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Signal transduction via FcγR and Mac-1 α-chain in monocytes and polymorphonuclear leucocytes

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SUMMARY

Some (VIM12, Leu-15, 5A4.C5), but not all, Mac-1-specific monoclonal antibodies (mAb) induced a clear respiratory burst in unprimed monocytes but not in unprimed polymorphonuclear leucocytes (PMN). We showed that this monocyte stimulation occurred via formation of Mac-1 mAb–FcγRI or Mac-1 mAb–FcγRII complexes, as human monomeric IgG1 could completely block the respiratory burst induced by the murine IgG2a subclass anti-Mac-1 mAb Leu-15 and the FcγRII-specific mAb IV.3 inhibited respiratory burst formation by IgG1 subclass anti-Mac-1 mAb VIM12 and 5A4.C5, respectively. $F(ab')_2$ fragments of mAb VIM12 did not stimulate. This association between Mac-1 and FcγRII may be due to a near spatial association between these molecules in monocytes, as we observed partial inhibition of FITC-labelled anti-FcγRII mAb IV.3 binding after prior incubation with mAb VIM12. If monocytes were preincubated with mAb IV.3 or aggregated IgG, there was partial inhibition of mAb VIM12 binding. The non-stimulating anti-Mac-1 mAb (JML.H11,44, OKM1, LM2/1, Mo1) did not show any significant competition with mAb IV.3 binding to FcγRII. Both non-stimulating CD18-specific mAb, however, showed strong competition with mAb IV.3 binding to FcγRII. On unprimed PMN, the situation was different. No Mac-1-specific mAb induced a respiratory burst and there was no competitive inhibition between anti-Mac-1 mAb and antibodies binding to FcγRII. In interferon-γ (IFN-γ)-primed PMN, however, we observed a functional association between Mac-1 and FcγRI as IgG2a subclass mAb Leu-15 induced a respiratory burst which could be inhibited by monomeric human IgG1, as observed in monocytes. However, no other anti-Mac-1 mAb was able to induce a respiratory burst in IFN-γ-primed PMN. Therefore, a similar signal transducing capability may exist between Mac-1 and FcγRI on both monocytes and PMN, despite a different relationship between Mac-1 and FcγRII on these cell populations. As no Mac-1 β-chain-specific (CD18) mAb were able to induce a respiratory burst in monocytes, despite being able to interact with FcγR via their Fc regions, as detected by competition with mAb IV.3 for binding to FcγRII, we conclude that intracellular signalling via Mac-1 mAb–FcγRII complexes requires the α-chain.

INTRODUCTION

Mac-1 (CD11b/CD18, complement receptor 3; CR3) is a glycoprotein member of the leucocyte integrin family consisting of a two-chain αβ heterodimer of 170,000–95,000 MW expressed on polymorphonuclear leucocytes (PMN), monocytes and natural killer (NK) cells. A number of different ligands has already been identified for Mac-1. Among them are intracellular adhesion molecule-1 (ICAM-1),[1] iC3b,[2] Factor X,[3] fibrinogen[4] and a lectin-like ligand binding site.[5] The binding sites for these ligands, except for the lectin-like binding site, have been mapped to the I region of the α-chain.[6] Monoclonal antibodies (mAb) are available which recognize these or part of these determinants and can block the binding of most of these natural ligands.[6] The functional role of Mac-1 also includes mediating homotypic[7] and heterotypic cell adhesion, such as by PMN to activated endothelial cells.[8] Lack of β_2 integrin expression, as in leucocyte adhesion deficiency syndrome,[9] results in impaired phagocyte migration and pus formation, resulting in recurrent bacterial infections.

We have established that the anti-Mac-1 antibody VIM12, which specifically recognizes the α-chain of the Mac-1 molecule,[6] binds to the C-terminal region, does not inhibit the binding of any of the natural ligands tested[6] and also does not inhibit formyl-methionyl-leucyl-phenylalanine (FMLP)-induced PMN aggregate formation, unlike other CD11b mAb.[6]

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Abbreviations: CD, cluster of differentiation; CR3, complement receptor 3; CyB, cytochalasin B; DCFDA, 2′7′ dichlorofluorescein diacetate; FcγR, immunoglobulin G Fc receptor; FITC, fluorescein isothiocyanate; FMLP, formyl-methionyl-leucyl-phenylalanine; IFN-γ, interferon-γ; mAb, monoclonal antibody; MFI, mean fluorescence intensity; PE, phycoerythrin; PMN, polymorphonuclear leucocytes; ROS, reactive oxygen species; SAM, $F(ab')_2$ fragments of sheep antibodies specific for mouse immunoglobulins; SEM, standard error of the mean; SRBC, sheep erythrocytes.

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Functional synergism between Mac-1 and FcγR has been suggested by the observation of the inhibitory effect of anti-Mac-1 mAb on FcγR-mediated phagocytosis by monocytes of IgG-coated sheep red blood cells (SRBC) or IgG aggregates,[10,11] although it is unclear which FcγR are involved. We were thus interested to examine the functional relationship between Mac-1 and immunoglobulin G Fc receptor I (FcγRI) and FcγRII expressed on both monocytes and PMN in terms of signal transduction leading to a respiratory burst.

MATERIALS AND METHODS

Preparation of peripheral blood leucocytes

Monocyte-enriched fractions were prepared from the peripheral blood of healthy volunteers. Isolation of the peripheral blood mononuclear phagocyte cell fraction was by buoyant density gradient centrifugation over Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) and subsequent removal of T cells by rosette formation with SRBC. PMN were collected after addition of 2% dextran in phosphate-buffered saline (PBS) to blood to give a final concentration of 0·5% followed by incubation for 45 min at room temperature, after which PMN were purified from the plasma-rich layer by density gradient centrifugation over Ficoll–Hypaque. Contaminating erythrocytes were removed by flash lysis for 10 seconds in distilled water. When indicated, PMN were incubated for 20 hr in the presence of 10^3 U/ml IFN-γ (Bender and Co., Wien, Austria) in RPMI-1640 supplemented with 10% fetal calf serum, gentamycin and amphotericin B, in a fully humidified atmosphere of 5% CO_2 at 37° to induce PMN to express the high-affinity receptor for human monomeric IgG. Expression of the FcγRI on monocytes or IFN-γ-stimulated PMN was detected by immunofluorescence staining of cells with the FcγRI-specific mAb 32.2 with flow cytometric analysis.

Monoclonal antibodies

Monoclonal antibodies used in this study from our laboratory were VIT8b (CD8; IgG1), VIT6 (CD1a; IgG2a), VIT-200 (CD45; IgG2a), VID1 (HLAD; IgG1) and VIM12 (Mac-1; IgG1). Antibodies used from other sources were as follows: Leu-15 (Mac-1; IgG2a; Becton Dickinson, Mountain View, CA), JMLH11 (Mac-1; IgG1; M. M. Yokoyama, Fukuoka, Japan), mAb 44 (Mac-1; IgG1; N. Hogg, London, U.K.), LM2/1 (Mac-1; IgG: ATCC, Rockville, MD), OKM1 (Mac-1; IgG2b; Ortho Pharmaceuticals, Raritan, NJ), Mol (Mac-1; IgM; R. F. Todd, Ann Arbor, MI), 5A4.C5 (Mac-1; IgG1; L. K. Ashman, Adelaide, Australia), BMA 0310 (CD11c; IgG1; Behringwerke, Germany), M 232 (CD18; IgG1; M. Bernard, Villejul, France), MHM 23 (CD18; IgG1; A. McMichael, Oxford, U.K.), IV.3 (FcγRII; IgG2b; Medarex Inc., Lebanon, NH), 32.2 (FcγRI; IgG1; Medarex Inc. and MEM-15 (CD14; IgG1; V. Hořejši, Prague, Czech Republic).

Preparation of VIM12 F(ab′)$_2$ fragments

Preparation of F(ab′)$_2$ fragments of VIM12 was by using immobilized pepsin and the recommended protocol from Pierce (Pierce Europe, Oud-Beijerland, The Netherlands).

Assessment of respiratory burst

To assess mAb-induced generation of intracellular production of reactive oxygen species, we used a flow cytometric method first described by Bass *et al.*[12] and used subsequently in our laboratory[13] for PMN and further modified for application to monocytes. Briefly, monocytes and PMN (10^6) were incubated for 30 min on ice with purified mAb in 100 μl of PBS, pH 7·2 (without sodium azide), to give a final concentration of 10 μg/ml, followed by washing twice with PBS. Cells were incubated in 100 μl of Earle's balanced salt solution (Flow Laboratories Ltd, Irvine, U.K.) containing 10 μg/ml cytochalasin B (CyB; Sigma, Diesenhofen, Germany) with 2′7′ dichlorofluorescein diacetate dye (DCFDA; Eastman Kodak, Rochester, NY) (10 μg/ml) with a further incubation for 10 min on ice. After addition of 50 μl of Earle's buffer containing CyB (10 μg/ml), the cells were further incubated for 45 min at 37°. In some experiments, FMLP (10^{-6} M) was included in Earle's buffer instead of purified mAb. In other experiments, monomeric human IgG1 (0·01%) was included at this stage to blockade binding of murine IgG2a subclass mAb to FcγRI. Human IgG1 was centrifuged at 100,000 *g* for 30 min to remove aggregates. Finally, 200 μl of PBS containing 0·5 mM EDTA was added. All experiments were analysed within 30 min of the end of the experiment by a FACS 440 or a FACScan (Becton Dickinson, Mountain View, CA) where non-monocytic or non-PMN cells were excluded from analysis according to morphological criteria. Fluorescence intensity of DCFDA-labelled cells was recorded on a linear scale. Reactive oxygen species (ROS) production by monocytes and PMN was expressed as the percentage increase of mean fluorescence intensity (MFI) compared to the negative control mAb, according to the formula below:

$$\text{percentage increase} = \frac{\text{MFI}_{\text{mAb}} - \text{MFI}_{\text{co-mAb}}}{\text{MFI}_{\text{co-mAb}}} \times 100.$$

A greater than 100% increase in MFI was inferred as positive.

IgG Fc receptor and Mac-1 blocking studies

Purified monocytes or PMN (10^6) were incubated for 30 min on ice with an isotype control mAb or mAb VIM12 to give a final concentration of 10 μg/ml in 100 μl, followed by washing twice. Afterwards, the cells were further incubated at 37° for 40 min in the presence or absence of CyB (10 μg/ml) and then stained with FITC-conjugated F(ab′)$_2$ fragments of sheep antibodies specific for mouse immunoglobulins (SAM; An der Grub, Vienna, Austria) as a negative control, FITC-labelled FcγRII-specific mAb IV.3 (10 μg/ml final concentration) or phycoerythrin (PE)-conjugated anti-Mac-1 mAb Leu-15 (final dilution 1:20). Alternatively, monocytes or PMN were preincubated with unlabelled mAb IV.3 or heat-aggregated human IgG and then with FITC-labelled anti-Mac-1 or CD18 mAb. After washing three times, cells were then analysed by FACScan with linear amplification.

RESULTS

Mac-1-specific mAb induce intracellular ROS formation in monocytes but not in PMN

Incubation of freshly prepared, unstimulated monocytes with Mac-1-specific mAb VIM-12 (IgG1), 5A4.C5 and mAb Leu-15 (IgG2a) compared to isotype control non-binding mAb induced a respiratory burst with intracellular ROS production in about 60% of individuals tested ($n = 13/22$), which corresponds to the population with a FcγRII polymorphism able to bind mouse IgG1 or IgG2b;[14] whereas incubation with mAb Leu-15 stimulated monocytes from all individuals. All other Mac-1-specific

Table 1. Antibody-induced reactive oxygen species production in unstimulated monocytes and PMN

Monoclonal antibody	Percentage increase in MFI (+SEM)	
	Monocytes	PMN
VIM12	426±219	9±7
Leu-15	346±86	2±6
JML.H11	11±9	10±44
44	32±9	9±45
OKM1	18±23	1±15
LM2/1	31±37	2±13
Mo1	8±11	24±10
5A4.C5	339±115	15±15
MHM 23	14·3±34·1	24±8
M 232	14±12	16±13
VIT-200	3·3±4·6	ND
BMA 0310	10·4±10·5	ND
VID1	160·7±33·6	ND

Monocytes and PMN were incubated with the mAb listed above and their intracellular ROS production detected by oxidation of DCFDA, which was measured by flow cytometer (FACScan; Becton Dickinson). Data show mean values of experiments made with 22 different donors and express the percentage increase in MFI±SEM, indicating ROS production compared to a non-binding isotype control mAb.

mAb did not induce a respiratory burst (Table 1). Results of a typical experiment are shown (Fig. 1b,c). Addition of CyB during incubations led to enhanced production of ROS, indicating that an intact cytoskeleton is not necessary for signal transduction and that the non-cytoskeleton-associated subpopulation of Mac-1 molecules may be important for signalling via FcγR. CyB was thus included in all subsequent experiments. Both IgG1 subclass CD18 mAb were unable to stimulate a respiratory burst (Table 1). Other cell-binding mAb of the same isotypes were also unable to stimulate a respiratory burst (Table 1 and Fig. 1).

Freshly isolated PMN which express cell-surface Mac-1 molecules were also analysed for intracellular ROS production after addition of anti-Mac-1-specific mAb. We found no comparable stimulation of freshly isolated PMN after incubation with any mAb. Results from a typical experiment are shown (Fig. 1e, f). As cross-linking of FcγRII on PMN by mAb IV.3 and subsequently with SAM could stimulate intracellular ROS production in both monocytes and PMN (data not shown), and incubation with FMLP (10^{-6} M) (Fig. 1d) could also stimulate PMN, we consider that under our experimental conditions PMN are able to produce ROS. Therefore, we conclude that binding to the anti-Mac-1 mAb VIM12, 5A4.C5 and Leu-15 to monocytes but not unprimed PMN can transduce a signal leading to respiratory burst with intracellular ROS production.

Analysis of anti-Mac-1 mAb–FcγR complex formation

Our observation of respiratory burst induction by the IgG1 subclass mAb VIM12 and 5A4.C5 and the IgG2a subclass mAb Leu-15 indicated that formation of Mac-1 mAb–FcγR complexes able to transduce a signal may be occurring in monocytes but not PMN. To investigate this, we performed a series of

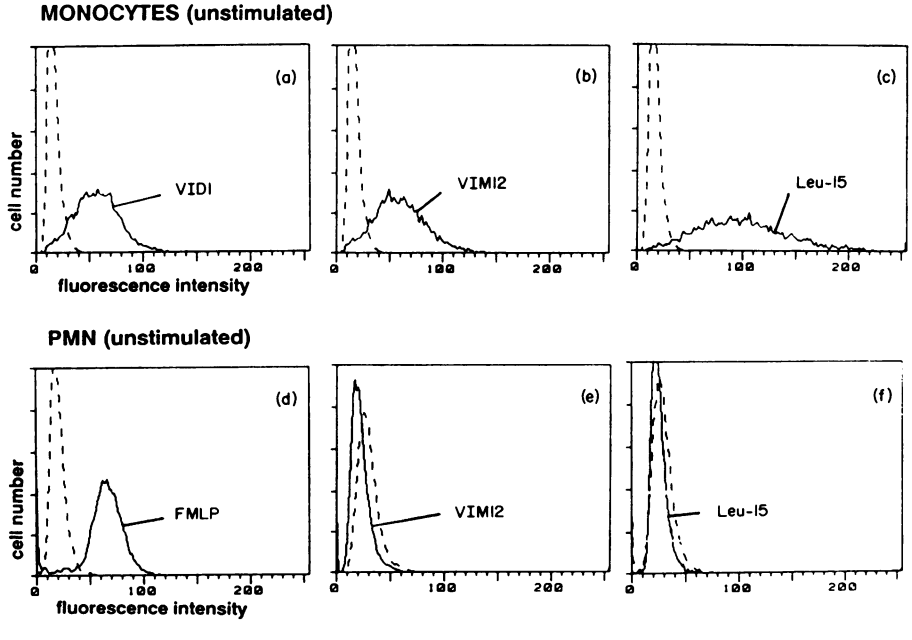

Figure 1. ROS production after incubation of unstimulated monocytes with mAb (10 μg/ml) (a) VID1, (b) VIM12 and (c) Leu-15 and unstimulated PMN with (d) 10^{-6} M FMLP, (e) VIM12 and (f) Leu-15 compared to negative control cell binding mAb (- - -) MHM 23 (a,b,d,e) and VIT-200 (c,f). The production of intracellular ROS was measured by oxidation of intracellular DCFDA and measured by flow cytometry (FACScan; Becton Dickinson) using linear amplification.

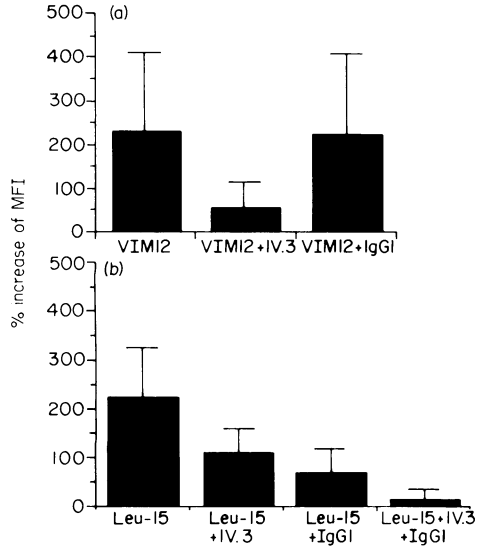

Figure 2. Inhibition of intracellular ROS production by unstimulated monocytes after incubation with mAb (10 μg/ml) (a) VIM12 only, with prior incubation with anti-Fcγ RII mAb IV.3 followed by mAb VIM12, or after incubation with mAb VIM12 and subsequently monomeric human IgG1 (0·01%), and after incubation for 30 min with (b) mAb Leu-15 only, with prior incubation with mAb IV.3 (10 μg/ml), after incubation with Leu-15 and subsequently monomeric human IgG1 (0·01%) and after prior incubation with mAb IV.3 then mAb VIM12 and finally with monomeric human IgG1 (0·01%). The production of ROS was detected by oxidation of intracellular DCFDA and measured by flow cytometry (FACScan) with linear amplification.

experiments in which we blockaded the IgG Fc portion binding site of FcγRII with mAb IV.3, and FcγRI with monomeric human IgG1 as previously reported,[15] before adding anti-Mac-1 mAb VIM12 AND Leu-15, respectively. Prior incubation of monocytes with mAb IV.3 almost totally blocked the stimulation of intracellular ROS production in monocytes by mAb VIM12 in responders, but incubation with monomeric human IgG1 (up to 1 mg/ml) had no effect (Fig. 2a). This indicates that formation of a Mac-1 VIM12–FcγRII complex is necessary to induce intracellular ROS production by mAb VIM12-bound monocytes. Stimulation of monocytes by mAb Leu-15 could be

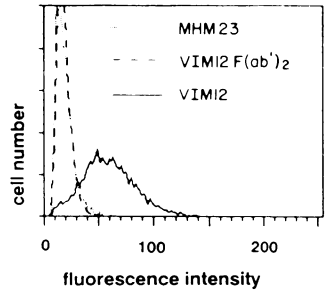

Figure 3. Respiratory burst induction by unstimulated monocytes after incubation with intact mAb VIM12 (10 μg/ml), $F(ab')_2$ fragments of mAb VIM12 (20 μg/ml) and negative control cell binding mAb MHM 23 (10 μg/ml). The production of ROS detected by oxidation of intracellular DCFDA was measured by flow cytometry (FACScan; Beckton Dickinson) with linear amplification.

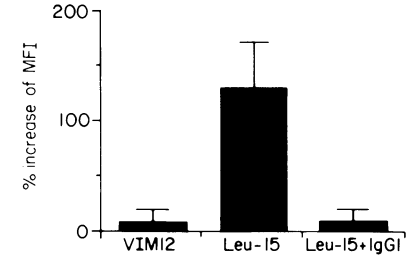

Figure 4. Inhibition of respiratory burst induction by PMN after stimulation with 10^3 U/ml IFN-γ for 20 hr and subsequent incubation with mAb VIM12 (10 μg/ml), Leu-15 (10 μg/ml) or Leu-15 (10 μg/ml) followed by further incubation with monomeric human IgG1 (0·01%). The production of ROS was detected by oxidation of intracellular DCFDA, as measured by flow cytometry (FACScan; Becton Dickinson) using linear amplification.

partially blockaded by prior incubation with either monomeric human IgG1 or mAb IV.3 and totally with both (Fig. 2b), indicating that both Mac-1–Leu-15-FcγRI and Mac-1–Leu-15–FcγRII complexes can induce intracellular ROS production in monocytes.

$F(ab')_2$ fragments of mAb VIM12 did not stimulate intracellular ROS production by monocytes, further indicating a requirement for the antibody Fc region for formation of a Mac-1–mAb–FcγR complex able to stimulate ROS production (Fig. 3).

Stimulation of ROS production by mAb Leu-15 in IFN-γ-treated PMN

Freshly isolated PMN, despite their expression of FcγRII and Mac-1, could not be stimulated with intact anti-Mac-1 antibodies (Fig. 1e, f). As IFN-γ-stimulated PMN express the high-affinity IgG Fc receptor[16] (FcγRI) able to find the Fc region of mouse IgG2a,[17] we were interested to determine whether PMN expressing FcγRI could be stimulated by the IgG2a mAb Leu-15 to produce intracellular ROS. We thus stimulated PMN

Table 2. Binding of anti-FcγRII mAb IV.3 on monocytes following preincubation with anti-Mac-1 mAb

Monoclonal antibody	Without CyB	With CyB
VIM12	44 ± 11*	57 ± 5
5AF.C5	46 ± 5	44 ± 6
Leu-15	101 ± 7	60 ± 5
JML.H11	96 ± 5	111 ± 13
44	57 ± 8	72 ± 1
OKM1	90 ± 1	100 ± 7
LM2/1	65 ± 15	63 ± 13
Mo1	104 ± 8	105 ± 7
MHM23	31 ± 4	35 ± 2
M232	30 ± 9	29 ± 15

Results show percentage binding of FITC-labelled anti-FcγRII mAb IV.3 alone compared to prior incubation with anti-Mac-1 or CD18 mAb or to a non-binding isotype control mAb in the presence or absence of CyB (10 μg ml).

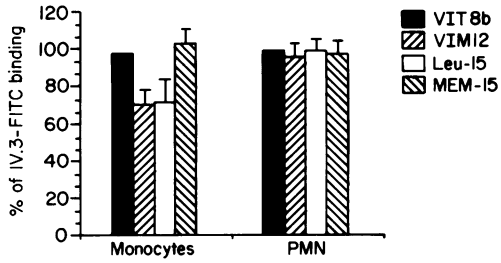

Figure 5. Inhibition of binding of FITC-conjugated mAb IV.3 (10 μg/ml) on unstimulated monocytes and PMN after prior incubation with mAb VIM12 (10 μg/ml) or Leu-15 (10 μg/ml) as well as the negative control non-cell binding and cell binding mAb VIT8b (10 μg/ml) and MEM-15 (10 μg/ml), respectively. Fluorescence staining of cells was measured by flow cytometry (FACScan; Becton Dickinson).

overnight with 10^3 U/ml IFN-γ, which resulted in *de novo* expression of FcγRI detected by binding mAb 32.2 (data not shown). These stimulated PMN, when incubated with Leu-15, but not other murine IgG2a isotype mAb such as VIT-200 (data not shown) or with the other anti-Mac-1 mAb, showed a clear production of intracellular ROS (Fig. 4). This ROS production by PMN could be blocked by prior incubation with monomeric human IgG1 but not with mAb IV.3 (Fig. 4), confirming that formation of a Mac-1–Leu-15–FcγRI complex but not a Mac-1–Leu-15–FcγRII complex could transduse a signal to induce ROS production by PMN. As the other anti-Mac-1 mAb could not stimulate IFN-γ-primed PMN, this indicates that there is no functional change in the relationship between Mac-1 and FcγRII in these PMN.

Association between Mac-1 molecules and FcγR

We were interested to study whether the close functional association between Mac-1 molecules and FcγRI and FcγRII on monocytes and between Mac-1 and FcγRI on PMN is also reflected in a nearby spatial association, as previously suggested in studies showing iC3b-coated particles inhibiting binding of IgG to FcγR on monocytes.[11] To examine if such an association exists between Mac-1 molecules and FcγRI or FcγRII on monocytes or PMN, we incubated cells with mAb VIM12 or VIT8b (isotype negative control) followed by washing, and then a further incubation in the presence or absence of CyB at 37°, followed by staining with FITC-conjugated SAM, with FITC-labelled mAb IV.3, or with PE-conjugated Leu-15. In other experiments, cells were firstly incubated with mAb IV.3 or aggregated human IgG, followed by FITC-conjugated VIM12. In four of six individuals, we observed that incubation of the VIM12-labelled monocytes resulted in an approximately 30–50% decrease in FITC-labelled mAb IV.3 binding, compared to monocytes incubated firstly with non-binding negative control mAb VIT8b only (Table 2 and Fig. 5a). Both CD18 mAb, however, showed a greater inhibition of mAb IV.3 binding than did mAb VIM12 (Table 2). No comparable decrease was observed in PMN (Fig. 5b) preincubated with mAb VIM12. Binding of PE-labelled Leu-15 and staining with FITC-conjugated SAM was diminished to an equal extent. Inclusion of CyB (10 μg/ml) during incubation with mAb had no effect on competitive binding of mAb (Table 2). Preincubation of monocytes with mAb IV.3 or human aggregated IgG resulted in a partial decrease in binding of VIM12 in monocytes only (Table 3). We consider that there is steric competitive inhibition between binding of stimulating IgG1 subclass anti-Mac-1 mAb and antibodies to FcγRII in monocytes but not PMN.

Table 3. Binding of anti-Mac-1 mAb after prior incubation of monocytes or PMN with anti-FcγRII mAb IV.3 or heat-aggregated human IgG

Antibody	IV.3	Aggregate
Monocytes		
VIM12	60·5±8	63·5±8
Leu-15	103±3	94±6
JML.H11	90·5±5	91±13
44	71±24	95±8
LM2/1	94±20	105±8
Mol	84±4	102±14
PMN		
VIM12	101±13	102±14
Leu-15	101±5	118±5
JML.H11	111±24	102±8
44	91±4	100±6
LM2/1	94±4	99±16
Mol	116±14	111±18

Results show percentage binding of anti-Mac-1 mAb alone, compared to prior incubation with mAb IV.3 (10 μg/ml) or heat-aggregated human IgG (0·01%) before incubation with anti-Mac-1 mAb.

DISCUSSION

Recently, much work has been focused on obtaining more insights into the mechanism of complex patterns of effector cell activation via immunoglobulin receptors and adhesion molecules. Some researchers have reported that IFN-γ-stimulated U937 cells can be stimulated to produce superoxide after cross-linking FcγRI[18] and FcγRII[19] with mAb, whereas others report that mAb-induced cross-linkage of other surface molecules, such as CD13, CD14 and MHC class II molecues,[20] CD43 molecules[21] and CD31 molecules,[22] with FcγR results in enhanced superoxide or hydrogen peroxide production by human monocytes. Macintyre *et al.*[19,20] proposed formation of an antigen–mAb–FcγR complex which can transduce a signal resulting in superoxide production by monocytes. Signal transduction leading to intracellular ROS production after cross-linking of surface molecules by mAb binding to PMN has already been reported by us via CD24 molecules.[13] Berton *et al.*[23] report that cross-linkage of LFA-1 and gp150/95, but not Mac-1, by immobilized mAb could stimulate ROS production by neutrophils.

We, however, have observed that unstimulated monocytes are able to produce intracellular ROS after cross-linkage of Mac-1 with IgG1 subclass anti-Mac-1 mAb VIM12 and 5AF.C5 (Table 2, Fig. 1). The variability evident in our results is probably due to the influence of the non-responder population.[14] As $F(ab')_2$ fragments of mAb VIM12 were unable to stimulate ROS production (Fig. 3) and preincubation of monocytes with FcγRII Fc region-specific mAb IV.3 could block mAb VIM12-induced respiratory burst induction (Fig. 2),

we consider that Mac-1 mAb–FcγRII complexes can transduce a signal in unstimulated monocytes. Our results confirm those of Macintyre *et al.*[20] who reported that the anti-Mac-1 mAb 44 was unable to stimulate IFN-γ-treated monocytes. Anti-Mac-1 mAb vary in their effects on cell function.[6,24,25] There is clearly an epitope-dependent stimulation for the anti-Mac-1 mAb which depends upon the Mac-1-bound antibody forming a complex with FcγR (Figs 2 and 3, Tables 2 and 3). Curiously, mAb VIM12 and 5A4.C5 bind to the C-terminal region and I domain, respectively[6] and so are clearly unrelated epitopes, which highlights the importance of interaction of these mAb with the FcγR. We are, however, able to differentiate the role of the α- and β-chains of Mac-1 in signal transuction leading to a respiratory burst in monocytes, as while the β-chain-specific mAb are able to consistently competitively inhibit binding of FcγRII Fc region-specific mAb IV.3 to monocytes and thus are able to form an $\alpha\beta_2$ integrin–mAb–FcγRII complex, they cannot induce a respiratory burst in monocytes. Therefore, signalling via the Mac-1–mAb–FcγR complex leading to intracellular ROS production in monocytes requires the Mac-1 α-chain.

Our result showing lack of respiratory burst induction in PMN by anti-Mac-1 mAb is in agreement with Berton *et al.*[23] However, our results that anti-gp150/95 and CD18 mAb were unable to induce a PMN respiratory burst contrast those of Berton *et al.*[23] This may be due to epitope differences between the mAb used or that our mAb were in soluble form whereas those of Bertoni *et al.*[23] were immobilized allowing more stable cross-linkage. There was no difference between responsiveness of monocytes and PMN if the cells had been prestimulated with IFN-γ and the IgG2a subclass mAb Leu-15 used (Fig. 4). As mAb Leu-15 was able to induce a respiratory burst in IFN-γ-stimulated PMN, which could be only blockaded by monomeric human IgG1, whereas mAb VIM12 interacting with FcγRII alone was not able to stimulate a respiratory burst, it indicates that the FcγRI on IFN-γ-stimulated PMN may not require co-signalling via FcγRII, as has been reported previously for the FcγRIIIB.[26,27] This is consistent with the observation of Akerley *et al.*[17] that mAb 197, able to cross-link FcγRI via both its Fab and Fc regions, stimulated a respiratory burst in IFN-γ-activated PMN. IFN-γ stimulation of PMN did not enable mAb VIM12 or 5A4.C5 to induce ROS production.

Since investigations of Fc receptor-dependent functions blockadable by anti-Mac-1 mAb have generally been undertaken by using rosette formation with rabbit IgG-coated SRBC,[10,28,29] identification of which FcγR is involved in Mac-1-dependent functions was unclear. Studies of Zhou *et al.*[30] indicated that CR3 can be co-capped with FcγRIIIB on PMN, indicating a close association between these structures; however, no such study has been made for CR3 and FcγRI or FcγRII. From our experiments we find that both FcγRI and FcγRII are involved functionally with Mac-1 in monocytes. Our observation of a different functional association between Mac-1 and FcγRII on monocytes and PMN (Fig. 5), due to a near spatial association, is consistent with the observation of Graham *et al.*,[29] who found that while mAb against Mac-1 could inhibit phagocytosis of IgG-sensitized erythrocytes by both monocytes and PMN, they could only inhibit binding of these erythrocytes to monocytes and not to PMN, thus showing a dissociated phagocyte–target interaction and phagocytic capability involving Mac-1 and FcγR in PMN but not monocytes. In this study, identification of the FcγR involved was not made.

The role of CyB in enhancing intracellular ROS production by monocytes with Mac-1 molecules cross-linked with mAb VIM12 and Leu-15 may be due to inhibition of intracellular degradation of reactive oxygen intermediates.[31] CyB did not improve competitive binding of anti-Mac-1 mAb with anti-FcγRII mAb or heat-aggregated antibodies, or enable cross-linkage with FcγRI on monocytes; nor did it alter cross-linkage of mAb Leu-15 to FcγRI on IFN-γ-stimulated PMN (data not shown). We speculate that a Mac-1–mAb–FcγRI or –FcγRII complex on monocytes may mimick a foreign organism with surface-fixed complement (iC3b molecules) or carbohydrate structures recognized by the Mac-1 lectin-like binding site,[5] as well as surface-bound IgG resulting in stimulation of a respiratory burst.

In conclusion, we show that Mac-1 bound by mAb VIM12 or 5A4.C5 (with FcγRII only) or Leu-15 an interact with either FcγRI or FcγRII, or both, and transduce a signal leading to intracellular ROS production in unstimulated monocytes, but not in unstimulated PMN. In PMN, only after stimulation with IFN-γ, is Mac-1 bound by mAb Leu-15 able to interact with FcγRI to transduce a signal resulting in intracellular ROS production.

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