Impairment of neutrophil Fc γ receptor mediated transmembrane signalling in active rheumatoid arthritis

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Abstract

Neutrophil Fc γ receptor (Fc γ R) signalling responses were compared in healthy subjects, patients with definite rheumatoid arthritis (RA), ankylosing spondylitis, and osteoarthritis. The patients with A were subdivided into those with active synovitis and those with quiescent disease. Basal intracellular calcium ion concentrations in patients with inactive RA were significantly higher than in control subjects, which in turn were greater than in patients with active RA. Transient cytosolic calcium ion fluxes were observed after binding Fc γ RII or Fc γ RIII with specific monoclonal antibodies and cross linking with the F(ab')₂ fragment of antimouse IgG. Response times were significantly faster for Fc γ RII than for Fc γ RIII. Peak concentrations of intracellular calcium ions after neutrophil stimulation were comparable for Fc γ RII and RIII in healthy subjects. Neutrophils in patients with ankylosing spondylitis and osteoarthritis responded to Fc γ R triggering, but in the group with active RA fluxes of calcium ions were severely depressed. Neutrophils isolated from patients with RA with quiescent disease showed exaggerated responses when compared with controls. Expression of all three Fc γ R types on neutrophils from patients with active RA, as measured by monoclonal antibody binding, was comparable with control cells. Impairment of neutrophil Fc γ R cytosolic signalling in active RA could reflect a receptor signalling defect with potential effects on Fc mediated functions, or a fundamental defect in calcium ion homeostasis within these cells.

It is becoming apparent that the part played by the polymorphonuclear neutrophilic leucocyte (neutrophil) in the development of rheumatoid arthritis (RA) is multifaceted and not confined to the release of lysosomal enzymes and oxygen radicals into the synovial fluid causing joint damage.¹ Neutrophils are capable of generating proinflammatory cytokines such as interleukin 1,² and can be stimulated to produce effector functions which include antibody dependent cell cytotoxicity and opsonin independent bacterial phagocytosis via surface receptors for the Fc portion of IgG.³ There is conflicting evidence as to whether blood neutrophil functions are defective in RA. The balance of existing in vitro data favours normal chemotaxis and phagocytotic behaviour,⁴ though functions may be impaired in vivo in the presence of high titres of rheumatoid factor or immune complexes, possibly due to Fc γ receptor modulation.⁵

Neutrophils are capable of expressing three types of Fc γ receptor. Fc γ receptor type I (Fc γ RI), the only high affinity receptor ($K_a=5 \times 10^8$ mol⁻¹ l) for monomeric IgG, is expressed at very low levels by resting neutrophils but can be induced by proinflammatory mediators such as γ interferon to between 5000 and 20 000 sites per cell. Fc γ receptor type II (Fc γ RII) is constitutively expressed at between 30 000 and 60 000 sites per cell and the glycosyl phosphatidylinositol linked Fc γ receptor type III (Fc γ RIII) is the most abundant receptor, normally expressed at about 100 000-300 000 molecules per cell.³

Fc γ receptors are capable of NADPH oxidase activation leading to oxygen radical production⁶ and the release of lysosomal enzymes.⁷ It has been proposed that the release of soluble Fc γ RIII following activation may also affect the regulation of immunoglobulin production by plasma cells.⁸

Many cell surface receptors on a wide variety of cell types transduce signals mediating the cell's response via inositol 1,4,5-trisphosphate release.⁹ Inositol trisphosphate binding to a specific receptor on endoplasmic reticulum results in a rapid release of calcium ions from intracellular stores.^{10 11} Intracellular calcium ion release following cross linking of the low affinity Fc γ RIII on neutrophils has been shown,¹² though these workers did not observe such a response to stimulation through the Fc γ RII. The transmembrane signalling system triggered by Fc γ receptors on neutrophils is complex. Phagocytosis of IgG opsonised particles is accompanied by a transient increase in cytosolic calcium and the formation of inositol trisphosphate.¹³ Priming by interleukin 1 and tumour necrosis factor, however, leads to a more pronounced activation through a protein kinase C mediated pathway.¹⁴

The relative contribution that Fc γ RII and Fc γ RIII make to neutrophil IgG Fc mediated functions is gradually being determined. Fc γ RIII does not appear capable of mediating neutrophil cytotoxicity towards hybridoma cells,¹⁵ whereas it is the sole Fc receptor implicated in opsonin independent bacterial phagocytosis. Fc γ RIII specific lysis of chick erythrocytes has been shown with hetero-antibodies to Fc γ RIII.¹⁶ Fc γ RII is capable of triggering the neutrophil respiratory burst and also evokes neutrophil degranulation,⁷ whereas Fc γ RIII is only capable of mediating degranulation.¹⁷

We have approached the question of whether the control of Fc receptor mediated neutrophil functions are defective in RA at the receptor or

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signal transduction level by measuring intracellular calcium ion mobilisation events in response to cross linking of the membrane spanning Fcγ RII and the glycosyl phosphatidylinositol linked Fcγ RIII.

Patients and methods

Peripheral blood was obtained from 26 healthy donors, 14 patients with definite RA, four patients with ankylosing spondylitis, and three with osteoarthritis. The 14 patients with RA were divided into two groups according to the degree of activity of synovitis at the time the blood was collected. Seven patients with six or more tender swollen joints, together with two out of three of erythrocyte sedimentation rate >28 mm/hour, more than 45 minutes early morning stiffness, or nine tender swollen joints were classified as having 'active synovitis'. Seven patients with no tender swollen joints, erythrocyte sedimentation rate <28 mm/hour, and no early morning stiffness were classified as an 'inactive disease' group. Patients with overt joint infections or sepsis were excluded from the study. The table summarises age and sex distributions of the different groups studied.

Blood from these subjects was collected into heparinised polypropylene tubes. Neutrophils were isolated by density centrifugation over a Ficoll meglumine M85 gradient.¹⁸ The neutrophil suspension was removed and washed. After an initial wash in phosphate buffered saline, erythrocyte contamination was removed by lysis using a 10 second exposure to distilled water before the addition of an equal volume of double strength phosphate buffered saline. A further phosphate buffered saline wash was followed by resuspension in RPMI 1640 containing 10% fetal bovine serum at a cell concentration of 5×10^6 /ml. All these procedures were carried out at ambient temperature. Microscopic analysis showed less than 5% contamination of this population by mononuclear cells. The viability of the cells was greater than 98%.

Antibodies recognising the two low affinity receptors for the Fc portion of IgG were used in this study. The murine IgG2b antibody IV.3 recognising Fcγ RII (CD32) was obtained in purified form, as were the murine IgG2a antibodies 32.2 and 3G8 recognising Fcγ RI (CD64) and Fcγ RIII (CD16) respectively (Medarex, West Lebanon, NH, USA). No endotoxin contamination was detected in any of the antibody preparations used.

Intracellular calcium ion concentrations, $[Ca^{2+}]_i$, were measured using the fluorescent calcium binding probe indo-1 (Molecular

Probes Junction City, OR, USA).¹⁹ Neutrophils were incubated with a 20 µg/ml final concentration of monoclonal antibody together with a 1 µmol/l final concentration of the acetoxymethyl ester of indo-1 for 30 minutes at 37°C in RPMI 1640. After loading the cells were washed once in Hanks's HEPES buffered saline before being resuspended in the same medium to a cell concentration of 2.6×10^6 cells per ml.

A Shimadzu model 540 recording spectrofluorimeter with a thermostatically controlled stirred cell was used to monitor calcium binding to indo-1. The wavelength used for excitation was 355 nm (slit width 5 nm) with fluorescence emission detected at a wavelength of 400 nm using the same slit width.

Cells were preincubated for five minutes to allow for temperature equilibration at 37°C. Basal fluorescence was monitored by a chart recorder for a further two to three minutes before Fcγ receptors were cross linked by the addition of 30 µg of F(ab')₂ fragment of sheep antimouse IgG (Sigma Chemical, Poole, Dorset, United Kingdom). The fluorescence intensity of emission was monitored for at least eight minutes after stimulation. These signals were calibrated to determine intracellular calcium ion concentrations. A value for maximal fluorescence emission (F_{max}) was obtained by the addition of 0.5% Triton X-100 to lyse the cell suspension. This was carried out in the presence of 10 µmol/l diethylenetriaminepentaacetic acid. Minimum fluorescence (F_{min}) was measured by chelating calcium ions to less than 10 nmol/l by the addition of 10 mmol/l EGTA and 40 mmol/l TRIS. Basal and stimulated intracellular free calcium ion concentrations were then estimated using the dissociation constant for calcium binding to indo-1 of 250 nmol/l and the following equation²⁰

$$[Ca^{2+}]_i = K_d \times (F - F_{min}) / (F_{max} - F)$$

Basal and peak stimulated intracellular calcium ion concentrations were expressed in nmol/l and intergroup comparisons made using the Mann Whitney U test assuming a non-parametric data distribution. Spearman's rank correlation coefficient was used to determine the degree of correlation between variables.

The expression of neutrophil Fcγ receptors was measured by surface staining and flow cytometry. Twenty microlitres of medium containing neutrophils at a final concentration of 5×10^7 per ml in a 20 µl volume were reacted with the three previously described monoclonal antibodies that recognise the three types of human Fcγ receptors. The indirect staining method used, employing the F(ab')₂ fragment of FITC conjugated rabbit antimouse IgG to detect monoclonal binding, has been described elsewhere.²¹

Mean fluorescence intensity was analysed using an Ortho 50H cytofluorograph plus a 2150 computer (Becton Dickinson). The relative number of Fcγ receptors of each type per cell could then be calculated by reference to a calibration graph of FITC coated latex beads coated with defined numbers of FITC molecules (Flow Cytometry Standards, Research Triangle

Age and sex data for the control subjects and patients used in the study (see text for classification criteria for active and inactive rheumatoid arthritis (RA))

Group	Age (years)		Sex ratio (M:F)
	Median	Range	
Healthy controls (n=27)	40	21-62	11:16
Inactive RA (n=7)	69	51-72	4:3
Active RA (n=7)	51	36-67	0:7
Ankylosing spondylitis (n=4)	40	37-55	4:0
Osteoarthritis (n=3)	55	40-87	1:2

Park, NC, USA) according to the method of LeBouteiller *et al.*²²

Results

Intracellular calcium ion concentrations were measured in resting neutrophils from subjects in all control and patient groups. Incubation of indo-1 loaded cells with either monoclonal antibody IV.3 or 3G8 had no effect on the basal calcium ion concentration and did not result in a mobilisation of calcium ions in either the healthy control or patient groups. Median (interquartile range) basal intracellular calcium ion concentrations for the control group were 217 (125) nmol/l. Values for the ankylosing spondylitis group were similar at 210 (71) nmol/l; however, basal intracellular calcium ion concentrations in the two RA groups were significantly different from control subjects. The inactive RA group at 327 (184) nmol/l was significantly higher ($p < 0.05$) than control values and the active RA group 102 (67) nmol/l was significantly lower than control values ($p < 0.01$).

The sole addition of $F(ab')_2$ fragment of anti-mouse IgG to neutrophils had no effect on the intracellular calcium ion concentrations. However, when cells were preincubated with either monoclonal IV.3 or 3G8, the addition of the cross linking second antibody resulted in mobilisation of calcium ions. Figure 1 shows

Figure 1 Neutrophil calcium ion fluxes in response to cross linking. (A) $Fc\gamma$ RII, (B) $Fc\gamma$ RII in the presence of 10 mmol/l EGTA, and (C) $Fc\gamma$ RIII. Cells were isolated from a healthy subject. Basal and peak intracellular calcium ion concentrations are expressed in nmol/l. The peak on the extreme right represents maximum amount of released calcium ions (F_{max}).

typical calcium ion fluxes by control neutrophils triggered via $Fc\gamma$ RII or RIII. The presence of 10 mmol/l EGTA in the incubation buffer to chelate extracellular calcium had no effect on the flux resulting from $Fc\gamma$ receptor crosslinking, indicating that all calcium ion movements were from intracellular stores.

For control neutrophils, the peak $[Ca^{2+}]_i$ reached was not statistically different with respect to the Fc receptor type triggered. Median peak fluxes were 1098 (1215) nmol/l for $Fc\gamma$ RII and 660 (413) nmol/l for $Fc\gamma$ RIII. Differences were observed, however, in the time taken to reach peak calcium ion concentrations. Peak intracellular calcium ion concentration occurred 35.5 (16) seconds after $Fc\gamma$ RII triggering but was significantly retarded at 72 (20) seconds for $Fc\gamma$ RIII ($p < 0.001$).

Comparative calcium ion responses to $Fc\gamma$ RII triggering between controls and patient groups are shown in Fig 2A, which demonstrates the relative lack of response in a patient with RA with active synovitis compared with a control response, one from a patient with quiescent disease, and a patient with ankylosing spondylitis. A similar pattern of response was observed for $Fc\gamma$ RIII triggering, in which there is a complete absence of response by active RA neutrophils (fig 2B). Figure 3 summarises the median differences in response between groups. Patients with inactive disease showed exaggerated responses to cross linking of both receptor types, but owing to a large interquartile range the differences were not statistically significant. The patients with active synovitis showed a marked impairment in peak calcium ion flux for both Fc receptor types; $Fc\gamma$ RII was less than the controls, ankylosing spondylitis, and inactive RA ($p < 0.05$); $Fc\gamma$ RIII was less than controls and inactive RA ($p < 0.001$) and less than ankylosing spondylitis ($p < 0.01$). Responses in the ankylosing spondylitis group, though generally lower than in control subjects for both $Fc\gamma$ receptor types, were not statistically significant. Spearman's rank correlation analysis of basal and peak flux calcium ion concentrations showed a strong positive correlation between these variables for both $Fc\gamma$ RII ($r_s = 0.576$, $p < 0.001$) and $Fc\gamma$ RIII ($r_s = 0.665$, $p < 0.001$). Cell surface staining using monoclonal antibodies to the three $Fc\gamma$ receptor types was used to ascertain whether the reduction in calcium ion fluxes by active RA neutrophils was due to decreased expression of $Fc\gamma$ receptors. Flow cytometric analysis standardised by FITC labelled calibration beads showed that the expression of neutrophil $Fc\gamma$ receptors of all three types was not significantly different when cells from control subjects were compared with those from patients with active RA (fig 4).

Discussion

$Fc\gamma$ RII and RIII expressed on peripheral blood neutrophils are capable of triggering a transient intracellular calcium ion mobilisation which appears not to involve membrane ion channels. This observation is at variance with that of Kimberley *et al* who used a similar protocol and

Figure 2 (A) Neutrophil calcium ion fluxes in response to cross linking Fcγ RII. Comparison of fluxes obtained by triggering cells isolated from a healthy control, a patient with RA with active synovitis, a patient with RA with quiescent joint inflammation, and a patient with ankylosing spondylitis (AS). (B) Calcium ion fluxes in response to cross linking Fcγ RIII. Comparison of fluxes from a healthy control, a patient with RA with active synovitis, a patient with RA with quiescent joint inflammation, and a patient with generalised osteoarthritis (OA). Basal and peak intracellular calcium ion concentrations are expressed in nmol/l. The peak on the extreme right represents maximum amount of released calcium ions (F_{max}).

reported calcium ion fluxes in response to Fcγ RIII but not to Fcγ RII cross linking.¹² Significant Fcγ RII mediated fluxes, however, were reported by these workers in two of 14 experiments. We report a significant calcium flux in all 26 healthy subjects studied after cells were triggered via either receptor. The responses were specific, only being observed when receptors were cross linked with monoclonal antibodies to the Fc receptor and then the F(ab')₂ fragment of antimouse IgG. Responses were not seen to the second antibody alone, to isotype mouse antibody controls or when the monoclonal antibodies were incubated with cells without having been cross linked. The characteristics of the calcium ion fluxes obtained by cross linking the two Fcγ receptors differed in the time to peak response but not in the overall intracellular calcium ion concentration.

This could imply that Fcγ RIII may be signalling through an additional intermediate mechanism. Evidence exists that neutrophil Fcγ RIII signalling leads to priming for subsequent enhanced phagocytosis via Fcγ RII and a different transduction route.²³ Intracellular calcium ion fluxes were severely depressed when neutrophils from patients with RA with active synovitis were triggered via Fcγ RII or RIII. This impairment correlated with depletion of basal intracellular calcium ion concentration indicating a defect in intracellular calcium ion homeostasis or sequestration of free calcium ions which may influence the cell's ability to give a subsequent ion flux. The depletion of neutrophil free calcium was only observed in the context of active inflammatory synovitis. Conversely, inactive disease was accompanied by an abnormally high free intracellular calcium

Figure 3 Comparison of peak intracellular calcium ion concentrations in controls, patients with active and inactive RA, and those with ankylosing spondylitis (AS). Fcγ RII responses are denoted by light hatched bars and Fcγ RIII by dark hatched bars. The active RA group showed significantly impaired fluxes. *Fcγ RII less than controls, ankylosing spondylitis, and inactive RA ($p < 0.05$). **Fcγ RIII less than controls and inactive RA ($p < 0.001$); less than AS ($p < 0.01$).

Figure 4 Expression of neutrophil Fcγ RI, RII, and RIII measured by monoclonal antibodies 32.2 (open bars), IV.3 (light hatched bars), and 3G8 (heavy hatched bars). No significant differences were observed between groups. Results expressed as mean (SD) FITC equivalent binding sites per cell.

ion concentration. In normal circumstances calcium ions regulate their own release from intracellular stores¹⁰ and calcium ion concentrations are under strict homeostatic control. Aberrant intracellular calcium ion concentration would profoundly affect the ability of the neutrophil to respond to a stimulus transduced via calcium ion release. It may be argued that the lack of calcium ion flux is the consequence of previous activation of neutrophils by immune complexes generated within the inflamed synovium and released into the exudate. We have shown that the lack of response is not due to the loss of surface

receptors capable of being cross linked. All three Fc receptor types were expressed at the same level in active RA neutrophils when compared with controls, suggesting that the receptors had not been modulated by immune complexes, as receptor ligation results in the internalisation of Fcγ RII and the shedding of Fcγ RIII.⁸ Also, responses within the normal range by neutrophils from patients with active ankylosing spondylitis would suggest that lack of response is not the result of a chronic inflammatory process per se. Emerging views on the neutrophil in the development of RA have suggested a part in the antagonism of the inflammatory response including the release of interleukin 1 antagonists²⁴ and the ability to down regulate lysosomal enzyme activity.²⁵ If intracellular calcium ion mediated signalling pathways are involved in neutrophil priming before activation, depleted basal intracellular calcium ion concentration and thence impaired calcium ion mobilisation could have important consequences for the anti-inflammatory activities of neutrophils. An impairment of Fc receptor mediated phagocytosis would also affect the efficiency of removal of potentially damaging immune complexes and could contribute to the chronicity of the inflammatory response. Further studies are in progress to find the correlation between these intermediaries of signal transduction and endpoint pro- and anti-inflammatory neutrophil functions.

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