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The Anti-Fc γ RIII MAb 3G8 Induces Neutrophil Activation Via a Cooperative Action of Fc γ RIIIb and Fc γ RIIa

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Human neutrophils express two types of Fc γ receptors, the transmembrane Fc γ RIIa and the glycan-phosphatidylinositol-anchored Fc γ RIIIb, that show synergism in provoking a cellular response. To analyse further the requirements for this synergism to occur we used the monoclonal antibody 3G8, directed against Fc γ RIII. This antibody is able to induce neutrophil activation, as measured by an increase in the intracellular free Ca²⁺ concentration and homotypic neutrophil aggregation, but only when the Fc part of the antibody is able to interact with Fc γ RIIa. We observed that binding of the Fab parts of 3G8 mAb to two Fc γ RIIIb molecules and binding of the Fc part to one Fc γ RIIa molecule is required, because a bispecific antibody, 2B1, in which only one 3G8 Fab is present, did not induce neutrophil activation. Moreover, engagement of one Fc γ RIIa molecule and two Fc γ RIIIb molecules on the same cell is instrumental to achieve activation of the mAb 3G8. The activation of neutrophils by the 3G8 antibody represents a further example of synergistic activation of neutrophils via Fc γ receptors. © 1997 Elsevier Science Ltd

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INTRODUCTION

Human neutrophils express two classes of Fc receptors for IgG on their cell surface, namely Fc γ RIIa and Fc γ RIIIb. Per neutrophil, 10 000–20 000 Fc γ RIIa molecules and 100 000–300 000 Fc γ RIIIb molecules are expressed on the plasma membrane (Huizinga *et al.*, 1989). Binding of IgG-coated particles or cells to these receptors induces phagocytosis, generation of superoxide and release of enzymes from intracellular granules into the phagocytic vacuole (Van de Winkel and Capel, 1993). Fc γ RIIa is a transmembrane molecule, whereas Fc γ RIIIb on neutrophils is anchored via a glycan-phosphatidylinositol moiety in the mem-

brane (Huizinga *et al.*, 1988). Clustering of both Fc γ RIIa and Fc γ RIIIb induces neutrophil activation. One of the intracellular signals that is induced is a rise in the cytosolic free Ca²⁺ concentration (Hundt and Schmidt, 1992; Kimberly *et al.*, 1990; Vossebeld *et al.*, 1995). We have described previously that the heterotypic clustering of Fc γ R on neutrophils evokes a synergistic rise in the intracellular concentration of Ca²⁺ as compared to separate Fc γ RIIa or separate Fc γ RIIIb cross-linking. Heterotypic Fc γ R cross-linking induces a more efficient mobilization of Ca²⁺ from intracellular stores and stimulation of Ca²⁺ influx. Probably, these two processes are linked according to the model of capacitive Ca²⁺ entry (Putney, 1990), in which depletion of the intracellular Ca²⁺ stores generates a signal that activates Ca²⁺

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influx. The Ca^{2+} influx is only observed under conditions of heterotypic $\text{Fc}\gamma\text{R}$ cross-linking and not under conditions of separate $\text{Fc}\gamma\text{R}$ cross-linking (Vossebeld *et al.*, 1995).

The anti- $\text{Fc}\gamma\text{RIII}$ mAb 3G8 has been shown to induce neutrophil activation via simultaneous engagement of both $\text{Fc}\gamma\text{RIIa}$ and $\text{Fc}\gamma\text{RIIIb}$ (Edberg and Kimberly, 1994; Edberg *et al.*, 1995). We investigated the mechanism of this activation by comparison of the effects induced by 3G8 with that of the bispecific antibody 2B1, in which one 3G8 Fab has been replaced by a Fab directed against the neu oncogene (Weiner *et al.*, 1993). We also investigated whether $\text{Fc}\gamma\text{RIIa}$ and $\text{Fc}\gamma\text{RIIIb}$ need to be engaged on the same cell to achieve neutrophil activation. The activation of neutrophils by the intact mAb 3G8 provides an useful tool to gain further insight into the mechanism of receptor cross-linking.

MATERIALS AND METHODS

Materials

Indo-1/AM was from Molecular Probes, Eugene, OR, U.S.A. Pepsin and papain were from Sigma Chemical Co., St. Louis, MO, U.S.A. Fluorescein diacetate was from Nutritional Biochemicals Corporation, Cleveland, OH, U.S.A. Hydroethidine was from Polyscience, Inc., PA, U.S.A. Fluorescein isothiocyanate was from Serva, Heidelberg, Germany. Incubation medium contained 132 mM NaCl, 6 mM KCl, 1 mM CaCl_2 , 1 mM MgSO_4 , 1.2 mM potassium phosphate, 20 mM Hepes, 5.5 mM glucose and 0.5% (w/v) human serum albumin (pH 7.4).

Antibodies

As anti- $\text{Fc}\gamma\text{RIII}$ monoclonal antibodies (mAb), 3G8 (mIgG1) (Fleit *et al.*, 1982) and CLBFCgran1 (mIgG2a) (Werner *et al.*, 1985) were used. As anti- $\text{Fc}\gamma\text{RII}$ monoclonal antibody, IV.3 (mIgG2b) was used (Rosenfeld *et al.*, 1985). The mAbs were purified from culture supernatant by precipitation with 50% saturated ammonium sulphate and subsequent protein A affinity chromatography. F(ab')_2 fragments were made by digestion of intact antibodies with 2% (w/w) pepsin at pH 3.7 for 16 hr at 37°C. Fab fragments were prepared by digestion of intact antibodies with 2% (w/w) papain in phosphate-buffered saline (PBS) containing 5 mM EDTA and 10 mM cysteine for 90 min at 37°C. The reaction was terminated

by adding 20 mM iodoacetamide. Fc parts were removed by protein A affinity chromatography. IV.3 mAb was fluorescein isothiocyanate (FITC)-labelled by incubation for 2 hr with FITC at pH 9.5 (4-fold molar excess of FITC). Free FITC was removed by gel filtration over a Sephadex G25 column. The bispecific antibody 2B1 binds to the *c-erbB2* gene product of the HER2/neu proto-oncogene and to CD16. The murine hybridoma 520C9 and 3G8 were the parental clones. 2B1 was produced by fusion of the parental hybridoma clones as described (Shi *et al.*, 1991; Weiner *et al.*, 1993; Hsieh Ma *et al.*, 1992). The bispecific F(ab')_2 $\text{Fc}\gamma\text{RII}\times\text{Fc}\gamma\text{RIII}$ was prepared by biochemical cross-linking of the two Fab fragments and was characterized as described before (Vossebeld *et al.*, 1995). In short, Fab fragments of IV.3, prepared by digestion with papain as described above, were incubated for 5 hr under regular shaking at room temperature with a 10-fold molar excess of the heterobifunctional cross-linker *N*-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP) (Pierce, Rockford, IL, U.S.A.) added from a stock solution in ethanol. To remove unbound SPDP, the reaction mixture was passed over a G-25 Sephadex column equilibrated with PBS. Fab'-SH fragments of 3G8 were prepared by reducing F(ab')_2 fragments, obtained by pepsin digestion as described above, with 15 mM 2-mercaptoethanol for 30 min at 30°C. The reduced product was passed over a G-25 Sephadex column in PBS to remove the 2-mercaptoethanol and was immediately added to the IV.3 Fab-SPDP fragments. After the column step, a sample of 3G8 Fab'-SH was taken and incubated for 14 hr to control for spontaneous reoxidation of the Fab'-SH fragments. Analysis on SDS-PAGE showed that this was not the case. The mixture of Fab'-SH and Fab-SPDP fragments was concentrated to one-third of the original volume in C30 Amicon microconcentrators (Amicon, Beverly, MA, U.S.A.). After 14 hr of incubation at room temperature, the mixture was passed over an FPLC Superose 12 column equilibrated with PBS. Appropriate fractions were pooled and analysed on SDS-PAGE.

As CD11b antibody, 44A (mIgG1) was used (Buyon *et al.*, 1989), and as CD18 antibody, MHM23 (mIgG1) (Dako, Glostrup, Denmark) was used. The control antibody mIgG1 was from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Cross-linking of antibodies bound to neutrophils was performed with polyclonal goat-anti-mouse-immunoglobulin (GAM) F(ab')₂ fragments (Jackson ImmunoResearch, West Grove, PA, U.S.A.).

Cell isolation

Human neutrophils were isolated from peripheral blood of healthy donors. Blood was anti-coagulated in 0.4% (w/v) trisodium citrate and centrifuged over Percoll with a specific gravity of 1.076 g/ml (1000g, 18 min, 20°C). After lysis of the erythrocytes in the pellet fraction with ice-cold lysis buffer containing 155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA (pH 7.4), the granulocytes were washed twice with PBS and resuspended in incubation medium. The granulocytes consisted of 90–95% neutrophils; the remaining cells were eosinophils.

Ca²⁺ measurements

Intracellular Ca²⁺ was measured as described before (Koenderman *et al.*, 1989). Neutrophils (2×10^7 /ml in incubation medium) were loaded with indo-1 by incubation with 1 μ M indo-1/AM for 45 min at 37°C. The cells were washed and resuspended in incubation medium (10^7 /ml) and kept at room temperature. The cells were diluted five times in incubation medium and were prewarmed at 37°C before transfer to a cuvette. Fluorescence measurements were performed with a spectrofluorometer (model RF-540, Shimadzu Corporation, Kyoto, Japan). The contents of the cuvette were magnetically stirred and the cuvette holder was kept at 37°C. The excitation and emission wavelengths were 340 and 390 nm, respectively. Calibration of indo-1 fluorescence as a function of [Ca²⁺] was determined by first adding digitonin and thereafter Mn²⁺. To saturate all trapped indo-1 with Ca²⁺, digitonin (10 μ M) was added to the cell suspension. Subsequently, the indo-1 fluorescence was quenched with MnCl₂ (0.5 mM) (Gryniewicz *et al.*, 1985). For the calculation of [Ca²⁺], a dissociation constant of 250 nM was used for the indo-1/Ca²⁺ complex (Bijsterbosch *et al.*, 1986).

Assessment of Ca²⁺ influx was carried out with the Mn²⁺ quenching technique, as described before (Vossebeld *et al.*, 1995). For this purpose, an emission wavelength of 446 nm instead of 390 nm was chosen, resulting in the complete absence of fluorescence changes upon addition of a Ca²⁺ mobilizing stimulus (data

not shown). Just prior to each measurement, cells were diluted five times in incubation medium without Ca²⁺ to 2×10^6 /ml (to favour Mn²⁺ entry) (Demaurex *et al.*, 1994). The cells (2×10^6 /ml) were prewarmed at 37°C. Two minutes prior to addition of the stimulus, 0.5 mM MnCl₂ was added to the indo-1 loaded neutrophils (2×10^6 /ml) and fluorescence changes were recorded with time as described above.

Measurement of Fc interaction with Fc γ RIIIa on human neutrophils

Human neutrophils were incubated with a mIgG1 control, 2B1 bispecific antibody or 3G8 mAb (5 μ g/ml) for 30 min at 4°C. Cells were washed and subsequently incubated with IV.3-FITC (5 μ g/ml) for 30 min at 4°C. After washing of the cells, binding of the fluorescent antibody was measured by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, U.S.A.).

Determination of neutrophil aggregation with FACS analysis

Neutrophil aggregation was measured by double colour FACS analysis, as previously described (Kuypers *et al.*, 1990). In short, neutrophils (5×10^6 /ml in incubation medium without Ca²⁺) were labelled with fluorescein diacetate (FDA) (0.66 μ g/ml) or hydroethidine (HE) (160 μ g/ml) for 30 min at 37°C. Cells were washed and resuspended in incubation medium at 2×10^6 /ml. Equal volumes of FDA-labelled and HE-labelled neutrophils were mixed in a stirred cuvette that was thermostatted at 37°C. After a 5-min preincubation, the stimulus was added. After various times, 50 μ l of sample was taken and immediately fixed in paraformaldehyde [1% (w/v) in incubation medium]. The samples were measured in a FACScan (Becton Dickinson). Double-coloured aggregates were calculated as a percentage of the total coloured events counted.

Statistical analysis

For statistical analysis, paired Student's *t*-tests were performed. *P* values exceeding 0.05 were considered not significant.

RESULTS

Involvement of both Fc γ RIIIa and Fc γ RIIIB in 3G8 stimulation

We examined the mechanism by which intact anti-Fc γ RIII mAb 3G8 induces neutrophil

Fig. 1. Increase in the intracellular Ca^{2+} concentration by intact 3G8 mAb. Indo-1-loaded neutrophils ($2 \times 10^6/\text{ml}$) were activated by intact 3G8 mAb ($5 \mu\text{g}/\text{ml}$), or activated by intact 3G8 mAb after blockade of $\text{Fc}\gamma\text{RII}$ by IV.3 Fab (anti- $\text{Fc}\gamma\text{RII}$) ($5 \mu\text{g}/\text{ml}$), as indicated by the arrows. Intracellular Ca^{2+} was measured as described in Materials and Methods. This figure is representative for three independent experiments.

activation. Intact 3G8 mAb induced a transient increase in intracellular free Ca^{2+} ions ($[\text{Ca}^{2+}]_i$) in human neutrophils (Fig. 1 and Table 1), whereas F(ab')_2 fragments of this antibody did not (Table 1). The F(ab')_2 fragments of mAb 3G8 did bind to the neutrophils (data not shown). Because the antibody is of the subclass mouse IgG1, binding of the Fc part to $\text{Fc}\gamma\text{RIIa}$ can be expected (Kurlander, 1983). Indeed, preincubation of the cells with Fab fragments of an anti- $\text{Fc}\gamma\text{RII}$ mAb completely blocked the Ca^{2+} response induced by intact 3G8 (Fig. 1 and Table 1), but not the binding of 3G8 to the cells (data not shown). Thus, the Fc part of this

Fig. 2. Increase in the intracellular Ca^{2+} concentration by bispecific antibody 2B1. Indo-1-loaded neutrophils ($2 \times 10^6/\text{ml}$) were activated by bispecific antibody 2B1 ($5 \mu\text{g}/\text{ml}$) followed by cross-linking with GAM F(ab')_2 ($50 \mu\text{g}/\text{ml}$), as indicated by the arrows. Intracellular Ca^{2+} was measured as described in Materials and Methods. This figure is representative for three independent experiments.

antibody is involved in this activation. In addition, preincubation of the cells with Fab fragments of another anti- $\text{Fc}\gamma\text{RIII}$ mAb (CLBFcgran1), an antibody that is able to interfere with the binding of 3G8 mAb (Fleit *et al.*, 1989), completely abolished the 3G8 mAb-induced Ca^{2+} response (Table 1).

Minimal requirements of 3G8 stimulation

We next compared the effect of mAb 3G8 with that of the bispecific antibody 2B1. This antibody is derived from the 3G8 clone directed against $\text{Fc}\gamma\text{RIII}$, and the 520C9 clone directed against the *c-erbB2* gene product. Interestingly, the 2B1 antibody was not able to induce a Ca^{2+} response (Fig. 2). This bispecific antibody induced an increase in cytosolic Ca^{2+} concentration only upon cross-linking with GAM F(ab')_2 (Fig. 2). GAM F(ab')_2 alone did not induce a Ca^{2+} response (data not shown).

Both parental antibodies of 2B1 are of the mIgG1 subclass, so the 2B1 is also a mIgG1 mAb. Nevertheless, we checked whether the 2B1 bispecific antibody is able to bind to $\text{Fc}\gamma\text{RIIa}$ via its Fc domain. 2B1 bispecific antibody competed efficiently with binding of FITC-labelled anti- $\text{Fc}\gamma\text{RII}$ mAb to neutrophils, suggesting that the 2B1 bispecific antibody is indeed able to bind to $\text{Fc}\gamma\text{RIIa}$ via its Fc domain (Table 2).

Another way of engaging $\text{Fc}\gamma\text{RIIa}$ and $\text{Fc}\gamma\text{RIIIb}$ was achieved by the use of a bispecific F(ab')_2 directed against $\text{Fc}\gamma\text{RII}$ and $\text{Fc}\gamma\text{RIII}$. This bispecific F(ab')_2 was unable to generate a Ca^{2+} response (data not shown), supporting the idea that two $\text{Fc}\gamma\text{RIII}$ binding sites in addition to Fc binding to $\text{Fc}\gamma\text{RIIa}$ are required to elicit a Ca^{2+} response in human neutrophils. As we have shown before, this bispecific F(ab')_2

Table 1. Increase in intracellular Ca^{2+} concentration by intact 3G8 mAb

Treatment	$[\text{Ca}^{2+}]_i$ increase (nM)
3G8	212 ± 27 ($n = 9$)
IV.3 Fab + 3G8	0 ± 0 ($n = 3$)
CLBFcgran1 Fab + 3G8	0 ± 0 ($n = 3$)
IV.3 Fab	0 ± 0 ($n = 5$)
3G8 F(ab')_2	0 ± 0 ($n = 8$)

Indo-1-loaded neutrophils ($2 \times 10^6/\text{ml}$) were treated with 3G8 mAb ($5 \mu\text{g}/\text{ml}$) with or without pretreatment with Fab fragments of IV.3 mAb (anti- $\text{Fc}\gamma\text{RII}$) ($5 \mu\text{g}/\text{ml}$) or CLBFcgran1 Fab (anti- $\text{Fc}\gamma\text{RIII}$) ($5 \mu\text{g}/\text{ml}$) for 5 min at 37°C . Cells were also incubated with 3G8 F(ab')_2 or IV.3 Fab (all $5 \mu\text{g}/\text{ml}$). Intracellular Ca^{2+} was measured as described in Materials and Methods. Peak increases above resting values of the initial response under various conditions were calculated. Values are given as the mean $\pm$ SEM. The intracellular Ca^{2+} concentration in resting cells was 58 ± 7 nM ($n = 9$).

Table 2. Binding of Fc parts of antibodies to Fc γ RIIa

	IV.3-FITC binding
mIgG1	58 $\pm$ 1
2B1	24 $\pm$ 3*
3G8	22 $\pm$ 5*

Neutrophils were incubated with the isotype-matched control mIgG1, 2B1 bispecific antibody or 3G8 mAb for 30 min at 4°C, were washed and were incubated with FITC-labelled anti-Fc γ RII mAb (IV.3). After washing, cell-bound fluorescence was measured by FACS analysis. Values are given in mean fluorescence intensity (mean $\pm$ SEM from three independent experiments).

*Significantly different from mIgG1 control ($P < 0.05$).

induces an increase in cytosolic Ca²⁺ concentration only upon cross-linking (Vossebeld *et al.*, 1995).

Induction of Ca²⁺ influx

We have shown previously that heterotypic Fc γ R cross-linking induces Ca²⁺ influx from the external environment in addition to Ca²⁺ mobilization from stores. This is different from separate Fc γ RIIa and separate Fc γ RIIIb cross-linking, in which only Ca²⁺ mobilization from stores is induced (Vossebeld *et al.*, 1995). The intact mAb 3G8 also induced an influx of Ca²⁺ from the extracellular medium, indicating that the increase in intracellular Ca²⁺ concentration is partly caused by Ca²⁺ influx. This influx can be blocked by pretreatment of the cells with anti-Fc γ RII Fab fragments (Fig. 3).

Engagement of three Fc γ R on the same cell

We next investigated whether cross-linking of Fc γ RIIa or Fc γ RIIIb by the 3G8 mAb on the same cell was necessary for activation. For this purpose, experiments were performed in which a population of indo-1-loaded cells was mixed with a population of unloaded cells, as shown in Fig. 4. Preincubation of the indo-1-loaded cells with anti-Fc γ RIII Fab (CLB Fcgran1 Fab) and the unloaded cells with anti-Fc γ RII Fab (IV.3 Fab), a situation in which only Fc γ RIIa cross-linking on the indo-1-loaded cells takes place [see Fig. 4(B)], completely blocked the 3G8-induced Ca²⁺ response. Preincubation of the indo-1-loaded cells with Fab fragments of anti-Fc γ RII Fab (IV.3 Fab) and unloaded cells with Fab fragments of anti-Fc γ RIII Fab (CLB Fcgran1 Fab), a situation in which only Fc γ RIIIb cross-linking can occur on the indo-1-loaded cells [see Fig. 4(C)], also almost completely blocked the 3G8-induced neutrophil activation. The residual response was partly caused by the inability of anti-Fc γ RII Fab (IV.3

Fab) pretreatment, which necessarily includes a washing step, to block Fc γ RIIa completely (a response of 12 $\pm$ 8 nM is left over). These experiments show that both Fc γ RIIa and Fc γ RIIIb have to be cross-linked on the same cell to elicit a Ca²⁺ response.

To establish whether 3G8 mAb was able to activate the cell by forming a trimeric complex of one Fc γ RIIa and two Fc γ RIIIb molecules or whether neutrophils having bound 3G8 could cross-link Fc γ R on other neutrophils [as in Fig. 4(B) and (C)], we investigated the dependence of the cell concentration for this activation. Lowering the concentration of cells did not diminish the Ca²⁺ response by intact 3G8 mAb (data not shown). The same is true for another stimulating agent fMLP. Taken together, these results strongly suggest that mAb 3G8 bound to one neutrophil is able to induce neutrophil activation.

Induction of functional responses

To investigate whether this way of Fc γ R cross-linking also induces functional responses, we measured homotypic aggregation of these cells. Indeed, we found that intact 3G8 mAb induced aggregation of neutrophils (Fig. 5). This cell aggregation is an activation-dependent process, because it did not occur at 4°C (data not shown). More specifically, this aggregation depended on activation of the β_2 integrin, CR3

Fig. 3. Effect of intact 3G8 mAb on Ca²⁺ influx. Indo-1-loaded neutrophils were pretreated (A) with or (B) without IV.3 Fab (5 μ g/ml) and were activated by intact 3G8 mAb (5 μ g/ml). Ca²⁺ influx was measured by Mn²⁺-dependent quenching of indo-1 fluorescence, as described in Materials and Methods. At time zero, 0.5 mM MnCl₂ was added, and 2 min later 3G8 was added (first arrow). To quench indo-1 fluorescence completely with Mn²⁺, digitonin (10 μ M) was added (second arrow). This figure is representative of three independent experiments.

Fig. 4. Effect of separate FcγR cross-linking by intact 3G8 mAb on the Ca^{2+} response. Heterotypic FcγR cross-linking was performed by mixing 10^6 indo-1-loaded neutrophils with 10^6 unloaded neutrophils without preincubation of the neutrophils. (A) The control response in which both FcγRIIa and FcγRIIIb can be cross-linked. (B) FcγRIIa cross-linking on indo-1-loaded cells was performed by preincubation of indo-1-loaded cells with CLBFCgran1 Fab and preincubation of unloaded cells with IV.3 Fab. (C) FcγRIIIb cross-linking on indo-1-loaded cells was performed by preincubation of indo-1-loaded cells with IV.3 Fab (anti-FcγRII Fab) and preincubation of unloaded cells with CLBFCgran1 Fab (anti FcγRIII Fab). After washing, the cells were treated with intact 3G8 (5 μ g/ml). Intracellular Ca^{2+} was measured as described in Materials and Methods. Values are given as the mean $\pm$ SEM of five independent experiments.

(CD11b/CD18), because both an antibody directed against CD18 (MHM23) and an antibody directed against CD11b (44A) were able to block this response in contrast to an isotype-matched control antibody [Fig. 5(A)]. In accordance with the inhibition of the Ca^{2+} response, pretreatment with IV.3 Fab inhibited the 3G8-induced aggregation [Fig. 5(B)]. The 2B1 bispecific antibody did induce some aggregation, but much less than the parental antibody 3G8 [Fig. 5(B)].

DISCUSSION

The monoclonal antibody 3G8, directed against FcγRIII, induces a transient elevation of $[Ca^{2+}]_i$ in human neutrophils. This activation requires heterotypic clustering of both FcγRIIa and FcγRIIIb by binding of the Fc region of 3G8 to FcγRIIa and binding of the Fab regions to FcγRIIIb. The interaction between FcγRIIa and FcγRIIIb induces a more efficient Ca^{2+}

mobilization than that observed after cross-linking of these receptors separately, as we have described before (Vossebeld *et al.*, 1995). In addition, we also found that 3G8 induced Ca^{2+} influx from the extracellular medium, which is in accordance with our previous findings that heterotypic FcγR cross-linking of both FcγRIIa and FcγRIIIb induces Ca^{2+} influx, in contrast to separate FcγRIIa or separate FcγRIIIb cross-linking. The stimulation of neutrophils by 3G8 represents a special case of synergistic activation by the cooperative action of FcγRIIa and FcγRIIIb. Such a cooperative action between FcγRIIa and FcγRIIIb has been suggested to be important for activation of PMN by immune complexes from synovial fluid from patients with rheumatoid arthritis (Robinson *et al.*, 1994).

The bispecific antibody 2B1, only differing in one Fab fragment from 3G8, did not induce a Ca^{2+} response in neutrophils. This bispecific antibody did bind to FcγRIIa via its Fc domain

(Table 2). The affinity of 2B1 for Fc γ RIII does not differ substantially from that of 3G8, as shown by Scatchard analysis of 2B1 binding to human peripheral blood lymphocytes (Weiner *et al.*, 1993).

Additionally, we have shown that engagement of both Fc γ RIIa and Fc γ RIIIb on the same cell was necessary to perform neutrophil activation via intact 3G8 mAb. Our mixing

experiments, in which only Fc γ RIIa cross-linking or only Fc γ RIIIb cross-linking by 3G8 mAb takes place, suggest that both Fc γ RIIa and Fc γ RIIIb have to be cross-linked on the same cell to induce a Ca²⁺ response in neutrophils.

The 3G8-induced Ca²⁺ response is not dependent on the concentration of neutrophils, which indicates that Fc γ R cross-linking by another neutrophil opsonized with 3G8 is probably not required. This means that 3G8 mAb activates neutrophils by cross-linking one Fc γ RIIa molecule with two Fc γ RIIIb molecules into a trimeric receptor complex. The inability of either bispecific antibody 2B1, bispecific F(ab')₂ directed against Fc γ RII and Fc γ RIII, or 3G8 F(ab')₂ to induce a Ca²⁺ response suggests that this trimeric complex is the minimal requirement to elicit neutrophil activation. In theory, a trimeric complex formed from two Fc γ RIIa molecules together with one Fc γ RIIIb molecule might also be capable of inducing neutrophil activation. However, treatment of human neutrophils with mAb IV.3 does not result in a measurable neutrophil activation (data not shown). The finding that cross-linking of two Fc γ RIIIb molecules together with one Fc γ RIIa molecule generates a receptor complex, that is able to start the intracellular processes leading to the induction of Fc γ R-mediated neutrophil activation, provides more insight into the mechanism of receptor cross-linking. We do not know the mechanism by which such a trimeric receptor complex, consisting of two Fc γ RIIIb and one Fc γ RIIa, induces neutrophil activation. Because both Fc γ RIIa- and Fc γ RIIIb-induced signalling involve protein tyrosine phosphorylation (Connelly *et al.*, 1991), a transphosphorylation event might play a role.

Some variation is found in the response to intact 3G8 mAb between different donors, probably caused by a difference in phenotype of Fc γ RIIa (Edberg *et al.*, 1995). There are two different forms of Fc γ RIIa, the high-responder and the low-responder form, which differ in their ability to bind mIgG1 (Tax *et al.*, 1983). However, this variation could also be due to the NA1 and NA2 phenotype of Fc γ RIIIb, which have been suggested to differ in their ability to induce synergism between Fc γ RIIa and Fc γ RIIIb (Salmon *et al.*, 1995).

In addition, we showed that intact mAb 3G8 also induces functional responses, such as neutrophil aggregation, as recently shown by others (Stöckl *et al.*, 1995). Binding of the intact

Fig. 5. Effect of intact 3G8 mAb on neutrophil aggregation. (A) FDA- and HE-labelled neutrophils were mixed and were preincubated (▲) without antibody, (○) with mIgG1, (■) with 44A or (△) with MHM23 (all 5 μ g/ml). At time zero, 3G8 (5 μ g/ml) was added. (B) Cells were preincubated (▲) without antibody or (■) with IV.3 Fab fragments (5 μ g/ml) at 37 °C for 5 min. At time zero, 3G8 (5 μ g/ml) was added and samples were taken at the times indicated. Cells preincubated without antibody were also treated (□) with 2B1 bispecific antibody (5 μ g/ml). As control (●), no antibodies were added. Results shown are the mean of percentage of double-coloured events $\pm$ SEM of three independent experiments.

mAb 3G8 also induced a respiratory burst activity, which amounts to about 30% of an fMLP response (Reumaux *et al.*, 1995). Like the Ca^{2+} response, the homotypic aggregation requires Fc binding to $\text{Fc}\gamma\text{RIIa}$, because the 3G8-induced aggregation was blocked by anti- $\text{Fc}\gamma\text{RII}$ Fab. In accordance with the inability of the bispecific antibody 2B1 to induce a Ca^{2+} response, the bispecific antibody 2B1 was hardly able to induce neutrophil aggregation. This 3G8-induced aggregation is an activation-dependent process that is dependent on CR3 interactions. Probably, $\text{Fc}\gamma\text{R}$ cross-linking causes an increase in the avidity of CR3 for its ligand (Buyon *et al.*, 1988), which is then able to bind to yet unknown counter structures (Kuypers *et al.*, 1990).

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