Effect of iC3b binding to immune complexes upon the phagocytic response of human neutrophils: synergistic functions between FcγR and CR3

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Abstract **We compared the phagocytosis of immune complexes (IC) and iC3b-opsonized derivatives (iC3b-IC) by human neutrophils. The phagocytosis of iC3b-IC via FcγR and CR3 was much greater than that of IC via FcγR alone. Adding ethanol to the cells decreased iC3b-IC phagocytosis to that of IC, which was not affected by these reagents, suggesting that the enhanced phagocytosis is attributable to CR3-mediated phospholipase D activation. The IC phagocytosis was inhibited more effectively by anti-FcγIIIB, whereas the iC3b-IC phagocytosis was partly inhibited only by anti-FcγRII. The main FcγR might differ in IC and iC3b-IC phagocytosis.**

Key words: Neutrophil; Phagocytosis; FcγR; CR3; Phospholipase D

1. Introduction

Phagocytosis is a fundamental effector function of human neutrophils that can be triggered by receptors for the Fc region of IgG, FcγRII (CD32) and FcγRIIIB (CD16), and the receptor of C3, CR3 [1]. FcγRII is a transmembrane glycoprotein of 40 kDa [2] and FcγRIIIB is a glycosyl phosphatidylinositol-linked membrane protein of 50–70 kDa [3]. Whether FcγRII and FcγRIIIB differ in ligand binding specificity as well as in their ability to induce phagocytosis, has been the focus of considerable investigation. When immune complexes (IC) activate the complement system, they become opsonized with C3b, which is subsequently converted into iC3b by complement factors I and H [4]. CR3 (CD11b/CD18) is a member of integrin family and is responsible for the binding and phagocytosis of iC3b-opsonized IC, although signals through CR3 alone are too weak to induce a significant phagocytic response in resting neutrophils [5]. In addition to the iC3b-binding domain, CR3 on neutrophils has a lectin-like domain through which it also acts as a pluripotent receptor in the recognition of zymosan [6], β-glucan [7], and *Escherichia coli* [8].

The role of FcγR in the phagocytic response of neutrophils has mostly been studied by the use of IC or IgG-coated particles [9–12]. There are several lines of evidence suggesting that CR3 is concerned in the FcγR-mediated cellular responses to IC by neutrophils. CR3-deficient neutrophils obtained from a patient with leukocyte adhesion deficiency syndromes showed depressed ability to phagocytose IgG-coated erythrocytes [13,14]. CR3 cocaps with FcγRIIIB through the lectin-like domain, and saccharides that inhibit cocapping also decrease IC-dependent transmembrane signaling and superoxide production [15,16]. Since FcγRIIIB is a glycosyl phosphatidylinositol-anchored membrane protein, CR3 is proposed to act as a membrane transducer for FcγRIIIB [16]. Furthermore, most of the IC formed in situ appear to be opsonized with iC3b before being processed by neutrophils [4]. Thus, iC3b-IC seems to be a useful ligand with which to assess FcγR-to-CR3 interaction in the phagocytic response of neutrophils. However, little is known about the contribution of CR3-derived signal transduction to FcγR-mediated cellular responses using the ligand, iC3b-IC.

This study was designed to compare the phagocytic responses to IC, iC3b-IC, and iC3b-IC[F(ab′)$_2$] by neutrophils, in which iC3b-IC is supposed to cross-link FcγR and CR3, whereas IC and iC3b-IC[F(ab′)$_2$] bind with only FcγR and CR3, respectively. In addition, CR3-mediated cellular responses are reportedly coupled to the activation of phospholipase D (PLD) [17]. Thus, ethanol and propranolol, which inhibit PLD-mediated cellular responses in neutrophils [18,19], were used to evaluate the effects of CR3-mediated responses upon phagocytosis of IC and iC3b-IC by neutrophils.

2. Materials and methods

2.1. Preparation of human neutrophils

Human peripheral blood was collected from healthy volunteers in acid citrate-dextrose. Neutrophils were isolated by sedimentation using Dextran T-500 and Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), followed by the hypotonic lysis of contaminating erythrocytes as reported [12,20].

2.2. Antibodies

The Fab fragment of a monoclonal antibody (mAb) (IV.3) to FcγR II and the F(ab′)$_2$ fragment of a mAb (3G8) to FcγRIII were purchased from Medrex (New Hampshire, USA). The mAb to CR3 (#44) was obtained from Leinco Technologies (Ballwin, USA). Human anti-tetanus toxoid IgG was obtained from Green Cross (Osaka, Japan). The pepsin fragment F(ab′)$_2$ of human anti-tetanus toxoid IgG was prepared by incubating anti-tetanus IgG with pepsin at a substrate/enzyme ratio of 50:1 (w/w) at pH 4.5 for 24 h at 37°C. The digest was passed through a column containing protein A-Sepharose, and the eluate was collected and used as F(ab′)$_2$. The absence of uncleaved IgG in the F(ab′)$_2$ preparation was confirmed by SDS-PAGE.

2.3. Preparation of IC and FITC-labeled IC

FITC-labeled or -unlabeled IC were prepared as reported [12,21], with FITC-labeled or -unlabeled tetanus toxoid and human anti-tetanus toxoid IgG. Tetanus toxoid, which was a gift from Prof. T. Kinoshita, the Research Institute for Microbial Diseases, Osaka University, was labeled with fluorescein isothiocyanate (FITC, Sigma Chemi-

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Abbreviations: IC, immune complexes; iC3b-IC, iC3b-opsonized IC; PLD, phospholipase D; FITC, fluorescein isothiocyanate; FIC, FITC-labeled IC; mAb, monoclonal antibody.

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cal Co.) as reported [12]. FITC-labeled IC[F(ab')$_2$] (FIC[F(ab')$_2$]) was prepared with FITC-labeled tetanus toxoid and anti-tetanus F(ab')$_2$ at equivalence as described above.

2.4. Preparation of iC3b-FIC and iC3b-FIC[F(ab')$_2$]

Fifty micrograms of FIC or FIC[F(ab')$_2$] was incubated with 300 μl of human serum without or with 10 mM EGTA-5 mM $MgCl_2$ for 60 min at 37°C. During this incubation, complement activation proceeded on FIC and FIC[F(ab')$_2$] to opsonize the complexes with C3b, which was immediately converted into iC3b, a true ligand for CR3.

2.5. SDS-PAGE

Samples were reduced with 2-mercaptoethanol and resolved by SDS-PAGE [22]. Gels were stained with Coomassie brilliant blue. Molecular mass markers were obtained from Pharmacia (Uppsala, Sweden), and iC3b was prepared by incubating C3b with factor H and I [23].

2.6. Assay of association and phagocytosis

The association and phagocytosis of FIC derivatives was measured by a flow cytometer as described [12,21]. Neutrophils (5×10^5 cells) were brought to 250 μl with Krebs-Ringer-phosphate buffer and mixed with FIC derivatives at a final concentration of 25 μg/ml (FIC equivalent determined by densitometry after SDS-PAGE). After an incubation for 20 min at 37°C, the cells were washed twice, then the amounts of cell-bound FIC were determined using a flow cytometer (EPICS-CS Coulter Co.). For the phagocytosis assay, neutrophils (5×10^5 cells) in 250 μl of Krebs-Ringer-phosphate buffer were incubated with FIC at 25 μg/ml for 20 min at 37°C, then chilled at 0°C. The cells were washed twice with cold phosphate-buffered saline, suspended in 20 mM sodium acetate buffer, pH 4.5, containing 150 mM NaCl and 0.5 mg/ml of Trypan blue to quench the fluorescence at the cell surface. The unquenchable fluorescence due to intracellular FIC was determined by flow cytometry.

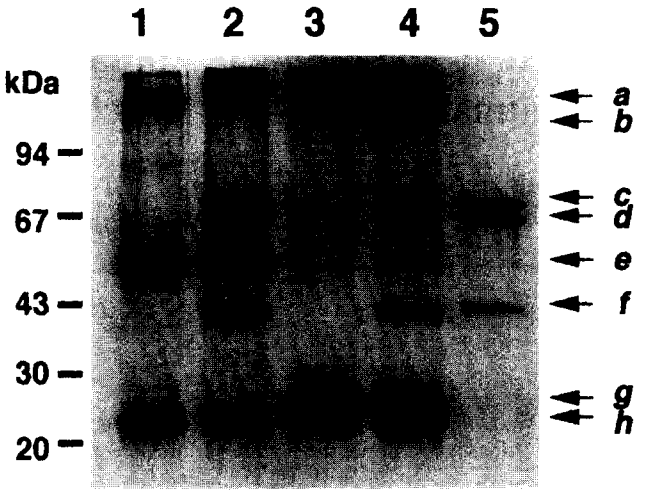

Fig. 1. SDS-PAGE analysis of FIC, iC3b-FIC, FIC[F(ab')$_2$] and iC3b-FIC[F(ab')$_2$]. FIC or FIC[F(ab')$_2$] was incubated with human serum containing 10 mM EGTA-5 mM $MgCl_2$ for 60 min at 37°C. After washing, these ligands, FIC (1), iC3b-FIC (2), FIC[F(ab')$_2$] (3), iC3b-FIC[F(ab')$_2$] (4), and iC3b (5), were resolved by SDS-PAGE (10% gel) under reducing conditions. The gels were stained with Coomassie brilliant blue. Molecular mass markers in kDa are shown on the left. Arrows at the right margin: (a), covalent complex of iC3b α_1 chain and IgG H chain; (b), covalent complex of iC3b α_1 chain and F(ab')$_2$ Fd chain; (c), iC3b β chain; (d), iC3b α_1 chain; (e), IgG H chain; (f), iC3b α_2 chain; (g), F(ab')$_2$ Fd chain; (h), IgG L chain.

3. Results

3.1. Effect of the opsonization of FIC with iC3b upon phagocytosis by neutrophils

Incubating FIC[F(ab')$_2$] as well as FIC with human serum results in the covalent binding of C3b through activation of the complement pathway. The C3b on FIC[F(ab')$_2$] and FIC is immediately cleaved by complement factors, H and I, to form iC3b-FIC[F(ab')$_2$] or iC3b-FIC; iC3b is composed of three disulfide-linked polypeptide chains, α_1 (68 kDa), α_2 (43 kDa), and β (70 kDa) [24]. SDS-PAGE of the serum-treated, reduced complexes revealed bands of 70 and 43 kDa, that corresponded to the β and α_2 chains, respectively, of iC3b (Fig. 1). The α_1 chain of iC3b was not detectable, because iC3b binds through the α_1 chain with the H chain of IgG or the Fd of F(ab')$_2$. Judging from the relative dye intensities of the H chain of IgG (the Fd of F(ab')$_2$) and the β chain of iC3b, the apparent molar ratio of IgG and iC3b was assumed to be 1:1. These iC3b-opsonized ligands as well as free FIC were used to assess CR3- and FcγR-dependent phagocytosis by neutrophils.

Table 1
Interaction of three ligands with neutrophils

Stimulants	Association (F.I.)	Phagocytosis (F.I.)	Incorporated F.I. (%)
FIC	32.8 ± 9.4	4.2 ± 0.8	12.9
iC3b-FIC	176.2 ± 32.6	38.0 ± 7.8	21.6
iC3b-FIC[F(ab')$_2$]	6.6 ± 2.9	2.0 ± 0.5	30.0

Neutrophils (5×10^5 cells) were incubated with each of the three ligands for 20 min at 37°C. In the phagocytosis assay, the cell suspensions were then rapidly chilled to 0°C in an ice bath, mixed with Trypan blue at pH 4.5 to quench the extracellular fluorescence, and analyzed by flow cytometry. The percent of incorporated fluorescence was the ratio of Phagocytosis F.I./Association F.I. at 37°C for 20 min. The data are the means with S.E.M. for three experiments

The phagocytosis of these ligands was time-dependent (2.5–20 min) (data not shown). Table 1 summarizes the association and phagocytosis of the three ligands when assayed after 20 min incubation. The association and phagocytosis of iC3b-FIC was much greater than those of either FIC or iC3b-FIC[F(ab')$_2$] alone. However, simultaneously incubating iC3b-FIC[F(ab')$_2$] (25 μg/ml)-treated neutrophils with unlabeled IC (50 μg/ml) did not enhance the phagocytosis of iC3b-FIC[F(ab')$_2$] (data not shown). These results suggested that the enhanced phagocytosis of iC3b-FIC requires the cross-linking of CR3 and FcγR on neutrophils. Table 1 also demonstrates the ratio of intracellular to cell-bound fluorescence intensity, which reflects the rate of incorporation of receptor-bound ligand. The highest fluorescence ratio was given by iC3b-FIC[F(ab')$_2$], which had the lowest level of association as well as phagocytosis. This suggested that CR3-mediated phagocytosis proceeds more rapidly than that mediated by FcγR, although CR3-dependent iC3b-FIC[F(ab')$_2$] association with neutrophils is lower than the FcγR-dependent FIC association with the cells.

3.2. Effect of ethanol and propranolol upon FcγR- and CR3-mediated phagocytosis

CR3-mediated cellular responses are reportedly coupled with the activation of PLD, which hydrolyzes phosphatidylcholine to accumulate diglyceride [17]. In the presence of ethanol, PLD activation results in the formation of phosphatidylethanol through a transphosphatidylation reaction together with a decrease in phosphatidic acid generation [18]. We investigated the effect of ethanol upon the phagocytosis of iC3b-FIC[F(ab')$_2$] by neutrophils. Phagocytosis of the ligand by neutrophils

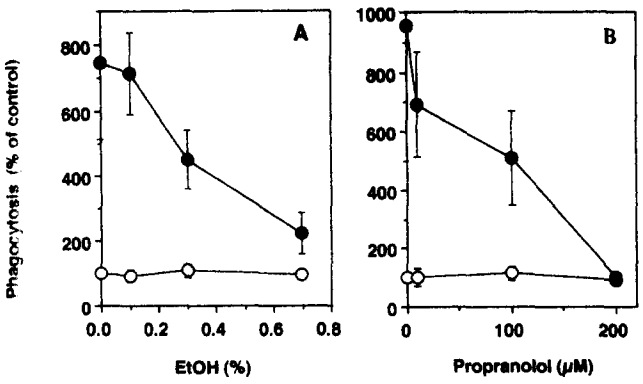

Fig. 2. Effect of ethanol or propranolol on phagocytosis of FIC or iC3b-FIC by neutrophils. Neutrophils (5×10^5 cells) were incubated with various concentrations of ethanol (A), or propranolol (B) for 5 min at 37°C, then with FIC (○), iC3b-FIC (●) for 20 min at 37°C. The data are the mean with S.E.M. of three experiments.

was dose-dependently inhibited by ethanol (data not shown): 88% inhibition was caused in cells preincubated with 0.7% ethanol. On the other hand, the enhanced phagocytosis of iC3b-FIC by neutrophils decreased with increasing ethanol concentration, close to the level of FcγR-mediated phagocytosis of FIC, which was not inhibited by ethanol (Fig. 2A). Exposure to 0.7% ethanol did not affect the viability of neutrophils and the association of FIC and iC3b-FIC. In addition, propranolol, which inhibits the production of diglyceride from phosphatidic acid [19,25], suppressed the enhanced phagocytosis of iC3b-FIC to the same level as that of FIC (Fig. 2B). Thus these results suggested that the accumulation of diglyceride by PLD is critical for the enhanced phagocytosis of iC3b-FIC by neutrophils.

3. Effect of anti-FcγR and anti-CR3 on phagocytosis of FIC and iC3b-FIC by neutrophils

Resting neutrophils prepared from peripheral blood express two types of FcγR, FcγRII (CD32) and FcγRIIIB (CD16). Incubating neutrophils with anti-FcγRII (IV.3) and anti-FcγRIIIB (3G8) decreased the phagocytosis of FIC to 86 and 39%, respectively, of that of control cells (Table 2). Incubating neutrophils with both anti-FcγR completely inhibited phagocytosis. These results suggested that although both FcγRII and FcγRIIIB are concerned in the phagocytosis of FIC, FcγRIIIB is the main receptor in the phagocytosis of FIC. On the contrary, the phagocytosis of iC3b-FIC was inhibited partially by anti-FcγR II (47%) but not by anti-FcγRIIIB, which did not inhibit the anti-FcγRII-resistant phagocytosis. These results suggested that different subclasses of FcγR are responsible for the processing of FIC and iC3b-FIC. Anti-CR3, mAb 44, inhibited the phagocytosis of iC3b-FIC to 50% of that of control. The anti-CR3-resistant phagocytosis of iC3b-FIC was inhibited by anti-FcγRII and -IIIB to 14% of that of control. These results suggest that FcγRII- and CR3-mediated cellular responses contribute equally to the phagocytosis of iC3b-FIC by neutrophils. To confirm the role of the CR3-mediated cellular response in the phagocytosis of iC3b-FIC, ethanol was used as an inhibitor of CR3-mediated responses. The anti-FcγRII-resistant phagocytosis of iC3b-FIC was almost completely inhibited by ethanol.

4. Discussion

It has been proposed that synergism between FcγR and CR3 is important in the processing of IC by neutrophils. We investigated the functional association between FcγR and CR3 using iC3b-opsonized IC, because IC formed in situ are likely to be in the form of iC3b-IC. We are the first to assess synergism between FcγR and CR3 in the phagocytic responses of human neutrophils using the FITC-labeled ligands; FIC, iC3b-FIC, and iC3b-FIC[F(ab')$_2$].

Neutrophils showed enhanced association and phagocytosis of iC3b-FIC compared with those of FIC. The independent activation of FcγR and CR3 of neutrophils by IC and iC3b-FIC[F(ab')$_2$] did not enhance the CR3-mediated phagocytosis of iC3b-FIC[F(ab')$_2$] to the level of iC3b-FIC, suggesting that the cross-linking of CR3 and FcγR by iC3b-FIC is required for the enhancement of CR3-mediated phagocytosis. Evidence that the enhanced phagocytosis of iC3b-FIC is decreased by treating neutrophils with ethanol or propranolol to the level of phagocytosis of FIC suggests that CR3-mediated signal transduction is responsible for the enhanced phagocytosis of iC3b-FIC.

CR3-dependent cellular responses are mediated by PLD activation [17], and exposure to ethanol results in a decrease in phosphatidic acid generation [18]. The phagocytosis of iC3b-FIC as well as iC3b-FIC[F(ab')$_2$] was inhibited by incubating neutrophils with ethanol, though ethanol did not affect FcγR-mediated phagocytosis. These results suggested that FcγR and CR3 use different signal transduction systems for their phagocytic responses. Propranolol, which inhibits the production of diglyceride from phosphatidic acid [19,25], also inhibited the CR3-mediated phagocytosis, indicating that the activation of protein kinase C by diglyceride is responsible for the CR3-mediated phagocytosis.

An additional effect of the iC3b-binding to IC is the shift of the major FcγR responsible for the phagocytic response. Phagocytosis of FIC by neutrophils was inhibited by anti-FcγRIIIB more effectively than anti-FcγRII, suggesting that

Table 2
Effect of anti-FcγRs, anti-CR3 and ethanolon the phagocytosis of FIC or iC3b-FIC by neutrophils

Stimulants	mAb or ethanol (EtOH)	Phagocytosis (% of control)
FIC	none	100
	IV.3	86.2 ± 14.2
	3G8	39.4 ± 7.3
	IV.3 + 3G8	2.6 ± 1.1
iC3b-FIC	none	100
	UV.3	47.8 ± 3.1
	3G8	105.1 ± 1.5
	44	49.7 ± 12.5
	IV.3 + 3G8	48.7 ± 5.0
	IV.3 + 3G8 + 44	14.4 ± 4.4
	EtOH	38.6 ± 1.9
	IV.3 + EtOH	7.0 ± 1.5
	3G8 + EtOH	45.1 ± 2.7
	IV.3 + 3G8 + EtOH	6.2 ± 1.2

Neutrophils (5×10^5 cells) were incubated with the indicated anti-FcγRs or anti-CR3 (2 μg/ml) for 30 min at 4°C. Cell suspensions were incubated with 0.7% ethanol for 5 min at 37°C, then with each of the stimulants at 37°C for 20 min. The data are the means with S.E.M. of three experiments and are given as the percentage of the phagocytic activity obtained from non-treated cells

FcγRIIIB is the major receptor responsible for the phagocytosis of IC. This supported the observations of Brunkhorst et al. [9], but seems to be in conflict with that of Huizinga et al. [10] and Edberg and Kimbery [11], who showed that FcγRII is much more efficient than FcγRIIIB in initiating the phagocytosis of IC. The reason for this discrepancy would be due to the difference in the size of IC; the latter groups used a large IC composed of *Staphylococcus aureus* [10] and erythrocytes [11]. Blocking FcγRII with mAb IV.3 is reportedly imperfect because the mAb IV.3 is easily expelled from the FcγRII by excess IC [10]. This could explain the partial inhibition of phagocytosis of iC3b-FIC by mAb IV.3. The reason for the differential binding of FIC and iC3b-FIC to FcγRII and FcγRIIIB is difficult to explain. However, it may be that the covalent binding of iC3b to FIC induces a conformational change in the Fc domain to alter the binding affinity between the Fc and FcγR. Evidence that the binding of iC3b-FIC to neutrophils is temperature dependent and requires divalent cations (data not shown), as is the interaction between iC3b and CR3 [26], may reflect a decrease in the affinity between FcγR and the Fc domain of iC3b-FIC. At any rate, FcγRII but not FcγRIIIB was functionally associated with CR3 during the processing of iC3b-opsonized IC by neutrophils.

Zhou and Brown have reported that a respiratory burst occurs when human neutrophils are plated on a surface coated with both anti-CR3 and anti-FcγRIIIB mAb [27]. This respiratory burst stimulated by synergy between FcγRIIIB and CR3 is completely inhibited by anti-FcγRII. Further analysis reveals that FcγRII is phosphorylated on tyrosine only when both CR3 and FcγRIIIB are ligated [27]. It is possible that tyrosine-phosphorylation of FcγRII enhances the binding between FcγRII and iC3b-FIC. Further investigations are required to understand the precise molecular mechanism of the synergy between FcγR and CR3 in the activation of neutrophils effector functions, including phagocytosis.

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