

Omalizumab increases the intrinsic sensitivity of human basophils to IgE-mediated stimulation

Donald W. MacGlashan, Jr, MD, PhD, and Sarbjit S. Saini, MD *Baltimore, Md*

Background: Treatment of allergic patients with omalizumab results in a paradoxical increase in their basophil histamine release (HR) response *ex vivo* to cross-linking anti-IgE antibody. It is not known whether this change in response is associated with an increase in intrinsic cellular sensitivity, which would be a paradoxical response.

Objective: We sought to determine whether the increase in response to anti-IgE antibody is a reflection of an increased cellular sensitivity expressed as molecules of antigen-specific IgE per basophil required to produce 50% of the maximal response.

Methods: Patients were treated with omalizumab or placebo for 12 weeks (NCT01003301 at ClinicalTrials.gov), and the metric of basophil sensitivity was assessed at 4 time points: baseline, 6 to 8 weeks, 12 weeks (after which treatment stopped), and 24 weeks (12 weeks after the end of treatment).

Results: As observed previously, treatment with omalizumab resulted in a marked increase in the maximal HR induced by cross-linking anti-IgE antibody. This change was accompanied by a marked shift in intrinsic basophil sensitivity, ranging from 2.5- to 125-fold, with an average of 6-fold at the midpoint of the treatment to 12-fold after 12 weeks. The magnitude of the increase in cellular sensitivity was inversely related to the starting sensitivity or the starting maximum HR. The increased cellular sensitivity also occurred when using leukotriene C₄ secretion as a metric of the basophil response. Twelve weeks after the end of treatment, cellular sensitivity was found to shift toward the baseline value, although the return to baseline was not yet complete at this time point.

Conclusions: Treatment with omalizumab results in a markedly increased sensitivity of basophils to IgE-mediated stimulation in terms of the number of IgE molecules required to produce a given response. These results provide a better quantitative sense of the phenotypic change that occurs in basophils during omalizumab treatment, which has both mechanistic and clinical implications. (*J Allergy Clin Immunol* 2013;■■■:■■■-■■■.)

Key words: Human, basophil, allergy, Fc receptors

Abbreviations used

ED₅₀: Effective density for 50% maximum response
 HR: Histamine release
 HSA: Human serum albumin
 IL-3R: IL-3 receptor
 LTC₄: Leukotriene C₄
 PAG: PIPES-albumin-glucose
 PIPES: Piperazine-N,N'-bis(2-ethanesulfonic acid)
 Syk: Spleen tyrosine kinase

Omalizumab is a recent addition to the therapies being used to treat asthma. It works by reducing free IgE levels sufficiently to reduce the presence of IgE on the cell surfaces of mast cells, basophils, and any other cells expressing IgE receptors. It has been established for many years that one consequence of the reduction in free IgE levels is a parallel reduction in FcεRI levels on cells that express this receptor.¹⁻⁴ Reduction in FcεRI levels occurs because bound IgE stabilizes FcεRI on the cell surface; unbound receptor is internalized and degraded.^{5,6} The combined reduction in free IgE and FcεRI levels is necessary to blunt IgE-mediated secretion from mast cells and basophils because these cells are exquisitely sensitive to stimulation. For example, fewer than 2000 antigen-specific IgE molecules are needed for a half-maximal response to antigen, but even 100 to 500 molecules are sufficient to initiate some secretion.⁷ With starting densities of 150,000 IgE molecules per basophil, considerable reduction in free IgE and FcεRI levels is required to achieve less than 500 IgE molecules per cell. A parameter that is highly relevant to achieving suppression of cell-surface antigen-specific IgE is the specific/total IgE ratio; low ratios allow for more rapid suppression of cell-surface densities.^{8,9}

However, recent studies have identified an unexpected effect occurring during treatment with omalizumab. Two clinical studies have now shown that peripheral blood basophils respond better to stimulation with a panspecific cross-linking anti-IgE antibody during omalizumab treatment.^{10,11} One reason for not observing a decreased response to a cross-linking anti-IgE antibody is that the dosing table for omalizumab is not designed to strongly reduce free IgE levels, and this condition produces a basophil with densities of total cell-surface IgE sufficient for a maximal response. However, this only explains the absence of reduction in response to anti-IgE antibody and not an increase. An increased response suggests that other changes are occurring. Previous studies have established a modest linkage between the maximal amount of histamine release (HR) that can be induced by an optimal stimulus and the sensitivity of the cell to stimulation.⁷ Cellular sensitivity is defined as the number of antigen-specific IgE molecules per basophil required to induce 50% of the maximal response (when stimulated with an optimal concentration of antigen). Although there is some linkage between these 2 parameters of IgE-mediated secretion in basophils, the linkage is weak and can be broken by exposing the cells

From the Department of Medicine, Johns Hopkins Asthma and Allergy Center, Johns Hopkins University.

Supported by National Institutes of Health grant AI070345.

Disclosure of potential conflict of interest: D. W. MacGlashan, Jr, has been supported by one or more grants from the National Institutes of Health. S. S. Saini has been supported by one or more grants from and has received one or more consulting fees or honoraria from Novartis and Genentech and has received royalties from UpToDate.

Received for publication January 3, 2013; revised March 15, 2013; accepted for publication April 18, 2013.

Corresponding author: Donald W. MacGlashan, Jr, MD, PhD, Department of Medicine, Johns Hopkins Asthma and Allergy Center, Johns Hopkins University, Unit Office 3, 5501 Hopkins Bayview Circle, Baltimore, MD 21224. E-mail: dmacglas@jhmi.edu. 0091-6749/\$36.00

© 2013 American Academy of Allergy, Asthma & Immunology
<http://dx.doi.org/10.1016/j.jaci.2013.04.056>

to certain conditions. For example, maximum release can be increased when basophils are exposed to cytokines^{12–14} and, based on the signaling involved, increased without significant increases in cellular sensitivity,^{15–19} so that it is possible that the weak linkage that is seen in the general population⁷ does not apply when subjects are being treated with biological or pharmacologic agents. Furthermore, a determination of the magnitude of the shift in sensitivity would also have additional implications regarding the underlying mechanisms of the change. On a more practical level, the metric of cellular sensitivity is the more interesting number for the purposes of developing a quantitative sense of what is happening to basophils (or mast cells) during omalizumab treatment and for predicting how the competing effects of cell-surface IgE reduction and changes in cellular sensitivity will affect the cell's response.¹¹ Therefore it was of interest to determine whether treatment with omalizumab resulted in an increase in cellular sensitivity (fewer molecules needed for release) as a first step in a mechanistic study of the underlying basis for the change in maximal response.

METHODS

Reagents and buffers

Common materials are purchased as described in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Piperazine-N,N'-bis(2-ethanesulfonic acid) (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% human serum albumin (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. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. Lactic acid buffer was used for removing endogenous cell-bound IgE (0.01 mol/L lactic acid, 0.14 mol/L NaCl, and 0.005 mol/L KCl, pH 3.9).^{7,20}

Basophil HR

Venous blood was drawn into syringes containing PBS-EDTA, and basophils were isolated with a 2-step Percoll-based density gradient. A portion of the enriched basophils was treated with lactic acid (see above) for 10 seconds to remove some of the endogenously bound IgE, thus exposing unoccupied receptors for greater sensitization.²⁰ Both lactic acid–treated cells and nontreated cells were divided into aliquots for serial concentrations of sensitizing IgE antibody. The concentrations of sensitizing antibody were chosen to provide a large range of IgE densities, as well as to provide some overlap in the IgE densities found on cells treated with lactic acid versus those that were not treated with lactic acid. This was to ensure that the treatment with lactic acid did not alter the sensitivity/function of the treated basophils, as noted previously.⁷ In general, overlap was accomplished with the chosen sensitization concentrations and also demonstrated that the lactic acid treatment did not significantly alter the sensitivity of the cells. The cells were sensitized with dilutions of an antibody specific for a peptide of the HIV protein gp120 in a buffer containing 10 µg/mL heparin, which aids sensitization, and 1 mmol/L EDTA at 4°C for 1 hour. For cells not treated with lactic acid, the concentrations of gp120-specific IgE were 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, and 0.625 µg/mL, and for the lactic acid–treated cells, the concentrations of gp120-specific IgE were 0.625 µg/mL, 0.31 µg/mL, and 0.16 µg/mL. One aliquot of cells was not sensitized; these cells provided background binding for the flow cytometric measurements of bound gp120-specific IgE and were also used for testing 2 concentrations of anti-IgE antibody (6061). The gp120-specific antibody was biotinylated. This biotinylation provided a way to assess the gp120-specific IgE levels of the cells after sensitization by means of flow cytometry, and the same streptavidin–Alexa Fluor 647 used for flow cytometry (see the [Methods](#) section in this article's Online Repository for additional details of flow cytometry) was also used to stimulate the basophils because streptavidin is naturally tetrameric. After washing the sensitized cells, a portion

were fixed for subsequent flow cytometry. The remaining cells from each sensitization condition were stimulated in PAGCM buffer with either an optimal concentration of streptavidin–Alexa Fluor 647 (1:500,000 dilution of stock reagent, as determined in pilot studies) or anti-IgE (0.5 µg/mL HP6061; Hybridoma Reagent Laboratory, Baltimore, Md) for 45 minutes at 37°C. Automated fluorimetry was used to measure histamine levels in cell-free supernatants.²¹ 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 HR from cells in buffer alone as follows: [(Stimulated HR – Spontaneous release)/(Lysed HR – Spontaneous HR)].

Omalizumab study

The details of this study are very similar to those of a previously published study.⁹ The current study is registered at ClinicalTrials.gov under the identifier NCT1003301. Briefly, adults 18 to 50 years old were chosen for their sensitivity to cat allergen. Informed consent was obtained through a protocol approved by the Johns Hopkins Hospital Institutional Review Board and the Data Safety Monitoring Boards of the National Institute of Allergy and Infectious Diseases. After testing for inclusion criteria, adults were entered into the study after a 2-week interval and randomized 1:1 to omalizumab/placebo treatment. Omalizumab was administered according to the manufacturer's dosing table. The basophil sensitivity assay was performed before drug administration and repeated at 6 to 8 weeks, after 12 weeks, and in some subjects 3 months after the cessation of treatment with omalizumab. After the study was initiated, it was decided that basophils would also be assessed for both FcεRI and spleen tyrosine kinase (Syk) expression by means of flow cytometry, and leukotriene C₄ (LTC₄) release was measured in supernatants used for histamine analysis. These later subjects also returned for an assessment 3 months after the cessation of treatment.

RESULTS

Since its initial development,²² the method for measuring intrinsic basophil sensitivity has been improving as a metric of the basophil IgE-mediated response.²³ Notably, the method has become based on flow cytometric measurements of the antigen-specific IgE densities (rather than elution and measurement in RAST assays). For this study, additional refinements were added to reduce the requirement for larger amounts of blood and to potentially allow the method to be used with more generally available reagents. The basis of the method is to sensitize basophils with a specific IgE at different densities and to measure both the amount of IgE actually bound to FcεRI and the basophil response to antigenic stimulation after sensitization. Sensitization with the same IgE throughout a study eliminates concerns that the natural IgE might change its characteristics (eg, affinity and epitope profile) during treatment. The Results section in this article's Online Repository at www.jacionline.org describes the changes incorporated into the current method.

Because different subjects' basophils display different cellular sensitivity values^{22,23} and we were interested in detecting the average shift in sensitivity in a population of subjects, an analytic approach was developed that optimally detected the shift and allowed averaging of disparate starting values of sensitivity. The Results section in this article's Online Repository describes the 2 analytic methods used to evaluate the results (see [Fig E1](#) in the article's Online Repository at www.jacionline.org). [Fig 1](#) shows the results for sensitivity measurements made during the omalizumab study. For the 9 subjects who completed the study while receiving placebo, there is no statistical difference in the sensitivity curves when comparing the starting curve with the curves generated at the midpoint of the study or the final visit during treatment. In contrast, there were marked shifts in sensitivity in the basophils of subjects treated with omalizumab. The sensitivity shifted 6-fold at the midstudy assessment and 12-fold at the final on-treatment assessment. Sigmoid

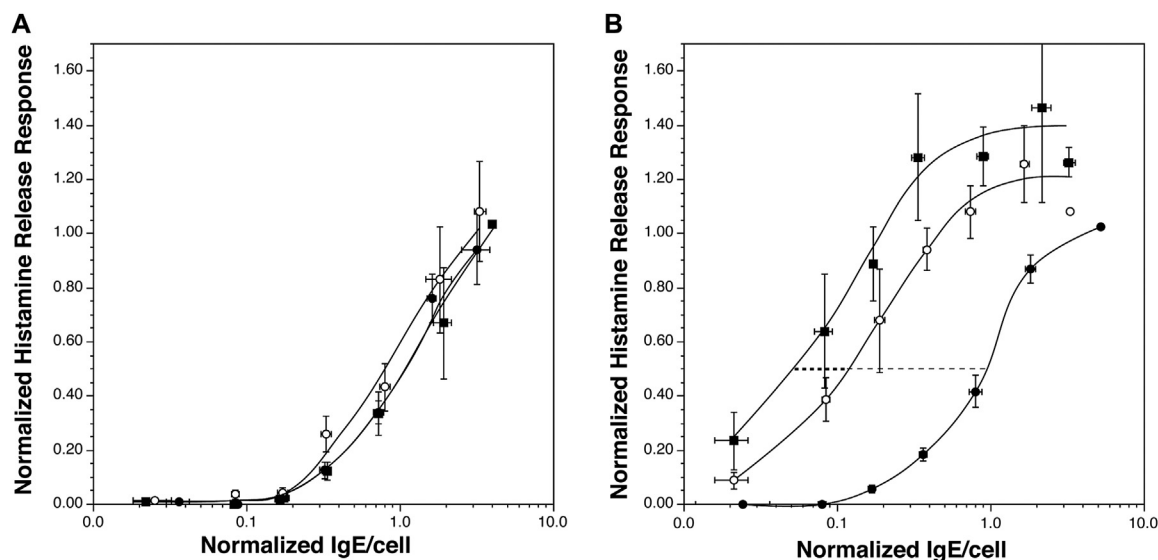


FIG 1. Sensitivity curves for subjects treated with omalizumab or placebo. **A**, Placebo-treated subjects ($n = 9$). *Solid circles*, Average baseline sensitivity curve; *open circles*, average sensitivity curve at the midpoint of the study (approximately 6–8 weeks); and *solid squares*, average sensitivity curve at approximately 12 weeks just before cessation of drug dosing. **B**, Omalizumab-treated subjects ($n = 10$). *Solid circles*, Average baseline sensitivity curve; *open circles*, average sensitivity curve at the midpoint of the study (approximately 6–8 weeks); and *solid squares*, average sensitivity curve at approximately 12 weeks. The dotted line provides a visual reference for the change in sensitivity.

fit values of the effective density for 50% of maximum (ED_{50}) were 1.03 ± 0.11 , 0.18 ± 0.06 , and 0.08 ± 0.03 at baseline, midpoint, and final measurements, respectively. Fig E2 in this article's Online Repository at www.jacionline.org shows individual data for the treated group and the curve fit (plus fit envelopes) for both the treated and placebo groups. Likewise, the maximum HR response increased from 1.0 at baseline to 1.55 ± 0.21 and 1.55 ± 0.14 at the midpoint and final measurements, respectively (this corresponded to 58%, 89%, and 89% HR for a maximum in absolute terms).

In previous studies¹⁰ the fold increase in HR to anti-IgE antibody was inversely correlated with the starting response to anti-IgE antibody. Likewise, the shift in sensitivity was inversely related to the starting absolute sensitivity (Fig 2, A). The shift in sensitivity was also related to the starting maximal response (Fig 2, B).

Basophils release several types of mediators, but the signal transduction mechanisms differ somewhat in specifics so that correlations in the magnitude of the response for each type of mediator can be somewhat noisy. This is particularly true for the relationship between the release of LTC_4 and histamine.²⁴ However, as shown in Fig 3, the basophil also became more sensitive when LTC_4 was the measured outcome. Likewise, the maximum release of LTC_4 increased during treatment. In the same subset of subjects ($n = 7$), $Fc\epsilon RI$ and Syk levels (both determined by means of flow cytometry) at week 12 were 0.28 ± 0.04 -fold and 2.1 ± 0.5 -fold baseline values, respectively.

Previous studies have suggested that cessation of omalizumab treatment results in a slow multimonth return of function consistent with the 3- to 4-week half-life of circulating IgG antibodies.² Fig 4 shows that 3 months after stopping omalizumab, basophil sensitivity has shifted in the direction of baseline sensitivity, although the shift is only about 50% complete.

Anti-IgE antibody has been used to assess the general basophil IgE-mediated response in previous studies, and the concentrations chosen for previous studies were based on pilot work with

nonallergic subjects. In those studies it was surprising to find that both patients with cat allergy and those with peanut allergy showed a somewhat lower mean effective concentration for the anti-IgE antibody being used, and during treatment, the results from an abbreviated range of concentrations suggested that the point of optimum HR shifted. Mechanistically, this was unexpected, and therefore in the current study, in addition to the sensitivity measurements presented above, 3 donors were examined with a much broader dose-response curve to anti-IgE antibody. Fig E3 in this article's Online Repository at www.jacionline.org shows that response to anti-IgE antibody increased, as found previously, and that there is a shift rightward in the median effective concentration for the response consistent with shifts found for cat and peanut antigens.

DISCUSSION

These studies demonstrate that treatment with omalizumab increases intrinsic basophil sensitivity and that this occurs for both HR and LTC_4 release. The change in sensitivity was reversible, although it appears to require several months because there was only a 50% recovery after 3 months off the drug. It should be noted that an increase in sensitivity is generally offset by significant decreases in total and especially allergen-specific IgE antibody levels on the cell surface so that the final effect on the allergen-specific cell response is often seen as a reduction in the dose-response curve for a specific allergen.^{9,25,26} The change in the allergen full dose response appears to be a good indicator of the efficacy of treatment^{11,25,27–29} because the standard allergen dose-response curve captures several changing parameters of the response (reduction in cell-surface allergen-specific IgE levels, any potential changes in IgE affinity, and changes in intrinsic cellular sensitivity). However, in isolation an allergen dose-response curve might mask the potentially important changes to intrinsic cellular function that also occur during treatment. A good example of this can be seen in studies of peanut allergy,

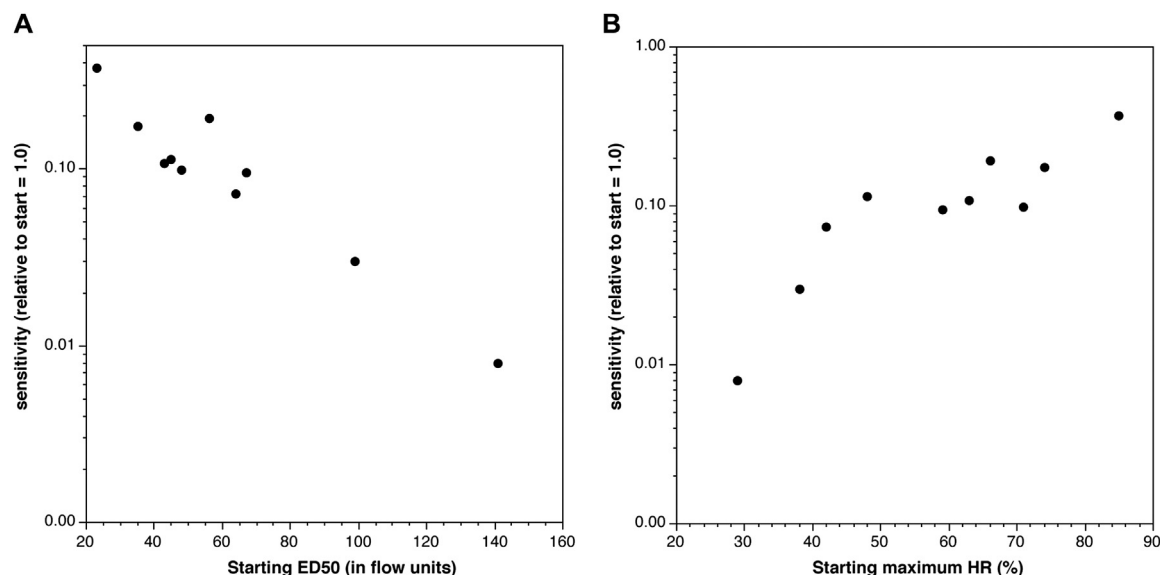


FIG 2. Relationships in change in sensitivity and starting sensitivity or starting maximum HR. **A**, Starting sensitivity (ED_{50}) as measured in units of mean fluorescence intensity from flow cytometric measurements. **B**, Relationship between starting maximum HR and change in sensitivity.

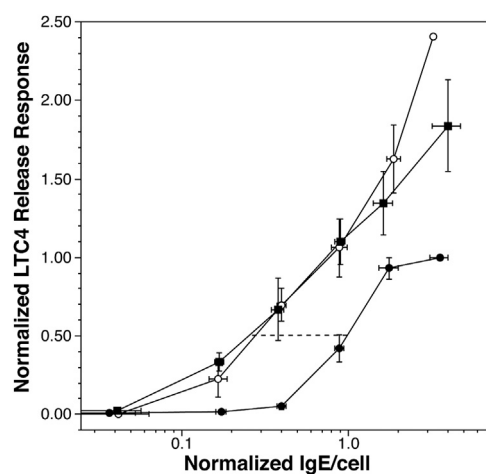


FIG 3. Sensitivity measurements using LTC_4 secretion as the outcome. LTC_4 levels were measured in the same supernatants in which HR was measured. The data are from only subjects treated with omalizumab ($n = 7$) and for whom data were available at each of the 3 time points (baseline, 6 weeks, and 12 weeks).

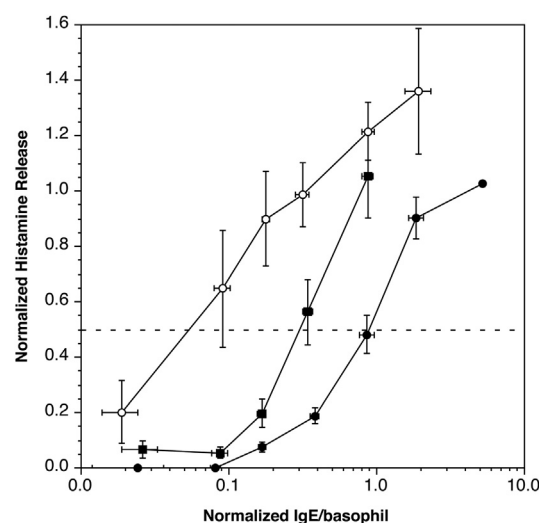


FIG 4. Recovery of baseline sensitivity after cessation of omalizumab treatment ($n = 7$). *Solid squares*, Average baseline sensitivity curve for the 7 subjects who completed the recovery period; *open circles*, average sensitivity curve at 12 weeks; and *solid circles*, average sensitivity curve 3 months after cessation of omalizumab.

in which the concentration of allergen needed to elicit basophil HR can shift rightward (ie, higher concentrations, as in the current study; data not shown), but the overall dose-response curve and its maximum are highly enhanced (also see the anti-IgE dose response curve, see Fig E3). Recent studies of peanut-sensitive subjects demonstrate that the changes in the intrinsic IgE-mediated response (as assessed by increases in the maximum response to anti-IgE antibody) work against the efficacy of omalizumab to modulate the basophil response and might provide a predictor of the clinical response as well.¹¹ We have recently determined that there were 2 factors that contributed to differences in suppression of the basophil response: the ratio of allergen-specific IgE to total IgE and the size of the increase in cellular sensitivity. If the allergen-specific IgE/total IgE ratio is low enough (<4%), even considerable enhancement in cellular sensitivity

is not sufficient to prevent suppression when already low densities of allergen-specific IgE are reduced by omalizumab. In addition to these factors, not all rightward shifts in the dose response result from a simple change in IgE density. Changes in the shape of the dose-response curves could also occur because the ratio of Syk and receptor density increases.³⁰ Finally, because it is not known how much allergen is found in the environment of basophils or mast cells, it is not yet clear which aspects of the dose-response curves are relevant to efficacy. However, without knowledge of the change in sensitivity, it is not possible to even guess how the various factors will compete. Therefore demonstrating that the basophil becomes 3- to 125-fold more sensitive to antigen-driven secretion provides a framework for future

studies of the underlying mechanisms that influence the efficacy of this therapeutic approach, as well as highlighting a potentially important process that regulates natural basophil sensitivity. Studies are underway to determine whether the information about the competing effects of change in sensitivity and IgE suppression have predictive value for choosing the patients to be treated with omalizumab.

The basis for an increase in the basophil response during treatment with omalizumab is currently unknown, although previous studies of both patients with cat allergy and those with peanut allergy noted an increase in the expression of Syk that was correlated with the maximum response to anti-IgE antibody.^{10,11} A similar change was also noted in the current study. Our previous study (and all subsequent studies, including this one) also demonstrated that the response to a non-IgE-dependent stimulus, N-formyl-methionyl-leucyl-phenylalanine, was not changed,¹⁰ suggesting that the effects of omalizumab are restricted to IgE-mediated signaling steps. It has also been suggested that studies of sustained aggregation during the maturation of basophils from CD34 progenitors might be applicable to this situation.³¹ In previous studies sustained aggregation during maturation resulted in “basophils” that expressed normal levels of histamine, granules, and FcεRI but showed reduced Syk expression. It has been speculated that treatment with omalizumab might relieve a state of chronic aggregation in allergic patients, although the nature of chronic aggregation has yet to be identified. The full extent of the changes occurring in basophils both in the CD34 culture studies and in patients treated with omalizumab has not been explored, but the changes in Syk levels are consistent with an increase in cellular responsiveness. The current studies now put a number on the change in cellular responsiveness, a more useful number in the context of understanding the magnitude of the change in the cell's IgE-mediated response. After approximately 12 weeks of omalizumab, basophils became 12-fold more sensitive, but the most extreme change was nearly 125-fold (Fig 2, A). On the basis of previous calibrations of the IgE antibody used for sensitization and an average ED₅₀ of 62 flow units in this study, this value corresponds to approximately 1100 molecules of IgE per cell. This appears somewhat more sensitive than previous baseline estimates of average basophil sensitivity, but the antigen in this case, streptavidin, was a somewhat better stimulus than the antigen used in previous studies (see this article's Online Repository at www.jacionline.org). A 12-fold change in sensitivity implies a cell that is capable of 50% of its maximal response (which has also increased) with only 100 antigen-specific IgE molecules per cell. Our previous modeling of IgE-mediated signaling in basophils³² suggests that a 2-fold change in Syk level alone (the amount observed in the previous cat and peanut studies, as well as this study) cannot readily account for a 12-fold change in sensitivity. The unexpected size of the increase in sensitivity suggests that future studies should examine the basophil for other changes. For example, models of IgE-mediated secretion³¹ suggest that a change in levels of both Syk and an Src family kinase, such as Lyn, might be sufficient to cause this kind of enhancement. There is some support for this possibility because of the 20 different signaling species that have been examined for downregulation by IgE-dependent aggregation,⁷ only Syk, Lyn, and FcεRI levels were altered. The loss of Syk is considerably greater than the loss of Lyn, but it is possible that during maturation of basophils (see the discussion above), modulation of Lyn might also occur during any sustained aggregation that can be relieved by treatment with omalizumab.

A feature of the changing basophil response that has been consistent in all our studies is the inverse relationship between the

response before treatment and its increase during treatment.^{10,11} This now applies to the sensitivity metric. Indeed, the correlation between sensitivity before treatment and the fold change in sensitivity was remarkably high. This property of the change supports the concept that the natural (unmodulated) basophil response is some fixed value but that there is heterogeneity of response in the population because of some process that is sensitive to omalizumab. If this process is related to chronic or sustained aggregation (during maturation?), then these results suggest that the unmodulated state of basophils would include an IgE-mediated response that leads to nearly 100% HR and a sensitivity on the order of 100 molecules per cell of antigen-specific IgE. In our recent study of peanut-sensitive patients, there was an indication that this increasing sensitivity mitigated against the efficacy of the drug.¹¹ This would stand to reason if the basophil plays a role in expression of food allergies. However, these observations suggest that the linkage between basophil function and disease response to omalizumab is possibly different from the linkage between the basophil response and disease severity. The linkage between basophil response and disease severity is not strong.³³⁻³⁹ For treatment to reveal that the change in basophil response has an influence on the efficacy of treatment suggests there are other factors involved. One speculation is that the end-organ responses in each subject slowly adapt to the basophil's behavior so that the target organs adapt over time to the subject's natural basophil responsiveness. This might wash out a relationship between basophil responsiveness and disease expression in a cross-sectional study but make it possible to find the relationship revealed when the basophil's response is rapidly changed in a longitudinal study.

In summary, this third study of basophil responsiveness during treatment with omalizumab shows not only that the change in maximum release is a repeatable feature of basophil responsiveness but also that a marked change in the intrinsic sensitivity of the basophil occurs. One interpretation of the various results is that omalizumab alters a process that serves to variably suppress the natural sensitivity and responsiveness of basophils. The magnitude of the increase is greater than current models of IgE-mediated secretion would predict for an increase in Syk expression alone.

We thank Valerie Alexander for her technical assistance in this study.

Key messages

- Treatment induces a large increase in the intrinsic sensitivity of basophils to IgE-mediated stimulation, an increase that translates to basophils initially requiring thousands of allergen-specific IgE per cell to basophils requiring IgE densities in the low hundreds to obtain equivalent mediator release.
- On the basis of current understanding about IgE-mediated secretion, the magnitude of the increase in sensitivity is much greater than can be accounted for by the concurrently measured increase in Syk expression, suggesting that there are additional changes in the cell that account for the increase.

REFERENCES

1. MacGlashan Dw Jr, Bochner BS, Adelman DC, Jardieu PM, Togias A, McKenzie-White J, et al. Down-regulation of FcεRI expression on human basophils during *in vivo* treatment of atopic patients with anti-IgE antibody. *J Immunol* 1997;158:1438-45.

2. Saini SS, MacGlashan DW Jr, Sterbinsky SA, Togias A, Adelman DC, Lichtenstein LM, et al. Down-regulation of human basophil IgE and FcεRIα surface densities and mediator release by anti-IgE infusions is reversible in vitro and in vivo. *J Immunol* 1999;162:5623-30.
3. Prussin C, Griffith DT, Boesel KM, Lin H, Foster B, Casale TB. Omalizumab treatment downregulates dendritic cell FcεpsilonRI expression. *J Allergy Clin Immunol* 2003;112:1147-54.
4. Gomez G, Jogie-Brahim S, Shima M, Schwartz LB. Omalizumab reverses the phenotypic and functional effects of IgE-enhanced Fc epsilonRI on human skin mast cells. *J Immunol* 2007;179:1353-61.
5. MacGlashan DW Jr, Xia HZ, Schwartz LB, Gong JP. IgE-regulated expression of FcεRI in human basophils: control by regulated loss rather than regulated synthesis. *J Leukoc Biol* 2001;70:207-18.
6. Borkowski TA, Jouvin MH, Lin SY, Kinet JP. Minimal requirements for IgE-mediated regulation of surface Fc epsilon RI. *J Immunol* 2001;167:1290-6.
7. 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.
8. Johansson SG, Nopp A, Oman H, Ankerst J, Cardell LO, Gronneberg R, et al. The size of the disease relevant IgE antibody fraction in relation to "total-IgE" predicts the efficacy of anti-IgE (Xolair) treatment. *Allergy* 2009;64:1472-7.
9. 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;125:889-895.e7.
10. Zaidi AK, Saini SS, MacGlashan DW Jr. Regulation of Syk kinase and FcRβ expression in human basophils during treatment with omalizumab. *J Allergy Clin Immunol* 2010;125:902-8.e7.
11. MacGlashan DW Jr, Savage J, Wood RA, Saini S. Suppression of the basophil response to allergen during treatment with omalizumab is dependent on two competing factors. *J Allergy Clin Immunol* 2012;130:1130-5.
12. Schleimer RP, Derse CP, Friedman B, Gillis S, Plaut M, Lichtenstein LM, et al. Regulation of human basophil mediator release by cytokines. I. Interaction with antiinflammatory steroids. *J Immunol* 1989;143:1310-7.
13. Kurimoto Y, de Weck AL, Dahinden CA. The effect of interleukin 3 upon IgE-dependent and IgE-independent basophil degranulation and leukotriene generation. *Eur J Immunol* 1991;21:361-8.
14. MacGlashan D Jr. Subthreshold desensitization of human basophils re-capitulates the loss of Syk and FcεRI expression characterized by other methods of desensitization. *Clin Exp Allergy* 2012;42:1060-70.
15. Miura K, MacGlashan DW Jr. Dual phase priming by interleukin-3 for leukotriene C4 generation in human basophils. *J Immunol* 2000;164:3026-34.
16. Miura K, Lavens-Phillips S, MacGlashan DW Jr. Localizing a control region in the pathway to LTC4 secretion following stimulation of human basophils with anti-IgE antibody. *J Immunol* 2001;167:7027-37.
17. Miura K, Saini SS, Gauvreau G, MacGlashan DW Jr. Differences in functional consequences and signal transduction induced by IL-3, IL-5 and NGF in human basophils. *J Immunol* 2001;167:2282-91.
18. MacGlashan DW Jr. Two regions of down-regulation in the IgE-mediated signaling pathway in human basophils. *J Immunol* 2003;170:4814-925.
19. Vilarino N, Miura K, MacGlashan DW Jr. Acute IL-3 priming up-regulates the stimulus-induced Raf-1-Mek-Erk cascade independently of IL-3-induced activation of Erk. *J Immunol* 2005;175:3006-14.
20. 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.
21. Siraganian RP. An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal Biochem* 1974;57:383-94.
22. MacGlashan DW Jr. Releasability of human basophils: cellular sensitivity and maximal histamine release are independent variables. *J Allergy Clin Immunol* 1993;91:605-15.
23. MacGlashan DW Jr, Vilarino N. Nonspecific desensitization, functional memory and the characteristics of SHIP phosphorylation following IgE-mediated stimulation of human basophils. *J Immunol* 2006;177:1040-51.
24. MacGlashan DW Jr, Peters SP, Warner J, Lichtenstein LM. Characteristics of human basophil sulfidopeptide leukotriene release: releasability defined as the ability of the basophil to respond to dimeric cross-links. *J Immunol* 1986;136:2231-9.
25. 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.
26. Nopp A, Cardell LO, Johansson SG, Oman H. CD-sens: a biological measure of immunological changes stimulated by ASIT. *Allergy* 2009;64:811-4.
27. Dahlen B, Nopp A, Johansson SG, Eduards M, Skedinger M, Adedoyin J. Basophil allergen threshold sensitivity, CD-sens, is a measure of allergen sensitivity in asthma. *Clin Exp Allergy* 2011;41:1091-7.
28. Glaumann S, Nopp A, Johansson SG, Rudengren M, Borres MP, Nilsson C. Basophil allergen threshold sensitivity, CD-sens, IgE-sensitization and DBPCFC in peanut-sensitized children. *Allergy* 2012;67:242-7.
29. Noga O, Hanf G, Kunkel G, Kleine-Tebbe J. Basophil histamine release decreases during omalizumab therapy in allergic asthmatics. *Int Arch Allergy Immunol* 2008;146:66-70.
30. Nag A, Faeder JR, Goldstein B. Shaping the response: the role of FcεpsilonRI and Syk expression levels in mast cell signaling. *IET Syst Biol* 2010;4:334-47.
31. Ishmael SS, MacGlashan DW Jr. Syk expression in peripheral blood leukocytes, CD34+ progenitors, and CD34-derived basophils. *J Leukoc Biol* 2010;87:291-300.
32. Vilarino N, MacGlashan DW Jr. Actin cytoskeleton-dependent down-regulation of early IgE-mediated signaling in human basophils. *J Leukoc Biol* 2004;75:928-37.
33. Busse WW, Swenson CA, Sharpe G, Koschat M. Enhanced basophil histamine release to concanavalin A in allergic rhinitis. *J Allergy Clin Immunol* 1986;78:90-7.
34. Gaddy JN, Busse WW. Enhanced IgE-dependent basophil histamine release and airway reactivity in asthma. *Am Rev Respir Dis* 1986;134:969-74.
35. Casolaro V, Spadaro G, Marone G. Human basophil releasability. VI. Changes in basophil releasability in patients with allergic rhinitis or bronchial asthma. *Am Rev Respir Dis* 1990;142:1108-11.
36. Youssef LA, Schuyler M, Gilmartin L, Pickett G, Bard JD, Tarleton CA, et al. Histamine release from the basophils of control and asthmatic subjects and a comparison of gene expression between "releaser" and "nonreleaser" basophils. *J Immunol* 2007;178:4584-94.
37. Norman PS, Lichtenstein LM, Ishizaka K. Diagnostic tests in ragweed hay fever. A comparison of direct skin tests, IgE antibody measurements, and basophil histamine release. *J Allergy Clin Immunol* 1973;52:210-24.
38. Bruce CA, Rosenthal RR, Lichtenstein LM, Norman PS. Diagnostic tests in ragweed-allergic asthma. A comparison of direct skin tests, leukocyte histamine release, and quantitative bronchial challenge. *J Allergy Clin Immunol* 1974;53:230-9.
39. Paterniti M, Kelly DC, Eckman JA, Sterba PM, Hamilton RG, Bochner BS, et al. Cat allergen-induced blood basophil reactivity in vitro predicts acute human nasal allergen challenge responses in vivo. *Clin Exp Allergy* 2011;41:963-9.

METHODS

The following were purchased: PIPES, BSA, EDTA, D-glucose, and erythrosin B (Sigma-Aldrich, St Louis, Mo); crystallized HSA (Miles Laboratories, Elkhart, Ind); FCS (BioWhittaker, Walkersville, Md); Percoll (Pharmacia, Piscataway, NJ); anti-IL-3 receptor (IL-3R)/CD123 (9F5) phycoerythrin-conjugated; anti-mouse IgG₁ Alexa Fluor 647, anti-mouse IgG_{2a} Alexa Fluor 647, and anti-mouse IgG_{2b} (Molecular Probes/Invitrogen, Carlsbad, Calif); and anti-human IgE monomeric IgM (6061P; Hybridoma Reagent Laboratories).

Flow cytometry

All analyses were performed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, Calif). Fixed mixed leukocytes were labeled with anti-IL-3R-phycoerythrin antibody and streptavidin-Alexa Fluor 647. Leukocytes prepared by using a 2-step Percoll gradient do not generally contain plasmacytoid dendritic cells, which express IL-3R at levels similar to those seen in basophils, and therefore these preparations did not need a second antibody to gate plasmacytoid dendritic cells. Forward/side-scatter parameters, in addition to IL-3R positivity, were used to gate basophils for measurement of streptavidin binding. Day-to-day instrument variability was monitored with CaliBRITE APC calibration beads (BD Biosciences).

Calibration of the SE44B signal by using flow cytometry

RBL-SX38 cells (transfected with human FcεRI, gift of Dr Jean-Pierre Kinet, Harvard, Boston, Mass) were loaded with SE44B to calibrate flow cytometric results with absolute gp120-specific IgE density. A portion were analyzed by using flow cytometry, as above, and a portion were treated with acetate buffer to dissociate bound IgE, levels of which were measured in a total IgE assay (ImmunoCAP; Phadia, Uppsala, Sweden), as described previously.^{E1} The density of cell-surface IgE was calculated from the total IgE measurement and a count of the cells used for dissociation. The flow cytometric results were then equated with this measured density of IgE/basophil.

RESULTS

Changes to the flow cytometry-based measurement of intrinsic sensitivity

Basophils were sensitized with a biotinylated IgE to refine the method. For detection by means of flow cytometry, streptavidin-Alexa Fluor 647 was used to measure the level of sensitization, and for mediator release, the same streptavidin-Alexa Fluor 647 was used to stimulate the cells (streptavidin is a naturally tetrameric molecule) after titrating to determine an optimal concentration for stimulation. The current method was cross-calibrated with prior methods to relate the results to previous measurements of basophil sensitivity. First, the flow cytometric signal strength (after calibrating the signal against a standard fluorescent bead) was calibrated against the absolute density of IgE by comparing signals with a previously calibrated set of receptor reagents (see the Methods section). Second, antigens have different abilities to induce secretion so that in absolute terms greater or fewer antigen-specific IgE molecules are needed

to induce the same amount of HR from the same basophil (a good example is that bivalent penicillin is 10-fold less capable of inducing HR than multivalent penicillin-HSA). The previous method for assessing sensitivity used a multivalent gp120-ovalbumin conjugate as a stimulus for gp120-specific IgE-sensitized cells. The biotinylated IgE used for these studies was the same gp120-specific IgE antibody used previously for cellular sensitivity measurements so that streptavidin as an antigen could be compared directly with gp120-ovalbumin as an antigen. For equal HR, streptavidin required 3.5 ± 0.2 -fold ($n = 4$) less cell-bound IgE than gp120-ovalbumin.

Analytic method

The data are first normalized to a unit-less sensitivity value. Fig E1 illustrates this method. After generating a sensitivity curve before the start of omalizumab, the midpoint of the curve is determined (Fig E1, A, shows a typical curve). Starting with the midpoint density, all IgE density data for a given subject's response are divided by this value. Likewise, the maximum HR at any point in the curve or the maximum release induced with anti-IgE antibody (usually the best response) is used as the divisor for each value of HR. The resulting curve, by definition for the baseline curve, has its midpoint at 1.0 on the x-axis and a midpoint response of 0.5 (Fig 1, B). Fig E1, C, shows that starting data points for 19 curves all lie on a single curve for averaging purposes. In other words, a curve fit is possible on the entire data set with minimal variance in the parameters of the curve fit (the midpoint on the x-axis and the maximum response are 2 parameters of the sigmoid function used for fitting). For each subject, subsequent sensitivity determinations for the same subject use the subject's starting midpoint values as divisors to transform subsequent curves (for that subject) in the context of the starting curve. Fig E1, C, also shows one other choice made for presenting the results. The alternating gray bands as background on the plot show regions in which data were averaged (both x- and y-axis averaging) to generate a single point with error bars in both directions. This is a visual tool for presentation and is used for presentation in Fig 1, but for the final analysis, each time point in the study (baseline, midpoint, and final) and all the data for omalizumab-treated ($n = 10$) subjects (Fig E2, K) were included in a general curve fit to a sigmoid function, which produces the desired ED₅₀ for the group's average response (much like the baseline fit curve shown in Fig E1, C, is the function fit for the data shown). A similar grouping for the placebo-treated subjects ($n = 9$) generated their average response at each time point in the study (Fig E2, L).

REFERENCE

- E1. MacGlashan DW Jr, Vilarino N. Nonspecific desensitization, functional memory and the characteristics of SHIP phosphorylation following IgE-mediated stimulation of human basophils. *J Immunol* 2006;177:1040-51.

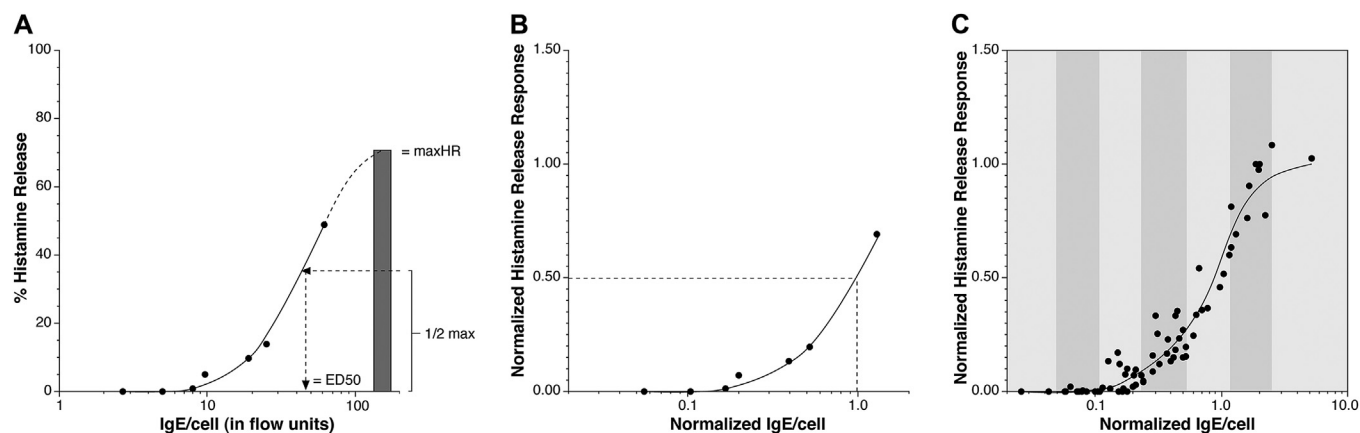


FIG E1. Example of data analysis for the determination of changes in basophil sensitivity. **A,** An example data set from one of the subjects in the study. Data represent the baseline sensitivity curve. **B,** Normalization of the curve in Fig E1, A, using the maximal HR and ED₅₀ points defined in Fig E1, A. **C,** Normalized results from all 19 baseline curves in the study are combined into one baseline curve and data fit by using a sigmoid function. The *gray vertical bands* in the plot represent regions in which results are averaged into a single data point for the purposes of presentation (see text).

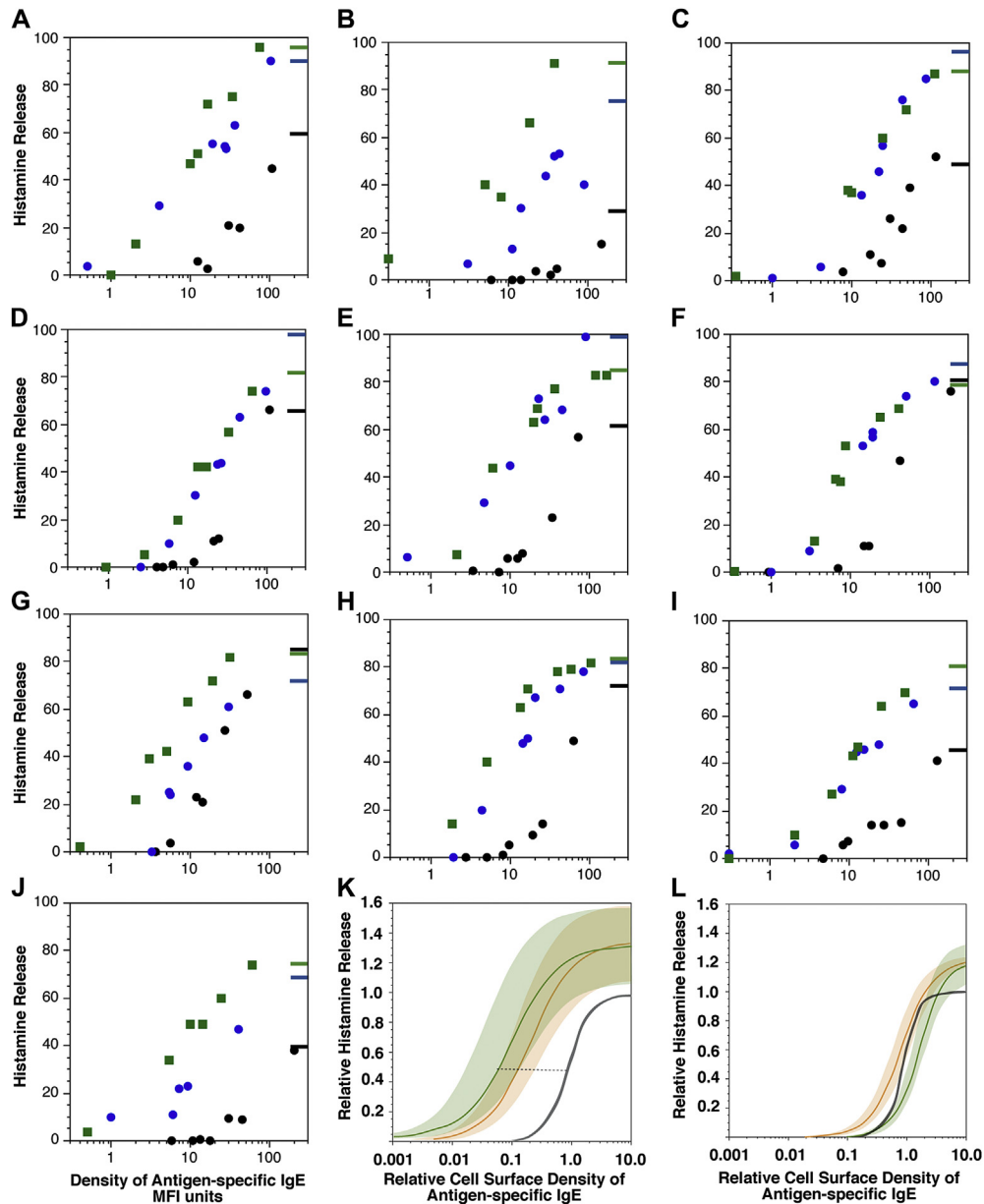


FIG E2. Individual plots for the treated group ($n = 10$). **A-J**, The abscissa is expressed in flow cytometric (net) mean fluorescence intensity units (see text for calibration), and the ordinate is expressed as percentage HR at the baseline visit (*black circles*), midpoint visit (*blue circles*), and final visit (*green squares*). Along the right side of each panel are *colored tick marks* (same color assignments) that indicate the maximum anti-IgE antibody-induced release for the 3 time points. **K** and **L** show the fitting results for all 10 treated or 9 placebo data sets, and the *shading* represents the error envelope around the central fit curve: *black* shows the baseline visit, *orange* is the midpoint visit, and *green* is the final time point visit.

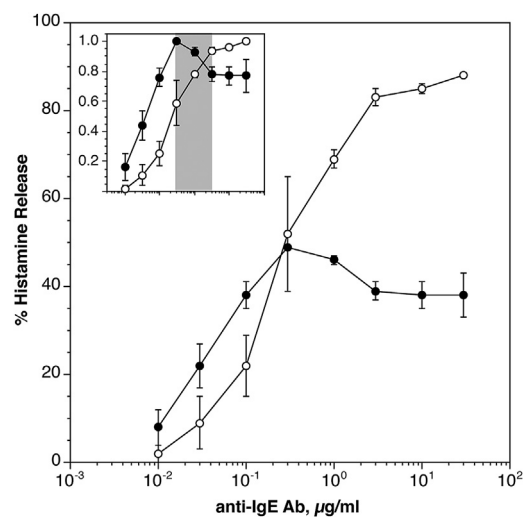


FIG E3. Anti-IgE dose-response curves at baseline and 12 weeks after treatment with omalizumab ($n = 3$). *Solid circles*, Baseline; *open circles*, 12 weeks. The *inset* shows the same data normalized to visually appreciate the curve shift. The *dark gray band* in the inset shows the choices of anti-IgE antibody concentration used in previous studies.