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Two distinct regions of FcγRI initiate separate signalling pathways involved in endocytosis and phagocytosis

W.Davis, Patrick T.Harrison[1], M.J.Hutchinson[1] and Janet M.Allen[1,2]

The Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG and [1]Division of Biochemistry and Molecular Biology and Department of Medicine and Therapeutics, The University of Glasgow, Glasgow G12 8QQ, UK

[2]Corresponding author

W.Davis and P.T.Harrison contributed equally to this work

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Cross-linking of the high affinity receptor for IgG, FcγRI, can result in both endocytosis of immune complexes and phagocytosis of opsonized particles in myeloid cells, although the cytoplasmic domain of the receptor lacks the tyrosine activation motif which has been implicated in signal transduction triggered by cross-linking of other Fc receptors. To identify the structural determinants of FcγRI-mediated ligand internalization, we have expressed FcγRI or truncated versions of FcγRI in COS cells, either alone or in the presence of the FcεRI γ subunit (which contains a classical tyrosine activation motif and associates with FcγRI in myeloid cells), and assessed their ability to mediate endocytosis and phagocytosis. We have found that FcγRI alone (in the absence of the γ subunit) is capable of mediating endocytosis in COS cells and that the process occurs via a novel, tyrosine kinase-independent signalling pathway. Activation of this pathway following cross-linking appears to require only the receptor extracellular domain. In contrast, FcγRI phagocytic function in COS cells is dependent on an interaction between the receptor transmembrane domain and the γ subunit and is mediated by recruitment of tyrosine kinase activity. Our data therefore indicate that distinct domains of the receptor regulate ligand internalization following receptor cross-linking by either immune complexes (endocytosis) or opsonized particles (phagocytosis) and that these functions are mediated by different intracellular signalling pathways.

Key words: endocytosis/FcγRI/γ subunit/phagocytosis

Introduction

Fc receptors (FcγRs) specific for immunoglobulin G (IgG) are expressed on the surface of a wide range of leucocytes and play a pivotal role in linking the cellular and humoral arms of the immune response. Three classes of FcγRs have been identified and cloned in humans and mice (FcγRI, FcγRII and FcγRIII), each with a variety of isoforms. The classification is based upon differences in cell-type distribution, protein and gene structure and affinity for IgG. Of the three receptor subtypes, only FcγRI has sufficiently high affinity to bind monomeric IgG; FcγRII and FcγRIII only bind IgG complexes (for reviews see Ravetch and Kinet, 1991; van de Winkel and Capel, 1993).

Cross-linking of FcγR results in two types of biological response; cell activation and ligand internalization. Cell activation is mediated by a characteristic signal transduction cascade, dependent on tyrosine phosphorylation (of receptor subunits and other cytosolic substrates) as an obligatory early event (for review see Lin *et al.*, 1994). This cascade triggers a variety of cellular effector functions, which include superoxide formation, protease release, induction of nitric oxide synthase and release of inflammatory mediators. FcγR-mediated ligand internalization may occur via either of two distinct processes: phagocytosis or endocytosis. Phagocytosis (internalization of IgG-coated particles >1 μm in diameter) is distinguished from endocytosis (internalization of smaller particles or soluble immune complexes) by a requirement for actin polymerization and by absolute inhibition at temperatures below 18°C (for review see Silverstein *et al.*, 1989). All three classes of FcγR have been shown to mediate ligand internalization by either process in leucocytes (Anderson *et al.*, 1991; Amigorena *et al.*, 1992a; Ghazizideh and Fleit, 1994; Harrison *et al.*, 1994a).

Since the cloning of FcγRs, much work has focused on defining the structural determinants for ligand internalization of the two low affinity receptors (from humans and mice) by expression of single receptors and receptor mutants in FcγR-negative cell lines. These studies have indicated that conserved sequences in the cytoplasmic tail of FcγRII play an essential role in mediating efficient endocytosis and phagocytosis (Odin *et al.*, 1991; Amigorena *et al.*, 1992a; Miettinen *et al.*, 1992; Bonnerot and Amigorena, 1993). In contrast, the cytoplasmic domain of murine FcγRIII, which lacks these sequences, is unable to mediate endocytosis in the absence of an accessory molecule (previously identified as the γ subunit of the high affinity IgE receptor, FcεRI), which contains the sequences necessary for endocytosis (Amigorena *et al.*, 1992b). Structural determinants of FcγRI-mediated ligand internalization have not yet been clearly defined. The cytoplasmic domain of human FcγRI also lacks the conserved sequences which have been implicated in FcγRIIa-mediated ligand internalization (Allen and Seed, 1989), however, it has recently been demonstrated that human FcγRI associates with the FcεRI γ subunit in the myeloid cell line U937, suggesting that this subunit may also function as an accessory molecule in FcγRI-mediated ligand internalization (Ernst *et al.*, 1993; Duchemin *et al.*, 1994).

In this study we have transiently expressed human FcγRI, FcγRIIa and the FcεRI γ subunit, as well as FcγRI truncation mutants and chimeric receptors, in COS cells

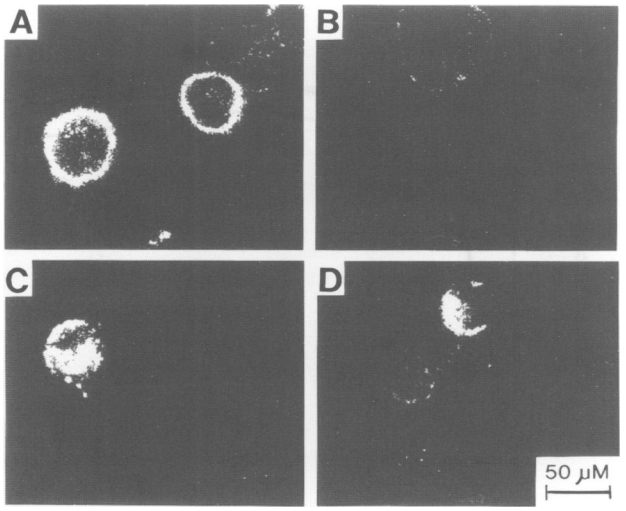

Fig. 1. Cross-linking of FcγRI-bound IgG results in its redistribution to a punctate array within COS cells. Cells were prebound to IgG at 4°C and then warmed to 37°C in the absence (**A** and **B**) or presence (**C** and **D**) of cross-linking antibody. Cells were then washed with PBS at pH 7 (A and C) or pH 2.5 (B and D). IgG was visualized using fluorescein-conjugated anti-human IgG.

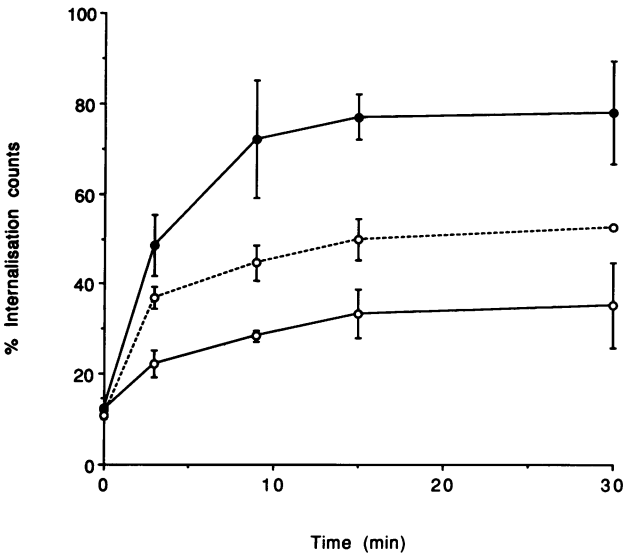

Fig. 2. Time course of the accumulation of cross-linked FcγRI–IgG complexes. COS cells expressing FcγRI were labelled with ^{125}I-labelled IgG at 4°C and then rapidly warmed to 37°C in the presence (closed circles) or absence (open circles) of cross-linking antibody. Non-acid-releasable counts were determined at the time points indicated and expressed as a percentage of total initial cell-associated counts (mean ± SD from at least three separate experiments). Also shown is the time course of FcγRI–IgG accumulation when cells were incubated (without cross-linking antibody) in the presence of 0.6 mM primaquine (open circles, dotted line).

(an FcγR-negative simian fibroblast line), in order to investigate the structure–function basis of FcγRI-mediated ligand internalization. We show that FcγRI is able to mediate both endocytosis and phagocytosis in transfected COS cells, but that these processes depend on different domains of the molecule and are mediated by different intracellular signals. Thus, FcγRI-mediated accumulation of immune complexes requires only the extracellular domain of the receptor and is not dependent on tyrosine kinase activity. In contrast, ingestion of opsonized particles mediated by FcγRI depends on tyrosine kinase activation and requires the presence of a signal transducing subunit which is not present in COS cells; this function may be performed by the FcεRI γ subunit, whose interaction with FcγRI requires the presence of the receptor transmembrane domain, but is independent of the cytosolic domain.

Results

FcγRI mediates efficient endocytosis in COS cells

In order to investigate the structural determinants of FcγRI-mediated internalization of immune complexes, it was necessary to establish whether COS cells (FcR-negative simian fibroblasts) transiently transfected with FcγRI could mediate endocytosis in a similar manner to that previously observed in the human monocyte cell line U937 (Harrison *et al.*, 1994a), where FcγRI is endogenously expressed.

Initially, we used fluorescent confocal microscopy to determine whether COS cells transiently expressing FcγRI (COS-FcγRI) accumulate cross-linked IgG complexes. COS-FcγRI cells were incubated in the presence of IgG at 4°C for 15 min and then rapidly warmed to 37°C in the presence or absence of cross-linking antibody. After fixation, cells were permeabilized and IgG was visualized using fluorescein-conjugated goat anti-human IgG. In the absence of cross-linking antibody, FcγRI-bound IgG was distributed evenly around the surface of the cell (Figure 1A). Treatment with acidified phosphate-buffered saline (PBS) was found to remove IgG from these cells (Figure 1B), indicating that the bulk of the immunoglobulin was bound upon the external face of the plasma membrane. In contrast, the addition of cross-linking antibody resulted in relocation of the plasma membrane-bound IgG to an intracytoplasmic compartment deep within the cell (Figure 1C). It was not possible to remove this internalized material from the cell with acidified PBS (Figure 1D), indicating that little IgG remained exposed on the plasma membrane. The internalization process was entirely temperature-dependent, since incubation with cross-linking antibody at 4°C caused the IgG to clump on the cell surface, whilst remaining accessible to the acid wash (data not shown).

The time course of IgG accumulation by COS-FcγRI cells in response to receptor cross-linking, as detected by an increase in non-acid-releasable counts following occupation of FcγRI with ^{125}I-labelled IgG, is shown in Figure 2. Cross-linking resulted in rapid accumulation of ^{125}I-labelled IgG; ~80% of initial cell-associated counts was internalized in less than 15 min. In the absence of cross-linking, accumulation of IgG was much slower, reaching ~30% within 15 min. Figure 2 also shows accumulation of ^{125}I-labelled IgG, in the absence of cross-linking, when cells were incubated in the presence of 0.6 mM primaquine. This compound is a well-established inhibitor of receptor recycling (Reid and Watt, 1990; Keller *et al.*, 1992; Harrison *et al.*, 1994a); accumulation of label (^{125}I-labelled IgG), at an initial rate similar to that observed in the presence of cross-linking antibody, therefore indicates that FcγRI–IgG is continually internalized and rapidly recycled to the cell surface with the ligand intact, in a similar manner to that recently

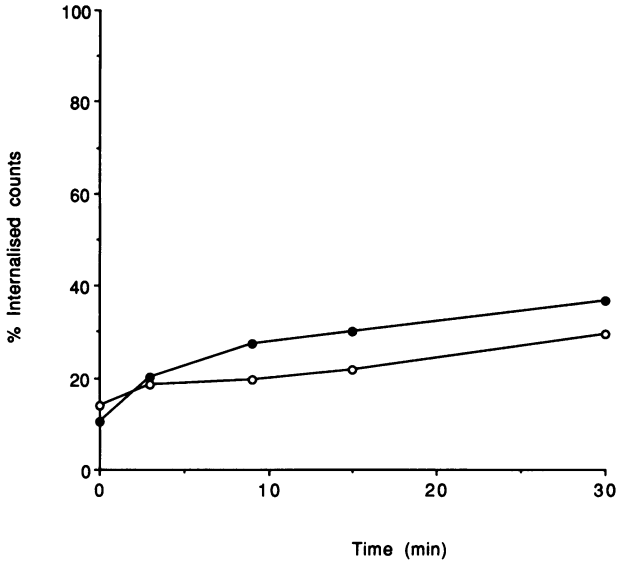

Fig. 3. Time course of the accumulation of cross-linked murine FcγRI–IgG complexes. COS cells expressing murine FcγRI were labelled with ^{125}I-labelled IgG at 4°C and then rapidly warmed to 37°C in the presence (closed circles) or absence (open circles) of cross-linking antibody. Non-acid-releasable counts were determined at the time points indicated and expressed as a percentage of total initial cell-associated counts (mean from three separate experiments).

demonstrated in U937 cells (Harrison *et al.*, 1994a). Thus, COS-FcγRI cells accumulate cross-linked IgG-containing complexes by a similar two-step mechanism to that which occurs in U937 cells.

Murine FcγRI does not mediate efficient endocytosis in COS cells

The time course of labelled IgG accumulation by COS cells transiently expressing murine FcγRI (COS-mFcγRI) is shown in Figure 3. In marked contrast to human FcγRI, murine FcγRI does not mediate significant intracellular accumulation of label following addition of cross-linking antibody. This experiment also represents an effective negative control; the observed lack of intracellular accumulation of IgG, despite use of identical reagents and a receptor with indistinguishable affinity for IgG (Sears *et al.*, 1990), clearly shows that a specific internalization signal is responsible for the observed increase in non-acid-releasable counts following cross-linking of labelled IgG bound to human FcγRI expressed in COS cells.

FcγRI and FcγRIIa are able to mediate internalization of monoclonal antibody-containing complexes in COS cells

In order to compare the processes of endocytosis mediated by FcγRI and FcγRIIa in transfected COS cells, we have used ^{125}I-labelled specific monoclonal antibodies to tag the receptors. Figure 4A shows that the kinetics of FcγRI-mediated accumulation of cross-linked monoclonal antibody complexes are essentially identical to those observed for FcγRI-mediated accumulation of cross-linked IgG complexes (Figure 2). Accumulation of cross-linked monoclonal antibody complexes by FcγRIIa (Figure 4B) is somewhat slower, taking 30 min (as opposed to 15 min) to reach ~80% internalization. Figure 4A and B also shows the rates of accumulation of cross-linked mono-

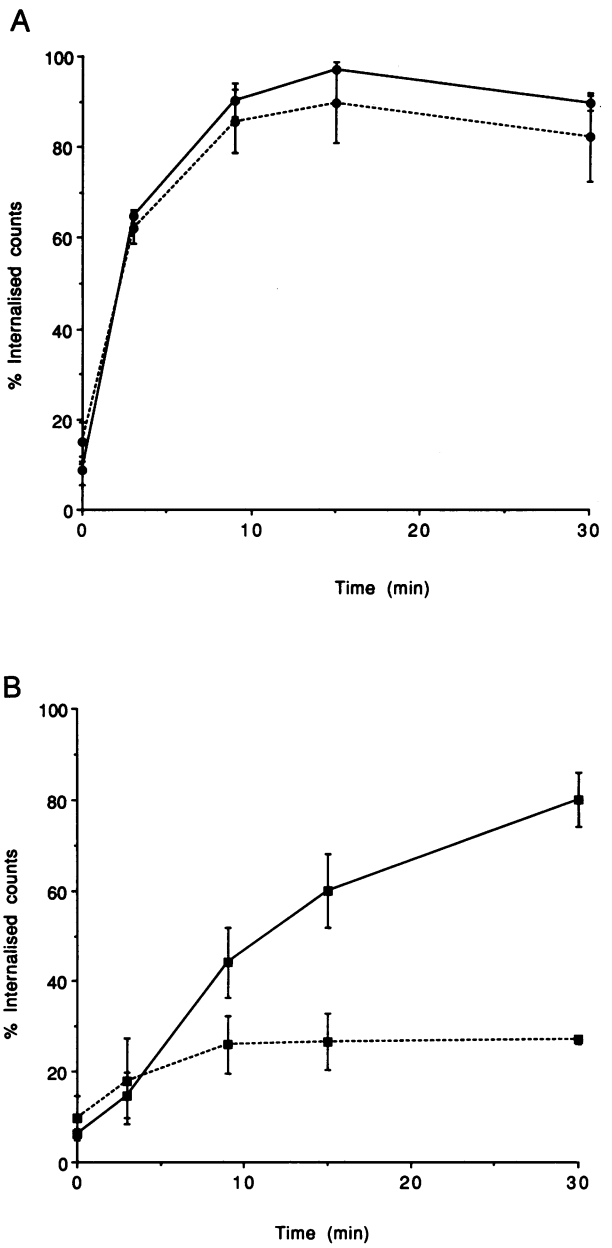

Fig. 4. Time course of the accumulation of cross-linked monoclonal antibody-containing complexes by COS cells expressing FcγRI and FcγRII. COS cells expressing FcγRI (**A**; solid line) or FcγRII (**B**; solid line) were labelled with ^{125}I-labelled monoclonal antibodies (mAbs 22 and 2E1 respectively) at 4°C and then rapidly warmed to 37°C in the presence of cross-linking antibody (anti-mouse IgG1 and 2a respectively). Non-acid-releasable counts were determined at the time points indicated and expressed as a percentage of total initial cell-associated counts (mean ± SD from at least three separate experiments). Dotted lines show results of experiments performed following 30 min pre-incubation of cells in the presence of 0.37 mM genistein.

clonal antibody complexes by each receptor following pre-incubation of the cells with the tyrosine kinase inhibitor genistein. Accumulation of cross-linked monoclonal antibody complexes mediated by FcRγIIa is almost completely inhibited by genistein (Figure 4B). This sensitivity to genistein inhibition is similar to data recently reported for FcRγIIa-mediated endocytosis in the myeloid cell line THP-1 (Ghazizideh and Fleit, 1994) and implies that

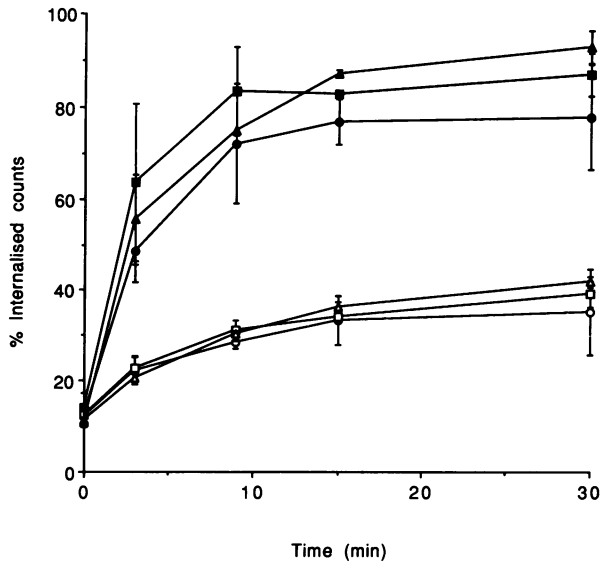

Fig. 5. Time course of the accumulation of cross-linked FcγRI-Manx and FcγRI-GPI. COS cells expressing wild-type FcγRI (circles), FcγRI-Manx (squares) or FcγRI-GPI (triangles) were labelled with ^{125}I-labelled IgG at 4°C and then rapidly warmed to 37°C in the presence (closed symbols) or absence (open symbols) of cross-linking antibody. Non-acid-releasable counts were determined at the time points indicated and expressed as a percentage of total initial cell-associated counts (mean ± SD from at least three separate experiments).

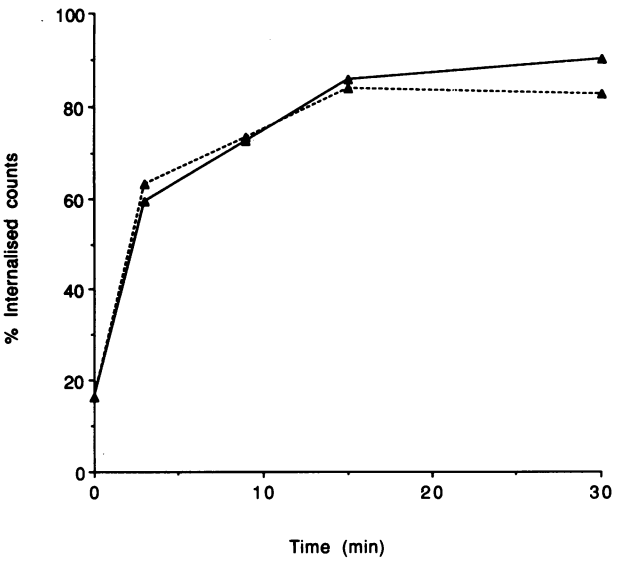

Fig. 6. Time course of the accumulation of cross-linked FcγRI–FcγRII in the presence and absence of genistein. COS cells expressing FcγRI–FcγRII were labelled with ^{125}I-labelled IgG at 4°C and then rapidly warmed to 37°C in the presence of cross-linking antibody. Non-acid-releasable counts were determined at the time points indicated and expressed as a percentage of total initial cell-associated counts (mean of two separate experiments). Dotted lines show results of experiments performed following 30 min pre-incubation of cells in the presence of 0.37 mM genistein.

FcγRIIa expressed in simian fibroblast cells is able to mediate endocytosis by recruitment of similar tyrosine kinase activities to those utilized by FcγRIIa in human myeloid cells. FcγRI-mediated endocytosis, in contrast, is not affected by genistein (Figure 4A). This genistein insensitivity has also been observed in myeloid cells (IFN-γ treated U937 cells; Harrison *et al.*, 1994a) and it therefore appears that similar (tyrosine kinase-independent) signal transduction pathways are involved in FcγRI-mediated endocytosis in both cell types. These observations thus confirm that transfected COS cells represent an effective model system for the study of FcγR-mediated endocytosis.

Endocytosis is not dependent on the cytoplasmic and transmembrane domains of FcγRI

In order to assess the dependence of FcγRI-mediated endocytosis on the presence of the receptor transmembrane and cytoplasmic domains, the cytoplasmic domain deletion mutant (FcγRI-Manx) and the glycosylphosphatidyl-inositol anchored version of the receptor (FcγRI-GPI) were expressed in COS cells and assayed for their ability to mediate the internalization of cross-linked IgG complexes. As shown in Figure 5, the time course of IgG accumulation by COS cells expressing either truncation mutant (in response to receptor cross-linking) was essentially identical to that observed with wild-type FcγRI. As with wild-type FcγRI, endocytosis mediated by FcγRI-Manx and FcγRI-GPI was unaffected by pre-incubation of cells with genistein (data not shown). These data show that the extracellular domain of FcγRI is in itself able to mediate endocytosis in an identical manner to the whole receptor and therefore suggest that the transmembrane and cytoplasmic domains of FcγRI are not required for endocytic function.

Replacement of the extracellular domain of FcγRIIa with that of FcγRI results in a receptor that has the endocytic properties of FcγRI

A chimeric receptor consisting of the extracellular domain of FcγRI fused to the transmembrane and cytoplasmic domains of FcγRIIa was constructed in order to determine whether the extracellular domain of FcγRI is able confer the endocytic properties of FcγRI to a chimeric receptor with unrelated transmembrane and cytoplasmic domains. Figure 6 shows that this is indeed the case; the kinetics of label accumulation and insensitivity to genistein inhibition observed in COS cells expressing the chimera are indistinguishable from those observed for wild-type FcγRI. This confirms that the determinant for FcγRI endocytic function lies within the extracellular domain of the receptor.

FcγRI is unable to mediate efficient phagocytosis in COS cells

In order to determine whether the structural determinants of FcγRI-mediated phagocytosis are the same as those for endocytosis, we assessed the ability of COS cells transfected with FcγRI, FcγRI-Manx and FcγRI-GPI to mediate phagocytosis, using the assay described. COS cells transfected with each of the three clones showed equivalent rosetting (data not shown). Following a brief hypotonic shock [to lyse non-phagocytosed sheep red blood cells (SRBC)], however, only very few COS cells with internalized SRBC were observed and, in these cells, we never observed more than one SRBC per cell (Figure 7C and E). No difference was seen between FcγRI and

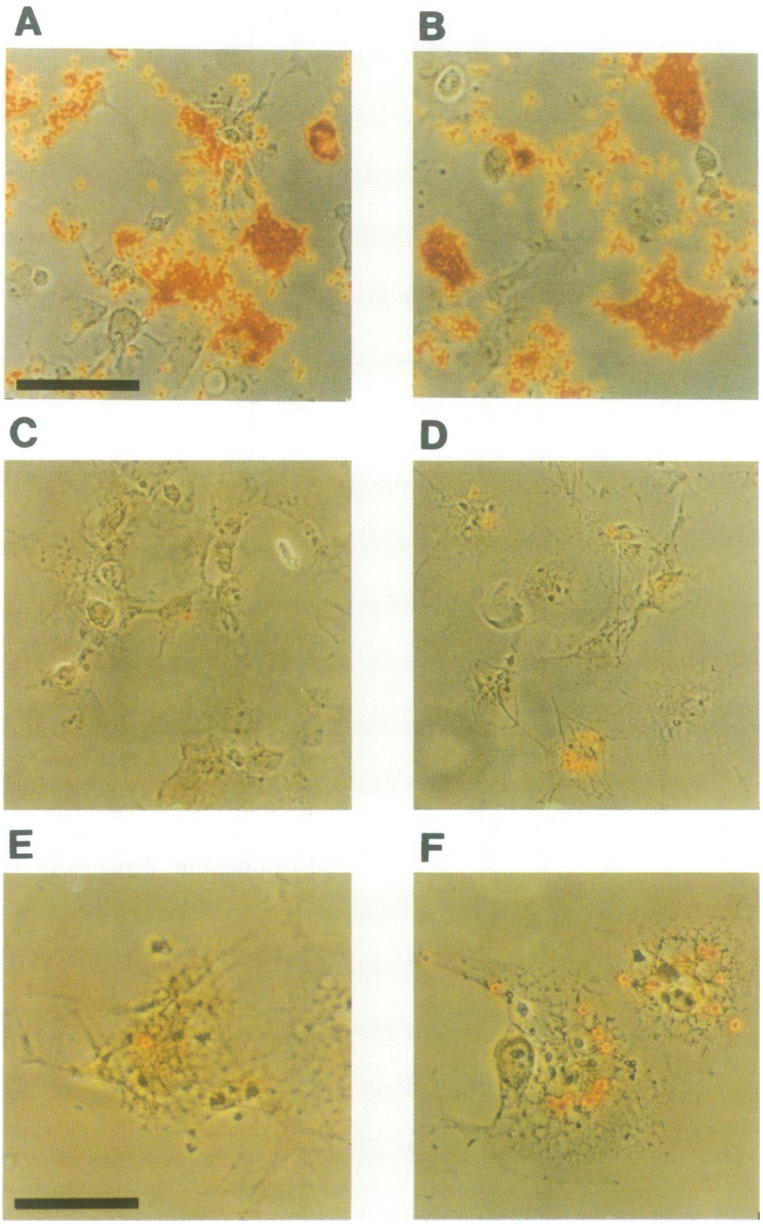

Fig. 7. Rosetting and internalization of opsonized SRBC by COS cells expressing FcγRI alone or FcγRI and the γ chain. COS cell monolayers expressing either FcγRI alone (**A**, **C** and **E**) or FcγRI and γ chain (**B**, **D** and **F**) following 4 h incubation with opsonized SRBC. Non-adhered SRBC were washed off to determine rosetting (A, B); surface-bound SRBC were lysed by hypotonic shock to determine internalized SRBC (C–F). Low power magnification (scale bar = 100 μm, A–D) was used to determine rosette function and number of COS cells with internalized SRBC; high power magnification (scale bar = 50 μm, E–F) demonstrates increased phagocytic capacity of individual cells expressing γ chain and FcγRI.

the two truncated versions of the receptor. No internalized SRBC were found in populations of cells incubated at 4°C or in cells treated with cytochalasin B (data not shown), showing that actin polymerization is necessary for the observed internalization. However, it is not clear whether these 'positives' are the result of occasional SRBC phagocytosis mediated by FcγRI or of the inaccessibility of a few SRBC to the hypotonic shock following partial envelopment by COS cells. We conclude that FcγRI alone (whilst capable of mediating immune complex internalization) is at best able to mediate only low levels of phagocytosis in transfected COS cells. This suggests a requirement for an accessory molecule, not present in COS cells, for efficient FcγRI-mediated phagocytosis.

Efficient FcγRI-mediated phagocytosis is dependent upon the presence of the γ chain and tyrosine kinase activity in COS cells

FcγRI has been shown to be physically associated with the γ chain of FcεRI; this subunit also appears to be involved in signal transduction following FcγRI cross-linking in U937 cells (Duchemin *et al.*, 1994). In order to assess the ability of the γ chain to confer phagocytic function on FcγRI in COS cells, we performed co-transfection experiments with FcγRI and γ chain. We found that the presence of co-transfected γ chain did not alter the rosetting observed (Figure 7A and B), but dramatically increased the number of COS cells with internalized SRBC. Thus, in COS cells transfected with

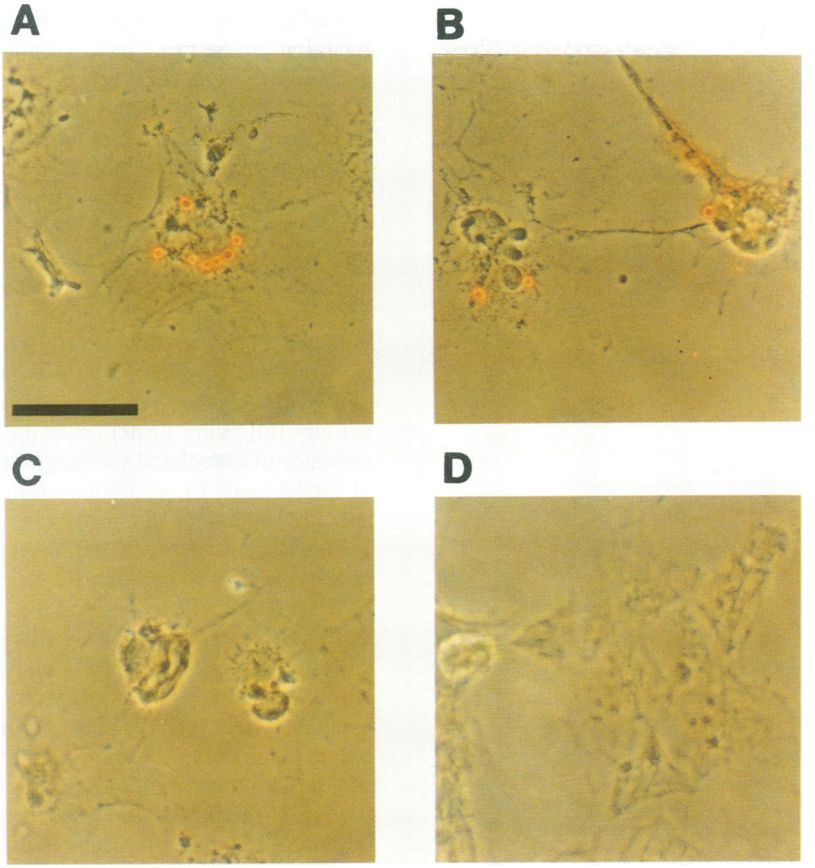

Fig. 8. Inhibition of phagocytosis in COS cells expressing FcγRI and the γ chain. Internalized SRBC in single COS cells: (**A**) incubated for 4 h with opsonized SRBC at 37°C; (**B**) as (A), but cells incubated in the presence of 0.37 mM genistein, following 2 h pre-incubation in this concentration of genistein; (**C**) as (A), but cells incubated in the presence of 0.1 mM cytochalasin B following pre-incubation in this concentration of cytochalasin B for 2 h; (**D**) as (A), but incubation was carried out at 4°C. Scale bar = 50 μm in all cases.

FcγRI alone we consistently observed only one or two positive cells in five examined fields of view (representing fewer than 1% of FcγRI-expressing cells), whilst, in contrast, in cells co-transfected with FcγRI and γ chain, five examined fields of view typically contained a total of 15–20 positive cells (see Figure 7C and D), depending on transfection efficiency. The mean phagocytic index (i.e. internalized SRBC per positive cell) was also drastically increased to 9.8 ± 1.4 (from 1 with FcγRI alone) (Figure 7E and F and Table I). Pretreatment of cells with the tyrosine kinase inhibitor genistein reduced the mean phagocytic index in FcγRI/γ co-transfectants to levels comparable with the background observed in FcγRI single transfectants (2.3 ± 0.44; Figure 8B and Table I). In transfectants pretreated with cytochalasin B (or incubated at 4°C) phagocytosis was completely abolished (Figure 8C and D). These observations indicate that the γ chain is capable of functioning as an accessory molecule in FcγRI-mediated phagocytosis and that the process of phagocytosis is dependent upon recruitment of tyrosine kinase activity by the γ chain, following cross-linking of FcγRI with opsonized SRBC.

The γ chain interacts with FcγRI in the transmembrane domain

The two tail-truncated versions of the receptor, FcγRI-Manx and FcγRI-GPI, were used in co-transfection experiments with the γ chain to determine which domain(s) of

Table I. Effect of the transmembrane and cytoplasmic regions of FcγRI on the interaction with the γ chain to mediate phagocytosis in COS cells

	Control cells (PI[a] mean ± SEM)	Genistein-treated cells (PI[a] mean ± SEM)
FcγRI (wt) + γ	9.8 ± 1.4	2.3 ± 0.44
FcγRI-Manx + γ	9.95 ± 1.56	2.75 ± 0.42
FcγRI-GPI + γ	2.12 ± 0.48	No positives

[a]Mean phagocytic index (number of internalized SRBC per positive COS cell) for cells co-transfected with either FcγRI or one of its truncated versions and the γ chain for control and genistein-treated cells. Data expressed as mean ± SEM

FcγRI is necessary for functional association with the γ chain (and hence for efficient FcγRI-mediated phagocytosis). Again, equivalent rosetting was seen in all cases (data not shown). FcγRI-Manx/γ co-transfectants showed equivalent SRBC phagocytosis to that observed with FcγRI/γ co-transfectants (Figure 9A and B), however, FcγRI-GPI/γ co-transfectants were unable to mediate efficient phagocytosis (Figure 9C and Table I). Incubation at 4°C or treatment with cytochalasin B again abolished all internalization (not shown). The dramatic fall in phagocytic index, to essentially background levels (2.12 ± 0.48), observed in the FcγRI-GPI/γ chain co-transfectant implies that the transmembrane domain, but not the cytosolic domain, of FcγRI is required for interaction with the γ

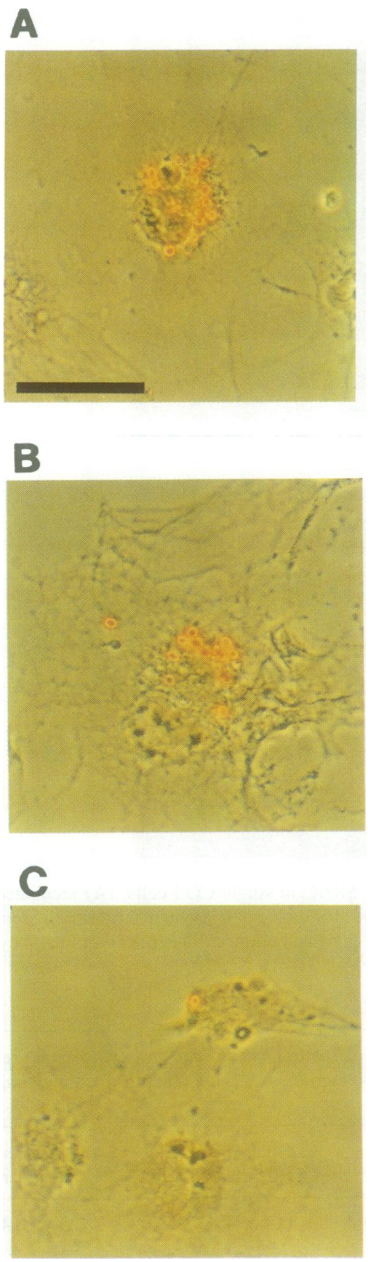

Fig. 9. Phagocytosis in COS cells co-transfected with FcγRI truncation mutants and the γ chain. (**A**) FcγRI and γ chain; (**B**) FcγRI-Manx and γ chain; (**C**) FcγRI-GPI and γ chain. Scale bar = 50 μm in all cases.

chain and therefore contains the recognition sequences involved in FcγRI−γ interaction.

Discussion

In this study we have used COS cells transiently transfected with cDNAs encoding FcγRI and truncation mutants of FcγRI to determine which regions of the receptor are necessary to mediate efficient endocytosis of immune complexes and phagocytosis of opsonized particles.

Initially, we have demonstrated that FcγRI-transfected COS cells accumulate cross-linked IgG complexes in a similar way to that previously reported in the human myeloid cell line U937 (Harrison *et al.*, 1994a). Thus, using confocal microscopy we observe that cross-linking of surface-bound IgG causes relocation of the receptor−ligand complex to an intracytoplasmic compartment deep within COS cells (Figure 1), whilst measurement of non-acid-releasable labelled IgG over a 30 min time course shows essentially complete internalization of label within 15 min (Figure 2). We have also shown that this redistribution is unaffected by treatment of the cells with 0.37 mM genistein, suggesting that it is independent of tyrosine kinase activation, again in a similar manner to U937 cells. In conjunction, these results show that the recently demonstrated physical and functional association of FcγRI with the FcεRI γ subunit (Ernst *et al.*, 1993; Duchemin *et al.*, 1994) is not necessary for the receptor to mediate endocytosis. Thus, whilst recruitment of tyrosine kinase activity following FcγRI cross-linking may depend on the presence of associated γ chain, it appears that cross-linking of FcγRI leads to accumulation of immune complexes by an independent mechanism. Use of labelled monoclonal antibodies has also enabled us to compare the process of endocytosis mediated by FcγRI with that mediated by FcγRIIa in transfected COS cells. Figure 3 shows that the kinetics (and genistein insensitivity) of accumulation of label following cross-linking of FcγRI-bound monoclonal antibody 22 are indistinguishable from those observed using labelled IgG (Figure 2). Cross-linking of FcγRII-bound monoclonal antibody leads to a somewhat slower accumulation of immune complexes, which, in contrast to FcγRI-mediated endocytosis, is almost completely inhibited by genistein. These findings are similar to those reported previously for FcγRII-mediated endocytosis in the human myeloid cell line THP-I (Ghazizideh and Fleit, 1994). It thus appears that the human myeloid cell Fc receptors (in contrast to murine FcγRI; see Figure 3) are both capable of coupling to the appropriate signal transduction cascade intrinsic to the simian fibroblast cellular apparatus; heterologous expression of FcγRI and FcγRIIa in COS cells therefore provides a valid and appropriate model for the study of FcγR-mediated endocytosis.

Having established the validity of COS cells as a model system, we have employed the assay using radiolabelled IgG to study the kinetics of immune complex accumulation by COS cells expressing two truncation mutants of FcγRI, namely the tail-minus mutant FcγRI-Manx (which lacks all but four of the amino acid residues of its cytosolic domain) and the glycosylphosphatidylinositol anchored receptor FcγRI-GPI (which lacks both transmembrane and cytosolic domains). Similar kinetics of accumulation to wild-type FcγRI are observed with both these mutants (Figure 5); this strongly suggests that the receptor is able to mediate endocytosis independently of either its transmembrane or cytosolic domains and that the determinant for endocytic function therefore lies within the extracellular domain. This conclusion is further supported by our data showing that replacement of the extracellular domain of FcγRIIa with that of FcγRI is sufficient to confer the endocytic properties of FcγRI on the resultant chimeric receptor (Figure 6). The data also imply that the determinants of endocytic function of FcγRII, which are present in the cytoplasmic domain of the chimera, are overridden by the FcγRI extracellular domain-dependent endocytic signal which is generated by cross-linking the chimeric receptor.

Previous studies have suggested that COS cells are not able to mediate efficient phagocytosis of opsonized red blood cells when transfected with FcγRI (Indik *et al.*, 1991). Our data (Figure 7C) are in agreement with these conclusions. It is clear, however, that FcγRI is able to mediate phagocytosis of opsonized particles in myeloid cells, under appropriate conditions (Anderson *et al.*, 1991). It has also been shown that COS cells are able to phagocytose opsonized SRBC when transfected with FcγRIIa (Indik *et al.*, 1991), suggesting that the cellular functions required for engulfment of cell-attached particles (i.e. actin polymerization-dependent formation of pseudopodia) can be performed by COS cells and also that they are activated appropriately (in a similar manner to that observed in macrophages) following FcγRIIa cross-linking. It therefore seems likely that an accessory molecule not present in COS cells is required for FcγRI phagocytic function. We have investigated a possible role for the γ subunit of FcεRI by performing co-transfection experiments; Figure 7 shows that COS cells transfected with cDNAs encoding both FcγRI and γ efficiently phagocytose opsonized red blood cells, demonstrating that the γ chain confers phagocytic potential to FcγRI and suggesting that the FcγRI−γ interaction may be required for FcγRI-mediated phagocytosis in myeloid cells. Involvement of the γ chain as an accessory molecule suggests that FcγRI-mediated phagocytosis is likely to depend on tyrosine kinase activation; indeed it has recently been shown that γ chain phosphorylation is a critical early event (occurring independently of actin polymerization) during FcγR-mediated phagocytosis in murine macrophages (Greenberg *et al.*, 1994). Inhibition of opsonized SRBC uptake following pretreatment of COS cells with genistein (Figure 8) shows that efficient FcγRI-mediated phagocytosis in COS cells is also critically dependent on tyrosine phosphorylation.

Comparison of amino acid sequences has suggested that those Fc receptors that interact with the γ chain do so via their transmembrane domains (Ernst *et al.*, 1993; Lin *et al.*, 1994). To investigate the dependence of the γ chain interaction on the presence of the FcγRI cytosolic and transmembrane domains, we again performed co-transfection experiments with the truncation mutants FcγRI-Manx and FcγRI-GPI. Whilst FcγRI-Manx/γ co-transfectants were able to mediate efficient phagocytosis, FcγRI-GPI/γ co-transfectants were not (Figure 9). Thus the cytosolic domain of FcγRI is not required for interaction with the γ chain, but the transmembrane domain is; FcγRI interaction with the γ chain is therefore likely to be via its transmembrane domain.

In summary, we have shown that FcγRI is able to mediate both endocytosis and phagocytosis in transfected COS cells, but that these processes depend on different domains of the molecule and are mediated by different intracellular signals. Thus, FcγRI-mediated ingestion of opsonized particles (in common with the processes of ligand internalization mediated by other Fc receptors) depends on tyrosine kinase activation and requires the presence of an associated subunit with a signal transducing cytoplasmic domain. We have shown that this signal transducing function may be performed in COS cells by the FcεRI γ subunit, whose interaction with FcγRI requires the presence of the receptor transmembrane domain, but is independent of the cytosolic domain. This strongly suggests that the γ subunit, which is endogenously expressed in myeloid cells such as U937 and is up-regulated by IFN-γ in concert with FcγRI (Durden *et al.*, 1994), also functions as an accessory subunit for FcγRI-mediated phagocytosis in myeloid cells. In marked contrast, FcγRI-mediated accumulation of immune complexes requires only the extracellular domain of the receptor and is not dependent on tyrosine kinase activation. This suggests that cross-linking of the FcγRI extracellular domain transduces a non-tyrosine kinase-dependent signal which results in immune complex accumulation in COS cells (and, by extension, in U937 cells, where FcγRI-mediated endocytosis is also tyrosine kinase-independent). This model implies that the extracellular domain of FcγRI is able to interact with the extracellular domain of an unidentified, transmembrane accessory protein (present in both U937 cells and COS cells) whose cytoplasmic domain is responsible for transducing a signal (leading to intracellular accumulation of immune complexes) following receptor cross-linking. The fact that this signal is independent of tyrosine phosphorylation shows that it differs fundamentally from those mediating ligand internalization via other Fc receptors (and indeed from that mediating phagocytosis via FcγRI); the precise nature of the signalling components (and indeed of the transmembrane accessory molecule) await identification.

We conclude that, in marked contrast to FcγRIIa, which mediates both endocytosis and phagocytosis by recruitment of tyrosine kinase activity (dependent on the cytoplasmic domain of the receptor), FcγRI utilizes distinct intracellular pathways to mediate these two processes. Both pathways are triggered by FcγRI cross-linking, although their initiation is dependent on the presence of different domains of the receptor. Phagocytosis is dependent on an interaction with the FcεRI γ subunit, mediated by the transmembrane domain of FcγRI, and is critically dependent on tyrosine kinase activation. The endocytic pathway, uniquely for Fc receptor-mediated signalling, is independent of tyrosine phosphorylation and requires only the extracellular domain of the receptor.

Materials and methods

Cells and cell culture

COS-7 cells (obtained from B.Seed) were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. COS cells were plated at a density of ~4×10^7 cells/100 mm plate 1 day before transfection.

Antibodies

^{125}I-labelled IgG was obtained from Amersham International. Monoclonal antibodies 22 (a gift from Medarex) and 2E1 (Serotec) were labelled with [^{125}I]NaI (Amersham International) as described (Fraker and Speck, 1978).

Transient expression

The SV40-based expression vector CDM (Seed and Aruffo, 1987) was used for the transient expression of the cDNAs of all clones in COS cells using the DEAE−dextran method (Allen and Seed, 1989). cDNAs for human FcγRI (p135; Allen and Seed, 1989) and human FcγRIIa (PC23, Stengelin *et al.*, 1988) were available in CDM. The cDNA for murine FcγRI (D13-2, Sears *et al.*, 1990) was available in the vector pGEXII (a kind gift from Dr D.W.Sears) and subcloned into CDM prior to use. The cDNA for the γ chain of FcεRI was available in the vector pSVL (a kind gift from Dr J.-P.Kinet). All experiments were performed

2 or 3 days post-transfection, when surface expression is maximal. Transfection efficiency was routinely of the order of 20–30% as assessed by immunofluorescence and FACS analysis (data not shown).

Mutant and chimeric receptor construction

To create the mutant FcγRI-Manx, a 400 bp *Sna*BI–*Not*I fragment containing 59 of the 60 amino acids of the cytoplasmic domain of FcγRI (in CDM) was excised, the *Not*I sticky end was converted to a blunt end and the two ends were ligated. The resultant clone comprises the entire extracellular and transmembrane domains of FcγRI, but only the first residue of the cytoplasmic domain (R) plus three additional residues (PRL) and an in-frame stop codon from the CDM vector. The mutation was confirmed by dideoxy sequencing.

Construction of a glycosylphosphatidylinositol-linked version of FcγRI (FcγRI-GPI) is described elsewhere (Harrison *et al.* 1994b). FcγRI–FcγRII was constructed by PCR amplification of the transmembrane and cytoplasmic domains of FcγRIIa. The forward primers for the amplification of FcγRII (5′-GGGCAGAGATCTACCAATGGGG-3′) contained a *Bgl*II site. The reverse primer (5′-GCTAACTAGAGAACCC-ACTG-3′) hybridizes to the CDM vector. The PCR fragment, which contains an internal *Not*I, site was then subcloned between the *Bam*HI and *Not*I sites of FcγRI-GPI (in CDM) to create a clone (FcγRI–FcγRII) comprising the extracellular domain of FcγRI fused to the transmembrane and cytoplasmic domains of FcγRII. The amino acid sequence at the junction is WFH (FcγRI)–MDL (linker)–PMG (FcγRII).

Expression levels of the mutant and chimeric receptors and of murine FcγRI, as indicated by mean channel fluorescence in positive cells, was comparable with that for wild-type FcγRI (data not shown).

Determination of internalization by confocal microscopy

The FcγRI-mediated internalization of IgG complexes was measured as previously described (Harrison *et al.*, 1994a). Briefly, adherent COS cells transiently expressing FcγRI were removed from 100 mm plates using 1 mM EDTA. Cells were resuspended in RPMI, 25 mM HEPES, 5% fetal calf serum (FCS) and allowed to adhere to poly-L-lysine coated glass 'multitest' slides (ICN). Slides were cooled to 4°C and cells incubated for 45 min with 13 μg/ml human polyclonal IgG (Serotec) in PBS, 1% bovine serum albumin (BSA). Attached cells were washed in PBS, 1% BSA and RPMI, 25 mM HEPES, 5% FCS was added in the presence or absence of cross-linking antibody (goat anti-human IgG, Fab specific). Slides were either warmed to 37°C for 10 min and returned to 4°C or kept at 4°C throughout. Cells were washed at 4°C using PBS at either pH 7.4 or pH 2.5, fixed, permeabilized and then blocked with 20% FCS in PBS.

IgG was visualized using fluorescein (FITC)-conjugated goat anti-human IgG (γ-chain specific). Immunoreactive FcγRI was labelled with a mixture of anti-FcγRI monoclonal antibodies 22 and 32.2 (a kind gift from Medarex) and visualized with FITC-conjugated goat anti-mouse IgG. Images were obtained using a Molecular Dynamics laser scanning confocal microscope with excitation at 488 nm and emission detected above 530 nm. Equatorial optical slices through the cells are displayed.

Determination of internalization using radiolabelled antibodies

FcγR-mediated internalization of antibody complexes using radiolabelled antibodies was measured as previously described (Harrison *et al.*, 1994a). Briefly, transfected COS cells were removed from 100 mm plates using 1 mM EDTA and washed once in PBS. Cells were then resuspended in RPMI, 25 mM HEPES, 5% FCS and incubated in the presence of ^{125}I-labelled antibodies on ice for 20 min to label the surface receptor. COS cells expressing FcγRI, FcγRI mutants and FcγRI chimeras were incubated with either ^{125}I-labelled IgG (final concentration 2 nM) or ^{125}I-labelled anti-FcγRI monoclonal antibody (~200 nM iodinated 22 in the presence of 5 mM IgG). COS cells expressing FcγRII were incubated in the presence of ^{125}I-labelled anti-FcγRII monoclonal antibody (2E1). Cells were washed, resuspended in RPMI, 25 mM HEPES, 5% FCS, incubated in the presence or absence of cross-linking antibody or primaquine/genistein (see below), rapidly warmed to 37°C and assayed at various time points. Duplicate aliquots (~10^6 cells) were diluted 20-fold into ice-cold isotonic PBS (pH 2.5) at each time point. Following a 5 min incubation on ice, cells were pelleted and the cell-associated counts determined in a γ counter. The initial total cell-associated counts were determined by dilution of two additional aliquots into PBS (pH 7.4) at the zero time point. Non-specific binding was negligible. Treatment with PBS (pH 2.5) routinely removed more than 85% of surface-bound ^{125}I-labelled antibodies. Data is expressed as a percentage of total initial cell-associated counts and presented as the mean ± SD from at least three separate experiments.

Freshly prepared primaquine was used at a final concentration of 0.6 mM. Cross-linking antibodies used were: whole antiserum to human IgG developed in goat (final concentration 1:100) or an IgG fraction of goat antiserum to murine IgG1 or IgG2a (as appropriate, final concentration 1:500). In some experiments cells were pretreated with genistein (Sigma) at a final concentration of 0.37 mM for 30 min; this concentration of genistein was then maintained in all subsequent steps prior to final wash.

Determination of phagocytosis of opsonized sheep red blood cells

Washed SRBC were opsonized by incubation at 4°C for 30 min with a sub-agglutinating concentration of a polyclonal rabbit antiserum raised to SRBC stroma (Sigma). After washing in ice-cold PBS, 1% BSA, the opsonized SRBC were resuspended in DMEM, 10% calf serum to achieve a haematocrit of 0.5–1.0%. This red cell suspension was then used to replace the normal culture medium of the COS cell monolayers (grown on glass coverslips) and the cells were incubated for a further 4 h at 37°C in 7% CO_2 (or at 4°C). Surface-bound SRBC were lysed by hypotonic shock (1 mM PBS, pH 2.5) and the COS cells were then fixed in 0.5% glutaraldehyde in 0.2 M PBS (pH 7.4). Internalized SRBC were visualized by staining for myeloperoxidase activity, using hydrogen peroxide and *o*-diansidine as substrates (Whyte *et al.*, 1993). Positive cells were then identified by light microscopy and the number of internalized SRBC per positive cell counted. A mean phagocytic index was calculated by averaging the data acquired from 20 positive cells. In some experiments cells were pretreated with genistein at a final concentration of 0.37 mM for 30 min (again, this concentration of genistein was maintained in all subsequent steps) or pretreated with (and maintained in) cytochalasin B (Sigma) at a final concentration of 0.1 mM for 2 h.

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