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Expression of murine Fc receptors for IgG

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by

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Abstract

EXPRESSION OF MURINE Fc RECEPTORS FOR IgG

by

Ronda E. Schreiber

Advisor: Professor Jay C. Unkeless

There are two distinct genes encoding murine low affinity Fc receptors for IgG ($Fc_\gamma RII$), $muFc_\gamma RII_\alpha$ and $muFc_\gamma RII_\beta$, which are transcribed in specific cell lineages. $Fc_\gamma RII_\alpha$ transcripts are present in macrophages, NK cells, and mesangial cells; $Fc_\gamma RII_\beta$ transcripts are expressed in $Fc_\gamma R$-bearing lymphocytes and macrophages. A sandwich enzyme-linked immunosorbent assay (ELISA) was devised to quantify the predicted $Fc_\gamma RII_\alpha$ protein. The ELISA is specific for $Fc_\gamma RII_\alpha$, and does not detect the closely related $Fc_\gamma RII_\beta$ protein. Upon stimulation with IFN-γ of the $Fc_\gamma RII_\beta$-negative macrophage cell line, J774a cells expressed enhanced levels of $Fc_\gamma RII_\alpha$. Peritoneal macrophages synthesized varying amounts of $Fc_\gamma RII_\alpha$. High levels of $Fc_\gamma RII_\alpha$ were observed in resident and thioglycollate-elicited peritoneal macrophages, but no $Fc_\gamma RII_\alpha$ was detected in Bacillus Calmette Guerin (BCG)-elicited macrophages. J774a cells stimulated with rIL-6 bound roughly 2-fold more anti-$Fc_\gamma RII$ mAb 2.4G2 IgG than unstimulated controls. However, the $Fc_\gamma RII_\alpha$-specific ELISA showed no change in the amount of $Fc_\gamma RII_\alpha$ expressed. A probe encompassing the extracellular coding sequence of $Fc_\gamma RII_\beta$ hybridized to two distinct transcripts that were elevated in rIL-6-stimulated J774a cells. One of these

transcripts had the same mobility in electrophoresis as $Fc_\gamma RII_\alpha$, whereas the other transcript was larger. Since it was confirmed, with an $Fc_\gamma RII_\beta$-specific probe, that J774a cells do not make $Fc_\gamma RII_\beta$ mRNA, the larger transcript must encode a novel $Fc_\gamma RII$. It is likely that the increased level of binding of the anti-$Fc_\gamma RII$ mAb 2.4G2 to rIL-6-induced cells represents translation of a $Fc_\gamma R$ distinct from $Fc_\gamma RII_\alpha$ or $Fc_\gamma RII_\beta$. The existence of such a receptor introduces further complexity to the heterogeneity of the murine $Fc_\gamma R$ family.

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V. ABBREVIATIONS

ADCC	antibody dependent cell cytotoxicity
BCG	Bacillus Calmette Guerin
BSA	bovine serum albumin
$Fc_\gamma R$	Fc receptor for IgG
FCS	fetal calf serum
GPI	glycan phophatidylinositol
HRP	horseradish peroxidase
KLH	keyhole limpet hemocyanin
mAb	monoclonal antibody
PIPLC	phosphatidylinositol phospholipase C
r	recombinant
sc	subcutaneous

VI. INTRODUCTION

Receptors for the Fc portion of IgG ($Fc_\gamma R$) are important mediators of immune function. $Fc_\gamma R$'s are expressed on macrophages, polymorphonuclear cells (PMN's), NK cells, mesangial cells, platelets, and B and T cells (reviewed in Unkeless, 1988, 1989). Several different $Fc_\gamma R$'s have been described in both human and mouse systems (Unkeless, 1988,1989). Upon activation, $Fc_\gamma R$'s on macrophages and neutrophils mediate a variety of functions, such as phagocytosis and antibody-dependent cell cytotoxicity (ADCC), as well as the secretion of lysosomal hydrolases, reactive oxygen metabolites and inflammatory mediators (Nathan, 1980a). Ig synthesis by B cells is regulated by soluble $Fc_\gamma R$'s released from monocytes and T cells (Gisler, 1975; Neuport-Sates, 1977,1979; Calvo, 1986). $Fc_\gamma R$ on B cells influences B cell transformation (Parker, 1983,1984,1985).

A. Fc$_\gamma$R Function

1. Human Fc$_\gamma$R's

The family of human $Fc_\gamma R$'s consists of three types of members (Unkeless, 1989) (Table 1). **HuFc$_\gamma$RI** is expressed as a 72,000 dalton protein on both macrophages and monocytes and binds monomeric human IgG and murine IgG2a with high avidity (Ka = 1-3 x 10^8 M^{-1}) (Huber, 1971). HuFc$_\gamma$RII (CD32), a 40,000 Dalton protein, is found on monocytes, platelets, neutrophils, B cells, and the K562 cell line (Tetteroo, 1987) and binds IgG complexes with low avidity (Ka = ~ 2 x 10^6 M^{-1}) (Jones, 1985). **Fc$_\gamma$RII** is highly polymorphic and is encoded by three distinct

genes. $Fc_\gamma RIII$ (CD16), which binds human IgG1 and IgG3 with a Ka ~ 5×10^5 M^{-1} is expressed on macrophages, NK cells, neutrophils, eosinophils, and on some T cells (Tetteroo, 1987). The molecular weight of $Fc_\gamma RIII$ differs within and between cell types on which it is expressed, as will be discussed in this section.

Table 1 **HUMAN $Fc_\gamma R$ FAMILY**

Ligand and affinity	Gene	Distribution
$Fc_\gamma RI$ High avidity		**Monocytes/Macrophages**
IgG1 > IgG3 > IgG4 > > IgG2		
$Fc_\gamma RII$ Low avidity	**$Fc_\gamma RIIa$**	**Monocytes/Macrophages**
IgG1 > IgG2 > IgG4 > > IgG3		**Neutrophils, Leukemia**
	$Fc_\gamma RIIa'$	**Monocytes, B cells, Neutrophils, Placenta**
	$Fc_\gamma RIIb$	**Monocytes, B cells, Neutrophils, Lymphoid/Myeloid cells Platelets**
$Fc_\gamma RIII$ Low avidity	**$Fc_\gamma RIII1$**	**Neutrophils**
IgG1, IgG3	**$Fc_\gamma RIII2$**	**NK cells**
	?	**Macrophages Eosinophils T cells (some)**

Because the same cell types express different forms of human $Fc_\gamma RII$ and $Fc_\gamma RIII$, functions mediated by these receptors can not be attributed to an individual $Fc_\gamma R$ at this time.

$Fc_\gamma RI$ mediates ADCC in monocytes. Graziano and Fanger (1987) demonstrated that myelomas bearing anti-hu$Fc_\gamma RI$ or anti-$Fc_\gamma RII$ mAbs are targets for ADCC by monocytes. Moreover, IFN-γ treatment of U937 monocytic-like cells resulted in enhanced cytotoxicity by mouse IgG2a, which, of all the mouse isotypes, binds most avidly to $Fc_\gamma RI$ (Akiyama, 1984). On the other hand, this receptor probably does not play a singular role in mediating ADCC. Ceuppens et al. (1985) report that a family of four individuals express monocytes which are incapable of binding IgG2a with high avidity. However, these people maintain a normal capacity to fight infection, suggesting that other mechanisms are capable of mediating ADCC.

The neutrophil $Fc_\gamma RII$ also mediates ADCC. Upon IFN-γ treatment of neutrophils, the cytotoxic potential of this receptor is enhanced, although the lymphokine does not induce expression of $Fc_\gamma RII$ (Graziano, 1985).

$Fc_\gamma RII$ mediates phagocytosis by neutrophils, as demonstrated by Huizinga et al. (1989). Furthermore, these investigators suggest that $Fc_\gamma RIII$, also expressed on neutrophils, is not required for phagocytosis, since there was equal inhibition of phagocytosis by cells treated with anti-$Fc_\gamma RII$ antibody or anti-$Fc_\gamma RII$ antibody in combination with anti-$Fc_\gamma RIII$ antibody. Of the two $Fc_\gamma R$'s expressed on neutrophils ($Fc_\gamma RII$ and $Fc_\gamma RIII$), $Fc_\gamma RII$ plays a major role in mediating superoxide release by these cells. Tosi and colleagues have shown that exposure of neutrophils to elastase

causes reduced expression of $Fc_\gamma RIII$, but not $Fc_\gamma RII$ (Tosi, 1988). They found that O^-_2 production is not inhibited under these conditions, whereas a 79% decrease in IgG-induced O^-_2 release is observed upon inhibition of $Fc_\gamma RII$ with anti-$Fc_\gamma RII$ mAb IV-3. The relative importance of $Fc_\gamma RII$ versus $Fc_\gamma RIII$ in triggering the neutrophil oxidative burst was also observed by Huizinga et al. (1988). They isolated neutrophils from patients with paroxysmal nocturnal haemoglobinuria. The neutrophils, which were devoid of $Fc_\gamma RIII$, were able to mediate normal release of O^-_2.

Crosslinking of either $Fc_\gamma RII$ or $Fc_\gamma RI$ on monocytes also triggers the oxidative burst (Anderson, 1986). In these studies, superoxide was generated by aggregation of $Fc_\gamma RII$ or $Fc_\gamma RI$ with F(ab')2 fragments of either murine anti-$Fc_\gamma RII$ mAb IV-3 or anti-$Fc_\gamma RI$ mAb 32, respectively, and anti-murine IgG. Pfefferkorn and Fanger (1989) confirmed these findings with respect to $Fc_\gamma RI$, and also showed that in order for O^-_2 to be continuously generated, $Fc_\gamma RI$ must be crosslinked with ligand at subsaturating concentrations. Saturation of receptor with ligand resulted in a transient burst of O^-_2 release.

The monocytic $Fc_\gamma RII$ is also important in mediating a specific T cell proliferative response. Studies by Looney and colleagues (1986) revealed that this response is elicited by the crosslinking of anti-CD3 antibody to CD3 on T cells via the monocyte $Fc_\gamma RII$. This T cell response is not observed in all individuals, however (Tax, 1983; Clement, 1985; Anderson, 1987). This is due to a structural polymorphism in which the $Fc_\gamma RII$'s of non-responders and responders exhibit

distinct isoelectric focusing patterns (Anderson, 1987). This polymorphism was recently found to be highly complex, (Brooks, 1989) as described in detail later in this section.

$Fc_{\gamma}RIII$ (CD16) is also polymorphic, with regard to function and structure. NK cells and macrophages express a transmembrane-anchored form of $Fc_{\gamma}RIII$. In contrast, $Fc_{\gamma}RIII$ on neutrophils is anchored to the membrane via a glycan phosphatidylinositol (GPI) linkage (Selvaraj, 1988; Huizinga, 1988; Ravetch, 1989; Scallon, 1989; Ueda, 1989). Huizinga (1988) reported that stimulation of neutrophils with the chemotactic peptide formyl-methionyl-leucyl-phenylalanine results in release of $Fc_{\gamma}RIII$ from the neutrophil membrane, suggesting that this form of $Fc_{\gamma}RIII$ is stimulated in response to infection.

Polymorphisms of $Fc_{\gamma}RIII$ also exist within cell lines. These forms have been identified by SDS-PAGE analysis of the core proteins. Two receptor types are distinguishable on neutrophils (19 KDa and 21 KDa) and NK cells (20 KDa and 24 KDa) (Werner, 1986; Edberg, 1989). The pattern of receptor expression on neutrophils correlated with NA1 and NA2 allotype markers, whereas both the 20 KDa and 24 KDa $Fc_{\gamma}RIII$ types were expressed on NK cells, regardless of the allotype. On the contrary, cultured monocytes express only one species of $Fc_{\gamma}RIII$.

$Fc_{\gamma}RIII$ present on NK cells appears to be important in mediating NK cell cytotoxicity, since ligand occupation of the receptor results in enhanced lytic ability of the NK cells (Anegon, 1987). These investigators found that occupation of $Fc_{\gamma}RIII$ with ligand induced transcription of interleukin 2 receptor, IFN-γ, and TNF,

all of which activate NK function.

$Fc_\gamma RIII$ on macrophages in the spleen and Kuppfer cells in the liver are probably the primary receptor responsible for clearance of large immune complexes. Clarkson et al. (1986a) demonstrated in chimpanzees that the anti-$Fc_\gamma RIII$ mAb 3G8 inhibited in vivo clearance of autologous erythrocytes coated with antibody directed against a minor blood group antigen. MAb 3G8 has been tested as a potential therapeutic treatment for individuals with immune thrombocytic purpura, a disease in which patients exhibit high levels of anti-platelet antibody Clarkson, 1986b). Treatment of one patient resulted in a dramatic rise in platelet levels, which returned to normal levels within 2 weeks. A less dramatic response to secondary treatment with mAb 3G8 in this patient may be due to the synthesis of antibodies against murine IgG. Such a response may be a serious limitation in using 3G8 as a therapeutic treatment. $Fc_\gamma RIII$ expressed on macrophages mediates antibody-dependent enhancement of HIV infection (Homsy, 1989). Of the macrophage $Fc_\gamma R$'s, this function is limited to $Fc_\gamma RIII$, since antibodies which bind $Fc_\gamma RIII$, but not $Fc_\gamma RI$ or $Fc_\gamma RII$ inhibit the enhancement. However, the authors suggest that enhancement of HIV infection is also mediated by a different $Fc_\gamma R$ on $CD4^+$ lymphocytes, because IgG aggregates, but not anti-$Fc_\gamma RIII$ antibody could inhibit the function.

Soluble $Fc_\gamma R$'s have been detected by several investigators, although their biochemical characterization is still incomplete. The human monocyte-like cell line, U937, was shown to release a soluble $Fc_\gamma R$ which inhibited B cell IgG synthesis

(Calvo, 1986). In addition, a soluble $Fc_\gamma R$ has also been isolated from human serum. This soluble receptor also inhibits IgG production in vitro (Sandor, 1986).

II. Murine $Fc_\gamma R$'s

The family of murine $Fc_\gamma R$'s is comprised of several members (Table 2). $Fc_\gamma RI$, expressed on macrophages, binds monomeric murine IgG2a and human IgG1 with high avidity (Ka = 1 x 10^8 M^{-1}), in addition to immunoglobulin aggregates, and is trypsin-sensitive (Unkeless, 1977). $Fc_\gamma RII$'s, which are trypsin-resistant, bind IgG complexes with low avidity (Ka = 1 x 10^6 M^{-1}) (Unkeless, 1977; Diamond, 1980). $Fc_\gamma RII_\alpha$ is expressed on macrophages, mesangial cells, NK cells, and B and T cells. B and T cells express both $Fc_\gamma RII_\beta 1$ and $Fc_\gamma RII_\beta 2$, however, macrophages only synthesize $Fc_\gamma RII_\beta 2$ (Ravetch, 1986,1989; Santiago, 1989). A third $Fc_\gamma R$, $muFc_\gamma RIII$, which binds murine IgG3 aggregates, has been identified on macrophages by Diamond (1981). Finally, the presence of a soluble form of a $muFc_\gamma R$ isolated from T cells and B cells has been reported by several laboratories (Gizler, 1975; Loube, 1988; Pure, 1984; Khayat, 1984).

The number of receptor sites for the individual receptors has not been determined, since antibodies specific to the individual $muFc_\gamma R$ to not exist. However, Unkeless (1977) measured the binding of IgG to $Fc_\gamma R$ on the macrophage line, J774, and found that there are 200,000 binding sites for monomeric IgG2a, whereas there are roughly 800,000 binding sites for IgG complexes on the same isotype.

Table 2 **MURINE $Fc_\gamma R$ FAMILY**

	Ligand	Gene	Distribution
$Fc_\gamma RI$	High avidity IgG2a		Macrophages
$Fc_\gamma RII$	Low avidity IgG2a, IgG2b, IgG1	$Fc_\gamma RII_\alpha$	Macrophages NK cells, Mesangial cells
		$Fc_\gamma RII_\beta 1$	B cells, T cells
		$Fc_\gamma RII_\beta 2$	B cells, T cells Macrophages
		$Fc_\gamma RII?$	Macrophages, ?
$Fc_\gamma RIII$	IgG3	$Fc_\gamma R?$	Macrophages, ?

As is true of the human $Fc_\gamma R$ family, murine $Fc_\gamma R$'s mediate a variety of cellular responses. It is noteworthy that the functional studies of murine $Fc_\gamma R$'s reported to date have not elucidated the specific roles of the individual mu$Fc_\gamma R$. This ambiguity is due, in part, to the multiplicity of genes encoding the different $Fc_\gamma R$.

Murine macrophage $Fc_\gamma R$'s mediate phagocytosis. Ralph et al. (1980) reported that phagocytosis is stimulated by all classes of IgG on the mouse macrophage. These results, however, do not elucidate which $Fc_\gamma R$(s) mediate this

function, since $Fc_\gamma RI$, $Fc_\gamma RII_\alpha$, $Fc_\gamma RII_\beta 2$, and $Fc_\gamma RIII$, are coexpressed on the macrophage. More recently, Weinschanck (1988) reported that IFN-$_\gamma$-treated macrophages that expressed induced levels of $muFc_\gamma RII_\alpha$ mRNA, also exhibited enhanced phagocytosis. These data suggest that $muFc_\gamma RII_\alpha$ mediates phagocytosis. The studies discussed in the previous section by Miettinen (1989) indicate that $Fc_\gamma RII_\beta 2$ also mediates endocytosis of IgG complexes, since receptor-minus chinese hamster ovary cells transfected with $Fc_\gamma RII_\beta 2$ exhibit internalization of immune complexes into lysosomes.

Initial studies demonstrating the role of murine macrophage $Fc_\gamma R$'s in ADCC were performed by Cerrotoni et al. (1974). Nathan et al. (1979a,b) investigated the mechanism of this cytotoxicity, and found that is was mediated by H_2O_2, released by the activated macrophages. This cytotoxicity was later shown to be mediated by $Fc_\gamma RII$ (Nathan, 1980b). In these experiments, extracellular lysis by activated macrophages of tumor cells coated with homologous antibody was investigated. Nathan and colleagues showed that ADCC mediated by BCG-elicited macrophages was inhibited 70% by mAb 2.4G2, an antibody specific for murine $Fc_\gamma RII$ molecules. More recently, Perussia et al. (1989) reported that ADCC of antibody-coated target cells is mediated by $Fc_\gamma RII$-bearing NK cells, since mAb 2.4G2 inhibited this effect. Since $Fc_\gamma RII_\alpha$, but not $Fc_\gamma RII_\beta$ mRNA is detected in NK cells, either $Fc_\gamma RII_\alpha$ or some other, as yet unidentified $Fc_\gamma RII$ mediates ADCC by NK cells. Likewise, Kuziel and Tucker (manuscript in preparation) have shown that murine dendritic epidermal cells, in which $Fc_\gamma RII_\alpha$ transcripts are uniquely expressed, mediate ADCC.

Another function mediated by $Fc_\gamma R$ is the release of arachidonic acid (20:4) metabolites. These inflammatory mediators include prostaglandin (Humes, 1980) and leukotriene (Rouzer, 1980a). Studies performed by Bonney et al. (1978, 1979) showed that binding of immune complexes to mouse peritoneal macrophages induced phospholipase activity. This leads to the oxygenation of arachidonic acid via the cycloxygenase and lipoxygenase pathways (Bonney, 1978, 1979; Rouzer, 1980b), resulting in the formation of prostaglandins and leukotrienes, respectively.

$Fc_\gamma RII$ expressed on B cells are important regulatory molecules. Phillips and Parker (1983, 1984, 1985) have demonstrated that B cell blastogenesis is inhibitable by concurrent occupation of membrane IgM (or IgD) and $Fc_\gamma R$. This inhibition requires both membrane proteins to be occupied, however, since treatment of cells with anti-IgM (or anti-IgD) antibody F(ab'2) induces blastogenesis. Inhibition of blastogenesis was blocked with mAb 2.4G2, demonstrating that $Fc_\gamma R$ must be occupied by ligand for the anti-IgM-induced inhibition of blastogenesis to occur. This phenomenon was confirmed by Schad and Phipps (1989), who noted that B cell mitogenesis was inhibited by simultaneous occupation of Ig and $Fc_\gamma RII$ with monomeric ligands.

A soluble $Fc_\gamma R$ originating from LPS-induced but not uninduced mouse splenic B cells was described by Pure et al. (1984). This soluble receptor was reactive with mAb 2.4G2, indicating that it is a member of the $Fc_\gamma RII$ subfamily of murine $Fc_\gamma R$'s. In addition, they and others (Khayat, 1984) isolated a soluble $Fc_\gamma RII$ in normal mouse serum, as identified by its reactivity with mAb 2.4G2.

Soluble $Fc_\gamma R$'s derived from T cells are presumed to act as regulators of antibody production by B cells. Fridman et al. initially isolated a soluble $Fc_\gamma R$ secreted by alloantigen-activated mouse T cells (Gisler, 1975). This soluble receptor inhibited the production of Ig by mouse spleen cells. A similar soluble factor with the same suppressive function was isolated from L5178Y thymoma cells as well as an $Fc_\gamma R$-positive T cell clone (Neuport-Sautes, 1977, 1979). Analysis of the T cells revealed that soluble $Fc_\gamma R$ could be induced by treatment of cells with Ig. The specificity of the soluble $Fc_\gamma R$ was dependent upon the Ig used for induction. When IgG1 was used as the T cell stimulant, rosette-formation of IgG1-senisitized erythrocytes, but not IgG2a or IgG2b-sensitized erythrocytes was inhibited by the isolated immunoglobulin binding factor. On the other hand, when T cells were stimulated with IgG2a, a factor was secreted from the cells which specifically suppressed erythrocyte rosette formation on IgG2a or IgG2b-bearing spleen cells, but not IgG1-bearing spleen cells, erythrocytes (Lowty, 1983).

B. $Fc_\gamma R$ structure

I. Murine $Fc_\gamma R$'s

There are three mu$Fc_\gamma RII$ transcripts encoded by two distinct genes, $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$ (Ravetch, 1986; Hibbs, 1986; Lewis, 1986). All three transcripts have been transfected and expressed in $Fc_\gamma R$-negative cell lines and have been shown to encode $Fc_\gamma RII$ molecules which bind IgG1/2b/2a immune complexes (Ravetch, 1986; Weinschanck, 1988, Miettinen, 1989). The cellular distributions of $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$ mRNA are distinct. Whereas the $Fc_\gamma IIR_\alpha$ transcript is expressed in

macrophages, NK cells, and mesangial cells (Ravetch, 1986; Santiago, 1989; Perussia, 1989), the $Fc_\gamma RII_\beta$ transcript is synthesized in macrophages and B and T cells. There are two transcripts of the $Fc_\gamma RII_\beta$ gene: The $Fc_\gamma RII_\beta 2$ transcript is present in macrophages, as well as lymphocytes and lymphoid cell lines, whereas the $Fc_\gamma RII_\beta 1$ transcript is found only in lymphocytes and lymphoid cell lines (Ravetch, 1986).

Although transcripts for the $muFc_\gamma RII_\alpha$ and $muFc_\gamma RII_\beta$ genes have been isolated, the translated proteins have only been indirectly detected by the binding of IgG complexes or mAb 2.4G2 to $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$ transfected cell lines (Ravetch, 1986; Weinschanck, 1988). Analysis of tryptic peptides isolated from $Fc_\gamma RII$ from S49.1 cells disclosed amino acid identity between tryptic fragments from the purified $Fc_\gamma R$ and the sequence deduced from the $muFc_\gamma RII_\beta$ cDNA (Pan, 1987); however, no amino acid analysis of the native $Fc_\gamma RII_\alpha$ protein has been reported.

The structures of all three $Fc_\gamma RII$'s are distinct, although they exhibit marked homology to each other (Figure 1). Based on their cDNA sequences, the predicted amino acid sequences of the extracellular domains of $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$ are 95% homologous, and are composed of two repeated Ig-like domains, each containing two N-linked glycosylation sites and two cysteine residues, which form intrachain disulfide bonds (Qu, 1988). The transmembrane domains of both $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$ are preceeded by a serine and threonine-rich region. No homology exists, however, between the leader sequences, the transmembrane, and the cytoplasmic domains of $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$. The transmembrane domain of $Fc_\gamma RII_\alpha$ is noteworthy for

containing a negatively charged aspartate. The cytoplasmic domain of $Fc_\gamma RII_\alpha$ is short and basic, consisting of 26 amino acids. The sequence encoding the intracytoplasmic domain of $Fc_\gamma RII_\beta 1$ is comprised of 92 amino acids, and is acidic. The $Fc_\gamma RII_\beta 2$ sequence is identical to $Fc_\gamma RII_\beta 1$, with the exception of a 46 amino acid insertion present in the cytoplasmic region of $Fc_\gamma RII_\beta 1$, which is absent in $Fc_\gamma RII_\beta 2$.

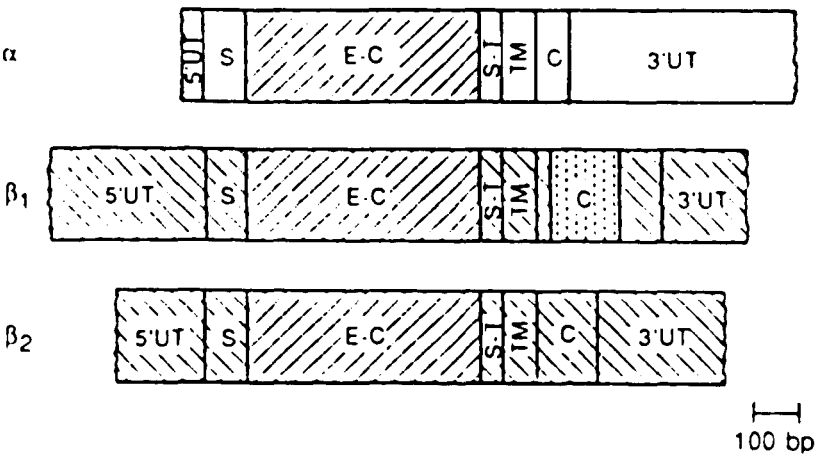


Fig.1. **Structure of the $Fc_\gamma R$ cDNA's of macrophage and T cells. The genes $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$ are 95% homologous in their extracellular domains (E-C). $Fc_\gamma RII_\beta 1$ and $Fc_\gamma RII_\beta 2$ are alternatively-spliced transcripts encoded by the $Fc_\gamma RII_\beta$ gene. They are identical except in their cytoplasmic domain (C), where there is a 138-nucleotide insertion present in the $Fc_\gamma RII_\beta 1$ sequence which is absent in the $Fc_\gamma RII_\beta 2$ sequence.**

MuFc$_\gamma$RII$_\alpha$ shares homology with several receptors. It is most homologous to human Fc$_\gamma$RIII (Simmons, 1988), with which it shares 62% nucleotide and 63% amino acid sequence homology. The two receptors are most similar in their transmembrane domains, where there is 81% homology, including flanking regions surrounding the conserved charged aspartyl group. The transmembrane domain of muFc$_\gamma$RII$_\alpha$ is also homologous to that of the high affinity rat basophilic Fc$_\epsilon$R. These two sequences share an identical stretch of 8 amino acids surrounding the conserved aspartate residue.

Fc receptor for IgE (Fc$_\epsilon$RI) may help elucidate the structure and function of Fc$_\gamma$RII$_\alpha$. Comparison of the predicted Fc$_\gamma$RII$_\alpha$ protein and the α subunit of Fc$_\epsilon$RI reveals an overall nucleic acid homology of 49% with 32% amino acid identity (Kinet, 1987). The two areas of greatest homology between the two proteins are the NH_2-terminal region, where there is 71% amino acid homology, and the transmembrane domain, in which there is an identical stretch of 8 amino acids surrounding a common aspartate residue between the two genes. Fc$_\epsilon$RI is a multisubunit structure which consists of the ligand-binding, membrane-spanning α subunit, and three additional subunits (Metzger, 1986). These latter subunits, 1 β and 2 disulfide-linked γ proteins, are not exposed at the cell surface. All three subunits are physically associated and are required for ligand binding (Miller, 1989). The transmembrane domain of the α chain is one region which may associate with the β and γ subunits by electrostatic interactions with the charged aspartyl group. Since the transmembrane domain of Fc$_\gamma$RII$_\alpha$ is homologous to the membrane-

spanning domain of $Fc_{\epsilon}RI$, the multi-subunit $Fc_{\epsilon}RI$ complex is a suitable paradigm for analyzing the structure and function of $Fc_{\gamma}RII_{\alpha}$. Indeed, Ra (1989) recently reported that the γ subunit of the $Fc_{\epsilon}RI$ complex associates with $Fc_{\gamma}RII_{\alpha}$.

The extreme structural differences between the cytoplasmic and transmembrane domains of $Fc_{\gamma}RII_{\alpha}$ and $Fc_{\gamma}RII_{\beta}$ suggests that the two receptors function distinctively. The unusual presence of a charged aspartate residue in the predicted transmembrane domain of $Fc_{\gamma}RII_{\alpha}$, but not $Fc_{\gamma}RII_{\beta}$, suggests that the two receptors may interact differently within the membrane.

Analysis of transfected $Fc_{\gamma}RII_{\beta}1$ and $Fc_{\gamma}RII_{\beta}2$ function by Miettinen et al. (1989) disclosed an important relationship between the structure of the $Fc_{\gamma}RII_{\beta}1$ and $Fc_{\gamma}RII_{\beta}2$ cytoplasmic domains and their function. They found that $Fc_{\gamma}RII_{\beta}2$, but not $Fc_{\gamma}RII_{\beta}1$, mediates endocytosis of ligand by chinese hamster ovary cells and subsequent fusion to lysosomes. Apparently, $Fc_{\gamma}RII_{\beta}2$ possesses a domain required for delivery to coated pits which is interrupted by the 46 amino acid insert in the cytoplasmic domain of $Fc_{\gamma}RII_{\beta}1$.

The ligand binding domains of $Fc_{\gamma}R$'s recognize different epitopes on IgG2a and IgG2b (Diamond, 1985). Using cyanogen bromide fragments of these antibodies, it was demonstrated that one fragment from the $C_{\mu}2$ domain of IgG2b bound macrophage $Fc_{\gamma}RII$, whereas a fragment from both the $C_{\mu}2$ and the $C_{\mu}3$ domains of IgG2a bound $Fc_{\gamma}RI$. The $C_{\mu}2$ fragment of IgG2b did not inhibit the binding of the $C_{\mu}2$ fragment of IgG2a. The $C_{\mu}3$ fragment of IgG2a enhanced the binding of the $C_{\mu}2$ fragment of IgG2a. These competition studies suggested that the

IgG2a fragments were binding to a different receptor than were the IgG2b fragments. Binding of the IgG2a fragments was inhibited by exposure to trypsin. Since $Fc_\gamma RI$, but not $Fc_\gamma RII$ is trypsin sensitive, the authors concluded that the $Fc_\gamma RI$ recognized the two IgG2a fragments. It is important to note that at the time these studies were performed, the complexity of the $Fc_\gamma R$ structures was not fully realized. Therefore, although these data demonstrate that different IgG domains are recognized on the IgG2a and IgG2b isotypes, it is not clear to which $Fc_\gamma R(s)$ the IgG2a and IgG2b fragments bound.

II. Human $Fc_\gamma R$'s

Although the structure of the high avidity murine $Fc_\gamma RI$ has not been determined, the high avidity human $huFc_\gamma RI$ structure has been elucidated (Allen, 1989; Peltz, 1989). Its cDNA sequence predicts an integral membrane protein, with a single transmembrane domain and a short, highly charged cytoplasmic domain. The ectodomain of $huFc_\gamma RI$ consists of three Ig-like domains, each containing two N-linked glycosylation sites and two cysteine residues, which presumably form intrachain disulfide bonds. There is considerable homology between the first and second Ig-like domain of $huFc_\gamma RI$ and the corresponding $muFc_\gamma RII$ Ig-like domains, but there is little similarity between the third Ig-like domain of $huFc_\gamma RI$ and either Ig-like domain of $muFc_\gamma RII_\alpha$ or $muFc_\gamma RII_\beta$. It is likely that this third Ig-like domain bestows the high-affinity for IgG to $huFc_\gamma RI$, since preliminary studies by Seed et al. (unpublished results) have shown that point mutations in this domain cause reduced affinity of the receptor for IgG. It remains to be determined if a similar domain is

present in the high affinity murine $Fc_{\gamma}RI$.

Analysis of $Fc_{\gamma}RI$-binding regions on the Ig molecules has recently been reported by Duncan et al. (1988), who identified a single amino acid residue in the $C_{H}2$ region of IgG1 and that of murine IgG2a which is required for high affinity binding to $Fc_{\gamma}RI$. An additional epitopes is involved in the binding of monomeric IgG2a to mu$Fc_{\gamma}RI$, as reported by Diamond et al. (1985), who demonstrated that IgG2a binding was inhibited by aggregated cyanogen bromide fragments from both the $C_{H}2$ and $C_{H}3$ domains of IgG2a.

Three distinct genes encoding $Fc_{\gamma}RII$ have recently been isolated from monocyte, macrophage and B cell lines (Brooks, 1989). Each of these genes bears strong homology to the murine $Fc_{\gamma}RII_{\alpha}$ and $Fc_{\gamma}RII_{\beta}$ genes. Receptors encoded by these three genes ($Fc_{\gamma}RIIa$, $Fc_{\gamma}RIIa'$, and $Fc_{\gamma}RIIb$), all bind immune complexes and are recognized by $Fc_{\gamma}RII$ mAb's. The predicted structures of $Fc_{\gamma}RIIa$ and $Fc_{\gamma}RIIa'$ are 95% identical throughout their sequences. The extracellular domain of $Fc_{\gamma}RIIb$ and the initial part of the intracytoplasmic domain is homologous to that of $Fc_{\gamma}RIIa$ and $Fc_{\gamma}RIIa'$, but the remaining portion of the cytoplasmic tails differ between $Fc_{\gamma}RIIa$ and $Fc_{\gamma}RIIa'$ and the $Fc_{\gamma}RIIb$ receptors. The structures of $Fc_{\gamma}RIIb$ and murine $Fc_{\gamma}RII_{\beta}$ are highly conserved. Transcripts from both species of receptors are alternatively spliced in their first cytoplasmic exon. Both noncoding and coding regions of murine $Fc_{\gamma}RII_{\beta}2$ and $Fc_{\gamma}RIIb2$ cDNAs are identical. $Fc_{\gamma}RIIa'$ is similar to murine $Fc_{\gamma}RII_{\beta}$ genes and the human $Fc_{\gamma}RII_{\beta}$ genes, except in its cytoplasmic domain. Finally, $Fc_{\gamma}RIIa$ is similar to the human $Fc_{\gamma}RIIb$ genes and the murine

$Fc_\gamma RII_\beta$, in addition to the murine $Fc_\gamma RII_\alpha$ gene.

The individual human $Fc_\gamma RII$ transcripts have distinct patterns of expression (Brooks, 1989). Transcripts encoding $Fc_\gamma RIIa$ have been found in monocyte-like cell lines, HL-60 cells differentiated with DMSO, erythroleukemic cell lines, as well as neutrophils, adherent macrophages, and chronic myelogenous leukemia cells. $Fc_\gamma RIIa'$ transcripts were found to be expressed in neutrophils, B lymphocytes, cultured adherent monocytes, U937 cells, and placenta cells, but not in T cells. Finally, $Fc_\gamma RIIb$ expression has been measured in many lymphoid and myeloid cell lines, as well as in B lymphocytes, cultured adherent monocytes, and neutrophils.

Functional consequences of the structural polymorphism of human $Fc_\gamma RII$ expressed in monocytes have been observed. As noted previously in this section, Anderson (1987) initially observed a correlation between a polymorphism in $Fc_\gamma RII$ protein and its ability to mediate an anti-CD3-specific T cell proliferative response. Clark (1989) extended these findings by comparing the cDNA sequences from monocyte responder and nonresponders. The $Fc_\gamma RII$ encoded by the nonresponding individuals differed from the responder's receptor by a single base substitution resulting in a change from histidine to arginine.

Human $Fc_\gamma RIII$ (CD16) is also polymorphic. $Fc_\gamma RIII$ expressed on NK cells and macrophages is a membrane-spanning receptor, whereas $Fc_\gamma RIII$ expressed on neutrophils is anchored via a glycan-phosphatidyl-linkage, devoid of a cytoplasmic tail (Selvaraj, 1988; Huizinga, 1988; Ravetch, 1989; Scallon, 1989; Ueda, 1989). Analysis on a molecular level revealed that the transcript encoding the neutrophil

receptor terminates 4 amino acids after the predicted transmembrane domain. The NK cell $Fc_\gamma RIII$ transcript codes for 21 additional amino acids, which comprises the putative cytoplasmic domain.

Edberg et al. (1989) have analyzed the core $Fc_\gamma RIII$ protein on both cell types. They observed that the neutrophils of individuals homozygous for either NA1 or NA2 $Fc_\gamma RIII$ allotypes expressed one species of receptor, whereas those people heterozygous for the allotype expressed both $Fc_\gamma RIII$ types. Moreover, analysis of all NK cells, regardless of allotype, revealed the presence of two distinct $Fc_\gamma RIII$ types which were distinct from those of the neutrophil.

C. Fc$_\gamma$R Cycling

It has been suggested that the fate of murine macrophage $Fc_\gamma RII$ varies depending upon the valency of the bound ligand. Mellman et al. (1983) observed that murine $Fc_\gamma RII$-mediated phagocytosis of immune complexes resulted in an irreversible depletion of >50% of $Fc_\gamma RII$ from the macrophage plasma membrane. Once internalized, both receptor and immune complexes were degraded rapidly in the lysosome, with a $T_{½}$ of < 2 h versus the $T_{½}$ of ~ 10 h in the absence of ligand (Mellman, 1983, 1984b). The receptor is not degraded, however, upon binding of monomeric IgG, but both $Fc_\gamma R$ and ligand are recycled to the surface (Kurlander, 1983; Jones, 1985). Mellman et al. (1984a) observed that $Fc_\gamma RII$ occupied with monovalent Fab fragments of mAb 2.4G2, is internalized, transported to endosomes, and rapidly delivered back to the surface. $Fc_\gamma RII$ bound by multivalent preparations of mAb 2.4G2 Fab, however, resulted in the transport of the receptor-ligand complex

to the lysosome (Mellman, 1984a; Ukkonen, 1986).

D. Fc$_\gamma$R Signalling

In order to fully understand how Fc$_\gamma$R's function, their signaling mechanisms must be ascertained. Knowledge of the mechanism(s) by which Fc$_\gamma$R's transduce their signals is limited. Receptor crosslinking is probably required for Fc$_\gamma$RII-mediated phagocytosis to occur. As stated previously, occupation of Fc$_\gamma$RII with multivalent ligand triggers endocytosis of the receptor-ligand complex to the endosome, followed by fusion with the lysosome, whereas binding of monomeric ligand to Fc$_\gamma$RII results in transport of the bound receptor to the endosome and back to the membrane (Kurlander, 1983; Jones, 1985; Mellman, 1984a; Ukkonen, 1986). These data suggest that in order for Fc$_\gamma$RII-ligand complexes to be phagocytosed, receptor-crosslinking must occur.

Crosslinking of human Fc$_\gamma$RI and of Fc$_\gamma$RII is necessary for receptor-mediated release of H_2O_2. Anderson et al. (1986) demonstrated that incubation of monocytes with F(ab')2 anti-Fc$_\gamma$RI or anti-Fc$_\gamma$RII mAbs caused the release of superoxide only when the antibodies were crosslinked by anti-murine IgG. These findings were confirmed and extended by Pfefferkorn and Fanger (1989), who observed that the release of O^-_2 mediated by Fc$_\gamma$R on monocytes continued until ligand binding stopped. A direct relationship was observed between the moles of O^-_2 released and the moles of antibody bound to the receptor, suggesting that each Fc$_\gamma$RI, when activated, triggers a part of the signal to release O^-_2.

The role of ion fluxes in signalling Fc$_\gamma$R-mediated events has been

investigated by several laboratories. Aderem and colleagues have studied the role of Na^+ in phospholipase activation (which leads to the release of arachidonic acid) by binding of immune complexes to mouse peritoneal macrophages (1986). They observed that there is a Na^+ requirement for this event that can be bypassed by a Ca^{2+} influx mediated by an ionophore. Ca^{2+} is necessary for this release of arachidonic acid, they found, since EGTA inhibits its formation. From this data, they proposed that the triggering of phospholipase activity by muFc$_\gamma$R requires a series of sequential events including a Na^+ influx followed by an increase in intracellular Ca^{2+}.

Studies by Suzuki suggest that the muFc$_\gamma$RII possesses phospholipase A_2 activity. Such a phenomenon could explain the activation of phospholipase by immune complex binding observed by Aderem (1986). Suzuki and colleagues describe the isolation of two distinct IgG binding proteins from both a mouse macrophage line, P388D1 (Fernandez-Botran, 1985; Suzuki, 1982) and mouse peritoneal macrophages (Nitta, 1984). Purification of cell lysates on a phosphatidyl choline-analogue column yielded a protein which specifically bound IgG2b aggregates and possessed phospholipase A_2 activity, whereas passage of cell lysates over a column containing IgG aggregates produced a protein which bound IgG2a complexes only. The PC-binding protein described by Suzuki (Fernandez-Botran, 1985) differs from Fc$_\gamma$RII isolated from other laboratories (Hibbs, 1985; Lane, 1982; Mark, 1985; Mellman, 1980), however. Whereas the PC-binding protein is 38KD, the Fc$_\gamma$RII protein described by others is 65KD. The isoelectric points of the two proteins differ

as well. Finally, the quantities obtained per cell equivalent from purified by Suzuki were 20x greater than what has been previously reported to exist on any mouse macrophage line (Unkeless, 1975,1977,1979).

The role of calcium mobilization from intracellular pools ($[Ca^{2+}]_i$) has been studied by several investigators. Two groups reported that a rise in $[Ca^{2+}]_i$ upon $Fc_\gamma R$ ligation is associated with receptor-mediated phagocytosis in both mouse macrophages (Young, 1984) and in human neutrophils (Lew, 1985). No rise in $[Ca^{2+}]_i$ is observed, however, during $Fc_\gamma R$ ligation in mouse peritoneal macrophages (McNeil, 1986). More recently, DiVirgilio (1988) used several methods to clamp and buffer $[Ca^{2+}]_i$ mouse macrophages, in order to establish unequivocally the role of $[Ca^{2+}]_i$ in $Fc_\gamma R$-mediated phagocytosis. Very low levels of $[Ca^{2+}]_i$ (1-10 nm) were observed. At these levels, they demonstrated that phagocytosis was not inhibited. Pfefferkorn investigated the effect of monovalent cation fluxes on $Fc_\gamma R$-mediated phagocytosis. She demonstrated that cation fluxes were not required for the initiation of either phagocytosis or for O_2^- generation by $Fc_\gamma R$ (1984).

Although ion fluxes are not required for the induction of phagocytosis by $Fc_\gamma R$, cellular proteins may be involved in signal transduction of $Fc_\gamma R$. The role of G proteins in this event is compelling, and several laboratories have investigated $Fc_\gamma R$/G protein associations. Gresham et al. (1987) suggested that G proteins are involved in triggering phagocytosis by the human neutrophil $Fc_\gamma R$, presumably $Fc_\gamma RII$. They reported that both cholera toxin and pertussis toxin, which bind different classes of G proteins, can inhibit $Fc_\gamma R$-mediated stimulated, but not

unstimulated phagocytosis. Furthermore, they showed that this inhibition is not due to the loss of $Fc_{\gamma}R$ expression. The induction of phagocytosis by phorbol esters, which activate protein kinase C, is not inhibited by either toxin. Therefore, the authors suggest that one or more G proteins and protein kinase C may play a role in phagocytosis.

In addition, Feister and colleagues reported that G proteins are involved in the triggering of the oxidative burst by the human neutrophil $Fc_{\gamma}RII$ (1988). Using pertussis toxin, they demonstrated that $Fc_{\gamma}RII$-mediated superoxide production was almost completely inhibited, whereas receptor-stimulated degranulation was only partially inhibited. These data suggest that two mechanisms of signal transduction are operative in $Fc_{\gamma}RII$-mediated event, one of which involves G proteins.

VII. EXPRESSION OF MURINE Fc RECEPTORS FOR IgG

Two murine $Fc_\gamma RII$ genes, $muFc_\gamma RII_\alpha$ and $muFc_\gamma RII_\beta$, encode distinct transmembrane $Fc_\gamma RII$ proteins (Ravetch, 1986; Lewis, 1986; Hibbs, 1988). The predicted amino acid sequences of the extracellular domains of $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$ are 95% homologous, and are composed of two repeated Ig-like domains, each containing two disulfide-linked cysteine residues and two glycosylation sites. No homology exists, however, between the leader sequences, the transmembrane, or the cytoplasmic domains of $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$. The $Fc_\gamma RII_\alpha$ transcript is expressed in macrophages (Ravetch, 1986), NK cells (Perussia, 1989), and mesangial cells (Santiago, 1989); the $Fc_\gamma RII_\beta$ transcript has been found in macrophages, B cells, and T cells (Ravetch, 1986). There are two transcripts of the $Fc_\gamma RII_\beta$ gene: $Fc_\gamma RII_\beta 1$ contains a 46 amino acid insertion in its cytoplasmic domain which is absent from $Fc_\gamma RII_\beta 2$. Whereas the $Fc_\gamma RII_\beta 2$ transcript is present in B cells, T cells, and macrophages, the $Fc_\gamma RII_\beta 1$ transcript is found only in lymphocytes (Ravetch, 1986). The translated proteins have been indirectly identified by the binding of monoclonal antibody (mAb) 2.4G2, which binds to both $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$ (Unkeless, 1979), or IgG complexes to cell lines transfected with $Fc_\gamma RII_\alpha$ or $Fc_\gamma RII_\beta$ (Ravetch, 1986; Weinschanck, 1988). There is identity between sequences of tryptic peptides isolated from $Fc_\gamma RII$ purified from the S49.1 T cell line and the sequence predicted from the $Fc_\gamma RII_\beta$ cDNA sequence (Pan, 1987), but no amino acid analysis of $Fc_\gamma RII_\alpha$ protein has been reported.

In order to analyze the expression of $Fc_\gamma RII_\alpha$ protein, peptides were synthesized peptides in the cytoplasmic domain of $Fc_\gamma RII_\alpha$ that were predicted by the cDNA sequence, and used the peptides coupled to keyhole limpet hemocyanin (KLH) to elicit anti-peptide IgG. The anti-peptide antibody was used in a sandwich ELISA to demonstrate that $Fc_\gamma RII_\alpha$ is translated in J774a, a macrophage cell line that synthesizes $Fc_\gamma RII_\alpha$ mRNA, as well as in peritoneal macrophages. Our results suggest a positive correlation between the level of $Fc_\gamma RII_\alpha$ expression and phagocytosis. Finally, I found that rIL-6 enhances the expression of a $Fc_\gamma R$ that bears the mAb 2.4G2 epitope but that is distinct from $Fc_\gamma RII_\alpha$.

MATERIALS AND METHODS

Animals. Female CD2F$_1$/J mice (The Jackson Laboratory, Bar Harbor, ME) were used at 10 to 16 weeks of age. Female New Zealand white rabbits (The Jackson Laboratory) were used at 20 weeks of age.

Cell lines and culture conditions. J774A (Weinschanck, 1988), S49.1 and L5178Y (Horibata, 1970) cell lines were grown in suspension culture in alpha-modified MEM (αMEM) (Hazleton, Lenexa, KA) supplemented with 3% FCS (Flow labs, McLean, VA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Mouse peritoneal macrophages were cultured in Dulbecco's modified Eagle's MEM (DMEM) supplemented with 10% FBS and maintained as described below. Cell lines treated with lymphokines were cultured in αMEM supplemented with 10% FCS and maintained as described below.

Cell lysate preparation. Cells (10^7) were harvested and centrifuged at 400 x g. Cell pellets or membranes (10^7 cell equivalents) were lysed in PBS containing 0.5% NP-40, 1mM diisopropylfluorophosphate (Sigma) and 0.2 U/ml aprotinin (Sigma). Lysates were cleared of nuclei and cell debris by centrifugation ((10,000 x g, 15 min.)

Membrane isolation. Cell membranes were isolated according to the method of Jesaitis et al (1983) and Huey et al. (1985). Briefly, 3 x 10^9 cells were suspended in

40 ml of 10mM HEPES, pH7.4, 0.34 M sucrose, 0.1 mM $MgCl_2$, 1 mM EDTA, 10mM dithiothreitol, and 100 KIU/ml aprotinin (Sigma Chemical Co., St. Louis, MO). The cells were ruptured by nitrogen cavitation after equilibration of cells with nitrogen (400 psi, 20 min, 4°). After low-speed centrifugation (10 min at 800 X g) to remove intact cells and nuclei, the supernatant was centrifuged (43,000 X g, 25 min) and the pellet was resuspended in 10 ml of 10% sucrose in HME buffer (20 mM NaHepes, 2 mM $MgCl_2$, 1 mM EDTA, pH 8.0). This was layered on a discontinuous sucrose gradient layered with 12 ml of 40% sucrose, 10 ml of 30% sucrose, and 10 ml of 20% sucrose in all diluted in HME buffer and centrifuged (100,000 X g, 1 h). Four distinct bands were detected, and the two intermediate bands were collected and stored at -70° for use in ELISA.

Peritoneal cells. Resident peritoneal macrophages were isolated from untreated mice. Thioglycollate-elicited macrophages were obtained 4 days after i.p. injection of 1 ml of thioglycollate broth. BCG-elicited macrophages were obtained from mice injected 29 days previously and boosted on day 21 with 10^7 live organisms of BCG Pasteur strain 1011, obtained as a gift from Dr. North, Trudeau Institute (Saranac Lake, NY). Organisms were sonicated prior to use with a microprobe in a sonicator (Ultrasonics, Inc., Farmingdale, NY) set at 2 for 2 min. Peritoneal cells were obtained by lavage with 5 ml of cold PBS containing 10 mM EDTA. Cells were washed with PBS and plated in 100 cm dishes at 2 x 10^7 cells/dish in DMEM containing 10% FCS. Differential counts of mouse peritoneal cells were made using

Wright's stain. BCG-infected animals yielded 2.5 x 10^6 cells/mouse with 25% macrophages. Mice injected with thioglycollate broth yielded 6 x 10^5 cells/ mouse with 70% macrophages, 3% polymorphonuclear leukocytes (PMN's), and 27% lymphocytes. Untreated mice yielded 2 x 10^6 cells/mouse with 45% macrophages, 19% PMN's and 36% lymphocytes. After 2 hours, non-adherent cells were removed. After the adherence step, the BCG-activated cells were 80% macrophages, and the thioglycollate-stimulated and resident peritoneal cells were 90% macrophages. Fresh medium was added, and macrophages were harvested 18 hours later.

Antibodies. The rat anti-Fc_γRII mAb 2.4G2 was purified from culture supernatant on a rabbit anti-rat agarose column (Sigma) as follows. Culture supernatant from hybridoma cells (5 x 10^5 cells/ml DME) was collected by centrifugation at 400 x g and passed over the column. Unbound material was removed from the column with PBS until optical density at 280 nm of buffer passed through the column was equal to that of the washing buffer. The bound Ig was eluted from the column with 0.1 M glycine-HCl, pH 2.5, neutralized with an equal volume of 0.1 M Tris, pH 8.5, and dialyzed versus PBS.

Both rabbit anti-Fc_γRII and normal rabbit IgG were purified from serum by affinity chromatography on a protein A column (Pierce, Rockford, IL). Serum diluted 1:1 in PBS was passed over the column twice, and all subsequent steps were identical to those described for purification of mAb 2.4G2. Biotinylated goat anti-rabbit and anti-rat antibodies and strepavidin horseradish peroxidase (HRP) were

purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Peptide synthesis, conjugation and immunization. All L-amino acids and blocked amino acids were from Peninsula (Belmont, CA). Peptide sequences RNLQTPREYWRKSLC (A) and KSLSIRKHQAPQDKC (B) were synthesized stepwise by the solid-phase methods (Merrifield, 1963), using a N-t-BOC-S-4-methyl benzyl-L-cysteine as the solid phase Merrifield resin support. After synthesis, deblocking, and HF cleavage (Tam, 1983), the peptides were shown to be of satisfactory purity by HPLC on a VYDAC C18 reverse phase column eluted with a 0-50% acetonitrile gradient containing 0.1% trifluoroacetic acid. Amino acid analyses of the peptides gave the expected amino acid ratios. The peptides were coupled to KLH by the carboxyl terminal cysteine residues using the bifunctional reagent m-maleimidobenzoyl-N-succinimide ester (MBS) (Pierce) (Lerner, 1981). KLH (5 mg in 0.5 ml of PBS, pH 7.4) was acylated with 2 mg MBS in 50 µl DMF (30 min, room temperature). Free and/or hydrolyzed MBS were removed by gel filtration over a Sephadex G25 column in 10 mM phosphate buffer (pH 6). The activated protein fractions were pooled and incubated with 10 mg peptide A or B (3 h, room temperature, pH 7.4). The peptide-KLH conjugate was dialyzed against 150 mM NaCl, 10mM phosphate (pH 7.2) and used for immunization.

Isolation of anti-peptide Ig. Rabbits were immunized s.c. with 1 mg of KLH-

conjugated peptide in 1 ml of complete Freund's adjuvant and were boosted two weeks later s.c. with 0.5 mg conjugated peptide in 1 ml of incomplete Freund's adjuvant. The animals were bled 2 and 4 weeks after the latter injection. Anti-peptide IgG was isolated from the serum by affinity chromatography over a peptide-Sepharose 4B column (Pharmacia Fine Chemicals, Uppsala, SW). The bound Ig was eluted from the column with 0.1 M glycine-HCl, pH 2.5, neutralized with an equal volume of 0.1 M Tris, pH 8.5, and dialyzed against PBS.

Lymphokine induction. Rat recombinant IFN-γ (rIFN-γ) (Amgen, Thousand Oaks, CA) had a specific activity of 5×10^5 mouse U/ml. The following lymphokines were provided by Dr. Mark Garnick (Genetics Institute, Cambridge, MA): Human recombinant IL-6 (rIL-6), produced in *Escherichia coli*, 5×10^6 U/mg , human recombinant macrophage colony stimulating factor (rM-CSF) produced in CHO cells, (1:300,000 dilution yields 1\2 maximal stimulation of colony formation), and human recombinant granulocyte/macrophage colony stimulating factor (rGM-CSF), produced in CHO cells, 1.7×10^7 U/mg. Human recombinant TNF-α (rTNF-α), 3×10^5 U/mg, produced in yeast, was a gift from Dr. Anthony Cerami (Rockefeller University, New York, NY). Transforming growth factor-β (TGF-β), purified from porcine platelets, was purchased from R & D Systems (Minneapolis, MN). Mouse rIL-2 and rIL-3 were purchased from Genzyme Corp (Boston, MA). Mouse IFN-α, and IFN-β were purchased from Lee Biomolecular (San Diego, CA).

J774a or S49.1 cell lines were grown in suspension cultures (2×10^5 cells/ml)

in αMEM containing 10% FCS in the presence or absence of 200 U/ml rIFN-γ. Other lymphokines were added for 24-96 h to J774a cells initially seeded at 2 x 10^5 cells/well in 24 well cluster plates (Linbro, McLean, VA).

Fc$_\gamma$RII$_\alpha$ ELISA. Fc$_\gamma$RII$_\alpha$ in cell or membrane lysates was measured by ELISA. Cell lysates were cleared of nuclei and cell debris by centrifugation (10,000 x g, 15 min.) Fc$_\gamma$RII$_\alpha$ in cell lysates was measured by sandwich ELISA. Serial dilutions of cell or membrane lysates in 0.5% Nonidet P-40 (NP-40) diluted in PBS (50 μl/well) were incubated in flat bottom 96-well microtiter plates (Falcon Pro-bind, Beckton-Dickenson Laboratories, Oxnard, CA) previously coated overnight with mAb 2.4G2 IgG (5 μg/ml PBS, 50 μl/well) in PBS and blocked with 3% PBS/BSA. Fc$_\gamma$RII$_\alpha$ was detected by addition of rabbit anti-peptide Ig (5 μg/ml PBS, 50 μl/well) followed by sequential additions of biotinylated goat-anti-rabbit IgG and strepavidin-HRP (in PBS, at manufacturer's suggested dilution). Bound strepavidin-HRP was assayed colorometrically and developed as described previously (Khayat, 1987). Alternatively, cell lysates were incubated in microtiter plates coated with rabbit anti-Fc$_\gamma$RII IgG (5 μg/ml PBS, 50 μl/well), and Fc$_\gamma$RII$_\alpha$ was detected by sequential additions of biotinylated (Berman, 1980) rabbit anti-peptide Ig and strepavidin-HRP.

Fc$_\gamma$RII ELISA. Fc$_\gamma$RII in cell lysates was measured by sandwich ELISA. Serial dilutions of cell lysates prepared as described above were incubated in microtiter plates coated overnight with mAb 2.4G2 (5 μg/ml PBS, 50 μl/well). Fc$_\gamma$RII was

detected by addition of rabbit anti-Fc$_\gamma$RII IgG (5 μg/ml PBS, 50 μl/well) and the assay was developed as described above.

Iodination and binding studies. MAb 2.4G2 IgG (50 μg) was iodinated with 1 mCi of [^{125} I] Na (Amersham Corp., Arlington Heights, IL) using tubes coated with 1 μg iodogen (Pierce) as described (Fraker, 1978). Briefly, antibody was incubated with [^{125}I] Na for 5 min at 4°. Unincorporated [^{125}I] Na was removed by gel filtration on a G-25M column (Pharmacia) by passage of PBS containing 1 mg/ml BSA (PBS-BSA) over the column. Fractions (0.5 ml) were collected and those containing labeled antibody were detected with a geiger counter. The final specific activity of labeled antibody, which was determined by gamma spectrometry (RiaGamma, LKB, Gaithersburg, MD) was 6 x 10^6 cpm/μg mAb protein. Binding of [^{125}I]-2.4G2 mAb to adherent cells was done on ice in 24 well cluster plates (Linbro). Cells were incubated with [^{125}I]-2.4G2 (0.5 ml, 5.5 μg mAb 2.4G2/ml, 5.4 x 10^5 cpm/μg) in PBS-BSA. Background was determined in a parallel incubation with [^{125}I]-2.4G2 of low specific activity (0.5 ml, 200 μg 2.4G2/ml, 1.5 x 10^4 cpm/μg). After incubation for 1 h, the initial labeling solution was aspirated, and the wells were washed three times with PBS-BSA by immersing plates in buffer. Cells were removed with cotton swabs, which were assayed by gamma spectrometry.

Plasmid isolation Plasmids were isolated from bacteria using a modification of the alkaline lysis technique of Birnboim & Doly (1979), followed by CsCl gradient

ultracentrifugation. 1 ml of L broth (per liter: 10 gm Bacto-Tryptone; 5 gm Yeast Extract; 10 gm NaCl; 1 gm glucose) containing ampicillin (50 μg/ml) was inoculated with bacteria from a frozen stock. This seed culture was grown overnight in sterile Falcon 2059 tubes at 37° in a Queue Shaker rotating at 225 rpm. 100-200 μl of this seed culture were transferred to 1.5 liter growth media (L-broth with 50 μg/ml ampicillin) and grown overnight as described for seed culture. Bacteria were harvested by centrifugation in 250 ml bottles in a fixed angle rotor (4000 x g, 20 min., 4°). The bacterial pellet was resuspended in 12 ml of GET buffer (25 mM Tris-HCL; 10mM EDTA; 1% Glucose, pH 8.0) and kept on ice for 15 min. This suspension was incubated with 0.5 ml of lysozyme [25 mg lysozyme (Calbiochem)/ml GET, 15 min, 4 °]. Subsequently, 27 ml of fresh alkaline lysis solution (0.2 M NaOH; 1% SDS) was added to mixture and incubated for 15 min at 4 . This incubation was followed by addition of 20.25 ml of cold 3 M KOAc, pH 4.8 (prepared according to Maniatis et al. (1982). This was thoroughly mixed and incubated for 15 min. at 4o. After centrifugation of the mixture in a fixed angle rotor (20,000 x g, 20 min., 4°), the supernatant was passed through cheesecloth into a 50 ml Nalge 3139 tube. 42 ml of isopropanol was added, and the material was precipitated (room temperature, 20 min.). After centrifugation in a swinging bucket rotor (16,000 x g, 30 min., room temperature), the pellet was air dried for 10 min. and resuspended in 5 ml of TEN10 (10 mM Tris-HCl; 1 mM EDTA; 10 mM NaCl, pH 8.0) containing 150 μl of RNAase A (10 mg/ml deionized water) and incubated for 60 min. at 37°. The solution was sequentially extracted as follows: 18 ml of

Phenol:Chloroform:Isoamyl Alcohol (25:24:1 volume ratio) was added to the solution and centrifuged in a swinging bucket rotor (1700 x g, 5 min., room temperature). To supernatant, Chloroform:Isoamyl Alcohol (24:1 volume ratio) was added and spun as in previous extraction. Supernatant was precipitated by addition of 18 ml isopropanol (20 min., room temperature). The precipitate was pelleted in a swinging bucket rotor (16,000 x g, 30 min., room temperature). The pellet was resuspended in 20 ml of 51% weight/volume Cesium Chloride:TE solution (10 mM Tris-HCl; 1 mM EDTA, pH 8.0). This solution was transferred to 2 Beckman polyallomer Quick Seal Ti75 tube with a syringe capped with 16 gauge needle. 300 µl of Ethidium Bromide (10 mg/ml deionized H_2O) was added to the tubes, which were then heat-sealed and centrifuged in a Beckman Ti75 rotor (55,000 rpm, 14 h, 20°). Plasmid band was withdrawn from tube under long-wave ultraviolet light, with a 23 gauge needle attached to a 3 ml syringe. After transfer to a 50 ml tube, the solution was extracted 3 times with water-saturated butanol (40 ml). The aqueous solution was diluted with 2 volumes of sterile deionized water after transfer to a clean tube. Material was precipitated from solution by addition of 1/9th volume of 3 M NaOAc, pH 5.2 and 2 volumes of 95% ethanol (2 h, -20°). The DNA was pelleted in a swinging bucket rotor (16,000 x g, 30 min, 4°) vacuum dried for 30 min., and resuspended in TE. Quantity and purity of DNA was determined by spectrophotometry (260 and 280 nm wavelengths) and restriction digestion analysis.

Restriction enzyme digestions Enzymes used in restriction digests were purchased

from either New England Biolabs (Beverly, MA) or Boehringer Manheim Biochemicals (Indianapolis, IN), according to manufacturer's instructions. DNA inserts were separated agarose gel electrophoresis [(1.5% low melt agarose in TE, 10 μl ethidium bromide/100 ml gel). After separation, the appropriate DNA band was visualized under short wave ultraviolet light, and cut from gel. Isolation of DNA from gel was achieved using the technique of Maniatis (1982). Recovery of DNA was quantified by comparing DNA of known quantity to purified insert on agarose gel electrophoresis.

Labeling of cDNA inserts. cDNA inserts were labeled by a modification of the nick translation method described by Maniatis (1982). [^{32}P] dCTP and nick translation kit (containing enzymes and unlabeled nucleotides) were purchased from Amersham. DNA (100 ng, 0.5-1 mg/ml) was incubated with 5x buffer (4 μl), enzyme solution (2 μl), [^{32}P] dCTP (5-8 μl), and deionized water to a total volume of 20 μl. Reaction mixture was incubated at 14° for 2 h, and then stopped by addition of 1 μl EDTA (0.5 M). Unincorporated [^{32}P] dCTP was removed by passage of solution over a spun column, which was made as follows by a modification of the technique described by Maniatis (1982). A 3 ml syringe was packed with Sephadex G-50 (Pharmacia) by centrifugation (200 x g, 20 sec.). Column was blocked by addition of 10 μl of sheared/denatured salmon sperm DNA (10 mg/ml TE) in 1 ml TE, followed by centrifugation (200 x g, 2 min.). 2 μl of tRNA (5 mg/ml TE) was added to DNA solution, which was then added to the column. After centrifugation (400

x g, 5 min), specific activity of labeled DNA was determined using a scintillation counter (1217 RackBeta, LKB Pharmacia). Incorporation of 10^8 cpm/μg DNA was acceptable for use in northern analysis.

cDNA probes. After isolation of plasmid DNA as described above, restriction digestion was performed as follows. The $Fc_\gamma RII_\alpha$ cDNA insert including the 5' untranslated and signal regions, and the first 43 base pairs encoding the receptor was isolated by digestion of plasmid 4018 (Ravetch, 1986) with Bam H1 and Pst 1. The $Fc_\gamma RII_\beta$ cDNA insert was isolated by digestion of plasmid 3901 (Ravetch, 1986) with Pst 1, resulting in a 285 base pair cDNA containing the 5' untranslated region. The $Fc_\gamma RII$ cDNA insert corresponding to the 5' extracellular coding region was isolated by digestion of plasmid 3901 with Bam H1 and Bgl II.

Northern blot analysis. Total cellular RNA was isolated from cells after guanidinium thiocyanate lysis and centrifugation through 5.7 M cesium chloride as described by Chirgwin (1979). Total RNA was electrophoresed on a 1.2% agarose gel and transferred to nitrocellulose (Maniatis, 1982). Blots were hybridized with [^{32}P]-labeled cDNA probes at 42° for 18 hr in a solution containing 50% formamide, 10% dextran sulfate, 5x SSCPE, (1X SSC is 0.15 M NaCl, 0.015 M Na citrate, 0.01 M KH_2PO_4, 0.001 M EDTA), 1X Denhardts (0.02% polyvinylpyrrolidone, 0.02% Ficoll, and 0.02% BSA), 100 μG/ml of sonicated salmon sperm DNA, and 10^6 cpm/ml of ^{32}P-cDNA probe (17). Blots were exposed to Kodak XAR film and 2

intensifying screens (Dupont Cronex Lightening Plus) after washing in 0.1X SSC containing 0.1% SDS at 50°. Densitometry was performed using an LKB densitometer (Ultroscan, XL).

PIPLC digestion. Control or rIL-6 stimulated adherent J774a cells (10^6) in 24 well plates were incubated (1 h, 37°) with *Bacillus thuringiensis* phosphatidylinositol-specific phospholipase C (PIPLC) (with a final activity of 5 μmol/min/ml measured by hydrolysis of phosphatidyl inositol) generously provided by M. Low (Columbia University, New York, NY) (Low, 1988). After washing plates with PBS-BSA, cells were incubated with [^{125}I]-2.4G2 (0.5 ml, 5.5 μg 2.4G2/ml, 5.5 x 10^5 cpm/μg). Nonspecific binding was determined in a parallel incubation with equivalent radioactivity of [^{125}I]-2.4G2 of low specific activity (200 μg 2.4G2/ml, 1.5 x 10^4 cpm/μg). As a positive control for PIPLC activity, we measured by FACS the binding of the anti-CD16 FITC-mAb 3G8 to PIPLC-digested and control human neutrophils.

RESULTS

Expression of $Fc_\gamma RII_\alpha$ mRNA. To examine the expression of $Fc_\gamma RII_\alpha$, I used the macrophage-like cell line, J774a, which synthesizes $Fc_\gamma RII_\alpha$ transcript, but not $Fc_\gamma RII_\beta$ mRNA (Weinschanck, 1988). This was confirmed by Northern blot analysis on total RNA from J774a cells using $Fc_\gamma RII_\alpha$-specific and $Fc_\gamma RII_\beta$-specific cDNA probes (Fig. 2A and B). Incubation of the J774a cells with IFN-γ resulted in a 4-fold increase in the level of $Fc_\gamma RII_\alpha$ mRNA, whereas IFN-γ did not induce $Fc_\gamma RII_\alpha$ mRNA in either J774a or S49.1 cells (Fig. 2A and B). Only the $Fc_\gamma RII_\beta$ transcript was expressed in S49.1 cells (Fig. 2B), as reported previously (1).

An ELISA for $Fc_\gamma RII_\alpha$ I developed an antibody that would bind specifically to $Fc_\gamma RII_\alpha$ by immunizing rabbits with KLH-conjugated synthetic peptides corresponding to the cytoplasmic domain of $Fc_\gamma RII_\alpha$ (Figure 3). Peptide A, RRNLQTPREYWRKSLC, is the predicted membrane-proximal sequence from residues 206 to 220 and peptide B, KSLSIRKHQAPQDKC, is the predicted carboxyl-terminal sequence from residues 218 to 231 (Ravetch, 1986). Both peptides had cysteine (not present in the $Fc_\gamma RII_\alpha$ sequence) added at the COOH-terminus to facilitate coupling to proteins. Purification of antisera from the rabbit injected with peptide A yielded 1 mg of specific IgG/ml sera and antisera from the rabbit injected with peptide B yielded 0.4 mg of specific IgG/ml sera.

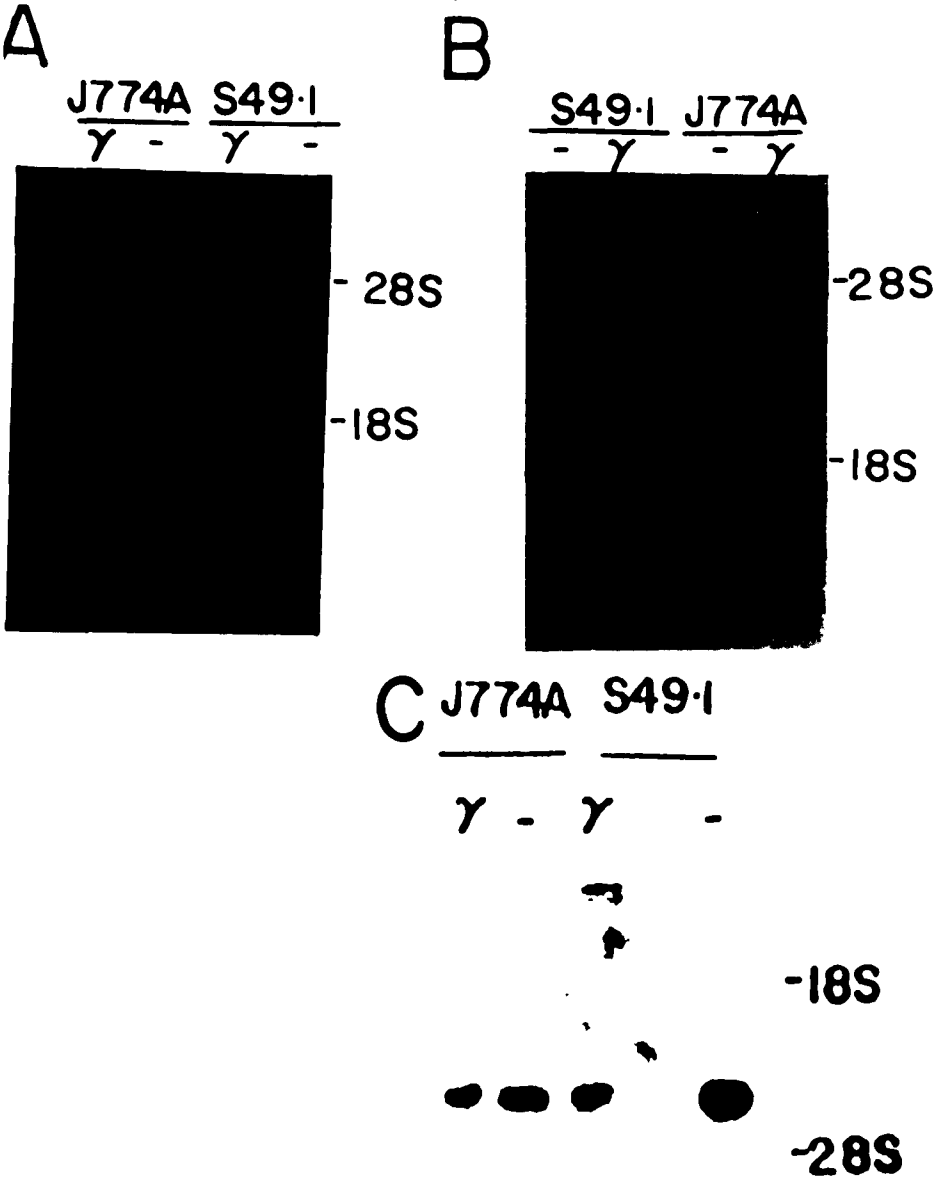


Fig. 2 **Effects of IFN-γ on $Fc_\gamma RII$ mRNA levels in J774a and S49.1 cells. Cells were grown in suspension culture in the presence or absence of IFN-γ (100 U/ml) for 24 h before the preparation of RNA and Northern analysis as described in Materials and Methods. Two blots were prepared with 10 μg of total RNA loaded in each lane and hybridized with $Fc_\gamma RII_\alpha$-specific (A) and $Fc_\gamma RII_\beta$-specific (B) cDNA probes. Blots were exposed on XAR film for 18 hours (A) and 66 hours (B). Blots were stripped and rehybridized with an α-actin cDNA probe for normalization of RNA (C).**

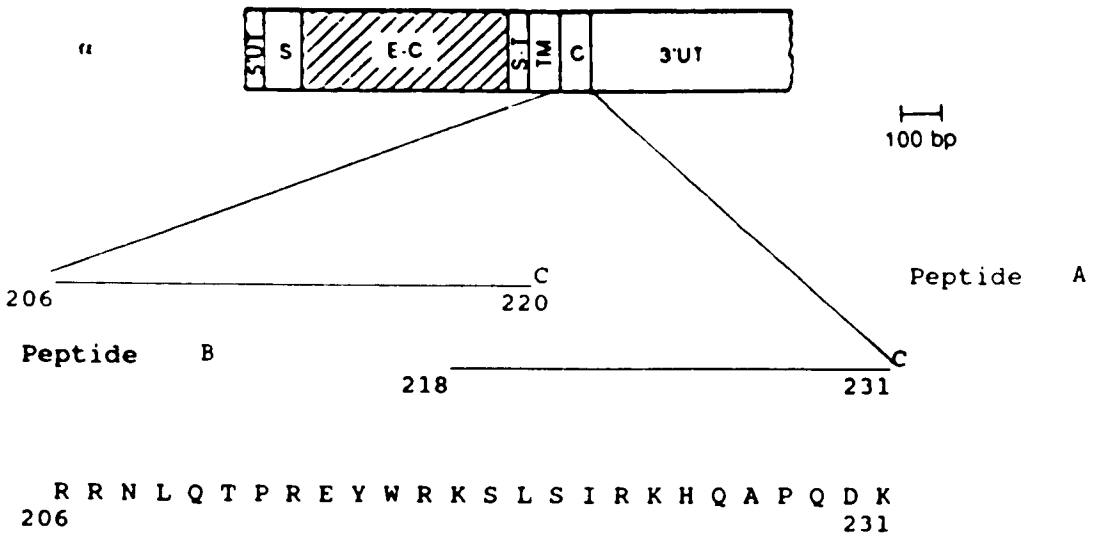


Fig.3 **Sequence of synthetic peptides. Peptides A and B were synthesized as predicted from the cytoplasmic $Fc_{\gamma}RII_{\alpha}$ cDNA sequences indicated.**

I used these anti-peptide sera in a sandwich ELISA for $Fc_\gamma RII_\alpha$. Either anti-$Fc_\gamma RII$ mAb 2.4G2 or a rabbit antiserum directed against the extracellular domain of $Fc_\gamma RII_\beta$ (Qu, 1988) was adsorbed onto microtiter plates which were then incubated with the J774a and S49.1 cell lysates. $Fc_\gamma RII_\alpha$ that was bound to the plate was then visualized with anti-peptide IgG. Only antisera elicited by immunization with the carboxyl terminal peptide, peptide B, gave a signal over background in this assay. The conformation(s) of peptide A recognized by the anti-peptide antibody may differ from the conformation(s) in the native protein. The ELISA is specific for $Fc_\gamma RII_\alpha$, and does not give a positive signal with lysates of S49.1 cells, which express $Fc_\gamma RII_\beta 1$ and $Fc_\gamma RII_\beta 2$ mRNA (Fig. 4a). The same specificity was observed when the ELISA plates were coated with either anti-$Fc_\gamma RII$ mAb 2.4G2 (Fig.4a), or with a polyclonal rabbit sera elicited by immunization with a truncated $rFc_\gamma RII_\beta$ that has the transmembrane and cytoplasmic domains deleted (Fig. 5a). In parallel with the increased abundance of $Fc_\gamma RII_\alpha$ mRNA levels observed after IFN-γ stimulation of J774a cells (Fig. 2a), a great increase in $Fc_\gamma RII_\alpha$ was observed in lysates from IFN-γ-stimulated J774a cells relative to unstimulated cells (Figs. 4b, 5b). ELISA of $Fc_\gamma RII_\alpha$ in a membrane fraction of J774a cells resulted in reduced background relative to that in total cell lysates, suggesting an enrichment of $Fc_\gamma RII_\alpha$ in the membrane fraction (Fig. 6). These experiments demonstrated that $Fc_\gamma RII_\alpha$ protein is translated as predicted by its mRNA transcript, with a cytoplasmic tail and extracellular domain.

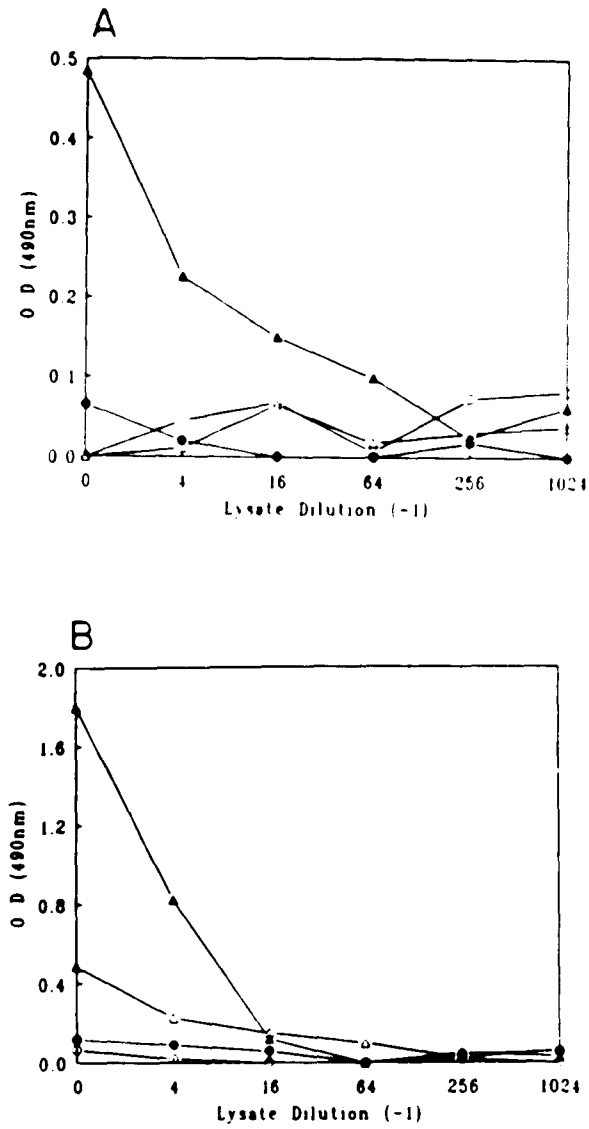


Fig. 4 **Detection of $Fc_{\gamma}RII_{\alpha}$ by ELISA. Cells grown as in Fig. 1 were lysed and assayed on microtiter plates coated with mAb 2.4G2 as described in Materials and Methods. (A) Specificity of anti-peptide B antibody. J774a cell lysates (▲,△) and S49.1 lysates (•,○) were incubated with either anti-peptide B IgG (▲,•) or normal rabbit Ig (△,○). (B) IFN-γ elevation of $Fc_{\gamma}RII_{\alpha}$ expression. Lysates of J774a cells (▲), J774a cells induced with 100 U/ml IFN-γ (▲), S49.1 cells (○), and S49.1 cells induced with IFN-γ (•) were assayed with anti-peptide B antiserum. The data are representative of three experiments.**

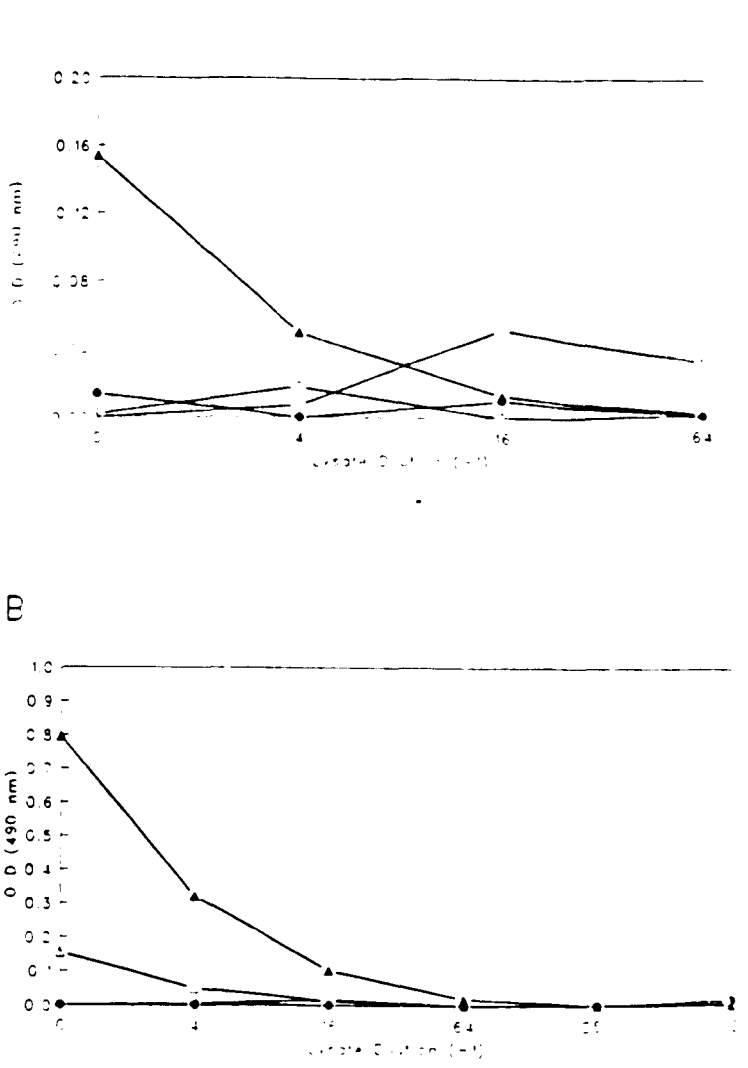


Fig. 5 **Detection of $Fc_{\gamma}RII_{\alpha}$ by ELISA. Cells grown as in Fig. 1 were lysed and assayed on microtiter plates coated with rabbit anti-$Fc_{\gamma}RII$ antibody as described in Materials and Methods. (A) Specificity of anti-peptide B antibody. J774a cell lysates (▲ ,△) and S49.1 lysates (•,◦) were incubated with either anti-peptide B IgG (▲ ,•) or normal rabbit Ig (△ ,◦). (B) IFN-$_{\gamma}$ elevation of $Fc_{\gamma}RII_{\alpha}$ expression. Lysates of J774a cells (△), J774a cells induced with 100 U/ml IFN-$_{\gamma}$ (▲), S49.1 cells (◦), and S49.1 cells induced with IFN-$_{\gamma}$ (•) were assayed with anti-peptide B antiserum. The data are representative of three experiments.**

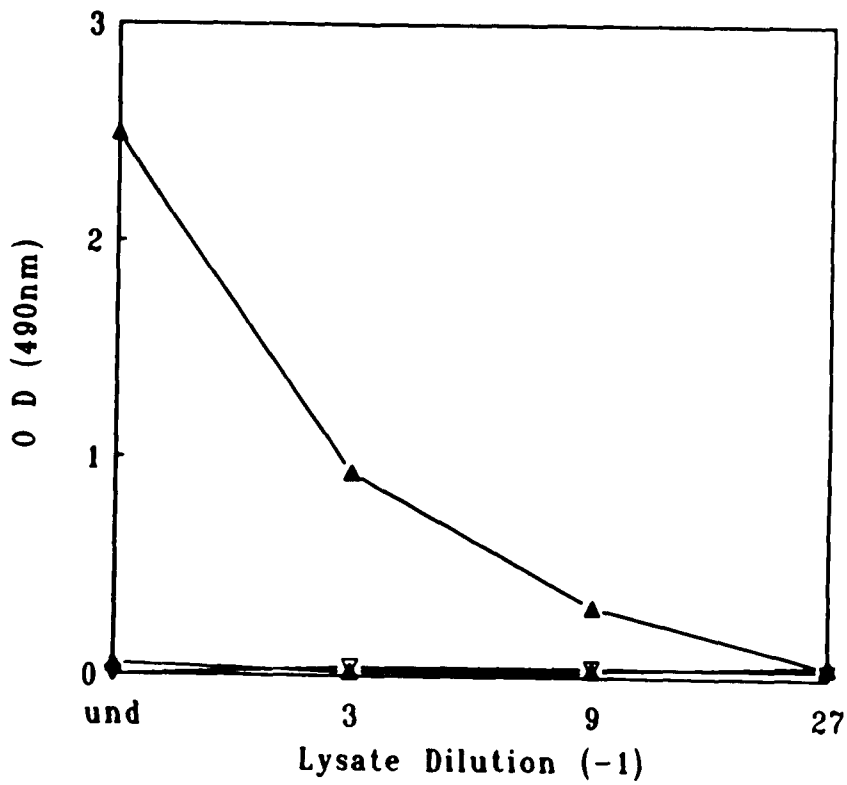


Fig. 6 Detection of $Fc_{\gamma}RII_{\alpha}$ in membrane lysates. Membranes were isolated from cells in suspension culture, lysed in NP-40-containing buffer, and assayed for $Fc_{\gamma}RII_{\alpha}$ as in Fig. 2. Binding of anti-peptide B antibody to J774a (▲), S49.1 (▼), and L5178Y ($Fc_{\gamma}RII$ negative) cells (△).

Expression of $Fc_\gamma RII_\alpha$ in Peritoneal Macrophages. I quantified the expression of $Fc_\gamma RII_\alpha$ in resting peritoneal macrophages and macrophages elicited with viable BCG or thioglycollate broth. Resident and thioglycollate-elicited macrophages expressed equivalent levels of $Fc_\gamma RII_\alpha$, but no $Fc_\gamma RII_\alpha$ was detectable in BCG-elicited macrophages (Fig. 6). This is in dramatic contrast to the results of Ezekowitz et al. (1981), who found roughly equivalent levels of binding of mAb 2.4G2 to resident peritoneal macrophages and BCG-elicited macrophages.

I next examined the regulation of $Fc_\gamma RII_\alpha$ synthesis by different lymphokines. Initial screening of relative $Fc_\gamma RII$ levels was done by measuring the binding of mAb [^{125}I]-2.4G2 to viable J774a cells treated with physiologic doses of the different lymphokines. Most lymphokines had no effect on the binding of [^{125}I]-2.4G2. However, the ratios of [^{125}I]-2.4G2 bound to rIL-6 induced/control J774a cells was 1.9 ± .22 (Table 3). Initial experiments were done with 500 U/ml rIL-6, but the effect was also observed with 25 U/ml of rIL-6. The experiments were reproducible with different lots of rIL-6 and were repeated 6 times, with an average ratio of [^{125}I]-2.4G2 bound to rIL-6-induced/control cells of 1.8 ± .22. Increased expression of $Fc_\gamma RII$ was evident within 48 h of rIL-6 addition, and was stable at 96 h. Since intact cells were used in this assay, only plasma membrane $Fc_\gamma RII$ was measured. To determine if the increase in $Fc_\gamma RII$ represented an increase in total cellular $Fc_\gamma RII$, the levels of $Fc_\gamma RII$ in total cell lysates of rIL-6-treated versus untreated J774a cells were compared. I observed parallel increases in mAb 2.4G2 binding to intact cells (Fig. 8a) and to whole cell lysates (Fig 8b) after rIL-6 stimulation.

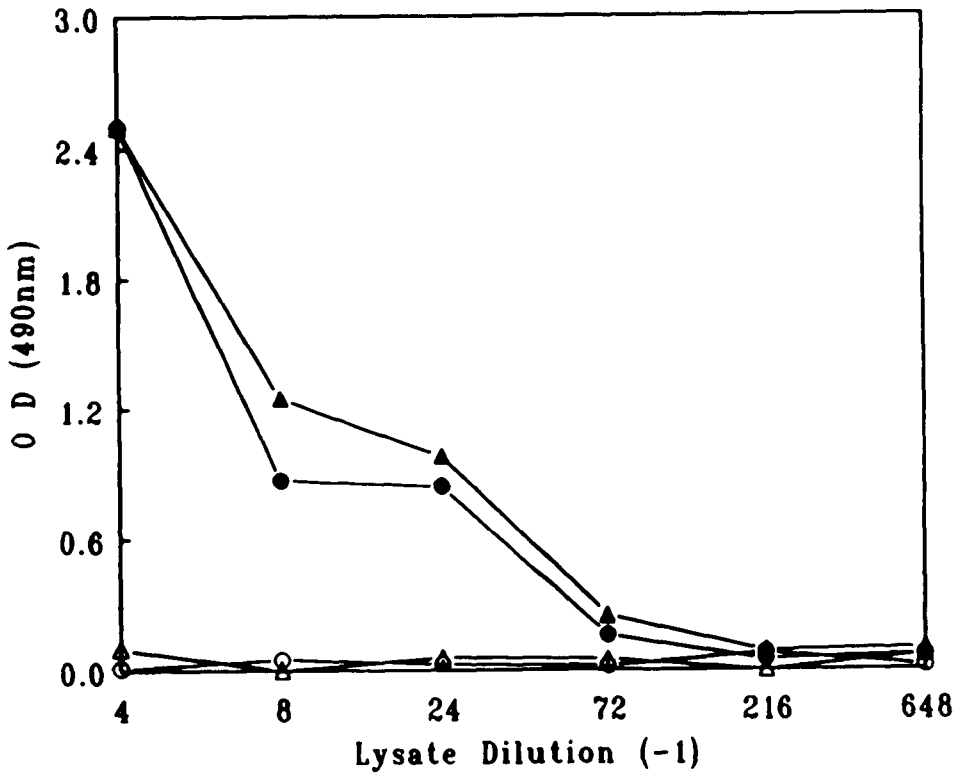


Fig. 7 $Fc_\gamma RII_\alpha$ expression in different peritoneal macrophages populations. Macrophages were isolated by peritoneal lavage, and non-adherent cells removed after incubation in culture plates for 2 h. The adherent cells were lysed as before and assayed for $Fc_\gamma RII_\alpha$ ELISA as in Fig. 2. Thioglycollate-elicited macrophages (▲), BCG-elicited macrophages (●), resident peritoneal macrophages (○), and S49.1 cells (△).

Table 3. Effect of lymphokines on $Fc_\gamma RII$ expression.

Lymphokine	Units/ml (or dilution)	Bound mAb 2.4G2 ($ng/10^6$ cells)	
		24 h	48 h
Experiment 1			
Control	—	16.5 ± 0.1	18.2 ± 0.2
Interleukin 6	500	18.4 ± 1.7	29.4 ± 0.3
Interleukin 2	500	16.3 ± 0.1	16.8 ± 0.6
Interleukin 3	100	17.3 ± 0.1	18.2 ± 0.2
Granulocyte/Macrophage CSF	100	17.9 ± 0.4	16.8 ± 0.1
Macrophage CSF	1:300,000	17.1 ± 0.6	17.1 ± 0.6
Tumor Necrosis Factor α	100	19.1 ± 1.1	18.8 ± 0.7
Interferon β	100	15.9 ± 1.3	18.3 ± 0.9
Interferon α	100	18.0 ± 0.6	19.7 ± 1.5
Experiment 2			
Control	—	21.0 ± 1.7	N.D.
Interleukin 4	100	24.0 ± 1.0	N.D.
Transforming Growth Factor - β	100	22.7 ± 0.6	N.D.

Effect of lymphokines on $Fc_\gamma RII$ expression. J774a cells were cultured in the presence or absence of various recombinant or purified lymphokines at 100 U/ml, unless otherwise stated, for 48 hours. Cells were incubated with [^{125}I]-2.4G2 and unlabeled 2.4G2 (0.5 ml, 5.2 μg/ml, 3.9 x 10^5 cpm/μg) as described in Materials and Methods. The data are representative of three experiments.

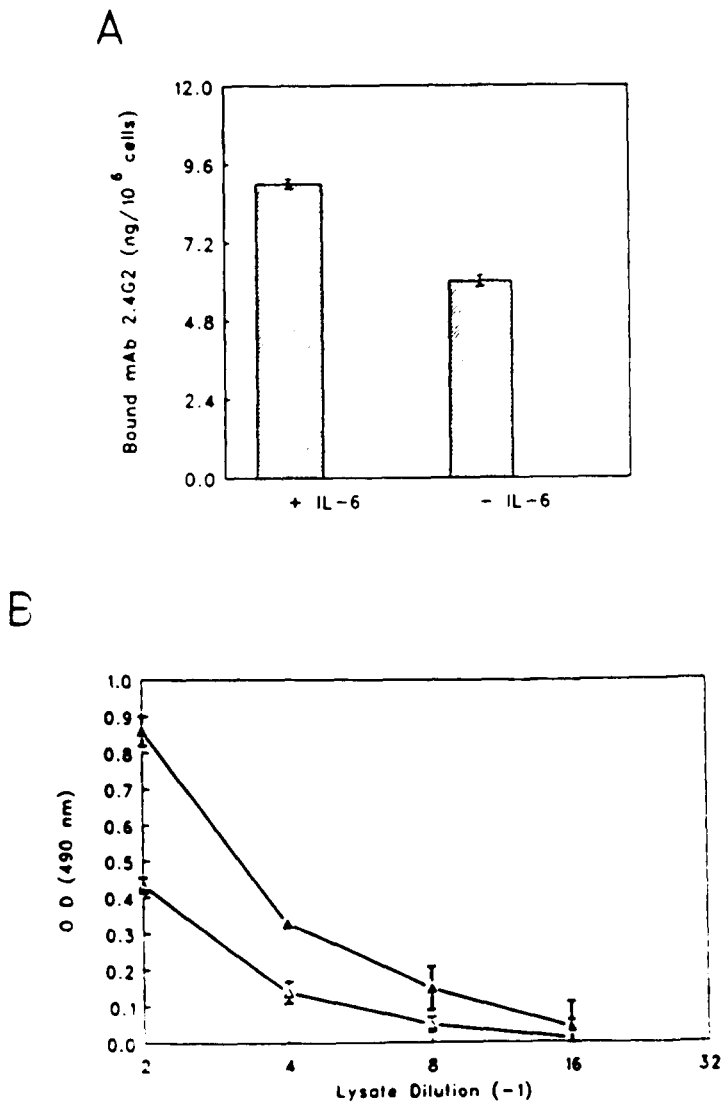


Fig. 8 **Effect of rIL-6 on $Fc_\gamma RII$ expression. J774a cells were cultured in the presence or absence of 25 U/ml of rIL-6 for 48 h. (A) Plasma membrane $Fc_\gamma RII$. Intact cells were incubated with [^{125}I]-2.4G2 (0.5 ml, 5.5 μg 2.4G2/ml, 3.4 x 10^5 cpm/μg) in PBS-BSA as described in Materials and Methods. The data are representative of six experiments. (B) Total cellular $Fc_\gamma RII$. J774a cell lysates were prepared from the rIL-6-stimulated (▲) and control groups (△) and binding of mAb 2.4G2 was measured in an ELISA as described in Materials and Methods.**

Northern analysis of total RNA revealed that rIL-6 stimulation resulted in an increased abundance of $Fc_\gamma RII_\alpha$ mRNA (Figs. 9a, 11a), but not $Fc_\gamma RII_\beta$ (Fig. 9b) mRNA in J774a cells. I expected to observe a comparable rise in $Fc_\gamma RII_\alpha$ protein, but analysis of lysates from four independent experiments by ELISA showed the same level of $Fc_\gamma RII_\alpha$ protein in rIL-6 induced and control J774a cells (Fig.10). The ELISA is clearly sensitive enough to detect small changes in $Fc_\gamma RII_\alpha$ expression (Figs. 4,5,7,8).

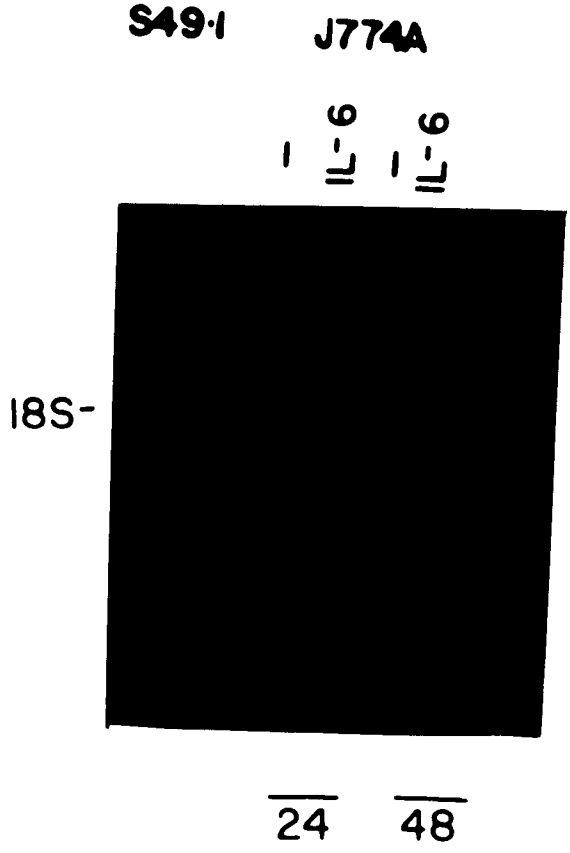
Were there a glycan-phosphatidylinositol anchored form of $Fc_\gamma RII_\alpha$, the $Fc_\gamma RII_\alpha$ ELISA would not detect it, since the cytoplasmic domain would be absent. I looked for GPI-linked $Fc_\gamma RII$ by measuring binding of [^{125}I]-2.4G2 to rIL-6 induced versus uninduced J774a cells that had been digested with PIPLC. To confirm activity of the PIPLC, I examined release of human $Fc_\gamma RIII$ (CD16) from neutrophils. No changes in binding of [^{125}I]-2.4G2 were observed after digestion with PIPLC of either control or rIL-6 induced J774a cells. This was not due to inactive PIPLC, since the human neutrophils showed a dramatic decrease in mAb FITC-3G8 binding to $huFc_\gamma RIII$ (CD16) after digestion with PIPLC as reported previously (Selvaraj, 1988; Huizinga, 1988; Ravetch, 1989; Scallon, 1989; Ueda, 1989).

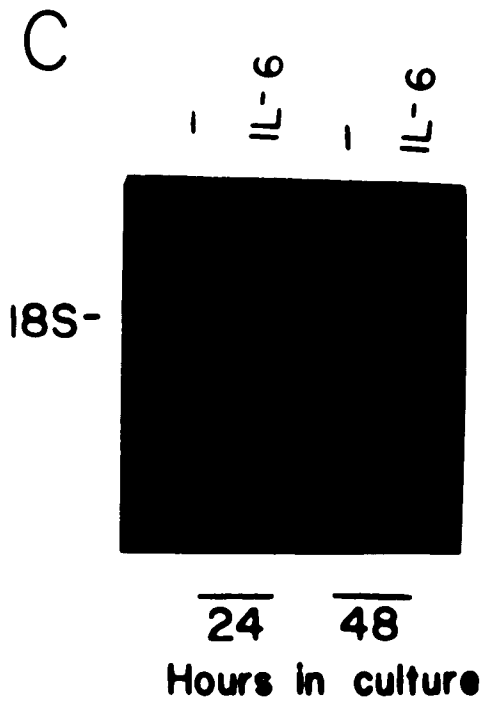
Because rIL-6 induced J774a cells lack $Fc_\gamma RII_\beta$ mRNA, do not express higher levels of $Fc_\gamma RII_\alpha$ protein, but express higher levels of $Fc_\gamma RII$ as determined by mAb 2.4G2 binding, I probed RNA from rIL-6-stimulated cells for the presence of a novel $Fc_\gamma RII$ mRNA. I used a cDNA probe encoding the NH_2-terminal Ig-like domain from residues 16 to 80 of $Fc_\gamma RII_\beta$, which should hybridize to all $Fc_\gamma RII$

transcripts. In addition to the $Fc_{\gamma}RII_{\alpha}$ mRNA, this cDNA probe hybridized with a mRNA about the same size as the $Fc_{\gamma}RII_{\beta}1$ mRNA (Fig. 9C). The amount of the larger transcript was elevated at 48 h by induction of J774a cells with IL-6 (Fig. 11b). A cDNA probe from the 5' untranslated region of $Fc_{\gamma}RII_{\beta}$ failed to hybridize to this larger transcript (Fig. 9B), so it cannot be either $Fc_{\gamma}RII_{\beta}1$ or $Fc_{\gamma}RII_{\beta}2$. I suggest that translation of this novel $Fc_{\gamma}RII$ mRNA results in a receptor bearing a mAb 2.4G2 epitope.

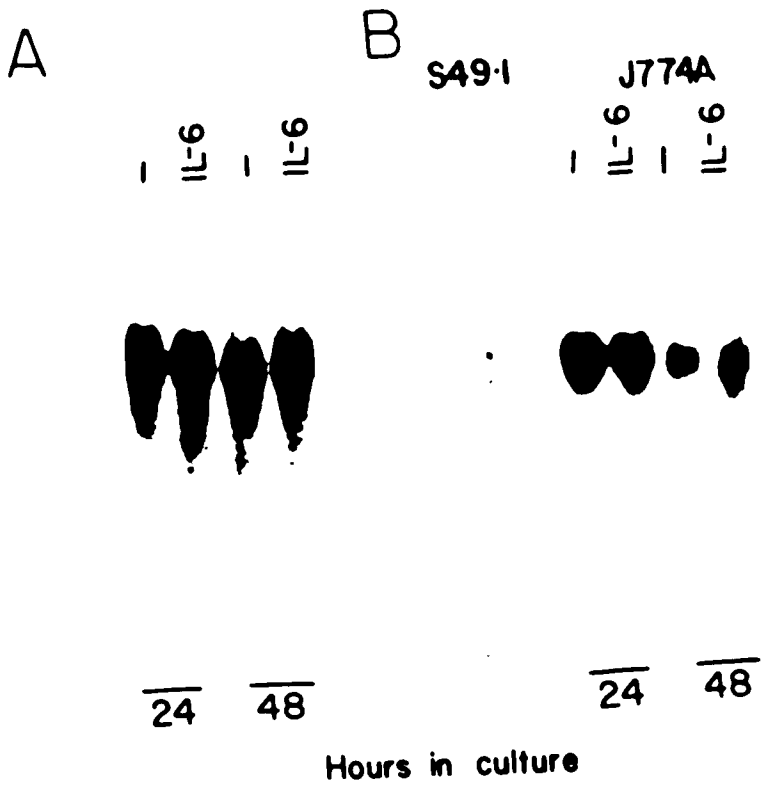
Fig.9 Northern blot analysis of $Fc_\gamma RII_\alpha$ mRNA expression. J774a cells were incubated in the presence or absence of 25 U/ml of rIL-6. Two blots were prepared with 50 μg of total RNA loaded in each lane and hybridized with (A) $Fc_\gamma RII_\alpha$-specific and (B) $Fc_\gamma RII_\beta$-specific cDNA probes described in Materials and Methods. (C) Blot A was stripped and reprobed with an $Fc_\gamma RII$ encoding the NH_2-terminal Ig-like domain from residues 16 to 80 of $Fc_\gamma RII_\beta$ as described in Materials and Methods. (D) Blots A and B were exposed for 1 week, stripped and reprobed with a glucose phosphate dehydrogenase (GPDH) cDNA probe for normalization of RNA.

B




C
-
IL-6
-
IL-6
18S-
24
48
Hours in culture

D



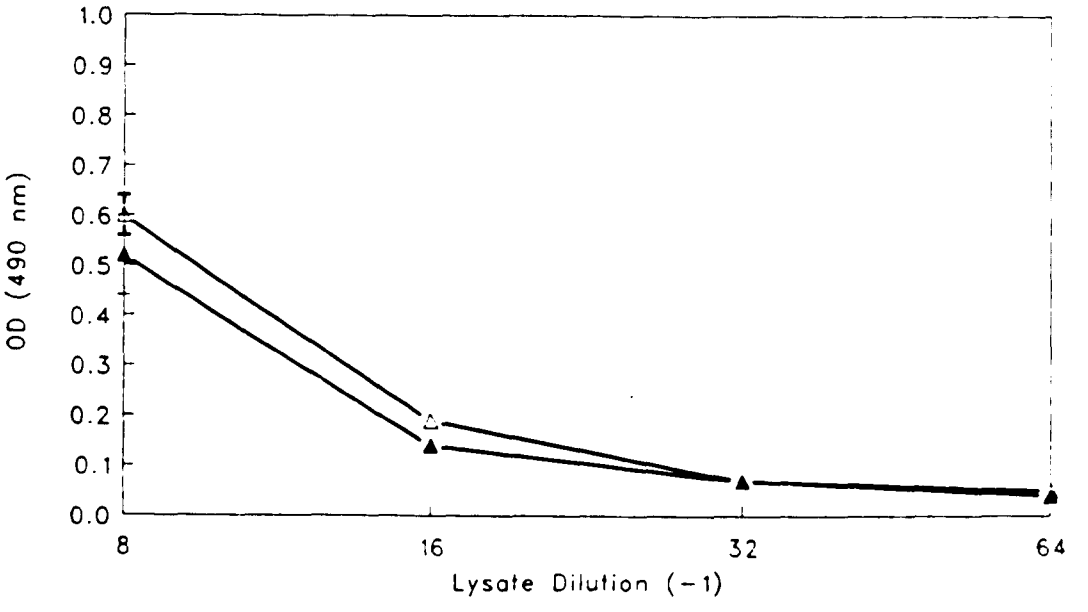


Fig.10 **$Fc_\gamma RII_\alpha$ expression in IL-6-induced J774a cells. J774a cells were cultured in the presence or absence of 25 U/ml of rIL-6 for 48 h. J774a cell lysates were prepared from the rIL-6-stimulated (▲) and unstimulated groups (△) and $Fc_\gamma RII_\alpha$ was measured in ELISA using mAb 2.4G2-coated wells as described in Materials and Methods. The data are representative of four experiments.**

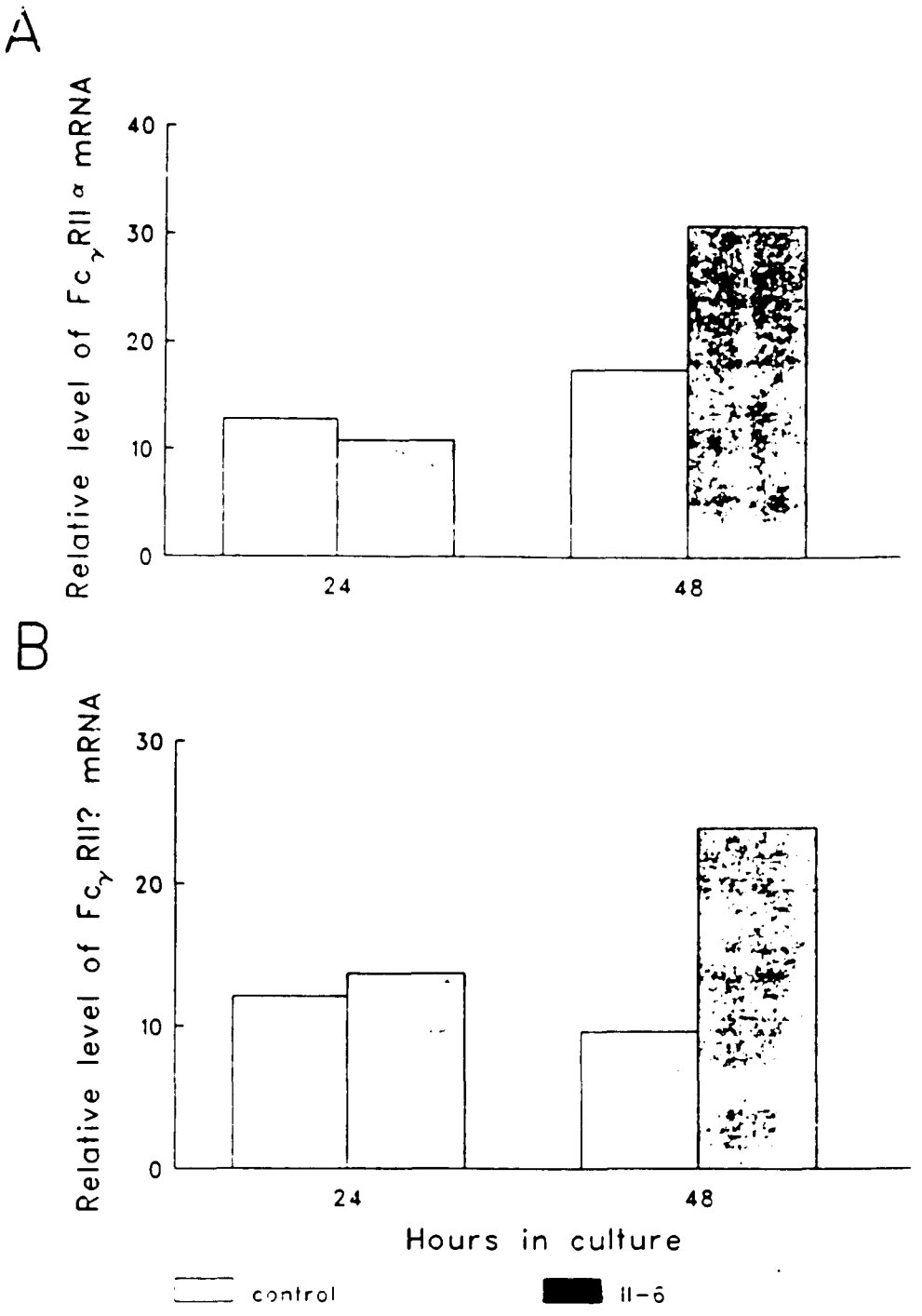


Fig. 11 $Fc_\gamma RII$ mRNA levels in IL-6-induced J774a cells. Densitometry of the autoradiographs shown in (A) Fig. 9a and (B) 9c was performed as described in Materials and Methods.

DISCUSSION

Two distinct murine $Fc_{\gamma}RII$ genes, mu$Fc_{\gamma}RII_{\alpha}$ and mu$Fc_{\gamma}RII_{\beta}$, are transcribed in macrophages, NK cells and mesangial cells (Ravetch, 1986; Lewis, 1986; Hibbs, 1986; Santiago, 1989), or in macrophages and lymphocytes (Ravetch, 1986), respectively. Amino acid analysis of $Fc_{\gamma}RII$ from the S49.1 cell line shows that the native protein matches exactly that predicted by the $Fc_{\gamma}RII_{\beta}$ cDNA sequence (Pan, 1987), but similar identification of the native $Fc_{\gamma}RII_{\alpha}$ protein sequence has not been obtained. Though $Fc_{\gamma}RII_{\alpha}$ and $Fc_{\gamma}RII_{\beta}$ are quite similar in their extracellular domains, differences in their cytoplasmic domains should allow for the detection of the native $Fc_{\gamma}RII_{\alpha}$ protein, if present.

I have demonstrated that $Fc_{\gamma}RII_{\alpha}$ is synthesized as a full length, membrane-spanning protein upon analysis by ELISA of J774a cells expressing the $Fc_{\gamma}RII_{\alpha}$ mRNA. In this system, the cytoplasmic domain of $Fc_{\gamma}RII_{\alpha}$ is bound by the antiserum directed against the 15 carboxyl terminal residues, whereas the ectodomain of the receptor is recognized by either mAb 2.4G2 or a rabbit anti-$Fc_{\gamma}RII$ antibody elicited by a truncated r$Fc_{\gamma}RII_{\beta}$ that contains only the ectodomain of $Fc_{\gamma}RII_{\beta}$ (Qu, 1988).

Immunization of rabbits with either peptide A or peptide B, corresponding to distinct cytoplasmic domains of $Fc_{\gamma}RII_{\alpha}$, resulted in the generation of anti-peptide B Ig, which reacted with native receptor and anti-peptide A Ig, which did not bind $Fc_{\gamma}RII_{\alpha}$. Several features may influence the ability of anti-peptide antibodies to bind

the native sequence. Hopp & Woods (1981) initially observed that many antigenic determinants of a protein are within or adjacent to highly hydrophilic regions of the protein. In addition, Tainer (1984) reported that anti-peptide antibodies generated against regions of high atomic mobility, or flexibility, react strongly with the native protein, whereas anti-peptide antibodies synthesized against peptides corresponding to well-ordered regions of the protein are non-reactive with the protein. Based on their correlative studies between the antigenicity of a site and its accessibility to large spheres, Novotny et al. (1986) theorized that the flexibility of antigenic determinants are a consequence of their accessibility to antibody domains and that this accessibility is a more consistent predictor of antigenicity than is flexibility. Consistent with this idea are the data of Fieser et al. (1987), which suggest that the there is a positive correlation between the ability of the peptide to adopt secondary structure at the epitopes and its success in eliciting anti-peptide antibodies that are reactive with protein.

These factors need to be considered to explain why peptide B, corresponding to the fifteen amino acids most proximal to the carboxyl terminus of $Fc_\gamma RII_b$, induced the synthesis of antibodies which were reactive with the native protein, whereas peptide A, which matches the fifteen amino acids adjacent to the transmembrane domain of the native protein, did not elicit antibodies which recognized the intact protein. It is possible that the peptide B cognate is more accessible to the antibody-binding domain than is the region on the receptor corresponding to peptide A, since peptide B includes the free COOH-terminal

residue of the receptor. Both peptides are hydrophilic, suggesting that this factor is not responsible for the differences in antibody recognition of the cognate sequence.

Using the $Fc_\gamma RII_\alpha$ ELISA, I have demonstrated that the level of $Fc_\gamma RII_\alpha$ expression varies in different populations of macrophages. Ezekowitz et al. (1981) measured the binding of mAb 2.4G2 to macrophage populations, and reported that BCG-elicited and resident macrophages had roughly the same number of mAb 2.4G2 binding sites, which was ~3 fold lower than that of thioglycollate elicited macrophages. Upon analysis of macrophages at the level of individual $Fc_\gamma RII$ gene expression, however, I found that resident and BCG-elicited macrophages differ dramatically, since the latter do not synthesize $Fc_\gamma RII_\alpha$ protein. Resting and thioglycollate-elicited peritoneal macrophages are both actively phagocytic compared to BCG-elicited macrophages, which are poorly phagocytic (Ezekowitz, 1981). This suggests that $Fc_\gamma RII_\alpha$ may play an important role in phagocytosis.

IFN-γ stimulation caused a 4-fold elevation of the amount of $Fc_\gamma RII_\alpha$ mRNA and a higher level of $Fc_\gamma RII_\alpha$ protein relative to control cells. Furthermore, Weinshanck demonstrated that the increased level of $Fc_\gamma RII_\alpha$ transcript parallels enhanced phagocytosis of opsonized sheep red blood cells by J774a cells. As suggested by the pattern of $Fc_\gamma RII_\alpha$ expression I observed in peritoneal macrophages, this positive correlation between the phagocytic capacity of the IFN-γ-induced J774a cell and its level of $Fc_\gamma RII_\alpha$ expression implicates $Fc_\gamma RII_\alpha$ as a mediator of phagocytosis by macrophages.

IFN-γ stimulation of monocytes, macrophages, and neutrophils mediates a

variety of cellular effects. Nathan (1983) demonstrated that treatment of human macrophages with rIFN-γ resulted in enhanced oxidative metabolism and antimicrobial activity of the cell. Moreover, several groups examined the $Fc_\gamma R$ expression of in human neutrophils and monocytes, and found that rIFN-γ greatly increased the expression of $Fc_\gamma RI$ in both cell types (Perussia, 1983; Guyre, 1983; Petroi, 1988). This increased expression in the neutrophil correlated with enhanced phagocytosis and ADCC (Petroni, 1988).

These multiple effects of IFN-γ upon both $Fc_\gamma R$-mediated function and expression must be considered in relation to our finding that IFN-γ stimulates $Fc_\gamma RII_\alpha$ expression. The effect of IFN-γ upon receptor-mediated functions in J774a cells need to be analyzed. In addition, the influence of IFN-γ upon synthesis of $Fc_\gamma RI$ in murine macrophages needs to be addressed and correlated to the levels of receptor-mediated function.

The role of $Fc_\gamma RII_\alpha$ in receptor-mediated events other than phagocytosis is also unclear. I observed an absence of $Fc_\gamma RII_\alpha$ in BCG-elicited macrophages. These cells exhibit enhanced release of reactive oxygen intermediates and antibody dependent cell cytotoxicity (Nathan, 1977,1980). Therefore, the data suggest that $Fc_\gamma RII_\alpha$ may not be essential for triggering these cellular responses. On the contrary, Perussia et al. (1989) have suggested that $Fc_\gamma RII_\alpha$ on the surface of NK cells mediates ADCC of antibody-coated YAC-1 target cells, by demonstrating inhibition of ADCC with anti-$Fc_\gamma RII$ mAb 2.4G2. The receptor's role in this event is not definitive either, however, since other, as yet undescribed $Fc_\gamma RII$ molecule(s) may be expressed by these cells.

$Fc_\gamma RII_\beta$ expressed on macrophages may not be required in mediating some of these receptor-associated events. Weinschanck et al. (1988) demonstrated that resident peritoneal macrophages, when stimulated with IFN-$_\gamma$, exhibit low levels of $Fc_\gamma RII_\beta$ mRNA relative to unstimulated cells, while maintaining high $Fc_\gamma RII_\alpha$ mRNA levels. Since IFN-$_\gamma$ is known to enhance ADCC, phagocytosis, and the oxidative burst, $Fc_\gamma RII_\beta$ may not be required to mediate these responses, if the receptor protein levels correlate with the mRNA levels.

IL-6 is a multifunctional lymphokine (reviewed in Kishimoto, 1989). As a stimulator of cell growth, it acts upon myelomas, plasmacytomas, T cells, and mesangial cells. IL-6 also induces cell differentiation leading to Ig secretion in B cells, the synthesis of acute phase proteins in liver cells, and the cytotoxic potential in T cells. Most relevant to this discussion is the role of IL-6 in stimulating the secretion of Ig from B cells. This is interesting in light of the IL-6-enhanced levels of $Fc_\gamma RII$ that I observed. In vivo, this novel $Fc_\gamma RII$ may be induced in tandem with Ig secretion by IL-6 during an immune response to bind increased levels of immune complexes. Indeed, IL-6 has been shown to induce the differentiation of mouse myeloid leukemia cells (M1), which lead to enhanced phagocytosis and expression of $Fc_\gamma RII$ (Miyaura, 1988).

I observed that stimulation of J774a cells with rIL-6 resulted in a ~2-fold increase in $Fc_\gamma RII_\alpha$ mRNA, although this did not result in an elevation of cell $Fc_\gamma RII_\alpha$ protein titer. Ru et al. (1989) have demonstrated that the $_\gamma$ subunit of rat $Fc_\varepsilon RI$ associates with $Fc_\gamma RII_\alpha$. In addition, they have shown that the efficiency of

expression of surface $Fc_\gamma RII_\alpha$ in COS-7 cells transfected with $Fc_\gamma RII_\alpha$ cDNA was markedly increased when the cells were cotransfected with both $Fc_\gamma RII_\alpha$ and γ cDNA. This suggests that the expression of γ is the limiting factor in $Fc_\gamma RII_\alpha$ expression. It is possible that the absence of increased $Fc_\gamma RII_\alpha$ surface protein expression in IL-6-induced cells which exhibited enhanced $Fc_\gamma RII_\alpha$ transcript levels is due to a limiting amount of γ protein, which is not induced by IL-6.

Since the binding of mAb 2.4G2 to J774a cells was reproducibly induced ~2-fold by rIL-6, and the cells make no $Fc_\gamma RII_\beta$ mRNA, it is likely that the IL-6 may be regulating the synthesis of a previously undefined member of the $Fc_\gamma RII$ family. Indeed, a $Fc_\gamma RII_\beta$ cDNA probe encoding most of the NH_2-terminal Ig-like domain hybridized to a mRNA co-migrating with $Fc_\gamma RII_\alpha$, and to a larger, novel $Fc_\gamma RII$ mRNA species which was not detected with the $Fc_\gamma RII_\alpha$ or $Fc_\gamma RII_\beta$-specific probes. This novel transcript was enhanced in IL-6-stimulated versus untreated cells.

Recently, Hibbs et al. (1988) reported the amino acid sequence of two peptides which shared 80% homology to the $Fc_\gamma RII_\beta$ and $Fc_\gamma RII_\alpha$ sequences surrounding the two cysteine residues in the first Ig-like domain. Hogarth et al. (1989) then isolated, after hybridization with an oligonucleotide probe based on the novel $Fc_\gamma RII$ peptides, a cDNA encoding a new $Fc_\gamma R$ ($Fc_\gamma R_\gamma$), which shares only 43% amino acid homology with the ectodomains of $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII$. Since the amino acid sequence of $Fc_\gamma R_\gamma$ differs so markedly from $Fc_\gamma RII_\alpha$ and $Fc_\gamma RII_\beta$, it is unlikely that the novel mRNA species I observed encodes the $Fc_\gamma R_\gamma$, since the probe I used at high stringency was complimentary to the extracellular $Fc_\gamma RII_\beta$ sequence.

Clearly, in order to demonstrate that the mRNA I have described encodes a receptor which bears the mAb 2.4G2 epitope, the cDNA must be cloned and expressed.

In conclusion, I have demonstrated that $Fc_{\gamma}RII_{\alpha}$ is a membrane-spanning receptor that is expressed in a variety of macrophages. The positive correlation between $Fc_{\gamma}RII_{\alpha}$ expression and the cell's phagocytic capacity suggests that the receptor mediates endocytosis. In addition, I have detected the presence of a novel $Fc_{\gamma}RII$, which is likely to be an important mediator of immune responses.

VIII. EPILOGUE

I have shown that $Fc_\gamma RII_\alpha$ is expressed as a transmembrane protein in various macrophages. The results of these studies implicate $Fc_\gamma RII_\alpha$ as a mediator of phagocytosis in macrophages. I have also identified a novel mRNA species of $Fc_\gamma RII$ present in macrophages. These experiments suggest that the structure of the murine $Fc_\gamma R$ family is more heterogeneous than realized previously. It is probable that individual $Fc_\gamma R$ species function distinctively from one another. Experiments are now needed to ascertain the following:

1) cellular events mediated by the individual $Fc_\gamma R$'s;
2) regulation of receptor expression;
3) relationships between structure and function of the various $Fc_\gamma R$'s;
4) signal transducing mechanisms
5) cellular distribution of individual $Fc_\gamma R$'s

Upon a complete understanding of the biochemical and molecular intricacies of these receptors, we can begin to understand the fine tuning of the cells involved in the immune response.

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