

PURIFICATION OF HUMAN  $\gamma$  INTERFERON RECEPTORS  
BY SEQUENTIAL AFFINITY CHROMATOGRAPHY ON  
IMMOBILIZED MONOCLONAL ANTIRECEPTOR  
ANTIBODIES AND HUMAN  $\gamma$  INTERFERON

BY MICHEL AGUET AND GILLES MERLIN\*

*From the Institute of Immunology and Virology, University of Zürich, POB 8028 Zürich, Switzerland; and \*Unité Institut National de la Santé et de la Recherche Médicale 196, Institut Curie, 75231 Paris, France*

IFNs comprise three genetically determined classes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) and at least eight subtypes of IFN- $\alpha$  (1). IFN- $\alpha$  and - $\beta$  are produced in response to viral infections and compete for presumably common receptors (2, 3). IFN- $\gamma$  is a product of activated lymphocytes that differs structurally and functionally from IFN- $\alpha/\beta$  (4) and binds to distinct receptors (2, 5, 6). The binding properties of IFN receptors have been investigated on various cells; the structural elements of these receptors, however, have not been characterized as yet (for reviews see references 7, 8). Crosslinking experiments with  $^{125}\text{I}$ -labeled rIFN- $\gamma$  revealed ligand/receptor complexes within an  $M_r$  range between 70,000 and 140,000 (9–12). We now report the production of mAbs against IFN- $\gamma$  receptors of human Raji cells and the use of these antibodies together with immobilized recombinant human IFN- $\gamma$  (rhuIFN- $\gamma$ )<sup>1</sup> for sequential affinity purification of the receptor protein(s).

### Materials and Methods

*Interferon.* rhuIFN- $\gamma$  with a specific activity of  $2 \times 10^7$  U/mg of protein was kindly provided by Dr. C. Weissmann, Institute of Molecular Biology 1, University of Zurich. Labeling with  $^{125}\text{I}$  was carried out as follows: 25  $\mu\text{g}$  rhuIFN- $\gamma$  were reacted for 1 min at room temperature with 2 mCi carrier-free  $\text{Na}^{125}\text{I}$  (200 mCi/ml; Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland) in 100  $\mu\text{l}$  PBS, pH 7.2, after addition of 10  $\mu\text{l}$  0.05% wt/vol chloramine T. The reaction was stopped by addition of 10  $\mu\text{l}$  0.05% wt/vol sodium pyrosulfite and the labeled protein was separated from free iodine by gel filtration on a prepacked Sephadex G-25 PD-10 column (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with PBS containing 10% FCS (KC Biological, Inc., Lenexa, KS). The protein fraction was diluted 10-fold in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 10 mM Hepes buffer (Gibco Laboratories) and 10% FCS. The specific radioactivity of  $^{125}\text{I}$ -labeled huIFN gamma ranged from 30 to 50  $\mu\text{Ci}/\mu\text{g}$  of rhuIFN- $\gamma$  as determined by precipitation of proteins with 10% TCA. This range of specific radioactivity, which corresponds to a substitution ratio of about 0.3, was found to

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<sup>1</sup> *Abbreviation used in this paper:* rhuIFN- $\gamma$ , recombinant human interferon  $\gamma$ .

give the best ratio between specific and nonspecific high-affinity binding to cell surface receptors under saturating conditions. No loss of antiviral activity could be measured upon labeling. The sensitivity of the IFN assay does not allow detection of a loss of activity below 30%; it cannot be excluded, therefore, that labeled rhuIFN- $\gamma$  that shows specific binding to high-affinity cell surface receptors is in part biologically inactive. The binding properties relevant to the experiments reported herein remained unchanged upon storage at  $-70^{\circ}\text{C}$  for several months.

*Interferon Neutralization Assay.* rhuIFN- $\gamma$  was assayed on human WISH cells (Gibco Laboratories) challenged with vesicular stomatitis virus. WISH cells were grown as monolayer cultures in Dulbecco's modified MEM (Gibco Laboratories) supplemented with 10% FCS. 1 U/ml of huIFN- $\gamma$  is defined as the concentration that results in 50% protection from the cytopathic effect. Neutralization of this antiviral activity by mAbs was titrated by serial dilutions in cell culture medium of ascitic fluid against 4–8 U/ml of rhuIFN- $\gamma$ .

*Cells.* The continuous human Burkitt lymphoma lines Raji, P-3HR<sub>1</sub>, Daudi, Namalwa, and mouse L1210 cells were grown in nonagitated cultures in RPMI 1640 medium containing 10% FCS. Binding experiments and cell fractionation were carried out with exponentially growing cells expanded to a density of at most  $10^6$  cells/ml. Raji cells that were used for extraction of membrane proteins were grown in agitated suspension cultures of 5 liters in the same medium.

*Cell Lysates.* 5 liters of Raji cell cultures corresponding to  $5 \times 10^9$  cells were centrifuged at 400 g for 15 min at  $4^{\circ}\text{C}$  and the pellet was resuspended in 20 ml 10 mM Tris/Cl (pH 7.2)/100 U/ml aprotinin (Trasylol; Bayer, Leverkusen, Federal Republic of Germany). After 10 min incubation on ice, cells were subjected to Dounce homogenization and an equal volume of 10 mM Tris/Cl (pH 7.2)/0.5 M sucrose/100 mM KCl/10 mM MgCl<sub>2</sub>/2 mM CaCl<sub>2</sub>/100 U/ml aprotinin was added. This suspension was centrifuged at 2,000 g for 15 min at  $4^{\circ}\text{C}$  and the microsomal fraction contained in the supernatant was subsequently pelleted at 100,000 g for 90 min at  $4^{\circ}\text{C}$ . This membrane-enriched fraction was resuspended in 20 ml 10 mM Tris/Cl (pH 7.2)/150 mM NaCl (TBS)/0.1 M sucrose/100 U/ml aprotinin and kept frozen at  $-20^{\circ}\text{C}$ .

*Immunization and Cell Fusion.* Membrane-enriched fractions of  $\sim 5 \times 10^{10}$  human Raji cells were affinity purified on rhuIFN- $\gamma$  coupled to Affigel-10 as described below. One 4-wk-old female BALB/c mouse was immunized with the pooled eluates in CFA in four intraperitoneal injections at intervals of 2 wk. 3 d after the last injection,  $10^8$  spleen cells were fused with  $5 \times 10^7$  X63.Ag8.653 myeloma cells (kindly provided by Dr. H. Hengartner, Institute of Experimental Pathology, University of Zürich) according to established methods (13). Hybridomas were selected in Iscove's modified Dulbecco's medium (KC Biological, Inc.) supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100  $\mu\text{g}/\text{ml}$ ), glutamine (4 mM), sodium pyruvate (1 mM), 2-ME (50  $\mu\text{M}$ ), hypoxanthine (100  $\mu\text{M}$ ), aminopterin (0.4  $\mu\text{M}$ ), and thymidine (16  $\mu\text{M}$ ).

*Screening of Hybridoma Supernatants.* Hybridoma supernatants were first screened for competitive inhibition of binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  to Raji cells.  $2 \times 10^6$  Raji cells per well were incubated with 50  $\mu\text{l}$  of  $2 \times 10^{-10}$  M  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  in culture medium and 50  $\mu\text{l}$  of each hybridoma supernatant for 2 h at  $4^{\circ}\text{C}$  in U-bottomed microtiter plates. Nonspecific binding was determined by simultaneous addition of  $3 \times 10^{-8}$  M unlabeled rhuIFN- $\gamma$ . Subsequently, the cells were washed three times by centrifugation in the cold with culture medium and were transferred into counting vials. Binding of mouse mAbs to cells of the human lymphoma lines Raji, P-3HR<sub>1</sub>, Daudi, Namalwa, or to mouse L1210 cells was determined as follows:  $2 \times 10^6$  cells per well were incubated with 50  $\mu\text{l}$  of hybridoma supernatant for 2 h at  $4^{\circ}\text{C}$  and washed twice as described above. Subsequently, the cells were incubated with 100  $\mu\text{l}$  per well of a  $^{125}\text{I}$ -labeled affinity-purified sheep anti-mouse IgG antibody (Amersham Corp., Arlington Heights, IL; 13  $\mu\text{Ci}/\mu\text{g}$  of protein,  $\sim 10^5$  cpm per well) for 2 h at  $4^{\circ}\text{C}$  and were washed three times before transfer into counting vials.

*Affinity Chromatography.* Before conjugation to Affigel-10 (Bio-Rad Laboratories, Richmond, CA), ascitic fluid from mice carrying the hybridoma A6 (see Results) was dialyzed against 18% wt/vol Na<sub>2</sub>SO<sub>4</sub> at room temperature, and the precipitate was

dissolved and dialyzed against 0.1 M NaHCO<sub>3</sub>. Conjugation was carried out at a final protein concentration of 5 mg/ml by mixing 2 ml of wet gel with 2 ml of protein solution. This suspension was incubated overnight at 4°C under continuous gentle agitation. Subsequently, the gel was washed on a fritted glass funnel with ~100 ml of each of the following buffers: TBS/0.1% Triton X-100; 10 mM Tris/Cl (pH 7.2)/0.5 M KCl/0.5% Triton X-100 (buffer A); 10 mM Tris/Cl (pH 7.2)/150 mM NaCl/1% Triton X-100/1% sodiumdeoxycholate/0.1% SDS/10% glycerol (buffer B); 0.2 M glycine/HCl (pH 2.2)/0.1% Triton X-100 (elution buffer). After this initial washing procedure the gel was washed and stored in TBS at 4°C. rhuIFN- $\gamma$  was conjugated to Affigel-10 under the same conditions; 2 ml of rhuIFN- $\gamma$  containing 5 mg/ml of protein were dialyzed against 0.1 M NaHCO<sub>3</sub> and mixed with 2 ml of wet gel, and the suspension was incubated overnight at 4°C under continuous agitation. The subsequent washing steps were the same as for the conjugation of antibody A6 with one exception; since the biological activity of huIFN- $\gamma$  is lost at low pH, washing with elution buffer was omitted. For affinity purification a membrane-enriched fraction from  $2 \times 10^{10}$  Raji cells was lysed by addition of Triton X-100 to a final concentration of 1% (wt/vol). After incubation for 10 min on ice, lysates were clarified at 30,000 g for 15 min at 4°C and the supernatants were adsorbed at 4°C on 400  $\mu$ l Affigel-10 coupled to antibody A6 at a flow rate of 5 ml/h. Subsequently, the gel was washed by centrifugation with 10 ml TBS/0.1% Triton X-100, 3  $\times$  10 ml buffer A and 10 ml of buffer B. The gel was then transferred to a small disposable column (Bio-Rad Laboratories) and washed with 150 mM NaCl/0.1% Triton X-100 before application of 0.8 ml elution buffer. The eluate was immediately neutralized by addition of 200  $\mu$ l 0.5 M Tris base and eventually kept frozen at -20°C before further purification. The same protocol was applied for affinity purification of these eluates on rhuIFN- $\gamma$  coupled to Affigel-10. Final eluates were dialyzed against 50 mM Tris/Cl (pH 7.2)/0.1% Triton X-100 and stored at -20°C. To analyze the protein pattern the eluates were labeled with <sup>125</sup>I and subjected to SDS-PAGE. Briefly, 100  $\mu$ l aliquots were reacted for 1 min at room temperature with 0.5 mCi carrier-free Na<sup>125</sup>I (200 mCi/ml) after addition of 10  $\mu$ l 0.05% wt/vol chloramine T. The reaction was stopped by addition of 10  $\mu$ l 0.05% wt/vol sodium pyrosulfite and the labeled protein was separated from free iodine by gel filtration on a prepacked Sephadex G-25 PD-10 column (Pharmacia Fine Chemicals) equilibrated with 50 mM Tris/Cl (pH 7.2)/0.1% Triton X-100.

**SDS-PAGE.** SDS-PAGE was carried out according to Laemmli (14) with 10% polyacrylamide gels. Before electrophoresis samples were dialyzed against 50 mM Tris/Cl (pH 6.8)/0.1% Triton X-100 and diluted 3:4 in 0.250 M Tris/Cl (pH 6.8)/1% SDS/40% glycerol. For SDS-PAGE under reducing conditions samples were heated for 5 min at 96°C after addition of 2% 2-ME (Sigma Chemical Co., St. Louis, MO). Gels were stained with Bio-Rad Laboratories' silver stain according to the manufacturers protocol.

**Protein Transfer to Nitrocellulose.** Electroblotting to nitrocellulose (BA 85; Schleicher & Schuell, Inc., Keene, NH) was performed at 80 V/250-350 mA in 25 mM Tris/Cl/10 mM glycine (pH 8.3)/20% methanol for 90 min at 0-5°C. After electroblotting or direct application of 1- $\mu$ l samples (dot blot assay), the nitrocellulose was saturated overnight at room temperature in 10% wt/vol dry skim milk in PBS containing 0.05% wt/vol merthiolate (Eli Lilly and Co., Indianapolis, IN). To reveal the presence of rhuIFN- $\gamma$  binding protein nitrocellulose strips were incubated for 2 h at room temperature in  $2 \times 10^{-10}$  M <sup>125</sup>I-labeled rhuIFN- $\gamma$  in RPMI 1640 medium containing 10% FCS and 10 mM Hepes buffer, or in the various hybridoma supernatants. Nonspecific binding was determined in the presence of  $3 \times 10^{-8}$  M unlabeled rhuIFN- $\gamma$ . Before autoradiography on Fuji RX film nitrocellulose was washed three times for 10 min at room temperature with the same medium without IFN and once for 10 min at room temperature in TBS/0.05% (wt/vol) Tween 20.

## Results

**Screening and Characterization of mAbs.** <sup>125</sup>I-labeled rhuIFN- $\gamma$  binds to Raji cells with an apparent dissociation constant of  $10^{-10}$  M at 4°C as extrapolated

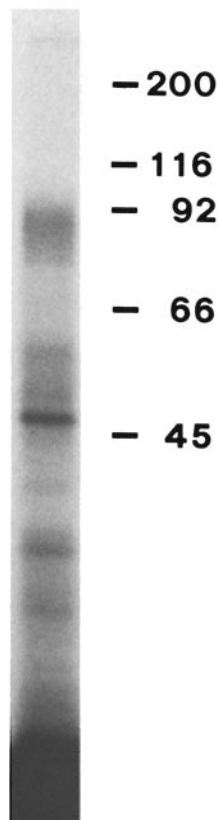
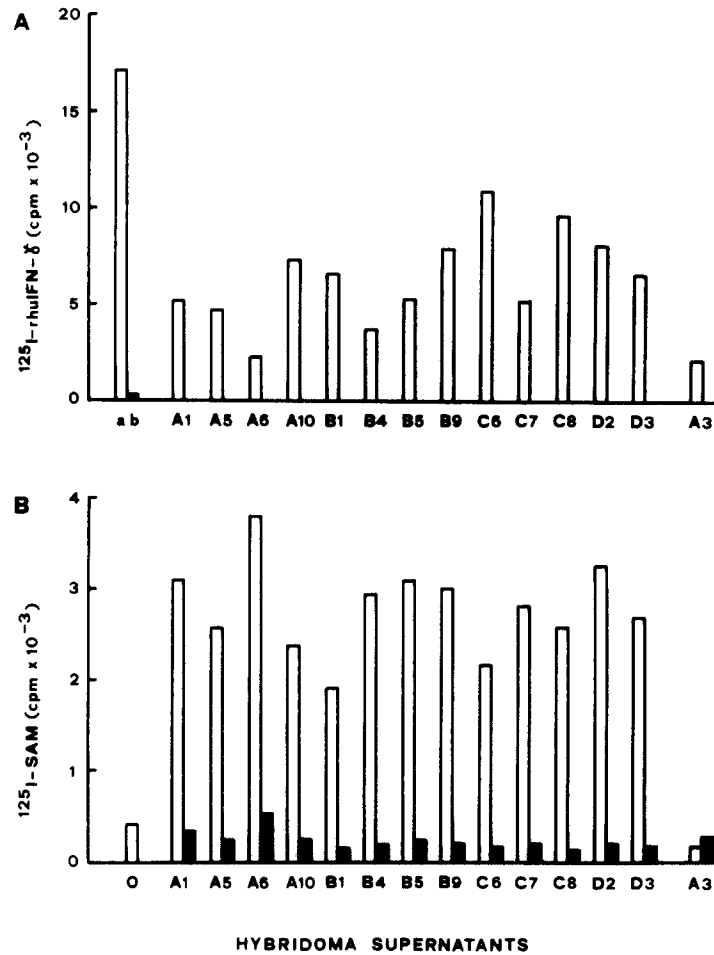


FIGURE 1. SDS-PAGE and autoradiography of an  $^{125}\text{I}$ -labeled eluate from a rhuIFN- $\gamma$  column after adsorption of solubilized Raji cell membranes. Analogous eluates were used to immunize a BALB/c mouse for inducing mAb against huIFN- $\gamma$  receptors. SDS-PAGE was carried out under nonreducing conditions as described in Materials and Methods. High molecular mass standards (Bio-Rad Laboratories) are indicated in kilodaltons.

from linear Scatchard plots. As compared with three other human lymphoid cell lines (P-3HR<sub>1</sub>, Daudi, Namalwa; Fig. 3) and also peripheral human leukocytes (6), Raji cells express the highest number of specific binding sites for rhuIFN- $\gamma$  ( $\sim 10^4$  sites per cell), while the dissociation constant is virtually the same for all these cells. Therefore, Raji cells that are easy to grow as large scale cultures were chosen as starting material for affinity purification of huIFN- $\gamma$  receptors. The enrichment of membranes by Dounce homogenization and differential centrifugation allows recovery of  $\sim 80\%$  of the cell surface receptors, as revealed by use of specifically bound  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  as a tracer. Affinity purification of Triton X-100 extracts of enriched Raji cell membranes from  $\sim 5 \times 10^{10}$  cells on a rhuIFN- $\gamma$  column yielded  $\sim 200\text{--}400 \mu\text{g}$  of eluted protein, as roughly estimated from silver-stained polyacrylamide gels. This eluate was used for immunization of one BALB/c mouse. The protein pattern of eluted  $^{125}\text{I}$ -labeled protein as revealed by SDS-PAGE is shown in Fig. 1. Retrospectively, as compared with the highly purified proteins after sequential affinity chromatography, the content of rhuIFN- $\gamma$  binding protein was estimated at  $<10\%$  of the total protein (Fig. 5A). mAbs were screened for inhibition of specific binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  to Raji cells as shown in Fig. 2A. 20 of 1,400 hybridoma supernatant fluids screened showed such an inhibitory effect. Among these, 13 contained antibodies that could recognize a cell surface antigen on Raji cells. This binding could be



**FIGURE 2.** (A) Screening of hybridoma supernatants for inhibition of specific binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  to Raji cells at a receptor saturating concentration ( $2 \times 10^{-10}$  M). (a) Binding in the absence of antibodies. (b) Nonspecific binding in the presence of excess unlabeled rhuIFN- $\gamma$  ( $3 \times 10^{-8}$  M). (B) Binding of mAbs to Raji cells as revealed by addition of  $^{125}\text{I}$ -labeled sheep anti-mouse IgG antibodies (SAM). Raji cells were preincubated with hybridoma supernatants or cell culture medium (O) without ( $\square$ ) or in the presence of  $3 \times 10^{-8}$  M unlabeled rhuIFN- $\gamma$  ( $\blacksquare$ ). After 2 h the cells were washed and incubated with  $^{125}\text{I}$ -labeled SAM.

inhibited competitively by excess unlabeled rhuIFN- $\gamma$  (Fig. 2B), but not by IFN- $\alpha 2$  (not shown), which interacts with distinct receptors (2). Seven additional antibodies such as antibody A3 (Fig. 2) failed to bind to Raji cells although they inhibited specific receptor binding of rhuIFN- $\gamma$ . These antibodies are specific for rhuIFN- $\gamma$  that was present as a contaminant in the immunogen (since biological activity and binding capacity of huIFN- $\gamma$  are lost irreversibly at low pH, washing of the affinity column with elution buffer had been omitted and therefore noncovalently bound rhuIFN- $\gamma$  was expected to contaminate the eluates to some extent). As exemplified with antibody A6 and confirmed for the other putative anti-huIFN- $\gamma$  receptor antibodies, the experiment depicted in Fig. 3 shows that the number of binding sites revealed by direct binding of  $^{125}\text{I}$ -

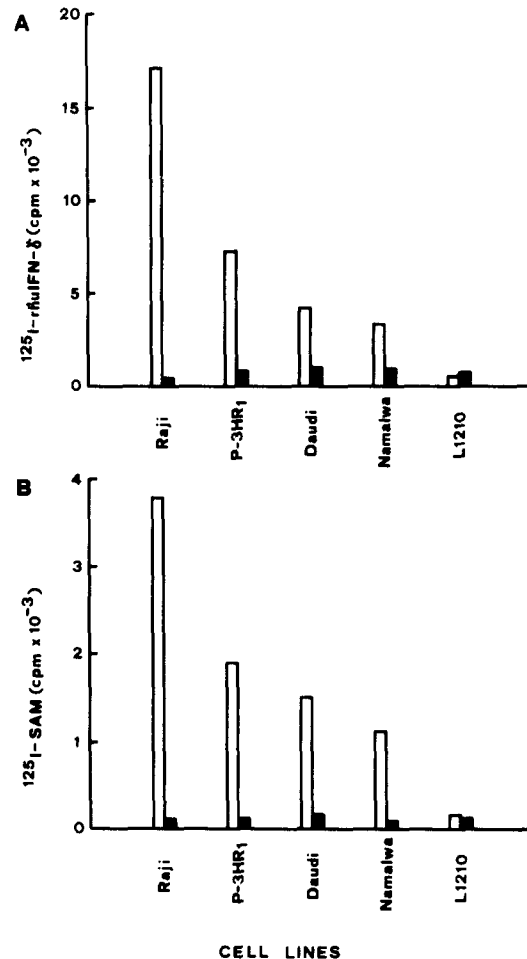


FIGURE 3. (A) Comparative binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  to cells of four human (Raji, P-3HR<sub>1</sub>, Daudi, Namalwa) and one murine (L1210) lymphoid cell line under the same conditions as in Fig. 2A ( $\square$ ). Nonspecific binding was determined in the presence of  $3 \times 10^{-8}$  M unlabeled rhuIFN- $\gamma$  ( $\blacksquare$ ). (B) Comparative binding of mAb A6 to the same cells as revealed by addition of  $^{125}\text{I}$ -labeled SAM (see Fig. 2B).

labeled rhuIFN- $\gamma$  correlates with the amount of antigen recognized by antibody A6 on four different human lymphoid cell lines. Mouse L1210 cells neither express rhuIFN- $\gamma$  binding sites nor antigens recognized by antibody A6. All mAbs selected belong to the IgG class. Preparative quantities of antibodies were purified from ascitic fluid from BALB/c mice injected intraperitoneally with the hybridomas. Ascitic fluid containing anti-receptor antibodies neutralizes the antiviral activity of rhuIFN- $\gamma$  on WISH cells with titers ranging from 1:640 to 1:2,560 against 8 U/ml of rhuIFN- $\gamma$ . None of the mAbs exhibit an antiviral effect on WISH cells.

*Affinity Purification of huIFN- $\gamma$  Receptor Protein.* Antibody A6 was selected for affinity chromatography since it gave the highest binding signal on Raji cells, both in hybridoma supernatants (Fig. 2B) and ascitic fluid (not shown). As shown

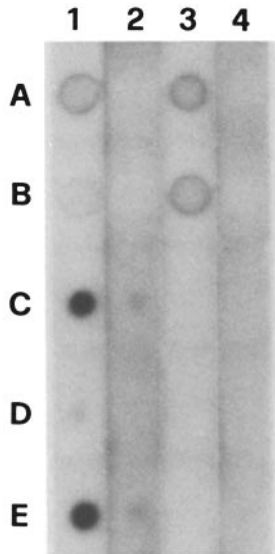


FIGURE 4. Sequential adsorption and elution of rhuIFN- $\gamma$  binding proteins from immobilized mAbs A6 (lanes 1 and 2) or A3 (lanes 3 and 4) and rhuIFN- $\gamma$ , as revealed in a dot blot assay. Nitrocellulose strips were incubated with  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  without (lanes 1 and 3) or in the presence of excess unlabeled rhuIFN- $\gamma$  (lanes 2 and 4), and were washed and incubated for autoradiography. (A) Triton X-100 extract of a membrane-enriched Raji cell fraction. (B) Same material after adsorption to the mAb columns. (C) Eluates from the antibody columns. (D) Eluates from the antibody columns after adsorption to immobilized rhuIFN- $\gamma$ . (E) Eluates from the rhuIFN- $\gamma$  column.

in Fig. 4, affinity purification of huIFN- $\gamma$  binding proteins from lysates of enriched Raji cell membranes can be followed in a simple dot blot assay; an A6 column retains a protein fraction that can bind rhuIFN- $\gamma$ , and this fraction can be quantitatively adsorbed on a rhuIFN- $\gamma$  column and retains its binding activity upon elution from this column. As a control, solubilized Raji cell membranes were adsorbed to immobilized antibody A3 that is specific for huIFN- $\gamma$  (Fig. 2). huIFN- $\gamma$  binding proteins could neither be adsorbed nor eluted from this column (Fig. 4). The protein pattern of  $^{125}\text{I}$ -labeled eluates as revealed by SDS-PAGE is shown in Fig. 5A. Sequential affinity chromatography on antibody A6 and rhuIFN- $\gamma$  columns yields two major protein species with approximate  $M_r$  of 90,000 (p90) and 50,000 (p50), respectively. Upon electroblotting to nitrocellulose, both p90 and p50 can bind  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  and this binding can be displaced by addition of unlabeled rhuIFN- $\gamma$  (Fig. 5B, lanes *a* and *b*) but not IFN- $\alpha$  (not shown). While the binding to p50 is always reproducible, the capacity of p90 to bind rhuIFN- $\gamma$  was found to depend on the batch of nitrocellulose used. As in the final eluate after sequential affinity chromatography on antibody A6 and rhuIFN- $\gamma$  columns, p90 and p50 are the only rhuIFN binding proteins revealed upon electroblotting of eluates from an antibody A6 column (Fig. 5B, lane *c*), and according to the dot blot assay, (Fig. 4) these proteins are no longer detectable in such eluates when these have been adsorbed on an rhuIFN- $\gamma$  column (not shown). Consistent with its capacity to inhibit the binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  to its cell surface receptors, antibody A6 can also specifically inhibit the binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  to p90 and p50 (Fig. 5B, lane *e*). SDS-PAGE and electroblotting to nitrocellulose of unadsorbed Raji membrane lysates reveal a very weak signal corresponding to p50 and no additional rhuIFN- $\gamma$  binding proteins can be detected (not shown).

To verify the results obtained with the mAb A6, the same purification procedure was repeated using antibody B9, which was chosen arbitrarily. It was

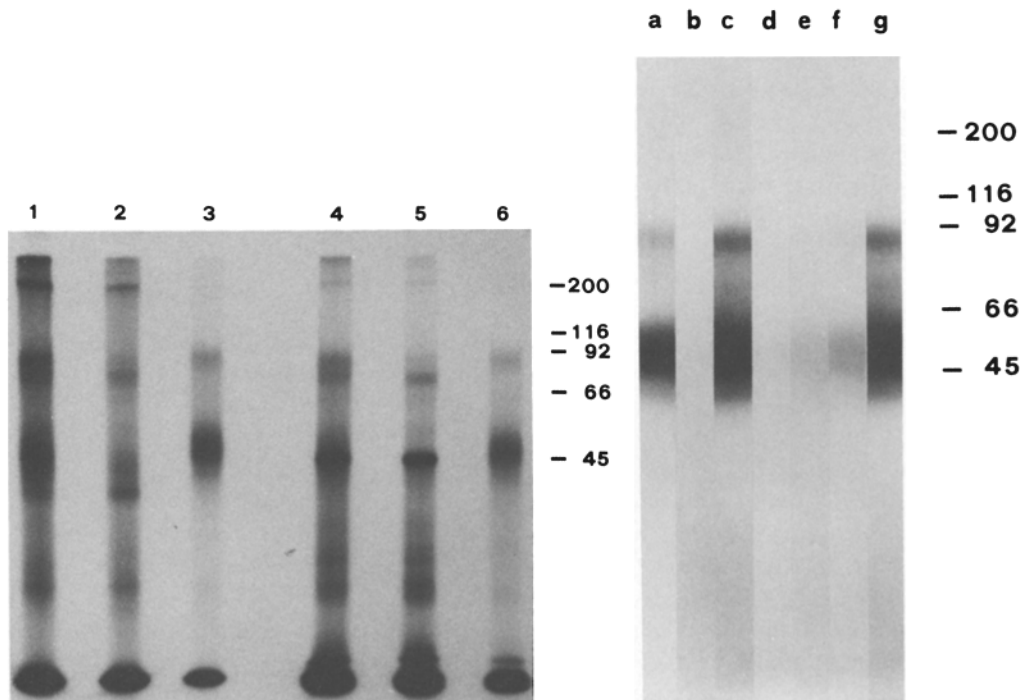


FIGURE 5. (A) SDS-PAGE and autoradiography of  $^{125}\text{I}$ -labeled sequential eluates from antibody A6 and rhuIFN- $\gamma$  columns. Lanes 1-3 were run under nonreducing conditions, lanes 4-6 under reducing conditions. High molecular mass standards (Bio-Rad Laboratories) are indicated in kilodaltons. Lanes 1 and 4; eluate from an antibody A6 column (see Fig. 4C); lanes 2 and 5, eluate from an antibody A6 column after adsorption to a rhuIFN- $\gamma$  (see Fig. 4D); lanes 3 and 6, final eluate from a rhuIFN- $\gamma$  column (see Fig. 4E). (B) Lanes a-b, binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  to proteins of the final eluate after sequential chromatography of solubilized Raji cell membranes on antibody A6 and rhuIFN- $\gamma$  columns. The eluate was subjected to SDS-PAGE under nonreducing conditions and electroblotted to nitrocellulose. Nitrocellulose strips were incubated with  $^{125}\text{I}$ -rhuIFN- $\gamma$  alone (a) or in presence of excess unlabeled rhuIFN- $\gamma$  (b); lanes c-g, binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  to electroblotted proteins eluted from an antibody A6 before further purification on an rhuIFN- $\gamma$  column. Nitrocellulose strips were incubated with  $^{125}\text{I}$ -rhuIFN- $\gamma$  alone (c), in the presence of excess unlabeled rhuIFN- $\gamma$  (d), in the presence of hybridoma supernatant A6 (e), in the presence of hybridoma supernatant B9 (f), or in the presence of a hybridoma supernatant containing nonspecific mAb (g). The binding proteins were visualized by autoradiography.

ascertained by determining their respective pI that antibodies A6 and B9 are indeed different. A superimposable pattern of purified proteins with an identical capacity to bind rhuIFN- $\gamma$  was obtained when antibody B9 was chosen for affinity chromatography instead of antibody A6 (not shown). Antibody B9 can also inhibit the binding of labeled rhuIFN- $\gamma$  to p90 and p50 (Fig. 5B, lane f), though to a lesser extent than antibody A6. This difference correlates with the different capacity of antibodies A6 and B9 to inhibit the binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  to cell surface receptors (Fig. 2A). It was confirmed by use of a  $^{125}\text{I}$ -labeled sheep anti-mouse IgG antibody that both antibodies A6 and B9 can bind to the same proteins as  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  (not shown).

Sequential affinity chromatography of a lysate of 80 ml containing about 10 mg/ml of protein and corresponding to about  $2 \times 10^{10}$  Raji cells yields 1 ml of a final eluate with about 500 ng of p50, as estimated on silver-stained polyacryl-



amide gels. The proportion of p90 varies from one preparation to another but never exceeds 30% of p50 (Fig. 5A). The yield and the quantitative enrichment of rhuIFN- $\gamma$  binding proteins after the various purification steps are difficult to evaluate. Based on an estimation of  $10^4$  receptor molecules per Raji cell, a membrane enriched fraction of  $2 \times 10^{10}$  cells would contain about 17  $\mu$ g of p50. Accordingly, the final recovery of 500 ng p50 would amount to  $\sim 3\%$ . As suggested by the different intensities of dots (Fig. 4) and also protein bands in autoradiography, rhuIFN- $\gamma$  binding proteins are markedly enriched upon purification on the antireceptor antibody column. Dilution series in the dot blot assay indicate that the enrichment after affinity chromatography on the antibody column is  $\sim 10$ – $20$ -fold (not shown). Clearly, rhuIFN- $\gamma$  binding proteins eluted from the antireceptor antibody column are recovered to  $>50\%$  upon adsorption and elution from a rhuIFN- $\gamma$  column (Fig. 4). Thus, when estimated in the dot blot assay, the recovery of rhuIFN- $\gamma$  binding proteins after sequential chromatography on both columns would amount to  $\sim 10\%$ .

Assuming that the estimation of 500 ng/ml of protein p50 in the final eluate is correct, this eluate should be able to neutralize the antiviral activity of  $\sim 3,000$  U/ml of huIFN- $\gamma$ , provided that the binding affinity of the soluble receptor is the same as for the native cell surface molecule. We were not able, however, to detect any IFN- $\gamma$  neutralizing activity of the huIFN- $\gamma$  binding proteins eluted from the affinity columns. The reasons for this are not known at present. It is possible that the dilution in the IFN neutralization assay of Triton X-100 containing eluates to detergent concentrations that are not cytotoxic accounts for a destabilization or a loss of affinity of soluble receptor proteins.

### Discussion

We have produced monoclonal anti-huIFN- $\gamma$  receptor antibodies as a tool for purification of receptor protein to analyze its structure and gene(s). A Triton X-100 extract of a membrane-enriched Raji cell fraction was affinity purified on a rhuIFN- $\gamma$  column and the eluate served as immunogen (Fig. 1). Raji cells were chosen for their comparatively high level of expression of specific huIFN- $\gamma$  binding sites (Fig. 3). Since binding of labeled rhuIFN- $\gamma$  was the only available receptor assay, mAbs were selected for inhibition of ligand binding to the receptor. Among such inhibitory antibodies some were found to bind rhuIFN- $\gamma$  that was contaminating the immunogen (see Results). The others are characterized by the following properties: they bind to the surface of cells that express huIFN- $\gamma$  receptors, but not to heterologous cells (Figs. 2 and 3). This binding is proportional to the number of IFN- $\gamma$  binding sites expressed on different cells (Fig. 3) and can be inhibited competitively by addition of rhuIFN- $\gamma$  (Fig. 2). Altogether these findings suggest that this second group of antibodies is interacting specifically with epitopes of IFN- $\gamma$  receptors. Concordant with the partial inhibition of ligand binding, ascitic fluid from hybridoma-carrying mice has some neutralizing effect on the antiviral activity of rhuIFN- $\gamma$  on WISH cells, suggesting that similar epitopes are recognized on these cells. Antibodies to receptors may mimic the action of peptide ligands such as epidermal growth factor (15, 16). Yet, none of the anti-huIFN- $\gamma$  receptor antibodies exhibit antiviral activity on WISH cells. Affinity purification of rhuIFN- $\gamma$  binding proteins from solubilized

Raji cell membranes can be easily followed in a dot blot assay (Fig. 4). Thus, a column containing the mAb A6 retains a protein fraction that, upon elution, is still able to specifically bind  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$ . This fraction can be reabsorbed and eluted from a rhuIFN- $\gamma$  column. As compared with the weak specific signal in the starting material, both eluates from antibody A6 and rhuIFN- $\gamma$  columns contain markedly concentrated rhuIFN- $\gamma$  binding proteins. SDS-PAGE of the final eluate reveals an enrichment of two main heterogeneous, probably glycosylated, protein species, p90 and p50 (Fig. 5A). A comparison of the labeled eluate from a rhuIFN- $\gamma$  column (Fig. 1) with the eluate obtained by sequential affinity chromatography on antireceptor antibody and rhuIFN- $\gamma$  columns (Fig. 5A) illustrates the contribution of immobilized antireceptor mAbs for additional purification of receptor proteins. A superimposable pattern of purified rhuIFN- $\gamma$  binding proteins was reproduced when a different antireceptor antibody (antibody B9) was chosen for affinity chromatography instead of antibody A6. It is also noteworthy that similar results were obtained when the purification sequence was inverted with an rhuIFN- $\gamma$  column preceding the antibody A6 column (data not shown).

Upon electroblotting to nitrocellulose, both proteins p90 and p50 can bind  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  (Fig. 5B). This binding is specifically displaceable by excess unlabeled rhuIFN- $\gamma$  and also by the anti-huIFN- $\gamma$  receptor mAbs, but not by control antibodies. Hence, it seems likely that p90 and p50 represent at least a part of huIFN- $\gamma$  receptors. The electrophoretic migration of both p90 and p50 was virtually the same under nonreducing and reducing conditions (Fig. 5A), and therefore, a simple monomer-dimer relationship between both proteins seems unlikely. The marked heterogeneity of p50 suggests that this protein is glycosylated. An additional, more homogeneous rhuIFN- $\gamma$  binding protein species seems to be hidden within the diffuse band of p50 (Fig. 5), and preliminary results obtained with two-dimensional electrophoresis confirm this observation. In view of its rather low molecular weight, p50 could be a degradation product; alternatively, it might represent a subunit of the huIFN- $\gamma$  receptor. At present, however, the relationship between p90 and p50 is unclear.

Several reports (9–11) on the analysis of crosslinked ligand-receptor complexes suggest an  $M_r$  range for huIFN- $\gamma$  receptors between 100,000 and 150,000. Recently, analogous crosslinking experiments (12) with various cell lines revealed a marked heterogeneity of ligand-receptor complexes with three main  $M_r$  species of 70,000, 92,000, and 160,000, respectively. It remains to be established whether these different species represent subunits or split products of the huIFN- $\gamma$  receptor. The similarity of p90 and the proteins that make up the diffuse p50 band with regard to binding of both rhuIFN- $\gamma$  and also the mAbs used for their purification suggests an extensive homology around the respective binding sites. Purified p90 and p50 separated by preparative SDS-PAGE and used as immunogens should give rise to mAbs with antireceptor properties similar to those described here, and this would underpin the role of these proteins as a part of the huIFN- $\gamma$  receptor. In addition, the characterization of a broader spectrum of epitopes could help to further elucidate the relationship between these various protein species.

### Summary

mAbs against human IFN- $\gamma$  (huIFN- $\gamma$ ) receptors were obtained by immunizing a BALB/c mouse with eluates from immobilized recombinant huIFN- $\gamma$  (rhuIFN- $\gamma$ ) on which lysates of enriched Raji cell membranes had been adsorbed. mAbs were selected for competitive inhibition of receptor binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$ . The following additional properties suggest that these antibodies are specific for huIFN- $\gamma$  receptors: they bind to the surface of human cells expressing IFN- $\gamma$  receptors but not to heterologous cells; this binding is inhibited competitively by addition of rhuIFN- $\gamma$ ; the number of binding sites revealed by direct binding of  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  correlates with the amount of antigen recognized by the mAbs on different cell lines. A Triton X-100 extract of a membrane-enriched fraction of human Raji cells was affinity purified with these mAbs and the eluates from such columns were further purified on immobilized rhuIFN- $\gamma$ . As revealed by SDS-PAGE, the final eluate contained two major protein bands with approximate  $M_r$  of 90,000 (p90) and 50,000 (p50), respectively. Both proteins were able to specifically bind  $^{125}\text{I}$ -labeled rhuIFN- $\gamma$  upon electroblotting to nitrocellulose. This binding could be inhibited by the huIFN- $\gamma$  receptor mAbs, suggesting that the same epitopes are recognized on p90, p50, and on the cell surface. Therefore, these proteins most likely represent at least a part of huIFN- $\gamma$  receptors.

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