

Suppression of the basophil response to allergen during treatment with omalizumab is dependent on 2 competing factors

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Background: A recent study of subjects with peanut allergy treated with omalizumab generated some results that were concordant with a study of subjects with cat allergy treated with omalizumab. However, there were differences that provided additional insight into the nature of the cellular responses in allergic subjects.

Objective: We sought to determine the cause for failure to suppress the allergen-induced basophil response during treatment with omalizumab.

Methods: Patients with peanut allergy were treated with omalizumab. Clinical, serologic, and cellular indices relevant to the response of the subjects and their peripheral blood basophil values (specific/total IgE ratio, cell-surface FcεRI expression, and histamine release responses to anti-IgE antibody or peanut allergen) were obtained at 3 times.

Results: After treatment, approximately 60% of the subjects' basophil responses to peanut allergen did not significantly decrease. In 40% of cases, the *in vitro* basophil response to peanut allergen increased 2- to 7-fold. The increases were associated with 2 primary factors: a high (>10%) specific/total IgE ratio and an increase in the intrinsic response of the basophil to IgE-mediated stimulation. The extent to which the basophil response to peanut allergen increased was inversely correlated with improvement in the patient's ability to tolerate ingestion of peanut.

Conclusion: The basophil response during treatment with omalizumab is a consequence of 2 competing factors: suppression of allergen-specific IgE on the cell surface versus increased intrinsic sensitivity to IgE-mediated stimulation. In subjects with peanut allergy, the basophil response appears to mitigate against the ability of omalizumab to improve the patient's tolerance of oral allergen. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Basophil, anti-IgE, peanut, spleen tyrosine kinase, IgE receptor

Abbreviations used

AUC:	Area under the curve
BHR:	Basophil histamine release
HSA:	Human serum albumin
OFC:	Oral food challenge
PAG:	PIPES-albumin-glucose
PIPES:	Piperazine-N,N'-bis(2-ethanesulfonic acid)
Pn-BHR AUC:	Area under the dose-response curve to peanut allergen
SPT:	Skin prick test
Syk:	Spleen tyrosine kinase

A longstanding issue in the field of allergic disease is concerned with the relative roles of mast cells and basophils in the expression of this family of diseases. This question was recently explored by using omalizumab to manipulate IgE levels in subjects with cat allergy.¹ The study design used observations that the basophil response to cat allergen would decrease much faster than the tissue mast cell response when subjects were started on omalizumab. It was found that when the basophil response was markedly decreased, the nasal mast cell response was not, but that clinical signs and symptoms during an experimental allergen challenge in the nose were decreased by 50%. One interpretation of this observation was that basophils contributed to the acute response to allergen in the nose.

This experimental design was extended to subjects with peanut allergy.² As in the previous design, each patient's basophil histamine release (BHR) response to peanut allergen was monitored after the start of omalizumab. When the response decreased to 20% of the baseline value before omalizumab, subjects were brought back to the clinic for the first posttreatment double-blind oral food challenge (OFC). Unexpectedly, approximately 60% of the subjects' basophils did not show a decreased response to peanut allergen when considering the entirety of the peanut dose-response curve, although there were decreases at the lowest suboptimal levels of stimulation. There appear to be reasons for these results, which will be explored in this report because they speak to a newly discovered characteristic of the system. In the aforementioned study of subjects with cat allergy, it was observed that although the basophil response to cat allergen generally decreased after starting treatment, the distribution of the decrease in the treated subjects was broad. In that study it was noted that for nearly all subjects, the expression of basophil spleen tyrosine kinase (Syk) increased.³ This early nonredundant tyrosine kinase is essential for early signal transduction and is required for IgE-mediated histamine release, as well as essentially all IgE-mediated functional outcomes in basophils. The increase in Syk expression was accompanied by an increase in the response of basophils to the panstimulus anti-IgE antibody (an aggregating anti-IgE antibody routinely used to assess the IgE-mediated response

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from human basophils). The increase in Syk expression was unexpected unless certain properties of basophil maturation were taken into account,⁴ but even with this consideration, the behavior was surprising and clearly represented a possible confounding process toward the efficacy of treatments such as omalizumab. Although results from cat allergy studies suggested that there might be a mitigating factor related to the basophil's intrinsic sensitivity, the current peanut study demonstrated that this effect could be a dominant aspect of the underlying biological response to omalizumab. This study will demonstrate how 2 biological processes, the specific/total IgE ratio and the omalizumab-induced increase in the basophil's IgE-mediated response, compete to alter the basophil's response to challenge with a specific allergen, peanut in this case. In addition, this analysis of the peanut response improves on the interpretation of the mechanisms underlying the clinical response presented in the clinical portion of the study.

METHODS

Study design

The study was a 6-month, open-label study of 14 subjects and is described in greater detail in a companion article.² Briefly, subjects were treated with omalizumab according to the package insert for a period of 6 months. Before the start of treatment, several weeks after the start of treatment, and 6 months after the start of treatment, blood was obtained and basophils were tested for IgE-mediated responses and FcεRI and Syk expression. Also before treatment, subjects were tested with a double-blind peanut OFC. During the first weeks of treatment, subjects returned for blood sampling at week 2 and then weekly until week 6 to monitor peanut allergen-induced BHR. When a subject's peanut allergen-induced BHR decreased to less than 20% of baseline values (assessed based on the area under the dose-response curve to peanut allergen [Pn-BHR AUC]), a second OFC (OFC2) and skin prick test (SPT) titration (peanut SPT) were performed. If a patient's Pn-BHR AUC had not decreased to less than 20% of baseline values by week 6, OFC2 was performed at week 8. Peanut-BHR AUC was followed monthly after OFC2 in those subjects whose Pn-BHR AUC had not decreased to less than 20% of baseline values. After 6 months of omalizumab, a final OFC (OFC3) and peanut SPT were completed.

Total and peanut-specific IgE levels

Total and peanut-specific IgE levels were measured with the ImmunoCAP 250 (Phadia, Uppsala, Sweden).

BHR

Venous blood was drawn into syringes containing PBS-EDTA, and basophils were isolated with a single Percoll-based density gradient (Pharmacia, Piscataway, NJ) with Accuspin separation tubes (Sigma-Aldrich, St Louis, Mo). Enriched basophils were enumerated and stimulated for BHR with anti-IgE (0.03–1 μg/mL; HP6061; Hybridoma Reagent Laboratories, Baltimore, Md) and peanut allergen (0.001–10,000 ng/mL) in duplicate for 45 minutes at 37°C by using calcium-containing buffers. Automated fluorometry was used to measure BHR in cell-free supernatants.⁵ Results for each stimulus are reported as a percentage of the total histamine content found in an aliquot of perchloric acid-lysed leukocytes after subtraction of spontaneous BHR from cells in buffer alone, as follows:

$$\frac{(\text{Stimulated histamine release} - \text{Spontaneous histamine release})}{(\text{Lysed histamine release} - \text{Spontaneous histamine release})}$$

In some analyses the results are reported as Pn-BHR AUC values. BHR was performed twice before treatment with omalizumab, at the screening during

recruitment, and on the first day (day 0 = baseline) of the study before drug administration. The fold change in either the clinical response or the basophil response was calculated. For the basophil response, the area under the curve (AUC) of the peanut basophil response at OFC2 versus OFC1 was calculated, and the fold change was calculated as follows:

$$\text{AUC}_{(\text{OFC2})} / \text{AUC}_{(\text{OFC1})}$$

For the clinical response, the ratio of the amount of peanut ingested before stopping the challenge at OFC2 versus OFC1 was calculated.

Flow cytometry

Flow cytometric methods relevant to a study of omalizumab treatment have been described in detail in a previous publication³ and are included in the **Methods** section in this article's Online Repository at www.jacionline.org.

Statistics

Figures show average data ± SEM with relevant comparisons using the Student *t* test. Pearson correlations and linear regression are used for relationships. For curve fitting, data for the dose-response curves shown in **Fig E2** were fit to a Gaussian function from which 50% of maximum and peak position were extracted.

RESULTS

As previously reported,² 6 of 14 subjects' basophil responses to peanut allergen stimulation (AUC of the full dose-response curve) decreased, whereas the 8 remaining subjects showed an increase that averaged 2.9-fold (95% CI, 2.29-fold to 3.41-fold). In our previous study of subjects with cat allergy,¹ only 2 of 12 of the subjects showed an increase in the basophil response to cat allergen, only 1 of which was significantly greater than a ratio of 1.0 (posttreatment/baseline). The difference in the 2 studies was unexpected, quite marked, and puzzling because omalizumab is largely expected to reduce the function of basophils and mast cells. The difference might derive from 2 sources of biological variation that now appear to be relevant to the function of basophils.

The density of cell-surface peanut-specific IgE was considerably greater during treatment than the density of cat-specific IgE observed in the cat study. **Fig 1** summarizes the comparison. The calculation is based on several pieces of information. First, the starting FcεRI density was greater in the subjects with peanut allergy (**Fig 1, A**). Second, the ratio of treatment FcεRI density/baseline FcεRI density was slightly higher in the peanut study (**Fig 1, B**). Third, the average peanut-specific/total IgE ratio was higher in the subjects with peanut allergy (**Fig 1, C**). With these 3 factors and 1 additional factor, the IgE/FcεRI ratio for different IgE concentrations (for peanut and cat studies, a factor of 0.5; see the **Results** section and **Fig E1** in this article's Online Repository at www.jacionline.org), the average cell-surface specific IgE density was approximately 7-fold higher in the peanut study than in the cat study (5040 vs 740 IgE molecules per cell, respectively). This difference is important because several studies have suggested that basophils respond maximally to stimulation with only 5000 antigen-specific IgE molecules per cell.⁶ Note also that this number represents the average for the study subjects and that the heterogeneity is important for understanding the detailed response (see below).

An unexpected observation from the cat allergy study was that most subjects receiving omalizumab demonstrated an increase in

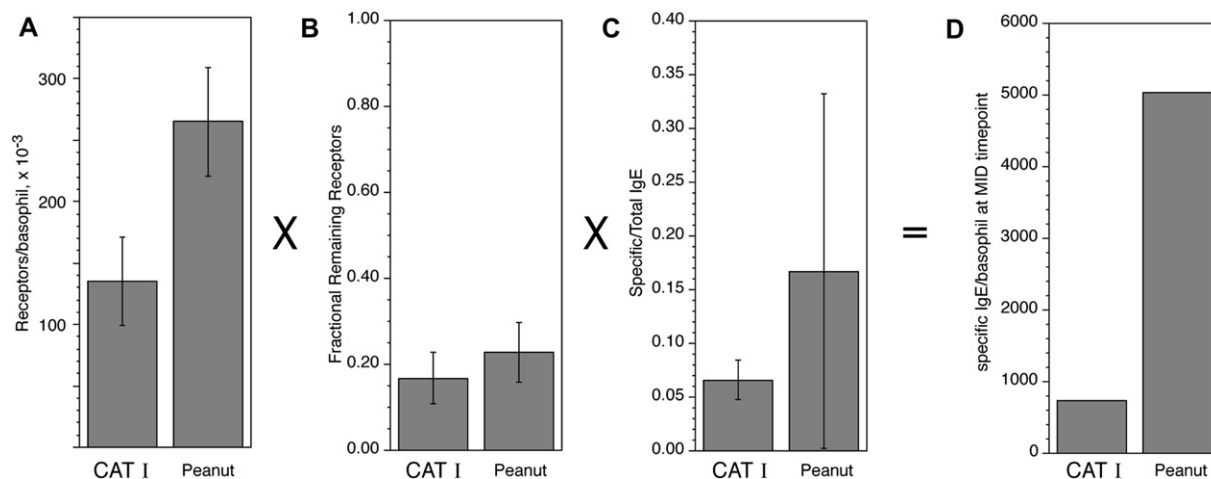


FIG 1. Summary of factors needed to calculate cell-surface allergen-specific IgE density after treatment with omalizumab: comparison of 2 studies, cat and peanut (the current study). **A-C**, Mean $\pm$ SD. **D**, Calculated allergen-specific IgE density, which is a product of Fig 1, A-C, and a fourth factor that is similar for the 2 studies (see this article's Online Repository).

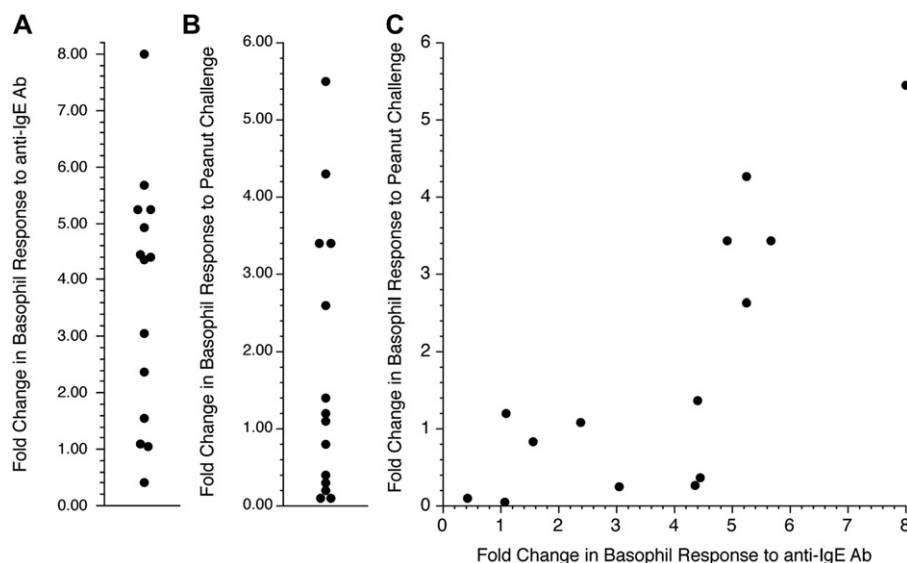


FIG 2. Distribution of responses obtained from the peanut I study. **A**, Distribution of the *in vitro* basophil response to anti-IgE antibody (Ab; AUC); fold change between the pretreatment response (baseline) and the midpoint response. **B**, Distribution in the *in vitro* basophil response to peanut allergen (AUC). **C**, Relationship between the distributions shown in Fig 2, A and B.

the basophil response to the panstimulus anti-IgE antibody (an aggregating anti-IgE antibody).³ This was accompanied by an increase in Syk expression in basophils.³ A similar observation was made in the peanut study, demonstrating that in subjects with food allergy, omalizumab also alters intrinsic responsiveness. In this study the AUC for the anti-IgE dose-response curve was used as a metric of the change for comparison purposes with the peanut dose-response curve. The average increase in response was 3.9-fold (Fig 2, A), and there was a correlation ($r = 0.729$) between the increase in the response to anti-IgE antibody and the change in the response to peanut allergen (Fig 2, C; Fig 2, B, also shows the distribution in peanut response). In a subset of subjects (those for whom Syk measurements were made, $n = 8$), Syk expression was measured in basophils by using flow cytometry.

As found in our previous cat study, Syk expression increased approximately 2-fold in these subjects, and the extent of Syk expression correlated with the increase in the anti-IgE response (Fig 3, A and B; also see the Results section in this article's Online Repository for a discussion of the Syk assay). There was also an inverse correlation between the starting anti-IgE response and the fold increase in the anti-IgE response (Fig 3, C).

The combined observations of high remaining peanut-specific IgE levels and an increase in the cell's responsiveness offer some insight into events occurring during treatment. The results suggest that 2 parameters determine the final response to peanut allergen: the specific/total IgE ratio and the increase in cellular responsiveness, as measured by the basophil response to stimulation with the pan-cross-linking stimulus anti-IgE. Fig 4 shows

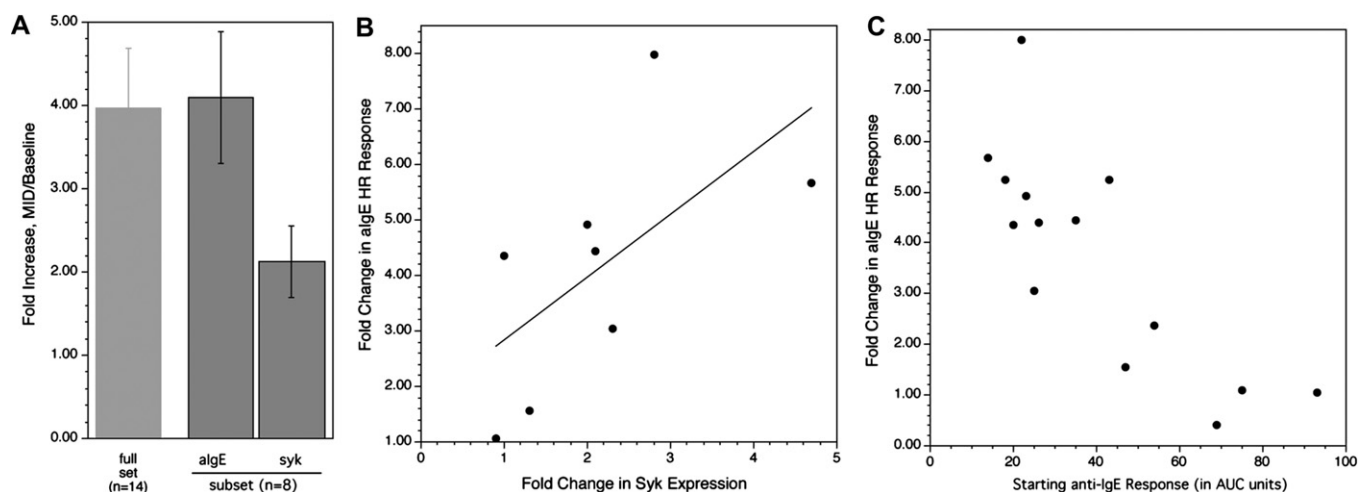


FIG 3. Changes in Syk expression during treatment and relationship of starting to final anti-IgE response. **A**, The far left column shows the average fold change in the basophil response to anti-IgE antibody, and the 2 right columns show the average fold change in the anti-IgE antibody response or Syk expression for the subset of 8 subjects in whom Syk expression was measured. **B**, Correlation between the fold upregulation of the anti-IgE antibody response versus change in Syk expression. **C**, Relationship between the starting anti-IgE antibody response and the fold change in this response at the midpoint visit during treatment with omalizumab. HR, Histamine response.

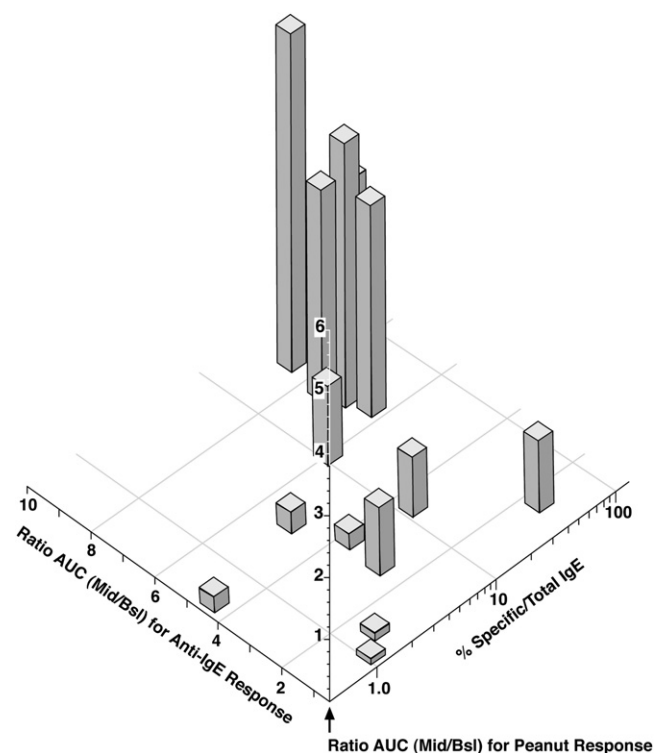


FIG 4. *In vitro* basophil response to peanut allergen stimulation (AUC of the full peanut dose-response curve) versus 2 parameters of basophil function, percentage of specific/total IgE, and fold increase in the basophil response to stimulation with the panstimulus anti-IgE antibody. Fold changes refer to the responses at the midpoint visit versus the response before (baseline [Bsl]) treatment with omalizumab (ie, Mid/Bsl). Each vertical bar represents the *in vitro* peanut response for each patient.

the 3-dimensional relationship. It is evident that there is interplay between suppression of the allergen-specific IgE on the cell surface and increased cellular sensitivity. In the worst cases (ie, a

high specific/total IgE ratio and increased cellular responsiveness) the peanut dose-response curves are markedly enhanced. An alternative perspective on this analysis is to categorize the specific/total IgE ratio and increased cellular responsiveness and average the peanut dose-response curves for the 4 groups generated by the category divisions. Fig E2 in this article's Online Repository at www.jacionline.org shows the results analyzed this way. The thresholds used to make the categories are suggestive: specific/total IgE ratios of less than 4% and changes in cellular responsiveness of less than 1.5-fold (to anti-IgE) result in a basophil response to peanut allergen that is markedly suppressed. Specific/total IgE ratios considerably greater than 4% and an increased cellular response greater than 4-fold result in an increased response to peanut allergen.

Parenthetically, it is apparent from the character of the dose-response curves shown in Fig 4 that peanut allergens are complex enough to generate some unusual patterns in these dose-response curves, notably a double maximum in some cases. In addition, as has been pointed out in the companion article for these studies,² a general behavior is for the curve to shift rightward during treatment, or, by using the nomenclature proposed by Nopp et al,⁷ CDsens shifts rightward. For simple antigens and monospecific antibodies, aggregation theory predicts a stable optimum for secretion throughout all densities of cell-surface IgE.⁸⁻¹⁰ However, peanut antigens are complex, and the IgE bound to the cell surface is composed of many epitope specificities; therefore the consequences of decreasing densities is less well defined. Fig E3 shows 1 experimental multivalent antigen dose-response curve at 2 widely different densities and demonstrates that the optimum does indeed shift rightward with lower densities of antigen-specific IgE (see the Discussion section).

The range of basophil responses led to the analysis shown in Fig 5. On the abscissa is plotted the fold increase in AUC for the peanut dose-response curve (when comparing the second challenge with baseline), and on the ordinate is plotted the log-fold change in peanut tolerated in the OFC for these same 2 time

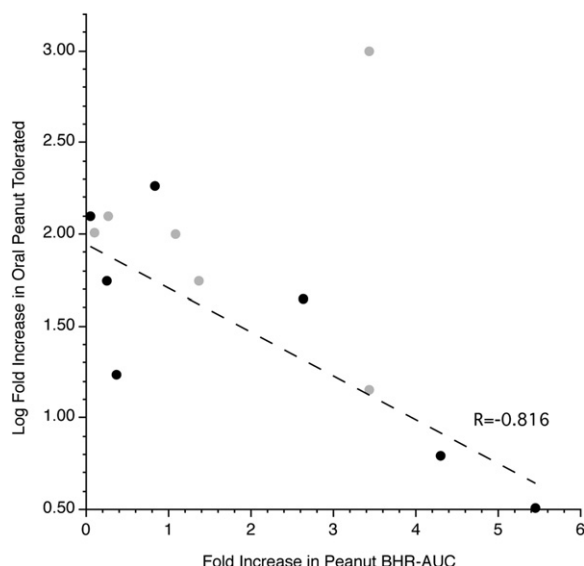


FIG 5. Relationship between the fold change in the *in vitro* basophil response to peanut allergen (AUC) and the log fold change in tolerated peanut ingestion during OFC (midpoint visit value/baseline visit value). Gray circles represent those subjects who reached the stopping rule for ingestion of peanut at the midpoint OFC, whereas black circles represent those subjects from whom what a ratio could be calculated. The linear regression was fit to the noncapped data points (solid circles).

points. The plot presents 2 groups of subjects, those for whom the ratio in OFC could be directly calculated because the 10-g stopping rule was not needed ($n = 7$, solid block symbols) and those for whom the ratio is not explicit because the stopping rule was imposed (and therefore the numerator of the ratio is necessarily fixed to 10 g, $n = 6$, light gray symbols). The regression/correlation was analyzed by using only the explicit ratio data (black symbols). The plot suggests that the more enhanced the basophil response, the more poorly the subject responds to omalizumab.

DISCUSSION

Recent studies exploring the immunologic changes that accompany treatment with omalizumab have provided some interesting insights into the behavior of basophils and their role in the allergic disease under study. By combining our experiences obtained from a cat allergy study¹ and the current peanut study,² it is possible to perceive a developing pattern. One of the most obvious patterns is the relationship of the basophil response to the specific/total IgE ratio. This is a logical link because this ratio determines how readily one can decrease basophil antigen-specific IgE densities during treatment to reach levels that are known to be suboptimal for IgE-mediated stimulation.^{6,11} The basophil is quite sensitive, and the sensitivity among subjects' basophils is quite heterogeneous. In the general population the average density of antigen-specific IgE that is required for 50% of maximal response is approximately 2000 molecules per cell, but this value varies by nearly 30-fold among subjects.^{6,11,12} There is a correlation between the maximum response induced by anti-IgE antibody and this intrinsic sensitivity,⁶ and from an as-yet-unpublished ongoing study of omalizumab's effects, we now know that the intrinsic sensitivity increases considerably (meaning that fewer IgE molecules per cell are needed for a response). The full basis for this change is not yet known, although the

increases in Syk expression are consistent with a more sensitive basophil.⁶

The observations that the response to anti-IgE antibody generally increases and that Syk expression increases have now been replicated in this peanut study. This appears to be a general property of basophils during treatment with omalizumab, although the cause of the change is not known. However, this property works against the effects of decreasing the antigen-specific IgE density on the basophil's cell surface. The results presented in Fig 4 highlight the competition between the 2 effects. It is notable that a marked increase in basophil response is possible (≤ 5 -fold when taking the dose-response curve as a whole). Coupling both a marked increase in cellular sensitivity and a very high specific/total IgE ratio (see also Fig E2, D, resulting in approximately 9000 peanut-specific IgE molecules per cell, or the points furthest from the origin in Fig 4) produces such an increase. In contrast, when the intrinsic sensitivity does not increase (see below) and the density of peanut-specific IgE is very low after treatment (Fig E2, A; approximately 750 molecules per basophil or the points nearest the origin in Fig 4), the peanut response is completely suppressed. On the basis of the results in Fig E2, B and C, it appears that the tipping point for suppression is an approximately greater than 5-fold change in basophil responsiveness or a greater than 10% peanut-specific/total IgE ratio, with some compensatory exchange between these 2 factors to produce a cell that is not suppressed. A specific/total IgE ratio of 10% might require only a modest increase in responsiveness (1.5- to 2.0-fold), whereas a ratio of specific/total IgE of 40% requires only a small change in responsiveness.

It was also useful to note that the unexpected characteristic of omalizumab treatment to increase Syk expression and IgE-mediated release was similar in both aeroallergen and food allergy conditions. One possible distinguishing aspect of these 2 types of allergy would relate to the avoidance that is possible in subjects with food allergy. Provided that the patient has no nonfood allergies, the immune system is not being constantly exposed to allergen, which might alter the character of the basophil response to omalizumab, as has been suggested in our previous study for cat or other perennial allergens. Surveys of the frequency of coincident allergies¹³ suggest that food-only allergies are likely rare, so that it is likely that even subjects with food allergy who are avoiding specific foods continue to be exposed to indoor or outdoor nonfood allergens.

The underlying mechanisms that cause a change in cellular responsiveness remain unknown, but in both the predecessor cat study³ and this study, there is an excellent inverse correlation between the starting responsiveness and the increase that occurs during treatment. The system is behaving as if the low starting response is under the control of an IgE-dependent mechanism because omalizumab relieves the suppression that might be inherent in these subjects. *In vitro* studies of basophil maturation have suggested that chronic stimulation can generate a basophil with normal granule and receptor expression but suppressed Syk expression.⁴ Therefore relief of chronic aggregation would be expected to reverse suppression but with undetectable changes in receptor density or granule expression because these are not altered by chronic aggregation. If this were an explanation, it would follow that some subjects experience a natural form of aggregation (that has yet to be identified) that is reversed by omalizumab.

From both the cat and peanut studies, it has also become apparent that, on average, the dose-response curve for the antigen

shifts rightward, both the peak response and the 50% point for the response (so-called CDsens^{7,14,15}). Both theoretic and experimental studies of simple bivalent haptens and monoclonal or polyclonal IgE antibodies specific for the haptens have shown that the cell-surface density of hapten-specific IgE does not shift the concentration for optimal histamine release because of the nature of the cross-linking reaction that occurs with bivalent haptens.⁸⁻¹⁰

However, normal antigens have multiple epitopes that bind to complex mixtures of epitope-specific IgE antibodies on the cell surface, and theoretic studies are less specific about a prediction for the behavior of the dose-response curve with changing cell-surface density. We examined a multivalent antigen with a polyclonal IgE (albeit probably close to monoclonal in its target epitope, penicillin) and found that with changing densities of penicillin-specific IgE, the optimum and CDsens values are shifted rightward with decreasing densities. Therefore the rightward shift observed during treatment with omalizumab is likely to be a simple reflection of the decreasing densities of either cat- or peanut-specific IgE. Whether the same rightward shift that occurs in tissue responses results from the same mechanisms is not yet clear.

In this peanut study all subjects experienced an improvement in their tolerance for orally ingested peanut. The fact that 60% of subjects' basophils showed no suppression but a marked increase in basophil response (when considering the entirety of the dose-response curve) gives the impression that the basophil response is not particularly relevant to the clinical response. The analysis shown in Fig 5 suggests that the clinical response might be mitigated by poor improvement in the basophil response. Approximately 25% of the subjects showed poor improvement on OFC when the basophil response was markedly enhanced. If this perspective is correct, the peanut study indirectly suggests the possibility that the mast cell response is largely corrected by treatment but that the basophil response might interfere with optimal improvement. There are indications from the cat study that a similar trend occurs (unpublished results); poorer improvement associates with less suppression of the basophil response. In the companion article describing the clinical outcomes of this study, it was noted that both the basophil and OFC dose-response curves shift rightward. From this perspective, the change in basophil response is consistent with the change in OFC response, albeit with changes that are not as great as observed in the OFC. This interpretation is consistent with a role for the basophil in the clinical response. However, this interpretation ignores the entirety of the peanut dose-response curve, and it cannot be known how much of the dose-response curve the basophil experiences *in vivo*. An alternative interpretation based on the available data and the analysis presented in Fig 5 continues to suggest a role for the basophil but one that modulates a suppressed intestinal mast cell response, sometimes opposing the apparent omalizumab-mediated suppression of this cell. These studies were not designed to distinguish between these 2 possible interpretations.

In summary, these studies highlight 2 competing factors that determine the nature of the antigen-specific IgE response in human basophils. Variability in the cell density of antigen-specific IgE (peanut in this study) as a result of variable

specific/total IgE ratios provides one determinant of the response. The second determinant, which opposes the effect of decreasing antigen-specific IgE levels, is an increased sensitivity of basophils to IgE-mediated stimulation.

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Key messages

- The *in vitro* IgE-mediated basophil response during omalizumab treatment increases for some subjects, and the variability in whether suppression or enhancement occurs results from 2 factors: the specific/total IgE ratio and changes in intrinsic basophil sensitivity.
- Variability in the basophil IgE-mediated response might predict the clinical efficacy of omalizumab.

REFERENCES

- Eckman JA, Sterba PM, Kelly D, Alexander V, Liu MC, Bochner BS, et al. Effects of omalizumab on basophil and mast cell responses using an intranasal cat allergen challenge. *J Allergy Clin Immunol* 2010;125:889-95, e7.
- Savage JH, Courneya J-P, Sterba PM, MacGlashan DW, Saini SS, Wood RA. Kinetics of mast cell, basophil, and oral food challenge responses in omalizumab-treated adults with peanut allergy. *J Allergy Clin Immunol* 2012. ■■■:■■■.
- Zaidi AK, Saini SS, MacGlashan DW Jr. Regulation of Syk kinase and FcRbeta expression in human basophils during treatment with omalizumab. *J Allergy Clin Immunol* 2010;125:902-8, e7.
- Ishmael SS, MacGlashan DW Jr. Syk expression in peripheral blood leukocytes, CD34+ progenitors, and CD34-derived basophils. *J Leukoc Biol* 2010;87:291-300.
- Siraganian RP. An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal Biochem* 1974;57:383-94.
- MacGlashan DW Jr. Relationship between Syk and SHIP expression and secretion from human basophils in the general population. *J Allergy Clin Immunol* 2007;119:626-33.
- Nopp A, Johansson SG, Ankerst J, Bylin G, Cardell LO, Gronneberg R, et al. Basophil allergen threshold sensitivity: a useful approach to anti-IgE treatment efficacy evaluation. *Allergy* 2006;61:298-302.
- Dembo M, Goldstein B, Sobotka AK, Lichtenstein LM. Histamine release due to bivalent penicilloyl haptens: control by the number of cross-linked IgE antibodies on the basophil plasma membrane. *J Immunol* 1978;121:354-8.
- Goldstein B, Dembo M, Sobotka AK, Lichtenstein LM. Some invariant properties of IgE-mediated basophil activation and desensitization. *J Immunol* 1979;123:1873-82.
- MacGlashan DW Jr, Dembo M, Goldstein B. Test of a theory relating to the cross-linking of IgE antibody on the surface of human basophils. *J Immunol* 1985;135:4129-34.
- Nopp A, Johansson SG, MacGlashan D Jr. Early signal protein expression profiles in basophils: a population study. *J Leukoc Biol* 2009;86:313-25.
- MacGlashan DW Jr. Releasability of human basophils: cellular sensitivity and maximal histamine release are independent variables. *J Allergy Clin Immunol* 1993;91:605-15.
- Arbes SJ Jr, Gergen PJ, Elliott L, Zeldin DC. Prevalences of positive skin test responses to 10 common allergens in the US population: results from the third National Health and Nutrition Examination Survey. *J Allergy Clin Immunol* 2005;116:377-83.
- Nopp A, Johansson SG, Ankerst J, Palmqvist M, Oman H. CD-sens and clinical changes during withdrawal of Xolair after 6 years of treatment. *Allergy* 2007;62:1175-81.
- Nopp A, Cardell LO, Johansson SG, Oman H. CD-sens: a biological measure of immunological changes stimulated by ASIT. *Allergy* 2009;64:811-4.

METHODS

Reagents and buffers

The following were purchased: piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), BSA, EDTA, D-glucose, and erythrosin B (Sigma-Aldrich); crystallized human serum albumin (HSA; Miles Laboratories, Elkhart, Ind); FCS (BioWhittaker, Walkersville, Md); Percoll (Pharmacia); anti-BDCA-2/CD303 (AC144; Miltenyi Biotec, Auburn, Calif); anti-IL-3R/CD123 (9F5) phycoerythrin conjugated; anti-mouse IgG₁ Alexa 647, anti-mouse IgG_{2a} Alexa 647, and anti-mouse IgG_{2a} (Molecular Probes/Invitrogen, Carlsbad, Calif); anti-human IgE monomeric IgM (6061P, Hybridoma Reagent Laboratories); and horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin antibody and horseradish peroxidase-conjugated Sheep anti-mouse immunoglobulin antibody (Amersham Life Sciences, Arlington Heights, Ill). The anti-FcεRIα antibody 22E7 was a gift from Hoffman-LaRoche (Basel, Switzerland). Anti-Syk antibody (4D10) was from Santa Cruz Biotechnology (Santa Cruz, Calif), and anti-FcεRIβ was from Upstate Biologicals (Lake Placid, NY).

PIPES-albumin-glucose (PAG) buffer consisted of 25 mmol/L PIPES, 110 mmol/L NaCl, 5 mmol/L KCl, 0.1% glucose, and 0.003% HSA. PAGCM was PAG supplemented with 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂. Labeling with antibodies for flow cytometry was conducted in PAG with 1 mmol/L EDTA containing 0.25% BSA in place of 0.003% HSA. ESB is a Novex electrophoretic sample buffer containing 5% 2-mercaptoethanol. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. Lactic acid buffer for removing endogenous cell-bound IgE was 0.01 mol/L lactic acid, 0.14 mol/L NaCl, and 0.005 mol/L KCl (pH 3.9).^{E1}

Flow cytometry of FcεRI expression

In previous studies we have calibrated the measurement of cell-surface FcεRIα that is detected by using flow cytometry with the anti-FcεRIα murine mAb 22E7 (which detects occupied and unoccupied FcεRIα) to report absolute densities of cell-surface receptor in some experiments.^{E2} Briefly, the flow cytometric measurements were calibrated by examining the fluorescence staining of 6 donors' basophils that spanned a moderate range of staining intensities (ultimately calibrated to be 8,000 to 140,000 FcεRI per basophil) and simultaneously assessing the receptor or IgE density by using the acetate elution method.^{E2} 22E7 labeling was compared with total FcεRI density by means of acetate elution (after sensitizing with PS myeloma IgE to fully sensitize all surface receptors so that IgE effectively measured total surface receptor) and was linear ($R = 0.963$). In pilot studies the effect of fixing the cells before labeling was examined. Mixed leukocytes obtained from a 1-step Percoll preparation were fixed according to the manufacturer's instructions (Fix and Perm Kit; Caltag, Carlsbad, Calif). After fixation, the cells were stored overnight in elutriation buffer (PAG containing 0.25% BSA in place of 0.003% HSA). The overnight blocking step was found to be necessary if the calibration of 22E7, as described above, is to be considered valid. With this step, no difference in calibration was detected.

Analysis of Syk protein expression by using intracellular flow cytometry

All analyses were performed on a BD FACSCalibur flow cytometer. The measurement of Syk protein expression by means of flow cytometry with the anti-Syk antibody 4D10 has been previously described and validated with respect to standard Western blotting.^{E3,E4} This method has been used on both impure and purified cell fractions with equivalent results. Briefly, fixed mixed leukocytes were labeled with anti-IL-3 receptor and anti-BDCA2 antibodies (phycoerythrin and fluorescein isothiocyanate, respectively) and permeabilized (Fix and Perm Kit, Caltag) in the presence of 4D10 or IgG_{2a} antibody. Cells were then incubated with an anti-mouse IgG_{2a}-Alexa 647 antibody to complete the labeling. Syk protein expression is reported as normalized net mean fluorescence intensity, with the difference between 4D10 and isotype-labeled cells corrected for instrument variability by using CaliBRITE APC calibration beads (BD Biosciences).

This peanut study was the first time that blood was drawn at an off-site location for subsequent analysis, including Syk measurement. We discovered

in the first samples being delivered that the Syk measurements appeared markedly suppressed relative to expectations for normal Syk levels in both basophils and plasmacytoid dendritic cells, which are measured simultaneously. It is now apparent that EDTA-chelated blood cannot be stored for very long without altering the ability of this assay to measure Syk expression. Separate testing indicated changes begin to occur within 30 minutes. The magnitude of the effect is different for different subjects, and therefore the primary effect is to introduce noise into the measurement. Therefore, for the peanut study, we were restricted to a subset of the subjects under study with samples that were known to have been delivered rapidly ($n = 8$).

RESULTS

Relationship between cell-surface IgE and FcεRI during treatment

One of the factors that is needed to determine antigen-specific IgE density on basophils during treatment with omalizumab in this study (because IgE densities were not measured) relates to the changing IgE/FcεRI ratio at low free IgE levels. We know from phase I studies of omalizumab that the IgE/FcεRI ratio decreases to less than 20% when free IgE levels are suppressed by greater than 99%. Under the current dosing tables, free IgE levels are not suppressed as effectively, and the IgE/FcεRI ratio (cell density) is generally around 50%. Using data from our recent cat study, in which both IgE and FcεRI densities (by using calibrated flow cytometry) were measured, Fig E1 shows the relationship between suppression of receptor expression (fraction of receptor after/before treatment) versus IgE/FcεRI ratio. From this plot, we can estimate that the ratio is approximately 0.50 in the peanut study with pre-FcεRI/post-FcεRI values of 0.23.

Categorizing the basophil response

To illustrate the competition between the specific/total IgE ratio and the change in intrinsic sensitivity, the subjects were grouped according to 2 criteria, resulting in 4 groups: peanut-specific/total IgE ratio of less than 4% or greater than 4% and enhancement of the anti-IgE response (as a metric of the change in the signaling apparatus, such as Syk expression) of less than or greater than 1.5-fold. These categorical thresholds were selected on the basis of previous observations in the cat allergy study of similar design.^{E5} For the specific/total IgE ratio thresholds, the less than 4% value is related to the observation that at less than 4%, the basophil response changes rapidly.^{E5} For the change in anti-IgE response, the value of 1.5 is based on the previous cat study's placebo-treated subjects, representing greater than 1 SD of the visit-to-visit variation greater than a value of no change.^{E5} The question is whether the current data can begin to sort out which process dominates the final basophil response. In Fig E2, A, the specific/total IgE ratio is low (<4%), and there is no significant increase in the anti-IgE response; therefore the cells are not more sensitive, and this component does not compete against the decreasing cell-surface IgE levels. The peanut dose-response curve is essentially flat after treatment. In an opposite sense, Fig E2, D, shows that the combination of a high specific/total IgE ratio and a marked increase in cellular sensitivity produces a cell that has a markedly enhanced peanut dose-response curve. The interesting division occurs with Fig E2, B and C. Fig E2, B, suggests that a sufficiently low specific/total IgE ratio dominates the increased cellular sensitivity, although even while suppressed it is apparent that there remains a significant response to peanut. After sorting 13 of the 14 responses into panels Fig E2, A, B, and D, there was only 1 patient who fell into the category defined in

Fig E2, C, and this result suggests that even with no change in cellular sensitivity, a very large specific/total IgE ratio results in no suppression of the peanut dose-response curve.

Dependence of antigen dose-response curves on cell-surface antigen-specific IgE density

Basophils were initially treated with weak lactic acid buffer (pH 3.7) to dissociate some cell-surface IgE for the generation of basophils with a high density of unoccupied receptors to examine the question of the IgE density dependence of the antigen dose-response curve.^{E1,E6} The cells were then resensitized at 2 density levels of penicillin-specific IgE.^{E7} Densities were measured based on elution of cell-bound IgE with pH 3.0 acetate buffer and measurement in penicillin-specific RASTs, which was combined with basophil counts to obtain density.^{E7} A very broad dose-response curve with benzyl-penicilloyl (BPO)(21)-HSA was examined in the subsequently washed cells. For these 4 experiments, the histamine release was normalized to the specific maximum histamine release found for a particular preparation. This step allowed all 4 experiments to be combined for curve fitting, despite absolute differences in the maximum histamine release from each preparation of basophils. Fig E3 shows both the individual data points derived from all 4 experiments (faded symbols of red or green) and the

Gaussian curve fit to the data points. The average density for basophils sensitized to a high level of BPO-specific IgE was $128,000 \pm 50,000$, and that for a low level of BPO-specific IgE was $15,000 \pm 5,000$. The peak response shifted rightward approximately 4.8-fold for the peak and 5.7-fold for the CDsens (point of 50% maximal response; 3.9 vs 21.9 ng/mL for high vs low, respectively).

REFERENCES

- E1. Pruzansky JJ, Grammer LC, Patterson R, Roberts M. Dissociation of IgE from receptors on human basophils. I. Enhanced passive sensitization for histamine release. *J Immunol* 1983;131:1949-53.
- E2. MacGlashan DW Jr. Endocytosis, re-cycling and degradation of unoccupied FcεRI in human basophils. *J Leukoc Biol* 2007;82:1003-10.
- E3. MacGlashan DW Jr, Ishmael S, Macdonald SM, Langdon JM, Arm JP, Sloane DE. Induced loss of Syk in human basophils by non-IgE-dependent stimuli. *J Immunol* 2008;180:4208-17.
- E4. Ishmael S, MacGlashan DW Jr. Early signal protein expression profiles in basophils: a population study. *J Leukoc Biol* 2009;86:313-25.
- E5. Eckman JA, Sterba PM, Kelly D, Alexander V, Liu MC, Bochner BS, et al. Effects of omalizumab on basophil and mast cell responses using an intranasal cat allergen challenge. *J Allergy Clin Immunol* 2010;125:889-95, e7.
- E6. MacGlashan DW Jr. Releasability of human basophils: cellular sensitivity and maximal histamine release are independent variables. *J Allergy Clin Immunol* 1993;91:605-15.
- E7. MacGlashan DW Jr, Lichtenstein LM. Studies of antigen binding on human basophils. I. Antigen binding and functional consequences. *J Immunol* 1983;130:2330-6.

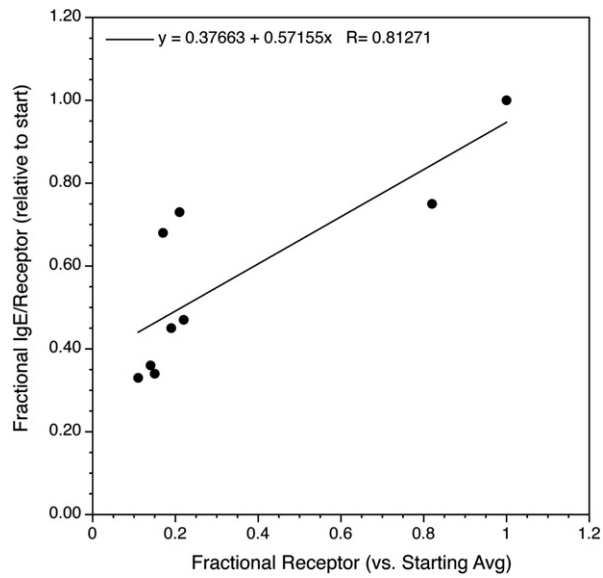


FIG E1. Relationship between the decrease in receptor expression on basophils during treatment with omalizumab and the ratio of occupied to total FcεRI.

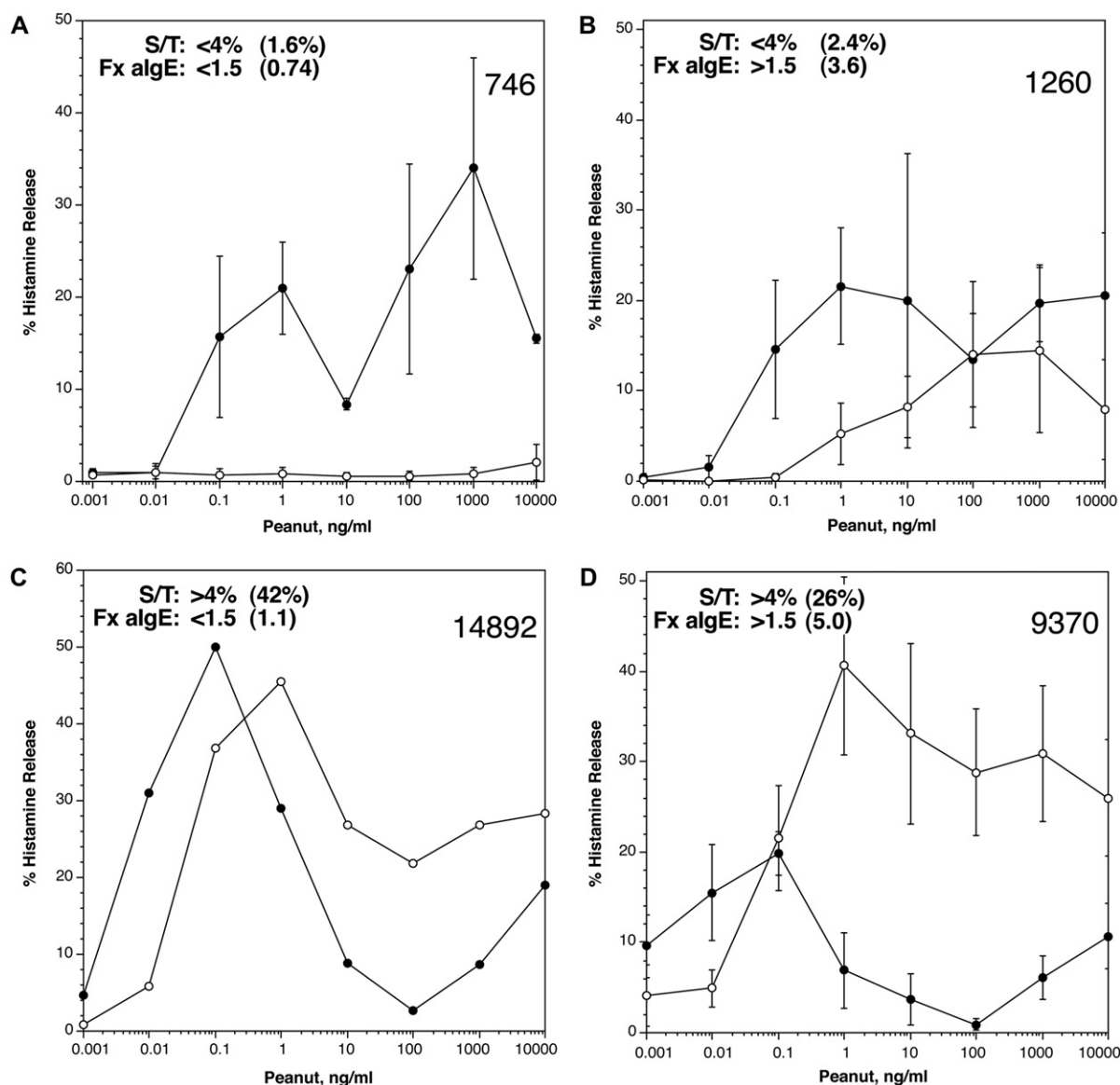


FIG E2. *In vitro* basophil response to peanut allergen stimulation divided according to both the peanut-specific/total IgE ratio and the relative increase in the response to anti-IgE antibody (midpoint/baseline). The dividing points for the specific/total ratios (*S/T*) were greater or less than 4% peanut specific. The thresholds for the increase in the anti-IgE response (*Fx algE*) were a less than 1.5-fold and greater than 1.5-fold change. Dose-response curves: *solid circles*, pretreatment or baseline; *open circles*, midpoint. Averages $\pm$ SEMs are shown. The numbers under the panel designation represent the average number of peanut-specific IgE molecules per basophil in the specific category. This number is calculated from the known specific/total IgE ratio and the remaining IgE measured on the basophils after treatment.

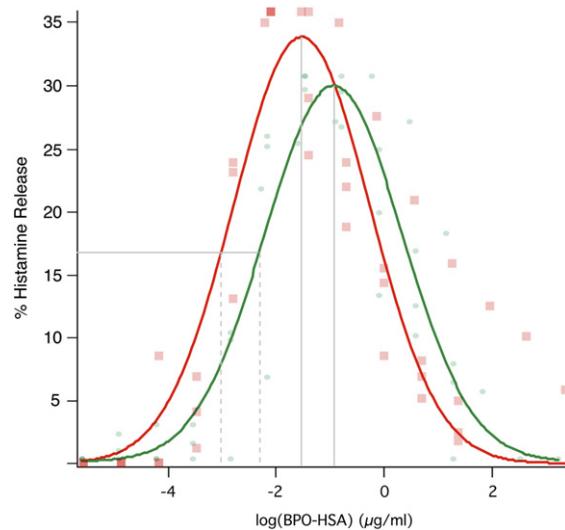


FIG E3. Dose-response curves to BPO(21)-HSA stimulation in basophils sensitized with 2 densities of BPO (penicillin)-specific IgE. The data for 4 different experiments are plotted as *faded symbols*, and heuristic Gaussian curves are fit to all the data points for the 2 conditions. The *vertical gray lines* mark the peaks of the fit curves, and the *dashed vertical gray lines* mark the point of 50% of the maximal response. The density of BPO-specific IgE is $15,000 \pm 5,000$ (*green line*) and $128,000 \pm 50,000$ (*red line*) for the 2 conditions.