

Inhibition of human cord blood-derived mast cell responses by anti-FcεRI mAb 15/1 versus anti-IgE Omalizumab

Irina Mirkina, Tamás Schweighoffer, Franz Kricek *

Novartis Institutes for BioMedical Research, Brunnerstraße 59, A-1235 Vienna, Austria

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Abstract

Aggregation of the α -chain of the high affinity IgE receptor (FcεRI α) on mast cells or basophils after cross-linking of receptor-bound IgE by its antigen or an anti-IgE antibody results in cell activation and release of inflammatory mediators. Omalizumab (Xolair®, Novartis Pharmaceuticals; Genentech Inc.) is a recombinant humanized anti-IgE mAb developed for the treatment of severe allergic asthma. It complexes with free serum IgE, which prevents its binding to FcεRI and thereby interrupts the allergic cascade. Administration of an inhibitory anti-FcεRI α mAb may represent an alternative strategy to neutralize IgE-mediated receptor activation. In the present report, for the first time, we have performed direct side of side comparison between the inhibitory anti-FcεRI α mAb designated 15/1 and Omalizumab for their effects on human cord blood-derived mast cells. We provide the first evidence that both 15/1 mAb and Omalizumab efficiently inhibit FcεRI-mediated human mast cell responses *in vitro* (degranulation, activation, release of IL-8 and IL-13, phosphorylation of Akt) and that mAb 15/1 is a non-anaphylactogenic antibody, which compared to Omalizumab, displays markedly higher inhibitory potency in the presence of high IgE levels.

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1. Introduction

Binding of human IgE by the heavy-chain constant region domain 3 (Cε3) to its high affinity receptor (FcεRI) is considered a critical event in the development of allergic reactions. In humans FcεRI is expressed as tetrameric ($\alpha\beta\gamma_2$) complex on tissue mast cells and blood basophils [1]. Aggregation of the α -chain of FcεRI (FcεRI α) by cross-linking of the receptor-bound IgE by a specific antigen (Ag) or an anti-IgE antibody results in cell activation and release of both preformed (e.g., histamine, serotonin, β -hexosaminidase, tryptase) and newly synthesized (e.g., leukotrienes) inflammatory mediators [1–4]. The IgE–FcεRI interaction is therefore an important target for anti-allergic interventions, and several strategies have emerged, which are aimed at disrupting this interaction.

Omalizumab (Xolair®, Novartis Pharmaceuticals, East Hanover, NJ; Genentech Inc., South San Francisco, CA) is a recombinant humanized monoclonal anti-IgE antibody

developed for the treatment of allergic diseases. This non-anaphylactogenic anti-IgE monoclonal IgG antibody recognizes the site of IgE binding to FcεRI within the Cε3 domain of IgE and works by forming complexes with circulating free IgE, thus preventing IgE from attaching to FcεRI on effector cells and thereby blunting the IgE-mediated inflammatory responses [5–8]. Treatment of patients with Omalizumab has been shown to cause a marked reduction in serum levels of free IgE, down-regulation of IgE receptors on circulating basophils and dendritic cells, reduction in tissue (nasal and bronchial) eosinophils, bronchial mast cells, as well as T cell and B cell numbers [5,8,9]. As a systemic anti-IgE agent, Omalizumab has demonstrated clinical efficacy in patients with moderate and severe allergic asthma and allergic rhinitis [6,9,10]. The exact dosage of Omalizumab is determined by bodyweight and serum total IgE levels [11,12]. The major drawback of therapeutic treatment with Omalizumab is the exclusion of patients with high body mass index or high IgE levels.

Signaling through the high affinity IgE receptor can also be prevented by monoclonal antibodies directed against different epitopes within FcεRI α . We have earlier demonstrated that an anti-FcεRI mAb designated as 15/1 inhibits IgE/Ag-triggered

* Corresponding author. Tel.: +43 1 866 34 357; fax: +43 1 866 34 727.

E-mail address: franz.kricek@novartis.com (F. Kricek).

histamine and leukotriene release from the rat basophilic leukemia cell line RBL-2H3, transfected with human FcεRI and from human peripheral blood basophils [1]. The epitope of mAb 15/1 is localized within the FG loop of the membrane proximal α2 domain and we have provided evidence that 15/1 competes with IgE for receptor binding [13].

In vitro studies which would directly compare the two above described strategies (the use of Omalizumab *versus* that of inhibitory anti-FcεRI mAb) have not been described yet. We therefore aimed at evaluating the effects of Omalizumab on human mast cells, the major effector cells of allergic reactions, in direct comparison to mAb 15/1. Our report provides the first evidence that, in IgE-sensitized human cord blood-derived mast cells (hCBMCs) stimulated with anti-IgE, both mAb 15/1 and Omalizumab inhibit degranulation, activation, release of IL-8 and IL-13, and inhibit phosphorylation of Akt. Moreover, we show that mAb 15/1 is a non-anaphylactogenic antibody and that in the presence of high IgE levels mAb 15/1 inhibits human mast cell responses more efficiently than Omalizumab.

2. Materials and methods

2.1. Monoclonal antibodies

The monoclonal anti-human IgE antibody Omalizumab (Xolair®) was from Novartis clinical batch No. M02971 and has been purified and analyzed according to the standard GMP production process. The monoclonal anti-human FcεRIα antibody 15/1, batch SDS653, was purified from Novartis stock hybridoma cells, clone 2/6/22, by immunoaffinity chromatography of the cell culture supernatant on anti-mouse IgG1 agarose. The protein concentration of the purified antibody, dissolved in phosphate buffered saline, was determined using the Bio-Rad Bradford BiG kit BR070405. Human IgE B11 and anti-hIgE mAb Le27 originate from Prof. B. Stadler, University Bern, Switzerland.

2.2. Purification of cord blood progenitors

Heparinized cord blood (approximately 40 ml from one delivery, obtained from Hospital Lainz, Vienna, Austria) was diluted 1:3 with HBSS buffer containing 2 mM EDTA, overlaid on Ficoll Paque™ Plus (Amersham Biosciences, Uppsala, Sweden) and centrifuged at room temperature for 20 min at 400 × g. Mononuclear cells at the interface were collected, and CD133+ early hematopoietic precursors were isolated from the mononuclear cells by magnetic cell sorting on a AUTOMACS magnetic separation column using a CD133+ isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the isolated CD133+ progenitor cells mounted to >97%.

2.3. Differentiation of cord blood progenitors into mast cells

CD133+ progenitor cells were grown in presence of SCF (100 ng/ml; BioSource International) and IL-6 (100 ng/ml; Novartis Pharma AG, Basel, Switzerland). Cytokines were applied in

Stem Span serum-free medium (Stem Cell Technologies) containing 100 μg/ml penicillin/streptomycin and 1% L-glutamine. Cultures were maintained at 37 °C and 10% CO₂ in 6-well plates at cell density 0.5 × 10⁶/ml. Cells were fed twice per week during the first 4 weeks of culturing, and thereafter once per week by removing half of the volume of the cytokine-supplemented medium and adding fresh medium containing 2 × cytokine cocktail. After 8 weeks, cultures were supplemented with 5% FCS (Gibco) and IL-4 (10 ng/ml, Novartis Institutes for Biomedical Research (NIBR) Vienna, Austria). At various time-points cultures were routinely examined for morphology, expression of mast cell tryptase, expression of hematopoietic cell surface antigens as described below (see Section 2.4).

2.4. Characterization of mast cell cultures (FACS analysis, histochemistry)

Expression of cell surface markers was examined by single-color flow cytometry. Cells (4 × 10⁵ per sample) were incubated with PE- or FITC-labeled mouse mAbs against human CD3, CD19, CD16, CD14, CD13, CD54 and CD117 (BD Pharmingen), PE-labeled mouse mAb against human CD63 (Immunotech), biotin-labeled mouse mAb against human FcεRIα (clone 15/1-2/6/22, NIBR, Vienna) for 30 min at 4 °C. After washing with PBS + 2% FCS, anti-FcεRIα-biotin stained cells were subsequently exposed to PE-labeled streptavidin (BD Pharmingen) for 30 min at 4 °C. Cells were analyzed by flow cytometry using Cell Quest (BD) software. In each experiment, cells were preincubated with human IgG (1 μg/ml) before exposure to mAbs to block non-specific antibody binding. Staining reactions were controlled using corresponding isotype-matched antibodies.

Mast cell morphology was examined after staining of cytopsin preparations with May-Gruenwald/Giemsa (Merk, Darmstadt, Germany). Cells (1 × 10⁵) were spun down onto a glass slide using a Cytospin3 cytocentrifuge (Shandon). The smears were air-dried, stained with May-Gruenwald for 5 min, washed with water and subsequently stained with Giemsa stain for 20 min. After washing with water, smears were air-dried and analyzed microscopically.

For immunostaining of mast cell tryptase, cytopsin preparations were prepared as described above, with the exception that polylysine-coated glass slides (Menzel GmbH, Braunschweig, Germany) were used. Smears were air-dried, fixed in acetone:methanol solution (1:1), washed in TBS buffer (pH 7.6) and incubated overnight at 4 °C with an anti-human mast cell tryptase mAb (DakoCytomation, Denmark). Immunoreactive cells were subsequently visualized by the alkaline phosphatase/anti-alkaline phosphatase (APAAP) method using the APAAP kit (DakoCytomation, Denmark) according to the manufacturer's instructions. For negative controls isotype-matched (mouse IgG1, DakoCytomation, Denmark) was used instead of anti-tryptase mAb.

2.5. Mast cell degranulation and activation assays

Mast cell degranulation was monitored by measuring the release of β-hexosaminidase into the cell culture supernatants,

whereas activation was assessed by flow cytometric measurement of CD63 expression on the cell surface. Briefly, mast cells were placed in 96-well V-bottom plates at 1.0×10^5 cells/well (in triplicates or quadruplicates for each sample) in assay medium (phenol red-free RPMI 1640 medium plus 0.1% BSA) supplemented with IL-4 (25 ng/ml) and FCS (5%). Cells were incubated with mAb 15/1 or Omalizumab at 0.01–1000 nM for 1 h, and subsequently sensitized overnight with human IgE B11 at either constant concentration (27 nM) or gradually increasing concentrations (1–243 nM). The following day cells were spun, rinsed with the assay medium and triggered with mouse anti-human IgE mAb Le27 at 5 µg/ml. After the incubation with anti-IgE at 37 °C for 2 h, cells were spun, cell pellets from triplicates were pooled and stained with anti-CD63 mAb or isotype-matched mAb (mouse IgG1) to determine the expression of CD63 by flow cytometry (see Section 2.4), whereas supernatants were collected for further measurement of β -hexosaminidase content.

Potential anaphylactogenicity of mAb 15/1 was assayed in absence of IgE. Cells were incubated overnight with full size mAb 15/1 or its Fab fragment at 0.01–1000 nM, or with an anaphylactogenic control anti-IgE receptor-specific antibody (Buehlmann Laboratories, Schoenenbuch, Switzerland) at 0.01–100 nM, and cell supernatants were collected for measurement of β -hexosaminidase release.

To measure β -hexosaminidase release, 90 µl of cell supernatants were transferred to 96-well flat bottom plates and mixed with 90 µl of chromogenic β -hexosaminidase substrate *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma, 1.3 mg/ml in 0.1 M sodium citrate buffer, pH 4.5). After incubation for 1.5 h at 37 °C, the reaction was stopped by the addition of 90 µl of 2 M NaOH, and the release of the product *p*-nitrophenol was determined by monitoring the O.D. at 405 nm in an ELISA reader. Data were expressed either as the percentage of the maximal β -hexosaminidase release after stimulation with anti-IgE minus spontaneously released material (no anti-IgE added) (as described in [14]), or in absolute values (O.D. at 405 nm). Similarly, data for CD63 expression were either calculated as percentage of the maximal value of mean fluorescence intensity (MFI) of staining with anti-human CD63 mAb after stimulation with anti-IgE minus the value of MFI for CD63 expressed spontaneously (no anti-IgE added), or in absolute values (MFI).

2.6. Cytokine release assay

To monitor cytokine release, cells were treated as described above (see Section 2.5). Supernatants were collected after 2, 6 and 24 h of triggering with anti-IgE. Cytokine content was assessed using Fluorokine® MAP multiplex kits (R&D Systems) for IL-8, IL-13, IL-5 and TNF- α on a Luminex analyzer. Each experimental sample was measured in triplicates. Data were expressed in absolute values (pg/ml).

2.7. Analysis of Akt phosphorylation

For phosphorylation experiments, cells were treated as described above (see Section 2.5), except that cells were trig-

gered with anti-IgE for 5 and 15 min, respectively. The reaction was terminated by the addition of 10 $\times$ TNE lysis buffer (500 mM Tris-HCl, pH 8.0, 1.5 M NaCl, 10% NP-40, 20 mM EDTA) supplemented with 10 mM sodium orthovanadate, 1 $\times$ phosphatase inhibitor cocktail set II (Calbiochem) and 1 $\times$ protease inhibitor cocktail (Roche). After removal of cell debris by centrifugation at 14,000 rpm for 5 min, aliquots of cell lysates (from 1×10^6 cells) were immunoprecipitated with immobilized anti-Akt mouse mAb (Cell Signaling). Total cell lysates and immunoprecipitated samples were resolved by 7.5% SDS-PAGE under reducing conditions and transferred onto Immobilon P membrane (Milipore). Immunoprecipitated proteins were probed with rabbit polyclonal anti-phospho Akt (Ser473) antibody (Cell Signaling), whereas total lysates were probed with rabbit anti-PI3 kinase p85 antiserum (Upstate) for normalization of loading densities. Immunoreactive proteins were visualized with Super-Signal West Pico Chemiluminescent Substrate (Pierce).

3. Results

3.1. Generation and characterization of human cord blood-derived mast cells

We have generated mast cells by growing human umbilical blood CD133+ progenitors in serum-free Stem Span medium in the presence of SCF and IL-6 for 0–8 weeks. After 8 weeks the cultures were additionally supplemented with IL-4 and serum. This method of combining a serum-free with a serum-supplemented culture systems was reported to support the formation of functional human mast cells [15]. After 10 weeks the cultures exhibited typical mast cell characteristics shown on Fig. 1 for one representative donor. Mast cell metachromasia was observed after staining of cytopsin preparations with May-Gruenwald/ Giemsa (Fig. 1a). Ninety percent of the cells were highly positive when immunostained with mast cell specific marker, an anti-human mast cell tryptase mAb (Fig. 1b). Ninety-two percent of the cells in the viable cell region R1 (Fig. 1c) expressed Fc ϵ RI α (Fig. 1d) and cells were positive for CD117 (c-kit) (Fig. 1e). From different donors, only those mast cell cultures which contained no less than 90% of cells positive for human mast cell tryptase and no less than 90% of cells positive for Fc ϵ RI have been further used in our functional assays. Cultures were negative for CD3, CD19, CD16, CD14 antigens, markedly positive for myeloid antigen CD13 and highly positive for the adhesion molecule CD54 (ICAM-1) (data not shown). These characteristics are in good accordance with published data on the phenotype of cultured mouse bone marrow-derived and human cord blood-derived mast cells [15–18]. Cells were kept in culture up to 20 weeks without any significant decrease in viability.

3.2. Effects of mAb 15/1 and Omalizumab on anti-IgE-induced degranulation, activation and cytokine release in CBMCs sensitized with a constant amount of IgE

CBMCs were pre-treated with mAb 15/1 or Omalizumab at 1000–0.01 nM prior to the overnight sensitization with IgE at

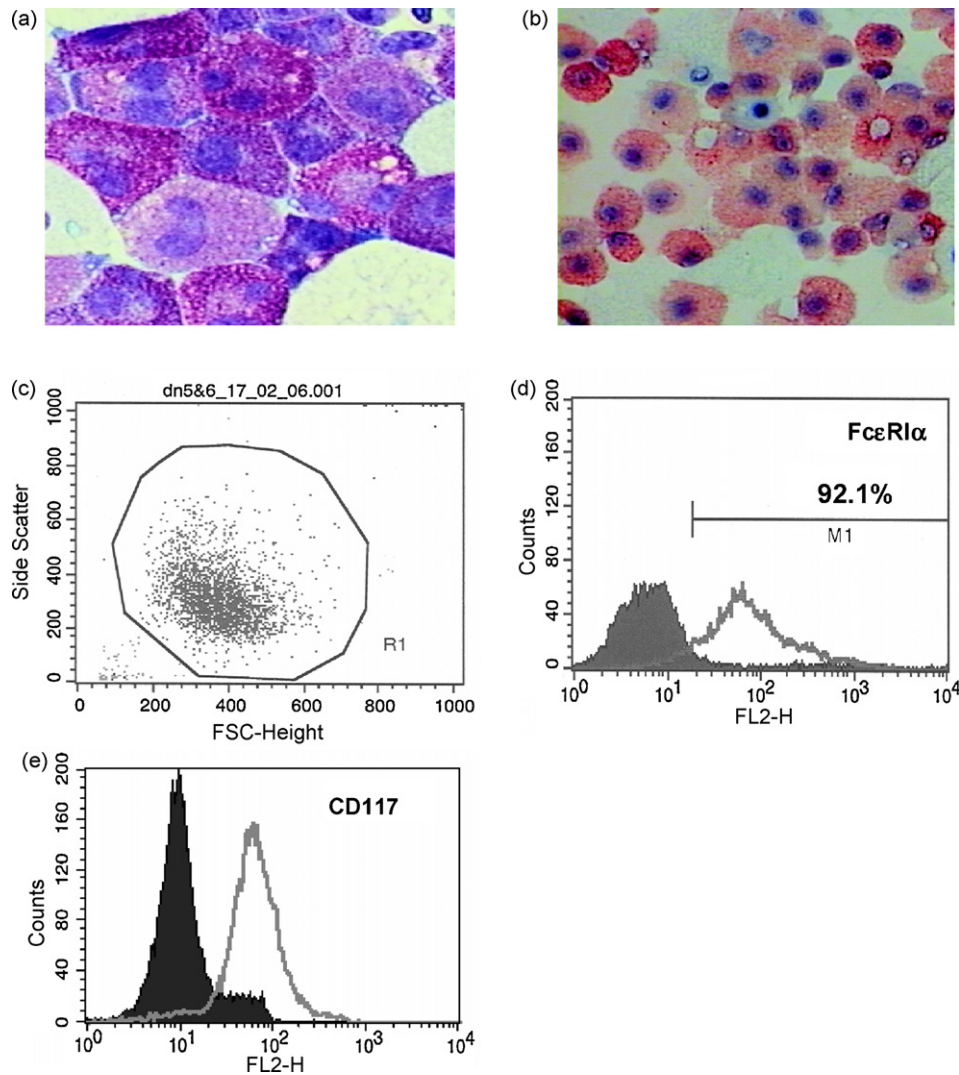


Fig. 1. Characterization of human cord blood-derived mast cell culture. (a) Smears were stained with Giemsa/May-Gruenwald. (b) Smears were immunostained with anti-human mast cell tryptase. (c–e) Cells were analyzed by flow cytometry for the expression of FcεRIα (d) or CD117 (c-kit) (e), respectively. Filled histogram plots correspond to isotype-matched control mAbs. Representative dot blot (c) shows forward (FSC) and side scatter (SSC). The viable cell region (R1) was gated on the basis of FSC and SSC. M1 marker on the histogram plot of (d) shows the percentage of gated cells, which express FcεRI. Data are from one representative donor out of four.

27 nM. Cells were triggered by cross-linking of FcεRI by the addition of the anaphylactogenic anti-IgE mAb Le27. Release of β-hexosaminidase, an enzyme stored in mast cell granules, into cell supernatant was monitored as a measure of degranulation (Fig. 2a), whereas the expression of CD63 marker at the cell surface was analyzed as a measure of activation (Fig. 2b). In the absence of mAb 15/1 and Omalizumab, stimulation of cells with anti-IgE induced a marked release of β-hexosaminidase and up-regulation of surface CD63: the increase in β-hexosaminidase release was up to six-fold in anti-IgE-stimulated cells (maximal release) compared to the cells non-treated with anti-IgE (spontaneous release) and the corresponding increase in CD63 expression was up to five-fold (data not shown). In addition, in the absence of mAb 15/1 and Xolair®, a fairly abundant level of released IL-8 (~4700 pg/ml) was detected in the mast cell supernatant already after 2 h of stimulation with anti-IgE (Fig. 2c) with further increase at 24 h (data not shown). Spon-

aneous release of IL-8 was negligible (not shown). IL-13 was undetectable 2 h after stimulation with anti-IgE (not shown), but substantial release of IL-13 (~215 pg/ml) was found 24 h after triggering (Fig. 2d). IL-13 in cells not treated with anti-IgE (spontaneous release) was below the detection limit (not shown).

The levels of released β-hexosaminidase, IL-8 and IL-13, as well as cell surface expression of the CD63 activation marker all showed a marked decrease in cells pre-treated with mAb 15/1 and Omalizumab (Fig. 2a–d). The inhibition of these responses by mAb 15/1 and Omalizumab was dose-dependent with calculated IC₅₀ of 15 nM (mAb 15/1) and 57 nM (Omalizumab) for the inhibition of β-hexosaminidase release (Fig. 2a), IC₅₀ of 1.5 nM (mAb 15/1) and 20 nM (Omalizumab) for the inhibition of CD63 cell surface expression (Fig. 2b), IC₅₀ of 0.06 nM (mAb 15/1) and 23 nM (Omalizumab) for the inhibition of IL-8 release (Fig. 2c) and IC₅₀ of 0.3 nM (mAb 15/1) and 14 nM (Omalizumab) for the inhibition of IL-13 release (Fig. 2d). Sim-

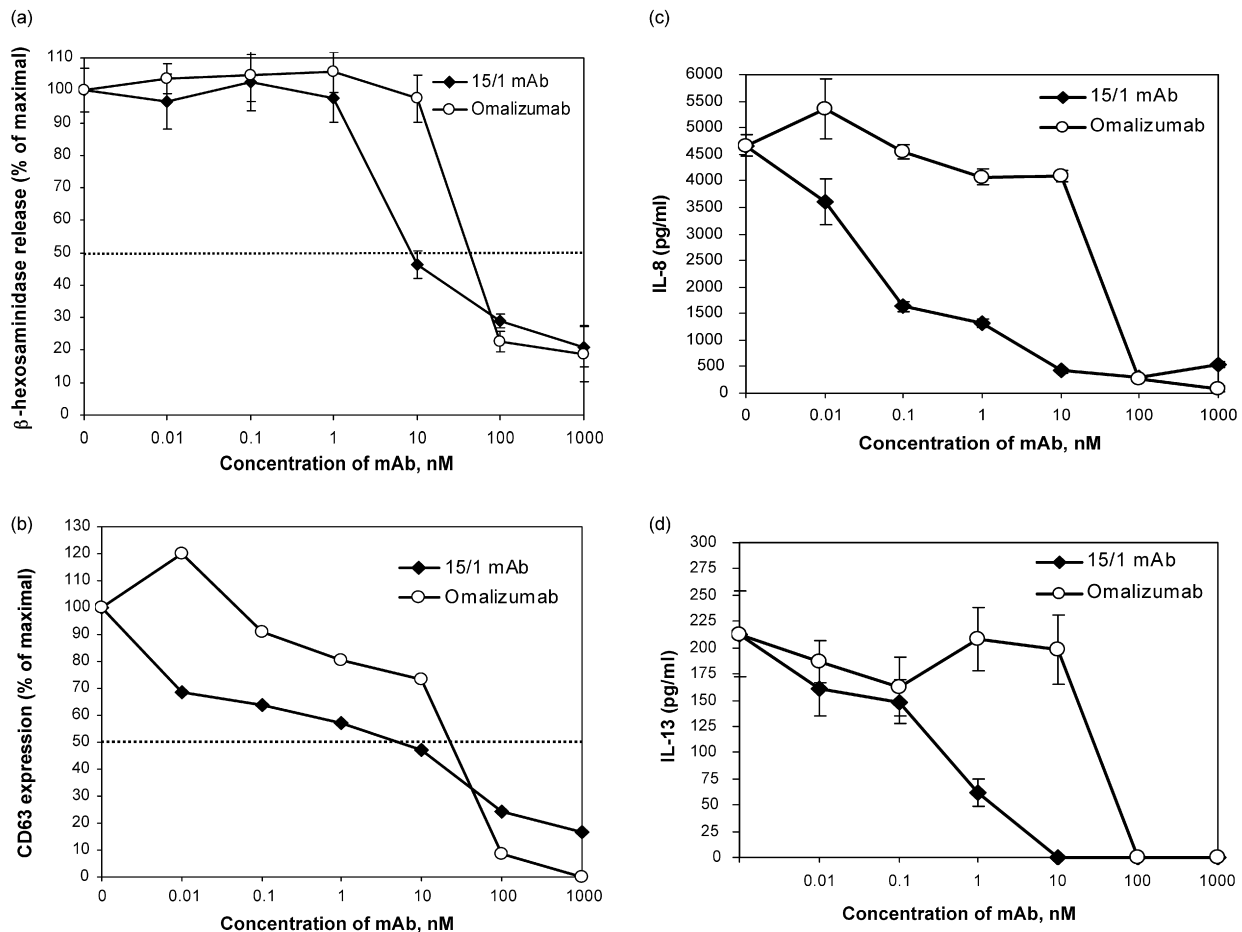


Fig. 2. Effect of mAb 15/1 and Omalizumab on IgE-dependent degranulation, activation and cytokine release of hCBMCs sensitized with constant amount of IgE. CBMCs were incubated with mAb 15/1 or Omalizumab (both at 0.01–1000 nM) for 1 h, followed by overnight sensitization with IgE (27 nM). The following day cells were stimulated by anti-IgE for 2 h. (a) β -Hexosaminidase release into the cell supernatant was monitored as a measure of degranulation. Results are expressed as means of three experimental samples run in parallel ($n = 3$) $\pm$ S.D., and are shown as percentage of maximal release upon stimulation by anti-IgE after subtraction of spontaneous release values obtained without stimulation by anti-IgE. (b) Pooled cell pellets from three parallel samples of stimulated cells were analyzed by flow cytometry for the expression of CD63 on the cell surface as a measure of cellular activation. Results are calculated as percentage of maximal MFI obtained after stimulation by anti-IgE and subtraction of MFI obtained with non-stimulated cells. (c and d) Cell supernatants collected after 2 and 24 h of triggering by anti-IgE, respectively, were assessed for cytokine content using Fluorokine[®] MAP multiplex kits. The release of IL-8 is shown in (c) and represents the absolute value of IL-8 (pg/ml) after 2 h of stimulation by anti-IgE, whereas the release of IL-13 is shown in (d) and represents the absolute value of IL-13 (pg/ml) after 24 h of stimulation by anti-IgE. Results are expressed as means ($n = 3$) $\pm$ S.D. Data are presented for one experiment out of three which were performed on human CBMCs generated from three different donors.

ilar to the data obtained for IL-8 and IL-13 (Fig. 2c and d), pre-treatment of cells with mAb 15/1 and Omalizumab caused a dose-dependent inhibition of the anti-IgE-triggered release of TNF- α and IL-5 (data not shown).

3.3. MAb 15/1 does not induce anaphylactogenic responses

Since mAb 15/1 is a bivalent anti-Fc ϵ RI α -specific antibody, it seemed likely that it might by itself have the capacity to cross-link IgE receptors thereby triggering anaphylactic reactions (e.g., cellular activation, mediator release) in mast cells in the absence of receptor-bound IgE. To find out whether mAb 15/1 possesses an agonistic activity on mast cells, we incubated CBMCs with serial concentrations of mAb 15/1, 15/1 Fab fragment or an anaphylactogenic anti-Fc ϵ RI α control antibody, in the absence of IgE. The following day the release of β -hexosaminidase and cell surface expression of

CD63 was measured. Within the broad concentration range analyzed, mAb 15/1 was not able to induce any substantial release of β -hexosaminidase (Fig. 3a) or increase in cell surface CD63 expression (Fig. 3b). As expected, no β -hexosaminidase release or up-regulation of CD63 on the cell surface was also detected in response to the monomeric Fab fragment of mAb (Fig. 3a and b) in agreement with its lack of cross-linking potential. In contrast to 15/1 antibody, a marked dose-dependent release of β -hexosaminidase and up-regulation of CD63 was found to be induced by the anaphylactogenic Fc ϵ RI-specific control antibody (Fig. 3a and b).

3.4. Effect of mAb 15/1 and Omalizumab on anti-IgE-induced Ser473 phosphorylation of Akt in CBMCs

Activation of PI3 kinase has been reported to occur upon the aggregation of Fc ϵ RI on mast cells [19]. We examined the

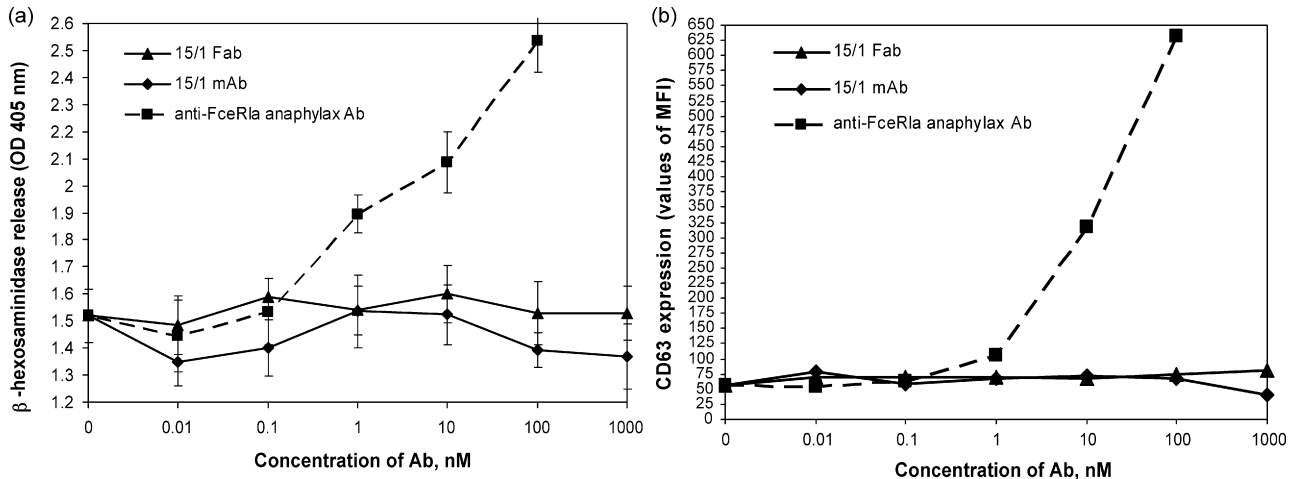


Fig. 3. mAb 15/1 and Fab fragment of 15/1 are not anaphylactogenic to human CBMCs. Human CBMCs were incubated with the indicated concentrations of full size mAb 15/1, purified Fab fragment of 15/1 or with an anaphylactogenic control antibody (anti-IgE-receptor stimulation control) overnight. (a) The release of β -hexosaminidase was measured in cell supernatants. Results are expressed in absolute values (O.D. at 405 nm) as means ($n=4$) $\pm$ S.D. (b) Cell pellets from four experimental samples run in parallel were pooled and analyzed for the cell surface expression of CD63. Results are expressed in absolute values (MFI). Data are presented for one experiment out of two, which were performed on human CBMCs generated from two different donors.

Ser473 phosphorylation of Akt as a measure of PI3 kinase activation in IgE-sensitized CBMCs stimulated with anti-IgE, and the effect of mAb 15/1 and Omalizumab on Akt phosphorylation. Cells were pre-treated with mAb 15/1 (30 nM) or Omalizumab (30 nM) prior to the overnight sensitization with IgE (27 nM). The following day cells were stimulated with anti-IgE for 5 and 15 min, respectively. Cell lysates were immunoprecipitated with anti-Akt mAb, and the blot was probed with anti-phospho (Ser473) Akt polyclonal Ab. No phosphorylation signal could be seen in CBMCs sensitized with IgE without subsequent receptor aggregation by anti-IgE (Fig. 4a, lane “IgE”). Stimulation with anti-IgE for 15 min led to the phosphorylation of Akt (Fig. 4a, lane “IgE + anti-IgE”). Phosphorylation of Akt was completely

abrogated in cells pre-treated with mAb 15/1 (Fig. 4a, lane “15/1 /IgE + anti-IgE”), and strongly reduced in cells pre-treated with Omalizumab (Fig. 4a, lane “Omalizumab/ IgE + anti-IgE”). Probing of total cell lysates with anti-PI3 kinase p85 antiserum confirmed equal sample loading of all lanes (Fig. 4b). No Akt phosphorylation was detected after 5 min of stimulation with anti-IgE (data not shown).

3.5. Impact of sensitization with increasing amounts of IgE on the inhibition of anti-IgE-triggered CBMCs responses by mAb 15/1 mAb and Omalizumab

According to clinical data, IgE levels vary in allergic individuals from relatively low (5–10 IU/ml) to highly elevated (exceeding 500 IU/ml) in severe allergy cases [11,20]. In order to mimic these different clinical conditions, we studied the inhibition of anti-IgE-induced responses by mAb 15/1 and Omalizumab in the presence of increasing amounts of IgE. Cells were pre-treated with mAb 15/1, isotype control mAb or Omalizumab (all at 30 nM) and sensitized with IgE ranging from 1 nM (80 IU/ml) to 243 nM (19440 IU/ml). The following day cells were stimulated with anti-IgE for 2 h. Without pre-treatment with the inhibitory antibodies, stimulation by anti-IgE induced a marked release of β -hexosaminidase (up to six-fold increase over the spontaneous release) (Fig. 5a).

Pre-treatment with mAb 15/1 resulted in a nearly complete block of β -hexosaminidase release at IgE levels ranging from 1 to 81 nM, and marked reduction (two-fold) of β -hexosaminidase release at the highest IgE level analyzed (243 nM) (Fig. 5a).

Pre-treatment with Omalizumab caused a nearly complete block of β -hexosaminidase release at IgE levels ranging from 1 to 3 nM, a two-fold reduction at 9 nM of IgE, and no reduction of β -hexosaminidase release if IgE levels were 27 nM and higher (81 and 243 nM) (Fig. 5a).

The release of IL-8 from mast cells triggered for 2 h with anti-IgE was analyzed at four sensitizing IgE concentrations

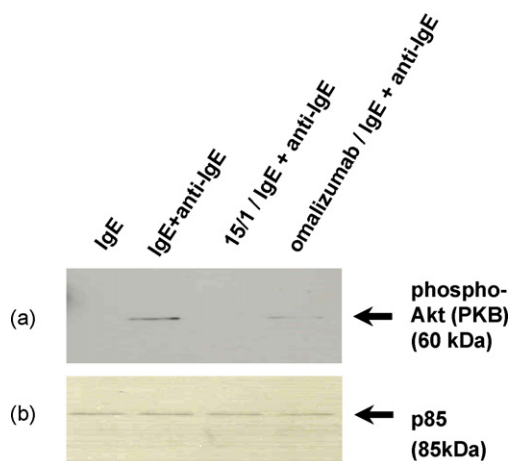


Fig. 4. Effect of mAb 15/1 and Omalizumab on IgE-dependent serine/threonine phosphorylation of Akt (PKB) in human CBMCs. Cells were incubated with mAb 15/1 (30 nM) or Omalizumab (30 nM) for 1 h followed by overnight sensitization with IgE (27 nM). The following day cells were stimulated by anti-IgE for 15 min and lysed. (a) Cellular lysates were immunoprecipitated with mouse anti-Akt mAb and membrane was probed with anti-phospho Akt. The size of the detected band corresponds to that of Akt (60 kDa). (b) Total lysates were probed with anti-PI3K p85 for normalization of the loading density.

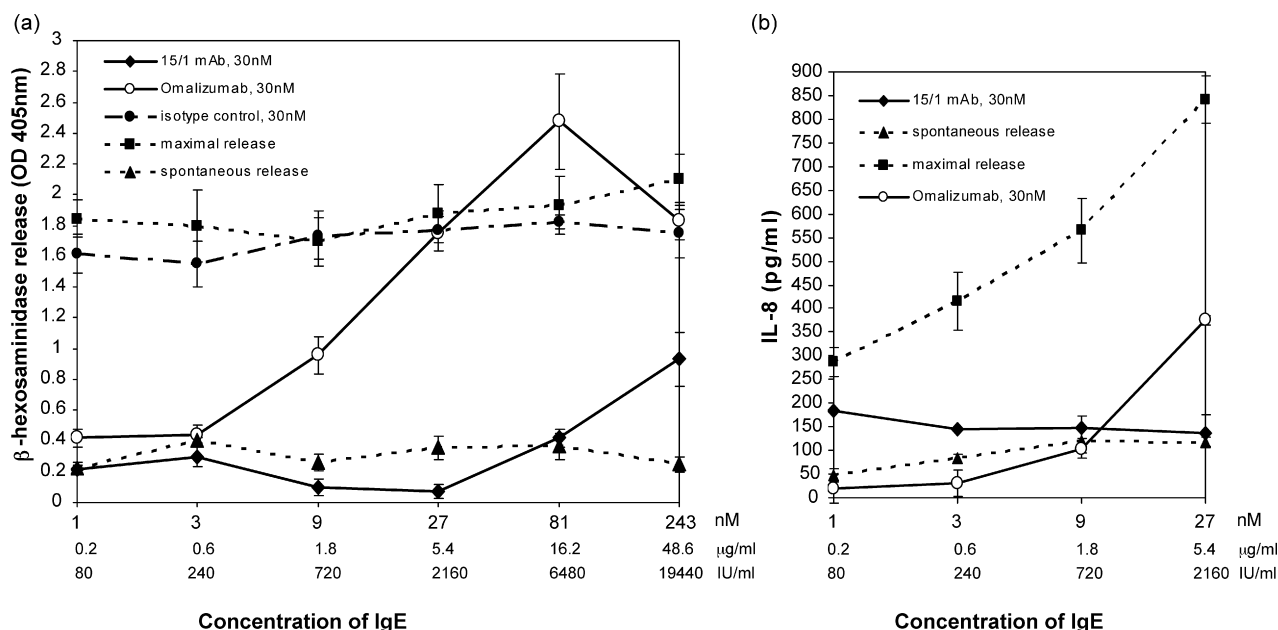


Fig. 5. Effect of sensitization with increasing amounts of IgE on the inhibition of anti-IgE-triggered CBMC responses by mAb 15/1 and Omalizumab. Human CBMCs were incubated with mAb 15/1 (30 nM) or Omalizumab (30 nM) or isotype-matched control mAb mouse IgG1 (30 nM) for 1 h and subsequently sensitized with increasing concentrations of IgE (as indicated on the figure) overnight. The following day cells were stimulated by anti-IgE for 2 h and cell supernatants were collected. (a) Cell supernatant were analyzed for β -hexosaminidase release. Results are expressed as means ($n=3$) $\pm$ S.D. in absolute values (O.D. at 405 nm). (b) Cell supernatants were analyzed for the release of IL-8. Results are expressed as means ($n=3$) $\pm$ S.D. in absolute values (pg/ml). Data are presented for one experiment out of two which were performed on human CBMCs generated from two different donors.

(1–27 nM). Release of IL-8 from anti-IgE-stimulated cells showed a strong IgE-dependence, with the most efficient release (~ 850 pg/ml) being seen at 27 nM of IgE (Fig. 5b). At all four concentrations of IgE (1–27 nM), treatment with mAb 15/1 (30 nM) efficiently inhibited the release of IL-8 (Fig. 5b). Again, similarly to the above described data for β -hexosaminidase release (Fig. 5a), pre-treatment with Omalizumab (30 nM) caused fairly efficient reduction in IL-8 level at IgE concentrations of 1, 3 and 9 nM, and two-fold decrease of inhibitory activity, if IgE concentration was 27 nM (Fig. 5b).

Thus, the inhibitory activity of Omalizumab on mast cell IgE-mediated responses showed a reverse correlation with the levels of IgE, with the inhibitory potential of Omalizumab being largely impaired, once the molar ratio of IgE to Omalizumab exceeded one to one, whereas the inhibition by mAb 15/1 was not significantly affected by high IgE levels.

4. Discussion

Interruption of the allergic cascade by complexing free serum IgE with the anti-IgE mAb Omalizumab (Xolair®) thus preventing IgE from being bound by the α -chain of its high affinity receptor (Fc ϵ RI α) is the therapeutic principle of a recently developed treatment of atopic disorders like severe allergic asthma. Theoretically, a similar inhibitory effect might be also achieved with an antibody that recognizes epitopes on Fc ϵ RI α , which are part of the IgE binding site or represent other effector regions involved in receptor signaling. The present report is the first to compare the anti-Fc ϵ RI α mAb to Omalizumab for their ability to inhibit IgE-dependent responses in human mast cells *in vitro*.

Mast cells used in this study were generated from human cord blood CD133+ progenitors by culturing the cells with SCF and IL-6 in serum-free medium for 8 weeks and additionally supplementing late mast cell cultures with serum and IL-4. Addition of IL-4 has been reported to promote the expression of Fc ϵ RI which is otherwise nearly absent on mast cells generated *in vitro* [15,21] and to enhance functional maturation (production of TNF- α , IL-5 and IL-13 and mediator release) in response to Fc ϵ RI aggregation [4,15,16,18,22–24]. Expression of c-kit (CD117) on our mast cell preparations was moderate (Fig. 1e) which is in agreement with reported observations that treatment of mast cells with IL-4 leads to down-regulation of CD117 [23] and that feeding mast cells with SCF (c-kit ligand) induces its internalization [18].

The first step of Fc ϵ RI activation is the formation of lipid rafts after cross-linking of multiple receptor molecules on the cell surface. Receptor cross-linking can occur either directly by anti-Fc ϵ RI α (auto)antibodies or by complexing receptor bound IgE molecules with multivalent antigens or anti-IgE antibodies. Clinically, the first mechanism can lead to the development of chronic idiopathic urticaria [21] whereas the second mechanism causes IgE dependent allergic diseases [21,25]. On the molecular level downstream receptor signaling triggers a series of events in mast cells which can be monitored to assess the biological effect of inhibitory compounds with the potential to treat the above mentioned diseases.

In our experiments, the release of β -hexosaminidase, an enzyme of mast cell granules, was used as a readout for mast cell degranulation. In parallel we have examined the cell surface expression of CD63 as a measure of mast cell activation.

CD63, also known as lysosome-associated membrane protein-3, is a member of the tetraspanin family of membrane-associated molecules [26] and is a well-established component of the late endosomal and lysosomal membranes [26]. In agonist-treated basophils, RBL-3H3 rat basophilic leukemia cells, mouse bone marrow-derived mast cells, eosinophils and neutrophils, CD63-containing granules were reported to be translocated from cytoplasm to the plasma membrane with subsequent fusion of granules to the plasma membrane [26,27]. Up-regulation of CD63 on the surface of stimulated granulocytes coincides with mediator release [26,27]. Therefore, in clinical studies CD63 is used as basophil activation marker to estimate the reactivity of patient's blood basophils to pollen allergens by flow cytometry [28,29]. In contrast to basophils, in human mast cells CD63 has been less extensively explored in context with agonist-induced cellular activation, but it has been mentioned as one of activation markers expressed on the surface of human cord blood derived mast cells, uterine mast cells and HMC-1 [30].

Our results provide first evidence that in IgE-sensitized human cord blood-derived mast cells both mAb 15/1 and Omalizumab efficiently and dose-dependently inhibit anti-IgE-triggered degranulation and CD63 cell surface expression. The calculated IC_{50} values show that mAb 15/1 inhibited degranulation more efficiently than Omalizumab by a factor of 3.8, which is in agreement with the different affinities of these antibodies for their target molecules: K_D mAb15/1 – Fc ϵ RI α = 0.8 nM; K_D Omalizumab – hIgE = 2.8 nM (unpublished data, surface plasmon resonance measurements with immobilized recombinant Fc ϵ RI α and human IgE, respectively). In contrast, the IC_{50} factor of mAb 15/1 and Omalizumab mounts to 13.3 for the inhibition of CD63 expression. Since activated mast cells are known to release pro-inflammatory cytokines (e.g., IL-8, TNF- α) as well as cytokines which affect the balance between Th1 and Th2 cells (e.g., IL-5, IL-13), we have also tested the effect of mAb 15/1 and Omalizumab on the anti-IgE induced release of the chemokine IL-8 and the Th2-cytokine IL-13 into the culture medium. Again, both antibodies completely inhibited cytokine release with mAb 15/1 being more efficient than Omalizumab by 2–3 orders of magnitude (Fig. 2c and d). The IgE concentration used in these experiments was kept constant (27 nM). Therefore, the relative differences observed for the anti-Fc ϵ RI α mAb versus the anti-IgE mAb on the different functional read-outs provokes the speculation that the molecular mechanism by which mAb 15/1 interferes with IgE receptor signaling is not exclusively restricted to the inhibition of IgE/IgE receptor binding as it is the case for the anti-IgE mAb Omalizumab. This assumption is further supported by the finding that mAb 15/1 by itself is not anaphylactogenic as shown in Fig. 3. Based on its divalent structure, the full size antibody 15/1 should have the intrinsic capability to trigger the IgE receptor by cross-linking in a similar way as the anaphylactogenic anti-Fc ϵ RI α reference mAb (Fig. 3) and the anti-IgE mAb Le27 that was used as triggering agent for IgE-sensitized mast cells in all experiments described in this report. ITIM activation by cross-linking with Fc γ RIIb co-expressed on the mast cell surface might represent such an additional mode of action for mAb 15/1. However, it seems unlikely due to insufficient cross-reactivity of the murine

mAb 15/1 constant region with the human Fc γ RIIb on mast cells. It should be noted that the experiment shown in Fig. 3 was performed IgE free on non-sensitized mast cells to allow full accessibility of mAb 15/1 to its epitope on Fc ϵ RI α . As expected, the purified monovalent Fab fragment of mAb 15/1 was also not anaphylactogenic (Fig. 3) although it retained its biological activity (Fc ϵ RI α binding and inhibition of sLT release from RBL-2H3-hu α cells, inhibition of β -hexosaminidase release from human CBMCs, data not shown).

PI3 kinase has been reported to be involved in IgE-dependent responses in mast cells [19,31]. As an indirect measure of PI3 kinase activation, the Ser/Thr phosphorylation of Akt (also known as protein kinase B, PKB) has been widely used [19,32]. Thus, we have monitored the Ser 473 phosphorylation of Akt after cross-linking of Fc ϵ RI with anti-IgE, and the impact of administration of mAb 15/1 and Omalizumab on Akt phosphorylation. Both 15/1 and, to a lesser extent, Omalizumab inhibited the anti-IgE-induced Ser473 phosphorylation of Akt (Fig. 4a), indicating that the administration of mAb 15/1 and Omalizumab impairs the PI3 kinase signal transduction pathway. The effects of mAb 15/1 and Omalizumab on other signaling pathways which could be potentially activated by Fc ϵ RI aggregation (e.g., tyrosine phosphorylation) await further investigation.

The efficacy of treatment with Omalizumab depends on the patient's body weight and baseline (pretreatment) serum total IgE levels. Therefore, the standard treatment regimen includes the determination of serum pretreatment IgE levels and subsequent proper adjustment of the necessary Omalizumab dosage [11,20]. Patients whose pretreatment serum IgE levels are highly elevated combined with higher body weights (>600–700 IU/ml for body weights >60–70 kg, >500–600 IU/ml for body weights >70–90 kg, >300–400 IU/ml for body weights >90 kg) do not qualify for treatment with Omalizumab because the volume of the drug would be too excessive and expensive [11,20]. Maximal decreases in free (uncomplexed) circulating IgE, viewed as one indicator of the effectiveness of Omalizumab administration, have been reported when *in vivo* Omalizumab: IgE molar ratios in blood of 10–20 are achieved [33]. The experiment shown in Fig. 5 is in agreement with these clinical findings. While at a 10-fold molar excess (over IgE) Omalizumab was fully active in suppressing mast cell degranulation, activity dropped to 50% at three-fold excess. At concentrations equimolar to- or lower than those of IgE, Omalizumab has lost its capacity to inhibit degranulation. As expected, IgE concentration dependence is much less pronounced for mAb 15/1, being still active at 19440 IU/ml IgE. At equimolar (to IgE) concentration mAb 15/1 was also significantly more potent than Omalizumab in inhibiting secretion of IL-8.

In summary, we have shown that an efficient inhibition of IgE-dependent *in vitro* responses in human mast cells can be achieved by both strategies (the use of Omalizumab and the use of anti-Fc ϵ RI inhibitory mAb). With respect to the expected IgE independence, a non-anaphylactogenic anti-Fc ϵ RI α mAb 15/1 shows an inhibition extent superior to that of Omalizumab under the high IgE conditions. Based on our data, we would expect anti-Fc ϵ RI α mAb to have an advantage over Omalizumab for allergic patients having highly elevated serum IgE level.

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