Studies of hamster-human and mouse-human somatic fibroblast hybrids and transfected mouse fibroblasts have demonstrated that signaling through the human interferon-γ receptor (hu-IFN-γR) requires the formation of a complex consisting of ligand (IFN-γ), a ligand binding receptor chain (IFN-γR1), and a signal transducing receptor chain (IFN-γR2). To date, the ability of this receptor complex to transduce the full repertoire of biological signals has been difficult to assess due to the limited number of activities that IFN-γ can exert on fibroblasts. The current report assesses the ability of hu-IFN-γR chains to transduce signals in the absence of background human gene products by expressing hu-IFN-γR2 in a transformed macrophage cell line (F10/96) derived from a hu-IFN-γR1 transgenic mouse. Our results indicate that F10/96 clones expressing both human receptor proteins bind hu-IFN-γ with an affinity comparable to that of human cells. Binding of either human or mouse IFN-γ to its respective receptor elicits classic IFN-γ responses such as up-regulation of major histocompatibility complex antigens, enhanced expression of IRF-1, and increased production of NO² radicals, interleukin-6, tumor necrosis factor-α, and granulocyte macrophage-colony stimulating factor. However, hu-IFN-γ could not fully protect the clones from cytotoxic effects of encephalomyocarditis virus and vesicular stomatitis virus while mo-IFN-γ could. These results demonstrate that while co-expression of hu-IFN-γR1 and hu-IFN-γR2 is necessary and sufficient for most IFN-γ-induced responses, it is not sufficient to confer a generalized antiviral state. These findings further suggest that additional species-specific accessory factor(s) are necessary for full signaling potential through the IFN-γ receptor complex. The nature and potential role of such factors in IFN-γR signaling is discussed.

Human interferon gamma (hu-IFN-γ) is a pleiotropic cytokine that influences a variety of biological responses including induction of antiviral states, antiproliferative activity, and immunomodulation within responsive cell types (1–5). An early event in each of these responses is the binding of hu-IFN-γ to the hu-IFN-γR1 chain (p90 chain; ligand binding chain) which is encoded by a gene localized on human Chromosome 6 (6). Previous experiments have shown that hamster-human somatic cell hybrids carrying human Chromosome 6 bind hu-IFN-γ with high affinity but do not exhibit detectable biological activity in response to hu-IFN-γ. If, however, both Chromosomes 6 and 21 are present, the hybrids responded to hu-IFN-γ by increasing MHC class I antigen expression (7). These observations were later confirmed by expression of the human (8) and mouse IFN-γR1 chains (9–13) in heterologous cells. The transfected human and mouse IFN-γR1 chains specifically bound hu-IFN-γ and mo-IFN-γ, respectively, but IFN-γ failed to induce biological responses. Subsequent analyses demonstrated that a functional receptor complex could be generated by expression of hu-IFN-γR1 in hamster-human somatic cell hybrids that harbored human Chromosome 21 (14), suggesting that hu-IFN-γ-mediated signal transduction requires species-specific accessory protein(s) encoded by Chromosome 21. This hypothesis was later confirmed by the cloning of the human and mouse IFN-γR2 chains (accessory factor-1, AF-1; also designated IFN-γR8 chain) (15, 16). Expression of hu-IFN-γR2 in hamster-human somatic cell hybrids harboring the hu-IFN-γR1 chain led to MHC class I induction in response to hu-IFN-γ (15). However, the ability of the two chains alone to transduce other IFN-γ-mediated signals was less clear. Previous results showed that the hu-IFN-γR2 chain was unable to confer full antiviral activity against vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) in response to hu-IFN-γ (15, 17–19). Others reported that hu-IFN-γ induces the expression of MHC antigens, 2',5'-oligoadenylate synthetase and protection or partial protection from EMCV and VSV cytopathic effect in a mouse-human somatic cell hybrid that harbored human Chromosome 21 and expressed a transfected hu-IFN-γR1 chain (20, 21). Nevertheless, since it appeared that the presence of both hu-IFN-γR1 and hu-IFN-γR2 chains in hamster cells could not confer sensitivity to hu-IFN-γ for VSV and EMCV protection, Cook et al. (18) postulated that two addi-

The abbreviations used are: hu-IFN-γ, human interferon-γ; mo-IFN-γ, mouse interferon-γ; MHC, major histocompatibility complex; EMCV, encephalomyocarditis virus; VSV, vesicular stomatitis virus; AF, accessory factor; kb, kilobase pair(s); TNF, tumor necrosis factor; IL, interleukin; G-CSF, granulocyte-colony stimulating factor; IRF-1, interferon regulatory factor-1.
tional accessory factors, AF-2 and AF-3, were required for protection of cells against EMCV and VSV, respectively. AF-2 was suggested to also be encoded by a gene on Chromosome 21 because hamster cells containing Chromosome 21 that were transfected with a vector expressing the hu-IFN-γR1 chain exhibited protection against EMCV, but not VSV in response to hu-IFN-γ (18, 19). AF-3 was suggested to be encoded by a different chromosome because VSV protection was not seen in these same cells.

To address the question whether more than one hu-IFN-γ receptor accessory factor is involved in the transduction of the different biological activities by hu-IFN-γ, we derived a mouse macrophage cell line (F10/96) from the bone marrow of a hu-IFN-γR1 transgenic mice. F10/96 was subsequently cloned and transfected with a hu-IFN-γR2 encoding vector. The resultant clones were used to assess the ability of hu-IFN-γR1 and hu-IFN-γR2 to bind ligand and transduce signals necessary for a variety of IFN-γ-induced biological responses.

MATERIALS AND METHODS

Cell Lines—Murine L929 (ATCC CCL 1), WEHI 164 line (ATCC CRL-1751), and human WISH (ATCC CCL 25) fibroblasts cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium supplemented with 2 mM l-glutamine, 10 mM sodium pyruvate, and 10% heat inactivated fetal bovine serum (RPMI complete medium). The NF580 G-CSF-dependent cell line (22) was grown in Iscove's modified Dulbecco's medium supplemented with 2 mM l-glutamine, 5 mM 2-mercaptoethanol (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 7% heat inactivated fetal bovine serum. All media and the associated supplements were from Life Technologies, Inc. (Paisley, Scotland) and 7% heat inactivated fetal bovine serum. All media and the associated supplements were from Life Technologies, Inc. (Paisley, Scotland).

Generation of Transgenic Mice Expressing Hu-IFN-γR1—Transgenic mice were generated by microinjection of a 42-kb SoI genomic fragment containing the gene for hu-IFN-γR1 into zygotes of B6D2F1 mice and re-implanted in B6CBAF1 foster mothers as described (23). Transgene positive mice (B6D2-hR1) were identified by Southern blot analyses in which a 9.5-kb hybridizing band was detected in EcoRI-digested tail DNA after hybridization with a 32P-labeled 1.4-kb cDNA probe encoding hu-IFN-γR1.

Primary Cell Culture and Immortalization of Macrophages—Bone marrow cells from B6D2-hR1 transgenic mice were plated at a density of 106 cells/ml in RPMI complete medium supplemented with 0.5 mM 2-mercaptoethanol. Cells were then infected with the M12-hN21 recombinant retroviral vector carrying an envnull-mycΔMYC fusion gene, as described (24, 25). The infection procedure was carried out as follows: 0.5 μl of supernatant from a 24-h subconfluent culture of Δ2-N11 producer cells was diluted 1:1 with complete medium containing 8 μg/ml Polybrene and incubated with 1–5 × 106 bone marrow cells. After 1 h at 37 °C, the supernatant was diluted with complete medium and half of it was regularly changed twice a week. Medium was supplemented with 10% L929 conditioned medium (containing macrophage-colony stimulating factor) in the first week after infection, reduced to 5% in the second week, and gradually eliminated in the following weeks. About 20 days after infection, the proliferating cells were replated and considered established as continuous cell lines after 10 further passages. Once established, the cells were cloned by limiting dilution technique and grown in complete medium without any additional growth factor.

Cytotransferometric Analysis of Immortalized Clones—Phenotypic analysis of immortalized clones was performed by immunostaining. The monoclonal antibodies 145-2C11 anti-mouse CD3, RM4-5 anti-mouse CD4, 53-6.7 anti-mouse CD8a, 30-H12 anti-CD90 (Thy-1.2), RA3-6B2 anti-CD45R (B220), 14-4-4S anti-mouse MHC class II antigens H-2^d, 2.4G2 anti-mouse CD3/CD16 (P3C6/IIH7 Receptor), M1/74 anti-CD11a (LFA-1α), M1/70 anti-mouse CD11b (Mac-1α chain), GL1 anti-CD86 (B7.2), and RB6-8C5 anti-mouse Gr-1 myeloid differentiation antigen were from PharMingen (San Diego, CA). The monoclonal antibodies M1/42.3.9.8.HLK anti-mouse MHC class I antigens (ATCC TIB 126), 33D1 anti-mouse dendritic cells (ATCC TIB 227), F4/80 anti-mouse macrophages (ATCC HB198), and N418 anti-mouse CD11c (ATCC HB224) and M1/9.3.4.HL.2 anti-mouse common leukocyte antigen (ATCC TIB 180) were prepared from hybridomas obtained from the Tissue Culture Collection (Rockville, MD). The monoclonal antibodies y999 and GR-20 are against hu-IFN-γR1 chain (26) and mo-IFN-γR1 chain (27), respectively.

Indirect immunofluorescence was performed as follows. After washing, 106 live cells were incubated for 30 min at 4 °C with 1 μg of antibody in phosphate-buffered saline with 10% normal mouse serum. Cells were washed and further incubated for 30 min with a fluorescein isothiocyanate-conjugated second step reagent: fluorescein isothiocyanate-conjugated goat anti-rat Ig, fluorescein isothiocyanate-conjugated anti-hamster Ig, or fluorescein isothiocyanate-conjugated anti-mouse Ig (PharMingen). The stained cells were resuspended in 0.3 ml of phosphate-buffered saline with 5% bovine serum albumin and analyzed with a FACScan flow cytometer (Becton Dickinson, Brea, CA, CA). Matched irrelevant murine Ig were used as negative controls.

Cytokines—Recombinant human IFN-γ (hu-IFN-γ) and recombinant mouse IFN-γ (mo-IFN-γ) were produced in Escherichia coli and purified to homogeneity as described (28). They displayed specific antiviral activity of about 7 × 105 and 5 × 105 units/mg of protein, respectively. Recombinant mouse TNF-α and IL-6 were purchased from PharMingen Inc., while recombinant human G-CSF (Neupogen) was produced by F. Hoffmann-La Roche.

Plasmids and Stable Transfections—The plasmid pJS4 is an expression vector containing the cDNA encoding the hu-IFN-γR2 chain (accessory factor-1, AF-1) (15); the plasmid pRc/CMV is an expression vector containing a neor gene for selection of stable transfecants (In Vitro Genetic Systems, Santee, CA). For stable transfections F10/96 were plated at a density of 2 × 105 cells/35-mm tissue culture plate and transfected using LipofectAMINE reagent (Life Technologies, Inc., Paisley, Scotland) according to the manufacturer's instructions. For each transfection 2 μg of pJS4 were co-transfected with 0.2 μg of pRc/CMV. Control clones expressing only the neor phenotype were transfected with pRc/CMV alone. Forty-eight hours after transfection the cells were trypsinized, split at a ratio of 1:10, and plated in medium containing 600 μg/ml antibiotic G418. Proliferating clones were isolated and subcloned 15–20 days later.

Northern Blot and Nucleic Acid Hybridization—Total RNA was prepared from cells according to a modification of the guanidine isothiocyanate method (29). RNA was electrophoresed through a 1.2% agarose gel with 6% formaldehyde and blotted onto Zeta-Probe synthetic nylon membranes. The membranes were hybridized with the following probes: human AF-1 cDNA, hu-IFN-γR2 cDNA (1.8-kb EcoRI fragment), mouse IRF-1 cDNA (960-base pair PvuII/BsmI fragment), and mouse β-actin cDNA (1.1-kb PstI fragment). Hybridization with random primed 32P-labeled cDNA probes was carried out overnight in 50% formamide, 30% formate buffer, 0.5 M NaH2PO4, pH 7.2, 7% SDS. The blots were washed at 65 °C in 1× EDTA, 40 μg/ml NaH2PO4, pH 7.2, 1% SDS and autoradiographed with Hyperfilm MP film and a Cronex Lightning Plus intensifying screens (DuPont, Wilmington, DE) at −80 °C for 24–48 h.

IFN-γ Labeling and Binding Assays—IFNs were iodinated with Na125I using the chloramine T procedure (30) to specific activities ranging from 2 to 6 × 106 cpm/mg of protein. No reduction in biological activity was observed after labeling. The binding assays were performed as described previously (26). Briefly, 105 cells were incubated in duplicate with various concentrations of 125I-huIFN-γ, with or without 100 μg/ml unlabeled IFN-γ, for 2 h on ice in the presence of washed three times and counted in a γ-scintillation spectrometer. Binding data were analyzed by Scatchard analysis.

Nitrite (NO2−), TNF-α, IL-6, and G-CSF Production—Macrophage cell clones were cultured (10 5 per ml) in RPMI-complete medium. After 1 h at 37 °C, an equal volume of medium or of medium with 10 μg/ml lipopolysaccharide and 100 units/ml IFN-γ was added and the plates were incubated for an additional 48 h at 37 °C. Supernatants were harvested and assayed for NO2−, TNF-α, IL-6, and G-CSF content. Nitrite was quantitated as described by Ding and co-workers (31). TNF-α was quantitated by TNF-α-mediated cytotoxicity using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide colorimetric assay on the TNF-α sensitive clone 13 of the WEHI 164 line as described by Espevik et al. (32). This cytotoxicity is inhibited by antibodies to TNF-α and can be quantitated by comparison to a standard curve of TNF-α to pg/ml concentration. IL-6 production was assayed by an enzyme-linked immunosorbent assay test with MPS-20F3 capture antibody and MPS-32C11 detecting antibody, according to the manufacturer's instructions (PharMingen). G-CSF was quantitated by the capacity of supernatants to support the proliferation of NFS60 cells. This G-CSF-dependent proliferation was quantitated by 3-[4,5-di-
Activities Transduced by the IFN-γ Receptor Complex

Fig. 1. Comparison of human and mouse IFN-γ binding to lymphocytes obtained from normal (B6D2) and transgenic mice (B6D2-hyR1) expressing hu-IFN-γR1. Binding of iodinated mouse (upper panel) and human (lower panel) IFN-γ was assessed on spleen (Spl) and thymus (Thy) cells. Binding was measured in the presence of unlabeled irrelevant protein (black bars), hu-IFN-γ (striped bars), and mo-IFN-γ (gray bars). Binding to human PBL was included as a positive control for hu-IFN-γ binding.

methyl thiazol-2-yli-2,5-diphenyl tetrazolium bromide colorimetric assay and calibrated by comparison to a standard curve of G-CSF to pg/ml concentration (33).

Antiviral Assay—Antiviral activity of human and mouse IFN-γ was determined in a bioassay based on the reduction of the cytopathic effect produced by the EMCV or VSV according to Familletti and coworkers (15) reported that the hu-IFN-γR2 mRNA contained the neo' gene or with pRc/CMV alone as negative control. Following G418 selection, four clones: Cl.10, Cl.13, Cl.14, and Cl.28 were found to express hu-IFN-γR2 as assessed by high stringency Northern analyses, while three clones: Cl.17, Cl.20, and Cl.26 were negative (Fig. 4). The high stringency of the washes after hybridization and the absence of any signal for clone Cl.Neo transfected only with the Neo' plasmid (Fig. 4, lane 1), excluded the possibility of a cross-hybridization with the endogenous mo-IFN-γR2 mRNA.

The expression of hu-IFN-γR2 mRNA in the clones varied with respect to the quantity of the transcript detected. In particular, Cl.10 and Cl.28 always produced a more intense band with respect to Cl.13 and Cl.14. The broad shape of the bands is unlikely to be due to mRNA degradation as revealed by the distinct band for β-actin mRNA. In a previous work, Soh and co-workers (15) reported that the hu-IFN-γR2 mRNA migrated slightly below the 18 S ribosomal RNA. In contrast, we observed that the stable transfection of the cDNA encoding the hu-IFN-γR2 produced a transcript that migrates just above the 18 S. This discrepancy is likely due to an acquisition of some vector sequences by the hu-IFN-γR2 mRNA.

The ability of the reconstituted hu-IFN-γR (co-expression of chains R1 and R2) on F10/96 mouse macrophages to transduce signals was initially assessed by observing the changes in cell surface MHC class I and class II antigens in response to exogenously added hu-IFN-γ. As shown in Figs. 5 and 6, addition of hu-IFN-γ to cultures of Cl.10 and Cl.28, but not Cl.Neo, increased the expression of MHC class I and class II antigens. Similar positive results were obtained for clones Cl.13 and Cl.14 (data not shown). As expected, Cl.Neo lacking hu-IFN-γR2 failed to respond to hu-IFN-γ but retained the ability to up-regulate expression in response to mo-IFN-γ. Taken together these results demonstrate that clones Cl.10, Cl.13,
Cl.14, and Cl.28 express hu-IFN-\( \gamma \)R1 and hu-IFN-\( \gamma \)R2 chains as defined by their ability to enhance MHC-associated antigen expression in response to hu-IFN-\( \gamma \).

The Hu-IFN-\( \gamma \)R2 Chain Does Not Contribute to Hu-IFN-\( \gamma \) Binding—There is little biochemical data on the involvement of hu-IFN-\( \gamma \)R2 in the binding of hu-IFN-\( \gamma \) in the context of a mouse cell. The availability of F10/96 expressing only the hu-IFN-\( \gamma \)R1 chain, and the clones expressing both chains (hu-IFN-\( \gamma \)R1 and hu-IFN-\( \gamma \)R2), allowed us to study the contribution of hu-IFN-\( \gamma \)R2 to the binding affinity of the receptor complex. As shown in Table I, the results of Scatchard analysis of ligand binding to either F10/96 cells (Cl.Neo) or hu-IFN-\( \gamma \)R2-vector transfected counterparts (Cl.10) demonstrated that all cells examined exhibited comparable capacity to bind hu-IFN-\( \gamma \) (\( K_d \approx 8 \times 10^{-11} \) M). This indicates that hu-IFN-\( \gamma \)R2 does not alter significantly the affinity of the receptor complex for ligand. A similar affinity was also observed for binding of hu-IFN-\( \gamma \) to human WISH cells.

Human IFN-\( \gamma \) Responsiveness of F10/96 Clones Expressing Both Hu-IFN-\( \gamma \)R1 and Hu-IFN-\( \gamma \)R2 Chains—Because the biological activity exerted by IFN-\( \gamma \) may vary according the type of target cells, it seemed inappropriate to define receptor competence on the basis of a single parameter such as enhanced expression of MHC products. Accordingly, we investigated the ability of reconstituted receptors on F10/96 macrophages to transduce signals for a variety of classically defined IFN-\( \gamma \)-induced responses. One such response is defined by the ability of IFN-\( \gamma \) to up-regulate expression of interferon regulatory factor-1 (IRF-1) mRNA, a potent regulator of IFN-inducible genes necessary for the establishment of IFN-\( \gamma \)-induced anti-viral and anti-proliferative responses (35). Using a protocol similar to that developed by Hemmi and co-workers (16) we demonstrated that exposure of clones (Cl.Neo, Cl.10, Cl.14, and Cl.28) to mo-IFN-\( \gamma \) for 20 h, dramatically increased the steady
state levels of the IRF-1 mRNA (Fig. 7). Human IFN-γ also induced IRF-1 expression, but only among those clones expressing both hu-IFN-γ-R chains. The ability to up-regulate expression requires hu-IFN-γ-R2 as demonstrated by Cl.Neo that responds to murine ligand only, despite expressing the hu-IFN-γ-R1 binding chain.

A second measure of IFN-γ-R-mediated signaling in macrophage populations is the ability of IFN-γ to induce production of the cytotoxic effector molecules TNF-α and nitrite (NO₂⁻). The activation of the inducible nitric oxide synthetase pathway by IFN-γ was determined by measuring NO₂⁻ release following co-stimulation with lipopolysaccharide and either human or mouse IFN-γ. Analyses of F10/96 clones Cl.10 and Cl.14 revealed comparable amounts of NO₂⁻ production when the clones were exposed to either species of IFN-γ (Fig. 8A). In contrast, Cl.Neo released NO₂⁻ in response to mo-IFN-γ only, further suggesting that both human chains are necessary for full signaling potential. Similar patterns of secretion are seen with TNF-α as measured by cytotoxic activity on WEHI 164.13 cells (Fig. 8B).

As an alternative measure of IFN-γ-mediated activation of F10/96, we have quantitated the production of IL-6 and G-CSF following co-stimulation with lipopolysaccharide (Fig. 8). Levels of secreted proteins were assessed by IL-6-specific enzyme-linked immunosorbent assay (Fig. 8C), and by bioassay measuring enhanced proliferation of NFS-60 cells in response to exogenous G-CSF (Fig. 8D). The results clearly demonstrate that IL-6 and G-CSF are secreted in response to both murine and human IFN-γ, although it should be noted that, in the case of G-CSF, significantly lower levels were detected when human IFN-γ was used compared to murine ligand. The reason for this difference remains unclear but appears to be a generalized phenomenon also affecting secretion of TNF-α and IL-6, albeit to a lesser extent than that seen with G-CSF.

Taken together, these results demonstrate that a variety of IFN-γ-mediated macrophage responses are critically dependent upon co-expression of hu-IFN-γ-R1 and hu-IFN-γ-R2. These receptor chains are both necessary and sufficient for hu-IFN-γ to up-regulate expression of MHC antigens, increase steady state levels of IRF-1, and enhance production of NO₂⁻ radicals, TNF-α, IL-6, and G-CSF.

Antiviral Activity of Hu-IFNγ on F10/96 Clones Expressing Both Subunits of the Hu-IFNγR Complex—A classic effect of IFN-γ treatment in vitro and in vivo is the induction of an antiviral state in which cells develop a transient resistance to infection by viruses that would otherwise be cytopathic. To determine if the reconstituted hu-IFN-γR expressed in F10/96 cells can transduce signals required for establishment of a resistant state, we attempted to infect F10/96 clones with either VSV or EMCV in the presence and absence of IFN-γ. The measure of resistance or protection used in these experiments is reported as the relative specific activity (units/mg of protein) of a stock solution of murine or human IFN-γ on the indicated cell types. One unit is equivalent to the amount of IFN-γ needed to confer 50% resistance to the cytopathic effects of a given virus. As shown in Table II, the mouse cell line L929 and human WISH cells are protected by mo-IFN-γ and hu-IFNγ, respectively, in a species-specific manner. Surprisingly, the F10/96-derived clones, which were consistently protected with mo-IFN-γ by virtue of their expression of mo-IFN-γ receptors, display a diverse response to hu-IFNγ. The control cells expressing hu-IFN-γ-R1 only (Cl.Neo) are not protected by hu-IFN-γ suggesting that, like other responses, the induction of an antiviral state requires the co-expression of hu-IFN-γ-R2. Among clones expressing both the chains of hu-IFN-γ-R (Cl.10, Cl.13, and Cl.14), protection from EMCV by hu-IFN-γ was evident, albeit at concentrations 100-fold higher than that needed to protect human WISH cells. Cl.14 and Cl.10 were unique in...
their ability to be protected from VSV-induced cytolysis, although it should be noted that the protection of Cl.10 was variable with resistance detected in only 2 out of 6 experiments. Cl.28 was not protected from either EMCV or VSV infection. These results demonstrate that the reconstituted hu-IFN-\(\gamma\)R is necessary but not sufficient to confer resistance to EMCV and VSV.

**DISCUSSION**

Early studies on the composition of the human IFN-\(\gamma\) receptor complex suggested that the hu-IFN-\(\gamma\)R2 chain encoded by human Chromosome 21 interacts with the extracellular domain of the hu-IFN-\(\gamma\)R1 chain in a species-specific manner (20, 21, 36–38). More recently, Kotenko et al. (38) demonstrated that this interaction is dependent on hu-IFN-\(\gamma\) as evidenced by the inability to cross-link hu-IFN-\(\gamma\) with ligand in the absence of hu-IFN-\(\gamma\)R1. Reconstitution of the receptor complex by transfection of hu-IFN-\(\gamma\)R1 into somatic cell hybrids harboring human Chromosome 21, or transfection of hu-IFN-\(\gamma\)R2 into hamster cells expressing hIFN-\(\gamma\)R1 rendered both cell types responsive to hIFN-\(\gamma\) as measured by up-regulation of MHC class I antigens (14, 15, 17, 19–21, 36, 37). Although functional, these receptor complexes were unable to transduce the IFN-\(\gamma\) signal to protect the cells from the cytopathic effects of VSV, and provided only partial resistance to EMCV (15, 19, 21). Taken together, these results raise several questions regarding the contribution of hu-IFN-\(\gamma\)R2 to the binding of ligand, as well as the spectrum of IFN-\(\gamma\) biological activities transduced by the reconstituted receptor complex.

To further study the functional properties of the hu-IFN-\(\gamma\)R2 chain in response to IFN-\(\gamma\), we derived a murine macrophage cell line, F10/96, from the bone marrow of B6D2-h\(\gamma\)R1 transgenic mice expressing the hu-IFN-\(\gamma\)R1 chain. This line and its derivative clones retain the macrophage phenotype and are particularly useful in that a wide variety of IFN-\(\gamma\) induced responses, not seen with other cells, can be studied and independently quantitated. As expected, F10/96 cells express only the hu-IFN-\(\gamma\)R1 chain of the human receptor complex and bind ligand with an affinity comparable to that observed for the endogenous receptor on human WISH cells (\(K_d = 8 \times 10^{-11} \text{ M}\)). In the absence of hu-IFN-\(\gamma\)R2, no biological responses were
noted except those transduced by mo-IFN-γ and its endogenous receptor.

The expression of both hu-IFN-γR subunits (e.g., hu-IFN-γR1 and hu-IFN-γR2) by F10/96 transfectants did not significantly alter the number of binding sites for hu-IFN-γ or the affinity of the receptor. We conclude that hu-IFN-γR1 is predominately responsible for ligand binding and that addition of hu-IFN-γR2 to the complex does not significantly affect the binding potential of hu-IFN-γR1. These findings are consistent with results reported for hamster (38) in which expression of hu-IFN-γR2 alone was not sufficient to allow ligand binding. The studies of Kotenko et al. (38, 39) showed that IFN-γ can be cross-linked to hu-IFN-γR1 and hu-IFN-γR2 receptor chains, but that the presence of the hu-IFN-γR1 chain is required for cross-linking to hu-IFN-γR2. Furthermore, the overall association constant for IFN-γ receptor binding does not significantly increase when both chains are present compared to when the IFN-γR1 chain only is present.

The functional studies on mouse macrophage clones expressing both hu-IFN-γR1 and hu-IFN-γR2 chains identified a number of biological activities that appear dependent on the presence of the second chain. The expression of both hu-IFN-γR subunits rendered the mouse macrophages sensitive to hu-IFN-γR2 to confer resistance in all mouse macrophage clones tested. In particular, only Cl.14 was protected from the cytopathic effects of both VSV and EMCV. In contrast, Cl.13 was protected from EMCV only, while Cl.28 was found susceptible to both viruses tested. Protection of Cl.10 from EMCV infection was not evident in all the experiments. It is noteworthy that the specific antiviral activity of the hu-IFN-γR determined on responsive mouse macrophage clones was at least 2 orders of magnitude less than the specific activity determined on human WISH cells. The failure of hu-IFN-γR2 to confer resistance in all clones tested may be explained in several ways. First, it is possible that qualitative or quantitative differences in the expression of human receptor chains may account for the inter-clonal variation in the ability of IFN-γ to induce antiviral states. Consistent with this view are our Northern analyses in which clones express distinct sizes and quantities of hu-IFN-γR2 transcripts. The relevance of these differences remains unclear since they do not appear to correlate with the ability of hu-IFN-γ to induce an antiviral state.

It is also possible that the inability of hu-IFN-γR1 and hu-IFN-γR2 to confer full IFN-γ responsiveness is due to the need for additional protein(s) that specifically confer antiviral states but are not required for IFN-γ-induced MHC antigen up-regulation, enhanced IRF-1 expression, or increased secretion of NO₂, TNF-α, IL-6, or G-CSF. Such proteins may be a novel accessory chain of the receptor complex as previously suggested by Cook et al. (17). Like IFN-γR1 and IFN-γR2 chains recruit, respectively, JAK-1 and JAK-2 into the ligand-assembled receptor complex (38, 40, 41), the putative accessory factor may start an independent signal transduction pathway for IFN-γ antiviral activity. Alternatively, the additional protein(s) may represent an independent membrane bound receptor that is induced by IFN-γ through the known IFN-γR1-R2 complex. The novel factor involved in the IFN-γ-induced antiviral activity may also be a cytoplasmic/nuclear protein that functions in parallel to the established JAK/STAT pathway that is responsible for many IFN-γ-induced responses. Regardless of the nature of this accessory protein it may not be species-specific.

![Graph](image)

**Fig. 7.** Effect of hu-IFN-γR KL on steady state levels of IRF-1 in F10/96 cells. Indicated clones were cultured in the absence or presence of 1000 units/ml of either hu-IFN-γ or mo-IFN-γ for 20 h. Total RNA was extracted and used for Northern blotting analysis. The same filter was sequentially hybridized with a mouse β-actin cDNA probe to demonstrate equal RNA loading.

![Graph](image)

**Fig. 8.** IFN-γ-induced secretion of NO₂, TNFα, IL-6, and G-CSF in F10/96 clones. Cells were cultured in the absence or presence of a mixture of 10 μg/ml lipopolysaccharide and 100 units/ml of either hu-IFN-γ or mo-IFN-γ for 48 h at 37°C. The concentration of NO₂, TNFα, IL-6, and G-CSF in the supernatants was assessed as described. Human IFN-γ (black bars) induces the production of NO₂ (panel A), TNF-α (panel B), IL-6 (panel C), and G-CSF (panel D) in mouse F10/96 macrophage clones Cl.10 and Cl.28 expressing both subunits of hu-IFN-γR. Mouse IFN-γ (gray bars) induces the production of NO₂, TNFα, IL-6, and G-CSF in either mock transected F10/96 cells (Cl.Neo) or in Cl.10 and Cl.14 cells. White bars show the basal production by non-activated cells.

| Table II | IFN-γ-induced protection of L929, WISH, and F10/96 clones from cytopathic effects of EMCV and VSV |
|----------|------------------------------------------------------------------------------------------|
| Treatment | L929                           | Wish                           | F10/96 clones          |
|          | EMCV | VSV | EMCV | VSV | EMCV | VSV | EMCV | VSV | EMCV | VSV | EMCV | VSV |
| Mo-IFN-γ | 5 × 10⁶ | 5 × 10⁶ | 0 | 0 | 3 × 10⁶ | 2 × 10⁶ | 10⁶ | 10⁶ | 10⁶ | 10⁶ |
| Hu-IFN-γ | 0 | 0 | 10⁷ | 10⁷ | 0 | 0 | 10⁵ | 10⁵ | 10⁵ | 10⁵ | 0 | 0 |

*Indicated cell types were treated with IFN-γ to evaluate protection against indicated viruses. The measure of resistance or protection used in these experiments is reported as the relative specific activity (units/mg of protein) of a stock solution of murine or human IFN-γ on the indicated cell types.
since hu-IFNγ can induce antiviral states in some clones. It is yet to be determined why the virus-resistant clones have retained expression of this putative chain while susceptible clones have not. Clearly additional analyses of extra- and intracellular signaling pathways are necessary to fully define both the composition of the hu-IFN-γR complex and the specificity of signals imparted by the receptor. The availability of the mouse macrophage cell clones expressing hu-IFN-γR1 and hu-IFN-γR2 in the absence of background human genes will be invaluable in addressing these and other questions relating to the diverse spectrum of IFN-γ-induced responses.

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