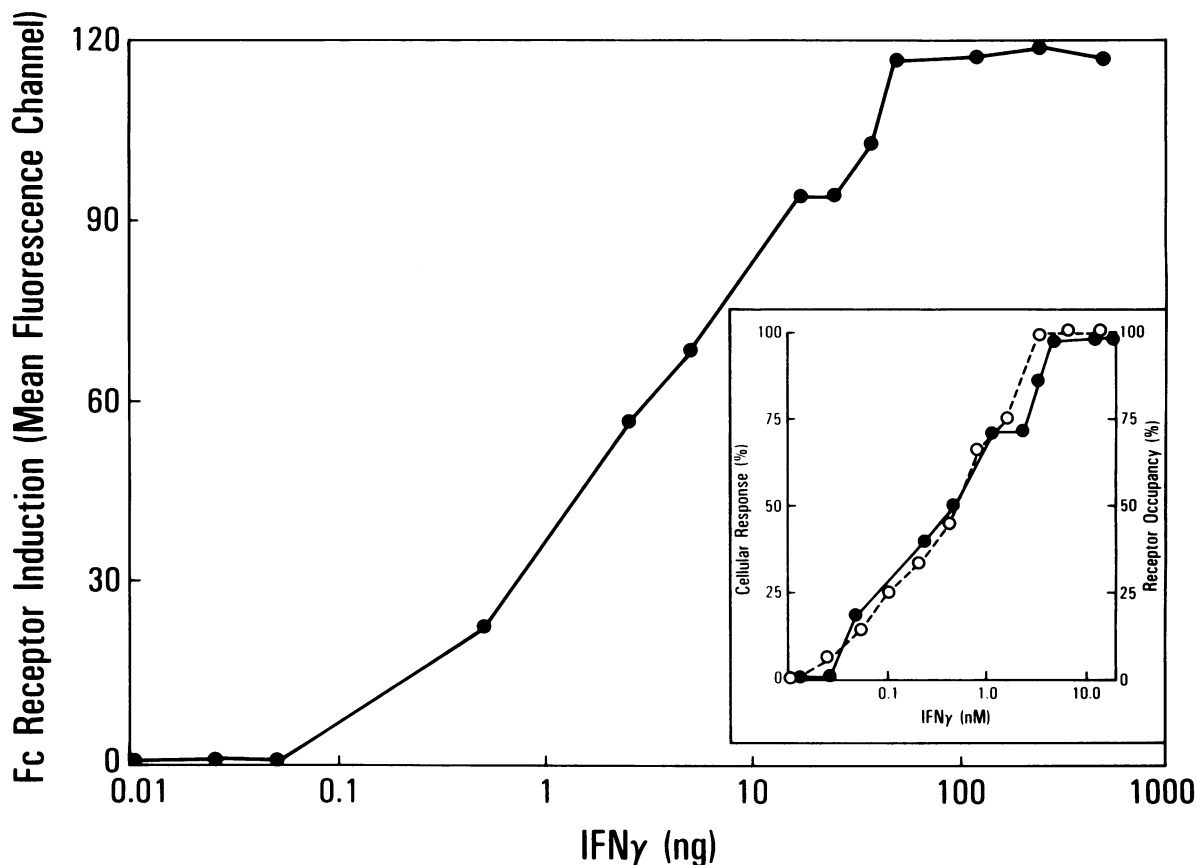
Figure 4. Correlation between IFNγ receptor occupancy and induction of Fc receptor expression on U937. Three-million cells were incubated in 10 ml with various amounts of IFNγ for 48 h at 37°C. Fc receptors were quantitated by direct immunofluorescence on a flow cytometer as described in Methods. Inset: Comparison of IFNγ receptor occupancy and induction of Fc receptors. The IFNγ input has been expressed in molarity and adjusted to 10⁶ cell/ml.

pronase-treated cells only 0.07 ng of ligand. These values represent an 87–95% reduction of IFNγ binding and suggest that the receptor is a protein.

Intracellular and extracellular distribution of IFNγ receptors. Saponin and digitonin have been used by others to permeabilize cells, and allow quantitation of intracellular (or cryptic) and extracellular receptors (18, 19). In order to obtain these values
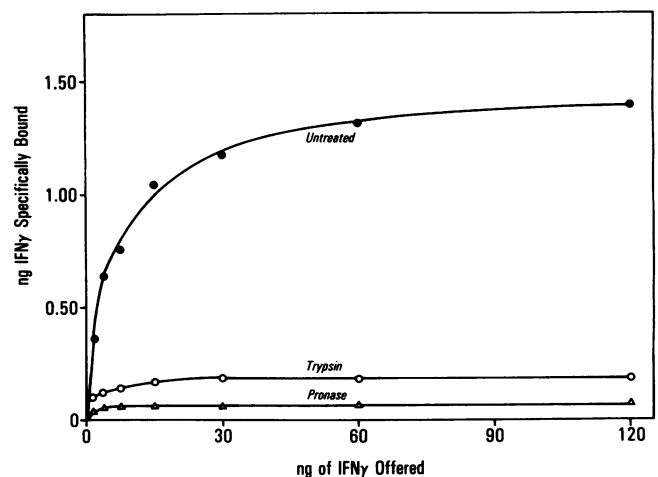
Table II. Cellular Differentiation Does Not Alter IFNγ Receptor Expression

Cell type	Number of experiments	Receptors/cell	$K_a \times 10^{-8}$ (M^{-1})
Monocytes:			
Control	2	7,900±400	4.9±1.2
After maturation		7,800±400	5.0±0.8
U937:			
Control	3	4,000±1,200	4.6±2.07
After induction		4,400±1,200	3.9±0.96
HL60:			
Control	2	3,600±800	4.4±0.7
After induction (PMA)		3,400±1,000	4.3±1.6
After induction (DMSO)		4,000±400	4.5±1.1

Maturation was induced in monocytes by culture in teflon beakers for 6 or 8 d. Maturation in U937 was induced by culture with PMA for 5 d. The promyelocytic cell line HL60 was differentiated into monocytic cells by incubation with PMA or into granulocytic cells by incubation with DMSO.

Figure 5. Effect of protease treatment on IFNγ binding by U937 cells. Three sets of 50 × 10⁶ cells were washed with EDTA and then incubated for 5 min with medium or 1 mg/ml of pronase or trypsin in a total volume of 50 ml. Cells were washed and IFNγ binding studies were carried out as outlined in Fig. 1.
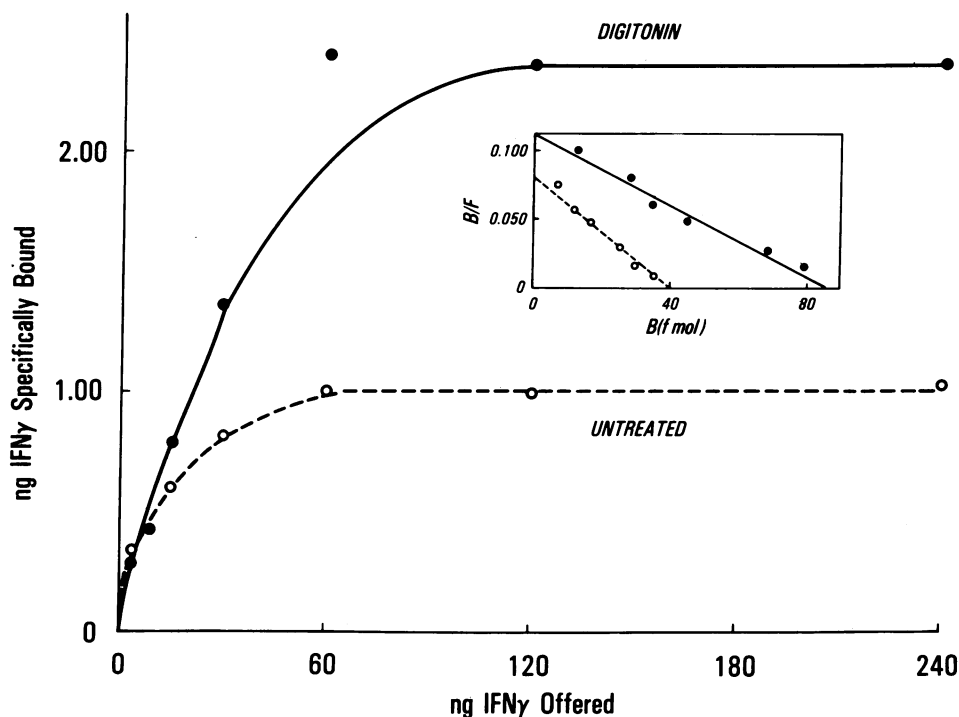
Figure 6. Binding of IFNγ to U937 cells in the presence and absence of 0.055% digitonin. Cells (5 × 10⁶) were incubated with ligand at 4°C for 2 h in a total volume of 1 ml. To one set of tubes, digitonin (14 mg/ml in ethanol) was added to a final concentration of 0.055%. Control tubes received an equivalent concentration of ethanol. The reaction was terminated by spinning the cells and counting the pellets and the supernatants.

for the IFNγ receptor, the binding studies were repeated on U937 with these agents. In the presence of 0.055% digitonin, U937 specifically bound 10,200 IFNγ molecules per cell, while untreated U937 bound only 4,700 IFNγ/cell (Fig. 6). Digitonin did not release prebound ¹²⁵I-IFNγ, indicating that the permeabilization procedure did not cause extraction of receptors. The binding affinity of the total receptor pool was comparable to the K_a values of only the external receptors. By calculation, the intracellular receptors were estimated to comprise 54% of the total cellular receptor pool. Similar values for the intracellular levels of receptor were obtained in two other experiments with digitonin (48 and 52%) or when 0.5% saponin was used as the permeabilization agent.

Receptor-mediated internalization of IFNγ. Whereas the binding of IFNγ to U937 was homogeneous at 4°C, it was bimodal at 37°C (Fig. 7, left). The lower affinity binding at 37°C (1.7 × 10⁹ M⁻¹) was of similar magnitude to that observed at 4°C (3.0 × 10⁹ M⁻¹). In addition, a high affinity binding of IFNγ to the cells was observed at the elevated temperature (2.3 × 10¹⁰ M⁻¹).

The high affinity binding observed at 37°C appeared to be a result of ligand internalization and not expression of different classes of receptors. This conclusion was reached because (a) purified U937 membranes bound IFNγ with a single affinity at either 4°C (1.2 × 10⁹ M⁻¹) or 37°C (1.5 × 10⁹ M⁻¹) (Fig. 7, right); (b) at either temperature, whole paraformaldehyde-fixed U937 displayed only a single binding affinity for ligand (0.9 × 10⁹ M⁻¹ at 4°C, 0.8 × 10⁹ M⁻¹ at 37°C); and (c) IFNγ internalization could be directly demonstrated at 37°C (Table III). In this last study, monocytes bound 33% more ligand at 37°C than at 4°C. When monocytes that had bound IFNγ at 4°C were stripped of surface receptors by trypsin treatment, the number of cell-associated IFNγ molecules decreased from 5,300 to 700/cell. In contrast to this result, cells that had been exposed to ligand at 37°C maintained 2,300 molecules of ¹²⁵I-IFNγ/cell

after enzyme treatment. When Scatchard analysis was performed on the reaction mixtures before and after trypsin stripping, an apparent 12.5-fold increase in the K_a was observed.

Degradation of internalized ¹²⁵I-IFNγ. The temperature dependent conversion of ¹²⁵I-IFNγ to TCA soluble degradation products is shown in Fig. 8. At either 4° or 37°C, cellular uptake of radiolabeled ligand reached a maximum after 10 min and remained essentially unchanged for the next 3.5 h. Maximum binding at 37°C (1.7 ng) was 30% greater than at 4°C (1.3 ng) (data not shown). At 4°C, no IFNγ degradation was observed
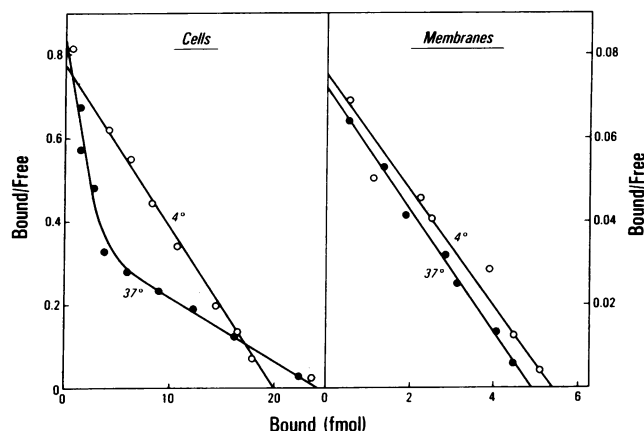

Figure 7. Effect of temperature on IFNγ interaction with whole U937 cells and membranes. (*Left*) U937 cells (5 × 10⁶) were incubated with ¹²⁵I-IFNγ at 4° or 37°C for 1 h in a total volume of 100 μl. Cell associated and free ligand were separated by centrifugation through pthalate oil. (*Right*) 200 μg of purified U937 membranes were incubated at 4°C or 37°C for 1 h in a total volume of 100 μl. Free and membrane-bound radiolabeled IFNγ were separated by ultracentrifugation. Nonspecific binding in both cases was determined by addition of 100-fold excess of unlabeled IFNγ.

Table III. Internalization of IFNγ by Monocytes

Incubation temperature	Trypsin stripping	IFNγ molecules/cell	$K_a \times 10^{-8}$ (M^{-1})
°C			
4	–	5,300	4.7
4	+	700	4.9
37	–	7,000	4.1
37	+	2,300	52.0

5×10^6 purified monocytes were adhered per well in six well tissue culture plates. Different amounts of IFNγ were added to the plated monocytes in a total volume of 1 ml. Two sets of monocytes were incubated for 2 h at 4°C and another two at 37°C. Then, one set of previously incubated monocytes at 4°C and another at 37°C were washed and treated with trypsin. The two other sets were only washed. Finally, 1% NP40 was added, cells were lysed, and the radioactivity associated with the cells counted.

over the 4-h period (Fig. 8). However, TCA soluble radioactivity became detectable in culture supernatants of monocytes held at 37°C for 2 h and increased linearly for the next 2 h. Based on the specific activity of the radioiodinated IFNγ, degradation was estimated to occur at a rate of 5,000 molecules/cell per h.

Characterization of the human monocyte IFNγ receptor by SDS-PAGE. The data presented thus far defined the IFNγ receptor of human mononuclear phagocytes by classical biochemical and functional criteria. We also wished to chemically define the receptor molecule. Initial attempts to directly radioiodinate the receptor on whole U937 cells and affinity purify it on IFNγ-coated beads were unsuccessful probably due to the limited number of receptor molecules expressed at the plasma membrane. However, since highly purified, radiolabeled ligand was available, we directed our efforts at affinity labeling the receptor by chemically cross-linking it to specifically bound ^{125}I-IFNγ. In preliminary experiments, cross-linking was confirmed by
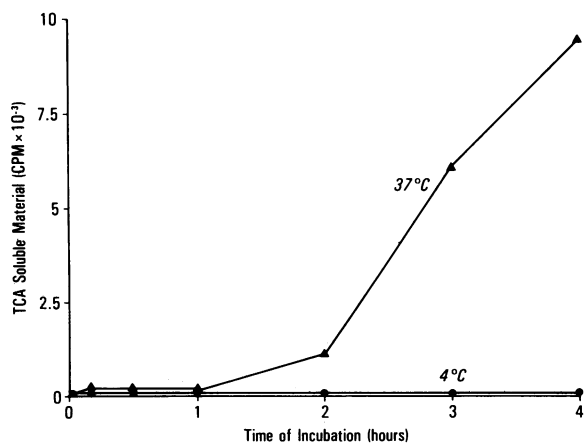

Figure 9. Purification of plasma membranes from U937 cells. Cells were prelabeled with ^{125}I-WGA subjected to N$_2$ cavitation, and purified by sucrose density gradient centrifugation for 1 h at 163,000 g. The distribution of ^{125}I-WGA, protein, and sucrose is shown as a function of the relative volume collected from the sucrose density gradient.

demonstrating a reduction in the dissociation rate of ligand from the cell surface (data not shown).

In order to minimize the possibility of proteolysis of the ligand-receptor complex (and reduce nonspecific labeling), plasma membrane-enriched fractions were prepared from U937 cells by N$_2$ cavitation and subsequent isopyknic sucrose density gradient sedimentation of the homogenate as outlined in the methods section. Plasma membrane recovery was determined by the presence of ^{125}I-WGA, which had been used in trace amounts to surface label the cells. 94% of the radioactivity applied to the gradients was sedimentable (total recovery was 95%) and recovered as a single band with a peak at a density of 1.286 g/cm (30% wt/vol sucrose) (Fig. 9). This fraction carried 23% of the total ^{125}I-WGA and only 5.6% of the total protein. Thereafter, the same fraction from gradients of unlabeled membranes was used as the membrane source.

Membrane preparations were incubated with ^{125}I-IFNγ, cross-linked, solubilized, and then analyzed by SDS-PAGE under reducing conditions. Fig. 10 depicts an autoradiograph of a

Figure 8. Degradation of IFNγ by monocytes. 5×10^6 of elutriation-purified cells were adhered per well in six well tissue culture plates. 200 ng of ^{125}I-IFNγ was added to each well and cultured at 4°C or 37°C for different periods of time. Then supernatants were harvested, mixed with FCS, and precipitated with TCA. TCA soluble and insoluble radioactivity was quantitated. Cells were washed, solubilized with 1% NP40, and radioactivity counted.
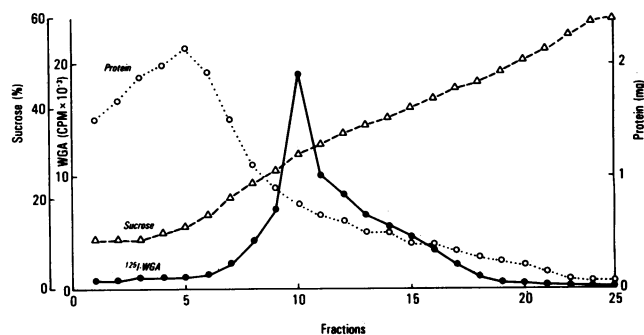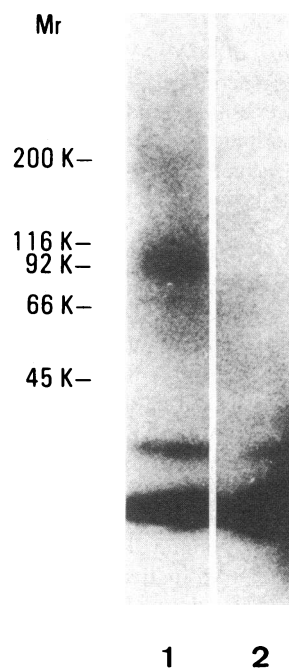

Figure 10. Cross-linking of ^{125}I-IFNγ bound to membranes of U937 cells and analysis by Na-DodSO$_4$/PAGE. 200 µg of purified membranes were incubated with 160 ng of ^{125}I-IFNγ for 2 h at 4°C, washed, and treated with 1 mM DSS. After washing, membranes were extracted and analyzed by NaDodSO$_4$/PAGE followed by autoradiography (lane *1*). In parallel, membranes were preincubated with a 100 excess cold IFNγ (lane *2*). K, hundred-thousand.

preparation cross-linked with 1 mM disuccinimidyl suberate (DSS). The sample in lane 1 represents membranes that were reacted with labeled IFNγ. A wide radioactive band is visible with an M_r of 94,000. This band has been observed in nine different experiments that used three different membrane preparations. The average M_r for the top and bottom portions of the band were 104,000±18,000 and 84,000±6,000, respectively. The band was not observed when the U937 membranes were pretreated with a 100-fold excess of unlabeld IFNγ (Fig. 10, lane 2), when the radioiodinated ligand was preincubated with a monoclonal anti-IFNγ (M125) that inhibited binding of IFNγ to cell surfaces (data not shown) or when the cross-linking agent was omitted (data not shown).

The two bands at the bottom of both tracks display M_r of 17,000 and 34,000, and are consistent with the presence of polypeptides, having molecular weights corresponding to a single IFNγ polypeptide chain and an internally cross-linked polypeptide chain dimer. These bands appear in the presence or absence of unlabeled IFNγ and are therefore presumed to be nonspecific and possibly represent free ligand carried through the washing procedure.

The high molecular weight band shows cross-linker selectivity. The band was observed only when DSS or EGS were used as linking agents but not when DMS was employed. DSS cross-linked preparations produced clearer autoradiographs than EGS samples. DSS cross-linking was dose dependent. At concentrations of 0.2 mM, no cross-linking occurred, while at high concentrations (30 mM), the samples were so heavily cross-linked that they did not enter the SDS gel. Optimal results were obtained at 1 mM linking agent.

In two experiments, whole U937 cells were used as the receptor source. The autoradiographs showed a complex band in which the M_r of the top was 107,000±10,000 and was 87,000±700 at the bottom. These results thus agree with those of the purified membrane preparations.

Discussion

The data presented in this report serve to define and partially characterize the IFNγ receptor on human mononuclear phagocytes. These studies represent a detailed analysis of the receptor on this cell type and are particularly warranted because monocytes and macrophages represent a major target of the immunomodulatory actions of IFNγ. Binding of radioiodinated, purified recombinant human IFNγ to human peripheral blood monocytes U937 and HL60 at 4°C was noncooperative, saturable, reversible, and of moderately high affinity (K_a = 0.5–1.0 × 10^9 M^{-1}). The interaction was specific because uptake of radiolabeled ligand was inhibited only by unlabeled natural or recombinant IFNγ (and not IFNα or IFNβ) or by monoclonal antihuman IFNγ, which blocked IFNγ-dependent antiviral activity. The mononuclear phagocyte receptor appeared to be a protein because IFNγ did not bind to cells treated with trypsin or pronase. At 37°C, surface-bound ligand was internalized and could not be eluted from the cells with trypsin. Intracellular IFNγ was degraded into TCA-soluble material at a constant rate of ~5,000 molecules/cell per h.

When binding was performed at 37°C, the Scatchard plot of the binding data was biphasic. This result could either indicate the presence of a second high affinity class of IFNγ receptors as suggested by Aiyer et al. (28), or that ligand internalization had occurred. The first possibility was excluded by the demonstration

that purified membranes and paraformaldehyde fixed U937 bound ligand in a unimodal manner at either 4° or 37°C.

Receptor expression and avidity were not influenced by cellular differentiation or activation, and even uncommitted precursor cells (such as HL60) carried IFNγ receptors on their surface. This observation is compatible with the previously reported ability of IFNγ to induce differentiation of precursor cells such as HL60 and U937 (29, 30), activate a variety of functions in mature human monocytes and macrophages (6), and cause terminal differentiation of monocytes to polykarions (31). Thus, the IFNγ receptor appears to be a ubiquitous component of the mononuclear phagocyte plasma membrane. This conclusion distinguishes the IFNγ receptor from Fc and C3 receptors, since the latter are expressed only on more differentiated forms of mononuclear phagocytes (21, 32).

The low magnitude of receptor expression on monocytes necessitated an indirect analysis approach to study the molecular weight of the receptor. Cross-linked preparations of radiolabeled ligand and purified plasma membranes migrated on SDS-PAGE as a wide band that spanned a molecular weight range of 87,000–104,000. While this apparent heterogeneity may reflect a high level of receptor glycosylation, it is more likely that it results from the formation of a cross-linked complex between the membrane component and either the internally cross-linked whole IFNγ molecule (consisting of two identical 17,000-mol wt polypeptide chains) or a single polypeptide chain subunit. Under the dissociating conditions used in the SDS-PAGE analysis, the complexes would therefore contain either a 34,000- or 17,000-M_r ligand component. By subtracting these values from the apparent molecular weights at the top and bottom of the band, the molecular weight of the receptor is estimated to be 70,000. The similarity of the molecular weight estimates made on different purified membrane preparations and whole cells suggests that receptor degradation did not occur during processing. However, the final assignment of a molecular weight for the IFNγ receptor on mononuclear phagocytes must await an analysis of the free molecule after documentation of its ligand binding capability. Attempts are currently being made in our laboratory to achieve these goals.

Interferon-γ induces a wide spectrum of biological activities in macrophages, including the induction of major histocompatibility complex class II antigens, Fc and C3bi receptors, cytocidal activity, and stimulation of IL1 production (6). Binding of IFNγ to its cell surface receptor represents the first step in the induction of the various macrophage cellular responses. In our experiments, the concentration of IFNγ needed to effect receptor occupancy closely correlated with that which induced Fc receptors on U937. Similar concentrations of IFNγ have been reported by others to induce responses in U937 or monocytes. Perusia et al. (26) and Guyre et al. (27) needed between 1 and 50 ng IFNγ/ml to induce 100% Fc receptor expression on U937 cells. Nathan et al. (33) reported that 1.2-nM concentrations of IFNγ (40.8 ng/ml) induced 50% of maximal toxoplasmacidal activity in monocytes. The latter experiments used human IFNγ that displayed a specific activity of 6 × 10^5 U/mg. Taken together, these results validate the biological relevance of the IFNγ receptor.

Over a period of 4 h, IFNγ was degraded at a linear rate of ~5,000 molecules/cell per h. This means that the receptors on the cell surface need to be replaced three times during this time period. This data is consistent with our previous report (7), which showed a similar pattern of IFNγ absorption by murine mac-

rophages. It is clear from these calculations that additional receptors must be recruited to the cell surface during the course of incubation. These may be derived from (*a*) a preexisting intracellular pool of receptors, (*b*) recycling of internalized receptors after the dissociation of bound ligand, or (*c*) *de novo* synthesis of additional IFNγ receptors. Increased receptor synthesis does not appear to be required, since cells treated with cycloheximide (a protein synthesis inhibitor) bound and degraded IFNγ in a normal fashion (data not shown). Experiments using permeabilized cells demonstrated the presence of cryptic receptors equal in the number to the receptors at the cell surface. In studies to be presented elsewhere, receptor recycling was evidenced by the ability of lysosomotropic agents to inhibit degradation (Celada, A., and R. D. Schreiber, manuscript in preparation). It is of interest to note that murine macrophages must be kept in contact with IFNγ for at least 4 h before tumoricidal activity can be initiated (7, 34–36). Similar observations have been reported for IFNγ-dependent induction of antiviral activity in fibroblasts (37). The possibility thus exists that ligand internalization may play a role in inducing certain cellular responses. However, other less complex functions, such as increased hydrogen peroxide production, appear to occur as a direct consequence of ligand-receptor interaction (33). Together, these results indicate that induction of complex biological functions in macrophages may require more than receptor-mediated signal transduction.

To date, an IFNγ receptor has been detected on several different human cell types, including fibroblasts (38–41), lymphoblastoid cells (42), epitheloid cells (43), and, in preliminary reports, U937 (44) and monocytes (45). At the present time, the relationship between the IFNγ receptors on these cells remains undefined. On the basis of the above studies, all cell types appear to carry comparable numbers of receptors (10^3–10^4/cell) that display similar binding affinities at 4°C ($K_a = 10^8$–10^9 M^{-1}). The cross-linked IFNγ-IFNγ receptor complex from WISH fibroblasts displayed an apparent molecular weight of 105,000 (40), and from lymphoblastoic cells, 125,000 (42). These values are comparable to the mean molecular weight of the U937-derived complex reported herein. Recent experiments, performed in our laboratory, which directly compared ligand binding to monocytes and fibroblasts, showed that IFNγ bound to the two cell types with identical affinities ($K_a = 1.5$ and 1.8×10^9 M^{-1}, respectively (Celada, A., and R. D. Schreiber, unpublished observation). Although this data suggests the presence of a single type of IFNγ receptor on different cells, definitive conclusions must await a full biochemical and genetic analysis of the receptor.

The identification of an IFNγ receptor on macrophages is the first step toward understanding macrophage activation at the molecular level. Work is currently in progress to fully characterize the receptor, determine its fate after receptor-ligand contact, and to assess whether receptor engagement is sufficient to initiate macrophage activation. These investigations should provide new insights into the role of IFNγ and activated macrophages in host defense.

Acknowledgments

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