Definition of Immunoglobulin A Receptors on Eosinophils and Their Enhanced Expression in Allergic Individuals

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Abstract

FcR receptors (FcR), detected by the binding of IgA and by anti-FcR antibodies, were found to be differentially expressed on eosinophils and neutrophils. Neutrophils were the major granulocyte population expressing FcR, and they expressed much higher levels of FcR than eosinophils. The expression of FcR by eosinophils could be upregulated approximately threefold by Ca2+ ionophore treatment in a dose- and time-dependent manner. This effect, which was blocked by a chelating agent, was not duplicated by other cellular stimuli. Eosinophils in allergic individuals displayed enhanced FcR expression, whereas neutrophils did not. The FcR on eosinophils had a higher molecular mass (70–100 kD) than those identified on neutrophils (55–75 kD). However, removal of N-linked carbohydrates from FcR of eosinophils and neutrophils revealed a major protein core of 32 kD for both cell types. The data indicate that expression of FcR molecules with a characteristic glycosylation pattern is upregulated on eosinophils in allergic individuals. (J. Clin. Invest. 1993, 92:1681–1685.) Key words: FcR receptor • IgA receptor • Fc receptor • eosinophil • allergy binding to specific FcR and FcR present on the cell surface (3, 4). However, IgA is the most abundant Ig isotype in the secretions (5) where eosinophils carry out many of their effector functions. It has been shown that IgA can bind to eosinophils (6, 7), which suggests they may possess IgA receptor(s), but FcR on eosinophils has not been identified and characterized.

An FcR on monocyte/macrophages and granulocytes has been defined as a variably glycosylated protein of 55–75 kD (8, 9) that can bind IgA1 and IgA2 antibodies via their Fc regions (9, 10). The open reading frame of the recently identified FcR gene encodes a transmembrane protein of approximately 30 kD, which has six potential sites for N-linked glycosylation in its extracellular region (11). The selective expression of FcR by myeloid lineage cells has been confirmed by analysis of FcR mRNA expression (11) and FcR molecules identified by monoclonal antibodies specific for native and recombinant FcR protein (12–14).

In this report, we have used the natural IgA ligand and anti-FcR mAbs to analyze the expression, regulation, and biochemical nature of FcR on eosinophils. FcR molecules were detected on eosinophils from all normal individuals following in vitro activation, whereas fresh eosinophils from allergic individuals frequently expressed FcR. The FcR molecules expressed by eosinophils differ from those on neutrophils and macrophages in that the former have a higher content of N-linked carbohydrate moieties.

Methods

Subjects. Heparinized blood samples were obtained from 45 adult individuals (27 male and 18 female). 22 had severe symptoms of allergic rhinitis and/or asthma. These allergic individuals were further selected on the basis of acute wheal and flare reactions in response to two or more allergens when tested by the prick method with extracts of house dust mite, grass pollen, ragweed pollen, tree pollen, and animal danders (15). The other 23 individuals had no history of allergy and were skin-test negative. None of the allergic subjects had received systemic corticosteroids or other medication at the time of blood collection.

Reagents. The following mouse antibodies were used: A3 (γ1x), A59 (γ1x), A62 (γ1x), and A77 (γ1x) mAbs specific for the FcR (13), the 32.2 (γ1x) mAb specific for the FcyR I (CD64) (Medarex, Inc., W. Lebanon, NH), the IV.3 (γ2b) mAb specific for the FcRII (CD32w) (ATCC), the 3G8 (γ1x) mAb specific for the FcγR III (CD16) (16), the W87 (γ2a) anti-CD10 mAb, the LeuM1 (μ) anti-CD15 mAb, and the My4 (γ1x) anti-CD14 mAb (Coulter Corp., Hialeah, FL). Control antibodies included an irrelevant mouse IgG2a (Becton Dickinson & Co., Mountain View, CA). HJH (γ1x) anti-Id (17), the Clq (μ) anti-chicken Ig mAb (18), and the MOPC 141 (γ2b) myeloma. FITC-labeled goat anti–mouse Ig antibodies lacking cross-reactivity with human Ig were from Southern Biotechnology Associates (Birmingham, AL). The human IgA myeloma proteins and F(ab)2 fragments of the corresponding goat anti–Id antibodies are described elsewhere (19).
Isolation of eosinophils and neutrophils. Red cells and granulocytes were first separated from mononuclear cells by centrifugation over Ficoll/Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradients. Granulocytes were isolated from the red cell pellet by differential sedimentation in 1.5% dextran in PBS (9). All reagents used for granulocyte isolation were prepared in pyrogen-free distilled water. Neutrophils and eosinophils of normal density were separated by using a discontinuous metrizamide gradient (20). Purity of eosinophils varied from 60–90%, whereas neutrophils were > 99% pure as determined by morphological characteristics following Giemsa staining. In some experiments, the eosinophils were further enriched (> 99.5%) by fluorescence-activated cell sorting, where eosinophils were selected on the basis of their light scatter characteristics and nonreactivity with anti-CD16 mAb (21, 22). Eosinophils were cultured for 1–18 h in RPMI 1640 supplemented with 25% of autologous human serum (and penicillin and streptomycin) in the presence or absence of various concentrations of Ca2+ ionophore (Ionomycin; Calbiochem, San Diego, CA). Other stimuli included PMA (2–1000 ng/ml), Con A (1–50 μg/ml; Sigma Chemical Co., St. Louis, MO), IFNγ (100 U/ml; Amgen Biologicals, Thousand Oaks, CA), rIFNγ (100 U/ml; Kyowa Hakko, Tokyo Japan), G-CSF (10–10000 pM), and GM-CSF (10–1000 pM). IL-1α (Immunex, Seattle, WA), IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, and TGFβ (Amgen Biologicals) were all used at concentrations of 10–1000 U/ml over a 12-h period of stimulation.

Immunofluorescence analysis of cells. In order to mask FcγR, the cells (1–2 × 10^6) were preincubated with 10 μl of aggregated human IgG (10 mg/ml) for 15 min at 4°C before incubation with 10 μl of test mAb (0.1 mg/ml) for 20 min at 4°C in PBS containing 10% FCS and 0.1% sodium azide. After extensive washing of the cells, FITC-labeled goat antibodies to mouse Ig (0.1 mg/ml) were used as the developing reagent. For detection of IgA binding, an indirect immunofluorescence assay (19) was employed. For this assay, cells were incubated with 10 μl of polymeric IgA (0.5 mg/ml) plus 10 μl of biotin-labeled F(ab′)2 fragments of the corresponding goat anti-IgA antibodies (0.5 mg/ml) for 20 min at 4°C. Phycoerythrin-labeled streptavidin (Becton Dickinson & Co.) was used as a developing reagent. Unrelated IgA myelomas were used as negative controls. Cells were analyzed by flow cytometry using a FACSscan® instrument (Becton Dickinson & Co.).

Immunoprecipitation analysis. Preparations of viable eosinophils (> 99.5% purity) and neutrophils (3–4 × 10^6 cells each) were surface labeled with Na235I (1 mCi; Amersharm Corp., Arlington Heights, IL) by the lactoperoxidase method (23). After washing, cells were lysed in 1 ml of 0.5% NP-40 in PBS containing 0.02% sodium azide, 1% aprotinin, 1 mM disopropylfluorophosphate, 5 mM iodoacetamide, and 1 mM PMFS (9, 13). Cell lysates were cleared by four separate incubations with 20 μg of an isotype-matched control mAb (JH3) plus 30 μl of goat anti-mouse Ig antibody-coupled Sepharose 4B beads (3 mg/ml packed beads) for 2–4 h at 4°C with constant rotation, and by two additional incubations with the anti-mouse Ig-coupled beads only. The absorbed lysates were divided into three aliquots that were incubated with control (JH3) or test (A59 or A62) mAb in the presence of 3 μl of anti-mouse Ig-coupled beads for 4 h at 4°C with constant rotation. After washing extensively with lysis buffer, bound materials were dissociated by addition of 0.5% SDS–0.1 M 2-mercaptoethanol and subjected to N-glycanase digestion as described (9). Digested and undigested materials were resolved by SDS–PAGE analysis (24) using 10% acrylamide. Molecular weight markers were purchased from Bio-Rad Laboratories (Richmond, CA).

Statistical analysis. The results were analyzed with the independent sample two-tailed Student's t test, and evaluation for correlation was conducted by linear regression analysis.

Results

Expression of FcγR on eosinophils from normal and allergic individuals. The eosinophil population was discriminated from the neutrophil population by differences in forward light scatter and side light scatter characteristics determined by flow cytometric analysis (21, 22). A forward-side scatter population distinct from neutrophils was first observed when eosinophils were partially purified on metrizamide gradients. Sorting for this population resulted in an enrichment in eosinophils to > 98% purity as determined by microscopic evaluation of stained cells. These forward-side scatter characteristics were then used to establish analytical gates for immunofluorescence analysis of eosinophils and neutrophils. The eosinophils were also distinguishable from neutrophils by their lack of or relatively weak expression of cell surface CD10, CD14, CD15, CD16, and CD64 antigens, whereas both cell types expressed CD52 and CD11b antigens.

To evaluate FcγR expression on blood granulocyte subpopulations, purified eosinophils and the purified neutrophils were examined for binding to either the polymeric IgA ligand or the anti-FcγR mAbs by indirect immunofluorescence. Whereas the eosinophils from an allergic individual expressed easily detectable levels of IgA binding and reactivity with the A59, A3, A62, and A77 anti-FcγR mAb, the eosinophils from a normal individual were noticeably less reactive in both assays (Fig. 1). The level of FcγR expression on normal eosinophils was approximately 10-fold lower than on neutrophils as estimated by mean fluorescence intensities (1.3±1.0 vs. 13±5; Fig. 2).

Because the FcγR was more easily identified on eosinophils from an allergic individual, a comparative analysis of anti-FcγR and anti-FcyR reactivity was performed using eosino-
phils and neutrophils from both allergic and normal individuals (Fig. 2). Anti-FcαR mAb reactivity was significantly increased ($P < 0.001$) for eosinophils from 22 allergic individuals (2.9 ± 1.9) as compared with eosinophils from 23 normal individuals (1.3 ± 1.0). FcγRIII expression was also higher on eosinophils from allergic individuals ($P < 0.002$), albeit at low levels relative to that on neutrophils (Fig. 2). In contrast, FcγRII expression was similar for both cell types in normal and allergic individuals.

Levels of IgA, IgG, and IgM were found to be in the normal range for all of the allergic individuals (data not shown), whereas increased IgE levels of > 100 μg/ml were found in more than half of the allergic individuals (12/22) and none of the controls (0/23). Significant correlation was not observed between FcαR levels on either cell type and serum Ig isotype levels. In addition, no significant differences were observed in neutrophil expression of FcαR and FcγR for normal and allergic individuals.

**Regulation of FcαR expression on normal eosinophils.** Highly purified eosinophils (> 99.5%) were examined for FcαR and FcγR expression before and after treatment with various cellular stimuli. FcαR expression was upregulated by approximately threefold following Ca$^{2+}$ ionophore stimulation (Fig. 3), whereas parallel experiments revealed no increase in the level of FcαR expression by cells of the U937 monocytic cell line (not shown). The increase in eosinophil FcαR expression was ionophore dose dependent with the maximal response occurring at a concentration of 10 μM. The increase in FcαR expression, which was maximal at 12 h after Ca$^{2+}$ ionophore stimulation, was inhibited by the chelating agent EGTA (Fig. 4). These results were obtained in experiments employing both the A59 and A62 anti-FcαR mAbs and the IgA ligand itself (not shown). In contrast, eosinophil FcγRII and FcγRIII levels were unaffected by Ca$^{2+}$ ionophore stimulation (data not shown).

FcγR expression by normal eosinophils was unaffected by similar treatment with a variety of other stimuli, including PMA, Con A, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IFN-α, -β, and -γ, TGFβ, G-CSF, and GM-CSF (data not shown). Changes were not observed even after exposure of the eosino...

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**Figure 2.** Expression levels of FcαR and FcγR on eosinophils and neutrophils from normal (○) and allergic (●) individuals. Purified eosinophils and neutrophils were stained with anti-FcαR (A59), anti-FcγRII (IV.3), anti-FcγRIII (3G8), or with isotype matched control mAbs as described in Fig. 1. FITC-conjugated goat antimouse Ig antibodies were used as a developing reagent. Immunofluorescence intensity was analyzed by flow cytometry using forward and side light scatter gates specific for each cell type. Results were expressed as the mean fluorescence intensity, which was estimated by: $x$ of staining with anti-FcR mAbs $- x$ of background control staining with isotype matched controls, in which $x$ indicates the computer-determined value for mean intensity for each fluorescence profile. The mean fluorescence intensities of FcαR and FcγRIII on eosinophils were statistically different in both groups of subjects ($P < 0.001$ and $< 0.002$, respectively). Note differences in the vertical scales for the panels.

**Figure 3.** The effect of calcium ionophore stimulation on FcαR expression by normal eosinophils. FACS-purified eosinophils (> 99.5% pure) incubated for 12 h with various doses of Ca$^{2+}$ ionophore (A) and for varying time intervals with 10 μM Ca$^{2+}$ ionophore (B) were washed and stained with an anti-FcαR mAb (A59) or the IgG1x control mAb (JH-3) as described in Fig. 1. The immunofluorescence intensity determined by flow cytometry was used to estimate an FcαR expression index ($x$ of staining of Ca$^{2+}$ ionophore-treated cells $- x$ of background control staining of Ca$^{2+}$ ionophore-treated cells)/($x$ of staining of nontreated cells $- x$ of background control staining of nontreated cells); $x$ indicates the computer-determined value for the mean fluorescence intensity of each FACS profile.

**Figure 4.** Calcium ionophore-induced upregulation of FcαR on eosinophils is blocked by the chelating agent EGTA. Blood eosinophils of 99.5% purity were stimulated with Ca$^{2+}$ ionophore (10 μM) in the presence or absence of EGTA (5 μM) for a 12-h period, washed, and stained with an anti-FcαR mAb (A59) as described in Fig. 1. The dotted histogram represents background fluorescence with a nonreactive, isotype-matched control mAb.
phil to cytokine combinations, such as IL-3, IL-5, and GM-CSF, which have been shown to induce eosinophilic differentiation (25). FceR expression reactivity was also unaffected when normal eosinophils were cultured in serum from allergic individuals.

**Biochemical nature of eosinophil FceR molecules.** Eosinophils highly purified (> 99.5% pure) by fluorescence-activated cell sorting were used for these studies in order to avoid neutrophil contamination, since the neutrophils express relatively high levels of FceR molecules. Eosinophils in sufficient numbers (~ 4 x 10^6) were obtained using blood samples from allergic individuals for high numbers of blood eosinophils expressing relatively high FceR levels. When iodinated cell surface proteins from the eosinophils were immunoprecipitated with A59 and A62 mAb, a broad 70–100-kD band could be identified (Fig. 5, lanes 9 and 11) whereas FceR molecules isolated from neutrophils had a molecular mass of 55–75 kD (lanes 3 and 5). Removal of N-linked carbohydrate moieties from the FceR molecules on eosinophils yielded the same major 32-kD protein core as that found on neutrophils (lanes 10 and 12 vs. lanes 4 and 6), whereas the minor 36-kD protein band seen in neutrophils was not observed in the N-glycanase-treated lysates of eosinophils. This result was confirmed using eosinophils from five other allergic individuals.

**Discussion**

The results of this study confirm that eosinophils can bind IgA (6, 7), and demonstrate their expression of FceR, albeit in lower levels than on neutrophils. Eosinophil expression of FceR was selectively enhanced by treatment with a Ca^{2+} ionophore, whereas FcyRII and FcyRIII expression were unaffected. Our results further suggest that FceR expression may be differentially regulated depending on the myeloid cell lineage. Treatment of monocytic cell lines (U937 and PLB985) with phorbol ester enhanced their expression of FceR (9, 13), but did not affect FceR expression by eosinophils. Conversely, while a Ca^{2+} ionophore enhanced FceR expression by eosinophils, this treatment did not alter FceR expression on U937 monocytoid cells. The existence of differential regulatory elements for FceR expression in different myeloid cell types is thus suggested by these results.

Since enhanced FceR expression by eosinophils can occur as a consequence of in vitro cell activation, the finding of enhanced FceR expression on eosinophils in allergic individuals may reflect in vivo activation. IgA is the major Ig isotype in the secretions where eosinophils carry out some of their effector functions (1–5) implying functional relevance of the FceR on eosinophils. Cytophilic IgA has been detected on the surface of intestinal eosinophils in parasite-infected mice (26). Moreover, treatment of eosinophils with secretory IgA antibodies resulted in the release of eosinophil-derived neurotoxin (7). In the latter study, secretory IgA was shown to initiate a signal for eosinophil degranulation that was two to three times more potent than that of IgG antibodies. FceR on activated eosinophils could thus play an effector role in host defense against helminth infections and in the pathogenesis of the inflammation in hypersensitivity diseases, such as asthma (27).

Not all allergic individuals exhibit upregulated FceR expression on the eosinophils. In approximately 25% of our patients with severe allergic rhinitis, asthma, or both, the eosinophils expressed FceR below or near the normal mean level. Neither serum IgE levels nor clinical status were obvious correlates with the eosinophil FceR levels, indicating further need for investigation into the basis for the FceR upregulation in allergic individuals.

The FceR molecules on eosinophils are closely related to the FceR on neutrophils, as both are recognized by monoclonal anti-FceR antibodies. However, we found that the FceR molecules isolated from eosinophils have higher molecular mass (70–100 kD) than those from neutrophils (55–75 kD). A previous report had suggested that a 55–60-kD IgA receptor exists on eosinophils (28) and, in preliminary immunoprecipitations of surface molecules from eosinophil preparations obtained by metrizamide gradients, we also identified FceR with molecular masses of 55–75 kD. In our studies, however, these FceR molecules were found to originate from contaminating neutrophils (5–10%), which express relatively high levels of FceR, and more rigorous eosinophil purification led to elimination of the 55–75-kD FceR and its replacement by the 70–100-kD FceR form.

The relative increase in FceR size on the eosinophil was found to be due to differential glycosylation of a single protein core. Removal of N-linked carbohydrates by N-glycanase digestion of FceR from eosinophils yielded a single protein band of 32 kD, whereas similar treatment of FceR from neutrophils gave rise to both 32- and 36-kD bands. This result was confirmed using two monoclonal anti-FceR antibodies, A59 and A62 (13), both of which are reactive with the recombinant product of the single FceR gene (11, 14). Our results therefore provide further evidence for molecular heterogeneity of FceR molecules on cells of neutrophil, monocyte/macrophage, and
eosinophil lineages, and suggest that differently glycosylated FcR are created by posttranslation modification. Because only one FcR gene can be identified with the available FcR cDNA probe, the theoretical possibility of two closely related genes with different numbers of glycosylation sites seems unlikely. Elucidation of the precise molecular basis for the differential glycosylation of FcR molecules on the different cell types is needed because differences in the carbohydrates moieties could influence ligand binding, thus affecting specific effector functions mediated by the different types of myeloid cells.

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