



Accumulated immune complexes of IgE and omalizumab trap allergens in an *in vitro* model

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ABSTRACT

The best understood mechanisms of omalizumab are that it neutralizes free IgE and down-regulates high-affinity IgE.Fc receptors (FcεRI) on basophils and mast cells. It has been proposed that since complexes of IgE and omalizumab are accumulated to 5–10 times the basal levels of IgE, they may trap incoming allergens, contributing to omalizumab's effectiveness. In order to investigate the ability of IgE:omalizumab complexes in trapping allergens and inhibiting basophil activation in an *in vitro* reconstitution model, the ability of IgE:omalizumab complexes to tie up antigen and hence inhibit (a) antigen binding to IgE bound by FcεRI, and (b) antigen-mediated activation of basophils, was examined. The free IgE was prepared by mixing different proportions of antigen-nonspecific IgE secreted by U266 cells and antigen-specific IgE, SE44 IgE, which recognizes a synthetic 15 a.a. peptide, R15K. The antigen was (R15K)₈-ova, i.e. ovalbumin conjugated with an average of 8 copies of R15K per molecule. The solid-phase FcεRI was a recombinant protein representing the extracellular portion of the α chain of the FcεRI receptor complex. The model FcεRI⁺ basophilic cell line was RBL.SX-38, a rat basophilic leukemic line transfected with the genes for α, β and γ subunits of human FcεRI. The results showed that the IgE:omalizumab complexes trapped increasing amounts of antigen with increasing (a) concentration of IgE, (b) proportion of antigen-specific IgE in total IgE, and (c) concentration of total immune complexes. Such trapping decreased the antigen-induced activation of FcεRI⁺ cells that had been pulsed with antigen-specific IgE, resulting in decreased mediator release. These results suggest that the rapidly accumulated IgE:omalizumab complexes in omalizumab-treated patients can capture allergens and consequently contribute to the pharmacological effects of omalizumab.

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1. Introduction

Omalizumab, a humanized monoclonal antibody (mAb) with a unique set of binding specificities toward human IgE [1], has been shown in numerous clinical studies to be efficacious and safe in treating various allergic diseases [2–6]. It has been approved for treating moderate-to-severe or severe allergic asthma in many countries. Omalizumab binds to an epitope on the CH3 domain of the ε chain, near the binding sites for the high-affinity IgE.Fc receptors (FcεRI) and for the low-affinity IgE.Fc receptors (FcεRII or CD23). While omalizumab can bind to free IgE in body fluids and membrane-bound IgE (mIgE) on B cells, it cannot bind to IgE bound by FcεRI on basophils and mast cells and by CD23 on various cell types [1].

The most understood pharmacological mechanisms of omalizumab are that it ties up free IgE, thus blocking its binding to FcεRI, and consequently decreasing the density of FcεRI on basophils and mast cells

[1,7,8]. As free IgE levels become very low and omalizumab is maintained in excess, the FcεRI on basophils and mast cells gradually become uncharged, and as bound IgE molecules dissociate from FcεRI due to thermodynamic equilibrium, they are tied up by excess omalizumab. Unoccupied FcεRI are unstable and are internalized, leading to their gradual removal from the surface of basophils, dendritic cells, and mast cells. Partly because basophils turn over with a short lifespan, the down-regulation of FcεRI on basophils is rapid, falling by 90% in 1–2 weeks [9,10]. However, the down-regulation of FcεRI on mast cells is slow, probably falling by a small extent in many weeks [9].

In several case series studies of omalizumab on atopic dermatitis, where many patients had serum IgE levels as high as 7000 to 35,000 IU/ml (10 to 50 times the approved ceiling of serum IgE of 700 IU/ml to be eligible to receive omalizumab), regular doses of omalizumab (maximum doses of 375 mg per 2 weeks) improved the symptoms in most of these patients [11–15]. It is probable that the administered doses of omalizumab were able to neutralize only a small part of the IgE produced in the intervals between omalizumab administrations, as free IgE levels were not changed in those patients whose IgE were measured [14]. If so, the administration of

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omalizumab should not have resulted in the depletion of free IgE and the consequent down-regulation of FcεRI. Furthermore, in a placebo-controlled clinical study of omalizumab in allergic rhinitis, some patients achieved symptomatic improvement within 1–2 weeks [10]. By this time, the FcεRI levels on mast cells in the nasal lining were probably not yet significantly down-regulated. In case studies of omalizumab on atopic dermatitis [13], chronic urticaria [16], allergic bronchopulmonary aspergillosis [17] and other conditions, symptomatic improvements were observed within two weeks after the first omalizumab injections. These various observations suggest that the improvement of symptoms in mucosal areas and skin was probably attributed to pharmacologic mechanisms other than the complete neutralization of free IgE and the down-regulation of FcεRI on mucosal or skin mast cells.

IgE in the blood has a relatively short half-life, about 2 days [18]. Thus, IgE is continually being replenished by IgE-producing plasma cells in the bone marrow and peripheral lymphoid tissues. Omalizumab has been approved only for patients with serum IgE in the 30–700 IU/ml range. Within this range, the recommended dosages of omalizumab are a function of the patient's serum IgE levels and are sufficient to bind to and tie up all IgE present at the time of the first omalizumab administration as well as the additional IgE produced during the intervals between omalizumab administrations [18].

In phosphate-buffered saline *in vitro* and in cynomolgus monkeys *in vivo*, IgE and omalizumab were found to form small immune complexes (IC) of various stoichiometry depending on their molar ratios, with the largest being a 3:3 structure [19,20]. In human serum, the largest complex was probably a 2:2 structure [21]. In patients, the IgE:omalizumab IC are soluble, have aggregate half-lives of about 21–24 days, and are accumulated to levels 5–10 times the basal level of IgE within one to two weeks of the first omalizumab administration [22,23]. While the IgE molecules in the IC cannot bind to FcεRI, their Fab arms can still bind to their specific antigens. Chang (one of the authors of this paper) proposed that the high-levels of IgE:omalizumab IC, relative to basal IgE levels, can compete for binding to allergens and hence attenuate allergenic reactions, especially in the early periods after the administration of omalizumab [1,5].

While the observations of symptomatic improvement upon the administration of omalizumab are consistent with the view that the accumulated IgE:omalizumab IC trap allergens and hence contribute to the antibody's pharmacologic effects, direct evidence that the complexes actually capture allergens is lacking. We therefore developed here an *in vitro* model to investigate the effect of IgE:omalizumab IC on antigen trapping and its consequences in reducing antigen binding to FcεRI on basophils and sensitization of these cells (Fig. 1).

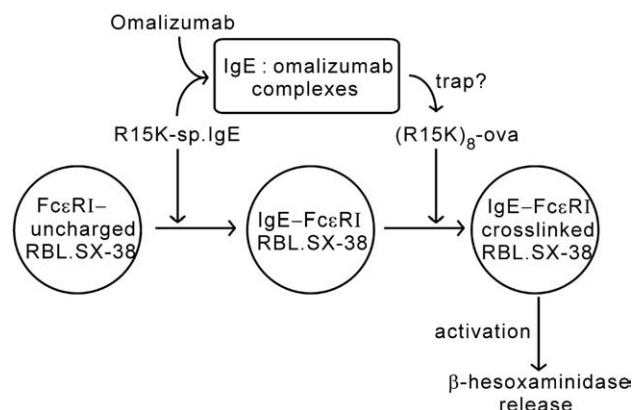


Fig. 1. Components that constitute the *in vitro* model employed in the present study for investigating the role of IgE:omalizumab IC on antigen trapping. Cells of the rat basophilic leukemic cell line RBL.SX-38 express human FcεRI. The model antigen (R15K)₈-ova is ovalbumin conjugated with 8 copies of 15 a.a. peptide, R15K. SE44-IgE, which is specific for R15K, is mixed with antigen-nonspecific U266-IgE at various proportions.

2. Materials and methods

2.1. Cell culture

Rat basophilic leukemia cell line, RBL SX-38, which expresses the α, β, and γ chains of human FcεRI [24], was a gift of Dr. Jean P. Kinet (Harvard Medical School). RBL SX-38 cells were cultured in EMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin and 1.2 mg/ml G418 (Gibco) as previously described. SE44, a transfectoma cell line expressing HIV-gp120-specific chimeric IgE with CH1-CH4 of human ε chain [25], was cultured in DMEM (Gibco) supplemented with 10% FBS, 1% penicillin/streptomycin, 1× HAT supplement and G418 at 200 μg/ml. U266B1, a human IgE-secreting myeloma cell line, was maintained in an RPMI 1640 medium containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% FBS.

2.2. Determining size of IgE:omalizumab IC by size exclusion chromatography

SE44 IgE and omalizumab were dissolved in phosphate-buffered saline (PBS) at a molar ratio of 1:2. After 30 min incubation, the IgE:omalizumab IC were analyzed by size exclusion chromatography (BioBasic HPLC L-2200, Thermo Scientific) using a BioBasic SEC-1000 analytical column (30 mm×7.8 mm, 1000 Å pore size; flow rate 0.5 ml/min, and running time 50 min).

2.3. Analysis of the binding of IgE:omalizumab IC and antigen with Protein A column and SDS-PAGE and Western blotting analysis

To detect the interaction of IgE:omalizumab IC with antigen, Protein A-Sepharose 4B beads were used to absorb omalizumab and its associated proteins. Subsequently, the absorbed proteins were subjected to (i) SDS-PAGE and (ii) Western blotting analysis. For these analyses, SE 44-IgE and omalizumab were mixed at a molar ratio of 1:2 and incubated at room temperature for 30 min. Next, 500 μl of (R15K)₈-ova (custom-made by Glyconex, Taipei, Taiwan) at 2 mg/ml was added and incubated at 37 °C for 30 min. The mixture was passed through a Protein A-Sepharose 4B column and the absorbed protein was processed for two separate 10% SDS-PAGE gels. In one SDS gel, the resolved proteins were stained with Coomassie blue. The proteins in the other SDS gel were transblotted onto a PVDF membrane (Hybond-P, GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and the presence of (R15K)₈-ova was identified by biotinylated SE44 IgE and avidin conjugated with horseradish peroxidase.

2.4. Measuring antigen trapping by IgE:omalizumab IC with ELISA

Recombinant His-human α subunit fusion protein, prepared by our laboratory, was diluted in coating buffer (0.1 M carbonate bicarbonate buffer, pH 9.6) at 20 μg/ml and 100 μl was transferred into each well of 96-well ELISA plates (Corning, New York, NY). After the plates were placed at 37 °C for 2 h and 4 °C overnight, the wells were washed three times with PBST (PBS containing 0.05% Tween 20) and incubated with 200 μl blocking buffer (PBS containing 5% bovine serum albumin (BSA) and 0.05% Tween 20) and incubated at 37 °C for 2 h, followed by washing three times with PBST buffer for 5 min. Subsequently, a total of 200 IU of IgE in 100 μl containing 0%, 10% or 30% SE44-IgE (the balance being U266-IgE) was added to each well and incubated for 1 h at room temperature. After the wells were washed 4 times with PBST, 90 μl of IgE:omalizumab IC at a molar ratio of 1:2 containing 0%, 10% or 30% SE44 IgE at a final concentration ranging from 1 to 32 times 200 IU/ml was added into each well. An aliquot of 10 μl of biotin-(R15K)₈-ova at 1 μg/ml was then added into each well. After the ELISA plates were incubated at 4 °C for 30 min, the

wells were washed six times with PBST and the bound biotin-(R15K)₈-ova was developed by horseradish peroxidase-conjugated streptavidin in a typical ELISA measuring absorbance at 405 nm.

2.5. Measuring antigen trapping by IgE:omalizumab IC by fluorescence flow cytometric analysis

RBL.SX-38 cells cultured in 15-cm culture dishes were detached in 2 mM EDTA/PBS buffer, washed, and resuspended in MEM with 10% FBS at 4×10^5 /ml. One milliliter of cell suspension was transferred to each 5-ml round bottom FACS tube. The RBL.SX-38 cells were spun down and medium removed, and 100 μ l of 200 IU/ml IgE in RPMI 1640 medium containing 0%, 10% or 30% SE44-IgE (the balance being U266-IgE) was added into each tube. After the cells were incubated at 37 °C for 2 h, the cells were spun down and the medium removed. As described in the section above, 90 μ l of IgE:omalizumab IC containing 0%, 10% or 30% SE44 IgE at a concentration ranging from 1 to 32 times 200 IU/ml was transferred to the tubes. Subsequently, 10 μ l of FITC-(R15K)₈-ova at 1 mg/ml or ovalbumin-conjugated FITC at 1 mg/ml (as negative control) was added into each tube. After incubation at room temperature for 30 min, the cells were washed twice with FACS buffer and resuspended in 0.5 ml FACS buffer. Mean fluorescence intensity was measured using a FACS Canto (BD Biosciences, Franklin Lakes, NJ). For each sample, the mean fluorescence intensity measured with FITC-(R15K)₈-ova was corrected against that taken with FITC-ova (as the background reading). The degree of inhibition of fluorescence intensity by IC in a sample containing SE44-IgE was then calculated as the % of decrease from the fluorescence intensity of a sample containing no SE44-IgE (only U266-IgE).

2.6. Measuring activation of RBL.SX38 cells by assaying β -hexosaminidase release

RBL.SX-38 cells were plated at a cell density of 4×10^4 cells/well in 100 μ l cell culture medium in each flat-bottomed well of 96-well microculture plates and incubated at 37 °C overnight. The medium was removed and 100 μ l of 200 IU/ml IgE containing a proportion of SE44-IgE (the balance being U266-IgE) was added into each well and the cells were incubated at 37 °C for 1 h. The medium was withdrawn and the cells were washed with Tyrode's/BSA washing buffer three times. Then, 90 μ l of IgE:omalizumab IC of varying proportions of SE44 IgE was transferred to the wells. Subsequently, 10 μ l (R15K)₈-ova

solution in serial dilution from 0.03 μ g/ml to 3 μ g/ml was added to each well. After the cells were incubated at 37 °C for 30 min, the plates were centrifuged at $260 \times g$ for 5 min at room temperature and 50 μ l of supernatant from each well was transferred into a new ELISA plate. The β -hexosaminidase activity was assayed as described elsewhere [26,27]. For 100% release, cells were lysed with 1% Triton X-100. In the first series of experiments, the proportion of free SE44-IgE in the total IgE was varied from 0.1% to 30% in half-log steps and the 90 μ l of IC solution was replaced with Tyrode's/BSA buffer. In another series of experiments, the proportion of free SE44-IgE in the total IgE solution was held at 10%, and the amount of added IgE:omalizumab (also using 1:2 molar ratio of 10% specific IgE) was varied from 1 to 32 times molar excess over the free IgE.

2.7. Statistical analysis

Data were expressed as means $\pm$ SEM, and the significance of differences between group means was determined with 2-way ANOVA using GraphPad Prism version 5.0 software (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. IgE:omalizumab IC are capable of binding to antigens

For most of the experiments performed in the present *in vitro* model studies, the IgE:omalizumab complexes were prepared at a 1:2 ratio. This reflects that patients are usually kept in omalizumab excess during most of the treatment period. Following the incubation of SE44-IgE and omalizumab at a molar ratio of 1:2, two major protein species were observed using HPLC-size exclusion chromatography (Fig. 2), one with molecular weight somewhat larger than that of IgM (950 kDa) and the other with molecular weight similar to thyroglobulin (670 kDa). These results suggest that IgE and omalizumab form 3:3 hexamers, with an estimated molecular weight of 1120 kDa, as previously reported [19]. The other major peak is most likely 2:2 tetramers of omalizumab:IgE immune complexes, with an estimated molecular weight of 680 kDa. Complexes of larger sizes were not observed.

In the *in vitro* reconstituted model adopted in these studies, the IgE was prepared by mixing different proportions of antigen-nonspecific IgE secreted by U266 cells and antigen-specific IgE secreted by SE44

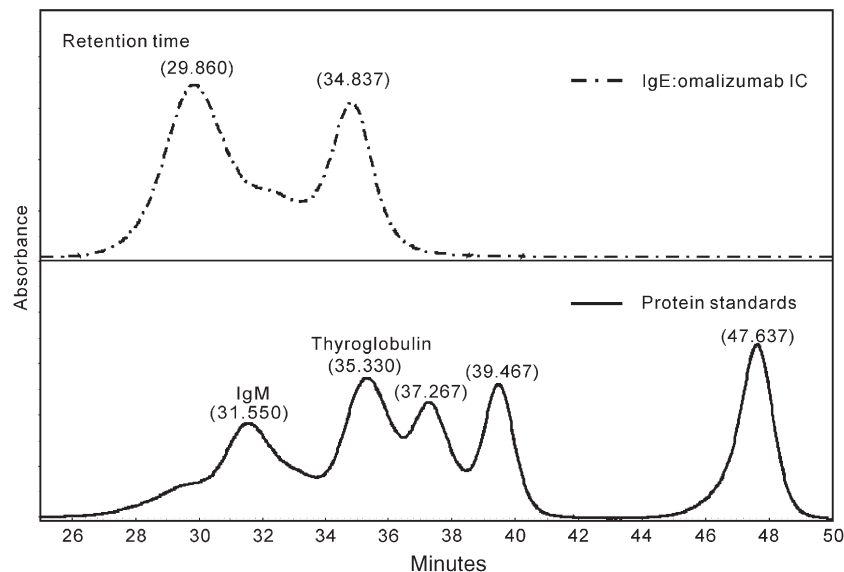


Fig. 2. HPLC-size exclusion chromatography of IgE:omalizumab IC. Mixture of IgE and omalizumab at a molar ratio of 1:2 (upper trace), and protein size standards from BioRad spiked with purified human IgM (lower trace) were subjected to HPLC-size exclusion chromatography as described in Materials and methods.

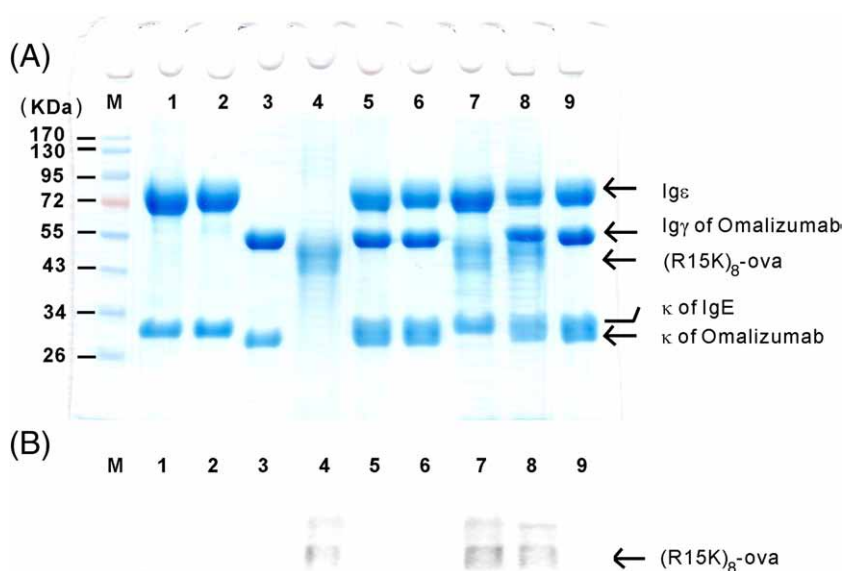


Fig. 3. (A) SDS-PAGE patterns of various mixtures of IgE, omalizumab, IgE:omalizumab IC, and (R15K)₈-ova. The protein absorbed by Protein A beads were subjected to 10% SDS-PAGE under reducing conditions and the resultant gels stained with Coomassie blue. Lane M, size markers; lane 1, SE44-IgE; lane 2, U266-IgE; lane 3, omalizumab (humanized IgG1); lane 4, (R15K)₈-ova; lane 5, SE44-IgE and omalizumab mixture, absorbed by Protein A-Sepharose 4B; lane 6, U266-IgE and omalizumab mixture, absorbed by Protein A-Sepharose 4B; lane 7, SE44-IgE and (R15K)₈-ova mixture, absorbed by omalizumab-Sepharose 4B; lane 8, SE44-IgE, omalizumab, and (R15K)₈-ova mixture, absorbed by Protein A-Sepharose 4B, lane 9, U266-IgE, (R15K)₈-ova, and omalizumab mixture, absorbed by Protein A-Sepharose 4B. (B) The presence of (R15K)₈-ova in lanes 4, 7 and 8 as revealed by Western blot analysis.

cells. The IgE secreted by SE44 is specific for a 15-a.a. peptide segment, R15K, of HIV gp120, while the antigen was (R15K)₈-ova. The SDS-PAGE patterns in Fig. 3 show that Protein A-Sepharose 4B resin could pull down SE44-IgE from the mixture of SE44-IgE and omalizumab (an IgG), indicating that SE44-IgE formed complexes with omalizumab (lane 5). Similarly, the SDS PAGE patterns show that SE44-IgE:omalizumab complexes could bind to (R15K)₈-ova (lane 8).

3.2. IgE:omalizumab IC inhibits antigen binding to IgE bound by α subunit of FcεRI

In this defined *in vitro* model, which is simpler than that in Fig. 1, the ability of increasing concentrations of immune complexes to trap antigen and hence inhibit the antigen binding to purified FcεRI protein on solid-phase substratum was studied. FcεRI comprises one α, one β, and two γ subunits, of which the α subunit is responsible for binding to IgE. In this set of experiments, recombinant α subunit of FcεRI was coated on ELISA plates and IgE containing 10% or 30% SE44-IgE (the balance being U266-IgE) at 200 IU/ml was bound to the α subunit. The IgE:omalizumab (1:2 mixture), in which the IgE contained either 10% or 30% SE44-IgE, was added to the wells at 0 to 32 times 200 IU/ml (total IgE concentration). In the present experiments, the amount of IC was varied between 0 and 32 times free IgE level, even though IC reaches maximally about ten times free IgE in patients. The IgE:omalizumab IC inhibited (R15K)₈-ova binding to the solid-phase IgE in a concentration-dependent fashion that was related to the total concentration of the IgE:omalizumab IC and to the proportion of SE44-IgE in total IgE (Fig. 4). IgE:omalizumab IC comprising entirely non-antigen-specific IgE (100% U266-IgE) did not inhibit (R15K)₈-ova binding to α-subunit bound SE44-IgE.

3.3. IgE:omalizumab IC inhibit binding of fluorescence-labeled antigen to IgE-saturated basophils

The RBL.SX-38 basophil line was used as a model for basophils. RBL.SX-38 cells were incubated with IgE containing various propor-

tions of SE44 IgE. The binding of FITC-conjugated (R15K)₈-ova to the RBL.SX-38 cells was measured by fluorescence flow cytometry. The fluorescence flow cytometric histograms in Fig. 5(A) show that IgE:omalizumab IC with SE44-IgE at 30% at various concentrations and FITC-(R15K)₈-ova caused varying degrees of fluorescence shift in staining RBL.SX-38 cells, which could be detected by the flow cytometric equipment FACS Canto. The presence of a 1 to 32 times molar excess of the IC caused increasing degrees of inhibition of binding of FITC-(R15K)₈-ova. Fig. 5(B) shows the degree of inhibition of (R15K)₈-ova binding to RBL.SX-38 cells by IgE:omalizumab IC with SE44-IgE at 30% and at 10% proportion of total IgE over a 1 to 32 molar excess range.

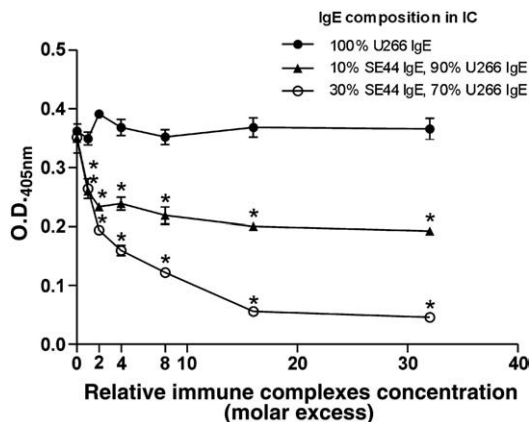


Fig. 4. The effects of IgE:omalizumab IC on inhibiting antigen binding to IgE bound by FcεRI α subunit. Recombinant human FcεRI α protein was coated onto wells of ELISA microplates, IgE at 200 IU/ml comprising either 0%, 10%, or 30% specific IgE, and 1:2 complexes of the same IgE mix at 0–32 times molar excess of the IgE concentration (200 IU/ml) were incubated together. Biotin-(R15K)₈-ova binding was then assayed by ELISA using HRP-streptavidin absorbance. Significance of differences between data obtained with IC-10% SE44-IgE or with IC-30% SE44-IgE and data obtained with IC-0% SE44-IgE was analyzed (**P* < 0.001).

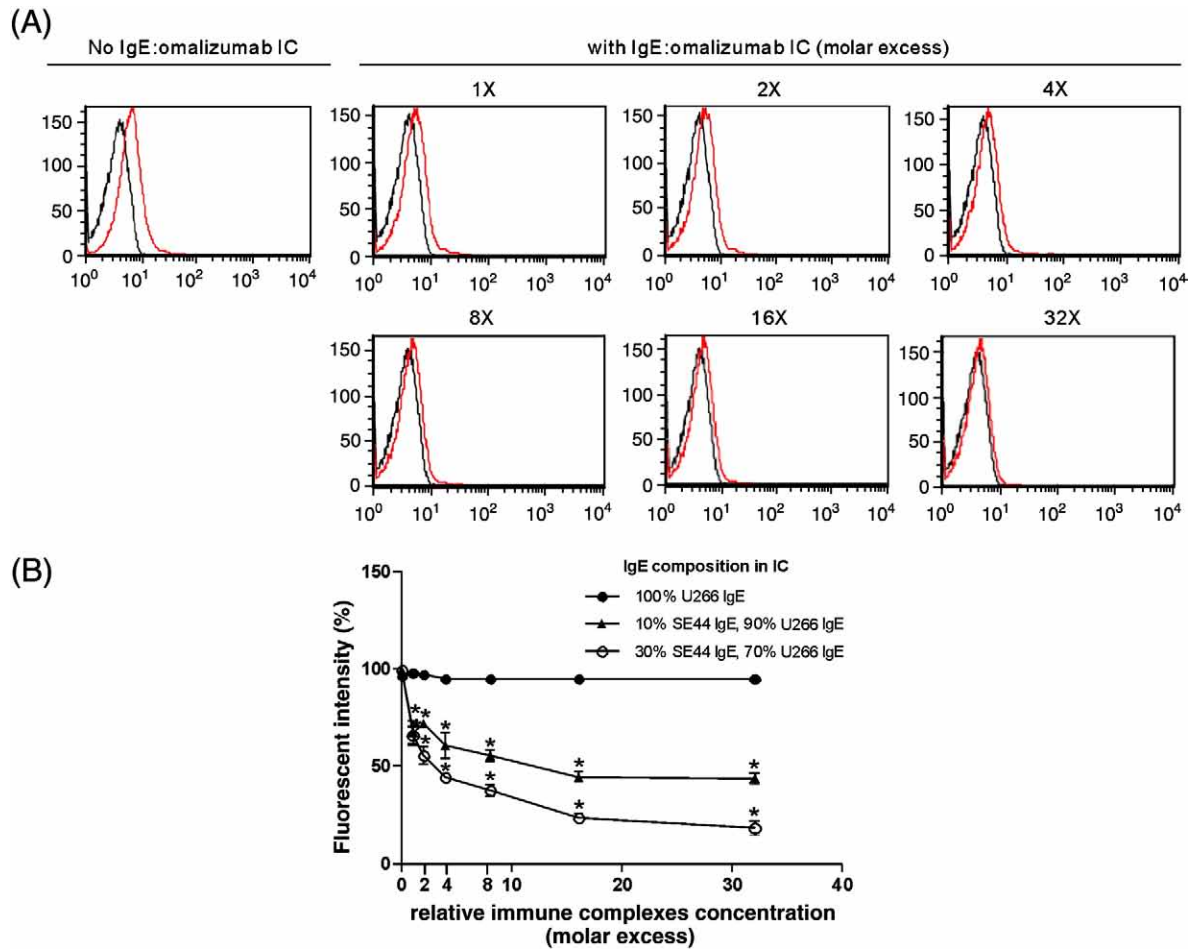


Fig. 5. The effects of IgE:omalizumab IC on inhibiting antigen binding to IgE bound by FcεRI on RBL.SX-38 cells. RBL.SX-38 cells were harvested from culture and suspended at 4×10^5 cells/ml in MEM medium containing 10% (v/v) heat-inactivated FBS, and 1 ml of the suspension was transferred into a 5 ml tube. IgE solutions and IgE:omalizumab mixtures were prepared as described in Fig. 4. RBL.SX-38 cells were stained with FITC-(R15K)₈-ova. The histograms and the degrees of fluorescence shift were measured by flow cytometry using FACS Canto as described in Materials and methods. (A) The fluorescence flow cytometric histograms with IgE:omalizumab IC containing SE44-IgE at 30% at various degrees of molar excess. (B) The degrees of inhibition of (R15K)₈-ova binding to RBL.SX-38 cells by IgE:omalizumab IC with SE44-IgE at 30% and 10%, respectively, over 1 to 32 molar excess range. (* $P < 0.001$).

3.4. IgE:omalizumab IC inhibited antigen-driven sensitization of RBL.SX-38 cells

To determine whether IgE:omalizumab IC could inhibit antigen cross-linking of IgE bound by FcεRI on RBL.SX-38 and hence the sensitizing of these cells, the release of β-hexosaminidase, as a quantitative indicator of the degranulation and sensitization of RBL.SX-38 cells, was measured by ELISA. In Fig. 6, RBL.SX-38 cells were incubated with 0.01 μg/ml–3 μg/ml of (R15K)₈-ova under different conditions of IgE:omalizumab IC. In Fig. 6(A), the total IgE was kept at 200 IU/ml, but the proportion of SE44-IgE in total IgE was varied; in Fig. 6(B), the SE44-IgE was kept at 10% of total IgE, but the total IgE (or IC) was varied from a 1 to 32 times molar excess of 200 IU/ml. The results presented in Fig. 6A show significant antigen-induced cross-linking of IgE-FcεRI on RBL.SX-38 cells and the subsequent activation of these cells when cultures were incubated with IgE containing 1%–30% SE44 IgE. The (R15K)₈-ova-induced β-hexosaminidase release from RBL.SX-38 cells was inhibited in a concentration-dependent fashion by IgE:omalizumab IC in the incubation medium (Fig. 6B). Compared with IgE:omalizumab IC that contained no SE44-IgE at a 32 times molar excess, IgE:omalizumab IC that contained 10% SE44-IgE at a 2-fold excess inhibited (R15K)₈-ova-induced sensitization of RBL.SX-38 cells significantly. As the IgE:omalizumab IC concentration increased, the degree of inhibition increased.

4. Discussion

The present study establishes an *in vitro* reconstituted model system that features the key molecular components of the IgE pathway in a patient upon treatment with omalizumab. One element of primary interest is the complexes formed by IgE and omalizumab, which accumulate rapidly in the blood and presumably body fluids in tissues upon treatment with omalizumab. In this model, the IgE consisted of antigen-nonspecific IgE from the U266 cell line and IgE specific for R15K peptide secreted by the SE44 cell line [25]. The present study illustrates that IC formed by IgE and omalizumab are capable of capturing a specific antigen, (R15K)₈-ova, and hence inhibiting the antigen binding to FcεRI on RBL.SX-38 cells, and ultimately reducing the antigen's ability to cross-link IgE on FcεRI and the consequent sensitization and degranulation of RBL.SX-38 cells. Our model studies provide support for the hypothesis described above. It should be noted that while the fluorescence shift as measured by the fluorescence flow cytometer in Fig. 5(A) is not large, the numbers of FcεRI occupied by SE44-IgE are sufficient for the antigen (R15K)₈-ova to activate the RBL.SX-38 cells.

It is conceivable that during an allergic response in an individual who has been sensitized to a certain allergen, the levels of allergen in the individual may rise to just above the threshold level. If IgE:omalizumab IC can reduce the effective concentration of incoming

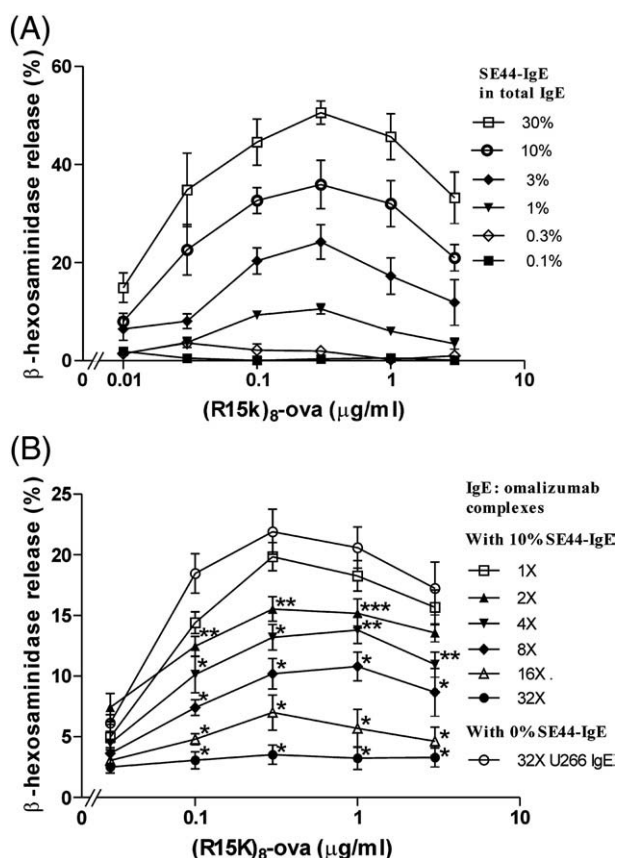


Fig. 6. Antigen-induced activation of RBL SX-38 cells pre-incubated with IgE containing different proportions of R15K-specific IgE. The degree of β -hexosaminidase release induced by (R15K) $_8$ -ova as a function of the proportion of specific IgE in total IgE (A). The effects of IgE:omalizumab IC in inhibition of (R15K) $_8$ -ova-induced activation of IgE-pulsed RBL SX-38 cells. The degree of inhibition of activation of β -hexosaminidase release was dependent on the concentration of SE44-IgE in the IgE:omalizumab IC. (B) Significance of differences between data obtained with IC-10% SE44-IgE at varying concentrations and data obtained with IC-0% SE44-IgE at 32 times molar excess was analyzed (* $P < 0.001$, ** $P < 0.01$, *** $P < 0.05$).

allergens to below threshold concentrations, the allergic reaction would not manifest. Because substantial reduction of Fc ϵ RI on mast cells takes time, if IgE:omalizumab IC do contribute pharmacologically to the therapeutic efficacy of omalizumab in diseases where mast cells play major pathogenic roles, the relative contribution of the IC's antigen trapping effect is probably most significant in the initial period after the administration of omalizumab.

In the *in vitro* model system employed in the present study, the presence of IgE:omalizumab IC was sufficient to reduce antigen-induced sensitization of RBL SX-38 cells without any reduction of Fc ϵ RI on the cells. It has been estimated that the amount of allergens required to cause an allergic response in a sensitized patient is minute. For example, when a patient sensitive to cat dander protein is suddenly exposed to a cat, such as in an allergen-provocation test, the patient may develop an allergic reaction in only a few minutes [28,29]. The amount of allergen inhaled by the patient in that time frame is minute. A patient with extreme sensitivity to peanut allergen may develop anaphylaxis upon the accidental ingestion of the amount of allergens contained in half a peanut [30–32]. It is plausible that the IgE:omalizumab IC, when accumulated to several times the basal levels of IgE, can compete effectively with Fc ϵ RI-bound IgE in binding to the incoming allergen and tie up a significant portion of it.

The trapping of allergen by IgE:omalizumab IC in blood circulation and allergen-exposed mucosal tissues should also inhibit allergen interactions with mIgE expressed on IgE-committed lymphoblasts and memory B cells. Furthermore, in the absence of omalizumab,

allergen may bind to IgE bound by CD23 on antigen presenting cells, which favors IgE responses [33,34]. In patients treated with omalizumab, which is an IgG1 antibody, the IgE:omalizumab may therefore divert the allergen to Fc γ receptors on a different set of antigen presenting cells. These various effects of the IgE:omalizumab IC may contribute to the attenuation of continual allergen-driven, IgE-biased immune responses.

We are currently collaborating with clinicians studying the effects of regular doses of omalizumab on allergic patients with very high IgE levels. Systematic analysis of free IgE, IgE:omalizumab IC, and Fc ϵ RI on basophils and mast cells in these patients may provide direct evidence that IgE:omalizumab IC can tie up allergens and contribute to the pharmacological mechanisms of omalizumab without the neutralization of free IgE and the down-regulation of Fc ϵ RI on basophils and mast cells.

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