Actin assembly is a crucial factor for superoxide anion generation from adherent human eosinophils

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Background: Cellular adhesion is crucial for eosinophil effector functions.
Objective: We sought to elucidate the role of the actin cytoskeleton in cellular adhesion and superoxide anion generation by human eosinophils.
Methods: Eosinophils were stimulated with platelet-activating factor (PAF) or complement component 5a on human serum albumin–coated plates with or without an actin-polymerization inhibitor, cytochalasin B (CB), or cytochalasin D (CD). Superoxide anion generation was measured on the basis of reduction of absorbance associated with cytochrome *c*. Eosinophil adhesion was assessed on the basis of eosinophil protein X content in adherent cells. Transient stimulus-induced increase of intracellular calcium and translocation of protein kinase C (PKC) βII, PKC δ, PKC ζ, and p47 phagocyte oxidase (a component of nicotinamide adenine dinucleotide phosphate oxidase) were also investigated.
Results: CB, CD, or antibodies against CD18 (the β2 chain of integrin, αMβ2) inhibited stimulus-induced eosinophil superoxide anion generation. Stimulus-induced eosinophil adhesion was unaltered by CB, whereas it was significantly suppressed by CD or anti-CD18 antibodies. Transient PAF-induced intracellular calcium increase was also unaffected by CB or CD, but stimulus-induced eosinophil shape changes and translocation of PKCs and p47 phagocyte oxidase to the cell membrane region were completely inhibited by CB. PAF-induced eosinophil degranulation was inhibited by CB, CD, or anti-CD18 antibodies, whereas complement component 5–induced degranulation was not suppressed by CB.
Conclusion: By itself, β2 integrin–dependent cellular adhesion is not sufficient for promoting eosinophil effector function. Adequate actin assembly is required for eosinophil adhesion and also for full superoxide anion generation in eosinophils. (J Allergy Clin Immunol 2003;112:126-33.)

Key words: *Eosinophils, activation, cytochalasin B, platelet-activating factor, complement component 5a, actin cytoskeleton, superoxide anion, protein kinase C, nicotinamide adenine dinucleotide phosphate oxidase*

Eosinophils play pivotal roles in allergic diseases and responses, such as bronchial asthma, allergic dermatitis, and parasitic infection.[1-3] In inflammatory conditions, eosinophils are induced to migrate from the bloodstream into extravascular spaces by various mediators released by T cells, mast cells, and other inflammatory cells. During these processes, eosinophils tether to, roll on, and firmly adhere to vascular endothelial cells through binding to adhesion molecules, such as selectins,[4] β4 integrins, and β2 integrins.[5] Eosinophils then release cytotoxic granule proteins and superoxide anion in response to various stimuli, resulting in tissue damage at inflammatory sites.[5]

In previous studies, inhibition of eosinophil adhesion by anti-CD18 (anti-β2 chain) or anti-CD11b (anti-αM chain) reduced eosinophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity.[6] We have reported that direct stimulation by CD11b in the absence of any other stimulus could induce eosinophil degranulation.[7] These findings indicate that cellular adhesion through integrins is crucial for promoting the effector functions of eosinophils.

Although signaling mechanisms concerning eosinophil activation are complex, including those that induce superoxide anion generation, the furthest downstream of these signals is recognized to be the superoxide anion–producing enzyme NADPH oxidase, which has been well characterized.[8-13] NADPH oxidase consists of both membrane components (p22, gp91, and Rap 1) and cytosolic components (p47 phagocyte oxidase [$p47^{phox}$], p67 phagocyte oxidase, Rac, and RhoGDI).[8] On NADPH oxidase activation (ie, superoxide anion generation), cytosolic components must be translocated to the membrane after their phosphorylation by means of various subtypes of protein kinase C (PKC) to enable them to associate with membrane components.[8-12] Further evidence shows that the cytoskeleton, including actin, is closely related to NADPH oxidase activation.[13,14]

Abbreviations used
C5a: Complement component 5a
CB: Cytochalasin B
CD: Cytochalasin D
DMSO: Dimethyl sulfoxide
EPX: Eosinophil protein X
FMLP: N-formyl-methionyl-leucyl-phenylalanine
HSA: Human serum albumin
NADPH: Nicotinamide adenine dinucleotide phosphate
$p47^{phox}$: p47 phagocyte oxidase
PAF: Platelet-activating factor
PKC: Protein kinase C

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Accumulating findings point to the importance of the cytoskeleton, including polymerized actin, in adhesion and movement of human neutrophils[15,16] and in secretion by mast cells.[17] In neutrophils cytochalasin B (CB), an actin elongation inhibitor, inhibited enhancement of N-formyl-methionyl-leucyl-phenylalanine (FMLP)–induced superoxide anion generation,[18] whereas other reports concluded that CB reduced NADPH activity.[19] On the other hand, in human eosinophils,o134 intracellular filamentous actin has been seen to increase at an early stage of activation.[16,19-22] CB, which enhances eosinophil activation when added in combination with FMLP,[23] partially inhibited complement component 5a (C5a)–induced eosino-phil adhesion to vascular cell adhesion molecule 1 but did not alter cellular adhesion against intracellular adhesion molecule 1, the counterligand of β2 integrin.[24] Thus, several reports have examined the role of the actin cytoskeleton in activation of human eosinophils, but details remain incompletely understood. Moreover, effects of CB on eosinophil effector functions are less clear than those on neutrophil effector functions.

In the present study, we used CB and another agent, cytochalasin D (CD), that cleaves filamentous actin to analyze eosinophil superoxide anion generation and adhesion in response to eosinophil activators, such as platelet-activating factor (PAF) or C5a, on a protein-coated surface. Our aim was to elucidate the relationship between the cytoskeleton and eosinophil activation. Furthermore, to determine whether CB affects the signaling cascade of eosinophil activation induced by PAF or C5a, we examined the effects of cytochalasins on translocation from the cytosol to the membrane of several subtypes of PKC during eosinophil activation, as well as translocation of the cytosolic NADPH oxidase component $p47^{phox}$.

METHODS

Reagents

PAF C-16 was obtained from Biomol Research Laboratories (Plymouth Meeting, Pa). C5a was purchased from Novabiochem (San Diego, Calif). CB and CD, obtained from Calbiochem (San Diego, Calif), were dissolved in dimethyl sulfoxide (DMSO) at 5 and 10 mg/mL, respectively. Anti-CD18 antibody (isotype $IgG_1\kappa$, clone L130) was from BD Pharmingen (San Diego, Calif), and $IgG_1\kappa$ mouse myeloma protein was purchased from ICN Pharmaceuticals Cappel Product (Aurora, Ohio). Fura-2 acetoxymethyl ester DMSO solution was obtained from Wako Pure Chemical Industries (Osaka, Japan). Alexa Fluor 532 phalloidin and Alexa Fluor 488 rabbit anti-goat IgG (H+L) were from Molecular Probes (Eugene, Ore). Anti-$p47^{phox}$ goat polyclonal antibody, anti-cPKC βII goat polyclonal antibody, anti-nPKC δ goat polyclonal antibody, anti-nPKC ζ goat polyclonal antibody, and goat IgG were from Santa Cruz Biotechnology (Santa Cruz, Calif). Globulin-free human serum albumin (HSA; A3782), cytochrome *c*, and DMSO were from Sigma (St Louis, Mo).

Cell isolation

Eosinophil purification was performed with minor modifications of a method previously described by using a magnetic cell separation system with anti-CD16 antibody–coated beads (Miltenyi Biotec GmbH, Bergish Gladbach, Germany).[25] Donors are all healthy, and different donors were used in replicate experiments. The purity of eosinophils counted with Randolph's stain was greater than 98%. The cell viability always exceeded 98%, which was determined by using trypan blue staining and flow cytometry with propidium iodide staining.

Superoxide anion generation

Superoxide generation from eosinophils was measured on the basis of reduction of cytochrome *c*, as previously described.[26,27] Purified eosinophils at 5×10^4 cells per well were placed on the HSA-coated wells of a 96-well, flat-bottom, tissue-culture plate. Then CB, CD, anti-CD18 antibody, and mouse $IgG_1\kappa$ used as an isotype-matched control at final concentrations of 5, 1, 5, and 5 μg/mL, respectively, was added, and the plate was incubated for 20 minutes at 37°C. After incubation, cytochrome *c* (final concentration, 80 μmol/L) was added, and the reactions were initiated by adding stimulus, PAF or C5a, at a final concentration of 1 μmol/L or 100 ng/mL, respectively. Immediately after addition of stimulus, the plate was loaded in an automated microplate reader (Wallac ARVO Sx-1420 multilabel counter; Perkin Elmer Life Sciences, Boston, Mass), and the absorbance at 550 nm was measured up to 180 minutes. During measurement, the plate was kept at 37°C. Superoxide anion generation was calculated with an extinction coefficient of 21.1×10^3 $cm^{-1}M^{-1}$ for reduced cytochrome c at 550 nm and was expressed as nanomoles of superoxide anion generation per 10^5 cells. All experiments were performed in duplicate.

Eosinophil adhesion assay

The numbers of adherent eosinophils were determined by measuring the contents of eosinophil protein X (EPX; Pharmacia-Upjohn, Tokyo, Japan) in adherent cells by means of RIA, as previously reported.[27] Briefly, in HSA-coated wells of a 96-well, flat-bottom, tissue-culture plate, eosinophils were added at 5×10^4 cells per well. Then CB, CD, anti-CD18 antibody, and mouse $IgG_1\kappa$ used as an isotype-matched control at final concentrations of 5, 1, 5, and 5 μg/mL, respectively, were added to the wells, and the plate was incubated for 20 minutes at 37°C. After incubation, the reactions were initiated by adding PAF or C5a at a final concentration of 1 μmol/L or 100 ng/mL, respectively. After 60 minutes, the supernatants were collected, and the plate was gently rinsed to remove nonadherent cells. Adherent cells were then lysed with 0.5% Nonidet P-40 detergent, and the EPX contents in the lysate were measured by means of RIA. All experiments were performed in duplicate. The percentage of adhesion was calculated as a ratio of EPX content in adherent eosinophils to total available EPX after incubation according to the following equation:

Percentage of adhesion = EPX in lysates of adherent cells after incubation/(Total EPX in lysates of cells before incubation – EPX release into supernatants during incubation) × 100.

Measurement of stimulus-induced intracellular calcium increase

Fura-2–loaded eosinophils (5×10^5 cells per well) were loaded into HSA-coated, black-bottom, cell-culture plates (FluoroNunc, flat-bottom, 96-well; Nalge Nunc International, Rochester, NY). Then CB or CD at a final concentration of 5 or 1 μg/mL, respectively, was added. Plates were loaded and kept for 15 minutes in an automated microplate reader (Wallac ARVO HTS-1420 multilabel counter; PerkinElmer Life Sciences, Boston, Mass) with temperature adjusted in advance to 37°C. Thirty seconds after measurement was initiated, 20 μL of PAF (final concentration, 1 μmol/L) was added to the measuring well, and measurement was continued up to 15 minutes. Intracellular calcium was determined as a fluorescence ratio between excitation at 340 and 390 nm.

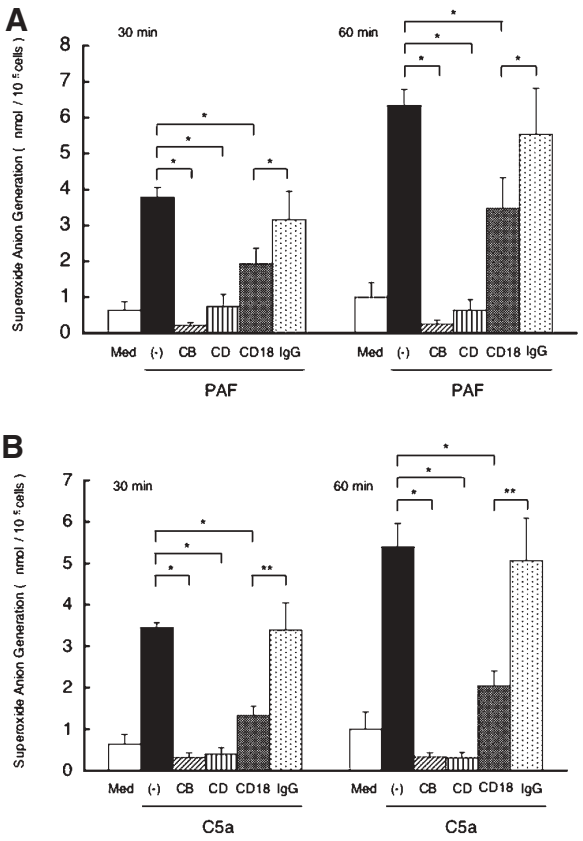

FIG 1. Inhibition of PAF- **(A)** or C5a-induced **(B)** eosinophil superoxide anion generation by CB, CD, or anti-CD18 antibody. The experiment was performed as described in the "Methods" section. The values of superoxide anion generation (in nanomoles per 10^5 cells) at 30 or 60 minutes after stimulation by PAF or C5a were presented as means ± SEM of 5 to 10 experiments. $*P < .01$, $**P < .05$ (significant difference).

Visualization of PKCs and NADPH oxidase p47^{phox} proteins

A suspension of 3×10^5 eosinophils in phenol red–free RPMI 1640 medium with 1 mmol/L HEPES was incubated with or without 5 μg/mL CB for 15 minutes in 37°C in an HSA-coated, 35-mm, glass-bottom dish (Matsunami, Osaka, Japan). After incubation, PAF or C5a was added at a final concentration of 1 μmol/L or 100 ng/mL, respectively. After 15 minutes at 37°C, attached cells were fixed with 3.7% (vol/vol) formaldehyde and permeabilized with 0.1% Triton X-100. Then cells were blocked, washed, and incubated for 45 minutes in the dark at 4°C with primary antibody (all dilutions, 1:100). Then cells were washed thoroughly and incubated for 45 minutes in the dark at room temperature with a mixture of Alexa Fluor 532 phalloidin (1 unit per dish) and secondary antibody (dilution, 1:200). Finally, the fluorescent signal was observed with an LSM 510 laser scanning microscope system (Carl Zeiss, Operkochen-Jena, Germany).

Eosinophil degranulation

Eosinophil degranulation was performed with slight modification from described previously.[26,27] Briefly, eosinophils were incubated with CB, CD, anti-CD18 antibody, or mouse $IgG_1\kappa$ and stimulated by PAF or C5a at a final concentration of 1 μmol/L or 100 ng/mL, respectively, as described in the adhesion assay. After 180 minutes' incubation, the supernatants were collected and stored at –20°C until they were assayed for EPX by means of RIA to quantitate eosinophil degranulation. Total cellular EPX contents were measured simultaneously by using supernatants from cells lysed with 0.5% Nonidet P-40 detergent. All experiments were performed in duplicate.

Statistical analysis

Normalized mean values from the indicated number of independent trials were averaged and expressed as means ± SEM. The statistical significance of the differences between various treatment groups was assessed with a paired or unpaired Student *t* test. Data with a *P* value of less than .05 were considered significant.

RESULTS

Effects of CB, CD, and anti-CD18 antibody on superoxide anion generation by human eosinophils

As shown in Fig 1, CB or CD inhibited PAF-induced (Fig 1, *A*) or C5a-induced (Fig 1, *B*) superoxide anion generation to less than the level of spontaneous generation. Anti-CD18 antibody suppressed stimulus-induced superoxide anion generation by approximately one half. The isotype-matched control antibody, mouse IgG1κ, did not significantly affect stimulus-induced superoxide anion generation. Additionally, the effects of CB on superoxide anion generation were consistent when 10-fold lower concentrations of PAF (100 nmol/L) or C5a (10 ng/mL) were used, although superoxide anion generation was weakened with these doses of stimuli (data not shown). CB or CD alone did not affect spontaneous superoxide anion generation, and DMSO vehicle (0.1%) did not affect spontaneous or stimulus-induced superoxide anion generation (data not shown).

Effects of CB, CD, and anti-CD18 antibody on eosinophil adhesion

As shown in Fig 2, both PAF (Fig 2, *A*) and C5a (Fig 2, *B*) induced significant eosinophil adhesion. Anti-CD18 antibody or CD inhibited stimulus-induced eosinophil adhesion significantly. Mouse IgG1κ, which was used as an isotype-matched control, did not alter eosinophil adhesion. On the other hand, CB, which suppressed PAF- and C5a-induced superoxide anion generation (Fig 1), did not affect PAF-induced eosinophil adhesion. Furthermore, although not to a statistically significant degree, CB slightly enhanced C5a-induced eosinophil adhesion. CB or CD treatment alone without stimulation did not significantly affect spontaneous eosinophil adhesion.

Effects of CB on intracellular calcium increase induced by PAF or C5a

To determine how CB inhibited PAF induction of effector functions, we examined the effect of CB on the PAF-induced increase in intracellular calcium levels. As shown in Fig 3, PAF-induced change of fluorescent ratio (f 340/f 390) was not altered by CB. Similarly, the C5a-induced increase in intracellular calcium was not affected by CB (data not shown). In addition, CD also did not affect PAF- or C5a-induced calcium increase (data not shown).

Effects of CB on PAF-induced translocation of NADPH oxidase p47^{phox}, PKC βII, PKC δ, and PKC ζ

Because CB did not affect the intracellular calcium increase induced by PAF, we next examined its effect on PAF- or C5a-induced activation of several PKCs and a component of NADPH oxidase, p47^{phox}. Because these molecules translocate from the cytosol to just beneath the cell membrane on activation, we assessed the intracellular location of these molecules 15 minutes after stimulation by using PAF or C5a with immunofluorescent staining and confocal laser scanning microscopy. As shown in Fig 4, NADPH oxidase p47^{phox}, PKC βII, PKC δ, and PKC ζ, all stained with green fluorescence, translocated from the cytosol to the cell membrane on stimulation with PAF or C5a. Fluorescent phallodin staining (displayed in red) indicated that on activation, eosinophils changed shape dramatically, with extension of filopodia. CB completely inhibited these shape changes of stimulated eosinophils. Furthermore, translocation of NADPH oxidase p47^{phox}, PKC βII, PKC δ, and PKC ζ also were completely inhibited. Similar results were obtained at 5 and at 30 minutes after stimulation (data not shown).

Effects of CB, CD, and anti-CD18 antibody on eosinophil degranulation

As shown in Fig 5, although some spontaneous degranulation had occurred by 3 hours, either PAF (Fig 5, *A*) or C5a (Fig 5, *B*) induced far more degranulation in eosinophils. Anti-CD18 antibody or CD completely inhibited eosinophil degranulation, whereas mouse IgG1κ did not affect stimulus-induced degranulation. CB inhibited PAF-induced degranulation, with a result approximating spontaneous degranulation. However, CB did not suppress C5a-induced degranulation. CB or CD treatment alone without stimulation did not significantly affect spontaneous degranulation.

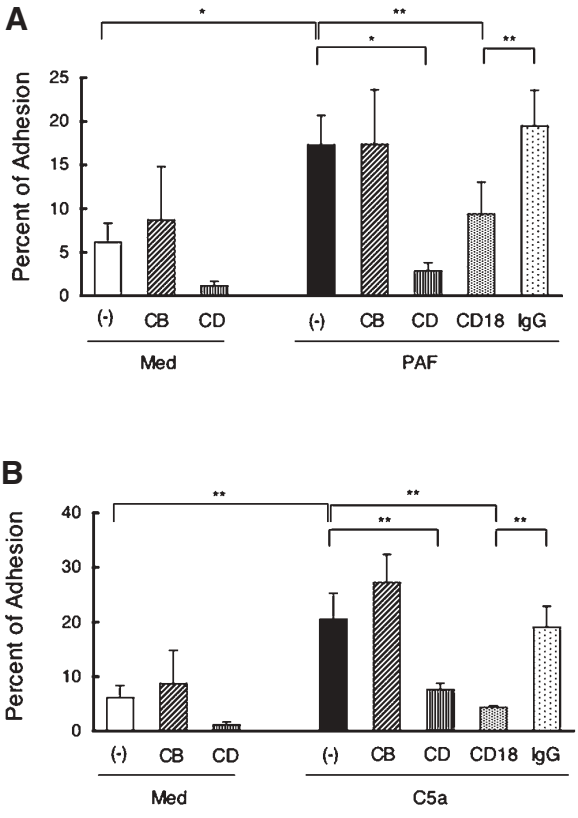

FIG 2. Effects of CB, CD, or anti-CD18 antibody on PAF- or C5a-induced eosinophil adhesion. Eosinophils were stimulated with PAF **(A)** or C5a **(B)** in the presence or absence of CB, CD, anti-CD18 antibody, or mouse $IgG_1κ$. Data are expressed as means ± SEM from 4 to 9 independent experiments. $*P < .01$, $**P < .05$ (significant difference).

DISCUSSION

In the present study, we showed that an anti-CD18 antibody that blocked the β chain of integrin αMβ2 and CD, which disrupts filamentous actin, similarly inhibited stimulus-induced cellular adhesion and subsequent effector functions of eosinophils. On the other hand, we found that inhibition of actin polymerization by CB markedly suppressed PAF-induced eosinophil effector functions, including superoxide anion generation and degranulation and C5a-induced superoxide anion generation, whereas PAF- or C5a-induced cellular adhesion to HSA was unaffected or slightly enhanced by CB. The dissociation between suppressed effector functions and unsuppressed cellular adhesion by CB is noticeable because previous studies have indicated that cellular adhesion and a subsequent outside-in signal are critical for effector functions of eosinophils.[7,27] Our result with anti-CD18 antibody confirmed these observations. Concerning the adhesion-effector function dissociation, we hypothesized that inhibition of actin polymerization by CB exerts inhibition at points relatively downstream from the outside-in signal, given that CB did not affect constitutive or PAF-induced expression of CD11b, a component of integrin αMβ2 (CD11b/CD18; data not shown) or the transient PAF-induced increase in intracellular calcium (Fig 3). Therefore we examined several PKCs, as well as p47^{phox}, a component of NADPH oxidase complex, as signaling molecules likely to be inhibited because these molecules are believed to be closely related to actin during activation.[10-12,28] In particular, NADPH oxidase p47^{phox} can be activated by actin, even in a cell-free system.[13]

Previous reports concerning human neutrophils indicated that activation of PKCs and p47^{phox} could be assessed on the basis of their translocation from cytosol fraction to plasma membrane fraction.[12] Accordingly, we evaluated effects of CB on translocation of PKC βII, PKC δ, PKC ζ, and p47^{phox} after stimulation by PAF or C5a by using immunofluorescence staining for these molecules. We performed staining for PKC βII, PKC δ, PKC ζ, and NADPH oxidase p47^{phox} by using the same procedure at 5, 15, and 30 minutes after stimulation with PAF or C5a. All of these molecules had translocated to just beneath the cell membrane at 5 minutes, where they

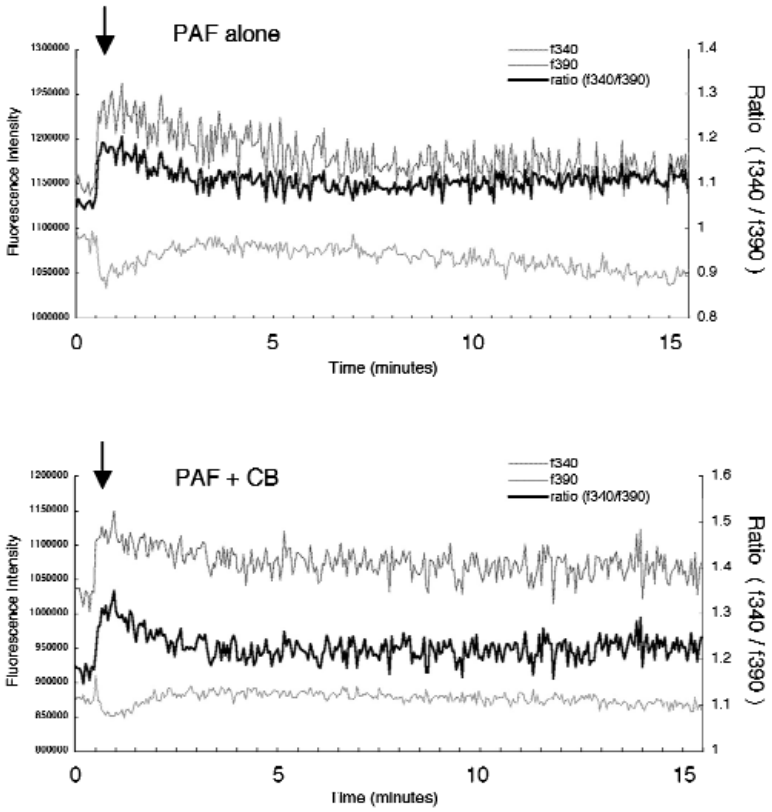

FIG 3. Effect of CB on PAF-induced intracellular Ca^{2+} response in human eosinophils. Fura-2–loaded eosinophils were incubated with *(lower panel)* or without *(upper panel)* CB. PAF was added at 30 seconds *(arrows),* and intracellular Ca^{2+} increase was measured as fura-2 fluorescence. The ratio between fluorescence at 340 nm and fluorescence at 390 nm was calculated. Representative tracings from one of 3 experiments done with cells derived from different donors are shown.

remained even at 30 minutes. This finding agrees with a report that in human neutrophils PKC and NADPH oxidase $p47^{phox}$ translocation occurs 3 minutes after stimulation with phorbol ester.[12] We chose the observation point of 15 minutes after stimulation because superoxide generation is accelerated at this point (data not shown), and translocation of these molecules, especially NADPH oxidase $p47^{phox}$, should strongly reflect their activation, as evidenced by superoxide generation. At all time points, CB inhibited PAF- or C5a-induced translocation of PKC βII, PKC δ, PKC ζ, and NADPH oxidase $p47^{phox}$ and also completely inhibited PAF- or C5a-induced morphologic changes in eosinophils. Taking these findings together, CB does not inhibit expression of β2 integrin, cell adhesion to HSA, or early intracellular calcium response. Instead, CB inhibits stimulus-induced PKC and NADPH oxidase $p47^{phox}$ activation downstream in an outside-in signaling pathway also involving morphologic changes of eosinophils, resulting in the suppression of superoxide anion generation. This is the first report on the mechanisms involved in the inhibitory effect of CB on superoxide generation in human eosinophils analyzing inhibited translocation of signaling molecules, such as the PKCs and $p47^{phox}$.

Stimuli-induced degranulation, another important effector function of eosinophils, was inhibited by CD, whereas it was inhibited by CB only when cells were stimulated with PAF but not with C5a. Although the exact mechanism is unclear, C5a-induced degranulation has been reported to be markedly enhanced by CB in human eosinophils.[29,30] Another report indicated that although CB inhibited C5a-induced superoxide anion generation, C5a-induced degranulation was enhanced,[31] which is consistent with our present result. Contrary to our present findings, however, another report maintained that PAF-induced degranulation was enhanced by CB.[32] Additionally, release of GM-CSF from human eosinophils induced by C5a or FMLP was found to be enhanced by CB.[33] On the other hand, eosinophil degranulation induced by eotaxin,[34] eosinophil granule proteins (eg, major basic protein), or eosinophil peroxidase was abolished with CB.[35] These conflicting lines of evidence suggest that the effects of CB on degranulation of human eosinophils depends on the secretagogues studied and what proteins were secreted by them. Concerning these complicated mechanisms, a previous report that showed FMLP-induced superoxide anion generation by human neutrophils to be enhanced by CB suggested that CB disrupt-

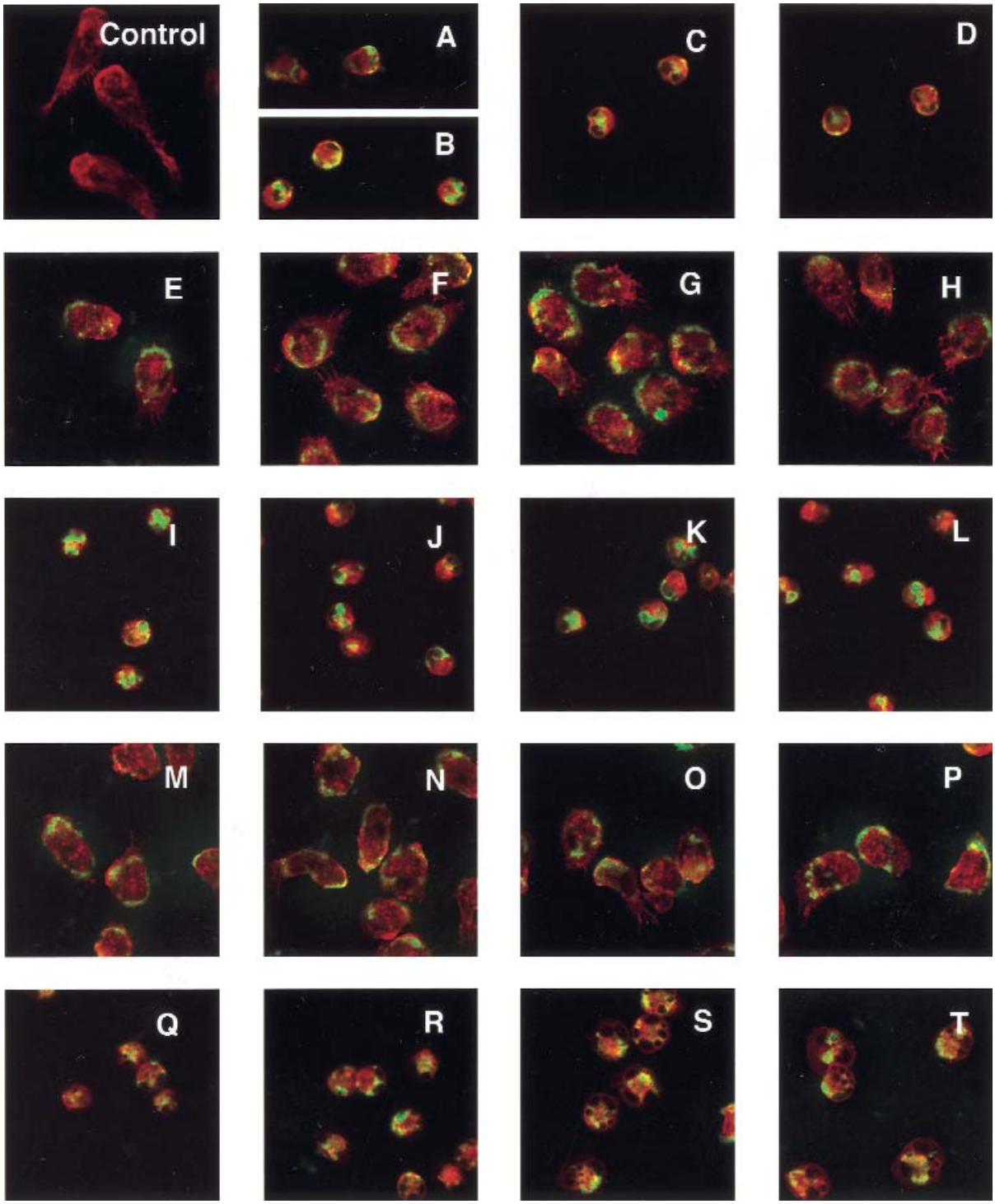

FIG 4. Effects of CB on PAF- or C5a-induced translocation of NADPH p47^{phox}, PKC βII, PKC δ, and PKC ζ in human eosinophils. Eosinophils were stimulated with 1 μmol/L PAF **(E-L)** or 100 ng/mL C5a **(M-T)** with (**I-L**, **Q-T**) or without (**A-D**, **E-H**, **M-P**) CB. *A* through *D* depict unstimulated eosinophils without CB. Cells were fixed and stained with p47^{phox}-specific (*A*, *E*, *I*, *M*, and *Q*), PKC βII–specific (*B*, *F*, *J*, *N*, and *R*), PKC δ–specific (*C*, *G*, *K*, *O*, and *S*), or PKC ζ–specific (*D*, *H*, *L*, *P*, and *T*) goat polyclonal antibody and then exposed to staining cocktail (containing labeled second ligands). *Control* shows the negative control. The experiments were repeated 3 to 5 times, with essentially identical results.

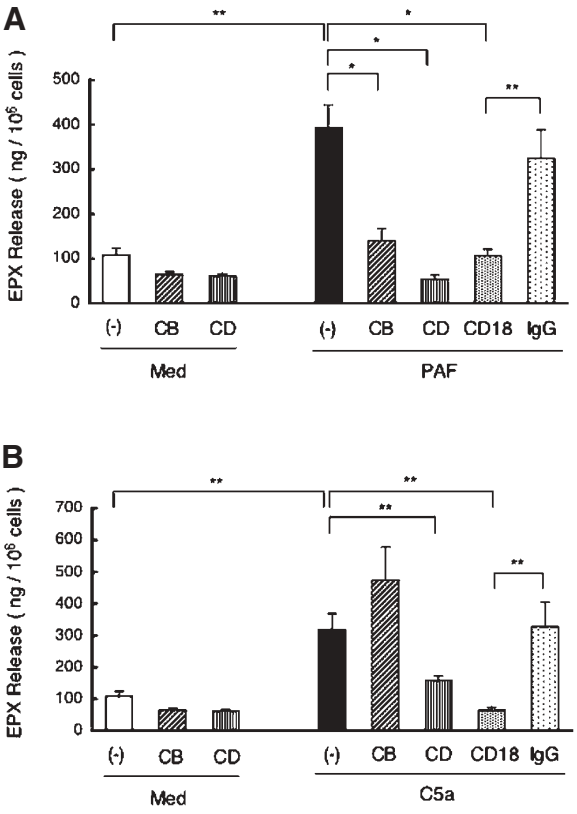

FIG 5. Effects of CB, CD, or anti-CD18 antibody on PAF- or C5a-induced eosinophil degranulation. Eosinophils were stimulated with PAF **(A)** or C5a **(B)** in the presence or absence of CB, CD, anti-CD18 antibody, or mouse $IgG_1\kappa$. The values are expressed as means ± SEM from 4 to 10 independent experiments. Total cellular EPX content was 2875.8 ± 354.5 ng/10^6 cells (mean ± SEM, n = 10). $P < .001$, $^{**}P < .05$ (significant difference).

ed an actin "barrier" surrounding intracellular particles that carried the FMLP receptor.[18] Subsequent overexpression of FMLP receptor and increased functional activity of FMLP resulted, thus upregulating FMLP-induced superoxide generation.[18] If the actin cytoskeleton downregulates receptor availability, such events are consistent with our present finding that C5a-induced degranulation was slightly enhanced by CB.

On the other hand, we found PAF-induced degranulation to be markedly reduced by CB. We speculated two mechanisms on conflicting eosinophil degranulation induced by PAF versus C5a in the presence of CB. First, as described above, CB might have upregulated C5a receptor availability but not PAF receptor availability through actin barrier disruption. Second, the intracellular signaling cascade mediating PAF-induced but not C5a-induced eosinophil degranulation might include PKCs or other molecules that are actin dependent, whereas C5a-induced eosinophil degranulation might involve molecules that are upregulated by actin disruption. However, C5a-induced intracellular calcium increase was not affected by CB (data not shown), suggesting that the possibility of the first speculation mentioned above might be low. In any case, further studies are needed to explain this difference concerning degranulation in detail. In addition, we attempted to confirm the effect of actin depolymerization on the functions of eosinophils by using another compound, CD. In some previous reports, it was shown that CD did not alter stimuli-induced eosinophil adhesion.[24,36] However, in our experiments CD reduced PAF- or C5a-induced cellular adhesion significantly, and the consequent superoxide anion generation and EPX release were also reduced. This discrepancy between CB and CD might be caused by the difference in their affinity for filamentous actin[37,38] and by the actin cleavage effect of CD, which does not occur with CB, whereas actin elongation (ie, addition of incoming globular actin to the barbed end of filamentous actin) is inhibited by both cytochalasins.[37] Taking these observations into consideration, it is reconfirmed that filamentous actin existing in eosinophils before stimulation (which is disrupted by only CD here) might be sufficient for stimuli-induced cellular adhesion, and furthermore, stimuli-induced elongation of filamentous actin (which is inhibited by both CB and CD) is critical for exerting the consequent superoxide anion generation from eosinophils.

In conclusion, we found that actin assembly and cellular adhesion are closely related to superoxide anion generation from human eosinophils. Both events are necessary for eosinophils to express full function. These results provide new evidence that the actin cytoskeleton might be useful as a therapeutic target for suppression of eosinophil functions involved in allergic inflammation.

Mechanisms of allergy

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