Allergen-specific IgG1 and IgG3 through $Fc_\gamma RII$ Induce Eosinophil Degranulation

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Abstract

Evidence suggests that eosinophils contribute to inflammation in bronchial asthma by releasing chemical mediators and cytotoxic granule proteins. To investigate the mechanism of eosinophil degranulation in asthma, we established an in vitro model of allergen-induced degranulation. We treated tissue culture plates with short ragweed pollen (SRW) extract and sera from either normal donors or SRW-sensitive patients with asthma. Eosinophils were incubated in the wells and degranulation was assessed by measurement of eosinophil-derived neurotoxin in supernatants. We detected degranulation only when sera from SRW-sensitive patients were reacted with SRW. Anti-IgG and anti-$Fc_\gamma RII$ mAb, but not anti-IgE or anti-$Fc_\epsilon RII$ mAb, abolished the degranulation. IgG-depleted serum did not induce degranulation; IgE-depleted serum triggered as much degranulation as untreated serum. Furthermore, serum levels of SRW-specific IgG1 or IgG3 correlated with the amounts of released eosinophil-derived neurotoxin. When eosinophils were cultured in wells coated with purified IgG or IgE, eosinophil degranulation was observed only with IgG. Finally, human IgG1 and IgG3, and less consistently IgG2, but not IgG4, induced degranulation. Thus, sera from patients with SRW-sensitive asthma induce eosinophil degranulation in vitro through antigen-specific IgG1 and IgG3 antibodies. These antibodies may be responsible for degranulation of eosinophils in inflammatory reactions, such as bronchial asthma. (*J. Clin. Invest.* 1995. 95:2813–2821.) Key words: bronchial asthma • eosinophils • immunoglobulin • allergen • degranulation

Introduction

Eosinophils play an important role in bronchial asthma (1). Eosinophils produce a number of lipid mediators and proteins that are involved in allergic diseases. However, evidence now available suggests that the most damaging eosinophil products to the host are cationic proteins. Eosinophil major basic protein (MBP)[1] and eosinophil peroxidase are toxic to human epithelial cells and pneumocytes (2, 3). MBP, eosinophil peroxidase, and eosinophil cationic protein damage tracheal epithelial cells by inducing ciliostasis and exfoliation (4). MBP causes bronchial hyperresponsiveness in primates (5). Both blood and sputa from patients with asthma contain elevated levels of MBP (6, 7), and MBP is localized to the damaged sites in the respiratory epithelium (8, 9). Clearly, eosinophils release their granule proteins into the inflamed tissues; however, the mechanism(s) which triggers the eosinophil activation and degranulation remains unclear.

The IgE-triggered release of mast-cell mediators in response to allergen is thought to be the primary event in hypersensitivity reactions such as bronchial asthma (10). Although it is clear that IgE is central to many allergic reactions, some observations suggest the existence of alternative and/or additional pathways of hypersensitivity reactions. First, mast cells can be sensitized and activated by IgG in vivo (11) and in vitro (12). Second, studies of passive cutaneous anaphylaxis in monkeys have shown that serum fraction containing IgG can sensitize recipient tissue, albeit in a less potent and relatively shorter-lived fashion than IgE (13). Third, late asthmatic reactions (LAR) to inhaled allergen challenge were associated with IgG antibody rather than IgE antibody (14, 15). Finally, significantly higher levels of IgE and IgG antibodies to inhaled allergen, such as dust mite antigen, were detected in children with asthma compared to normal subjects (16). Thus, IgG antibody, as well as IgE antibody to allergen, is implicated in the allergic reaction in bronchial asthma.

Eosinophils constitutively express Fc receptors (FcR) for IgG ($Fc_\gamma RII$/CD32) (17–20) and for IgA ($Fc_\alpha R$) (21). Indeed, eosinophils release granule proteins in vitro when stimulated by Sepharose 4B beads (Sigma Chemical Co., St. Louis, MO) coated with IgG, IgA, and secretory IgA (22). In contrast, anti-IgE as well as specific antigen, induced EPO release from hypodense eosinophils isolated from parasite-infected patients (23, 24); eosinophils expressed the low affinity FcR for IgE ($Fc_\epsilon RII$) and were activated through this receptor (25). However, $Fc_\epsilon RII$ on eosinophils has not been fully characterized and appears not to be identical to CD23, the low affinity IgE receptor on lymphocytes and monocytes (26). Eosinophils from patients with eosinophilia also express another lectin-type low affinity IgE-binding molecule, called Mac-2/ϵBP, and the cytotoxic function of eosinophils was abolished by the antibody against this molecule (27). More recently, it has been claimed that the high affinity IgE receptor, $Fc_\epsilon RI$, is present on eosinophils from eosinophilic patients, and various functions of eosinophils, including degranulation and parasite cytotoxicity, were mediated thorough this receptor (28). These results suggest that IgG, IgA including secretory IgA, and especially IgE are able to trigger eosinophil degranulation.

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1. *Abbreviations used in this paper:* DCS, defined calf serum; EDN, eosinophil-derived neurotoxin; FcR, Fc receptor; LAR, late asthmatic response; MBP, major basic protein; SRW, short ragweed pollen.

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To investigate the mechanism of eosinophil degranulation in bronchial asthma, we have established a model using short ragweed pollen (SRW) as an allergen and sera from SRW-sensitive patients with asthma as a source of Ig. Using this model, we studied the allergen-specific Ig and Ig-receptors on eosinophils responsible for allergen-dependent eosinophil degranulation.

Methods

Reagents. Endotoxin-free SRW (Lot No. L56-106) was obtained from Greer Laboratories, Inc. (Lenoir, NC). Globulin-free HSA was purchased from Sigma Chemical Co. Protein A was from Genzyme Co. (Cambridge, MA). ^{125}I-labeled protein A was prepared as described previously (29). Recombinant human IL-5 was a generous gift from Dr. Satwant Narula, Schering-Plough Research Institute (Kenilworth, NJ).

Purified human Ig. We used myeloma IgE purified from serum from a patient with multiple myeloma as described elsewhere (22) and IgE purchased from Cortex Biochem (San Leandro, CA). Purified serum IgG was purchased from Cappel-Organon Teknika Co. (Durham, NC). Purified human myeloma IgG1λ, IgG2λ, IgG3λ, and IgG4λ were purchased from Sigma Chemical Co.

Antibodies. Preparation of rabbit IgG anti-human IgE antibody and labeling with ^{125}I were as described previously (30). Goat IgG F(ab')2 anti-human IgG, goat IgG whole molecule anti-human IgE, goat IgG, mouse myeloma IgG1, and mouse myeloma IgG2b were purchased from Cappel-Organon Teknika Co. Goat IgG F(ab')2 was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Goat IgG F(ab')2 anti-human IgE was from DeaMed, Inc. (Windham, ME). Mouse IgG2b Fab fragment of anti-human Fc_γRII mAb (IV.3) and mouse IgG1 F(ab')2 anti-human Fc_γRIII mAb (3G8) were from Medarex, Inc. (West Lebanon, NH). Mouse IgG1 anti-Fc_ϵRII/CD23 mAb (9P.25) and mouse IgM antieosinophil Fc_ϵRII mAb (BB10CC2) were from Amac, Inc. (Westbrook, ME). Mouse IgG1 anti-human Fc_ϵRII/CD23 mAb (11/4) was obtained from Yamasa Shoyu Co., Ltd. (Chiba, Japan) and digested to Fab using ImmunoPure Fab Preparation Kit (Pierce Chemical Co., Rockford, IL). Mouse IgM, anti-mouse IgG peroxidase conjugate, and anti-human IgG2 mAb (HP-6014) were from Sigma Chemical Co. Anti-human IgG1 mAb (HP-6070), anti-human IgG3 mAb (HP-6050), and anti-human IgG4 mAb (HP-6023) were from Calbiochem Corp. (La Jolla, CA).

Sera. We obtained sera from 10 SRW-sensitive patients with asthma, who had not received immunotherapy, and from 8 normal subjects. We also used a pool of serum collected from 7 SRW-sensitive patients with asthma. All sera were stored in −20°C and used after filtration through a 0.22-μm membrane (Millipore Corp., Bedford, MA).

Measurement of SRW-specific Ig in sera. SRW-specific IgG and IgE in sera were determined by RIA and by the radioallergosorbent test (30), and SRW-specific IgG subclasses were determined by ELISA, as previously described (31). Briefly, wells of Immulon 4 microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with SRW by incubating 100 μl of SRW extract (100 μg/ml in 0.02 M carbonate buffer; pH 9.2) in each well for 4 h at room temperature. After incubation, wells were washed three times with PBS, filled with 100 μl of serum or serum dilution, incubated overnight at 4°C, and washed three times with PBS. To detect bound SRW-specific Ig, either ^{125}I-labeled protein A to measure IgG or ^{125}I-labeled rabbit anti-human IgE to measure IgE was added and incubated for 5 h at room temperature. After washing with PBS three times, radioactivity in each well was counted on a gamma spectrometer. The amount of SRW-specific antibodies was measured in counts per minute after subtracting background (no serum) cpm. To detect SRW IgG subclass antibodies, 100 μl of anti-human IgG subclass antibody was added to each well for 1 h at room temperature. Concentrations of antibodies used were: anti-IgG1 (10 μg/ml), anti-IgG2 (1:250), anti-IgG3 (25 μg/ml), anti-IgG4 (25 μg/ml). The specificities of these mAb were confirmed using purified myeloma IgG subclasses. After washing twice with PBS, 100 μl of peroxidase-conjugated goat anti-mouse IgG (1:250) was added to the wells and incubated for 1 h at room temperature. After washing twice with PBS, the bound anti-mouse IgG antibodies were detected using peroxidase substrate tablet set (0.05 M phosphate-citrate buffer, 0.4 mg/ml urea hydrogen peroxide, and 0.4 mg/ml *o*-phenylenediamine dihydrochloride, SIGMA FAST™; Sigma Chemical Co.). The levels of IgG subclass SRW-specific antibodies in each serum were expressed as sample absorbance (490 nm) minus background (no conjugated Ab) absorbance.

Preparation of IgG- or IgE-absorbed serum. Protein A coupled to cyanogen bromide-activated Sepharose 6B (Sigma) or anti-human IgE-coupled Sepharose 4B beads was used to deplete IgG or IgE from serum, respectively. Anti-IgE-coupled beads were prepared as previously reported (22). Protein-coupled beads were washed four times with PBS, mixed with an equal volume of serum, incubated overnight at 4°C with gentle shaking, and were removed by using Filter Sampler® (13 mm × 4 in; Baxter Scientific Products, McGaw Park, IL). The efficiency of depletion of IgE and IgG was examined by RIA, as described above.

Cell preparations. Eosinophil isolation was performed with minor modifications (32) of the method described by Hansel et al. using a magnetic cell separation system (MACS) (Becton Dickinson, San Jose, CA) (33). The purity of eosinophils counted by Randolph's stain was > 97%. PBMC were prepared by using Histopaque®-1077 (Sigma) by the manufacturer's method. In all experiments, PBMC and eosinophils were obtained from the same normal individuals. To test histamine release from basophils, peripheral blood leukocytes were isolated and cell-bound IgE was dissociated as described by Pruzansky et al. (34).

Histamine release assay. Leukocytes (treated to remove cell-bound IgE) were passively sensitized with SRW-specific IgE by incubating with pooled serum (diluted 1:1 with PBS) from SRW-sensitive patients with asthma for 2 h at 4°C. After passive sensitization, leukocytes were washed once with Pipes buffer and were suspended in HBSS at 2.5 × 10^6 cells/ml. Cells, 100 μl, were added to each well of U-bottom tissue culture plates (Catalogue No. 3799; Costar Corp., Cambridge, MA), and 100 μl of stimulants in HBSS, either 1 μg/ml SRW extract or 1 μg/ml goat anti-human IgE antibody, were added. Total leukocyte histamine was measured after addition of 100 μl of 0.5% NP-40 solution. After a 30-min incubation in a humidified incubator at 37°C and 5% CO_2, the plate was centrifuged at 200 *g* for 10 min at 4°C and supernatant histamine was determined using a Histamine EIA kit (Amac, Inc.) according to the manufacturer's instructions.

Eosinophil degranulation. To measure antigen-specific degranulation, wells of 96-well plates (Catalogue No. 3596; Costar Corp.) were coated with 100 μl of SRW extract (100 μg/ml in PBS) or control PBS for 2 h at 37°C. After aspiration, 100 μl HSA solution (2.5% in PBS) was added to each well and incubated for 1 h at 37°C to block nonspecific protein binding. Wells were washed once with 100 μl PBS and treated with 100 μl of diluted donor serum, as described above, for 2 h at 37°C. Unless indicated otherwise, sera were diluted to 10% in PBS. After aspiration and washing twice with PBS, 100 μl eosinophil suspension, 0.5 × 10^6 cells/ml in RPMI 1640 medium containing 25 mM Hepes, L-glutamine, sodium bicarbonate (Celox Co., Hopkins, MN), and 10% defined calf serum (HyClone Laboratories, Logan, UT) (RPMI-DCS), was added to the coated wells, followed by 100 μl PBMC suspended at 2 × 10^6 cells/ml in RPMI-DCS. Because IL-5 is implicated in eosinophil infiltration and activation in bronchial asthma (9), 100 μl IL-5 solution (2 ng/ml) was added to the wells instead of PBMC in some experiments. For tests of anti-human Ig antibodies, wells were treated with 100 μl antibody diluted in RPMI-DCS for 15 min at room temperature before eosinophil addition. For tests of anti-FcR antibodies, eosinophils or eosinophils with PBMC were preincubated for 1 h at 4°C with antibodies before being added to the serum-coated wells. Unless indicated otherwise, cells were cultured for 15 h in a humidified incubator at 37°C and

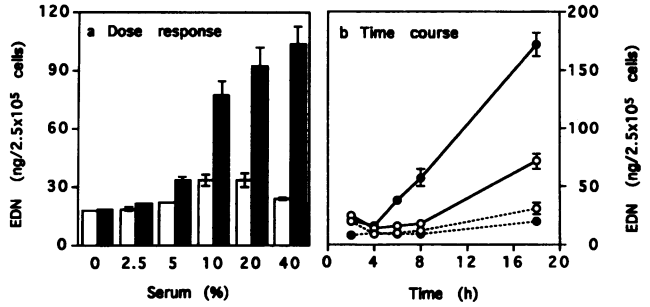

Figure 1. SRW allergen-induced eosinophil degranulation. (*a*) Dose-response of pooled sera from SRW-sensitive patients. Wells coated with SRW extract (*closed column*) or PBS (*open column*), were treated with pooled sera at concentrations indicated. Eosinophils (0.5×10^5 cells/well) were added to the wells and incubated for 15 h at 37°C in the absence of PBMC. (*b*) Time-course of antigen-induced eosinophil degranulation. Wells coated with SRW extract (——) or PBS (- - - - -), were treated with 10% pooled sera. Eosinophils were cultured in the wells for indicated times in the presence (*closed circle*) or absence (*open circle*) of PBMC. Results are presented as mean±SEM of triplicate analyses in one experiment.

5% CO_2. After incubation, supernatants were frozen at −20°C until assayed.

To examine eosinophil degranulation induced by purified Ig, wells were incubated with 100 μl Ig diluted in PBS for 2 h at 37°C and blocked as described above. Wells were washed with 100 μl PBS twice, and 100 μl of eosinophil suspension (0.5×10^6 cells/ml), and 100 μl of PBMC suspension (2×10^6 cells/ml), 100 μl of IL-5 solution (2 ng/ml), or 100 μl of RPMI-DCS were added to the wells and cultured for 15 h. In some experiments, eosinophils were preincubated with anti-FcR antibodies, as described above, to test the effect of these antibodies. Culture supernatants were collected as described above.

To measure eosinophil degranulation, eosinophil-derived neurotoxin (EDN) concentrations in the culture supernatants were measured by RIA, as described previously (22).

Results

Allergen-dependent eosinophil degranulation in vitro. We established an assay system for allergen-dependent eosinophil degranulation in vitro using SRW extract and sera from SRW-sensitive patients with asthma. Pooled serum from seven SRW-sensitive patients induced eosinophil degranulation in a dose-dependent manner (Fig. 1 *a*). In contrast, pooled serum in the absence of SRW did not induce degranulation, suggesting that allergen-specific factors in pooled serum are involved in eosinophil degranulation. The degranulation was time dependent and enhanced by PBMC as shown in Fig. 1 *b*. Wells coated with SRW extract, but without treatment with SRW serum pool, did not induce degranulation (data not shown). Table I shows results from 18 different eosinophil donors. The pooled serum from SRW patients induced eosinophil degranulation when plates were coated with SRW extract, and this degranulation was augmented by the presence of PBMC in the culture system. To investigate whether this eosinophil degranulation is specific for sera from SRW-sensitive patients, we compared the ability of sera from normal subjects ($n = 8$) and SRW-sensitive patients with asthma ($n = 10$) to induce degranulation. Sera from patients, but not those from normal donors, induced eosinophil degranulation ($P < 0.05$) (Fig. 2). Similarly, in the presence

Table I. Allergen-induced Eosinophil Degranulation in the Presence or Absence of PBMC

	EDN released (ng/2.5 × 10^5 cells)		
PBMC	− Allergen coating	+ Allergen coating	EDN ratio*
−	26.2±2.4	50.8±5.9‡	2.01±0.18
+	28.4±3.3	79.7±11.1‡	2.87±0.23§

Purified eosinophils were incubated for 15 h in the presence or absence of PBMC in wells which were pretreated with SRW, HSA, and 10% of pooled serum from SRW-sensitive patients in this order. Results are mean±SEM of 24 separate experiments from 18 eosinophil donors. * EDN ratio was calculated in each experiment by the following formula: (EDN ratio) = (EDN in supernatant of allergen-coated group)/(EDN in supernatant of allergen-uncoated group) as indicators of ability to induce eosinophil degranulation of the serum. ‡ Significantly higher than allergen-uncoated groups at $P < 0.0001$. § Significantly higher than groups without PBMC at $P < 0.0001$. Statistical analyses were performed by paired Student's *t* test.

of PBMC, we could detect significantly higher levels of degranulation with the sera from patients than with the sera from normal subjects (Fig. 2, $P < 0.05$). These results indicate that sera from SRW-sensitive patients induce eosinophil degranulation in an allergen-dependent manner.

Evidence for IgG and Fc_γRII-mediated eosinophil degranulation. To determine the Ig responsible for eosinophil degranulation, first, the levels of serum IgG and IgE antibodies were measured. Fig. 3 shows that both IgG and IgE SRW-specific antibodies were significantly higher in SRW-sensitive patients' sera than in sera from normal individuals ($P < 0.05$ in IgG, $P < 0.001$ in IgE); not surprisingly, the difference of IgE levels between sera from patients and normal individuals was striking. Analyses of the pooled serum from SRW patients revealed 642 cpm of IgG and 12,206 cpm of IgE SRW-specific antibody activity; these levels are similar to the average levels of patient sera in Fig. 3.

Second, the ability of anti-IgG and anti-IgE to block eosino-

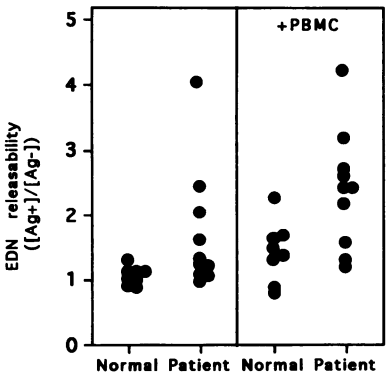

Figure 2. Eosinophil degranulation induced by sera of normal donors ($n = 8$) and SRW-sensitive patients with asthma ($n = 10$). The ability of each serum (10%) to induce degranulation was tested in the presence or absence of PBMC and expressed as EDN releasability (EDN ratio) which is calculated using the formula: (EDN ratio) = (EDN released from eosinophils in allergen-coated wells)/(EDN released from eosinophils in allergen-uncoated wells). These data were obtained from triplicate analyses in 14 separate experiments from 10 normal donors of eosinophils. Each serum was tested in one to three separate experiments. The patient group has a significantly higher EDN ratio than the normal group by Mann-Whitney U test ($P < 0.05$) in both conditions.

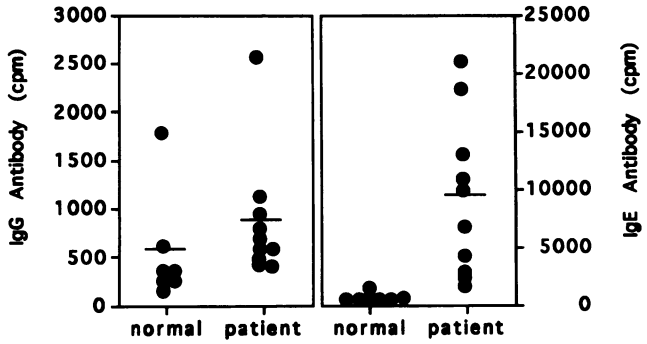

Figure 3. IgG and IgE SRW-specific antibodies in sera from normal donors ($n = 7$) and SRW-sensitive patients with asthma ($n = 10$). IgG and IgE antibodies were detected by RIA and radioallergosorbent test, respectively, using ^{125}I-labeled protein A or ^{125}I-labeled rabbit anti-human IgE. Horizontal bars show means of each group. Significantly higher amounts of specific IgG ($P < 0.05$) and IgE ($P < 0.001$) were present in the patient group by Mann-Whitney U test.

phil degranulation was tested. Wells coated with SRW extract and pooled serum were treated with goat IgG F(ab')2 fragments of anti-human IgG or anti-human IgE. Table II shows that the F(ab')2 fragment of anti-IgG inhibited the eosinophil degranulation to background levels (no allergen) even in the presence of PBMC. In contrast, the F(ab')2 fragment of anti-IgE had no effect on the eosinophil degranulation. When IL-5 was added to the incubation, degranulation was increased dramatically (64±10 and 203±58 ng/2.5 × 10^5 cells in the absence and presence of IL-5, respectively). Eosinophil degranulation in the presence of IL-5 was also inhibited by the F(ab')2 fragment of anti-IgG, but not anti-IgE (Table II). Furthermore, using eosinophils precultured for 4 d in the presence of IL-5 (0.5 ng/ml), only anti-IgG, and not anti-IgE, inhibited the allergen-induced degranulation (data not shown).

Third, the effect of specific removal of IgG and IgE was tested. IgG was specifically absorbed from the SRW-sensitive pooled serum by protein A–conjugated Sepharose beads. This IgG-deficient serum was unable to induce eosinophil degranulation irrespective of the presence of PBMC (Table III). In contrast, IgE-deficient serum, prepared using anti-human IgE-conjugated Sepharose beads, retained the ability to induce degranulation as potently as the control serum (Table III).

Fourth, to determine which FcR was mediating eosinophil degranulation, we studied the effects of the blocking of FcR by various anti-FcR antibodies on eosinophil degranulation. All antibodies we used are reported to inhibit the binding of their respective Ig to cell surfaces (17, 25, 35–37). As shown in Table IV, only anti-FcγRII antibodies showed potent inhibitory effects on degranulation in the presence and absence of PBMC. Although we also tested a higher concentration of anti-Fc$_\gamma$RIII (10 μg/ml), it showed no effect on eosinophil degranulation (data not shown). We could not detect any effects on eosinophil degranulation by various anti-Fc$_\epsilon$RII antibodies.

Fifth, to test whether IgE antibodies in pooled sera from SRW-sensitive patients were functional, peripheral blood leukocytes were passively sensitized with this serum pool and were stimulated by either SRW or anti-IgE. The basal histamine concentration in unstimulated supernatants was 0.44 nM; this corresponds to 2.2% release of total histamine. Basophils stimulated with 1 μg/ml of SRW released 5.93 nM histamine (30.1% of available histamine). Basophils stimulated with 1 μg/ml of anti-IgE released 2.66 nM histamine (13.5% of available histamine). Basophils stimulated with 1 μg/ml of anti-IgE released 2.66 nM histamine (13.5% of available histamine). Basophils incubated with either 1 μg/ml of control antibodies (goat purified IgG) or 1 μg/ml of SRW extract (after passive sensitization with serum from a healthy nonallergic donor) did not release histamine.

Finally, we tested whether the amounts of SRW-specific IgG and IgE in sera correlated with their abilities to induce degranulation; we found no significant correlations (data not shown). Experiments in the presence of PBMC showed similar findings. One explanation for the lack of correlation could be functional differences among the IgG subclasses (38). Therefore, we measured the levels of SRW-specific IgG subclasses in 10 sera from patients allergic to SRW and 6 sera from normal subjects and correlated levels of IgG subclasses with the sera's ability to induce degranulation. As shown in Fig. 4, we detected

Table II. Effect of Anti-Human Ig Antibodies on Eosinophil Degranulation

Allergen coating	Antibodies	EDN released (% of control)		EDN released (% of control)	
		Eosinophil	Eosinophil + PBMC	Eosinophil	Eosinophil + IL-5
–	–	39.2±4.6*	36.4±8.2*	37.4±4.6*	51.2±4.3*
+	–	100‡	100‡	100‡	100‡
+	Control Ab§	105.7±16.6	114.0±10.6	107.6±6.6	108.5±7.2
+	Anti-IgG	38.5±5.3*	42.7±12.3‖	40.8±8.0*	35.1±8.6*
+	Anti-IgE	116.6±21.2	128.5±21.2	157.3±7.0*	107.6±18.3

After treatment of wells with SRW extract, HSA, and 10% of pooled serum in this order, 100 μl of antibody solution (1 μg/ml) was added to the wells. 15 min later, 100 μl of eosinophils with or without PBMC suspension or IL-5 (2 ng/ml) was added to the wells and incubated for 15 h. Results are expressed as percentage of control in each experiment (no antibody treatment) and are presented as mean±SEM in four experiments from four different donors. Each of the experiments was performed in duplicate or triplicate. * Significantly different from controls at $P < 0.01$. ‡ The amounts of EDN released from eosinophils in control groups were 52.7±9.1 and 56.9±6.7 in the absence or presence of PBMC, respectively, and were 64.4±10.0 and 202.5±58.2 (ng/2.5 × 10^5 eosinophils; mean±SEM) in the absence or presence of IL-5, respectively. § Goat purified IgG F(ab')2 fragments were used as control antibodies. ‖ Significantly different from control at $P < 0.05$. Statistical analyses were performed by paired Student's t test.

Table III. Effect of IgG or IgE Removal on Eosinophil Degranulation

Allergen coating	Serum*	EDN released (% of control) Eosinophil	Eosinophil + PBMC
−	Control	51.3±5.1	37.9±4.4
+	Control	100‡	100‡
−	IgG absorbed	32.8±14.9	23.4±7.3
+	IgG absorbed	33.1±14.4§	27.3±8.5§
−	Control	59.2±13.2	41.7±8.8
+	Control	100‖	100‖
−	IgE absorbed	55.3±10.9	42.3±3.5
+	IgE absorbed	102.3±1.5	103.9±11.1

After treatment of wells with SRW extract, HSA, and a 1:10 dilution of SRW serum pool, eosinophils or eosinophils with PBMC were incubated for 15 h. Results are expressed as percentage of control (no Ig absorption) in each experiment and are expressed as mean±SEM of triplicate analyses in three separate experiments from three different eosinophil donors. Statistical analyses were performed by paired Student's *t* test between absorbed-serum group and corresponding control group. * Control sera for IgG absorption and for IgE absorption were from the same sources. IgG or IgE SRW-specific antibodies in sera were verified by RIA. The reactivities of IgG and IgE antibodies in the sera were as follows: for the IgG-absorbed serum, IgG, 618 cpm in control and 10 cpm in IgG-absorbed serum and IgE, 7,468 cpm in control and 5,660 cpm in IgG absorbed; for the IgE-absorbed serum, IgG, 516 cpm in control and 538 cpm in IgE-absorbed serum, and IgE, 3,014 cpm in control and none detected in IgE-absorbed serum. § Significant difference from allergen-coated control wells at $P < 0.05$. ‡ The amounts of EDN released from eosinophils in control groups were 74.1±22.5 and 81.2±19.3 (ng/2.5 × 10^5 eosinophils, mean±SEM) in the absence or presence of PBMC, respectively. ‖ The amounts of EDN released from eosinophils in control groups were 44.6±6.4 and 68.1±9.9 (ng/2.5 × 10^5 eosinophils, mean±SEM) in the absence or presence of PBMC, respectively.

Table IV. Effect of Anti-Fc Receptor Antibodies on Eosinophil Degranulation

Antigen coating	Antibodies	*n*	EDN released (% of control) Eosinophil	Eosinophil + PBMC
−	−	5	46.7±8.8*	45.0±8.2*
+	−	5	100‡	100‡
+	m IgG1§	5	104.8±8.2	112.0±11.7
+	m IgG2b§	4	96.3±3.5	115.3±14.5
+	m IgM§	2	86.1±1.3	127.2±7.1
+	anti-Fc_γRII	4	45.3±4.4*	57.9±9.2‖
+	anti-Fc_γRIII	3	105.4±13.0	97.9±13.0
+	anti-Fc_ϵRII(9P.25)	4	98.9±7.8	126.4±15.6
+	anti-Fc_ϵRII(11/4)	4	106.4±9.3	128.0±8.5
+	anti-Fc_ϵRII(BB10CC2)	2	97.7±20.6	103.7±21.4

Wells were treated with SRW extract, HSA, and 10% of pooled serum. Eosinophils or eosinophils with PBMC were preincubated with antibody (0.5 μg/ml) for 1 h at 4°C, added to wells and incubated for 15 h. The antibodies used in this experiment are as follows: anti-FcγRII (IV.3 mAb; mouse IgG2b Fab), anti-FcγRIII (3G8 mAb; mouse IgG1 F(ab')2), anti-Fc_ϵRII (9P.25 mAb; mouse IgG1, 11/4 mAb; mouse IgG1 Fab, BB10CC2 mAb; mouse IgM). Results are expressed as percentage of control (no antibody) in each experiment, and the results are presented as mean±SEM of triplicate analyses in two to five experiments (*n*) from five different normal eosinophil donors. * Significantly different from controls at $P < 0.01$. ‡ The amount of EDN released from eosinophils in controls in the presence and absence of PBMC were 86.2±30.6 and 68.4±18.6 (ng/2.5 × 10^5 eosinophils), respectively. § Mouse purified myeloma Ig whole molecules were used as control antibodies. ‖ Significantly different from control at $P < 0.05$. Statistical analyses were performed by paired Student's *t* test.

significant correlations between SRW-specific IgG1 or IgG3 levels and eosinophil degranulation. In contrast, specific IgG2 and IgG4 showed no significant correlations.

Ability of purified IgG, IgE, or IgG subclasses to induce eosinophil degranulation. The experiments described above suggest a role of SRW-specific IgG1 and IgG3, but not IgE, for the degranulation of eosinophils. To confirm this observation, first, wells of tissue culture plates were coated with purified IgG or IgE, and eosinophils were cultured for 15 h on these plates to test the ability of each Ig to induce eosinophil degranulation. We detected eosinophil degranulation by immobilized IgG in a dose-dependent manner (Table V; Experiment 1). However, the immobilized IgE did not induce eosinophil degranulation (Table V; Experiment 2), and this inability did not change even when eosinophils were cultured in the presence of PBMC in the IgE-coated wells (Table V; Experiment 3). In the presence of IL-5, IgG-induced degranulation was augmented by 300% or more, but again IgE failed to induce degranulation (Table VI). IgE from a different source also did not induce degranulation (Table VI). The experiments using eosinophils cultured for 4 d with 1 ng/ml of IL-5 showed similar findings. Second, to test the ability of each IgG subclass to induce degranulation, eosinophils were cultured for 15 h in wells coated with each IgG subclass. Among the IgG subclasses, IgG1 and IgG3 induced eosinophil degranulation from all of the individuals (Fig. 5); in contrast, IgG4 did not induce degranulation. The eosinophil degranulation response to IgG2 was heterogeneous; that is, only three out of eight individuals responded well to IgG2. Finally, the eosinophil degranulation induced by serum IgG, IgG1 and IgG3 was completely inhibited by anti-FcγRII (CD32) antibodies (data not shown).

Discussion

The mechanism of eosinophil degranulation in allergic diseases, such as bronchial asthma, is still unknown. To investigate this question, we established an assay system of allergen-dependent eosinophil degranulation in vitro. After coating the culture plate wells first with SRW extract and then with pooled serum from SRW-sensitive patients, eosinophil degranulation was observed to be concentration and time dependent (Fig. 1). SRW extract alone or pooled serum alone did not induce degranulation. Furthermore, sera from SRW-sensitive patients with asthma had a significantly higher activity to induce eosinophil degranulation than sera from normal donors (Fig. 2). These observations suggest that sera from SRW-sensitive patients contain an allergen-specific factor which induces eosinophil degranulation in vitro.

In atopic diseases, IgE is regarded as a critical Ig, and higher

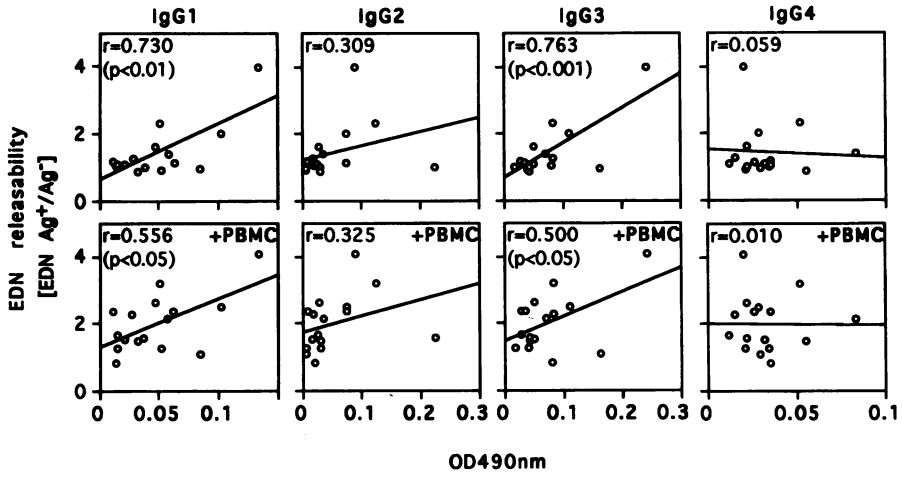

Figure 4. Correlations between the eosinophil degranulation and content of IgG subclass SRW-specific antibodies in sera from normal donors (n = 6) and SRW-sensitive patients with asthma (n = 10). The ability of sera to induce degranulation was assessed in the presence (*lower panel*) or absence (*upper panel*) of PBMC. Significant correlations were obtained for SRW-specific IgG1 and IgG3 both in the presence and absence of PBMC.

levels of mite-specific serum IgE are present in mite-sensitive patients with asthma compared with normal donors (16, 39, 40). On the other hand, elevated allergen-specific serum IgG was also detected in patients with asthma (16, 39–41). Likewise, we found significantly higher amounts of SRW-specific IgE and IgG in sera from SRW-sensitive patients with asthma (Fig. 3). Previously, using Ig conjugated-Sepharose beads, we found that IgG can trigger eosinophil degranulation; serum IgA also induced eosinophil degranulation, but less than IgG; IgE, IgD, and IgM did not cause eosinophil degranulation (22). Indeed, human eosinophils constitutively express the low affinity IgG FcR (FcγRII) on the plasma membrane (17, 19, 20, 42). On the other hand, others have reported both IgE-and IgG-induced eosinophil degranulation in rat (43) and human eosinophils (23, 24) using parasite antigens and sera from patients with parasitic infectious diseases. Furthermore, they reported that human eosinophils have both low affinity $Fc_\epsilon R$ ($Fc_\epsilon RII$) and high affinity $Fc_\epsilon R$ ($Fc_\epsilon RI$) (26–28).

Considering these results, we studied the contribution of IgG and IgE to allergen-dependent eosinophil degranulation. First, as shown in Table II, only the F(ab')2 fragment of anti-human IgG clearly inhibited the eosinophil degranulation; the F(ab')2 fragment of anti-human IgE had no effect on degranulation. Second, IgG-deficient pooled serum was unable to induce allergen-dependent eosinophil degranulation. On the other hand, IgE-deficient pooled serum did induce eosinophil degranulation (Table III). Third, we tested the effect of mAb to FcR on allergen-dependent eosinophil degranulation. As mentioned above, eosinophils constitutively express FcγRII, and their binding to IgG-coated surfaces and cytotoxic activities could be mediated through these receptors (17–19). Hartnell et al.

Table V. Ability of Immobilized-IgG or -IgE to Induce Eosinophil Degranulation

Experiment	Ig	PBMC	EDN released (ng/2.5 × 10^5 cells)
1	–	–	22.0±0.6
	IgG 1.2 μg/ml	–	28.5±1.2
	IgG 3.7 μg/ml	–	49.7±4.6
	IgG 11 μg/ml	–	101.6±6.6
	IgG 33 μg/ml	–	263.4±10.9
	IgG 100 μg/ml	–	303.7±14.0
2	–	–	17.2±0.6
	IgG 10 μg/ml	–	112.3±3.4
	IgE-PS 2 μg/ml	–	17.2±0.6
	IgE-PS 10 μg/ml	–	17.0±0.5
	IgE-PS 50 μg/ml	–	17.8±0.5
3	–	–	14.9±0.2
	IgG 100 μg/ml	–	139.0±0.5
	IgE-PS 100 μg/ml	–	17.8±1.8
	–	+	13.3±0.5
3	IgG 100 μg/ml	+	204.8±10.9
	IgE-PS 100 μg/ml	+	14.9±0.2

The wells were treated with 100 μl of each Ig solution at concentrations indicated and then with HSA to block any remaining protein binding sites. Eosinophils or eosinophils and PBMC were added to the wells and cultured for 15 h. Results are expressed as mean±SEM of triplicate analyses. Each experiment was done using eosinophils from different donors.

Table VI. Effect of IL-5 on Purified Human Ig-induced Eosinophil Degranulation

	EDN released (ng/2.5 × 10^5 cells)			
	Experiment 1		Experiment 2	
Ig	−IL-5	+IL-5	−IL-5	+IL-5
–	33.4±1.4	21.2±0.4	21.6±2.6	15.1±0.1
IgG	34.7±0.4	135.3±41.0	56.9±13.6	313.0±24.5
IgE-PS	30.5±0.9	20.7±2.0	23.0±3.7	13.7±0.4
IgE	32.5±3.7	18.4±0.5	17.1±1.5	12.4±0.3

Wells were treated with 100 μl of each Ig solution (10 μg/ml) followed by HSA to block any remaining protein-binding sites. Eosinophils were added to the wells and cultured for 15 h in the presence or absence of IL-5 (1 ng/ml). Results are expressed as mean±SEM of duplicate analyses. Each experiment was done using eosinophils from different donors. We used myeloma IgE purified from serum from a patient with multiple myeloma as described elsewhere (IgE-PS) (21) and myeloma IgE purchased from Cortex Biochem.

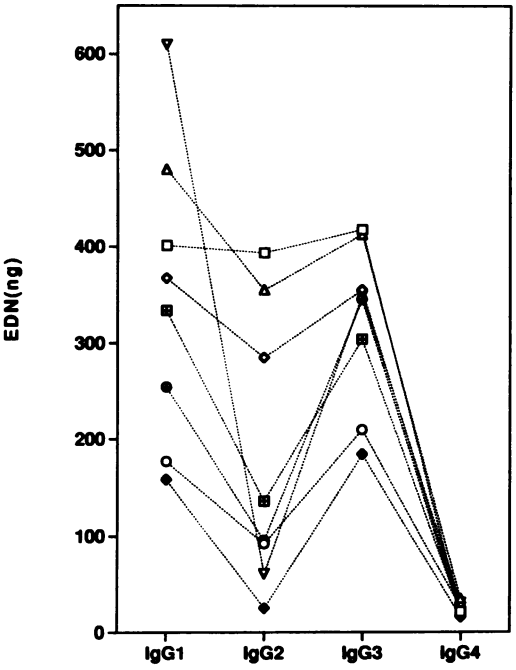

Figure 5. Ability of immobilized-IgG subclasses to induce eosinophil degranulation. The wells were coated with 50 μg/ml of each IgG subclass, and HSA was used to block active protein-binding sites. Eosinophils (0.5×10^5 cells/well) isolated from eight normal donors were added to the wells and incubated for 15 h at 37°C. Data are presented as released EDN (ng/2.5×10^5 cells) and the data points from each individual were connected by dotted lines. The basal EDN release of these experiments was 15.4±1.8 (ng/2.5×10^5 cells, mean±SEM).

(20) reported that FcγRIII was inducible on eosinophils, and leukotriene C_4 synthesis from eosinophils was induced by FcγRIII-mediated stimulation. Therefore, we tested the effects of anti-FcγRII and anti-FcγRIII on degranulation. Concerning $Fc_\epsilon R$, Capron et al. have reported the existence of $Fc_\epsilon RII$ on eosinophils, which is distinguishable from $Fc_\epsilon RII$/CD23 on B cells (26). Therefore, we also tested the effect of three mAb to $Fc_\epsilon RII$ on degranulation. Two of them were anti-CD23 and another was BB10CC2, namely the anti-$Fc_\epsilon RII$ on eosinophils (25). Among all these antibodies, only anti-FcγRII antibodies significantly inhibited the allergen-dependent eosinophil degranulation (Table IV). None of the anti-$Fc_\epsilon RII$ antibodies inhibited eosinophil degranulation. Previously, FcγRII-dependent eosinophil function was observed by three groups (17–19) using rosette formation assay and a cytotoxic assay. Our observation concurs with these latter reports and indicates the important role of $Fc_\gamma RII$ for eosinophil degranulation. Finally, we coated wells with purified human IgG or IgE. Eosinophils incubated with immobilized IgG degranulated in a dose-dependent manner. However, eosinophils incubated with immobilized IgE did not degranulate even in the presence of PBMC or IL-5 (Tables V and VI). Taken together, these experiments indicate that IgG anti-SRW antibodies, but not IgE antibodies, in patients' sera contribute to allergen-specific eosinophil degranulation and that the FcγRII on the eosinophil surface is involved in degranulation.

In addition to the experiments described above, we have also isolated hypodense eosinophils from four patients with the hypereosinophilic syndrome and performed experiments similar to those described in Tables II, III, and V. The results of these experiments did not differ from those using normal eosinophils, and no contribution of IgE to eosinophil degranulation was detected. Moreover, normodense eosinophils incubated with IL-5 also showed degranulation in an IgG-, not IgE-, dependent mechanism (Tables II and VI). We could not detect expression of $Fc_\epsilon RII$ on either normodense or hypodense eosinophils by FACS analyses, although we could detect a minimal amount of surface bound IgE (data not shown).

Human IgG consists of four subclasses, IgG1, IgG2, IgG3, and IgG4, and differences in their abilities to induce eosinophil activation were previously reported using a parasite-killing assay system (38). We tested their abilities to induce eosinophil degranulation by immobilizing each IgG subclass on the culture plate. As shown in Fig. 5, IgG1 and IgG3 induced eosinophil degranulation, whereas IgG4 did not. The ability of IgG2 to induce degranulation appeared to be dependent on donors of eosinophils. These findings are consistent with allelic polymorphism of the $Fc_\gamma RIIA$ gene (44), which results in a heterogeneic response to IgG2, while responses to IgG1 and IgG3 are always active (45). Furthermore, the IgG1- and IgG3-mediated eosinophil degranulation acted through FcγRII (data not shown). We detected significant correlations between the amounts of IgG1 or IgG3 anti-SRW antibodies and the ability of each serum to induce eosinophil degranulation (Fig. 4). These results suggest that IgG1 and IgG3 anti-SRW antibodies in patients' sera contribute to the allergen-dependent eosinophil degranulation.

Eosinophils likely play an important role in the pathogenesis of bronchial asthma, especially in LAR by releasing their cytotoxic granule proteins including MBP (1, 46). The LAR is usually observed several hours after allergen provocation in contrast to the immediate asthmatic response which occurs within 30 min (47–49). Several groups have reported on the possible role of allergen-specific IgE through the activation of mast cells (50–52) and other IgE-receptor bearing cells (53, 54) for the pathophysiology of LAR. According to our experiments, allergen-specific IgG may be another factor which is responsible for LAR. Allergen-specific IgG can induce eosinophil degranulation in vitro (Tables II–IV), and this IgG dependency is unchanged when eosinophils are primed by IL-5, the predominant eosinophil-active cytokine in bronchial asthma (55) (Table II). The time-course of allergen-dependent eosinophil degranulation is similar to that reported for LAR (Fig. 1 *b*). Furthermore, purified myeloma IgG1 and IgG3 are strong agonists for eosinophil degranulation in vitro (Fig. 5), and several previous studies have shown elevated levels of IgG1 in sera (40, 56) or bronchoalveolar lavage fluid (57) from patients with asthma. The production of IgG1 and IgG3, but not IgG2 or IgG4, is coordinately regulated by one of the Th2 cytokines, IL-10 (58); Th2 cytokines are implicated in the pathophysiology of bronchial asthma (59). In fact, Ito et al. (40) reported that the levels of allergen-specific IgG1 in sera correlated with a propensity to develop the LAR in mite-sensitive patients with asthma in vivo. Allergen-specific IgG, especially IgG1, may need to be evaluated as a possible Ig causing eosinophilic inflammation in various allergic diseases, such as bronchial asthma and hay fever.

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