FUNCTION AND REGULATION OF A MURINE MACROPHAGE-SPECIFIC IgG Fc RECEPTOR, FcγR-α

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Receptors for the Fc portion of IgG (FcγR)[1] provide a link between humoral and cellular immune response by targeting antibody/antigen complexes to effector cells. Crosslinking of these receptors on macrophages results in a wide array of cellular responses, which include phagocytosis, secretion of reactive oxygen intermediates, and lysosomal hydrolases, and ultimately mediates antibody-dependent cellular cytotoxicity (ADCC) (1). Studies on mouse macrophages have defined three distinct FcγRs: a low-affinity, trypsin-resistant receptor that binds IgG1/IgG2b (2, 3), a high-affinity trypsin-sensitive receptor for monomeric IgG2a (4), and a receptor for IgG3 (5). Characterization of the IgG1/IgG2b FcγR has been facilitated by the mAb 2.4G2, which blocks ligand binding to this receptor (6). In addition to its expression on macrophages, this FcγR is expressed on lymphocytes, where it displays broader ligand specificities, binding IgG1, IgG2b, as well as IgG2a (7). Its role on lymphocytes is not well defined but it is presumed to be involved in immune regulation (8).

This functional heterogeneity of the IgG2b/IgG1 FcγR has been addressed at the molecular level by the characterization of two genes that encode this receptor, referred to as FcγR-α and FcγR-β (9–11). These genes encode predicted transmembrane proteins with nearly identical extracellular domains, while differing completely in the transmembrane and cytoplasmic domains. Alternative splicing of the β gene results in two transcripts (β1 and β2) that differ in the cytoplasmic domain. The FcγR-α transcript is expressed only in macrophages, while the FcγR-β transcript is found in both macrophages and lymphocytes. Thus, the functional diversity displayed by this receptor on different cell types may result from the structural heterogeneity encoded in these two genes and their alternative transcripts.

To begin to address the role of this structural heterogeneity in mediating different FcγR functions, we have studied the contribution of FcγR-α and -β gene expression for two phenotypes, ligand specificity and macrophage activation. Through the transfection of these genes into FcγR negative cells, the contribution of different structural domains was assessed in determining ligand specificity. Further, we have iso-

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[1] *Abbreviations used in this paper:* α-MEM, alpha-modified minimum Eagle's medium; ADCC, antibody-dependent cellular cytotoxicity; FCγR, IgG Fc receptor.

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lated a mutant cell line derived from J774, which expresses only FcγR-α. This cell line is used as a model to investigate the function of the macrophage-specific FcγR-α. By treating those cells, as well as other macrophage-like cell lines and resident mouse peritoneal macrophages, with IFN-γ, we have assessed the role of these two genes in the process of macrophage activation. Treatment of macrophages with this lymphokine has been shown to result in increased phagocytosis of IgG-opsonized SRBC (12) and stimulation of the FcγR-mediated respiratory burst and ADCC (13). In this study we demonstrate the selective expression of FcγR-α induced by IFN-γ in these cell lines, which concommitantly become competent in the phagocytosis of antibody-coated particles. These data suggest that it is FcγR-α that plays a major role of binding antibody/antigen complexes and mediating phagocytosis by macrophages.

Materials and Methods

Cell Culture. The macrophage-like cell lines RAW 264.7 (14), J774 (15), and P388D1 (16) were grown in alpha-modified MEM (α-MEM) supplemented with 10% FCS (HyClone Laboratories, Logan, UT) heat inactivated at 56°C for 30 min, 100 U/ml penicillin, and 100 μg/ml streptomycin. Primary cultures of mouse peritoneal macrophages were established from pathogen-free female ICR mice (Charles River Breeding Laboratories, Kingston, NY) weighing 25-30 g as previously described (17). Cells were cultured overnight before use in α-MEM containing 10% FCS.

Gene Transfer. Coding sequences of the various cDNAs were cloned into the SmaI site of the eukaryotic expression vector pcEXV-3 (18). Transcription of the cloned sequences was under the control of the SV40 early promoter. LtK$^-$ cells were plated 24 h before transfection at 2×10^5 cells per 60-mm petri dish. The next day, the medium was removed and discarded, and the monolayers were washed twice with PBS. The cells were then incubated with serum-free DME containing DEAE-dextran (mol wt, 2×10^6) at a concentration of 150 μg/ml along with the plasmid DNA containing FcγR sequences at 8-15 μg/ml. After a 16-h exposure of the cultures to the DNA at 37°C in a CO_2 incubator, the cells were washed once with PBS and fed with α-MEM supplemented with 10% FCS. At ~60 h after the cultures were transfected, the cells were assayed for expression of surface FcγRs.

B78H1 cells were plated 24 h before transfection at 2×10^5 cells per 100-mm petri dish. High-molecular weight human DNA (19 μg) was mixed with pGCcos3neo (500 ng), the recombinant expression vector (1.5 μg) as described above, and was allowed to form calcium phosphate precipitates. 1 ml of precipitate was added per dish and incubated at 37° for 16 h. The medium was removed and replaced with selective medium containing 1 mg/ml of Geneticin (G418, Gibco, Grand Island, NY). Plates were refed every 2 d and resistant colonies were screened at ~10 d by rosetting with human erythrocytes conjugated with the mAb 2.4G2 (19). Alternatively, screening was performed with SRBC opsonized with rabbit antiserum to SRBC.

Preparation of Opsonized Erythrocytes, Rosetting, and Phagocytosis Assay. Sheep erythrocytes (Gibco) were first derivatized with the hapten TNP, as previously described (20). They were then incubated with the monoclonal anti-DNP U-7-6 (IgG1), U-7-27-1 (IgG2a), or U-12-5-1 (IgG2b) (gifts of Dr. Zelig Eschar, Weitzmann Institute, Israel) Opsonized SRBC were incubated with the transfected cell lines for 30 min at room temperature and then washed extensively with PBS. Rosetting for phagocytosis studies was performed with SRBC opsonized with rabbit serum to SRBC (Cappel Laboratories, Malvern, PA) at a non-agglutinating titer of antibody. After a 90-min incubation at 37°C, the macrophage cells were washed in PBS and then again in distilled water to hypotonically lyse extracellular rosetted SRBC. The cells were then fixed in 0.25% glutaraldehyde and were photographed.

IFN-γ Induction. The murine IFN-γ used in this study was highly purified recombinant protein synthesized in *Escherichia coli* and was generously provided by Genentech. The mouse IFN-γ had a specific activity of 1.9×10^7 U/mg. Highly purified mouse IFN-α and IFN-β were purchased from Lee-BioMolecular Research Laboratories, Inc., San Diego, CA. The mouse IFN-α had a specific activity of 1.7 × 10^6 International Reference Units per mg, and the mouse IFN-β had a specific activity of 5.3 × 10^7 International Reference Units per mg. The macrophage lines J774, RAW 264.7, and P388D1 were induced just before confluence in their regular cell growth media with either 200 U/ml IFN-γ or 500 U/ml IFN-α or -β. Resident mouse peritoneal macrophages were incubated in 100-mm culture dishes for 5 h after isolation. After attachment, the cells were washed twice with PBS and then incubated with 200 U/ml IFN-γ.

Iodination and Binding Studies. Purified Igs IgG1 (MOPC 21, Miles Laboratories Inc., Naperville, IL), IgG2a (UPC 10, Bionetics Laboratory Products, Kensington, MD), IgG2b (MOPC 195, Miles Laboratories Inc.), IgG3 (FLOPC 21, Bionetics Laboratory Products), and IgA (MOPC 315, Bionetics Laboratory Products) were iodinated using 50 μg of each protein and 1.0 mCi of ^{125}INa (Amersham Corp., Arlington Heights, IL) using Iodo-Beads (Pierce Chemical Co., Rockford, IL) as described (21). B78-FcγR-transfected cell lines were plated in 60-mm culture dishes and incubated with either monomeric ^{125}I-labeled Igs or heat aggregated Igs that were prepared by mixing an excess of nonlabeled Ig (1 mg/ml) with the corresponding ^{125}I-labeled protein for 45 min at 65°C. After incubation, the cells were washed three times with PBS and then solubilized with IN NaOH for gamma counting.

For direct binding studies on macrophage cell lines, 2.4G2 was iodinated as above and incubated in suspension with 10^6 cells per ml for 90 min at 4°C. Cells were washed by pelleting and resuspension, and bound radioactivity was determined by gamma counting. Indirect binding was performed with nonlabeled 2.4G2 and assayed with ^{125}I-labeled affinity-purified goat anti-rat IgG (Cappel Laboratories).

Northern Blot Analysis. RNA was isolated by the guanidine isothiocyanate procedure and was poly(A) selected (22). Poly (A^+) RNA was fractioned on a 1% agarose gel containing 2.2 M formaldehyde and was transferred to nitrocellulose (23). Nitrocellulose filters were hybridized at 42°C for 16 h in a solution containing 50% formamide, 10% dextran sulfate, 5 × SSC (1 × SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 1 × Denhardt's (0.02% polyvinyl-pyrrolidone, 0.02% Ficoll, and 0.02% BSA), and 200 μg/ml sonicated herring sperm DNA. The filters were washed at 50°C in 0.1 × SSC containing 0.1% SDS and exposed at −70°C to Kodak XAR film in the presence of intensifying screen (Cronex Lightning Plus).

Results

Expression of FcγR-α and FcγR-β by Transfection in FcγR-negative Cell Lines. The structural requirements for ligand binding of the FcγR-α and -β molecules was deter-

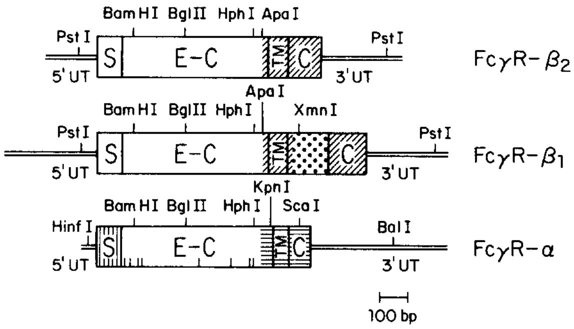
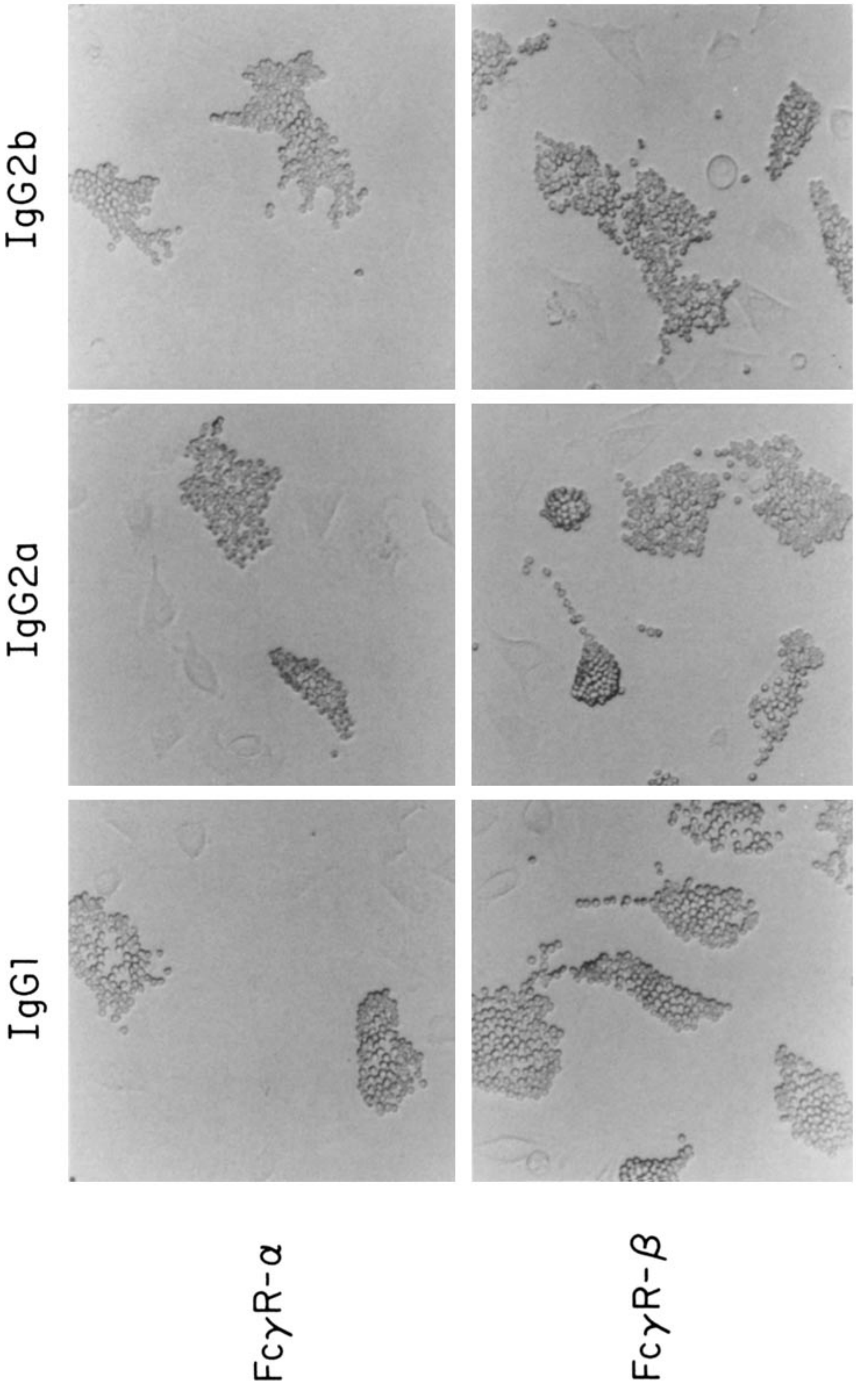


FIGURE 1. Structure and restriction map of FcγR cDNA. The coding region of the cDNA is divided into a proposed signal sequence (*S*), extracellular domain (*E-C*), transmembrane domain (*TM*), and a cytoplasmic domain (*C*). Vertical marks within the E-C of FcγR-α represent single amino acid changes between FcγR-α (*E-C*) and FcγR-β (*E-C*). Double lines are used to indicate 5' and 3' untranslated sequences (*UT*). Representative restriction sites are indicated. FcγR-β2, FcγR-β1, and FcγR-α are cDNA clones derived from lymphocyte and macrophage libraries (9). All cDNA were cloned into the expression vector pcEXV-3 for transfection experiments as described in Fig. 2. The structural relationships among these FcγR cDNA are schematically illustrated, demonstrating conservation of the E-C among all the transcripts, divergence of the S, TM, and C domains, and the alternative splicing relationship of β_1 and β_2 in the cytoplasmic domain.


IgG2b
IgG2a
IgG1
FcγR-α
FcγR-β

mined by expression of cDNA for α and β in FcγR negative cell lines. Expression of these cDNA sequences (Fig. 1) was achieved by inserting their entire coding sequences into an expression vector (pcEXV-3) (18) in which the SV40 early promoter is used to achieve transcription of the heterologous sequences. We have previously shown that the transfection of FcγR-β_2 resulted in the establishment of stable lines that avidly bound SRBC opsonized with rabbit IgG (9). Furthermore, a complete cytoplasmic domain is apparently unnecessary for the surface expression of the FcγR-β gene product, as determined by the expression of a truncated form of the FcγR-β_1 (data not shown). FcγR-α and -β share 95% identity in their extracellular domain (9) with no homology in either the serine/threonine rich region amino terminal to the transmembrane domain, the transmembrane domain, or the cytoplasmic domain. To determine the contribution of these structural differences to ligand binding, we have compared the FcγR-α and -β gene products as expressed by transfection of FcγR negative cells.

Expression of FcγR-α was achieved in mouse LtK$^-$ cells and COS (monkey kidney) cells and was detected by rosetting with IgG-opsonized sRBC 60 h after introduction of these genes in a transient expression system (not shown). The frequency of transient surface expression of the transfected FcγR-α was observed to be lower than that observed for FcγR-β, although the intensity of rosetting on an FcγR-α positive cell was the same as was observed for FcγR-β (see Fig. 2). This difference in transfection frequency of the FcγR-α and -β constructs may result from the contribution of 3′ untranslated sequences of FcγR-α (R. Weinshank, unpublished observations). Both receptors were inhibited in their ability to bind ligand by the mAb 2.4G2 (not shown), as predicted by their highly homologous extracellular domains (9). Thus, the structural requirements for immune complex binding are encoded in either FcγR-α or -β molecules alone.

IgG Subclass-specific Binding to FcγR-α and -β. Lymphocytes, which express FcγR-β only, have been reported to bind mouse IgG1, 2a, and 2b immune complexes, (7) while macrophages, which express FcγR-α and -β, display a more restricted binding with specificity for IgG1 and 2b (6). To determine if this observed difference in IgG immune complex binding to the FcγR is encoded by the structural differences found in FcγR-α and -β, the subclass specificity of IgG immune complex binding was determined on the transfectants expressing these molecules. Mouse mAbs of defined subclasses specific for DNP were reacted with SRBC derivitized with TNP (20). LtK$^-$ cells transiently transfected with FcγR-α or -β constructs were assayed for

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FIGURE 2. Subtype specificity of FcγR-α and FcγR-β_2 transiently expressed in transfected LtK$^-$ cells. An 1,100-bp HinfI-BalI fragment derived from the FcγR-α clone and a 1,300-bp PstI fragment derived from the FcγR-β_2 clone (9) were cloned into the SmaI site of the eukaryotic expression vector pcEXV-3. The recombinant plasmids were transfected onto mouse LtK$^-$ cells by the DEAE-dextran technique (45) using 3 μg/ml of pcEXV-3-β_2 and 15 μg/ml of pcEXV-3-α. After a 60-h incubation, the cells were assayed for isotype specificity using subclass-defined mAbs directed against the hapten DNP. SRBC were coupled to TNP (20) and incubated with the indicated mAb. The opsonized RBC were incubated with the transfected Ltk$^-$ cells for 30 min at room temperature and then washed several times with PBS. The cells were then fixed with 0.25% glutaraldehyde and scored for rosette formation. Identical results were obtained in COS (monkey kidney) cells; however, transfection of a 1,300-bp Pst I fragment of FcγRα in pcEXV-3 (9) did not result in observable surface expression of FcγR-α protein in Ltk$^-$ or COS cells, nor did expression of the 1,100-bp Hinf-Bal fragment result in expression of FcγR-α in B78H1 melanoma cells.

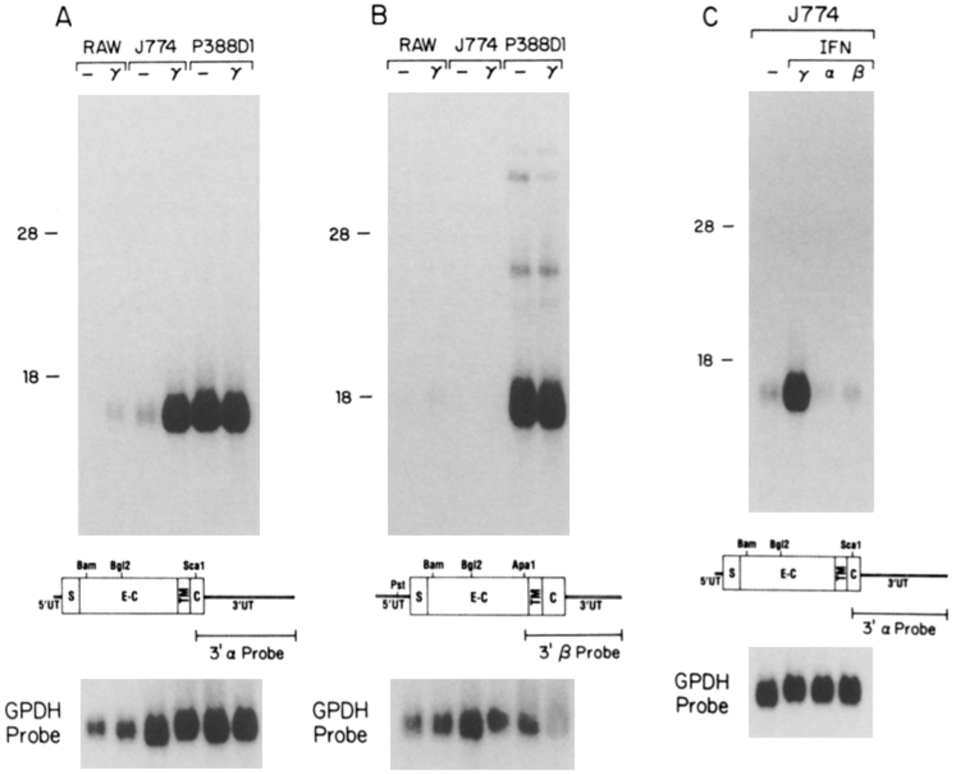

FIGURE 3. IFN inducibility and specificity of FcγR-α and FcγR-β transcripts in macrophage-like cell lines. Macrophage cell lines RAW 264.7, J774a, and P388D1 were maintained in culture as described in Materials and Methods. The cells were treated with 200 U/ml of IFN-γ for 18 h after which RNA blots were prepared as described and hybridized under stringent conditions either with the FcγR-α-specific probe (*A*) or the FcγR-β-specific probe (*B*). RNA was also isolated from J774a cells that had been treated with 500 U/ml of either IFN-α or IFN-β for 18 h (*C*). The ribosomal RNA markers are indicated. Poly(A)$^+$ RNA levels were normalized by reprobing the blots with GPDH.

binding to these subclass specific immune complexes. Binding was observed to IgG1-, IgG2a-, and IgG2b-coated SRBC for both FcγR-α and -β transfectants (Fig. 2), while no binding was observed for IgG3 or monomeric IgG (not shown). The binding of these IgG subclasses was inhibitable by the FcγR-specific mAb 2.4G2 (not shown). In addition to an identical specificity of binding, there were no differences in the numbers of bound SRBC for either receptor. Thus, the structural differences found between α and β do not contribute to differences in ligand specificity when expressed on heterologous cells.

Identical results were obtained in stable transfectants of B78H1 melanoma cells expressing the FcγRβ2 molecule. As was observed in the transient assays, IgG monomeric binding was not observed for this molecule (not shown), while binding of heat-aggregated IgG1, 2a, and 2b was observed. Again, similar to what was shown in the transient expression studies (Fig. 2), rosetting of this stable line with IgG1-, 2a-,

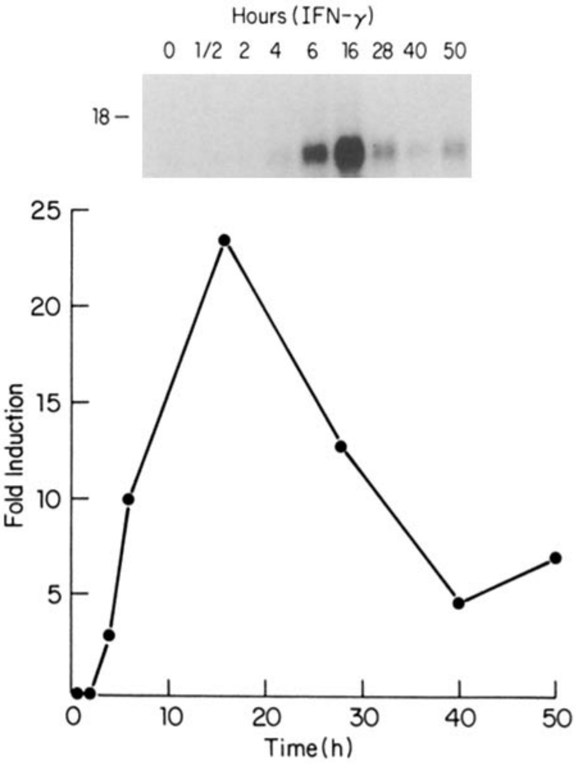

FIGURE 4. Time course for FcγR-α mRNA induction by IFN-γ in J774a cells. J774a cells were treated with 200 U/ml of IFN-γ for the indicated times, after which RNA blots were prepared as described and hybridized under stringent conditions with the FcγR-α-specific probe. Quantitation of the RNA blot was performed by densitometer scanning (fold induction = densitometer units induced/densitometer units uninduced) after normalization with the constitutive probe GPDH.

and 2b-coated SRBC was observed (not shown). No binding was detected for heat-aggregated IgG3 or IgA, consistent with the previous observation (5) that these isotypes bind to separate Fc receptors.

Differential Expression of FcγR-α and -β in Macrophage Activation. To investigate the contribution of α and β gene expression to different FcγR-mediated phenotypes, we have characterized their expression and regulation in macrophage activation. Previous studies have defined IFN-γ as a macrophage activating factor (MAF [25, 26]). Three macrophage lines were used in this study. Two of the cell lines, RAW 264.7 (14) and P388D1 (16), have previously been described. A third cell line is a spontaneous mutant derived from J774 (15). This cell line differs from its previous description (9) in that it no longer expresses FcγR-β. This cell line is designated J774a. Treatment with mouse IFN-γ at a concentration of 200 U/ml for 16 h induced the FcγR-α mRNA from undetectable levels in RAW 264.7 cells while inducing FcγR-α mRNA ~25-fold in J774a cells (Fig. 3 *A*). The macrophage-like cell line P388D1 expressed high levels of FcγR-α and -β mRNA constitutively that were unaffected by IFN-γ. RAW cells expressed low levels of FcγR-β mRNA that were similarly unaffected by IFN-γ, while J774a cells could not be induced to express detectable FcγR-β mRNA (Fig. 3 *B*). Induction of FcγR-α mRNA levels in J774a was specific for IFN-γ and could not be demonstrated with either mouse IFN-α or -β at the concentrations tested (Fig. 3 *C*). A time course for induction of FcγR-α mRNA in J774a cells was performed and demonstrated a threefold increase in steady-state message levels as early as

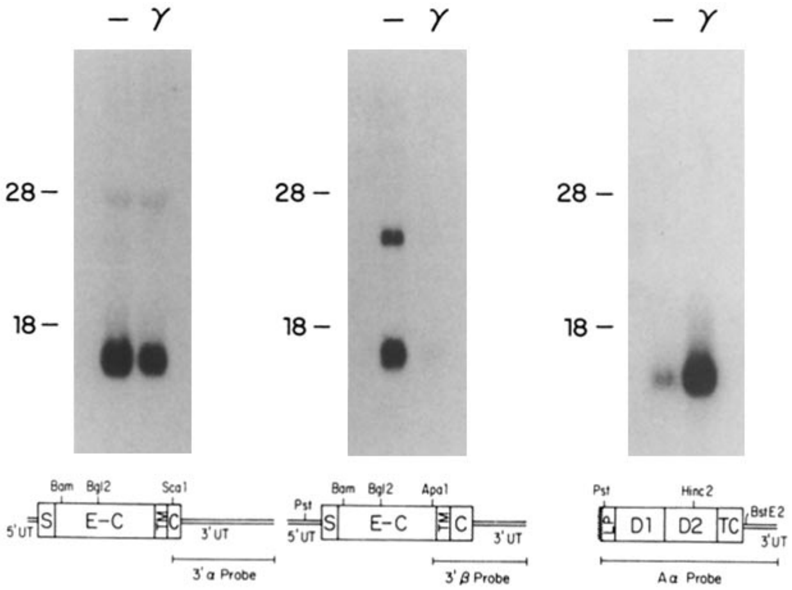

FIGURE 5. FcγR mRNA levels in resting and IFN-γ-activated mouse peritoneal macrophages. Primary cultures of peritoneal macrophages were treated with 200 U/ml of IFN-γ for 18 h, after which RNA was isolated from control and treated cells by the guanidine isothiocyanate procedure. Total RNA (10 μg) was fractionated on agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized under stringent conditions either with the FcγR-α-specific probe, the FcγR-β-specific probe, or a cDNA probe for the class II A-α murine Ia heavy chain (24). Hybridization to a larger RNA transcript (∼3.0 kb) with the 3′ FcγR-β gene-specific probe has been observed to be of variable intensity in macrophage-like cell lines and may represent either a β gene precursor transcript or crosshybridization to another related gene transcript. RNA levels were normalized by both ethidium bromide staining and reprobing the blots with a cDNA probe against glyceraldehyde 3-phosphate dehydrogenase, an enzyme that is not modulated by IFN (34). Quantitation of the RNA blot was performed by densitometer scanning.

4 h, with maximal induction of ∼25-fold at 16 h, after which a decline in these levels was observed (Fig. 4). The expression of FcγR-α was then maintained at a 5–7-fold increase after 50 h of continuous IFN-γ treatment.

To evaluate the significance of the FcγR-α induction in these macrophage-like cell lines, the effect of IFN-γ on FcγR-α and -β gene expression in mouse peritoneal macrophages was determined at 18 h of IFN-γ stimulation, the peak of the response observed in J774a cells. Both FcγR-α and FcγR-β mRNA were abundantly expressed in these cells (Fig. 5), with no modulation apparent by IFN-γ on FcγR-α expression, when normalized with a constitutive glycolytic enzyme probe GPDH. However, in the presence of IFN-γ, FcγR-β mRNA was decreased ∼10-fold. Class II murine major histocompatibility Aα mRNA (24) was induced 17-fold by IFN-γ in mouse peritoneal macrophages, indicating that IFN-γ-Rs were present and functional. Thus, the IFN-γ-activated peritoneal macrophage resembles the IFN-γ-stimulated J774a and RAW cell lines in expressing high levels of Fcγ-α mRNA and little or no FcγR-β mRNA. Similarly, thioglycollate-elicited macrophages demonstrate high levels of

TABLE I

Binding of ^{125}I-2.4G2 to IFN-γ-induced Macrophage-like Cell Lines

Nonlabeled Ig	J774a cells Control	J774a cells IFN-γ	RAW 264.7 cells Control	RAW 264.7 cells IFN-γ
No addition	12,069 ± 538	39,152 ± 2,711	16,546 ± 1,018	64,755 ± 3,806
2.4G2 (10 μg/ml)	4,413 ± 415	4,166 ± 287	5,263 ± 329	5,487 ± 717
IgG2a (10 μg/ml)	12,061 ± 1,093	38,231 ± 3,456	14,848 ± 1,286	56,927 ± 2,657

The macrophage-like cell lines were treated with 150 U/ml of IFN-γ for either 48 (RAW 264.7) or 96 h (J774a), after which they were removed from their culture flasks so that control and IFN-γ-treated cells could be normalized for cell number. RAW 264.7 cells (10^6/ml) and J774a cells (4×10^5/ml) were then incubated with ^{125}I-2.4G2 along with the indicated nonlabeled Ig for 90 min at 4°C. The cells were washed three times in PBS by pelleting and resuspension after which bound radioactivity was determined by gamma counting. Values are the mean ± SD for ^{125}I-2.4G2 binding, $n = 3$.

FcγR-α mRNA and little or no FcγR-β mRNA as determined by RNA in situ studies (Kirsch, I. R. and J. Ravetch, unpublished results).

IFN-γ Induces Surface Protein Expression and Ligand Binding Activity of the FcγR-α on J774a Cells. Induction of FcγR-α RNA by IFN-γ results in increased levels of FcγR-α protein, as demonstrated by the binding of ^{125}I-2.4G2 to the macrophage lines J774a and RAW (Table I). Both cell lines had detectable levels of the 2.4G2 epitope, which was induced by IFN-γ. After subtraction of the nonspecific binding components, induction of the 2.4G2 epitope was 4.5-fold on J774a cells and 5.2-fold on RAW cells (Table I). Identical results were obtained with ^{125}I-Fab 2.4G2, indicating that the induction was not a result of 2.4G2 binding by its Fc portion to IFN-stimulated cells (not shown). In addition, similar results were obtained using FcγR-α-specific antisera made against synthetic peptides corresponding to the cytoplasmic domain of FcγR-α (R. Weinshank, R. Schreiber, and J. C. Unkeless, unpublished observations). IFN-γ has been shown to result in increased expression of many proteins specifically induced by this lymphokine (27). To rule out an increase in 2.4G2 binding as a result of nonspecific changes in IFN-γ-treated cells, indirect binding studies were also performed on J774a cells using a panel of mAbs against a variety of macrophage surface markers (Fig. 6). A control mAb against a cytoplasmic enzyme, ornithine decorboxylase, exhibited no binding in the absence or presence of IFN-γ. Neither the Mac-1 determinant nor a protein modulated by parathyroid hormone (p150) was inducible by IFN-γ on these cells. Class II cell surface proteins (Ia) and the lymphocyte function-associated protein (LFA-1) were inducible by IFN-γ as previously reported (28–30). In addition, the protease-sensitive cell-surface glycoprotein found on macrophages (F4/80) was also inducible by IFN-γ. Surprisingly, the Thy-1 determinant was significantly induced by IFN-γ on J774a cells from undetectable levels.

Finally, to determine if the induction of FcγR-α mRNA and protein results in increased binding of IgG immune complexes to J774a cells, rosetting of IgG1-coated erythrocytes to J774a cells was performed. As seen in Fig. 7, binding was induced by IFN-γ and was inhibitable by mAb 2.4G2. The same observation was made using

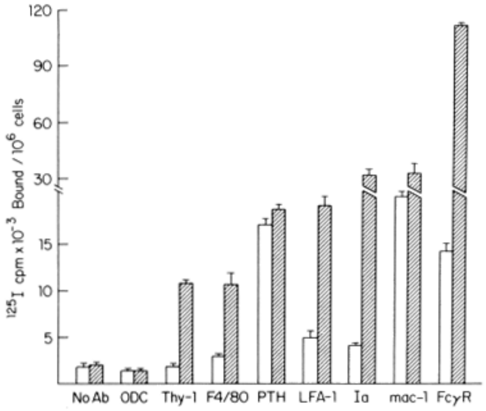

FIGURE 6. Comparison of FcγR-α induction by IFN-γ with other cell-surface markers on J774a cells. J774a cells were cultured in the presence (48 h) or absence of 200 U/ml IFN-γ. The cells were then removed from their flasks and normalized for cell number by coulter counting. 10^6 cells from control or IFN-γ treated culture flasks were incubated with rat mAbs with the following specificities: ornithine decarboxylase, (35), Thy-1 (36), F4/80 (37), p150-parathyroid hormone modulated protein (38), lymphocyte function associated (LFA-1) (39), class II surface molecule (Ia) (40), macrophage surface marker (Mac-1) (41), and FcγR (2.4G2) (6). The cells were incubated for 90 min at 4°C in an end-over-end mixer and then washed three times in PBS. Bound antibody was detected with affinity purified goat anti-rat IgG labeled with ^{125}I. After incubation, the cells were again washed three times and bound radioactivity was detected by gamma counting.

IgG2b-coated erythrocytes (data not shown), thereby establishing the FcγR-α protein induced by IFN-γ on these cells to be a functional FcR.

IFN-γ Induction of Phagocytosis. IFN-γ has previously been shown to activate macrophages leading to a variety of FcγR-mediated effector functions such as phagocytosis, the oxidative burst, and ADCC (13). In the absence of IFN-γ, J774a and RAW cells exhibited a minimal level of phagocytosis despite abundant rosetting with IgG-oponized SRBC (Fig. 8). P388D1 cells, however, were actively phagocytic in the absence of IFN-γ. After treatment with 200 U/ml of IFN-γ for 18 h, a dramatic increase of phagocytosis in RAW and J774a cells was observed (Fig. 8). This increase was reflected both in the number of macrophages within the population that had engulfed opsonized SRBC, as well as the number of protected SRBC per macrophage. P388D1 cells were actively phagocytic in the presence of IFN-γ, but not significantly different from the untreated P388D1 cells. Thus, the induction of phagocytosis in these cells parallels the induction of one of the two FcγR genes, FcγR-α.

Discussion

FcγRs occupy a central role in both the execution of cellular immune reactions and the coordination between humoral and cellular immune response systems. The diversity of mouse IgG subclasses is reflected in a diversity of IgG FcR. In addition to the existence of multiple receptors for different subclasses, FcγRs can distinguish between monomeric and immune-complexed Ig (4). The cell type specificity and differences in the functional domains of these receptors suggest that different FcR-mediated phenotypes may be triggered on specific cells in response to a common

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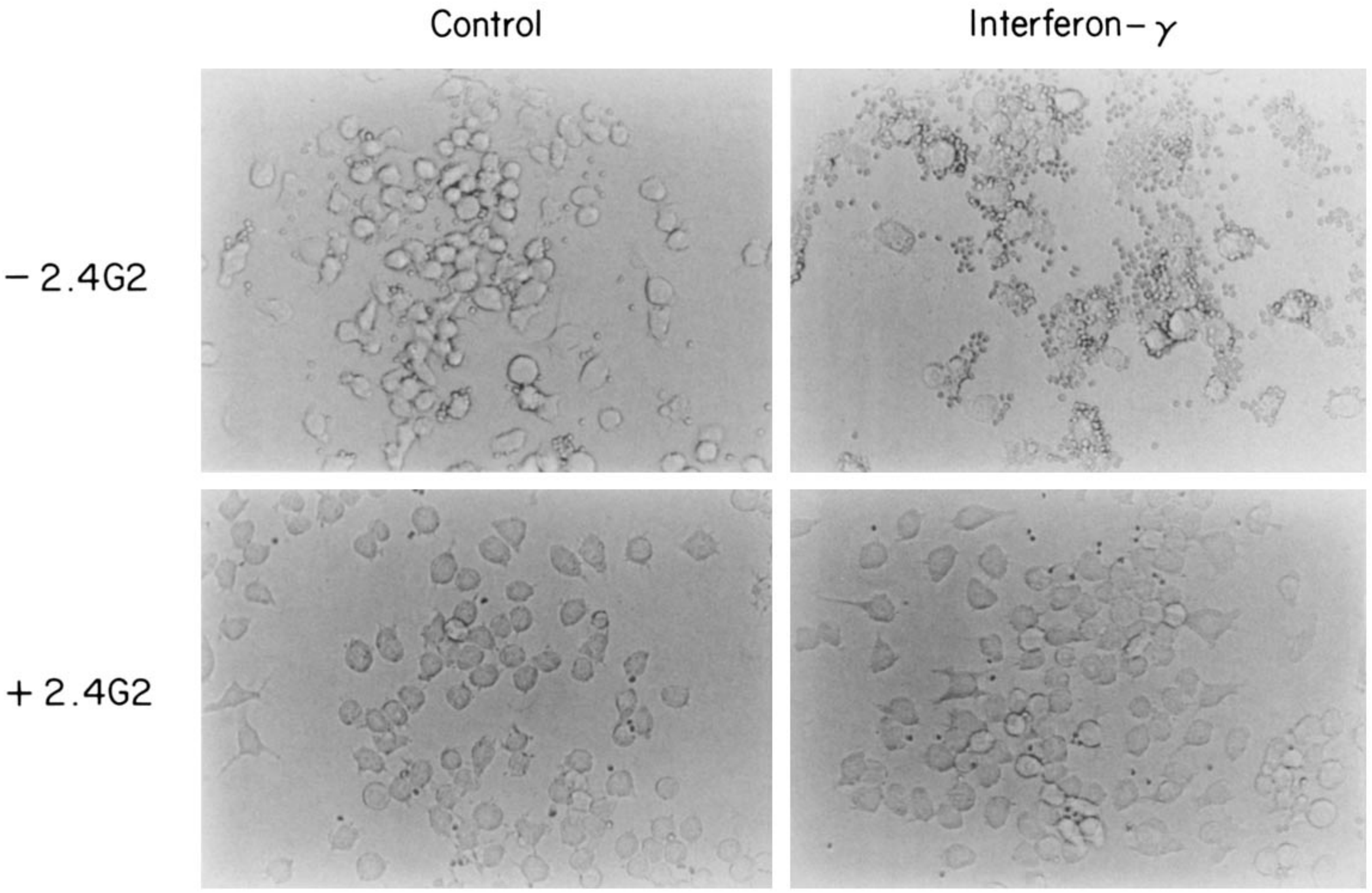
FIGURE 7. IFN-γ induction of ligand binding on J774a and competition with 2.4G2. J774a cells were treated with 200 U/ml IFN-γ for 48 h, after which control (untreated) and treated cells were preincubated with mAb 2.4G2 for 30 min at 37°C. TNP-SRBC opsonized with IgG1 were then incubated for 30 min at room temperature and then washed several times in PBS. The cells were then fixed in 0.25% glutaraldehyde and photographed.


Control
Interferon-γ
− 2.4G2
+ 2.4G2

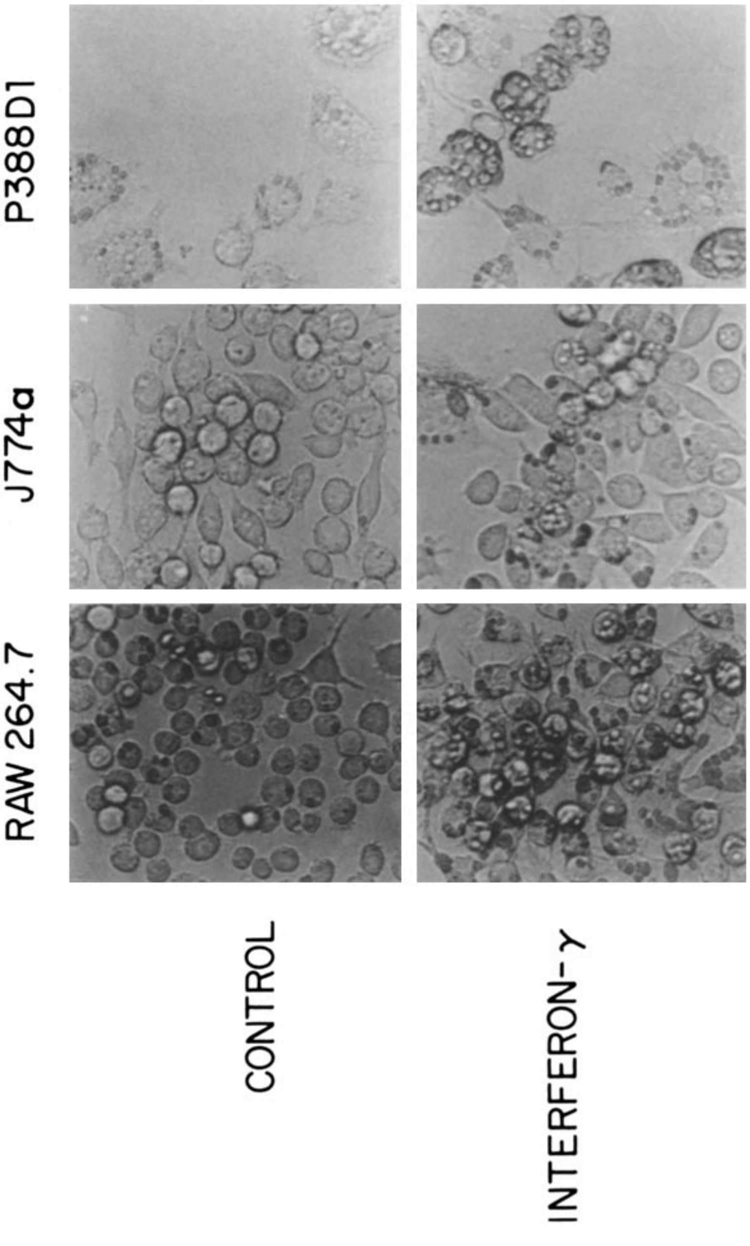


FIGURE 8. Effect of IFN-γ on phagocytic ability of mouse macrophage-like lines. The indicated cell lines, either treated with 200 U/ml IFN-γ for 48 h or untreated, were incubated with SRBC opsonized with anti-sRBC rabbit IgG at 37°C for 1 h, washed three times with PBS, and checked for rosetting. All lines demonstrated positive rosetting and were then washed rapidly three times with distilled H_2O to lyse external SRBC, fixed in glutaraldehyde and photographed. IFN-γ treatment dramatically increases phagocytosis in RAW and J774a lines, as determined by counting internalized SRBC. P388D1, in contrast, demonstrated little or no change in internalized SRBC.

ligand. The recent cloning of two FcγR genes and their transcripts (9–11) has facilitated the analysis of this complex receptor system. To begin to define the functional consequences of the structural complexity of this system, we have expressed cDNA encoding FcγR-α, -β_1, and -β_2 molecules by transfection of these clones into FcγR negative cell lines. We show here that both FcγR-α and FcγR-β cDNA expressed in FcγR negative lines are capable of the binding of IgG immune complexes, and that the requirements for ligand binding of each of the respective receptors are therefore encoded in a single gene. Both molecules bear the 2.4G2 epitope and are inhibited in their binding to ligand by this mAb. These FcγRs function as low-affinity binding receptors with a specificity not only for IgG1 and IgG2b, but also IgG2a. This broader specificity found for the FcγR molecules when analyzed by transfection is consistent with the observed specificity of this FcγR on lymphocytes (which express only FcγR-β) (7), and the behavior of this receptor after purification from macrophages (31). We propose that FcγR-α functions on macrophages in vivo with this specificity as well. The presence of the high-affinity IgG2a receptor on macrophages may compete for the binding of immune complexes of this subclass and result in a more restricted subclass specificity when analyzed by 2.4G2-inhibitable binding on macrophage lines. This is shown to be the case on resting mouse peritoneal macrophages. Without IFN-γ treatment, 2.4G2 can almost completely block IgG2a binding. In the presence of IFN-γ, which induces the high-affinity receptor (32, 33), 2.4G2 can no longer block IgG2a binding (data not shown). Similarly, thioglycollate-elicited macrophages possess a greater number of IgG2a receptors that are not competed by mAb 2.4G2 (not shown).

Since IFN-γ has previously been shown to induce the high-affinity FcγR, the selective induction of FcγR-α by IFN-γ observed in RAW and J774a cells raised the possibility that this molecule may be a component of the high-affinity IgG2a receptor. Three observations suggest that this is not the case. First, mouse LtK^- cells transfected with FcγR-α are incapable of binding monomeric IgG2a. Second, IgG2a binding was observed in RAW cells in the absence of IFN-γ induction (not shown), in which no FcγR-α mRNA could be detected (see Fig. 3 *A*), suggesting that the IgG2a monomeric receptor is separable from the FcγR-α moiety we describe here. Third, IFN-γ stimulation of peritoneal macrophages resulted in induction of an IgG2a high-affinity receptor, which was not inhibited by 2.4G2, yet FcγR-α mRNA was not induced in those cells, further suggesting that FcγR-α is not a component of a high-affinity IgG2a receptor. Finally, we can rule out the possibility that FcγR-β associates with FcγR-α to form a high-affinity binding site, since J774a cells lack FcγR-β, yet possess constitutive and inducible IgG2a receptors. Although the data presented here show that FcγR-α binds immune complexes and not monomeric IgG, we cannot rule out the possibility that some relationship exists between FcγR-α and other as yet unidentified proteins on macrophages that may alter either the specificity or affinity of this receptor.

To address the functional roles of FcγR-α and Fcγ-β, we have investigated the regulation of these two genes within the context of macrophage activation. Using highly purified mouse-IFN-γ, we have shown that the FcγR-α mRNA and protein are specifically induced in RAW 264.7 and J774a macrophage cell lines. The induction is dramatic in both cell lines (25-fold in FcγR-α mRNA in J774a cells) and specific for IFN-γ, among the cytokines tested. Interestingly, the maximal induction decreases in the continued presence of IFN-γ after 16 h but does not return to baseline, even after 50 h. Similar transient accumulation has been observed for other inducible genes (42, 43), and results, in part, from post-transcriptional mechanisms (44). Further studies involving transcriptional and post-transcriptional stabilization of FcγR-α mRNA will be necessary to determine the mechanism of this transient induction. Surface expression of FcγR-α protein was induced with IFN-γ on RAW and J774a cells as measured by increased levels of the 2.4G2 epitope as well as increased levels of a protein detected by FcγR-α-specific anti-peptide sera. Although mAb 2.4G2 recognizes both FcγR-α and FcγR-β, FcγR-β mRNA was not present or inducible in these cells. These data, in conjunction with the expression of the transfected FcγR-α molecule, shows that FcγR-α bears a 2.4G2 epitope. Functional ligand binding and inhibition by 2.4G2 on J774a cells confirms that FcγR-α is a functional low-affinity binding IgG FcR on macrophages.

The genetic heterogeneity of the molecules bearing the 2.4G2 epitope illustrates the limitations of using an Ab to define the regulation of this family of receptors. IFN-γ induces the FcγR-α molecule and leads to increased expression of the 2.4G2 epitope of J774a and RAW cells where FcγR-β expression is low or absent. However, this induction of the 2.4G2 epitope is not seen on peritoneal macrophages treated with IFN-γ, since the effect of this lymphokine is to down regulate FcγR-β gene expression, leaving FcγR-α expression at high levels. The net effect on peritoneal macrophages is to decrease the 2.4G2-bearing molecules on these cells, while dramatically altering the ratio of FcγR-α and -β gene expression, which is likely to contribute to their functional state.

Macrophage activation by IFN-γ leads to the phagocytosis of IgG-opsonized particles (12). When induced with IFN-γ, RAW 264.7 and J774a cells exhibit dramatic increases in their ability to engulf IgG-opsonized particles. This correlates with the induction of FcγR-α mRNA, protein, and IgG immune complex-binding in these cell lines. In cells such as P388D1 and resting peritoneal macrophages, which are actively phagocytic in the absence of IFN-γ, FcγR-α is already maximally expressed. When perintoneal macrophages are induced with IFN-γ, they not only continue to produce FcγR-α, but down regulate FcγR-β mRNA accumulation. FcγR-α, therefore, is coordinately induced with the appearance of phagocytosis or maintained at high levels in cells that are already phagocytic. It is likely that IFN-γ induces a variety of gene products that are involved in the ability of a cell to perform phagocytosis. The inducibility of FcγR-α and the subsequent increased binding capacity of a macrophage may not be sufficient alone to induce phagocytosis. Consistent with this notion, although abundant rosetting was detected on untreated RAW or J774a cells, no phagocytosis was observed until IFN-γ stimulation. It is likely that surface binding is not, in itself, sufficient to trigger phagocytosis in these macrophages lines, and induction of specific proteins by IFN-γ mediates this coupling to the cytoskeleton. Conversely, mouse LtK$^-$ cells, a fibroblast cell line, transfected with FcγR-α or -β, were not capable of phagocytosis despite high levels of IgG-coated SRBC binding. Therefore, the receptor itself may not be capable of mediating such an event. In a cell that is competent for phagocytosis, however, FcγR-α may provide the necessary coupling mechanism to mediate this complex process. The contribution of specific protein domains of FcγR-α and the role of IFN-γ-inducible components are currently under investigation to dissect the cellular machinery of phagocytosis.

Summary

Ligand binding specificities of two cloned murine FcγRs (FcγR-α, FcγR-β [9]) were determined by gene transfer into FcγR negative cell lines. Both receptors were expressed as full-length molecules capable of IgG immune complex binding that was inhibitable by the mAb 2.4G2. The ligand binding profiles of these receptors were indistinguishable whereby both bound immune-complexed mouse IgG1, IgG2a, and IgG2b, but not IgG3. Neither receptor could bind monomeric IgG2a, indicating these receptors to be low-affinity IgG Fc receptors.

Accumulation of the FcγR-α mRNA can be induced with murine IFN-γ at a concentration of 200 U/ml in the macrophage-like cell lines RAW 264.7 and J774a. The time course for induction indicates that the mRNA accumulation is transient but does not return to the uninduced level even after 50 h of treatment. FcγR-β mRNA was not induced by IFN-γ, rather its expression was down modulated in mouse peritoneal macrophages. Both RAW and J774a cells lines exhibited increased receptor levels after IFN-γ stimulation as measured by ^{125}I-2.4G2 and ligand binding. In the absence of IFN-γ, the RAW and J774a cell lines were minimally phagocytic, while P388D1 cells were actively phagocytic. In the presence of IFN-γ, however, RAW 264.7 and J774a cells were induced to become actively phagocytic. Induction of FcγR-α mRNA and protein by IFN-γ may be part of the process by which macrophages become activated to engulf antibody-coated particles.

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