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J Immunol 1999; 162:6901-6911; ;
http://www.jimmunol.org/content/162/11/6901

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Does IgE Bind to and Activate Eosinophils from Patients with Allergy?1

Hirohito Kita,2 Masayuki Kaneko, Kathleen R. Bar temes, Deborah A. Weiler, Andrew W. Schimming, Charles E. Reed, and Gerald J. Gleich

Human eosinophils have been reported to express both the mRNA and protein for the high affinity IgE receptor (FcεRI); it is speculated that this receptor plays a role in eosinophil mediator release in allergic diseases. However, questions still remain. How much of the FcεRI protein is actually expressed on the cell surface of the eosinophil? If they are present, are these IgE receptors associated with effector functions of eosinophils? To address these issues, we studied blood eosinophils from patients with ragweed hay fever. A high level of low affinity IgG receptor (FcγRII, CD32), but no expression of FcεRI, was detectable on the eosinophil surface by standard FACS analysis. However, after in vitro sensitization with biotinylated chimeric IgE (cIgE), cell-bound cIgE was detected by PE-conjugated streptavidin. This cIgE binding was partially inhibited by anti-FcεRI mAb, suggesting that eosinophils do express minimal amounts of FcεRI detectable only by a sensitive method. Indeed, FACS analysis of whole blood showed that eosinophils express ~0.5% of the FcεRI that basophils express. When stimulated with human IgE or anti-human IgE, these eosinophils did not exert effector functions; there was neither production of leukotriene C4 or superoxide anion nor any detectable degranulation response. In contrast, eosinophils possessed membrane-bound human IgG and showed functional responses when stimulated with human IgG or anti-human IgG. Thus, IgG and/or cytokines, such as IL-5, appear to be more important for eosinophil activation in allergic diseases than IgE.

Eosinophils play important roles in the pathophysiology of bronchial asthma and other allergic diseases (reviewed in Ref. 1). In such diseases, mediators released by T cells, epithelial cells, and other inflammatory cells induce migration of eosinophils from blood into the affected tissues (reviewed in Ref. 2). Subsequently, various stimuli trigger eosinophil activation, resulting in the local release of a series of inflammatory mediators, such as lipid metabolites (2, 3), superoxide anion (4), and toxic cationic granule proteins (5). Indeed, eosinophilic infiltration in the epithelium and submucosa of the airways has been a hallmark of the pathology of asthma and allergic rhinitis (6, 7). Correlations have been observed between the number of infiltrating eosinophils and asthma disease severity (6). In addition, pulmonary segmental allergen challenge in allergic individuals causes eosinophil recruitment into the airways; this is associated with the release of eosinophil granule proteins and an increase in vascular permeability (8, 9). In spite of this strong association between eosinophils and allergic diseases, the activation mechanism(s) of eosinophils in vivo are largely unknown.

A critical feature of the allergic response is the release of histamine and other inflammatory mediators from mast cells and basophils after allergen cross-links specific IgE Ab occupying high affinity IgE receptors (FcεRI) (10). Furthermore, epidemiological studies show a close correlation between serum IgE levels and the prevalence and severity of the bronchial asthma (11). Thus, there is converging evidence to support a role for IgE in the pathophysiology of allergic diseases in humans. Earlier, eosinophils were considered to express only low affinity IgE receptor (FcεRII) (12); however, recent studies by Gounni et al. (13) demonstrated that eosinophils express FcεRI and that they release inflammatory mediators and exert cytotoxicity to helminths through this receptor. Subsequent immunohistochemical and immunocytochemical studies on patients with allergic diseases showed that local allergen provocation induces expression of FcεRI by eosinophils infiltrating into the airways (14) and skin (15, 16). Furthermore, FcεRI was detected in blood eosinophils from patients with various allergic diseases (17). Therefore, it is reasonable to speculate that FcεRI plays a role in the eosinophil secretory process in allergic diseases.

There are caveats to this conception. Most of the patient studies were performed by histologic examination using in situ hybridization and immunocytochemical techniques. Therefore, little is known about the actual expression of FcεRI protein on the eosinophil surface. For example, in human T cells, the low affinity IgG receptor proteins (FcγRII, CD32) are stored within the cytoplasm but are not expressed on the cell surface (18), and their surface expression was detected only by a highly sensitive biotin-avidin flow cytometric technique (19). Furthermore, the functional significance of the FcεRI expression by eosinophils from patients with allergic diseases is also unknown. To address these questions, we studied blood eosinophils from patients with ragweed hay fever during the peak of the allergy season. We had three goals. First, we examined whether FcεRI proteins are expressed on the surface of the eosinophil, and we compared the expression levels to known FcεRI-bearing cells, such as basophils. Second, we examined whether FcεRI confers IgE binding to eosinophils. Finally, we examined whether IgE receptors stimulate mediator release by these eosinophils.

1 This work was supported by grants from the National Institutes of Health, AI 34577 and AI 34486, and by Mayo Foundation.

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Materials and Methods

Patients

Thirty patients, 18–60 yr of age, with moderate to severe ragweed allergic rhinitis and 14 healthy nonatopic subjects were studied. The patients had a history of ragweed hay fever, a positive skin test to ragweed extract, and an elevated level of IgE Ab for short ragweed (305–2190% of normal, median 1114%). Patients were excluded if they were receiving any forms of glucocorticoid treatment within the preceding 6 mo or immunotherapy within the preceding 12 mo. Each patient kept a diary of daily symptom scores for nasal congestion, pruritus, or discharge, ocular pruritus, eye watering, eye redness, and pruritus of the ear or palate to validate their clinical reaction to airborne ragweed allergen. Normal control subjects had no clinical or laboratory evidence of atopy. Blood samples were obtained from patients and control subjects during the peak of hay fever season in Rochester, MN (end of August) and used as described below. The study was approved by the Institutional Review Board of the Mayo Clinic and Mayo Foundation.

Antibodies

Anti-FcεRI α subunit mAb (15-1, mouse IgG1 (mlgG1)) and biotinylated chimeric mouse/human IgE monoclonal protein (cIgE) were kind gifts from Dr. J.-P. Kinet, Beth Israel Deaconess Medical Center, Harvard Medical School (Boston, MA). Concentration-response experiments to establish saturating concentrations for cIgE to sensitize human eosinophils in vitro and for 15-1 to inhibit the sensitization of cIgE were performed before this study began (our unpublished results); the saturating concentrations were ~25 μg/ml for cIgE and 10 μg/ml for 15-1 per 1 x 10^6 eosinophils. Anti-CD11b mAb (Bael1, mlgG1), anti-FcεRII (CD23) mAb (9P25, mlgG1), anti-CD25 mAb (B1.49.9, mlgG2a), anti-CD32 mAb (E1, mlgG2a), and anti-CD69 mAb (TP1.55.3, mlgG2b) were purchased from Immunotech (Westbrook, ME). Control mouse myeloma Ig, including mlgG1, mlgG2a, and mlgG2b, were obtained from ICN Pharmaceuticals (Costa Mesa, CA). PE-conjugated F(ab′)_2 fragments of sheep IgG anti-mlgG and PE-conjugated streptavidin were purchased from Sigma Chemical (St. Louis, MO) and Becton Dickinson (Mountain View, CA), respectively. FITC-conjugated goat IgG anti-human IgE, FITC-conjugated goat anti-human IgE, and FITC-conjugated goat anti-mouse IgM were from BioSource International (Camarillo, CA).

Reagents for cell functional analyses

We used myeloma IgE purified from serum from a patient with multiple myeloma as described elsewhere (5). Purified human IgE was also purchased from Cortech Biochem (San Leandro, CA). We performed all the experiments with both the IgE preparations and found that the results were virtually identical. Therefore, only results from our myeloma IgE preparation(s) are shown. Purified human serum IgG was purchased from ICN Pharmaceuticals. F(ab′)_1 fragments of goat IgG anti-human IgG and F(ab′)_2 fragments of goat IgG anti-human IgE were purchased from ICN Pharmaceuticals and DiaMed (Windham, ME), respectively. F(ab′)_2 fragment of goat IgM was obtained from Jackson ImmunoResearch Laboratories, West Chester, PA.

Cell preparation

Eosinophils were isolated from six patients and six normal subjects by a magnetic cell separation system (MACS, Becton Dickinson, San Jose, CA) as described previously with minor modifications (20). Briefly, 60 ml of venous blood anticoagulated with 50 U/ml heparin were diluted with PBS at a 1:1 ratio. Diluted blood was overlaid on isotonic Percoll solution (density, 1.085 g/ml, Sigma) and centrifuged at 1000 x g for 30 min at 4°C. The supernatant and mononuclear cells at the interface were carefully removed, and erythrocytes in sediment were lysed by two cycles of hypotonic water lysis. Isolated granulocytes were washed twice with PIPES buffer (25 mM PIPES, 50 mM NaCl, 5 mM KCl, 25 mM NaOH, 5.4 mM glucose, pH 7.4) containing 1% alpha calf serum (HyClone Laboratories; Logan, UT). An approximately equal volume of anti-CD16-conjugated magnetic beads (Miltenyi Biotec, Auburn, CA) was added to the cell pellet. After 60 min of incubation on ice, cells were loaded onto the separation column positioned in the strong magnetic field of the MACS. Cells were eluted three times with 5 ml of PIPES buffer with defined calf serum. The purity of eosinophils counted by Randolph’s stain was regularly ≥98%

Three times with 5 ml of PIPES buffer with defined calf serum. The purity of eosinophils counted by Randolph’s stain was regularly ≥98%.

Purified eosinophils were washed and suspended in reaction medium and then used immediately.

Basophils were obtained as the mononuclear cell layer after density gradient centrifugation from the same donor used for eosinophil isolation. Briefly, heparinized venous blood was centrifuged over Histopaque 1077 (Sigma), following the procedure recommended by the manufacturer. The mononuclear cell layer was collected, washed with PIPES buffer, suspended in reaction medium, and used immediately. The purity of basophils counted by Wright-Giemsa staining of cytopsin preparations was between 0.5% and 2%.

Flow cytometric analysis

Eosinophil activation markers. Expression of CD25, CD69, and CD11b on eosinophils was measured by flow cytometry as an indicator of eosinophil activation (21–23). To avoid purification-induced activation or inadvertent subset selection, total leukocyte samples were stained with mAb and eosinophils and were electronically gated during FACS analysis. Briefly, 5 gradients of heparinized whole blood from patients and normal subjects were incubated with 1 part of 6% hetastarch in 0.9% NaCl (Hesper, DuPont Pharmaceuticals, Wilmington, DE) for 45 min at 37°C to sediment erythrocytes. The buffy coat was collected, and the remaining erythrocytes were lysed by erythrocyte lysis solution (Becton Dickinson). Buffy coat leukocytes were washed with PBS containing 0.1% NaN3 and 1% BSA (PAB buffer). The cells were resuspended in PAB buffer at 2 x 10^6 cells/ml and plated in 96-well plates at 10 μl/well. One microgram of anti-CD11b, anti-CD25, anti-CD69, or isotype-matched control mouse Ig was added separately to individual wells and incubated for 30 min at 4°C. After washing, cells were resuspended with 100 μl of PE-conjugated F(ab′) fragments of sheep anti-mouse IgE diluted in PAB buffer at 1:400 and incubated in the dark for 30 min. The samples were washed and fixed with 1% paraformaldehyde. Purity analysis was performed within 2 h using a FACSscan flow cytometer (Becton Dickinson). Established programs were used to ensure consistent instrument settings (e.g., laser power, compensation, gain, scaling, etc.) among experiments. Two-dimensional dot-plot analysis of the green fluorescence intensity and side scatter revealed four distinct populations of leukocytes, including lymphocytes, monocytes, neutrophils, and eosinophils. Eosinophils were identified as a cell population with the strongest green autofluorescence and side scatter as previously described (24). Appropriateness of the identification of the eosinophil population was confirmed by positive staining for anti-CD9 mAb (Immuno-tech) as well as morphological analysis of cytopsin preparation of sorted cells. The cells were electronically gated on this eosinophil population, and the expression of activation markers was measured by examining the intensity of PE fluorescence. Fluorescence intensity was determined on the gated eosinophils using log-linear amplification and presented as the differences in the mean fluorescence channel (ΔMFC) between the test mAb (e.g., anti-CD11b) and isotype-matched control mouse Ig by Becton Dickinson Lysis II software.

Receptor expression

Expression of receptors for IgE and IgG, including FcεRI, FcεRII (CD23), and FcγRI (CD32), by eosinophils was examined by a standard FACS technique using isolated eosinophils. To release putative in vivo bound IgE molecules from the surface of the eosinophil, eosinophils were subjected to acid stripping as described previously (25). Briefly, isolated eosinophils were washed with cold 0.15 M NaCl. The pellets were treated with lactic acid buffer (130 mM NaCl, 5 mM KCl, 10 mM lactic acid, pH 3.9) for 3.5 min. Cells were washed with PAB buffer and suspended in the same buffer at 5 x 10^6 cells/ml. Aliquots (100 μl) of cell suspension were added to 15-ml polypyrrole conical tubes, mixed with saturating amounts of anti-FcεRI mAb (15-1), anti-FcεRII mAb (9P25), or anti-FcγRII mAb (2E1), or the same amount of control mouse Ig. After an incubation for 30 min on ice, the cell pellets were washed twice with cold PAB buffer and mixed with 100 μl of 1:400 dilution of PE-conjugated F(ab′)_2 fragments of sheep anti-mouse IgG. After an additional incubation on ice for 30 min in the dark, the cell pellets were washed twice with PAB buffer and fixed with 1% paraformaldehyde. Fluorescence analysis was conducted in a FACScan flow cytometer. Fluorescence intensity was determined on 10,000 cells from each sample using logarithmic amplification and presented as the differences in the mean fluorescence channel (ΔMFC) between the test mAb (e.g., anti-CD11b) and isotype-matched control mouse Ig by Becton Dickinson Lysis II software.

Ig binding

Binding of IgE or IgG to eosinophils and other leukocyte populations was examined before and after in vitro passive sensitization with these Ig. Isolated eosinophils or buffy coat leukocytes were obtained as described

3 Abbreviations used in this paper: mlgG, mouse IgG1; cIgE, chimeric mouse/human IgE; EDN, eosinophil-derived neurotoxin; HSA, human serum albumin; LT, leukotriene; PAB, PBS containing 0.1% NaN3, and 1% BSA; ΔMFC, differences in the mean fluorescence channel; EIA, enzyme immunoassay.
Eosinophil and basophil degranulation

Eosinophils and basophils were stimulated with anti-human IgE or anti-human IgG, either immobilized onto the wells of tissue culture plates or in solution, or FMLP (Calbiochem-Novabiochem, San Diego, CA) in solution. Polystyrene 96-well flat-bottom tissue culture plates were coated with 50 µg/ml Fab_{1,2} fragments of goat anti-human IgE, goat anti-human IgG, or goat IgG (control); blocked with 2.5% HSA; and washed with saline as described above. Plates used for anti-IgE, anti-IgG, or FMLP in solution were blocked with HSA. Freshly isolated eosinophils or basophil preparations were suspended in RPMI 1640 supplemented with 10 mM HEPES and 0.1% NaN₃ and resuspended in the medium at 5 × 10⁵ cells/ml and 1 × 10⁵ cells/ml, respectively. Aliquots of cell suspensions (100 µl) were added to the wells and stimulated with serial dilutions (Fab_{1,2} fragments of goat anti-human IgE, goat anti-human IgG or goat IgG control), or 1 µM FMLP (100 µl). Cells incubated in the wells coated with anti-IgE received 100 µM of medium alone. Cells were incubated for 60 min (basophil preparations) or 180 min (eosinophils) at 37°C and 5% CO₂. After incubation, supernatants were collected and stored at −20°C until assayed. To quantify basophil degranulation, the concentration of histamine in the sample supernatants was measured by histamine enzyme immunoassay (EIA) kit (Immunotech) following the procedure recommended by the manufacturer. To quantitate eosinophil degranulation, the concentration of eosinophil-derived neurotoxin (EDN) in the sample supernatants was measured by RIA. The RIA is a double-Ab competition assay in which radioiodinated EDN, rabbit anti-EDN, and burro anti-rabbit IgG are used, as reported elsewhere (5). Total cellular histamine and EDN contents were measured simultaneously using supernatants from cells lysed with 0.5% Nonidet P-40 detergent. The sensitivities of histamine EIA and EDN RIA were 0.2 nM and 2.0 ng/ml, respectively. All assays were done in duplicate.

Statistical analysis

Data are presented as mean ± SEM from the numbers of experiments indicated. Statistical significance was assessed using the Mann-Whitney U test or paired Student’s t test.

Results

Expression of activation markers by eosinophils from patients with hay fever

Generally, in Rochester, MN, ragweed hay fever season starts in early August, peaks at the end of August, and continues until the end of September. In the current study, patients’ symptoms increased in parallel to the pollen counts and peaked during the peak pollination period (late August through early September). Similarly, patients’ peripheral blood eosinophil counts increased significantly during the peak hay fever season from the values before the season (284 ± 41/µl at peak season vs 141 ± 19/µl before the season (early July); n = 30, p < 0.01). Initial FACS analyses of peripheral blood eosinophils validated in vivo “activation” of the patients’ eosinophils. These cells expressed significantly higher levels of several eosinophil cell surface activation markers, including CD25, CD69, and CD11b (21–23), compared with eosinophils from normal subjects (Fig. 1). The increased expression of CD11b was especially pronounced. In addition, when stimulated in vitro with 1 µM FMLP, patients’ eosinophils released significantly larger amounts of the eosinophil granule protein, EDN, than normal subjects’ eosinophils (27.3 ± 5.6 and 7.8 ± 1.1% of total EDN for patients and normal subjects, respectively; n = 6, p < 0.05). These findings suggest that blood eosinophils from patients with hay fever are “activated” compared with those from normal
Comparison of Responses to IgE by Eosinophils and Basophils

**Methods**

Leukocytes from patients with hay fever or normal subjects were stained with anti-CD25, anti-CD69, or anti-CD11b mAb or isotype-matched control mouse Ig followed by PE-conjugated F(ab’)2 fragments of sheep anti-mouse IgG. Cells were analyzed by FACScan flow cytometer and were electronically gated as described in Materials and Methods. Data are presented as the differences in the mean fluorescence channel (ΔMFC) between the test mAb (e.g., anti-CD11b) and isotype-matched control mouse Ig. Each dot represents the result from an individual subject. Significant differences between patients and normal groups are shown.

Expression of IgE and IgG receptors by eosinophils from patients with hay fever

We next examined whether patients’ eosinophils can bind IgE or IgG on their surfaces and whether they express receptors for these Ig. FACS analysis of cell-bound IgE with FITC-conjugated anti-IgE Ab showed that none or minimal amounts of IgE were present on the surface of freshly isolated eosinophils (Figs. 2 and 3A). When these eosinophils were incubated with IgE in vitro for 2 h, the amounts of bound IgE significantly increased (p < 0.01, Fig. 3A), suggesting that these eosinophils have the capacity to bind IgE. Freshly isolated eosinophils bound detectable amounts of IgG on their surface (Figs. 2 and 3A), and the levels increased further by in vitro incubation with IgG (Fig. 3A, p < 0.01). Thus, patients’ eosinophils were able to bind both IgE and IgG in vitro although the amounts of IgE bound in vivo were negligible.

Another question remains. Do eosinophils express Fc receptors accountable for binding these Ig? To detect IgE receptors, eosinophils were pretreated with lactic acid to remove any bound IgE and incubated with anti-receptor mAb and PE-conjugated secondary Ab. Surprisingly, no receptors for FcεRI or FcεRII were detected by 15-1 or 9P25 mAb, respectively, in any of the six patients (Figs. 2 and 3B). In contrast, FcγRII was easily detectable on the same eosinophils (Figs. 2 and 3B), consistent with the IgG binding capacity of eosinophils. The experiments were repeated without removing IgE from eosinophils; neither FcεRI nor FcεRII could be detected on these nonstripped eosinophils (data not shown).

Analysis of FcεRI expression by biotinylated cIgE

Perhaps we were unable to detect FcεRI on eosinophils because of the sensitivity limitations of the standard FACS technique. Therefore, to enhance the signals for FACS analysis, we removed any IgE bound to the cells by lactic acid treatment, then incubated them in vitro with biotinylated cIgE, and visualized them with PE-conjugated streptavidin. Fig. 4A shows that the binding of cIgE to eosinophils was clearly detectable. Furthermore, the cIgE binding was inhibited by pretreatment of cells with anti-FcεRI mAb (15-1) (Fig. 4B); the isotype-matched control Ig for anti-FcεRI did not affect cIgE binding to eosinophils. Similar analyses of eosinophils from six patients showed that this observation is reproducible and that the binding of cIgE was significantly blocked by anti-FcεRI mAb (Fig. 5A, p < 0.01). The inhibitory effects of anti-FcεRI mAb were variable among the patients, suggesting a wide variance in the expression levels of FcεRI. Interestingly, eosinophils from normal subjects also bound cIgE, and the binding was also inhibited partially but significantly by anti-FcεRI mAb (Fig. 5A, p < 0.05). The amounts of bound cIgE levels as well as the amounts of cIgE-binding inhibited by anti-FcεRI mAb tended to be higher in eosinophils from patients compared with eosinophils from normal subjects. Recent studies on mast cells and basophils show that the density of cell surface FcεRI is regulated by blood IgE levels (29, 30). In eosinophils, we also found that the expression of FcεRI, as estimated by anti-FcεRI-inhibitable binding of cIgE,
showed a strong linear correlation with serum levels of total IgE (Fig. 5B, \( r^2 = 0.941 \)).

**Whole blood analysis of FcεRI expression**

Although we could detect FcεRI expression by the clgE-biotin-streptavidin technique (Figs. 4 and 5), the failure to detect by the standard FACS technique (Figs. 2 and 3) suggested that the density of FcεRI expressed on the surface of the eosinophil is extremely low. Basophils are known to express a high density of FcεRI (31) and blood monocytes from patients with allergy also express FcεRI (32). Therefore, we compared the expression levels of FcεRI by eosinophils to other leukocytes. To this end, total leukocytes were sensitized with biotinylated clgE and visualized by PE-conjugated streptavidin and flow cytometry. The individual cell populations were electronically gated on their unique green autofluorescence and side scatter characteristics. This strategy avoided unexpected cell activation during the isolation procedures, avoided inadvertent subset selection, and enabled simultaneous analyses of

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**FIGURE 3.** Binding of Ig to eosinophils (A) and expression of Fc receptors by eosinophils (B). In A, freshly isolated eosinophils were divided into two portions. One portion of cells (presensitization) were stained immediately with FITC-conjugated goat anti-human IgE or FITC-conjugated goat anti-human IgG, or FITC-conjugated control Ab. The other portion of cells (postsensitization) were stripped with lactic acid and passively sensitized in vitro with human IgE (50 μg/ml) or human IgG (50 μg/ml) for 2 h at 4°C. Cells were then stained with FITC-conjugated goat anti-human IgE or FITC-conjugated goat anti-human IgG, or FITC-conjugated control Ab. Fluorescence analysis was conducted in a FACSscan flow cytometer. Data are presented as the differences in the mean fluorescence channel (ΔMFC) between the test mAb (e.g., anti-IgE) and control Ab. Each dot represents the result from an individual subject, and the results from the same subject are connected by a line. Significant differences between the groups are shown. In B, isolated eosinophils were treated with lactic acid to remove cell surface IgE. Cells were then stained with saturating amounts of anti-FcεRI mAb (15-1), anti-FcεRII mAb (9P25), anti-FcγRII (2E1), or isotype-matched control mouse Ig followed by PE-conjugated F(ab')2 fragments sheep anti-mouse IgG. Fluorescence analysis used a FACSscan flow cytometer. Data show the differences in the mean fluorescence channel (ΔMFC) between the test mAb (e.g., 15-1) and isotype-matched control Ab. Each dot represents the result from an individual subject.

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**FIGURE 4.** FACS analysis of clgE binding to eosinophils. Isolated eosinophils were treated with lactic acid to remove in vivo bound IgE molecules. Fluorescence analysis used a FACSscan flow cytometer, and a histogram of PE fluorescence is shown. A shows the binding of clgE to eosinophils. Cells were passively sensitized with medium alone (filled gray area) or 25 μg/ml biotinylated clgE (solid black line) for 2 h at 4°C and stained with PE-conjugated streptavidin. B shows the inhibitory effect of anti-FcεRI on eosinophil clgE binding. Cells were preincubated without Ab (gray area), with 10 μg/ml anti-FcεRI mAb (15-1, bold black line), or with 10 μg/ml isotype-matched control mouse Ig (thin black line) for 30 min at 4°C. Cells were then sensitized with 25 μg/ml biotinylated clgE for 2 h at 4°C and stained with PE-conjugated streptavidin.
different cell populations from the same sample. As shown in Fig. 6, A and B, green fluorescence/side scatter dot-plot of leukocytes incubated with PE-streptavidin alone without sensitization with cIgE revealed four distinct cell populations, including lymphocytes plus basophils, monocytes, neutrophils, and eosinophils. When cells were sensitized with biotinylated cIgE and stained with PE-streptavidin, we found that cIgE binds to all the cell populations to various degrees (Fig. 6 C). Notably, basophils were intensely stained, forming a unique population distinct from the lymphocyte cluster in PE fluorescence reading (Fig. 6 C) and even in the green fluorescence reading (Fig.

FIGURE 5. Binding of cIgE to eosinophils. In A, isolated eosinophils were treated with lactic acid to remove in vivo bound IgE molecules. Cells were preincubated with or without 10 μg/ml anti-FcεRI mAb (15-1), sensitized with 25 μg/ml biotinylated cIgE, and stained with PE-conjugated streptavidin. Fluorescence analysis used a FACScan flow cytometer. Data are presented as the differences in the mean fluorescence channel (ΔMFC) between test sample (e.g., biotinylated cIgE plus PE-conjugated streptavidin) and control (PE-conjugated streptavidin alone). Each dot represents the result from one subject, and data from the same subject are connected by a line. Significant differences vs without anti-FcεRI mAb pretreatment are shown. B shows the correlation between serum IgE concentrations and anti-FcεRI (15-1)-inhibitable binding of cIgE. Anti-FcεRI-inhibitable binding of cIgE was calculated as follows: (ΔMFC of cells preincubated without anti-FcεRI mAb and then incubated with biotinylated cIgE and PE-conjugated streptavidin) – (ΔMFC of cells preincubated with anti-FcεRI mAb and then incubated with biotinylated cIgE and PE-conjugated streptavidin). Each dot represents the result from one subject; ○, normal subjects; ●, data from patients with hay fever.

FIGURE 6. FACS analyses of whole blood for cIgE binding to leukocytes. Buffy coat leukocytes were treated with lactic acid buffer to remove in vivo bound IgE molecules. Cells were preincubated without (A–D) or with (E and F) anti-FcεRI mAb (15-1). Cells were then passively sensitized with (C–F) or without (A and B) 25 μg/ml biotinylated cIgE, and stained with PE-conjugated streptavidin. Fluorescence analysis used a FACScan flow cytometer. Two-dimensional dot-plot analysis of the green fluorescence intensity and side scatter of leukocytes showed four distinct populations (B), including lymphocytes (Ly), monocytes (Mo), neutrophils (Ne), and eosinophils (Eo). Basophils (Ba) fell within the population of lymphocytes. However, when stained with biotinylated cIgE and PE-conjugated streptavidin (D), basophils (Ba) formed one unique population in green fluorescence/side scatter dot-plot analysis (C) due to their extremely strong PE fluorescence. The identity of the individual cell populations was confirmed as described in Materials and Methods.
variable inhibitory effects (18.2% of inhibition, 5.5% of total histamine, n = 6). The effect of 10 μg/ml anti-IgE Ab in solution was weaker, but substantial amounts of histamine were released (51.3 ± 10.5% of total, n = 6). In contrast, 1 μg/ml anti-IgE Ab in solution failed to induce EDN release from eosinophils (Fig. 8B), and higher concentrations of anti-IgE Ab (50–50 μg/ml) did not induce EDN release from eosinophils (data not shown). Furthermore, immobilized anti-IgE Ab induced histamine release from basophils but not EDN release from eosinophils. In contrast, immobilized anti-IgG Ab induced both basophil histamine release and eosinophil EDN release.

Production of LTC4 and superoxide anion by eosinophils

The experiments described above suggest that a degranulation response is provoked by anti-IgE Ab in basophils, but not in eosinophils. Perhaps the amounts of IgE mounted on eosinophils are too small to trigger sufficient signals for cellular function when cross-linked by anti-IgE Ab. Furthermore, if the affinity of the IgE binding sites is low, the sites may be partially occupied and partially unoccupied in freshly isolated eosinophils (see Figs. 2 and 3). Therefore, to characterize completely the eosinophil responses to IgE, we stimulated eosinophils with IgG itself or anti-Ig Ab immobilized onto tissue culture plates and examined the production of an eicosanoid, LTC4. As shown in Fig. 9, ligation of unoccupied IgG receptors by immobilized IgG provoked LTC4 production in a concentration-dependent manner. In contrast, ligation of unoccupied IgE receptors (or binding sites) with immobilized IgE failed to induce LTC4 production. Similarly, ligation of cell-bound IgG by anti-IgG Ab stimulated eosinophil LTC4 production; however, anti-IgE Ab did not induce significant production of LTC4. Furthermore, as shown in Fig. 10A, eosinophils produced superoxide anion in an anti-IgG Ab-dependent manner.

Roles of IgE and IgG receptors for degranulation of basophils and eosinophils

In the next series of experiments, we examined the functional significance of IgE receptors for mediator release by eosinophils. The engagement of IgE receptors on basophils and mast cells by ligation of bound IgE with anti-IgE Ab is commonly used to trigger various effector functions of these cell types, including degranulation and production of lipid mediators and cytokines (reviewed in Refs. 33 and 34). Therefore, we investigated whether anti-IgE Ab induces degranulation of eosinophils and compared the responses of basophils and eosinophils using cells isolated from the same donors. When basophils were stimulated with 1 μg/ml anti-IgE Ab in solution, they released a large amount of histamine (Fig. 8A, 85.7 ± 4% of total histamine, n = 6). The effect of 10 μg/ml anti-IgE Ab in solution was weaker, but substantial amounts of histamine were released (51.3 ± 10.5% of total, n = 6). In contrast, 1 μg/ml anti-IgE Ab in solution failed to induce EDN release from eosinophils (data not shown). Furthermore, immobilized anti-IgE Ab induced histamine release from basophils but not EDN release from eosinophils. In contrast, immobilized anti-IgG Ab induced both basophil histamine release and eosinophil EDN release.

FIGURE 7. Binding of cIgE to leukocyte populations and the inhibitory effects of anti-FcεRI mAb (15-1). Buffy coat leukocytes were treated with lactic acid buffer, preincubated with or without FcεRI mAb, and sensitized with cIgE. The bound cIgE was visualized by PE-conjugated streptavidin and analyzed by a FACScan flow cytometer. Individual cell populations were electronically gated, based on their unique green fluorescence and side scatter characteristics, as shown in the dot plots of Fig. 6, B and D. PE fluorescence intensity was determined on 50,000 total cells from each sample using logarithmic amplification, which was converted to the linear equivalent by Becton Dickinson Lysis II software. Data are presented as the differences in the mean fluorescence intensity (ΔMFI) between test sample (e.g., biotinylated cIgE plus PE-conjugated streptavidin) and control (PE-conjugated streptavidin alone). Each dot represents an individual subject, and the statistical differences with or without preincubation with anti-FcεRI mAb were shown. ∆(W/O-With) value was calculated by subtracting (ΔMFI value without anti-FcεRI mAb pretreatment) from (ΔMFI value with anti-FcεRI mAb pretreatment) for each individual subject. Open bars and horizontal bars show the 25th to 75th percentile range and median of the data points, respectively.

6D). The distribution of cell clusters in green fluorescence/side scatter dot-plot did not change otherwise (Fig. 6D). Interestingly, when leukocytes were pretreated with anti-FcεRI mAb, the binding of cIgE to the basophil cluster was completely inhibited (Fig. 6E). In contrast, the inhibitory effects of anti-FcεRI mAb on cIgE binding to eosinophil and neutrophil clusters were not apparent in the dot-plot.

The quantitative analyses of cIgE binding and the inhibitory effects of anti-FcεRI mAb (15-1) are shown in Fig. 7. Binding of cIgE to leukocytes was observed in the following order: basophils (5062 ± 221) > neutrophils (346 ± 23) > eosinophils (124 ± 15) > monocytes (90 ± 9) (means ± SEM of ΔMFI, n = 27). Pretreatment of cells with anti-FcεRI mAb completely inhibited the cIgE binding to basophils (99.0 ± 0.1% of inhibition, n = 27), suggesting that FcεRI plays a major role in cIgE binding to basophils. The binding of cIgE to monocytes was inhibited moderately but significantly by anti-FcεRI mAb in 24 of 27 patients (45.0 ± 5.5% of inhibition, p < 0.01), suggesting roles for FcεRI and for other binding site(s) in cIgE binding to monocytes. In contrast, neutrophils anti-FcεRI mAb minimally affected the binding of cIgE (5.1 ± 5.3% of inhibition), and the inhibitory effects of anti-FcεRI mAb varied considerably among individuals as evidenced by a wide range of Δ(W/O-With) values. The effects of anti-FcεRI mAb on eosinophils were between those on monocytes and neutrophils, namely significant (p < 0.01) but small and variable inhibitory effects (18.2 ± 3.8% of inhibition). The binding of cIgE to leukocytes due to FcεRI was observed in the following order: basophils (5015 ± 223) > monocytes (40 ± 12) > eosinophils (25 ± 7) > neutrophils (13 ± 13) (means ± SEM of the ΔMFI with or without anti-FcεRI mAb). Thus, although the expression of FcεRI by eosinophils is detectable, the level is strikingly less than that by basophils and less than that by monocytes.
anion in a time-dependent manner when stimulated with immobilized IgG; immobilized IgE stimulated only minimal superoxide production. In addition, anti-IgG Ab, but not anti-IgE Ab, induced eosinophil superoxide production (Fig. 10B). Altogether, these findings suggest that eosinophil degranulation and production of LTC₄ and superoxide anion are only minimally induced by IgE-dependent stimuli, such as IgE or anti-IgE Ab, whereas these functions are induced vigorously by IgG-dependent stimuli.

Discussion

Eosinophils and their inflammatory products play important roles in the pathophysiology of bronchial asthma and other allergic disorders (reviewed in Ref. 1). Allergen-specific IgE is one of the major components in allergic diseases in humans, and the ligation of specific IgE bound to FcɛRI on mast cells and basophils triggers production and release of inflammatory mediators from these cells (reviewed in Refs. 33 and 34). Because eosinophils from hypereosinophilic patients may express FcɛRII (13), it has been suggested that FcɛRI plays an important role in mediator release by eosinophils in allergic disease (35). However, we have made four major observations suggesting that this is unlikely. First, by standard FACS analysis, eosinophils from patients with hay fever during the peak of the season express no or minimal levels of FcɛRI or FcɛRII on their surfaces (Figs. 2 and 3); however, these eosinophils do express eosinophil activation markers and FcɛRII. Second, eosinophils are able to bind IgE (Fig. 3); however, in contrast to basophils, the contribution of FcɛRI toward eosinophil IgE binding is minor (Fig. 7). Third, IgE-dependent stimuli fail to provoke the release of inflammatory mediators by eosinophils (Figs. 8, 9, and 10). Fourth, in contrast, eosinophils from the same donors express FcγRII (Fig. 2) and bind IgG (Fig. 3), and their effector functions are triggered by IgG-dependent stimuli (Figs. 8, 9, and 10). Therefore, eosinophils from patients with ragweed hay fever express negligible FcɛRI or FcɛRII. Unlike mast cells and basophils, factors other than IgE, such as IgG and cytokines, likely play roles in activation of eosinophils in allergic diseases.

The expression of IgE receptors by eosinophils has been a controversial issue. Murine eosinophils do not express FcɛRI or FcɛRII, which are the major IgE receptors on human eosinophils and human basophils. The absence of FcɛRI on murine eosinophils suggests that IgE receptors on human eosinophils are not functional in vivo. However, recent studies have shown that human eosinophils express FcɛRI on their surfaces and that FcɛRI is involved in the activation of eosinophils by IgE-dependent stimuli (36). This finding suggests that the expression of IgE receptors on human eosinophils is not a unique feature of human eosinophils, but rather a common feature of eosinophils from allergic diseases.
FceRII (36). Although human eosinophils were considered to express only the low affinity IgE receptor (FceRII) (12, 37–39), a more recent study by the same investigators demonstrated that eosinophils from hyper eosinophilic patients express FceRI (13). This latter report triggered clinical studies to examine eosinophil expression of FceRI. For example, the transcription of mRNA for α-, β-, and/or γ-chains of FceRI in eosinophils was detected during allergen-induced late phase cutaneous reactions in patients with allergy (16). In addition, local allergen provocation induced expression of FceRI protein by eosinophils infiltrating into the airways (14) and skin (15, 16). However, other investigators showed that the expression of FceRI protein was not detected on the surface of the eosinophil (40, 41). In addition, we showed that eosinophils from patients with hay fever express very low numbers of FceRI on their surface, which can be detected by the biotin-streptavidin technique but not by the standard FACS technique. Although the reasons for the discrepancies in our observations and some of the previous studies are unknown, we speculate that synthesis of receptor proteins and surface expression of the receptor proteins are regulated under different mechanisms. In other words, the detection of receptor proteins by immunohistochemistry may not necessarily represent the expression of the proteins on cell surface. For example, human T cells contain proteins for low affinity IgG receptor (FcγRII, CD32) within the cytoplasm, but these receptors are not expressed on the cell surface (18). A similar scenario is known for FcγRIII (CD16) in eosinophils (42). In addition, the earlier studies often used eosinophils from patients with hematologic or immunologic disorders, including idiopathic hypereosinophilic syndrome and lymphomas. Perhaps disease heterogeneity accounts for the receptor expression heterogeneity.

The strikingly different responses to IgE between basophils and eosinophils are apparent in this study. From the MFI of FACS analysis, ~50 times more clgE was bound to basophils than to eosinophils (Fig. 7). Furthermore, >99% of clgE binding to basophils was inhibited by anti-FceRI mAb pretreatment, suggesting that FceRI is the predominant IgE receptor in basophils. In contrast, anti-FceRII mAb only partially (18%) inhibited IgE binding to eosinophils. Thus, we estimate that eosinophils from these ragweed hay fever patients express ~0.5% of the FceRI compared with basophils. In addition, the basophil degranulation response was triggered by ligation of FceRI, either by anti-IgE Ab or by immobilized IgE; these same stimuli failed to induce degranulation or release of mediators from eosinophils. Interestingly, treatment of patients with atopy with humanized anti-IgE Ab resulted in ~96% decrease in the expression of FceRI and ~40% decrease in anti-IgE-induced histamine release response by basophils (30). Because eosinophils possess only ~0.5% of the FceRI possessed by basophils, this paucity of FceRI expression by eosinophils may explain the heterogeneous responses of eosinophils and basophils to FceRI ligation. Perhaps eosinophils lose or basophils acquire their capacity to express high levels of FceRI during their differentiation from common progenitors (43).

Then, the question remains as to which receptors or binding sites contribute to the binding of myeloma IgE (Figs. 2 and 3) and clgE (Figs. 4 through 7) by eosinophils. The results of our experiments and a review of the literature provide some insights. As shown in Figs. 2 and 3, none to minimal expression of FceRI or FceRII (CD23) was detectable using 15-1 or 9P25 mAb, respectively, and the standard FACS technique. These findings are also consistent with previous reports, demonstrating negligible binding of anti-FceRI mAb (clones 22E7 and 15-1) (17) and a panel of mAb against CD23 (44, 45) to eosinophils. Therefore, the expression levels of high affinity IgE receptor (FceRI) and low affinity IgE receptor (FceRII) on eosinophils are likely too low to play major roles in IgE binding. Furthermore, the minimal or lack of binding of IgE on eosinophils in vivo (Figs. 2 and 3) suggests that IgE binding to eosinophils is of low affinity. Besides these IgE Fc receptors, an IgE-binding molecule belonging to an S-type lectin family, called Mac-2/εBP, can also bind IgE through the carbohydrate recognition domain (46). Mac-2/εBP is expressed by various cell types, including neutrophils and eosinophils (47, 48). Indeed, Ab against Mac-2/εBP strongly inhibited IgE binding and IgE-dependent activation of human neutrophils (47). Therefore, by analogy to neutrophils, Mac-2/εBP is a good candidate molecule for IgE-binding sites on eosinophils. Our findings, showing large amounts of IgE binding to both eosinophils and neutrophils independent of FceRI (Fig. 7), are consistent with this speculation. Alternatively, IgE may bind nonspecifically through its carbohydrate moiety to lectin-like binding sites on the surface of the eosinophil.

Two other questions remain. Because previous histological studies (14–16) could not differentiate between the proteins within the cells and those on the cell surface, the first question involves the surface expression of FceRI by tissue eosinophils. As shown in Fig. 5B, the levels of FceRI expression by eosinophils show a positive correlation with serum IgE levels, suggesting that the expression of FceRI by eosinophils is regulated by IgE-dependent mechanisms similar to those in mast cells (29) and basophils (30). In addition, IL-4 enhances the expression of mRNA and protein for the α-chain of FceRI (40). It is well known that IL-4 is expressed in the local tissues of patients with allergy (49, 50); a more recent report suggests that IgE may be produced by B cells in the airway tissues in patients with allergy (51). Therefore, the tissue microenvironment may be optimal for FceRI expression, suggesting that the surface expression of FceRI by tissue eosinophils may be higher than the surface expression in peripheral blood eosinophils. On the other hand, even in the optimal tissue environment, the ability of eosinophils to express FceRI on their surfaces may be limited. For example, eosinophils obtained from bronchoalveolar lavage fluids from patients with allergy do not express detectable levels of FceRI on their surfaces (41). Eosinophils cultured for up to 11 days with myeloma IgE or IL-4, conditions known to up-regulate FceRI on basophils, failed to induce any detectable surface FceRI (41). Furthermore, Terada et al. (40) found that after culture with IL-4, eosinophil production of FceRI α-subunit protein was increased, but expression of FceRI was not detectable. In preliminary studies, we were not able to detect FceRI by standard FACS analysis on eosinophils cultured for 3 days with human myeloma IgE in the presence of various cytokines, such as IL-5, IL-3, IL-4, TNF-α and fibroblast supernatants (data not shown).

The second question concerns the interaction of IgE with other eosinophil agonists. We found that anti-IgE Ab or immobilized IgE by themselves failed to provoke eosinophil mediator release (Figs. 8 through 10). However, a previous report also suggests that IgE mediates eosinophil killing of schistosomula of Schistosoma mansoni when cells are stimulated with lipid mediators, such as platelet-activating factor or LTβ, without the apparent expression of FceRI (52). Therefore, IgE may collaborate with other eosinophil agonists to provoke some functions of the cells, such as cellular cytotoxicity. In preliminary studies, IgE did not enhance EDN release from eosinophils stimulated with platelet-activating factor or IL-5 (H. Kita and M. Muraki, unpublished observation). Furthermore, it is also possible that FceRI or IgE may be involved in other functions than secretory process of eosinophils, such as Ag presentation, as proposed in other cell types (53).

Remaining questions aside, our study clearly shows that the surface expression of FceRI by blood eosinophils from patients with hay fever is minimal and that, in contrast to IgG, IgE does not
mediate release of inflammatory mediators from these eosinophils. Therefore, previous observations regarding the expression of FcεRI and FcεRII by blood eosinophils from patients with eosinophilia may not be directly applicable to patients with allergy. Although our study may be limited because only patients with hay fever were studied, our observations raise questions about the current conception that eosinophil IgE receptors play a role in the pathophysiology of allergic diseases. The observations in IgE knockout mice, which demonstrated eosinophilic inflammation and increased bronchial hyperreactivity in the absence of IgE (54), may in part be applicable to humans; i.e., factors other than IgE may act as trigger(s) for mediator release by eosinophils in allergic inflammation.

Acknowledgments

We thank Dr. J.-P. Kinet for the kind gift of anti-FcεRI mAb (15-1) and biotinylated cIgE; Mr. James E. Tarara, Ms. Teresa J. Halsey, and Ms. Holly B. Lamb for their excellent technical assistance in flow cytometry; Ms. Cheryl Adolphson for editorial assistance; Ms. Linda Arneson for secretarial assistance; and Ms. Judith Blomgren and Ms. Kay Bachman for recruiting and obtaining blood samples from patients.

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