



Quantitation of serum IgE by using chimeras of human IgE receptor and avian immunoglobulin domains

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ABSTRACT

Anti-IgE therapeutics represent an efficient approach in the management of IgE-mediated allergic asthma. However, monitoring the reduction of IgE levels into a therapeutically efficient range requires the determination of residual serum IgE. We established an analytical approach to distinguish free and anti-IgE complexed serum IgE based on soluble derivatives of the human high-affinity IgE receptor. Soluble receptor derivatives represent an ideal means to analyze receptor antagonism by any ligand or blocking antibody. Therefore, the FcεRI ectodomain was fused with avian IgY constant domains that circumvent susceptibility to interference phenomena and improve assay performance. After production in HEK293 cells, subsequent characterization by enzyme-linked immunosorbent assay and immunoblotting confirmed the suitability of avian IgY constant domains for immobilization and detection purposes. To provide further insights into the different IgE reactivities, free allergen-specific IgE was also determined. Monitoring of sera from omalizumab-treated patients during the course of therapy revealed the applicability for assessment of omalizumab-complexed versus noncomplexed serum IgE. These parameters may allow correlation to clinical responses during anti-IgE therapy with the perspective of biomonitoring.

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Several immune-mediated diseases, including allergic hypersensitivity reactions [1], can be linked to circulating IgE antibodies. Although the least abundant serum isotype, IgE exhibits a variety of peculiarities regarding structure and effector functions, with major consequences concerning immune responses and pathologies. It acts as a key molecule in a network of proteins, including cellular receptors such as the high-affinity IgE receptor FcεRI, the low-affinity receptor CD23, and the IgE- and FcεRI-binding protein galectin-3 [2].

On crosslinking by allergens, IgE antibodies bound to FcεRI on mast cells and basophils trigger degranulation and release of proinflammatory mediators, leading to immediate reactions and initiation of biosynthetic pathways producing prostaglandins and leukotrienes [2]. Human FcεRI is expressed as a αβγ2 tetramer on mast cells and basophils and as a αγ2 trimer on antigen-

presenting cells (APCs)² such as dendritic cells, Langerhans cells, monocytes, eosinophils, platelets, and smooth muscle cells [3–5]. The extracellular domains of the ligand-binding α-chain display an outstanding affinity for IgE in the range of 10^{11} M^{-1} , providing the basis for an efficient loading of IgE with long-term stability on effector cells and resulting in a half-life of approximately 10 days [6].

Different strategies to therapeutically interfere with atopic levels of IgE and excess mast cell degranulation have been pursued, leading to the development of antagonistic antibodies [7]. Such antibodies bind to serum IgE and membrane-bound IgE on B cells with high affinity but not to either FcεRI- or CD23-bound IgE to prevent antigen-independent receptor crosslinking. Omalizumab (rhuMab E25, Xolair), a humanized kappa IgG1 antibody, was the

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² Abbreviations used: APC, antigen-presenting cell; FcεRIα-ecd, FcεRIα extracellular domain; cDNA, complementary DNA; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; PEI, polyethylenimine; NTA, nitrilotriacetic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RT, room temperature; AP, alkaline phosphatase; ABTS, 2,2-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEMA, immunoenzymometric assay; CCD, carbohydrate crossreactive determinant; RF, rheumatoid factor; HAMA, human anti-mouse IgG antibody.

first therapeutic IgE-neutralizing antibody that was approved for clinical use (in 2003 in the United States, in 2004 in the European Union). The antibody targets an epitope within the epsilon heavy chain constant regions to prevent interaction of serum IgE with Fc receptors on effector cells [8–10]. Apart from the initially intended purpose to reduce serum IgE concentrations, anti-IgE treatment also reversed phenotypic and functional effects of IgE-enhanced FcεRI levels on basophils [11] or mast cells [12] and receptor expression dropped to baseline levels within a few days. Both IgE and FcεRI reductions led to impaired activation of effector cells in the presence of allergen in a synergistic manner. Consequently, the histamine release of basophils, mast cell function, and even the chronic inflammatory process during the late phase were reduced in skin prick tests [11,13,14]. Unlike inhalative or topical treatments, anti-IgE promotes desensitizing effects systemically on both effector and regulatory cells of allergic inflammation.

Nevertheless, not all patients benefit from anti-IgE treatment, and the reasons for such treatment failures are still unknown. In vitro studies demonstrated that a reduction in free IgE to less than 10 ng/ml (4.16 IU/ml) was required to prevent IgE-mediated cross-linking of FcεRI and subsequent effector cell activation. It should be noted, however, that this sensitivity differs among individuals [15]. Based on the assessment of patients' clinical responses in phase I and II trials, clinical benefits were associated with an average reduction of serum IgE below 25 ng/ml (10.4 IU/ml) [16]. Therefore, the availability of reliable assays may help physicians to verify that free serum IgE concentrations indeed have declined into the therapeutically intended range. The experimental setup must ensure that the remaining free IgE can be discriminated from the huge amount of long-lasting IgE–omalizumab complexes [17].

To establish an efficient and reliable detection system enabling such a discrimination of free versus omalizumab-bound IgE, we fused the IgE-specific extracellular domains of the FcεRIα subunit with heavy chain constant regions of chicken IgY as domains for dimerization, immobilization, and detection. IgY is the major low-molecular-weight serum immunoglobulin in oviparous animals [18], and it has been shown that using IgY constant domains provides significant advantages in the field of diagnosis with respect to a decrease in nonspecific interactions. Here we present the generation of FcεRIα–IgY fusion proteins for the quantitation of free IgE versus omalizumab–IgE complexes and verified their applicability in the clinic for the characterization of serum samples from patients during anti-IgE therapy.

Materials and methods

Sera and patient samples

Serum was taken from patients with severe generalized atopic eczema and a history of allergic rhinoconjunctivitis, and allergic asthma was treated with a fixed schedule of 10 cycles of 150 mg omalizumab (Xolair, Novartis, Nuernberg, Germany) subcutaneously in 2-week intervals at every visit as reported previously. Patient numbers in our study correspond with the numbering mentioned there [19]. Serum samples were drawn 30 min before injection of omalizumab. Informed consent from the patients was obtained in accordance with the Declaration of Helsinki.

Cloning of FcεRI α-chain domains and IgY constant regions

The human FcεRIα extracellular domain (FcεRIα-ecl) was synthesized from complementary DNA (cDNA) derived from human peripheral mononuclear cells. The avian immunoglobulin constant domains were amplified from a cDNA library derived from chicken splenocytes. The FcεRIα-ecl was amplified without the original

signal sequence using one polymerase chain reaction (PCR) primer containing a *Pf*231I site (gatccgtacgtgtggggcagtcctcagaacctaagg) and another primer containing an *Sgs*I site (gatcggcgcgcccggagctttttattacagtaattgttag) and was introduced into pcDNA3.1/zeo containing a rat immunoglobulin leader sequence and the complete avian constant heavy chain or the avian Fc regions including a C-terminal 4 × His tag, respectively, as described previously [20]. Subsequently, the v2 domain was amplified using one primer containing an *Sgs*I site (gatcggcgcgcccgttagccccagag) and another primer containing a 4×His tag and an *Xba*I site (gatctctagatcagtgatgtgatggaactccgggcatcccttgacgtgat) and was introduced into pcDNA3.1/zeo containing a rat immunoglobulin leader sequence and the FcεRIα-ecl. All amplifications were performed using *Pfx* polymerase (Invitrogen, Karlsruhe, Germany) essentially as described by the manufacturer under standard reaction conditions.

Stable transfection of HEK293 cells

HEK293 cells (ATCC no. CRL-1573) were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Tissue culture reagents were obtained from Invitrogen. HEK293 cells were transfected with 2 µg of the expression vector using polyethylenimine (PEI, Sigma–Aldrich, Taufkirchen, Germany). Stable transfectants then were selected in DMEM supplemented with 10% (v/v) fetal calf serum containing 100 µg/ml zeocin as selection marker (Invitrogen). For secretory protein expression, transfected cells were grown for 3 days as an adhesion culture. The fusion protein secreted by transfected HEK293 cells was purified from the culture medium by affinity chromatography using Ni–NTA (nitrilotriacetic acid)–agarose (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

Assessment of immunoreactivity in ELISA

For assessment of immunoreactivity, purified FcεRI–IgY Fc (1 µg/ml diluted in 1% [w/v] bovine serum albumin [BSA] in phosphate-buffered saline [PBS]) was applied to microtiter plates previously coated with 100 µg/ml monoclonal antibodies of different isotypes [21,22] at 4 °C overnight and blocked with 2% (w/v) BSA–PBS at room temperature (RT) for 45 min. After incubation for 90 min at RT on a rocker platform, the wells were rinsed three times each with 0.1% (v/v) Tween–PBS and PBS and were further incubated with 100 µl of an anti-IgY (Fc-specific) alkaline phosphatase (AP) conjugate (diluted 1:10,000 in 1% [w/v] BSA–PBS, Rockland, Gilbertsville, PA, USA) for 45 min at RT on a rocker platform. The wells were rinsed again three times with 0.1% (v/v) Tween–PBS and PBS, and bound antibodies were visualized by the addition of 75 µl of a 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate solution (Sigma–Aldrich). Absorbance was determined at 405 nm after 15 min of incubation.

For determination of serum IgE, 100 µl of FcεRIα–IgY Fc (0.5 µg/ml in PBS) or omalizumab (10 µg/ml) was immobilized on microtiter plates. After incubation at 4 °C overnight, the wells were rinsed with wash buffer (reagent set B, BD Pharmingen, San Diego, CA, USA). Then dilutions of human myeloma IgE (Calbiochem, Nottingham, UK) or serum samples were added and incubated for 60 min at RT. For competition experiments, myeloma IgE (10 ng/ml) was applied in the presence of omalizumab at concentrations ranging from 0.02 to 200 µg/ml. After washing, 100 µl of biotinylated mouse anti-human IgE (BD Pharmingen, 1:250 in assay diluent) and streptavidin–horseradish peroxidase (HRP) conjugate (BD Pharmingen, 1:1000 in assay diluent) were added and further incubated for 30 min at RT. The wells were rinsed again, and bound IgE

was visualized by the addition of 75 μ l of ABTS substrate solution (Sigma–Aldrich). Absorbance was determined after 15 min and the addition of 50 μ l stop solution at 450/750 nm. Allergen-specific IgE was detected by the addition of biotinylated allergen extracts (Siemens Healthcare Diagnostics, Los Angeles, CA, USA) and streptavidin–HRP conjugate (BD Pharmingen, 1:1000 in assay diluent).

Quantitation of Fc ϵ RI α –IgY constructs was determined by sandwich enzyme-linked immunosorbent assay (ELISA, Chicken IgG Quantitation Kit, Biomol, Hamburg, Germany) according to the recommendations of the manufacturer.

Other methods

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), Western blotting, ELISA, and standard procedures in molecular biology essentially were performed according to established protocols [23]. For detection in immunoblot, anti-Fc ϵ RI α rabbit serum (Upstate, Lake Placid, NY, USA) and anti-rabbit IgG–AP conjugate (Sigma–Aldrich) or anti-IgY (Fc-specific) IgG–AP conjugate (Rockland) were used. Total serum IgE and allergen-specific IgE were determined using common laboratory analyzers (UniCAP250, Phadia, Uppsala, Sweden).

Results

Generation of recombinant Fc ϵ RI–IgY fusion constructs

The human Fc ϵ RI α -cDNA were amplified from cDNA derived from human peripheral mononuclear cells. Thereafter, the cDNA was introduced into expression vectors containing a rodent signal sequence and ν heavy chain constant regions (IgY) amplified from cDNA of chicken splenocytes and a C-terminal 4 $\times$ His tag to enable purification from culture supernatants under mild conditions [20].

After establishment of stable HEK293 cell lines, secretion of the fusion constructs (Fig. 1A) to the medium was analyzed by SDS–PAGE and Western blotting. As expected for the dimeric molecules, the apparent molecular masses were found to be in the range of 180 and 160 kDa for Fc ϵ RI–IgY and the Fc ϵ RI–IgY Fc construct containing only the IgY Fc region, respectively. Fc ϵ RI–IgY CH2 containing only the avian CH2 heavy chain region was detected both as a dimer and as a monomer. Due to its heterogeneity observed in

SDS–PAGE analysis, Fc ϵ RI–IgY CH2 was omitted from further characterizations. Quantitation of Fc ϵ RI–IgY and Fc ϵ RI–IgY Fc in the culture supernatant of adherent HEK293 transfectants by sandwich ELISA confirmed yields in the range of 200 ng/ml for Fc ϵ RI–IgY versus 4 μ g/ml for the miniaturized Fc ϵ RI–IgY Fc construct. After isolation from the culture medium by immobilized metal ion chromatography, SDS–PAGE and immunoblotting with subsequent detection using anti-Fc ϵ RI α rabbit serum and anti-rabbit IgG–AP conjugate or anti-IgY (Fc-specific) IgG–AP conjugate demonstrated homogeneity and the identity of both IgY fusion constructs (Fig. 1B).

Characterization of the receptor fusion molecules

All further experiments were performed with the recombinant Fc ϵ RI–IgY Fc construct due to its improved expression behavior. Immunoreactivity was analyzed by ELISA using Fc ϵ RI–IgY Fc as a detection moiety (Fig. 2A). As expected, signals were observed with immobilized recombinant human IgE [21] and murine myeloma IgE, but none of the other human immunoglobulin isotypes, including human IgA, IgM, and recombinant human IgG of all four subclasses [22], were detected. As expected, only those IgE constructs containing the ϵ -CH3 region essential for the receptor binding site were bound (data not shown).

The Fc ϵ RI–IgY chimera was employed as a detection moiety in immunoblotting (Fig. 2B). Using AlaBLOTs (allergen code I1, Siemens Healthcare Diagnostics) and a human monoclonal recombinant Api m 1-specific IgE antibody [21], Api m 1 was specifically detected in bee venom using both anti-IgE conjugate (left column in Fig. 2B) and Fc ϵ RI IgY/anti-IgY conjugate (right column in Fig. 2B).

Myeloma IgE concentrations were then determined in ELISA where IgE was captured from normal serum with Fc ϵ RI–IgY Fc and detected with anti-human IgE (Fig. 2C). Immobilized Fc ϵ RI–IgY Fc (Fig. 3A) or omalizumab (Fig. 3B) was used to capture myeloma IgE in the presence of increasing concentrations of either omalizumab or Fc ϵ RI–IgY Fc as competitors in solution. The mutual exclusion of either omalizumab or Fc ϵ RI–IgY Fc binding to the IgE epitope was confirmed; therefore, the general principle of providing a specific reagent that targets the IgE epitope defined by the native human IgE receptor was verified.

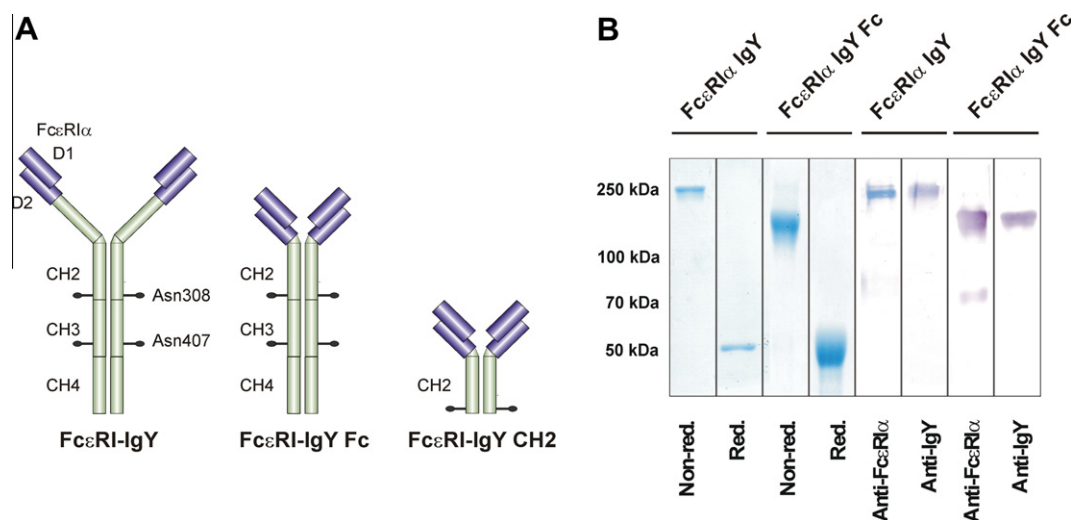


Fig. 1. Schematic representation and analysis of the recombinant fusion proteins. (A) Constant IgY regions are shaded gray, and extracellular domains D1 and D2 of the Fc ϵ RI α subunit are shaded darker gray. Glycosylation sites are indicated (black). (B) The fusion proteins were analyzed by SDS–PAGE and Coomassie staining under nonreducing and reducing conditions and immunoblotting. Detection was performed by using anti-Fc ϵ RI α rabbit serum and anti-rabbit IgG–AP conjugate or anti-IgY (Fc-specific) IgG–AP conjugate.

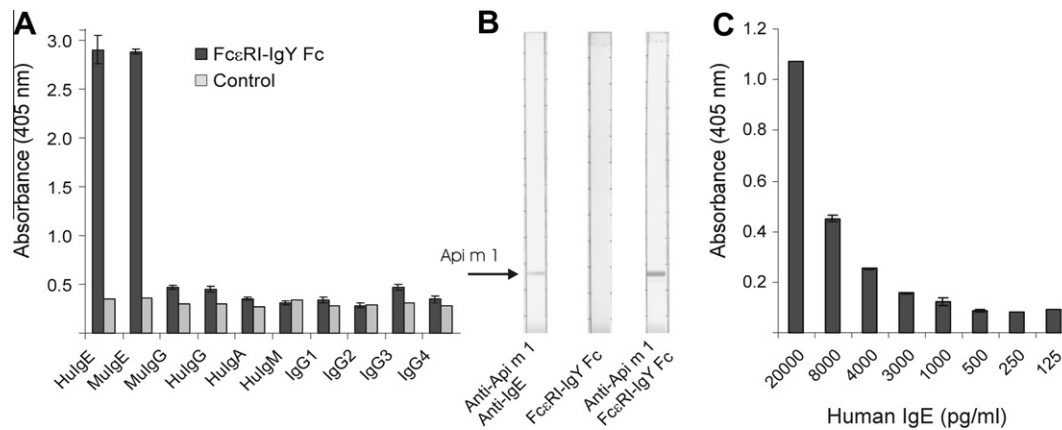


Fig. 2. Immunoreactivity and specificity of FcεRI-IgY Fc by ELISA. The specificity of FcεRI-IgY Fc as a detection moiety was assessed with different immobilized immunoglobulin isotypes (A) or with honeybee venom AlaBLOTs (allergen code I1) and monoclonal ApI m 1-specific IgE (B) and an anti-IgY AP conjugate. The immunoreactivity of immobilized FcεRI-IgY Fc was assessed using different concentrations of human myeloma IgE and biotinylated anti-IgE/streptavidin-AP conjugate (C).

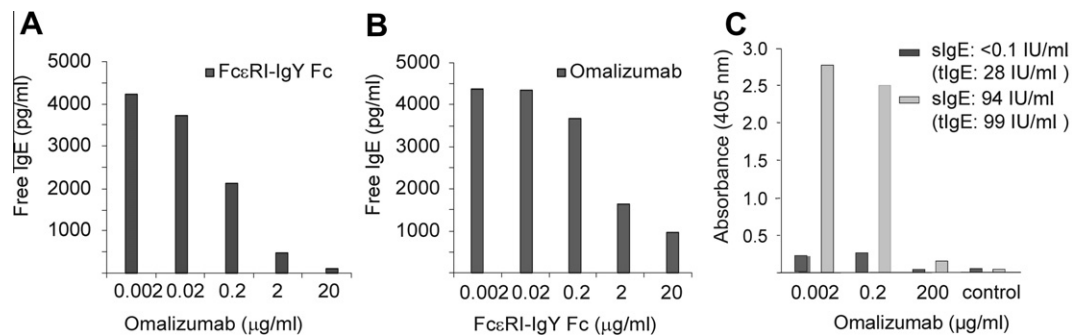


Fig. 3. Competition experiments. Immobilized FcεRI-IgY Fc or omalizumab (10 μg/ml) was incubated with 5 ng/ml nonspecific human myeloma IgE in the presence of omalizumab or FcεRI-IgY Fc as a competitor in solution. (A) Capture with FcεRI-IgY Fc and competition with omalizumab. (B) Capture with omalizumab and competition with FcεRI-IgY Fc. (C) Immobilized FcεRI-IgY Fc (10 μg/ml) was incubated with serum of a timothy grass allergic patient (allergen code G6). By using sera of two patients and omalizumab as a competitor in solution, noncomplexed serum IgE was determined using biotinylated timothy grass pollen extract and streptavidin-AP conjugate. sIgE, specific IgE; tIgE, total IgE.

To better define the nonbound serum IgE population, analyses on an allergen-specific level were performed. Therefore, serum IgE was captured via immobilized FcεRI-IgY Fc. Allergen-specific IgE was then detected by using biotinylated timothy grass pollen extract (*Phleum pratense*, allergen code G6) and a streptavidin-AP conjugate for detection (Fig. 3C). Signals were detected in serum of an allergic patient with high timothy grass pollen-specific IgE levels, but not in the control sample (96 kU/L and <0.1 kU/L, respectively, determined by UniCAP250, Phadia). Biotinylated house dust mite extract (*Dermatophagoides pteronyssinus*, allergen code D1) served as a control and did not produce any signals (data not shown). On the addition of omalizumab, free allergen IgE declined as expected and demonstrated the general feasibility of such analyses on a molecular level. In serum samples from atopic donors without grass pollen allergy, no free IgE could be detected when using biotinylated timothy grass pollen extract (data not shown).

Together, these data emphasize that the recombinant FcεRI-IgY fusion proteins constitute functional affinity molecules with authentic binding of IgE in different experimental setups allowing the reliable and efficient determination of noncomplexed IgE.

Free IgE assay using fusion molecules in omalizumab-treated patients

After evaluation of the basic characteristics of the FcεRI-IgY Fc construct, we monitored free versus omalizumab-complexed IgE levels in sera of patients undergoing omalizumab therapy over

time [19]. All patients displayed total serum IgE values above 1000 IU/ml before therapy and had a history of allergic rhinoconjunctivitis and allergic asthma. We determined free total IgE values in a setting similar to that in Fig. 3 with FcεRI-IgY Fc as a capture reagent (Fig. 4). As expected, levels of free IgE in all patients declined during the course of omalizumab-based intervention. Initial levels ranging from approximately 1500 to 5000 IU/ml reflected the highly atopic state of the individuals and corresponded to the presence of noncomplexed IgE. Omalizumab administration effectively suppressed free IgE levels in all serum samples, and this further persisted under therapy. However, after termination of therapy, elevated free IgE titers were reestablished, as observed in patient 5 in Fig. 5 (open symbols indicate termination of therapy). Therefore, coherent and robust results were obtained demonstrating the applicability and reliability of the FcεRI-IgY Fc molecule for the determination of unbound serum IgE.

Discussion

Anti-IgE treatment is designed primarily for reducing the level of free serum IgE and downregulating IgE receptors on effector cells. Accordingly, it was shown to improve symptoms, quality of life, and disease control (asthma exacerbations) in patients with concomitant asthma and persistent allergic rhinitis [13]. Although the causal role of IgE in allergic disease is well established, the relationship between free IgE and the reduction of clinical symptoms under anti-IgE therapy has not been evaluated accurately.

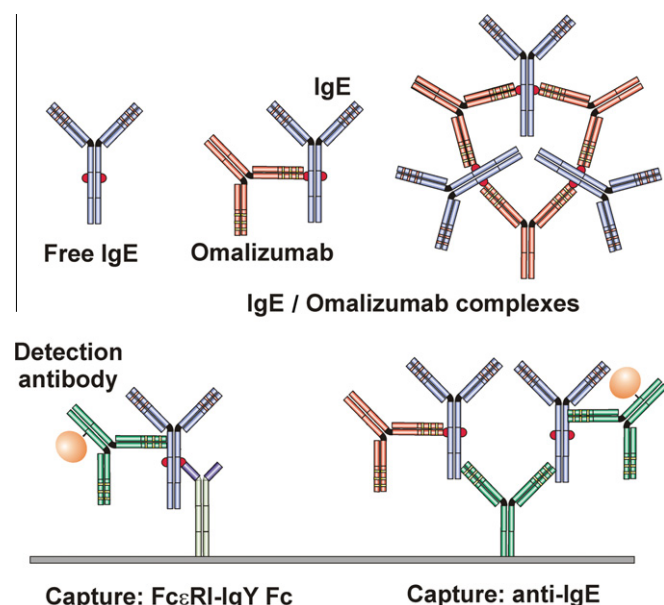


Fig. 4. Assay scheme for the determination of serum IgE in the presence of omalizumab. Fc ϵ RI–IgY Fc is used as a capture reagent for exclusive binding of free serum IgE (left). Anti-IgE antibodies (polyclonal or monoclonal), by contrast, are able to bind to free IgE and omalizumab-complexed IgE. Blue: human IgE; red circle: Fc ϵ RI binding site; red: omalizumab; green: nonhuman anti-IgE antibodies; yellow circle: enzyme conjugated to the antibody backbone. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Clinical trials in patients with allergic rhinitis and asthma have shown improvements rapidly after initiation of anti-IgE administration [24]. By this time, Fc ϵ RI on mast cells has been only marginally downregulated, and reduction of mast cell activation presumably has not yet been achieved. Thus, additional mechanisms must account for the therapeutic effects during the early period of anti-IgE treatment. One intriguing explanation relies on the formation of beneficial immune complexes of anti-IgE and IgE that rapidly accumulate up to 10 times the basal IgE levels in certain individuals [25]. The nature of these immune complexes formed *in vitro* ranges from a putatively cyclic hexamer to heterotrimeric structures, presumably consisting of 2:1 or 1:2 molecules of omalizumab/IgE [26,27]. Complex formation depends on the molar ratio of anti-IgE and IgE and may vary with the biochemical environment such as PBS versus serum [28]. These complexes show an increased functional affinity (avidity) and compete with allergen-specific receptor-bound IgE on mast cells and basophils for incoming allergen molecules, thereby preventing them from binding to receptor-bound IgE and inducing Fc ϵ RI crosslinking. In contrast to the potentially beneficial nature of these immune complexes, their generation and long-term persistence in the cir-

ulation was shown to interfere with the accuracy and reproducibility of total and allergen-specific IgE immunoassays [29].

Parallel to the development of omalizumab, a free IgE immunoassay was developed using an Fc ϵ RI–IgG chimera to capture non-complexed IgE [30]. The Fc ϵ RI–IgG “immunoadhesin” formed highly stable heterotrimeric complexes in solution [31], and its binding affinity was comparable to that described for the high-affinity Fc ϵ RI receptors on cultured cord blood basophils and transfected COS cells [32–34]. Although Fc ϵ RI–IgG appeared to be a useful reagent for the measurement of free IgE [35], it was never launched as a commercial assay. Although other assay setups using anti-IgE antibodies to discriminate between free and complexed IgE are generally feasible, IgE–Fc ϵ RI α interactions represent the underlying principle of IgE-mediated hypersensitivity. Because the IgE binding sites of Fc ϵ RI and omalizumab are not identical, other assays would be confined to the use of particular anti-IgE antibodies. By contrast, an assay on the basis of a soluble Fc ϵ RI chimera allows the analysis of a variety of anti-IgE therapeutics, including nonantibody molecules [7,36].

Hamilton and coworkers described the development and validation of free and total serum IgE immunoassays (IEMAs) that were designed specifically to evaluate the sera of omalizumab-treated patients [17]. These assays were based on the soluble human Fc ϵ RI α ectodomain produced via the baculovirus vector expression system in insect cells; however, when used as a capture moiety, immobilization abolished IgE reactivity [17]. Certain insect cell lines are known to provide immunogenic glycan structures containing α -1,3-core fucose [37], the hallmark of carbohydrate crossreactive determinants (CCDs). Especially food, pollen, and insect venom allergic patients are often characterized by the presence of CCD-specific IgE antibodies in their serum samples [38,39]. When using the heavily glycosylated Fc ϵ RI α ectodomains in immunoassays, this phenomenon might evoke erroneous results [40]. Therefore, to avoid such problems, we used a human cell line for recombinant production.

In this study, we generated Fc ϵ RI α chimeras by fusing the ligand-binding ectodomain with chicken IgY heavy chain regions as dimerization domains. Chicken-derived antibodies have increasingly proven to be valuable reagents in immunoassays because they offer many advantages over their mammalian homologues and do not interact with endogenous serum proteins well known to evoke assay interference. Rheumatoid factor (RF), heterophilic antibodies, human anti-mouse IgG antibodies (HAMAs), and complement components are considered as the most prominent causes of false-positive or false-negative results in immunoassays [41–43]. Furthermore, chicken IgY is not recognized by protein A, G, or L; thus, a combination of these standard detection conjugates in multiplex assays would be feasible. When produced in mammalian systems such as HEK293 cells, recombinant IgY retained its favorable properties [20,44]. After fusing the Fc ϵ RI extracellular domains with different IgY heavy chain domains, the comparative analyses of the constructs suggested that the secretion efficiencies

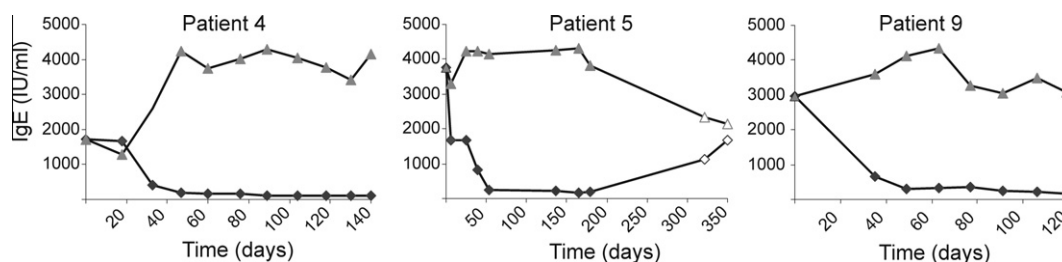


Fig. 5. Measurement of patient sera. Immobilized Fc ϵ RI–IgY Fc was incubated with patient sera treated with omalizumab (open symbols indicate that therapy has been completed). Noncomplexed serum IgE (diamonds) was determined using biotinylated monoclonal anti-IgE antibody, and streptavidin–AP conjugate with myeloma IgE was used as a standard (not shown). Total IgE (triangles) was measured by UniCAP250 (Phadia).

correlated primarily with their molecular masses, as was already shown for different IgG constructs [22]. FcεRI–IgY functioned as a detection or capture reagent for human myeloma IgE, and competition with omalizumab in solution further underlined its specificity.

Recently, this issue was addressed by establishing a pharmacokinetic–pharmacodynamic model of free IgE, and according to these studies a time- and concentration-dependent relationship between free IgE and clinical outcomes in patients with severe allergic asthma was observed [24,45]. In addition to the total IgE pool in the body, the quantitation of the specific IgE fraction may also be taken into account. Data on the informative value of this parameter, however, are still conflicting. Patients with high levels of mite-specific IgE profited most from omalizumab when the contribution to total specific IgE was low. However, no consistent predictive effect for the response to treatment was observed for IgE levels specific for individual allergens [46]. By contrast, in patients with seasonal allergic rhinitis, baseline-specific IgE, but not necessarily total IgE, correlated with symptom severity during anti-IgE treatment [47]. In cat allergic patients, the currently recommended doses of omalizumab eliminated IgE antibodies very efficiently and improved symptoms if the specific IgE antibody contributed less than 1% to the total IgE load but were not effective if the percentage was greater than 3% [48]. Apart from the possibility to determine serum IgE levels, the fate of local IgE within the target tissue of allergic responses remains elusive.

Using FcεRI–IgY as a capture moiety, we showed the determination of allergen-specific noncomplexed IgE by using biotinylated allergen extracts and enzyme-labeled streptavidin for detection in the presence of omalizumab as a competitor. Thus, a decline in allergen-specific free IgE as a parameter for patient selection for this kind of therapy can be further evaluated.

To benefit from anti-IgE therapy, monitoring of circulating total and allergen-specific IgE and free IgE concentrations may help to optimize dosing and maximize the efficacy of therapy. The best clinical outcomes are observed in those patients with a reduction to less than 25 ng/ml free IgE [49,50]. The optimal selection of patients on the basis of starting IgE values, however, is still under debate. It was previously reported that the efficacy of omalizumab was less in patients with low IgE levels (<75 kU/L) [13], but omalizumab was also proven to be effective in atopic patients with less than 30 kU/L [51]. In selected patients with generalized atopic eczema and high levels of total IgE, we observed surprising success with low-dose anti-IgE therapy [52]. Serum samples of three exemplary patients with severe generalized atopic eczema and a history of allergic rhinoconjunctivitis and allergic asthma under anti-IgE therapy were selected for evaluation of free IgE concentrations over time. All patients showed starting IgE levels greater than 1000 kU/L that rapidly dropped during therapy but rose again after termination of IgE suppression.

Even though the reasons for the ineffectiveness of anti-IgE therapy in some patients are still unknown, clinical effects might be attenuated because tissue mast cells lose their surface IgE very slowly, possibly within weeks, even when free serum IgE reaches low steady-state levels within hours of intravenous injection. Although IgE reduction persists for some time after discontinuation of therapy, IgE is continuously synthesized *de novo*. Consequently, serum IgE levels will eventually rise again after cessation of therapy [53], and skin test reactivity in a group of patients with perennial allergic rhinitis returned to prestudy baseline levels [54]. Thus, an adjustment of anti-IgE dosing and retreatment with maintenance doses at certain intervals appears to be reasonable [55].

These findings clearly emphasize the need to provide commercially available assay systems that are able to discriminate IgE–omalizumab complexes from noncomplexed IgE and, thus,

provide the basis to deduce adequate therapeutic regimens from residual-free IgE levels. Therefore, we established FcεRI–IgY chimeras allowing the discrimination and quantitation of nonbound and anti-IgE-complexed IgE in sera of patients undergoing anti-IgE therapy. As shown in different experimental setups, the extraordinary specificity and reactivity of native FcεRI is mirrored by our recombinant molecules when used as a soluble capture or detection reagent. Currently, a lateral flow assay that allows the determination of free IgE in serum samples within 10 min is under development as a point-of-care assay for clinicians. In selected patient sera, noncomplexed IgE declined rapidly, whereas total IgE remained rather stable.

This is due to the continuous production of IgE and, consequently, an accumulation of stable and long-lasting IgE–anti-IgE complexes. Of note, we assessed these changes in only a limited number of patients. On the basis of our pilot investigations, studies with larger cohorts may reveal the impact of free IgE during therapy and follow-up of treated patients against the background of different factors promoting or affecting anti-IgE therapy. Such studies may allow clinical interpretation of residual noncomplexed IgE as a biomarker to distinguish individuals responding to omalizumab treatment from nonresponders at an early time point in therapy.

In summary, we have established bivalent FcεRI fusion constructs with high specificity for human and murine IgE that may serve as ideal capture or detection reagents for the quantitation of free IgE levels in omalizumab-treated patients on a larger scale. Factors determining whether this translates into clinical benefit remain to be further investigated.

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References

- [1] F.D. Finkelman, D. Vercelli, Advances in asthma, allergy mechanisms, and genetics in 2006, *J. Allergy Clin. Immunol.* 120 (2007) (2006) 544–550.
- [2] H.J. Gould, B.J. Sutton, IgE in allergy and asthma today, *Nat. Rev. Immunol.* 8 (2008) 205–217.
- [3] S. Kraft, J.P. Kinet, New developments in FcRI regulation, function, and inhibition, *Nat. Rev. Immunol.* 7 (2007) 365–378.
- [4] J.P. Kinet, The high-affinity IgE receptor (FcεRI): from physiology to pathology, *Annu. Rev. Immunol.* 17 (1999) 931–972.
- [5] M. Iikura, M. Yamaguchi, K. Hirai, M. Miyamasu, H. Yamada, T. Nakajima, T. Fujisawa, C. Ra, Y. Morita, K. Yamamoto, Regulation of surface FcRI expression on human eosinophils by IL-4 and IgE, *Int. Arch. Allergy Immunol.* 124 (2001) 470–477.
- [6] T.W. Chang, The pharmacological basis of anti-IgE therapy, *Nat. Biotechnol.* 18 (2000) 157–162.
- [7] Z. Peng, Vaccines targeting IgE in the treatment of asthma and allergy, *Hum. Vaccin.* 5 (2009) 302–309.
- [8] L. Presta, R. Shields, L. O'Connell, S. Lahr, J. Porter, C. Gorman, P. Jardieu, The binding site on human immunoglobulin E for its high affinity receptor, *J. Biol. Chem.* 269 (1994) 26368–26373.
- [9] L.G. Presta, S.J. Lahr, R.L. Shields, J.P. Porter, C.M. Gorman, B.M. Fendly, P.M. Jardieu, Humanization of an antibody directed against IgE, *J. Immunol.* 151 (1993) 2623–2632.
- [10] R.L. Shields, W.R. Whether, K. Zioncheck, L. O'Connell, B. Fendly, L.G. Presta, D. Thomas, R. Saban, P. Jardieu, Inhibition of allergic reactions with antibodies to IgE, *Int. Arch. Allergy Immunol.* 107 (1995) 308–312.
- [11] D.W. MacGlashan Jr., B.S. Bochner, D.C. Adelman, P.M. Jardieu, A. Togias, J. McKenzie-White, S.A. Sterbinsky, R.G. Hamilton, L.M. Lichtenstein, Down-regulation of FcμRI expression on human basophils during *in vivo* treatment of atopic patients with anti-IgE antibody, *J. Immunol.* 158 (1997) 1438–1445.
- [12] G. Gomez, S. Jogie-Brahim, M. Shima, L.B. Schwartz, Omalizumab reverses the phenotypic and functional effects of IgE-enhanced FcεRI on human skin mast cells, *J. Immunol.* 179 (2007) 1353–1361.
- [13] S.T. Holgate, R. Djukanovic, T. Casale, J. Bousquet, Anti-immunoglobulin E treatment with omalizumab in allergic diseases: an update on anti-inflammatory activity and clinical efficacy, *Clin. Exp. Allergy* 35 (2005) 408–416.

- [14] R.J. van Neerven, E.F. Knol, A. Ejrnaes, P.A. Würtzen, IgE-mediated allergen presentation and blocking antibodies: regulation of T-cell activation in allergy, *Intl. Arch. Allergy Immunol.* 141 (2006) 119–129.
- [15] D.W. MacGlashan Jr., S.P. Peters, J. Warner, L.M. Lichtenstein, Characteristics of human basophil sulfidopeptide leukotriene release: releasability defined as the ability of the basophil to respond to dimeric cross-links, *J. Immunol.* 136 (1986) 2231–2239.
- [16] Genentech and Novartis Pharmaceuticals, Pulmonary–Allergy Drugs Advisory Committee meeting, 15 May 2003, <http://www.fda.gov/ohrms/dockets/ac/03/briefing/3952B1_01_Genentech-Xolair.pdf>.
- [17] R.G. Hamilton, G.V. Marcotte, S.S. Saini, Immunological methods for quantifying free and total serum IgE levels in allergy patients receiving omalizumab (Xolair) therapy, *J. Immunol. Methods* 303 (2005) 81–91.
- [18] S. Sun, W. Mo, Y. Ji, S. Liu, Preparation and mass spectrometric study of egg yolk antibody (IgY) against rabies virus, *Rapid Commun. Mass Spectrom.* 15 (2001) 708–712.
- [19] B. Belloni, M. Ziai, A. Lim, B. Lemerrier, M. Sbornik, S. Weidinger, C. Andres, C. Schnopp, J. Ring, R. Hein, M. Ollert, M. Mempel, Low-dose anti-IgE therapy in patients with atopic eczema with high serum IgE levels, *J. Allergy Clin. Immunol.* 120 (2007) 1223–1225.
- [20] K. Greunke, E. Spillner, I. Braren, H. Seismann, S. Kainz, U. Hahn, T. Grunwald, R. Bredehorst, Bivalent monoclonal IgY antibody formats by conversion of recombinant antibody fragments, *J. Biotechnol.* 124 (2006) 446–456.
- [21] I. Braren, S. Blank, H. Seismann, S. Deckers, M. Ollert, T. Grunwald, E. Spillner, Generation of human monoclonal allergen-specific IgE and IgG antibodies from synthetic antibody libraries, *Clin. Chem.* 53 (2007) 837–844.
- [22] I. Braren, K. Greunke, O. Umland, S. Deckers, R. Bredehorst, E. Spillner, Comparative expression of different antibody formats in mammalian cells and *Pichia pastoris*, *Biotechnol. Appl. Biochem.* 47 (2007) 205–214.
- [23] F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl, *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, 1994.
- [24] R.G. Slavin, C. Ferioli, S.J. Tannenbaum, C. Martin, M. Blogg, P.J. Lowe, Asthma symptom re-emergence after omalizumab withdrawal correlates well with increasing IgE and decreasing pharmacokinetic concentrations, *J. Allergy Clin. Immunol.* 123 (2009) 107–113.
- [25] J. Corne, R. Djukanovic, L. Thomas, J. Warner, L. Botta, B. Grandordy, D. Gygax, C. Heusser, F. Patalano, W. Richardson, E. Kilchherr, T. Staehelin, F. Davis, W. Gordon, L. Sun, R. Liou, G. Wang, T.W. Chang, S. Holgate, The effect of intravenous administration of a chimeric anti-IgE antibody on serum IgE levels in atopic subjects: efficacy, safety, and pharmacokinetics, *J. Clin. Invest.* 99 (1997) 879–887.
- [26] J. Liu, P. Lester, S. Builder, S.J. Shire, Characterization of complex formation by humanized anti-IgE monoclonal antibody and monoclonal human IgE, *Biochemistry* 34 (1995) 10474–10482.
- [27] F.M. Davis, L.A. Gossett, K.L. Pinkston, R.S. Liou, L.K. Sun, Y.W. Kim, N.T. Chang, T.W. Chang, K. Wagner, J. Bews, et al., Can anti-IgE be used to treat allergy?, *Springer Semin Immunopathol.* 15 (1993) 51–73.
- [28] B. Demeule, S.J. Shire, J. Liu, A therapeutic antibody and its antigen form different complexes in serum than in phosphate-buffered saline: a study by analytical ultracentrifugation, *Anal. Biochem.* 388 (2009) 279–287.
- [29] R.G. Hamilton, Accuracy of US Food and Drug Administration-cleared IgE antibody assays in the presence of anti-IgE (omalizumab), *J. Allergy Clin. Immunol.* 117 (2006) 759–766.
- [30] rhuMAB-E25 Study Group, Treatment of allergic asthma with monoclonal anti-IgE antibody, *N. Engl. J. Med.* 341 (1999) 1966–1973.
- [31] J. Liu, J. Ruppel, S.J. Shire, Interaction of human IgE with soluble forms of IgE high affinity receptors, *Pharm. Res.* 14 (1997) 1388–1393.
- [32] L. Miller, U. Blank, H. Metzger, J.P. Kinet, Expression of high-affinity binding of human immunoglobulin E by transfected cells, *Science* 244 (1989) 334–337.
- [33] T. Ishizaka, A.M. Dvorak, D.H. Conrad, J.R. Niebyl, J.P. Marquette, K. Ishizaka, Morphologic and immunologic characterization of human basophils developed in cultures of cord blood mononuclear cells, *J. Immunol.* 134 (1985) 532–540.
- [34] U. Blank, C.S. Ra, J.P. Kinet, Characterization of truncated α -chain products from human, rat, and mouse high affinity receptor for immunoglobulin E, *J. Biol. Chem.* 266 (1991) 2639–2646.
- [35] T.B. Casale, I.L. Bernstein, W.W. Busse, C.F. LaForce, D.G. Tinkelman, R.R. Stoltz, R.J. Dockhorn, J. Reimann, J.Q. Su, R.B. Fick Jr., D.C. Adelman, Use of an anti-IgE humanized monoclonal antibody in ragweed-induced allergic rhinitis, *J. Allergy Clin. Immunol.* 100 (1997) 110–121.
- [36] S.D. Mendonsa, M.T. Bowser, In vitro selection of high-affinity DNA ligands for human IgE using capillary electrophoresis, *Anal. Chem.* 76 (2004) 5387–5392.
- [37] M. Bencurova, W. Hemmer, M. Focke-Tejkl, I.B. Wilson, F. Altmann, Specificity of IgG and IgE antibodies against plant and insect glycoprotein glycans determined with artificial glycoforms of human transferrin, *Glycobiology* 14 (2004) 457–466.
- [38] R.C. Aalberse, J. Akkerdaas, R. van Ree, Cross-reactivity of IgE antibodies to allergens, *Allergy* 56 (2001) 478–490.
- [39] K. Hancock, S. Narang, S. Pattabhi, M.L. Yushak, A. Khan, S.C. Lin, R. Plemons, M.J. Betenbaugh, V.C. Tsang, False positive reactivity of recombinant, diagnostic glycoproteins produced in High Five insect cells: effect of glycosylation, *J. Immunol. Methods* 330 (2008) 130–136.
- [40] H. Seismann, S. Blank, I. Braren, K. Greunke, L. Cifuentes, T. Grunwald, R. Bredehorst, M. Ollert, E. Spillner, Dissecting cross-reactivity in hymenoptera venom allergy by circumvention of α -1, 3-core fucosylation, *Mol. Immunol.* 47 (2009) 779–808.
- [41] T.P. Vikinge, A. Askendal, B. Liedberg, T. Lindahl, P. Tengvall, Immobilized chicken antibodies improve the detection of serum antigens with surface plasmon resonance (SPR), *Biosens. Bioelectron.* 13 (1998) 1257–1262.
- [42] D. Carlender, A. Larsson, Avian antibodies can eliminate interference due to complement activation in ELISA, *Ups. J. Med. Sci.* 106 (2001) 189–195.
- [43] L.M. Boscato, M.C. Stuart, Heterophilic antibodies: a problem for all immunoassays, *Clin. Chem.* 34 (1988) 27–33.
- [44] K. Greunke, I. Braren, I. Alpers, S. Blank, J. Sodenkamp, R. Bredehorst, E. Spillner, Recombinant IgY for improvement of immunoglobulin-based analytical applications, *Clin. Biochem.* 41 (2008) 1237–1244.
- [45] P.J. Lowe, S. Tannenbaum, A. Gautier, P. Jimenez, Relationship between omalizumab pharmacokinetics, IgE pharmacodynamics, and symptoms in patients with severe persistent allergic (IgE-mediated) asthma, *Br. J. Clin. Pharmacol.* 68 (2009) 61–76.
- [46] U. Wahn, C. Martin, P. Freeman, M. Blogg, P. Jimenez, Relationship between pretreatment specific IgE and the response to omalizumab therapy, *Allergy* 64 (2009) 1780–1787.
- [47] Omalizumab Rhinitis Study Group, Specific IgE serum concentration is associated with symptom severity in children with seasonal allergic rhinitis, *Allergy* 63 (2008) 1339–1344.
- [48] S.G. Johansson, A. Nopp, H. Oman, J. Ankerst, L.O. Cardell, R. Gronneberg, H. Matsols, S. Rudblad, V. Strand, G. Stalenheim, The size of the disease relevant IgE antibody fraction in relation to “total-IgE” predicts the efficacy of anti-IgE (Xolair) treatment, *Allergy* 64 (2009) 1472–1477.
- [49] G. Hochhaus, L. Brookman, H. Fox, C. Johnson, J. Matthews, S. Ren, Y. Deniz, Pharmacodynamics of omalizumab: implications for optimised dosing strategies and clinical efficacy in the treatment of allergic asthma, *Curr. Med. Res. Opin.* 19 (2003) 491–498.
- [50] E. Adelroth, S. Rak, T. Haahtela, G. Aasand, L. Rosenhall, O. Zetterstrom, A. Byrne, K. Champain, J. Thirlwell, G.D. Cioppa, T. Sandström, Recombinant humanized mAb-E25, an anti-IgE mAb, in birch pollen-induced seasonal allergic rhinitis, *J. Allergy Clin. Immunol.* 106 (2000) 253–259.
- [51] J. Ankerst, A. Nopp, S.G. Johansson, J. Adedoyin, H. Oman, Xolair is effective in allergies with a low serum IgE level, *Intl. Arch. Allergy Immunol.* 152 (2010) 71–74.
- [52] A. Lim, S. Luderschmidt, A. Weidinger, C. Schnopp, J. Ring, R. Hein, M. Ollert, M. Mempel, The IgE repertoire in PBMCs of atopic patients is characterized by individual rearrangements without variable region of the heavy immunoglobulin chain bias, *J. Allergy Clin. Immunol.* 120 (2007) 696–706.
- [53] S.S. Saini, D.W. MacGlashan, S.A. Sterbinsky, A. Togias, D.C. Adelman, L.M. Lichtenstein, B.S. Bochner, Down-regulation of human basophil IgE and FC ϵ RI α surface densities and mediator release by anti-IgE infusions is reversible in vitro and in vivo, *J. Immunol.* 162 (1999) 5624–5630.
- [54] J. Corren, G. Shapiro, J. Reimann, Y. Deniz, D. Wong, D. Adelman, A. Togias, Allergen skin tests and free IgE levels during reduction and cessation of omalizumab therapy, *J. Allergy Clin. Immunol.* 121 (2008) 506–511.
- [55] S. Ogino, T. Nagakura, K. Okubo, N. Sato, M. Takahashi, T. Ishikawa, Retreatment with omalizumab at one year interval for Japanese cedar pollen-induced seasonal allergic rhinitis is effective and well tolerated, *Intl. Arch. Allergy Immunol.* 149 (2009) 239–245.