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Characteristics of CR3-mediated aggregation in human eosinophils: Effect of priming by platelet-activating factor

Leo Koenderman, PhD,* Taco W. Kuijpers, MD, Michela Blom, MSc, Anton T. J. Tool, MSc, Dirk Roos, PhD, and Arthur J. Verhoeven, PhD**
Amsterdam, The Netherlands

We have used double-color fluorescence-activated cell sorter analysis to characterize the homotypic aggregation response of human eosinophils (EOs). With this method, we demonstrate for the first time that EOs are able to form stable aggregates. The aggregation response induced by the phorbol ester, phorbol myristate acetate (PMA), was low and was not primed by platelet-activating factor (PAF). In contrast, the aggregation response induced by opsonized particles was markedly enhanced after priming with PAF. Additional experiments with several blocking monoclonal antibodies indicate that the CR3 receptor present on human EOs mediates the homotypic aggregation induced by PMA and by small opsonized particles through a putative "cell-adhesion" site on CR3, which binds to its counter structure on the opposing cell. The signal that initiates this binding event is generated after PMA addition or activation of the iC3b-binding site and is not sensitive for priming by PAF. The priming by PAF of the aggregation response induced by opsonized particles is restricted to an action on the iC3b-binding site, possibly by enhancing the affinity for its ligand. (J ALLERGY CLIN IMMUNOL 1991;87:947-54.)

From the Central Laboratory of The Netherlands Red Cross Blood Transfusion Service and Laboratory for Experimental and Clinical Immunology of the University of Amsterdam, Amsterdam, The Netherlands.

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Reprint requests: A. J. Verhoeven, PhD, c/o Publication Secretariat, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, PO Box 9406, 1006 AK Amsterdam, The Netherlands.

*Present Address: Dept. of Pulmonary Diseases, State University Hospital, Utrecht, PO Box 85500, Utrecht, The Netherlands.

**Dr. A. J. Verhoeven is a research fellow of the Royal Netherlands Academy of the Arts and Sciences.

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Human EOs are potent effector cells in the host defense against invading (micro)organisms, particularly in the killing of helminths.^{1,2} Moreover, it has been demonstrated that EOs are cytotoxic to tumor cells.³ Although this cell type has a beneficial role in normal individuals, the pathogenesis of several disorders is associated with excessive release of EO products. Especially in atopic disease, EOs are believed to play a detrimental role.⁴⁻⁶

EOs are chemotactic cells^{7,8} and release, on appropriate stimulation, the following inflammatory mediators: (1) preformed cytotoxic proteins, which can cause tissue destruction,³ (2) newly synthesized lipid mediators, which can cause tissue edema and constriction of smooth muscle, and (3) toxic oxygen metabolites generated by a membrane-bound reduced nicotinamide adenine dinucleotide phosphate oxidase.^{8,11}

In contrast to the abundance of data describing the role of the EO in inflammation, little is known about the regulation of this cell type. In particular, nothing is known about the aggregation response, which is believed to be important in the margination process.

In this study we have focused on the homotypic aggregation response of human EOs isolated from the peripheral blood of normal individuals. We have used a new method, which unequivocally determines the formation of cell aggregates. We demonstrated, in this study, for the first time, that EOs are able to form stable aggregates. Moreover, the aggregation response induced by opsonized particles is greatly enhanced after priming of the cells with PAF.

MATERIAL AND METHODS

Material

PMA (Sigma Chemical Co., St. Louis, Mo.) was dissolved in dimethyl sulfoxide at 1000 times the final concentration for cell incubations and was stored at -20°C . STZ was prepared as described previously.¹² Latex particles covered with purified iC3b were prepared by covalent coupling of F(ab')₂ fragments of the MAb anti-C3-1 (reacting with the C3d portion of C3 and recognizing iC3b) to the latex and subsequent binding of iC3b.¹³ PAF (L- α -phosphatidylcholine, β -acetyl, γ -O-hexadecyl) was obtained from Sigma Chemical Co. SFDA and HE were purchased from Molecular Probes (Junction City, Ore.) and Polysciences, Inc. (Warrington, Pa.), respectively. SFDA and HE were dissolved as described.¹⁴ Stock solutions of SFDA (5 mg/ml in dimethyl sulfoxide) and HE (1 mg/ml in Earle's medium, and 10% fetal calf serum) were kept at 4°C for periods of up to 3 weeks. The MAbs B2.12 (IgM isotype¹⁵) and 60.1 (IgG1 isotype¹⁶), CLB-LFA-1/2 (IgG2b isotype¹⁷) and Leu-M5 (IgG2b isotype obtained from Becton Dickinson, Mountain View, Calif.) are directed toward the

Abbreviations used

EO:	Eosinophil
PAF:	Platelet-activating factor
PMA:	Phorbol myristate acetate
STZ:	Serum-treated zymosan
MAB:	Monoclonal antibody
SFDA:	Sulfofluorescein diacetate
HE:	Hydroethidine
HSA:	Human serum albumin
FMLP:	<i>N</i> -formyl-methionyl-leucyl-phenylalanine
PK-C:	Protein kinase C

α -chains of CR3, LFA-1, and P150,95, respectively. CLB-LFA-1/1 (IgG1 isotype¹⁸) recognizes the common β -chain CD18.

All other chemicals were reagent grade. Incubation medium for the cell incubations contained 132 mmol/L of NaCl, 6.0 mmol/L of KCl, 1.0 mmol/L of CaCl_2 , 1 mmol/L of MgSO_4 , 1.2 mmol/L of potassium phosphate, 20 mmol/L of *N*'-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 5.5 mmol/L of glucose, and 0.5% (wt/vol) of HSA, pH 7.4.

Cell isolation

Blood was obtained from healthy volunteers. EOs were isolated from the buffy coat of 500 ml of blood anticoagulated with 13 mmol/L of trisodium citrate (pH 7.4), as described previously,⁸ with slight modifications. Briefly, the mononuclear cells were separated over isotonic Percoll (1.077 gm/ml, pH 7.4). After isotonic lysis of the erythrocytes in an ice-cold solution containing 155 mmol/L of NH_4Cl , 10 mmol/L of KHCO_3 , and 0.1 mmol/L of ethylenediaminetetraacetic acid (pH 7.2), the granulocytes were washed and suspended in Hanks' medium supplemented with HSA (0.5% wt/vol). Subsequently, the granulocytes were incubated for 30 minutes at 37°C to restore the initial density of the cells. After this incubation period, the granulocytes were washed and resuspended in phosphate-buffered saline supplemented with HSA (0.5% wt/vol) and trisodium citrate (13 mmol/L). After preincubation of the granulocytes for 5 minutes at 37°C , FMLP (10 nmol/L) was added to the cell suspension, and the incubation was continued for 10 minutes. Subsequently, the EOs were purified by centrifugation (15 minutes at 1000 g) over isotonic Percoll (1.082 gm/ml, pH 7.4). The EOs (purity, $96\% \pm 3\%$; $N = 9$) were washed and suspended in incubation medium and were kept at room temperature until use.

We have thoroughly investigated whether our isolation method leads to priming of EOs.^{8,19} We have not found any indications that EOs isolated from the blood of normal individuals are influenced by the FMLP treatment. Moreover, the present study also will demonstrate that EOs isolated

with this method are characterized by a resting phenotype and that they need priming for full activation.

Measurement of homotypic aggregation

Homotypic aggregation was measured by a double-color assay that was described previously in detail for human neutrophils.²⁰ Briefly, two separate suspensions of EOs (2×10^6 cells per milliliter) were colored fluorescent green or fluorescent red by incubation for 60 minutes with SFDA (100 $\mu\text{mol/L}$) or with HE (40 $\mu\text{mol/L}$), respectively. The cells were washed twice and were suspended in incubation medium. Equal amounts of fluorescent green and fluorescent red EOs were mixed (2×10^6 cells per milliliter). After 5 minutes of preincubation at 37° C (in some experiments with MABs present) in a reaction vessel that was thermostated and equipped with a stirring magnet, either PAF, 1 $\mu\text{mol/L}$, or solvent was added. After 2 minutes, STZ (1 mg/ml), PAF (1 $\mu\text{mol/L}$), PMA (100 ng/ml), or iC3b-latex (2×10^9 particles per milliliter) was added to activate the aggregation response. At the times indicated, samples were taken and immediately fixed with ice-cold paraformaldehyde (1% wt/vol in incubation medium). The samples were kept on ice and in the dark until analysis in an FACScan flowcytometer (Becton Dickinson). A gate, analyzing all aggregates, was set to avoid measurement events caused by the opsonized particles. The results are expressed as the percentage of double-colored events of the total of colored events.

RESULTS

Homotypic aggregation of human EOs induced by soluble stimuli and opsonized particles

As illustrated in Fig. 1, addition of the phorbol ester PMA (100 ng/ml) to human EOs led to a moderate aggregation response that was rather late in onset (>1 minute) and long lasting. In contrast, PAF (1 $\mu\text{mol/L}$) did not induce an aggregation response. Addition of opsonized particles (STZ or iC3b-latex) led to a small aggregation response. Hence, human EOs possess only a limited capacity to form aggregates on activation with a single stimulus, which is in marked contrast to human neutrophils.²⁰

Priming of the homotypic aggregation response of human EOs by PAF

Treatment of human EOs with PAF, which did not in itself cause aggregation of these cells (Fig. 1), resulted in a dose-dependent enhancement ("priming") of the aggregation response induced by STZ (Fig. 2). The optimal concentration of PAF for this effect to occur (1 $\mu\text{mol/L}$) also enhanced the aggregation induced by iC3b-latex (Fig. 3, A) but had no significant effect on the aggregation induced by PMA (Fig.

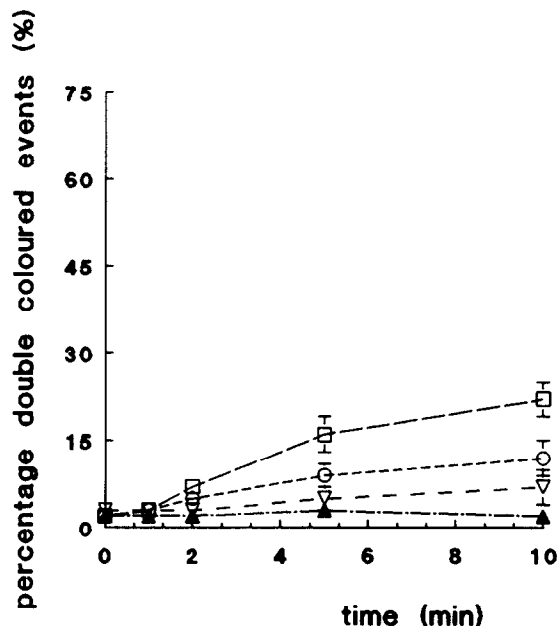


FIG. 1. Homotypic aggregation response of human EOs after activation with various stimuli. Equal amounts of fluorescent green and fluorescent red EOs were mixed and preincubated for 5 minutes at 37° C. Then, PAF, 1 $\mu\text{mol/L}$ ($\blacktriangle$); PMA, 100 ng/ml ($\square$); STZ, 1 mg/ml ($\circ$); or iC3b-latex, 2×10^9 particles per milliliter (∇), was added, and the incubation was continued for 10 minutes. Samples were taken just before adding the stimulus (designated as $t = 0$) and after 1, 2, 5, and 10 minutes. The samples were immediately fixed with ice-cold paraformaldehyde (1% wt/vol in incubation medium) and kept in the dark at 0° C until analysis in a flowcytometer. The results are expressed as the percentage of double-colored events of the total of colored events and are the means $\pm$ SEM of five different experiments.

3, B). Even when suboptimal doses of PMA were used, no priming was present (results not presented). Apparently, different mechanisms of aggregation are present, mediating either the opsonized particle-induced aggregation response or the phorbol ester-induced aggregation response.

The role of the adhesion molecules of the CD18 complex in the aggregation response of human EOs

Aggregation responses in human neutrophils are mediated by the CD18 complex of adhesion molecules.²¹ For this reason we evaluated the role of the CD18 complex in the aggregation response of human EOs. The three CD18 complex heterodimeric glycoproteins (LFA-1, CR3, and p150,95) consist of a specific α -chain noncovalently bound to an identical β -

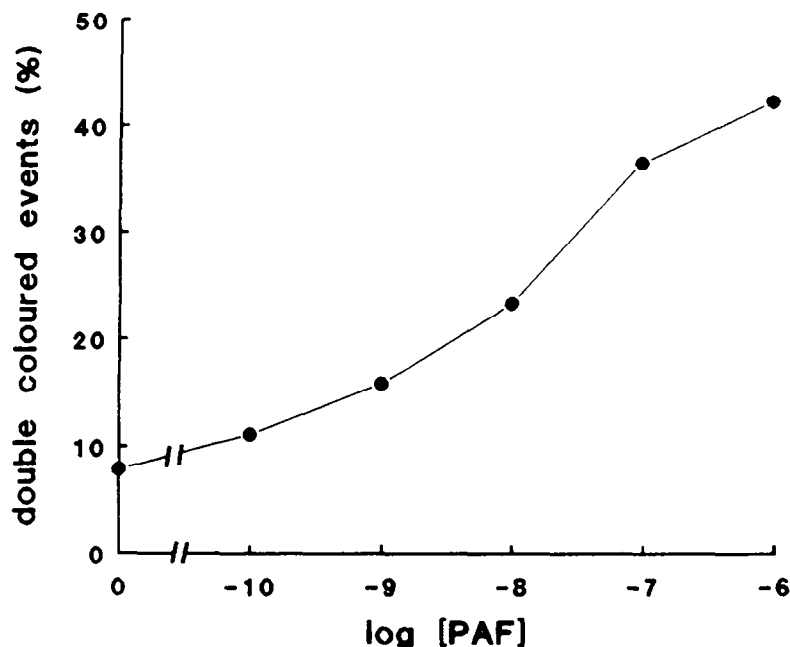


FIG. 2. The effect of different concentrations of PAF on the aggregation response induced by STZ. After 5 minutes of preincubation of the EOs at 37° C, various concentrations of PAF or buffer were added, and the incubations were continued for 2 minutes. Then, STZ (1 mg/ml) was added. Samples were taken 5 minutes after the STZ addition. The result depicted is the mean of two independent experiments with values differing <5%.

chain.²² Preincubation of human EOs with CLB-LFA-1.1, directed against the common β -chain, CD18, led to a potent inhibition of the aggregation response induced by PMA, indicating the crucial role of the CD18 complex in this response (Fig. 4, A). Of the MABs directed against the different α -chains, only MABs B2.12 (80% $\pm$ 5% inhibition) and 60.1 (>95% inhibition) directed against the α -chain of the CR3 receptor proved to be inhibitory in the aggregation response induced by phorbol esters. MABs against the α -chain of LFA-1 (CLB-LFA-1/2) and p150,95 (Leu-M5) were not inhibitory in the PMA-induced aggregation, although they have been demonstrated to be blocking agents in LFA-1 (van Lier R. Personal communication) and p150,95²³ dependent mechanisms, respectively (results not presented). These results indicate that the CR3 receptor is important in the aggregation response induced by phorbol esters, presumably by binding to its counter structure on the opposing cell. This is homologous to the situation in neutrophils.^{20, 24}

The aggregation response induced by STZ demonstrated other characteristics. The final aggregation induced by STZ in PAF-treated EOs was not influenced after preincubation with a saturating dose of CLB-LFA-1/1 directed against CD18 (Fig. 4, B).

Similar results were obtained in untreated EOs (not presented). In contrast, in the presence of MAB B2.12 against the α -chain of the CR3 receptor, which recognizes the iC3b binding site,¹⁵ the aggregation response induced by STZ was abolished in both PAF-treated EOs (Table I) and untreated EOs (results not presented).

Homotypic aggregation response of human EOs induced by latex particles covalently linked to iC3b fragments

The STZ particles we used were predominantly coated with iC3b fragments, although IgG molecules were also present.¹³ Because EOs express receptors for IgG (Fc γ R II), this opsonin might participate in the aggregation response. Moreover, STZ particles are rather large and might form bridges between different EOs. To circumvent these problems, we performed similar studies with small (0.8 μ m) latex beads covered with purified iC3b fragments, without IgG or C3b present.¹³ The aggregation response of human EOs induced by these beads demonstrated the same characteristics as the STZ-induced aggregation (Fig. 3, A; Table I), except that this response was virtually absent in the presence of MAB CLB-LFA1.1 (Fig. 4, B).

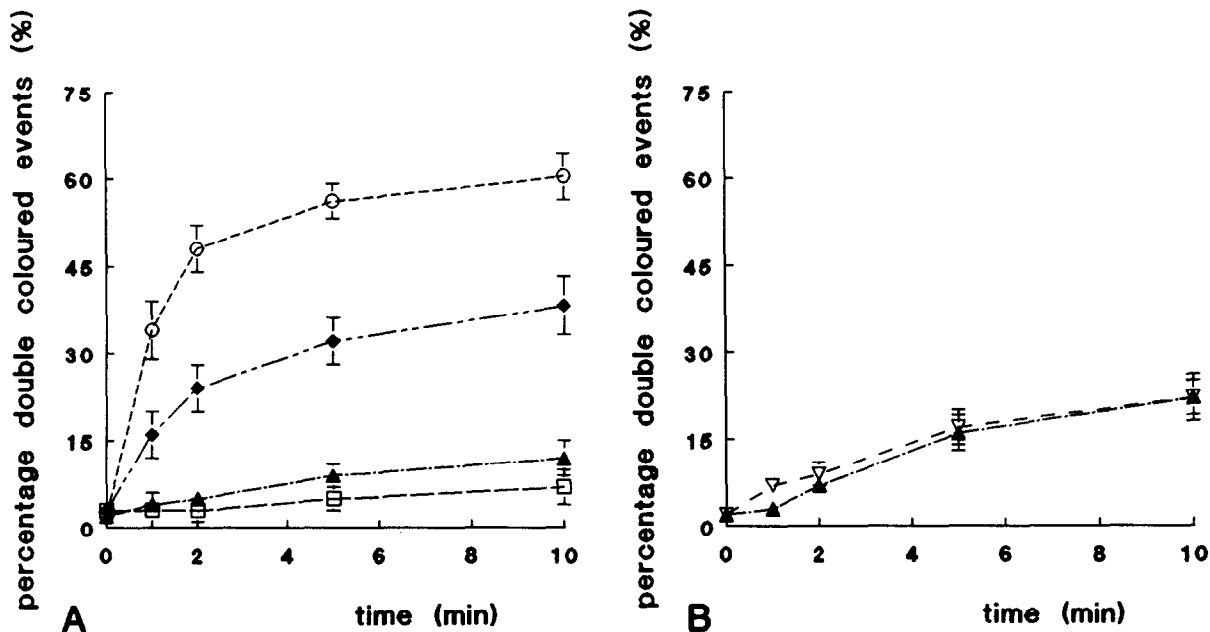


FIG. 3. The effect of priming by PAF on the homotypic aggregation response of human EOs. After a 5-minute preincubation of the EOs at 37° C, PAF (1 μ mol/L) or buffer was added, and the incubation was continued for 2 minutes. Then, STZ (1 mg/ml), iC3b-latex (2×10^9 particles per milliliter), or PMA (100 ng/ml) was added, and the incubation was continued for 10 minutes. Samples were taken and analyzed (details in the legend of Fig. 1). **A**, Untreated (▲) and PAF-treated (○) cells activated with STZ; untreated (□) and PAF-treated (◆) cells activated with iC3b-latex. **B**, Untreated (▲) and PAF-treated (▽) cells activated with PMA. Results are expressed as means $\pm$ SEM of five different experiments.

DISCUSSION

Although aggregation of EOs in analogy with human neutrophils²⁵ might be of importance for extravasation,²⁶ nothing is known about the aggregation response in human EOs. This finding is probably due to the difficulty to isolate pure EOs from peripheral blood of normal individuals and the limited sensitivity of the established turbidimetric method for determination of EO aggregation (results not presented). Therefore, we used an alternative method to measure unequivocally homotypic and heterotypic aggregation, which is described in more detail elsewhere.²⁰ This method is very sensitive and can even detect small cell clusters not readily detectable with aggregometry.

This study demonstrates that EOs isolated from normal donors have a low capacity to aggregate after activation with opsonized particles. Neutrophils aggregate very well under these conditions (results not presented). However, when EOs are pretreated ("primed") with PAF, which by itself does not induce aggregation, the aggregation response after activation with opsonized particles is markedly enhanced. The dose of PAF (1 μ mol/L), which is needed for optimal

priming of the aggregation response, is also needed for an optimal chemotaxis response²⁷ and for maximal priming of the respiratory burst.¹⁹ These observations support our previous conclusions^{8, 19} that the application of 10 nM of fMLP during EO purification does not cause priming of these cells. The PMA-induced aggregation appeared to be insensitive for priming by PAF, even at low concentrations of PMA. This result might suggest that another mechanism of aggregation mediates this response (see below).

At this time, our conclusions are not influenced by the fact that our method does not discriminate between large and small cell clusters. The small aggregation we have observed after PMA and STZ stimulation of unprimed cells consists of aggregates too small to be detected with turbidimetry (results not presented). The large increase in the aggregation response induced by opsonized particles in PAF-primed cells might even be underestimated by the formation of large aggregates.

The PMA-induced aggregation of human EOs is sensitive for inhibition by a MAb against CD18, indicating that under these conditions the aggregation response is mediated via the CD18 complex of adhe-

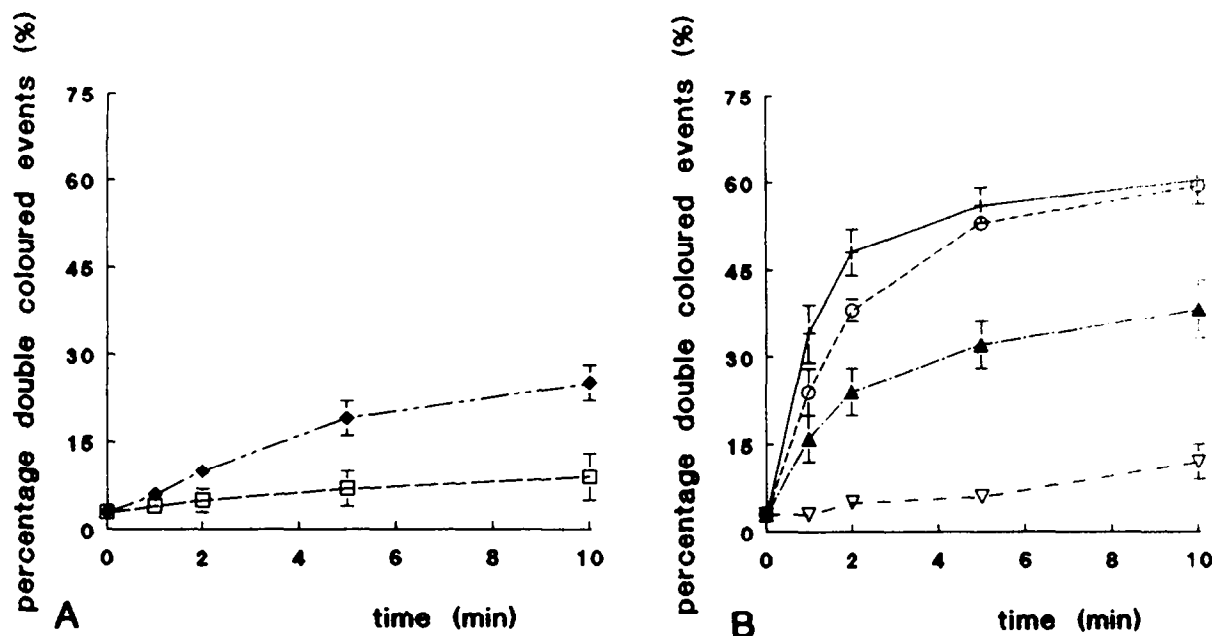


FIG. 4. The effect of blockade of the β -chain CD18 on the homotypic aggregation response of human EOs. After a 5-minute preincubation of the cells at 37° C in the presence or absence of the MAb CLB-LFA1/1 (100 μ g/ml), **A**, buffer or **B**, PAF (1 μ mol/L) was added, and the incubation was continued for 2 minutes. Then, PMA (100 ng/ml), STZ (1 mg/ml) or iC3b-latex (2×10^9 cells per milliliter) was added, and the incubation was continued for 10 minutes. Samples were taken and analyzed (details in the legend of Fig. 1). **A**, Untreated cells activated with PMA in the absence ($\blacklozenge$) or presence ($\square$) of CLB-LFA1/1. **B**, PAF-treated cells activated with STZ in the absence ($+$) or presence ($\circ$) of CLB-LFA1/1; PAF-treated cells activated with iC3b-latex in the absence ($\blacktriangle$) or presence (∇) of CLB-LFA1/1. Irrelevant IgG1 antibody did not have any effect on the responses observed. Results are expressed as mean $\pm$ SEM of five different experiments.

TABLE I. The effect of MAbs against CD18/CD11b on the opsonized particle-induced aggregation response of EOs primed by PAF

	STZ* (%)	iC3b particles* (%)
Control MAb	100	100
Anti-CD18 (CLB LFA-1/1)	99 $\pm$ 2†	25 $\pm$ 16‡
Anti-CD11b (B2.12)	17 $\pm$ 5§	28 $\pm$ 14‡

*After 5 minutes of preincubation of the EOs at 37° C, saturating amounts of MAbs were added. After an additional period of 5 minutes, PAF (1 μ mol/L) was added, and the incubation was continued for 2 minutes. Then, opsonized particles were added, and samples were taken after 10 minutes of activation.

†Not significantly different from control value.

‡ $p < 0.01$.

§ $p < 0.001$.

sion molecules (LFA-1, CR3, and p150,95). Of the MAbs against the different α -chains of this family of proteins, only MAbs against the CR3 proved to be inhibitory. These findings illustrate that the aggrega-

tion response induced by PMA is presumably mediated by a binding site on CR3 in close vicinity of the "lectinlike" binding site and the β -chain CD18. This hypothetical binding site, which we have addressed as "cell-adhesion site" in Fig. 5, binds to its ligand/counter structure on the opposing cell. This is in accord to the situation in human neutrophils.^{20, 24, 28} Under these conditions, the interaction between CR3 and its counter structure on the opposing cell is not sensitive for priming.

The aggregation response induced by iC3b-latex in PAF-primed EOs is like the PMA-induced response, inhibited in the presence of a MAb against CD18. This antibody does not prevent the binding of iC3b to its receptor (i.e., CR3), nor does it inhibit respiratory burst activation by these particles (not presented). These findings indicate that binding of iC3b to the CR3 receptor on PAF-primed EOs results in a signal, either intramolecular or via second messengers, that activates the binding between the "cell-adhesion site" on the CR3 receptor and its counter structure on the opposing cell. Possibly, PMA and iC3b activation use similar mechanisms to induce EO aggregation, for

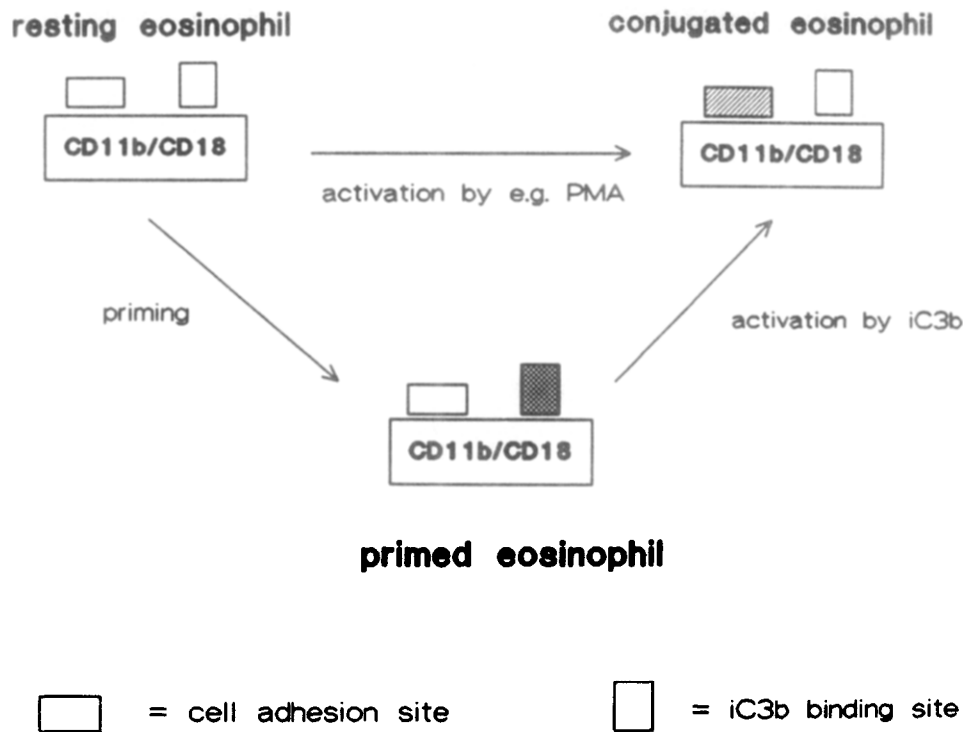


FIG. 5. Dual role of CR3 (CD11b/CD18) in homotypic aggregation in human EOs. The *hatching* indicates conformational changes of the binding sites.

instance, by inducing a conformational change of CR3.²¹

The sensitivity of the opsonized particle-induced aggregation response for priming by PAF, although the PMA aggregation response is not affected, indicates that the action of priming by PAF is restricted to the iC3b-binding site on the CR3 receptor. Possibly a change in affinity for iC3b is caused by priming (Fig. 5). This hypothesis is strengthened by the finding that during up regulation of CR3 receptors the 2-minute treatment of EOs with PAF does not occur (results not presented).

The aggregation response induced by STZ particles is, in contrast to the aggregation induced by PMA or iC3b-latex, almost insensitive for saturating amounts of MAb CLB-LFA-1/1 directed against CD18. Apparently, an additional type of cell-cell interaction is present under these conditions. The STZ-induced aggregation is inhibited in the presence of MAb B2.12, which recognizes the iC3b-binding site on the CR3 receptor (Table I). This finding, together with the size of the STZ particles, may indicate that the STZ particles form bridges between opposing cells by binding to the iC3b-binding sites on these cells. This bridge formation might be supported by interaction of the CR3 receptor with the proteoglycans present in zymosan, enabling the cells to escape from the inhibition

of MAbs directed against CD18. An alternative explanation for these data could be that binding of STZ particles reduces the binding of CLB-LFA-1/1 to the cells, allowing the cells to aggregate via CR3 binding to its counter structure.

As of now, not much is known about the signals responsible for the activation of the aggregation response in human EOs. Possibly, activation of the phospholipid/ Ca^{++} -dependent PK-C is a key event in the initiation of the aggregation response. However, preliminary studies with staurosporine, a very potent but not very specific inhibitor of PK-C,^{29, 30} indicate that the aggregation induced by opsonized particles in PAF-treated EOs is not dependent on PK-C activation (results not presented). Thus, another mechanism, strongly potentiated by priming, is likely to be involved.

In conclusion, this study demonstrates for the first time some characteristics of EO aggregation. The aggregation response of human EOs is very sensitive for priming by PAF when opsonized particles are used for activation. This phenomenon could modulate the adherent response toward opsonized targets, such as bacteria or helminths, and increase the cytotoxicity of the EOs, in concert with the priming of the respiratory burst¹⁹ and degranulation.³¹ However, when this enhanced cytotoxicity is caused in a nonspecific way in

diseases such as atopy, the primed EO participates in the pathogenesis of these syndromes. Studies on priming in vivo in atopy are currently under way in our laboratory.

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