IgM monoclonal antibodies recognizing Fc α R but not Fc γ RIII trigger a respiratory burst in neutrophils although both trigger an increase in intracellular calcium levels and degranulation

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The role of IgG receptor, Fc γ RIII, in the triggering of neutrophil functions has been controversial. Here we show that IgM monoclonal antibodies, Leu 11b and 1D3, recognizing Fc γ RIII, bind to human neutrophils triggering an increase in intracellular calcium concentration and release of myeloperoxidase upon degranulation but do not trigger a respiratory burst detectable as lucigenin-enhanced chemiluminescence. Although many fewer molecules of IgM monoclonal antibody, MY43, recognizing

Fc α R, bind to the same cells they trigger a much greater increase in intracellular calcium concentration, release of myeloperoxidase and a strong respiratory burst. Since the respiratory bursts triggered by IgG and IgA are equivalent, this demonstrates that Fc γ RII is responsible for the IgG-mediated response. IgM monoclonal antibody MC2, recognizing the abundant neutrophil cell-surface carbohydrate CD15, also triggers a small rise in intracellular calcium but no respiratory burst.

INTRODUCTION

The stimulation of neutrophils by aggregated immunoglobulins (immune complexes) is the central pathogenic event in a large number of inflammatory diseases. Neutrophils respond to stimulation by degranulation, which releases a battery of potent inflammatory mediators, and by the production of superoxide and other reactive oxygen species from activation of an NADPH oxidase, i.e. the so-called 'respiratory burst'. The two types of response might serve different functions and are probably under different control [1].

Cross-linking of Fc receptors by aggregated IgG or IgA or by monoclonal antibodies recognizing Fc γ R or Fc α R have been shown to cause both degranulation and activation of the respiratory burst. Studies on the stimulation of neutrophils by IgG are, however, complicated by the fact that there are two different receptors for IgG, Fc γ RII and Fc γ RIII, expressed constitutively on these cells. The relative role of Fc γ RII and Fc γ RIII in triggering neutrophil functions and their mechanism of action remains controversial.

Fc γ RII is a 40 kDa glycoprotein with low affinity ($K_a < 10^6$ M⁻¹) for monomeric IgG [1,2]. It possesses a transmembrane-spanning domain and a cytoplasmic tail [3], which is able to interact with G-proteins. This receptor is found to be functionally associated with the src-like tyrosine kinase fgr [4]. Fc γ RII is structurally related to the more abundant, low-affinity, Fc γ RIII (K_a for monomeric IgG $< 10^7$ M⁻¹) which, on neutrophils, is a 50–70 kDa glycosylphosphatidylinositol (GPI)-linked glycoprotein [5]. Neutrophil Fc γ RIII is thus unable to couple directly to G-proteins or the cytoskeleton. It has therefore been proposed that the role of Fc γ RIII might be to present ligand to the functionally active Fc γ RII [6,7]. This was suggested by the fact that neutrophils from patients with paroxysmal nocturnal haemoglobinuria [8,9], or neutrophils depleted of Fc γ RIII

using phosphatidylinositol-specific phospholipase C (PIPLC) or elastase [7], show little loss of normal function other than a reduced capability to bind dimeric IgG [9]. Although these results suggest that IgG-mediated activation of neutrophils is initiated primarily through the Fc γ RII, both receptors clearly bind immune complexes and there is evidence that Fc γ RIII is able to activate some cytotoxic mechanisms [10].

A number of studies using monoclonal antibodies recognizing Fc γ receptors have been undertaken to try to ascertain the role of Fc γ RIII. The results have been somewhat contradictory. One study using F(ab')₂ monoclonal antibodies against Fc γ RII and Fc γ RIII has suggested that cross-linking of Fc γ RIII triggers degranulation but does not trigger a respiratory burst [11]. Antibodies recognizing Fc γ RII appeared to trigger both, although triggering of the respiratory burst by anti-Fc γ RII required further cross-linking of the bound monoclonal antibody using F(ab')₂ anti-(mouse immunoglobulin). In contrast, in another study, cross-linked F(ab')₂ anti-Fc γ RII or anti-Fc γ RIII triggered a respiratory burst [12]. Fab fragments of monoclonal antibodies recognizing Fc γ RIII have also been shown to block the respiratory burst induced by soluble or insoluble immune complexes in a number of studies [12,13].

In recent years there have been major advances in our understanding of the mechanisms of signal transduction in neutrophils. Chemotactic agents such as *N*-formyl peptides or leukotriene B₄ [14,15] have been shown to cause rapid elevation of intracellular calcium, as well as initiating a respiratory burst. Indeed, it appears that intracellular calcium fluxes are essential for neutrophils to degranulate and activate the NADPH oxidase, since cells loaded with a calcium chelator [16] cannot respond with a respiratory burst.

The respiratory burst response to IgG immune complexes has been reported to be associated with small increases in intracellular calcium [13]. However, once again, studies on the stimulation of

Abbreviations used: DMSO, dimethylsulphoxide; FITC, fluorescein isothiocyanate; GPI, glycosylphosphatidylinositol; HBSS, Hanks balanced salt solution; PIPLC, phosphatidylinositol-specific phospholipase C; MPO, myeloperoxidase.

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calcium burst by specific Fc γ R_s have been contradictory [17–19].

Though there has been much work on the functional effects of Fc γ R_{III} cross-linking on neutrophils, most studies have used intact IgG monoclonals, or F(ab')₂, or Fab fragments. Results obtained with IgG monoclonal antibodies are open to the criticism that binding to receptor could be Fab- or Fc-mediated and F(ab')₂ fragments could contain undetectable levels of intact antibody. This might explain the contradictory findings using the same anti-receptor antibodies. In an attempt to study the triggering of neutrophils by Fc γ R as directly as possible, we have utilized two mouse IgM monoclonals against Fc γ R_{III}. These antibodies have the capability of cross-linking the receptors directly, because they are multivalent, and specifically, because there are no receptors for IgM on the neutrophil. The triggering was compared with that elicited by an IgM monoclonal antibody recognizing Fc α R.

MATERIALS AND METHODS

Materials

Dextran T70 and Ficoll 400 were obtained from Pharmacia, Milton Keynes, U.K.; Hanks balanced salt solution (HBSS), (10 ×) HBSS without Phenol Red, and calcium-free HBSS were from Gibco Life Technologies Ltd, Paisley, Scotland, U.K.; Fluo-3 AM from Calbiochem Novabiochem (UK) Ltd., Nottingham, U.K.; F(ab')₂ anti-(mouse IgM) from Caltag Laboratories. 'FACS lysing solution' was from Becton Dickinson, Oxford, U.K.; all other reagents were from Sigma.

Monoclonal antibodies

Anti-Fc γ R_{III} monoclonal antibody, Leu 11b [20], was purchased from Becton Dickinson; anti-Fc γ R_{III} monoclonal antibody, 1D3, and anti-Fc α R monoclonal, MY-43, were from the FcR panel of the Fifth International Workshop on Leucocyte Differentiation Antigens. 1D3 was prepared by Dr. J. D. Griffin, Dana Farber Inst., Boston, U.S.A. Fluorescein isothiocyanate (FITC)-1D3 was a gift from Dr. Robert Burnhouse, Coulter Corp., Hialeah, FL, U.S.A. MY-43 was prepared by Dr. Li Shen, Dartmouth Medical School, Hanover, NH, U.S.A. [21]. The anti-CD15 monoclonal antibody, MC2, was produced in this laboratory [22]. All monoclonal antibodies were diluted to give a stock solution (1 mg/ml IgM) which was sufficient to show saturation in flow cytometry as described below.

Purification of human neutrophils

Freshly heparinized whole blood (10 ml) from healthy volunteers was added to 5 ml of dextran T70 (6% w/v in 0.15 M NaCl) and allowed to sediment for 30 min at room temperature, to remove erythrocytes. The leucocytes in the supernatant were layered on to 10 ml of Lymphoprep (Nycomed Pharma AS, Oslo, Norway; density 1.077) on top of 15 ml of Ficoll-Hypaque (density 1.119) before centrifugation (700 g, 20 min, 22 °C). The cells at the Lymphoprep/Ficoll-Hypaque layer were removed, resuspended in assay buffer (1 × HBSS, 1% BSA, 20 mM Hepes, 10 mM D-glucose) and then centrifuged (200 g, 7 min, 22 °C). The pelleted neutrophils were resuspended in assay buffer and used immediately. Cells purified in this manner were 97% pure and seen to be > 95% viable using the Trypan-Blue-exclusion test.

Immunofluorescence flow cytometry

Aliquots (200 μ l) of whole heparinized blood were placed into tubes at 4 °C and 10 μ l of a 1:50 dilution of each IgM antibody

was added. After incubation at 4 °C for 1 h, the cells were washed by addition of 3 ml of assay buffer followed by centrifugation (200 g, 5 min, 4 °C). The supernatant was aspirated, and the cells resuspended in a 1:200 dilution of a FITC-conjugated goat anti-(mouse IgM) antibody, before incubation for a further 1 h at 4 °C. The cells were washed as described previously and then 2 ml of 'Fix and Lyse' was added before incubation for 20 min in the dark. The cells were centrifuged once more (200 g, 5 min, 4 °C), and pellet resuspended in 2% formaldehyde solution for analysis. The mean fluorescence of 10000 cells was measured using Lysis II software on a FACScan analyser.

Chemiluminescence measurements

The chemiluminescence assays were performed using a Dynatech ML1000, 96-well plate reader as previously described [23]. The plates were prepared as follows, 100 μ l of a 1:50 dilution of F(ab')₂ anti-IgM in assay buffer was incubated in the wells overnight at 4 °C. After washing the plates with PBS, 100 μ l of a working concentration of the IgM monoclonal antibodies was added before the plates were incubated overnight at 4 °C. After the plates had been washed thoroughly with PBS, 100 μ l of 100 mM lucigenin was added. An aliquot (50 μ l) of neutrophil suspension (1 × 10⁶ cells/ml in assay buffer) was added to each well and readings of chemiluminescence were taken every 90 s for 30 cycles.

Calcium measurement using Fluo-3 [24]

Purified human neutrophils were suspended in calcium-free assay buffer (calcium-free HBSS, 1% BSA, 20 mM Hepes, 10 mM D-glucose) at a concentration of 1 × 10⁷ cells/ml. Fluo-3 AM [1 mM dissolved in dimethylsulphoxide (DMSO)] was added to the cell suspension at a final concentration of 3 μ M. The cells were incubated at 37 °C in the dark for 30 min, then diluted to a concentration of 2 × 10⁶ cells/ml before incubation for a further 50 min at 37 °C in the dark, to allow total cleavage of the acetoxymethyl ester from the internalized dye. The cells were then pelleted (200 g, 5 min, 4 °C) and washed in assay buffer. They were then resuspended at 1 × 10⁶ cells/ml in assay buffer and stored at 4 °C until needed. For calcium measurements, the cells were warmed for 10 min to 22 °C. The fluorescence was measured continuously on the FACScan analyser using the Chronis software. Neutrophils were gated from their size/granularity ratio and the FL1 channel was set to read the Fluo-3 fluorescence. Acquisition was started with this fluorescence being the baseline, and after 30 s one of the mouse IgMs was added to give a final dilution of 1:200. The resulting fluorescence change was then measured for a further 330 s.

Degranulation

Samples for the enzyme assay were obtained by stimulating cells, 100 μ l of 1 × 10⁷ cells/ml, with a final dilution of 1:200 of the IgM monoclonals for various lengths of time at 37 °C. The reaction was stopped by centrifugation for 30 s in a microfuge, and the supernatant removed and stored on ice until required. An estimate of total enzyme content of the cells was achieved by lysing with addition of 0.2% Triton X-100.

Myeloperoxidase (MPO) activity was measured using a modification of the method of Henson et al. [25], the main difference being the use of the water-soluble substrate, orthophenylenediamine instead of orthodiansidine. The 100 μ l of supernatant containing the enzyme was added to 1.0 ml of freshly prepared 0.1 M sodium phosphate, pH 6.2, containing 0.5 mg/ml ortho-

phenylenediamine. The reaction was started with the addition of 0.1 ml of 0.06% H_2O_2 in distilled water. After the reaction had run for 60 min at 37 °C, it was stopped by the addition of 10 μl of H_2SO_4 . The absorbance (A_{480}) of the reaction products was measured within 30 min.

RESULTS

Binding of monoclonal antibodies to neutrophils measured by immunofluorescence

The binding of the IgM monoclonal antibodies to purified neutrophils was quantified by flow cytometry (Figure 1). At the concentrations used, the two IgM monoclonal antibodies recognizing Fc γ RIII, 1D3 and Leu11b, bound strongly to purified neutrophils and could be readily detected using the fluoresceinated anti-(mouse IgM) reagent (mean fluorescence 94 and 179 respectively). MC2, an IgM monoclonal recognizing the carbohydrate antigen, CD15, bound to a similar extent (mean fluorescence 179). My43, an IgM monoclonal recognizing Fc α R showed a much lower level of binding (mean fluorescence 57), although this was still above the level for the control antibody,

Figure 1 Binding of the IgM monoclonals to human neutrophils

Freshly purified human neutrophils incubated at 4 °C for 1 h with the IgM monoclonals, Leu11b (a) and 1D3 (b) against the Fc γ RIII, My43 (c) against the Fc α R, MC2 (d) against CD15, and a non-specific mouse IgM (e) as a control. The IgM binding was detected by incubation with an FITC-conjugated anti-(mouse IgM) second antibody, and measured on the FACScan using LYSIS II. This shows the results of a representative experiment out of six.

Figure 2 IgM stimulated lucigenin chemiluminescence

The lucigenin-enhanced chemiluminescence burst measured upon stimulation of neutrophils with the anti-receptor IgM monoclonals was measured on a 96-well plate luminometer. The IgM monoclonals were bound to the wells via F(ab')₂ anti-IgM. 50 μl of the cells, suspended at 1×10^6 cells/ml in HBSS, BSA, Hepes assay buffer were added to each well, and the chemiluminescence measured every 90 s for 30 cycles. My43 (●) was used to stimulate the Fc α R, Leu11b (◇) or 1D3 (◆) were employed to activate Fc γ RIII, and non-specific purified mouse IgM (□) was used to demonstrate the effects of IgM on the cells. The plot shows the activation curves of one experiment carried out on cells from one donor; this pattern was observed for ten different donors.

a purified mouse IgM myeloma of unknown specificity (mean fluorescence 44). Each of the monoclonal antibodies used in this study was at a final dilution, in the assay, of 1:1000 made from a stock solution containing 1 mg/ml IgM. Doubling of the antibody concentration did not result in additional binding of antibody to the cells, demonstrating that effective saturation of the receptors was occurring during functional assays detailed below.

Stimulation of neutrophil chemiluminescence by the IgM monoclonal antibodies

The stimulation of neutrophil chemiluminescence by the monoclonal antibodies bound to microtitre plates was measured using either luminol or lucigenin enhancement. Though both enhancing compounds showed the same results, it was found that lucigenin gave a larger overall burst response. In order to standardize the binding of the IgM antibodies to the plates, they were first coated with F(ab')₂ anti-IgM reagent. The F(ab')₂ elicited no chemiluminescent burst from the neutrophils. However, as can be seen from Figure 2, which is representative of a large number of experiments, neutrophils were stimulated by monoclonal antibody My43 (recognizing Fc α R) but not by 1D3 and Leu11b (recognizing Fc γ RIII) or MC2 (recognizing CD15). The same results were obtained when purified monoclonal antibodies were bound directly to the plates.

Stimulation of the release of intracellular calcium by IgM monoclonal antibodies

Purified neutrophils were incubated with the various IgM monoclonals and release of intracellular calcium was measured by the Fluo-3 technique. Again, the control, purified mouse IgM gave no response above the baseline. As seen in Figure 3, each of the IgM monoclonal antibodies recognizing cell-surface determinants were capable of eliciting a rapid and measurable release

Figure 3 The release of intracellular calcium on IgM stimulation

The change in intracellular Ca^{2+} concentration was measured on activation of the neutrophils using the fluorescent calcium indicator Fluo-3 with continuous measurement on the FACScan using the kinetics program, CHRONIS. Again the IgM anti-(Fc receptor) monoclonals were used to cross-link and activate the cells. The cells were suspended at 1×10^6 cells/ml in calcium-free medium and 30 s after the start of the experiment, ($\downarrow$), one of the IgM monoclonals was added, to give a final dilution of 1:200. The resulting increase in fluorescence was then measured for a further 330 s. Plot A represents the My43-stimulated response, traces B and C are Leu11b- and 1D3-stimulated responses respectively, and D is the non-specific IgM-stimulated trace. This shows the results of a typical experiment, one of six, with all measurements being carried out on a single donor's cells.

of calcium, suggesting that each is capable of cross-linking the respective receptor and transducing a signal to the cytoplasm. The largest calcium flux was caused by My43, the monoclonal recognizing $\text{Fc}\alpha\text{R}$. Although the monoclonals to $\text{Fc}\gamma\text{RIII}$ were incapable of initiating the respiratory burst, they were able to cross-link the receptor and cause a rise in intracellular calcium. The rise in intracellular calcium was smaller and slower than that

Figure 4 MPO release on neutrophil stimulation

The release of MPO upon degranulation was measured after stimulation of the cells with the anti-receptor monoclonals. The cells were stimulated by addition of the IgM antibodies to a final dilution of 1:200, and the reaction stopped by centrifugation. The enzyme content was measured using the method of Henson et al. [25], and a time course for release plotted for up to 10 min after stimulation. The graph shows the percentage release $\pm$ S.E.M. for six experiments. Once again, My43 ($\bullet$) was used to activate the $\text{Fc}\alpha\text{R}$ response, and Leu11b ($\blacklozenge$) used to activate the $\text{Fc}\gamma\text{RIII}$ response. In each case, baseline release of MPO from the cells occurring on incubation with control IgM has been subtracted.

from My43 stimulation. The inclusion of 0.5 mM EDTA in the external medium had no effect on the size of the initial burst of calcium triggered by monoclonals against $\text{Fc}\alpha\text{R}$ or $\text{Fc}\gamma\text{RIII}$, showing that both triggered the release of calcium from intracellular stores. The inclusion of 0.5 mM EDTA in the medium slightly enhanced the chemiluminescence burst elicited by the monoclonals against either receptor. The anti-CD15 monoclonal antibody stimulated a calcium flux intermediate between that of $\text{Fc}\alpha\text{R}$ and $\text{Fc}\gamma\text{RIII}$ (results not shown).

Stimulation of degranulation by IgM monoclonal antibodies

Purified neutrophils were incubated with the anti-receptor IgM monoclonals for various periods of time, up to 10 min, before the reaction was stopped by centrifugation. The MPO content of these supernatants was then measured. The rates of release of enzyme (expressed as a percentage of the total detergent-releasable enzyme content of the cells) from the neutrophils of six donors stimulated with My43 and Leu11b are seen in Figure 4. Both of the IgM monoclonals caused release of this enzyme, although to different levels; My43 addition to the cells resulting in a significantly greater release of MPO than Leu11b.

DISCUSSION

We have compared the binding and stimulation of neutrophils by IgM monoclonal antibodies recognizing $\text{Fc}\gamma\text{RIII}$ (1D3 or Leu11b), $\text{Fc}\alpha\text{R}$ (My43) and CD15 (MC2). It has been well documented that aggregation of $\text{Fc}\alpha\text{R}$ by My43 or aggregated IgA [23,26], will result in the release of oxygen radicals through the respiratory burst. The effects of cross-linking $\text{Fc}\gamma\text{RIII}$ using IgM monoclonal antibodies has not been previously reported.

Consistent with previous reports ([27] and references cited within) that the surface expression of $\text{Fc}\gamma\text{RIII}$ was around 20-fold greater than that of $\text{Fc}\alpha\text{R}$, binding of the anti- $\text{Fc}\gamma\text{RIII}$ monoclonal antibodies was much greater than the monoclonal antibody recognizing $\text{Fc}\alpha\text{R}$. Nevertheless, the anti- $\text{Fc}\alpha\text{R}$ monoclonal antibody elicited a strong respiratory burst, whereas neither of the monoclonals recognizing $\text{Fc}\gamma\text{RIII}$ elicited any. Since the respiratory bursts elicited by aggregated IgA and IgG are equivalent [23] these results indicate clearly that the respiratory burst triggered by IgG must be due to $\text{Fc}\gamma\text{RII}$.

The lack of triggering of a respiratory burst by anti- $\text{Fc}\gamma\text{RIII}$ antibody is consistent with the observation that F(ab')_2 fragments of the IgG monoclonal antibody recognizing $\text{Fc}\gamma\text{RIII}$ (CLBGran1) triggered degranulation but did not trigger a respiratory burst, whereas antibodies recognizing $\text{Fc}\gamma\text{RII}$ appeared to trigger both. Triggering of the respiratory burst by anti- $\text{Fc}\gamma\text{RII}$ required further cross-linking of the bound monoclonal antibody using F(ab')_2 anti-(mouse immunoglobulin) [11]. Our results contradict those of Crockett-Torabi and Fantone [12] who showed that cross-linked F(ab')_2 anti- $\text{Fc}\gamma\text{RII}$ or $\text{Fc}\gamma\text{RIII}$ (antibody 3G8) triggered a respiratory burst, while Fab fragments of monoclonal antibody recognizing $\text{Fc}\gamma\text{RIII}$ blocked the respiratory burst induced by soluble or insoluble immune complexes.

Although it did not trigger a respiratory burst, cross-linking of the GPI-linked $\text{Fc}\gamma\text{RIII}$ did trigger an increase in intracellular calcium, measured using the fluorescent dye Fluo-3, which demonstrates that cross-linking of the $\text{Fc}\gamma\text{RIII}$ receptors did mediate intracellular signalling. Our observations are consistent with a number of reports [18,28] which have shown that the IgG anti- $\text{Fc}\gamma\text{RIII}$ monoclonal 3G8 can trigger a rise in intracellular calcium that did not occur when the cells had been treated with PIPLC [17]. Cross-linking of neutrophil $\text{Fc}\gamma\text{RII}$ by monoclonal antibody IV.3 has been shown not to elicit a calcium signal [17].

Significantly, the concentration of intracellular calcium elicited by the anti-Fc α R antibody but not by anti-Fc γ RIII was greater than 250 nM, the threshold concentration of calcium reported to be necessary for activation of the oxidase [29].

Although triggering of a rise in intracellular calcium did not result in a respiratory burst, previous studies have shown that it stimulates actin polymerization and priming of phagocytosis mediated by Fc γ RII [30]. Our own results confirm the finding of Huizinga and colleagues [11] that cross-linking of Fc γ RIII results in degranulation. Fc γ RII and Fc γ RIII are thus both able to mediate the response of neutrophils to aggregated IgG but through different pathways. Fc γ RIII works in synergy with the Fc γ RII by facilitating the binding of IgG to Fc γ RII and priming the cells to respond with enhanced degranulation and phagocytosis. It is interesting that a single class of Fc α R appears to be capable of initiating the release of intracellular calcium, degranulation and a respiratory burst. The results suggest different pathways of intracellular signalling by the Fc γ Rs and Fc α R which require elucidation. It is of particular interest that the shapes of the curves for calcium release triggered by cross-linking of Fc α R and Fc γ RIII are very different. Since inclusion of EDTA in the extracellular medium had no effect on the size of the bursts, it appears that both are due to release of intracellular stores of calcium.

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