

Down-Regulation of FcεRI Expression on Human Basophils During In Vivo Treatment of Atopic Patients with Anti-IgE Antibody¹

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Treatment of allergic disease by decreasing circulating IgE with anti-IgE Abs is currently under clinical study. Based on previous unrelated studies, it appeared likely that FcεRI expression on basophils and mast cells might also be regulated by levels of circulating IgE Ab. Therefore, the expression of IgE and FcεRI on human basophils was examined in 15 subjects receiving humanized anti-IgE mAb intravenously. Treatment with the anti-IgE mAb decreased free IgE levels to 1% of pretreatment levels and also resulted in a marked down-regulation of FcεRI on basophils. Median pretreatment densities of FcεRI were ≈220,000 receptors per basophil and after 3 mo of treatment, the densities had decreased to a median of 8,300 receptors per basophil. Flow cytometric studies, conducted in parallel, showed similar results and also showed in a subset of 3 donors that receptors decreased with a $t_{1/2}$ of approximately 3 days. The responsiveness of the cells to IgE-mediated stimulation using anti-IgE Ab was marginally decreased (≈40%) while the response of the same cells to stimulation with dust mite Ag, *Dermatophagoides farinae*, was reduced by ≈90%. One possible explanation for these results is that FcεRI density is directly or indirectly regulated by plasma-free IgE levels. *The Journal of Immunology*, 1997, 158: 1438–1445.

Allergic disease depends on IgE Abs binding to high affinity receptors (FcεRI) located on tissue mast cells or circulating basophils. A critical component of the allergic response is the release of histamine and other mediators from these cells when allergen cross-links the specific IgE Abs occupying FcεRI. It is not surprising, therefore, that the most attractive strategy to prevent allergic disease has been to decrease the quantity of IgE. It is assumed that the amount of IgE bound to mast cells and basophils will, at some level, determine the sensitivity of these cells to Ag. Consequently, the expression of the receptor FcεRI should be an equally important determinant of the magnitude of the allergic response. Almost two decades ago, a study of human basophil receptor density noted a strong correlation between the plasma total IgE level and the number of FcεRI per basophil and a causal relationship was postulated (1). This and subsequent studies (2, 3) noted that FcεRI densities in allergic and nonallergic persons range from 10^4 to 10^6 receptors per basophil. Most allergic patients have basophils with several hundred thousand IgE receptors occupied with IgE Ab (4). Since the number of cell surface Abs required for half-maximal histamine release varies between several hundred and a few thousand (3, 5), strategies directed at lowering IgE are required to decrease the level by >99%.

More recent studies demonstrated that the number of unoccupied receptors generally remains constant across this entire range of total receptor densities (3). One hypothesis that would explain these observations is that basophils synthesize new receptors in response to IgE binding, thereby maintaining a relatively constant level of unoccupied receptor expression, but at the same time increasing total receptor density. However, it has been difficult to demonstrate this phenomenon in vitro, i.e., a dynamic control of receptor density by the presence or absence of IgE.

A humanized anti-IgE mAb, rhuMAb-E25, was recently developed. In a previously reported study, murine anti-IgE mAbs were produced by the immunization of mice with human IgE (6). The screening strategy employed in monoclonal selection identified an Ab that recognized IgE at the same site as FcεRI. Thus, this Ab has the following characteristics: it binds to free IgE and not to IgG or IgA, it blocks the binding of IgE to its specific high affinity receptor, it does not bind to basophil or mast cell-bound IgE, and it inhibits the synthesis of IgE in cultured IgE-producing cells. To make the molecule more suitable for continual administration to humans, the critical amino acids responsible for binding to IgE were grafted onto a consensus human IgG1 framework (7). This recombinant humanized anti-IgE mAb (rhuMAb-E25) demonstrated biologic properties similar to the murine anti-IgE mAb. rhuMAb-E25 was expected to be well tolerated in humans, since another Ab with the identical IgG1 framework but a different binding site (recombinant humanized anti-p185HER2 mAb) has been well tolerated when used for the long-term treatment of patients with breast cancer (8). Furthermore, since rhuMAb-E25 does not bind to IgE on mast cells and basophils, there was no presumed risk of cross-linking IgE molecules on these cells and inducing anaphylaxis. This toxicology trial with rhuMAb-E25 allowed us to test the hypothesis that reductions in plasma IgE would be associated with decreased expression of basophil FcεRI although these

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Table 1. Baseline characteristics of study subjects

Characteristics	Mean $\pm$ SD
Age	29.8 $\pm$ 6.3
Sex	
Male	7
Female	5
Weight	77.2 $\pm$ 16.3 kg
Baseline IgE	192 $\pm$ 79 IU/ml
Maintenance dose of biweekly rhuMab-E25	114.2 $\pm$ 40.4 mg

studies would not, in themselves, rule out other unknown effects of the treatment.

Materials and Methods

Subjects

Fifteen subjects were recruited through public advertisement for participation in an open-labeled, Phase I trial of i.v. rhuMab-E25. Twelve of these subjects were examined as described below while the remaining three began treatment later and were used for a series of experiments designed to follow FcεRI expression during the first 2 wk of treatment. Two additional dust mite-allergic subjects, recruited separately, were used as controls for changes in FcεRI during the time frame of the study. However, they were not subjected to any treatment regimen. All subjects complained of perennial nasal symptoms and fulfilled the following entry criteria: age range of 18 to 65, negative serum pregnancy test (βHCG) and not breast feeding at commencement of the study, usage of an acceptable method of contraception for women, weight within the 70 to 130% range of the ideal value for their height according to the 1983 Metropolitan Height and Weight Table, positive (≥ 5 mm wheal) epicutaneous skin test to either *Dermatophagoides farinae* or *Dermatophagoides pteronyssinus*, total serum IgE ranging between 187 and 1210 ng/ml (85–550 IU/ml), and a stable therapeutic regimen. Eleven subjects were also affected by asthma. Subjects were excluded if they had ever experienced anaphylaxis, if their perennial rhinitis symptoms included marked seasonal exacerbations, if they had to be hospitalized or visit the emergency department for asthma treatment within 6 wk before the initiation of the study, or if they had required mechanical ventilation for status asthmaticus in the year before the initiation of the study. In addition, we excluded persons whose baseline forced expiratory volume in 1 s (FEV₁) was $<50\%$ of the predicted value, those who had been on allergy immunotherapy in the year before the study, and those with a history of a medical illness that raised reasonable suspicion of possible interference with the safety outcomes of the study. Patients gave signed consent for this study as approved by the Johns Hopkins internal review board. The baseline characteristics of the 12 subjects used in the full study are shown in Table 1. In this study, two biweekly i.v. maintenance doses of rhuMab-E25 were administered: 0.015 mg/kg/IU/ml and 0.03 mg/kg/IU/ml. The two dose levels selected for this study were based on the pharmacodynamic (PD) data obtained in the complete phase IIa placebo-controlled trial in 181 dosed adults with allergic rhinitis.³ In this trial, a dosage of 0.0075 mg/kg/IU/ml/wk in a small number of patients suppressed serum-free IgE to ≤ 24 ng/ml. In the study reported herein, a twofold higher dosage group was also included to ensure adequate suppression of serum-free IgE concentrations, and to evaluate more fully the safety of high doses of rhuMab-E25 that may be required for clinical efficacy.

Buffers

PIPES (Sigma Chemical Co., St. Louis, MO) stock buffer was made up of 25 mM PIPES containing 110 mM NaCl, 5 mM KCl, and 40 mM NaOH, adjusted to pH 7.3 and stored at 10 times the above concentration. PAG⁴

buffer consisted of PIPES (1×) containing 0.003% human serum albumin (HSA) (Miles Laboratories Inc., Elkhart, IN) and 0.1% glucose. PAGCM buffer contained PAG with 1.0 mM CaCl₂ and 1.0 mM MgCl₂; PAG-EDTA contained PAG and 1.0 mM EDTA. Acetate elution buffer contained 0.05 M sodium acetate, 0.085 M NaCl, 10 mM EDTA and 0.03% HSA at pH 7.3.

Reagents

Polyclonal goat anti-human IgE was prepared as described previously (9); the Ab used for these studies represented the IgG fraction of goat serum prepared by DE-52 chromatography. Dust mite Ag (5000 PNU/ml, containing 0.4% phenol) *D. farinae* (Miles, Inc.) was obtained as the skin test reagent used to screen the donors in the study. This Ag was dialyzed against three changes of saline and finally 1× PIPES buffer using SpectroPor 10,000 MWCO tubing (Spectrum Medical Industries, Los Angeles, CA). The resulting Ag was tested on three types of donors, a nonreleaser (no IgE-mediated histamine release with anti-IgE Ab), a non-atopic donor highly sensitive to anti-IgE Ab and an atopic donor without skin test sensitivity to dust mites. The dialyzed Ag did not induce histamine release in these three types of donors, while it was an effective stimulus for basophils from dust mite allergic donors. The optimal concentration on the dose-response curve was determined to be ≈ 10 PNU/ml for dust mite allergic donors.

FMLP was obtained from Sigma Chemical Co. Benzylpenicilloyl (BPO)-specific IgE was partially purified from the serum of a penicillin-allergic patient by affinity methods previously described in detail (10). It is a combination of both IgG and IgE specific for penicillin and $>95\%$ of the IgE is specific for the penicillin hapten (11). BPO₁₁-HSA was prepared as described previously (12). Purified IgE (P.S. myeloma) was a gift from Dr. T. Ishizaka (13).

Cell preparation

Two types of basophil preparations were employed. For the receptor measurements by acid elution, venous blood was drawn by venipuncture and the cells were separated on a single-step Percoll gradient (14) (basophil purities of 0.5–2%). Briefly, blood was centrifuged over Percoll (sp. gravity 1.080), then the mononuclear cell layer was collected and washed with EDTA-saline, PAG-EDTA, and twice with PAG, and then used as described below. For the flow cytometric studies, the blood was diluted with Percoll to a final density of 1.065 g/ml and layered over Percoll at a density of 1.079 g/ml as described previously (15). After centrifugation at 450 × g for 15 min, the interface between the 1.065 g/ml Percoll/plasma upper layer and the 1.079 g/ml lower Percoll layer was harvested and washed as above (basophil purities of 3–15%).

General protocol

The protocol for receptor density measurements was conducted just before the start of treatment and 3 mo after the start of treatment. For receptor measurements by acid elution, washed mononuclear cells were divided into three portions: two-ninths for histamine release, two-ninths for endogenous IgE density measurements, and five-ninths for sensitization (see below) with subsequent IgE density measurements. The cells destined for a determination of histamine release were challenged as soon as their preparation was completed to minimize the effects of handling on intrinsic functional responses of the basophils. The cells destined for determination of endogenous IgE densities were resuspended in 5 ml of PAG buffer and incubated at 37°C for 30 min, after which they were centrifuged and resuspended in 1 ml saline. Further processing for IgE densities is described below. The cells destined for sensitization were prepared as described below.

Cell challenge

Dose-response curves for polyclonal anti-IgE Ab were examined to obtain the optimal or maximal release value. The response to two doses of *D. farinae* previously shown to be optimal and suboptimal for release (10 and 0.5 PNU/ml, respectively) was included. To determine whether the response of the basophils to a non-IgE-mediated stimulus remained constant throughout treatment, cells were challenged with 1 μM FMLP. Cells were resuspended in PAGCM, then challenged with stimulus for 45 min at 37°C at a final volume of 1 ml. All reactions, performed in duplicate, were stopped by centrifugation after 45 min and the supernatant was removed for histamine analysis. In each experiment, perchloric acid at a 1.6% final concentration was added to duplicate tubes to determine total histamine content. Histamine was assayed by an automated fluorometric technique (16) and the percentage of histamine release was calculated from the ratio

³ Casale, T. B., I. L. Bernstein, W. W. Busse, C. F. Laforce, D. G. Tindelman, R. R. Stoltz, R. J. Dockhorn, J. Reimann, J. Q. Su, R. B. Fick, and D. C. Adelman, 1996. Use of an anti-IgE humanized mAb in ragweed-induced allergic rhinitis. Submitted for publication.

⁴ Abbreviations used in this paper: PAG, 1× PIPES containing 0.003% human serum albumin and 0.1% glucose; PAGCM, PAG with 1.0 mM CaCl₂ and 1.0 mM MgCl₂; HSA, human serum albumin; EC₅₀, median effective concentration; PNU, protein nitrogen units; RIST, radio immuno sorbent test; RAST, radio allergo sorbent test; BPO, benzylpenicilloyl; BMDC, bone marrow-derived mast cells.

of sample to total histamine after spontaneous release was subtracted from both.

Sensitization

To determine both the total and unoccupied receptor densities, the cells were first sensitized with BPO-specific IgE. The cells were incubated with this reagent at 5 µg/ml for 20 min at 37°C in RPMI 1640 media (Life Technologies, Gaithersburg, MD) containing 1 mM EDTA and 10 µg/ml heparin (250 µl final vol). Previous studies demonstrated that sensitization with this concentration of BPO-specific IgE for this length of time effectively saturates unoccupied receptors and therefore allowed for the measurement of total receptors (by measuring the total IgE from saturated cells) as well as the measurement of the unoccupied receptor density (11). After washing once, the cells were layered over 1 ml of EDTA-chelated FCS (5 mM EDTA in heat-inactivated FCS) and centrifuged for 5 min to rapidly separate the cells from their diluted sensitization buffer. The cells were further washed twice in PAG, then resuspended in 5 ml of PAG buffer and incubated for 60 min at 37°C. This incubation step and the one used for unsensitized cells (see above) were incorporated to facilitate the elution of IgE from low affinity receptor binding, whether it be to FcεRII or FcγRII, as determined in previous studies. After centrifugation, the pellets were resuspended in 1 ml of saline.

Acetate elution

Before a final centrifugation of the cells suspended in saline (saline is used to eliminate the buffering capacity of PIPES, reducing its influence on the subsequent acetate elution), duplicate samples were taken for cell counts. For the cells that were sensitized and after centrifugation, a sample of the saline supernatant was kept for analysis of IgE to ensure that there was no carry-over from the sensitization step. None of these samples contained measurable BPO-specific IgE. The pellets were resuspended in ice-cold acetate buffer, pH 3.7, and incubated in an ice bath for 10 min. For the cells destined for a determination of endogenous IgE, the elution vol was 0.35 ml and for the cells used for total and unoccupied receptors the final vol of elution was 0.7 ml. After a brief centrifugation (15,000 × g), the supernatant was removed, and neutralized with 1 N NaOH. These samples were frozen immediately for later measurement of total and specific IgE by RIST or RAST (9). Acid elution was done in duplicate. In addition to these samples, each day of testing also included a sample of IgE treated with the acetate buffer (or untreated control) and these samples were frozen along with the cell elution samples. These non-cell-derived IgE samples were used to determine whether acid treatment and storage affected the measurement of IgE; however, no effect was observed. Basophil counts (alcian blue staining, see Ref. 17) made before elution were also obtained in duplicate. This method of acetate "stripping" determines the amount of IgE bound to both basophils and mononuclear cells, but previous studies have shown that it is the basophil that is the primary, if not sole, contributor to "stripped" IgE Ab for normal donors (11). The flow cytometric studies described below indicate that the monocytes of this study's donors did not have detectable IgE (see Discussion).

IgE measurement for cell studies

The eluted IgE was measured in either a total IgE RIST (endogenous IgE or total receptor measurement) or BPO-RAST (unoccupied receptors, see below). Cells counts (alcian blue-positive cells) obtained before elution allowed a calculation of the receptor density to be made (the amount of IgE measured by RIST or RAST divided by cell count with the result expressed as IgE molecules per basophil). This general procedure and the acetate elution technique have been described in detail previously (4, 5, 11, 12).

Serum IgE measurements

Human serum-free IgE levels were measured using a solid-phase ELISA. High binding flat-bottom polystyrene plates (Costar, Cambridge, MA) were coated overnight at 2 to 8°C with 100 ng of human IgE receptor α-chain IgG chimera (FcεRI-IgG) (18) in 100 µl carbonate buffer (pH 9.6), washed, and stored frozen. Samples were analyzed by standard ELISA methods. FcεRI-IgG captures only free IgE, that is, IgE that is not in a complex with rhuMAB-E25. After washing, captured IgE was detected with biotinylated monoclonal anti-human IgE and the color reaction generated with streptavidin-horseradish peroxidase and *o*-phenylenediamine/H₂O₂. The concentration of free IgE was calculated from a standard curve, using a four-parameter logistic log fit for the standards.

Flow cytometry

At various time points throughout the study, human basophils were isolated from 10 ml of venous blood by Percoll density gradient centrifugation (15).

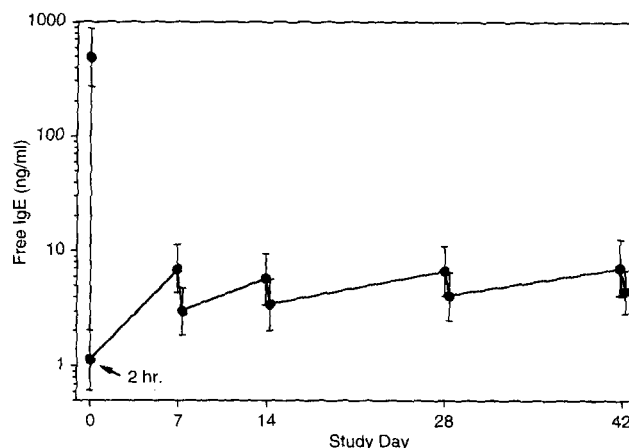


FIGURE 1. Free IgE Ab concentrations in patients undergoing anti-IgE Ab treatment. The plotted points represent the geometric means of data with the SD of the log-data also shown.

Basophil purity ranged between 1 and 9% (based on light microscopic counts after alcian blue staining). A flow cytometric technique incorporating light scatter characteristics was used to quantify cell surface IgE and FcεRI α-chain expression on basophils as described (2). Cell surface IgE was detected using FITC-conjugated polyclonal goat IgG anti-human IgE (Kirkegaard and Perry, Gaithersburg, MD); FITC-conjugated polyclonal normal goat IgG (Kirkegaard and Perry) was used as a control. To fully saturate FcεRI, separate aliquots of cells were passively sensitized with PS IgE myeloma (10 µg/ml, 37°C, 15 min in PAG/EDTA/heparin buffer; see above) before immunofluorescent labeling. Cell surface expression of FcεRI α-chain was detected using a mouse IgG1 anti-human FcεRI α-chain mAb (22E7, generously provided J. Kochan, Roche Pharmaceuticals, Nutley, NJ; see Ref. 19) and was compared with labeling with an identical concentration of irrelevant mouse IgG1 (Coulter, Hialeah, FL). The 22E7 Ab has been shown to recognize an epitope that is unaffected by FcεRI occupancy (19). Aliquots of cells were labeled in PBS containing 0.2% BSA with 4 mg/ml human IgG to minimize nonspecific binding to FcγR (2). Binding of monoclonals was detected using saturating concentrations of R-phycoerythrin-conjugated polyclonal goat anti-mouse IgG (Tago, Burlingame, CA). An EPICS Profile flow cytometer (Coulter, Inc., Hialeah, FL) was used to analyze fluorescent signals after excitation at 488 nm. Using "bitmap" gates to select for a population of cells that are predominantly basophils is possible because basophils have reasonably distinctive forward and side scatter characteristics. Since the cells were also enriched using a double Percoll gradient, these bitmaps can select a population of cells that is generally greater than 80% basophils, with the primary contaminants being lymphocytes. Monocytes were also gated for study by their distinctive forward/side scatter characteristics. Data is expressed as the mean fluorescence in labeled cells minus the mean fluorescence of IgG1 controls. Notably, incubation of fresh basophils from naive donors with up to 200 µg/ml of rhuMAB-E25 in vitro failed to alter labeling of basophils with FITC-anti-IgE or 22E7 Abs, suggesting that rhuMAB-E25 does not interfere with immune fluorescent labeling (data not shown). For some samples, 100 µl of whole blood was treated with perchloric acid and histamine content was analyzed as above. These samples were used to determine whether histamine content was measurable (i.e., the basophils were still present) even if IgE fluorescence became undetectable.

Statistics

A nonparametric Wilcoxon signed rank statistic was examined for each of the data sets although in some instances simple paired *t* tests were also applied. If error bars are shown, they represent the SEM.

Results

Patients were evaluated for serum titers of free IgE just before the first dose of anti-IgE Ab, 2 h following this first dose and every 2 wk subsequently. The data in Figure 1 show pretreatment, 2 h and 1, 2, 4, and 6 wk after the start of treatment for the 12 patients examined for FcεRI. Free IgE titers dropped rapidly after the first dose and although there was slight rebound after this first dose, the

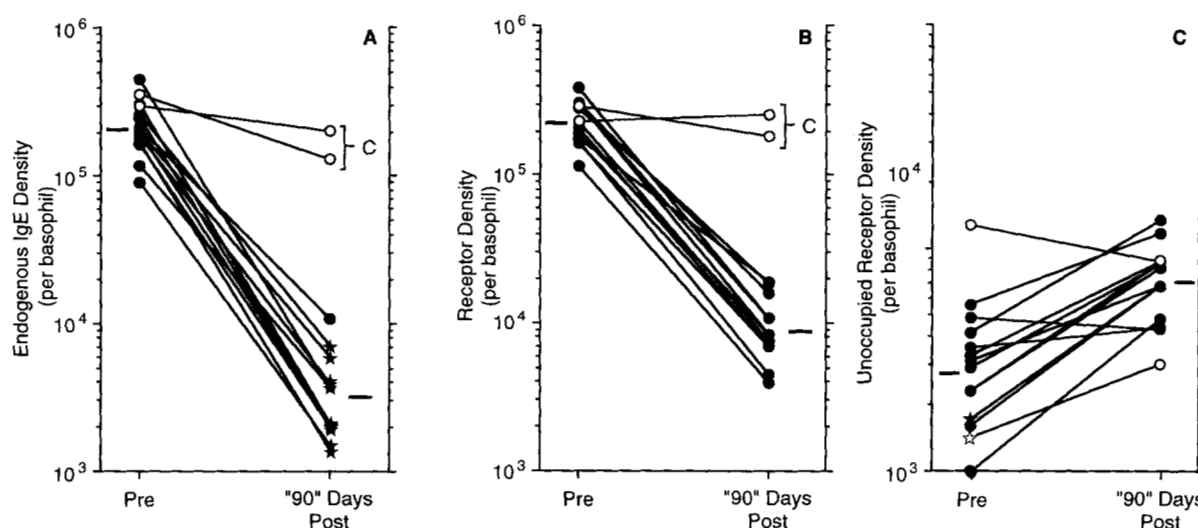


FIGURE 2. Expression of endogenous IgE and FcεRI on basophils of donors undergoing anti-IgE Ab treatment. A to C show the results for 12 treated patients and 2 controls. The data is generated using the acetate buffer "stripping" technique. The open symbols indicate the control patients, closed symbols indicate the treated patients. A, the results for endogenous IgE Ab. For 11 of the 12 subjects, IgE levels in the RIST were not above the negative controls of this assay. However, since basophils were counted, a maximum value for the IgE density could be assigned. The 0.512 ng/ml standard on the standard curve for the total IgE RIST, represented the least detectable concentration (≥ 2 SDs from negative controls). Therefore this value was used in the numerator of the expression to calculate IgE density (amount of IgE/basophil count). On the plot, stars represent data calculated in this manner. B plots the results of IgE measurements for cells fully sensitized and therefore essentially plots total receptor densities. C plots the BPO-specific IgE densities and therefore essentially plots unoccupied receptor densities.

levels remained essentially constant thereafter, averaging 1% of the pretreatment titers, decreasing from a geometric mean of 488 ng/ml to 4.8 ng/ml (222 to 2.2 IU/ml).

Figure 2 shows the results for the three receptor parameters measured. Figure 2A plots the basophil surface IgE densities for each patient. The pretreatment average was approximately 240,000 molecules per basophil (median = 220,000). At the 3 mo time point, the amount of IgE "stripped" from the cells and measured in the total IgE RIST was not detectable for most of the treated patients. As noted in the legend of Figure 2, it was possible to calculate the maximum level of expression for each patient at this time. The average of the data calculated in this manner was $\approx 2,200$ IgE molecules per basophil, corresponding to a greater than 99% decrease ($p = 0.0022$).

Figure 2B shows a similar set of data for sensitized cells, i.e., a measurement of IgE density after saturating the unoccupied receptors and therefore a measurement of total receptor density. It can be seen that all samples had measurable IgE after "stripping," indicating a detectable number of total receptors. The average IgE receptor number before sensitization was $\approx 240,000$ per cell (median = 220,000). This was reduced to $\approx 8,600$ IgE receptors per basophil at the 3 mo measurement (median = 8,300). Based on the above IgE and receptor density results, treatment with rhuMAB-E25 resulted in more than threefold more unoccupied than occupied receptors. Most importantly, however, like the serum-free IgE levels and the endogenous basophil surface IgE densities, total FcεRI receptor density had markedly decreased, by $\approx 97\%$ ($p = 0.0022$).

Figure 2C shows data for the unoccupied receptors before and during treatment, i.e., the "stripped" IgE from sensitized cells measured by the BPO-RAST. Since the IgE used for sensitization was nearly 100% specific for penicillin, this measurement determines the unoccupied receptor density. Before treatment, this density averaged ≈ 2700 /basophil. After treatment, the average increased to ≈ 7100 /basophil. This increase was statistically significant ($p =$

0.0029). The difference between total receptors (Fig. 2B) and unoccupied receptors (Fig. 2C) for the 3 mo time point, using the average values, suggests a value of ≈ 1500 endogenous IgE molecules, which is just below the maximal estimate obtained in Figure 2A. However, it should also be pointed out that this acetate "stripping" method of measuring receptors has an average error of $\approx 25\%$, so that the average total receptor density at 3 mo of 8600, if calculated from an individual experiment, would have a one SD error of ± 2200 . Therefore, the difference of 1500 should be interpreted cautiously. Not shown in the figure are the basophil counts, at the pretreatment and 3 mo time points; basophil numbers were not significantly different, with an average ratio 1.06 ± 0.11 ([counts at 3 mo]/[counts pretreatment]).

At three times during treatment, the basophils were also analyzed for IgE by flow cytometry (Fig. 3). Flow cytometry provided an important control to rule out the possibility that the absolute receptor measurements were also assessing high affinity binding of IgE on cells other than basophils, i.e., on monocytes. The enriched basophils were examined with or without prior sensitization with IgE myeloma to determine the endogenous expression of IgE or total FcεRI expression. As seen in Figure 3A, by 5 wk, and persisting at 10 wk, the cells of most donors expressed minimal endogenous IgE; most positively stained flow cytometric distributions became indistinguishable from control staining distributions ($p = 0.0022$). These points are plotted as a zero in the figure. Interestingly, the one subject with persistently detectable levels of IgE (Fig. 3A) had a definitely detectable level of IgE found by RIST (Fig. 2A). Total receptor expression after passive sensitization (Fig. 3B) also decreased markedly but was detectable at both 5 and 10 wk for about half the patients. Previous calibrations of the sensitivity of the flow cytometer for detecting basophil IgE demonstrated the minimum detectable expression to be 8,000 to 10,000 IgE molecules per basophil. Therefore, these undetectable levels of expression are compatible with the data shown in Figure 2 (the histamine content of these samples was similar to values obtained

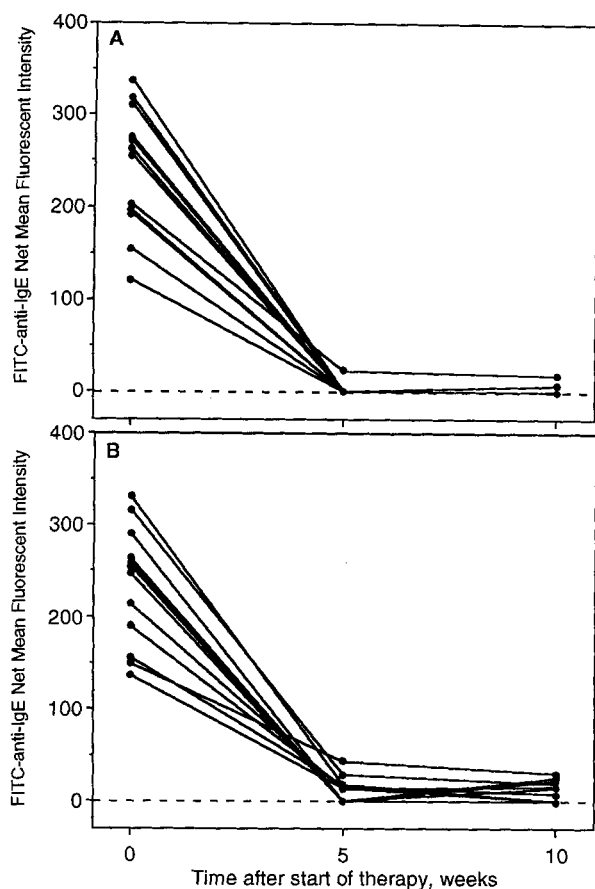


FIGURE 3. Levels of basophil IgE with and without sensitization as determined by flow cytometry. *A* shows the results for endogenous IgE expression (without sensitization) for 12 patients on treatment at three time points, and *B* shows similar data for cells first sensitized with IgE myeloma Ab (see text).

before treatment). Indeed, the sporadic net positive results (among patients) after sensitization are also compatible with the unoccupied receptor data in Figure 2, since the average total receptor density borders on the threshold of detection for the flow cytometer.

For the last three patients enrolled in the study, flow cytometric measurements were made several times during the first 2 wk of treatment to more carefully examine the kinetics of basophils IgE and FcεRI down-regulation. The dosing regimen was slightly different for these three patients and the timing of the doses is indicated in Figure 4. This figure demonstrates that a 50% decrease in both endogenous IgE density and total receptors occurred by approximately 3 days for all three patients. In these experiments, the total receptor expression was also determined more directly using an anti-FcεRIα subunit Ab, 22E7, which binds to both occupied and unoccupied receptors (Fig. 4*B*). Reduction in FcεRI paralleled the total receptor data obtained by first sensitizing the cells with IgE myeloma (Fig. 4*A*). Both endogenous IgE and FcεRI decreased at similar rates. We also noted that the flow cytometric distributions remained unimodal throughout the 2 wk time course.

Figure 5 illustrates the functional consequences of these changes on basophils as measured by their ability to secrete histamine in response to several physiologic stimuli. The basophils were assessed before and during treatment by challenge with: 1) dust mite Ag *D. farinae* at optimal and suboptimal doses; 2) a full dose-response to polyclonal goat anti-IgE Ab; and 3) a single concen-

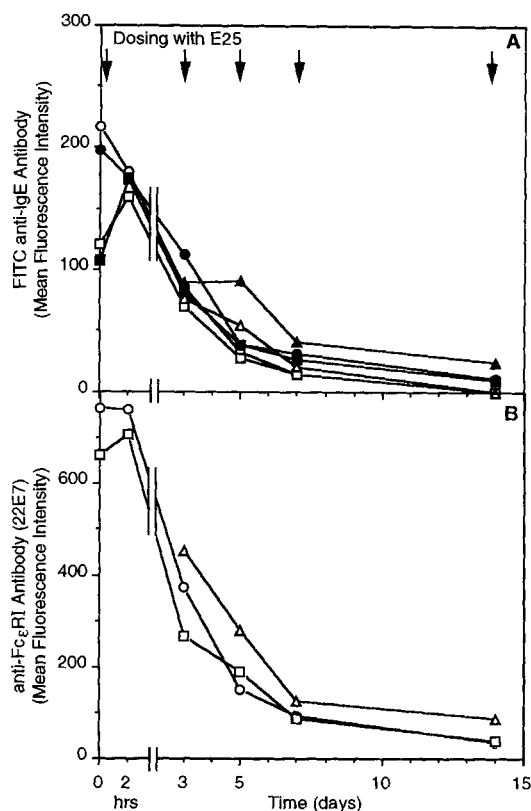
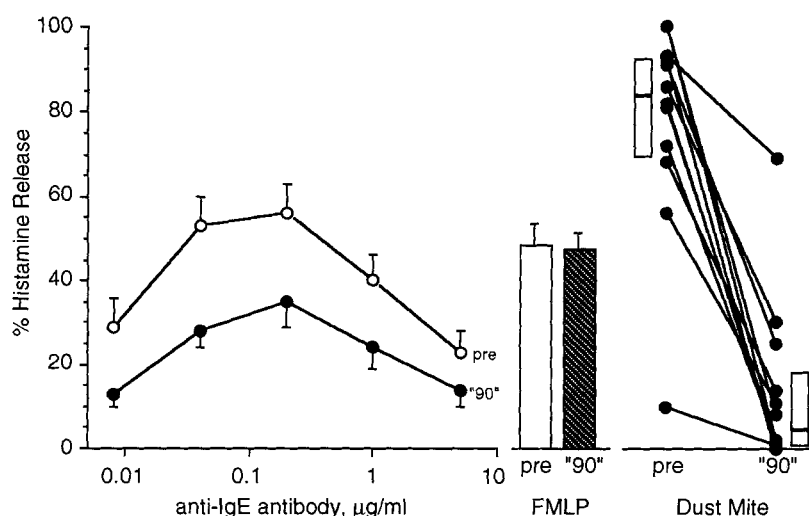


FIGURE 4. Flow cytometric measurements of endogenous IgE and FcεRI expression during the first 2 wk of treatment for three patients with the highest serum levels of IgE at enrollment (1093, 702, 823 ng/ml, respectively). *A* shows the results for IgE expression without prior sensitization (open symbols) and with prior sensitization (closed symbols) for three different subjects (square, circle, triangle). *B* shows the results for FcεRI expression as determined by the binding of the anti-FcεRIα Ab, 22E7. Note that the values at 0 and 2 h for subject Δ were not obtained. Values of zero in *A* represent undetectable compared with FITC normal goat IgG; mean fluorescence values for irrelevant IgG controls in *B* were 3 to 5. The dosing regimen is indicated by the arrows.

tration of FMLP. Since FMLP acts through a distinct receptor that has a markedly different signal transduction pathway from IgE-mediated release, the response to this stimulus was not expected to change during treatment and, as can be seen in the *middle panel*, it did not. In contrast, the response to dust mite Ag (the response to the optimal concentration is shown) decreased by $\approx 90\%$ (median ratio of postchallenge/prechallenge was 0.09, $p = 0.0022$). Moreover, in half the patients there was essentially no response. The response to the 20-fold lower suboptimal concentration of dust mite Ag was proportionally lower or zero (data not shown). The response of the two controls used in these studies was essentially unchanged (ratio of postchallenge/prechallenge was 0.83 ± 0.25 ; range, $n = 2$, data not shown). The response to anti-IgE Ab was also decreased in the treated patients but not to the same extent as Ag, with the peak response decreasing $\approx 40\%$ ($p = 0.045$) and no meaningful change in the character of the dose-response curve. There was significant variability among donor basophil responses, with nearly no change in the response to anti-IgE Ab in some donors, while in several cases the response to anti-IgE was nearly ablated. For seven of the patients at the 3 mo time point, we examined the response of the basophils that had been sensitized with

FIGURE 5. Histamine release response of basophils challenged with three secretagogues before treatment and 3 mo into treatment. The left portion of the figure shows the anti-IgE Ab dose-response curves at the two time points; pretreatment (○) and the 3 mo time point (●). The right side histograms show the responses to FMLP and dust mite Ag (*D. farinae* at 10 PNU/ml). The box plots show the median $\pm$ 25% of the median at the two time points.



anti-BPO IgE (for the receptor studies) to both an optimal concentration of anti-IgE (0.2 μ g/ml) and to an optimal concentration of BPO(11)-HSA. On average, the histamine release to BPO-HSA was $70 \pm 7\%$, essentially the same as the pretreatment response to the natural antigenic sensitivity, *D. farinae* ($78 \pm 7\%$). The response to anti-IgE Ab also increased to $52 \pm 8\%$, which is also the same as the pretreatment response to an optimal concentration of anti-IgE Ab ($53 \pm 7\%$). These studies indicate that the number of unoccupied receptors remaining on the treated patients' basophils were, when sensitized, sufficient to drive a normal response to both Ag and anti-IgE Ab. Correlation of the responses of the cells to remaining (endogenous) IgE densities is not possible because, as noted above, the 3 mo data generally only represented the calculated maximum possible IgE densities. There were two observations on a subset of the data that were worth noting. First, the only patient whose basophils had a measurable endogenous IgE density (at 5 and 10 wk in Fig. 3A and the closed circle point in Fig. 2A at "90" days) was also the only patient whose response to dust mite Ag was only marginally reduced. Second, the two patients whose estimated "maximal" endogenous IgE densities were the lowest (1400–1500/basophil) also showed strong responses to anti-IgE Ab but poor responses to dust mite Ag. Parenthetically, we also observed that mast cell function was markedly decreased: skin prick tests to *D. farinae* required ≈ 100 fold more Ag for a wheal and flare equal to pretreatment measurements ($p = 0.0007$).

Discussion

These studies demonstrate a remarkable down-regulation of Fc ϵ RI on basophils during a period of time when the free circulating titers of IgE reached a small fraction of the patients' pretreatment levels. The number of controls used was small, but previous studies have shown that IgE and Fc ϵ RI densities are relatively stable over long periods of time, varying at most two- to threefold, but usually varying no more than the error of this measurement technique (3).

Both measures of Fc ϵ RI expression on basophils, absolute quantitation by acetate stripping, and flow cytometry gave similar results. As noted in *Materials and Methods*, acetate stripping has been shown to measure IgE, in most instances, on basophils. The contribution of IgE bound to other cells, either through low or high affinity receptors, has been largely found to be minimal, based on direct observations of IgE by flow cytometry, immunofluorescence, or by comparisons of measurements for impure and purified basophils (4, 11, 20–25). However, for these studies, we also ex-

amined monocytes by flow cytometry and did not detect IgE or Fc ϵ RI. The caveat to this observation is that even the density of IgE shown to be present on the monocytes of atopic dermatitis patients is near the threshold of detection for many flow cytometers (2). It is therefore possible that we simply could not detect IgE on the patients of this study, while its presence would have some impact on absolute IgE measurements. However, even if the acetate-elution measurements were also detecting non-basophil high-affinity bound IgE, the primary conclusion that the receptor is down-regulated during treatment would still hold. It would be down-regulated on both basophils and monocytes. Furthermore, the flow cytometric measurements provide a clear indication that Fc ϵ RI on basophils is down-regulated to the same degree as the acetate-elution measurements.

The kinetics of the decrease in IgE expression, under conditions in which the free IgE concentration was less than 5 ng/ml, indicate that the dissociation constant for Fc ϵ RI was on a scale similar to that found for the interaction of rat IgE Ab and rat Fc ϵ RI ($t_{1/2} \approx 50$ –70 h) (26, 27). However, it is possible that this loss does not represent dissociation of IgE from the existing basophils but the replacement of an original pool of basophils with new basophils that have not experienced up-regulation of Fc ϵ RI because of the low plasma free IgE concentration. The observation that the flow cytometric distributions for samples taken within the first several days of treatment were unimodal suggests that the existing population was uniformly losing IgE.

The potential clinical implications of these findings are profound. In the thirty years since the Ishizakas' discovery that IgE is the causal Ab of allergic disease, one goal of research in this area has been to abolish allergic disease by decreasing the level of IgE. Studies along the way, relating basophil/mast cell function to IgE/Fc ϵ RI, indicated that this would be extremely difficult, since a $>99\%$ decrease in the plasma IgE level would be necessary. Results from the present study suggest that while this level of reduction in IgE level has now been achieved, the functional consequences might have been modest, as expected, were it not for the parallel decrease in receptor density. The two changes in the system, a decrease in both free IgE and Fc ϵ RI, support the potential for this therapeutic approach (7, 28). Indeed, in these studies, the basophil response to a major allergen was obviated in one-half of the patients and the mast cell response, as measured by skin tests, was markedly reduced. Since this treatment targets all IgE, one

would expect that the clinical response to this and all other allergens will be markedly decreased or eliminated in those patients.

These observations also have several implications for understanding the basic biology of the allergic reaction. The first derives from the changes in receptor density as they relate to the changes in function. Previous studies have noted that basophils have many unoccupied receptors. Basophils from a typical patient will give a half-maximal response to antigenic stimulation with a cell surface density of 2,000 Ag-specific IgE molecules (3, 5). As noted in this study, the starting density of IgE was approximately 240,000 and since the ratio of Ag-specific IgE to total IgE can be as high as 50% (in ragweed-allergic patients for example), there will be a surplus of antigen-specific IgE molecules. To reduce the presence of Ag-specific IgE on the basophil surface to levels significantly below this EC_{50} threshold, in the absence of a change in receptor density, total IgE titer would have to decrease much more than 100-fold. Factoring in the down-regulation of the receptor, however, suggests that the decrease in IgE titer need be less profound.

In this same context, previous studies of basophil sensitivity predicted the observed functional results. If endogenous IgE densities dropped to ≈ 1500 molecules/basophil, the response to anti-IgE Ab would be expected to decrease to $\approx 50\%$ of its starting value, since this stimulus should aggregate all cell surface IgE and the EC_{50} for a half-maximal response is ≈ 2000 molecules per basophil. On the other hand, with the Ag-specific IgE representing only a fraction of the total IgE Ab present on the cell surface (possibly 10% for dust mite Ag), the cell surface Ag-specific Ab density would be on the order of 150 molecules, far below the EC_{50} . Therefore, on theoretical grounds, the Ag-induced response should have decreased further than the anti-IgE response and the observed 90% decrease is predicted. The fact that the cells sensitized with BPO-specific IgE Ab showed a return of the response to pretreatment levels (both for anti-IgE and Ag) supports the prediction that the EC_{50} is around 2000 IgE molecules/basophil and reaches a maximum response by ≈ 5000 (3, 5).

The early studies of FcεRI densities on basophils showed a correlation between IgE titer and density but this correlational data lacked any data at the low end of the curve ($< 20,000$ receptors or plasma IgE < 3 ng/ml) (1). Subsequent anecdotal observations of patients with very low IgE titers showed that the lowest measured value of total receptor density was approximately 5,000. Our recent study of unoccupied receptor densities suggested that the basophil maintained a density of around 5,000 unoccupied FcεRI (3). Together, these observations led to the prediction that a basophil would express 5,000 to 10,000 receptors in the absence of circulating IgE. The current studies support this suggestion, since the total receptor density after 3 mo of treatment ranged from 3,000 to 18,000 (median of 8,300).

There are many possible mechanisms that might explain FcεRI down-regulation. It would be especially useful to differentiate between explanations that do or do not depend on free IgE levels. For example, immune complexes form in the blood of these subjects and the presence of the IgE/anti-IgE Ab complexes may somehow influence basophils or another cell through their Fcγ receptors. However, we are not immediately considering the possibility that there are other effects of the treatment that cause the reduction because we have found a similar down-regulation of FcεRI when basophils are cultured in the absence of IgE (manuscript in preparation). Although the *in vivo* study only examined the down-regulation of FcεRI, it seems reasonable to assume that if it were the free IgE Ab levels that were responsible for the down-regulation observed, that FcεRI expression is actually dynamically regulated by free IgE levels, both increases and decreases. The positive correlation previously found to exist between plasma IgE

levels and basophil FcεRI expression (1) supports this assumption as do recent *in vitro* experiments. Isotype-specific regulation of Fc receptors has been observed for IgE and FcεRII on B cells (29) and on T cells, Fc receptors for IgE, IgG and IgA are also regulated by their respective Ig isotype (29–32).

Working under the assumption that free IgE levels arbitrate FcεRI expression, there remain questions about which receptors and/or cells are involved. IgE might regulate the expression or secretion of another cell product that in turn regulates basophil FcεRI expression. However, there are several observations that suggest IgE might be interacting directly with the basophil. Studies using rat basophilic leukemia cells (RBL cells) have noted that there can be internalization of FcεRI receptors by endocytosis and that FcεRI with bound IgE Ab was less likely to undergo endocytosis (33). In this way, inhibition of endocytosis would allow an accumulation of surface receptors (until all internal receptors were expressed). While the degree of up-regulation induced by this mechanism does not appear sufficient to explain the large range of total receptor density observed among human basophil donors, it does indicate that the FcεRI can behave differently when its ligand is present in monovalent form. A similar stabilization of FcεRII by IgE occurs on rodent B cells although the mechanism appears to involve inhibition of proteolytic cleavage of FcεRII by IgE rather than changes in endocytosis or synthesis of FcεRII (34). Recently, we have observed that the presence of IgE could up-regulate the expression of FcεRI in cultures of 3-wk-old bone marrow-derived mast cells (BMMC) (35). We noted up to 8-fold increases in receptor expression over a 2-day period with IgE present in the culture media and this up-regulation was sensitive to cycloheximide. Whether or not this is a direct effect of IgE acting on the FcεRI of BMMC is unknown at this time. Since this was not a homogeneous population of mast cells, the effect on receptor expression could still have been indirect. The BMMC studies appeared to also exclude a role for FcγRII/III in the regulation of BMMC FcεRI expression but a role for non-FcεRI receptors (e.g., CD23) in the human basophil studies remains a possibility. Future studies of long term cultured human basophils should provide some insights into this aspect of FcεRI regulation.

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