

Original article

CD-sens and clinical changes during withdrawal of Xolair after 6 years of treatment

Background: Many clinical trials with omalizumab, Xolair, have been reported but the treatment period has always been short, i.e. < 12 months. After withdrawal, the clinical symptoms tend to return. A group of patients who stopped treatment after approx. 6 years allowed studies of the long-term effects of Xolair.

Methods: The patient's cat or mite allergen sensitivity was quantitated as basophil allergen threshold sensitivity, CD-sens, and immunoglobulin E (IgE) and IgE- and IgG4-antibodies were determined before start and during treatment withdrawal. Asthma severity was evaluated from forced expiratory volume (FEV), peak expiratory flow (PEF) and a questionnaire.

Results: At 6–14 months without Xolair 13 of the 18 cat and mite allergic asthmatics had either improved or remained the same as on treatment. Most of the patients were in a stable clinical condition reporting high quality of life, no increased nightly asthma attacks, no emergency visits as well as little or no increase in medication. The CD-sens to cat showed a peak 4 months after withdrawal but then decreased to levels below those of untreated patients with allergic asthma and at 12 months six of 14 had nonreactive basophils. Cat IgG4 antibody levels were higher than in cat allergies in general.

Conclusion: Most of the patients 12–14 months had, after closing of 6-year Xolair treatment, a surprisingly mild asthma. Interestingly, and probably contributing to the clinical results, a downregulation of basophil, and presumably also mast cell, reactivity, was seen.

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During the last years, the humanized monoclonal anti-immunoglobulin E (IgE) antibody omalizumab has been approved in the United States and European Union for the treatment of patients over 12 years of age with moderate to severe IgE-mediated allergic asthma. Omalizumab has been shown to decrease levels of circulating IgE by binding to the constant region (Cε3) of the IgE molecule and thereby blocking the structures responsible for the reaction with FcεRI and FcεRII receptors on mast cells and basophils (1). Several reports show a reduced level of free IgE by as much as 99% within 3 days followed by a 90% decrease in FcεRI expression on basophils after 7 days (2, 3), and by the time of nasal and skin prick tests (SPT) responses were significantly reduced (4). Withdrawal of omalizumab after a few months of treatment results in a return to pretreatment clinical state, FcεRI number and serum IgE levels within months (5).

The positive results on allergic disease are the most promising, and now when omalizumab is available for prescription, there is an urgent need for a simple test to select responders and to monitor treatment efficacy. There are several *in vivo* and *in vitro* methods to monitor

different immunological and inflammatory factors. Some of them, like the measurement of IgE, sometimes referred to as 'total IgE' and allergen-specific IgE antibodies in serum, are well standardized and easy to handle, although the results obtained do not mirror allergy-related inflammatory factors like cell responses and bronchial mucosal hyper-reactivity (6, 7). Other methods such as skin tests are simple and safe but not useful for quantitation of sensitivity as the precision is very low (8). Target organ allergen challenge tests, on the other hand, could be used but are difficult to handle, time-consuming, not very quantitative and potentially dangerous for the patient.

Over the last 10 years, flow cytometric methods allowing detection of activated basophils in blood have been developed and refined. By using a combination of CD63 as a basophil activity marker and CD203c for basophil identification, these methods allow objective analyses of a high number of basophils in a relatively short time (9, 10). Basophil allergen threshold challenge, CD-sens (11), as a measure of patient allergen sensitivity, correlated well with clinical tests for allergen sensitivity-like SPT titration (12), nasal allergen provocation (12) and bronchial

allergen provocation (unpublished). Thus, CD-sens is useful for monitoring the efficacy of different treatments, e.g. IgE-specific immunomodulation treatment (ESIT) with omalizumab or allergen-specific immunotherapy (ASIT), with small intra-assay/interassay variations (12).

The aim of this study was to follow basophil allergen sensitivity, CD-sens, serological parameters and the perception of symptoms and need for drugs in patients after withdrawal of omalizumab after a very long time of treatment, i.e. 6 years.

Material and methods

Subjects

The reference group consisted of patients (controls; $n = 15$) with a clinical history of moderate, well-controlled asthma and/or rhinoconjunctivitis and who tested positive for IgE antibodies to cat. All controls were drug free at the time of sampling and had not previously been treated with omalizumab or ASIT to cat.

The allergic patients (15 of the 18 were cat allergic and three of the 18 mite allergic), age (median) 48 years (range: 37–71), had received ESIT (omalizumab; Novartis, Basel, Switzerland) for 6 years as part of a prospective, clinical trial of severe allergic asthma, which needed high doses of inhaled, but no oral steroids (CIGE 0250011 followed by three extensions). As omalizumab had a most positive effect on their symptoms, quality of life (QoL) and need for 'heavy' drugs-like systemic steroids, they were, with continuing good result, provided with omalizumab until it was registered and available by prescription. During the drug withdrawal, they were analysed for basophil allergen sensitivity to cat and mite once a month for 6 months and a seventh time approximately 12–14 months after closing of the ESIT. After the last injection of omalizumab, they continued their previous asthma medication consisting of inhaled, but not systemic, corticosteroids and some other asthma drugs, but no antihistamines during 5 days prior to basophil testing. In a few cases, a sample was missing as the patient could not attend or could not abstain from antihistamines.

A serum sample of each patient obtained in 1998/1999 before the start of the clinical trial was kindly provided by Novartis (Basel).

All patients gave their informed consent to participate in the study, which was approved by the ethics committee of University of Gothenburg, Sweden.

Basophil activation

Basophil allergen threshold sensitivity analyses were performed as previously described (11, 12). Aliquots of 100 μ l heparinized blood were incubated with 100 μ l RPMI-HEPES (negative control; Gibco Ltd, Paisley, Renfrewshire, UK), 100 μ l fMLP 10^{-7} M (positive control; Sigma Chemical Co., St Louis, MO, USA), 25 μ l α -Fc ϵ RI (positive control; Böhlmann Laboratories, Basel, Switzerland) or 100 μ l allergen solution 0.001–10 000 SQU/ml (ALK-Abello A/S, Hørsholm, Denmark). After stimulation, cells were incubated with CD203c-PE (Immunotech, Marseille, France) for basophil identification and CD63-FITC (Immunotech) for detection of basophil activation. One tube containing blood and RPMI was directly incubated with α -IgE-FITC (Dako Cytomation, Glostrup, Denmark) and CD203c-PE. After antibody staining, the red blood cells were haemolysed by adding isotonic NH_4Cl -EDTA lysing solution. The leucocyte suspensions were washed once with 2 ml of phos-

phate-buffered saline (PBS), centrifuged and resuspended in PBS and analysed in a flow cytometer.

Flow cytometric analysis

Leucocytes were analysed and counted in an EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). The instrument was calibrated daily with two standardized fluorospheres, Flow-Check (Beckman Coulter) and Flow-Set (Beckman Coulter). The percentage of CD63 or IgE-positive cells in the basophil gate (determined by CD203c staining) was analysed. The percentage of CD63-positive basophils in the control samples was below 1.5%. The cut-off determining a positive test was set to 3% of CD63-positive basophils.

Definition of CD-sens

Basophil allergen threshold sensitivity, CD-sens, was analysed by flow cytometry after incubation with serial dilutions of cat or mite allergen. CD-sens was, as previously described (12), defined as the inverted value for the allergen concentration giving a 50% of maximum CD63% upregulation multiplied by 100, and used to describe a patient's allergen-specific sensitivity. The higher the CD-sens, the higher the basophil allergen sensitivity. The intra-assay CV was 13.1% and the interassay CV was 5.4% (11).

Calculation of allergen-binding activity

The CD-sens from untreated whole blood was compared with the CD-sens in blood samples where the plasma had been removed by washing the cells twice with PBS, 50 times the plasma volume, before allergen stimulation. The ratio CD-sens after/before washing was calculated and termed 'allergen-binding activity' (ABA).

Calculation of IgE per basophils

The number of IgE molecules per basophil was calculated using a FITC-conjugated antibody to IgE (see above) and compared with a standard curve of calibration beads (Dako Cytomation) as previously described (11).

Immunoglobulin analyses

The serum concentrations of IgE (kU/l) were determined by ImmunoCAPTM Total IgE (Phadia AB, Uppsala, Sweden) and of IgE- and IgG4-antibodies (kU_A/l) to cat dander (e1), or mite (d1) by ImmunoCAPTM Specific IgE (Phadia AB) according to the manufacturer's instructions. The relative IgE antibody concentration was expressed as absolute value in percentage of IgE.

Statistical analysis

Data are presented as 'medians; interquartile range'. Differences between the variables were first analysed by Friedman ANOVA. Paired comparisons over time within patients were performed using the nonparametric method Wilcoxon paired test and between patients and controls by using the nonparametric method Mann-Whitney *U*-test. Correlations between the different parameters were sought by calculating the Spearman correlation coefficient. Differences were considered statistically significant at $P < 0.05$.

Results

Symptoms and medication

All patients had received ESIT for 6 years with good results. During this period, their allergic symptoms were less pronounced than before treatment, with QoL regarded as very good, exacerbation attacks were rare and no emergency visits were needed. Their need for additional medication was limited; dosages of local steroids were reduced and no patient generally needed oral steroids, although occasionally taken in relation to exacerbations.

During the 12- to 14-month withdrawal period, the patients were clinically stable in their asthma, and no significant changes in forced expiratory volume in 1 s (FEV₁) could be detected between month 1 and year 1 (Fig. 1). Peak expiratory flow (PEF) measurements obtained at the last visit (months 12–14) were also reported to be good (103; 84–173 PEF, percentage predicted value).

At the visit 1 year after the end of ESIT, a clinical evaluation showed that 12 of the 18 patients judged their asthma symptoms to be less or the same as when they were on Xolair treatment (Fig. 2A). None of the patients reported that their nightly asthma exacerbations had increased after the Xolair withdrawal (Fig. 2B); instead three reported less or no nightly attacks. Only four of the 18 patients reported an increase in asthma medication after Xolair withdrawal (Fig. 2C) but none of them needed oral steroids. One patient had one attack during the year but did not need to seek medical help.

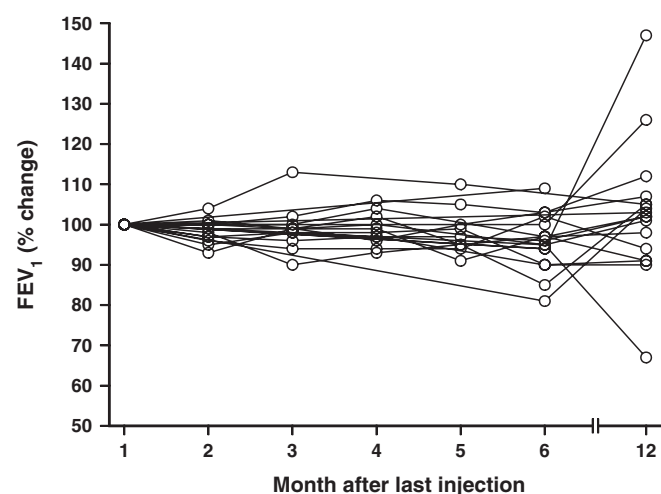


Figure 1. After 6 years with Xolair treatment, the medication was withdrawn and during the 12- to 14-month follow-up period, the patients' lung function was stable and no significant change in forced expiratory volume in 1 s was observed during the first year. Measures were performed on the 18 patients once a month during the first 6 months and a seventh measure was conducted approximately 12–14 months after withdrawal. The samples from each patient are linked.

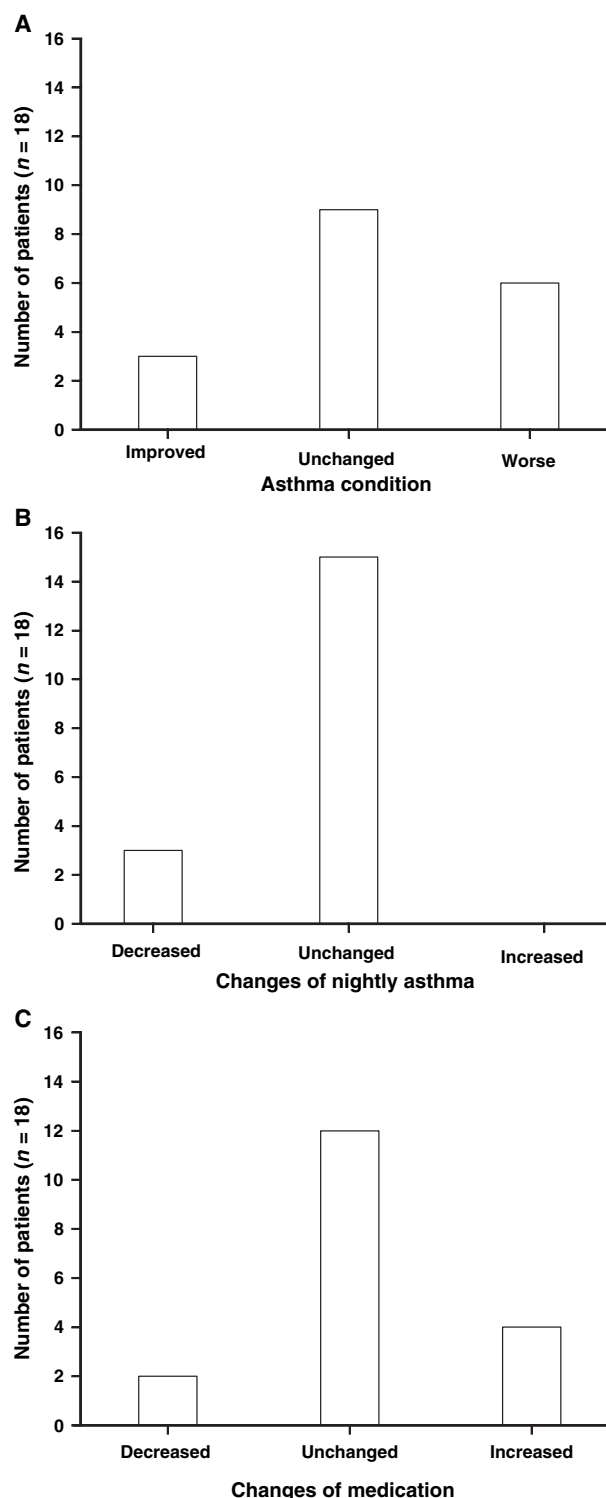


Figure 2. At 1 year visit after the end of immunoglobulin E-specific immunomodulation treatment, a clinical evaluation was performed by inviting the patients to answer a small questionnaire: 'Since you stopped the Xolair treatment 12 months ago: (A) How do you feel that your asthma has been? (B) Has your nightly asthma symptoms changed? (C) Has your asthma medication changed?'.

Basophil activity

When analysing basophils from the cat allergic ESIT-treated patients, it was found that during the 1-year follow-up period, the CD-sens to cat initially increased and at the most a 20 time rebound effect could be detected in samples taken for 4 months. Thereafter, CD-sens declined and at 1 year sampling six of 14 patients, one could not be accurately tested because of ongoing antihistamine treatment, had nonresponsive basophils (Fig. 3). Three mite allergic patients were not included in the CD-sens calculations as CD-sens values obtained with different allergens cannot be directly compared due to lack of standardization of allergen extracts. However, the CD-sens from all three mite allergic patients followed the same pattern as the cat allergic patients and they had only weakly positive CD-sens at 1 year sampling.

The cat allergic ESIT-treated patients had a significantly lower CD-sens at 1 month (0.10; 0.05–0.8; $P < 0.0001$), at 6 months (1.1; 0.2–3.8; $P < 0.001$) and at 1 year (0.3; 0–0.9; $P < 0.001$) after last Xolair dose when compared with the controls (6.8; 2.9–14.1; Fig. 4A). There was also a nearly significant difference between the ESIT-treated patients with active basophils and the controls (0.8; 0.4–3.3; $P = 0.06$) at the 1 year sampling.

CD-sens and clinical state

At 12 months, eight of the 15 cat allergic patients had a CD-sens < 0.4 , i.e. below the low quartile of the controls. During the withdrawal year, seven of these eight reported

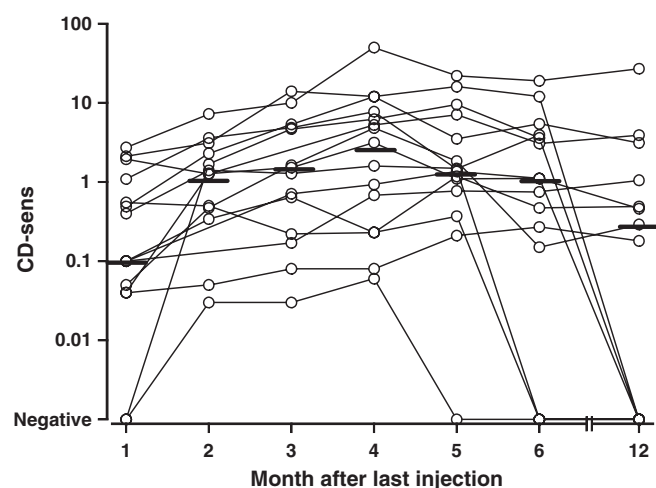


Figure 3. The CD-sens to cat was monitored during the 12- to 14-month withdrawal phase. During this period, a rebound effect could be detected, which reached at the most a 20-fold increase after 4 months and then declined over time. At each sample, the median is presented as a horizontal line. Samples were taken once a month during the first 6 months and a seventh sample was taken approximately 12–14 months after withdrawal.

less or the same asthma symptoms, none of them had increased nightly asthma attacks and seven were taking less or the same medication when compared to being on Xolair treatment.

Serological parameters

To be able to study whether ESIT has a direct effect on basophils or whether it influences serological mechanisms, a factor termed 'ABA' was calculated for cat allergy. There was a significantly higher level ($P < 0.05$) of ABA in 1 month sample (2.5; 1.9–4.5) from ESIT-treated patients when compared with that of the controls

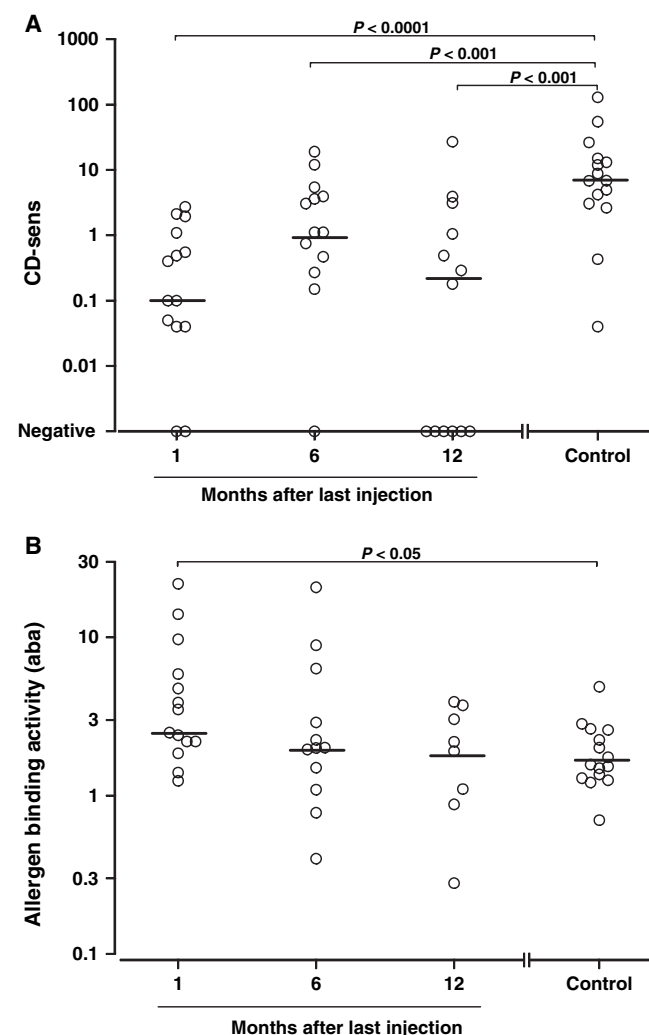


Figure 4. The CD-sens (A) and allergen-binding activity (ABA; B) was measured in samples from all cat allergic patients once a month during the withdrawal phase. Even 12–14 months after Xolair withdrawal, the CD-sens was significantly lower ($P < 0.01$) than that in the controls, whereas for ABA no significant change could be detected between immunoglobulin E-specific immunomodulation-treated patients and controls at any time point. At each sample, the median is presented as a horizontal line.

(1.6; 1.3–2.4). During the 6-month follow-up period, the ABA slightly decreased (2.0; 1.6–5.5) but was still higher than in the control group. Allergen-binding activity continued to decrease during the next 6–8 months and was as low as 2.1 (1.1–3.2) in the 1-year sample (Fig. 4B).

When comparing ABA with serum levels of IgG4 antibodies to cat, no significant correlation was found at any time point neither in the patients nor in the controls.

The level of IgG4 antibodies to cat was measured in serum samples collected during 1998/1999 before the start of the clinical trial and during 1-year withdrawal of Xolair. The levels of IgG4 antibodies to cat were the highest before Xolair treatment started and declined during treatment and the first 6 months of withdrawal and thereafter, a slight increase could be detected in the 1-year follow-up sample.

IgE on basophils and in serum

When analysing the number of IgE molecules on the basophils from the cat allergic patients (the mite allergic patients followed the same pattern), a significantly ($P < 0.0001$) lower number of IgE molecules was found in the 1-month sample from ESIT-treated patients (125 000; 72 000–179 000) when compared with the controls (644 000; 482 000–1 206 000). However, in samples taken at 6 and 12 months, this difference did no longer exist, 784 000; 623 000–1 050 000 and 1 037 000; 646 000–1 129 000, respectively, and the amounts of IgE on basophils were completely comparable to that of the controls.

In addition, the absolute levels of IgE antibodies to cat in ESIT-treated patients and in controls were measured. In the samples taken before Xolair treatment started, the absolute levels were lower (3.0; 1.7–6.9 kU_A/l) compared with those 1 month after last injection (7.0; 3.7–11.7 kU_A/l), most likely due to accumulation of IgE–omalizumab complexes with long half-time elimination. However, in the sample taken at 6 months after treatment, the levels had decreased (2.3; 1.5–7.6 kU_A/l) and were almost comparable to the levels in the controls (3.6; 1.5–7.2 kU_A/l) and this pattern continued in the sample taken at 12 months where a significant decrease was detected (1.9; 1.3–4.8 kU_A/l, $P < 0.05$). Interestingly, the relative concentration of IgE antibodies to cat, i.e. the percentage of IgE antibodies of IgE was not significantly different before, during or after Xolair administration.

Further, in the controls, in contrast to ESIT-treated patients at 1 year, there was a significant ($P < 0.05$) correlation between CD-sens and the relative, but not absolute, IgE antibody concentration to cat.

Discussion

A new approach to treatment of allergic disease was introduced during 2003 in the USA and during 2005 in Europe when a humanized, monoclonal antibody to IgE,

omalizumab, Xolair[®], became available. Injection of Xolair results in a rapid 99% decrease in free serum IgE within 3 days (2), followed by 99% and 90%, respectively, downregulation within 7 days of the high-affinity IgE receptor (FcεRI) on basophils (1, 3) and mast cells (3). As a result, IgE antibody-mediated allergic disease symptoms are diminished (4, 7, 13). Omalizumab is well tolerated and effectively prevents exacerbations in patients with severe, persistent asthma (14–16).

In the clinical trials, the patients were usually treated for a few months up to 1 year. When Xolair was withdrawn, the serum IgE levels and number of IgE receptors on the basophils returned to pretreatment values (5). Not surprisingly, also the clinical symptoms of asthma and allergic rhinitis usually return forcing the patient to start again with regular drugs, to increase the dosage of conventional drugs like antihistamines or local steroids and to seek medical help.

No study has reported the long-term effect of Xolair. In 1998, from Sweden, 22 patients were enrolled in the international omalizumab trial of severe allergic asthma, CIGE 0250011 followed by three extensions. The patients had perennial, allergic asthma that needed continuous treatment with rather high doses of local steroids. However, they were pleased with the effects of omalizumab and urged to be allowed to continue with the drug. This was accepted and they continued without any significant intervention until the summer of 2005, when Xolair became available by prescription in Sweden. It was then decided that before a decision was taken on prescribing the drug, the ongoing treatment should be closed and Xolair withdrawn.

This decision presented a unique opportunity to monitor clinical and immunological parameters during the withdrawal phase. Of the 22 patients, 18 accepted to participate in the withdrawal study. Cat and mite allergies were parts of the recruitment criteria and of the participants, 15 were cat and three were mite IgE sensitized. Basophil allergen threshold sensitivity, CD-sens (12), to both cat and mite were analysed but only cat CD-sens was used for further comparisons. The reason is that CD-sens of different allergens cannot generally be compared because of inadequate allergen extract standardization and, in addition, the number of mite allergic patients was too low. During the first 6 months after the last Xolair dosage, an expected increase in cat allergen sensitivity was seen. CD-sens peaked at 4 months, and was followed by a slow decrease but at 6 months, the mean value was still significantly higher than it was during treatment. However, CD-sens continued to decrease and at 12–14 months, seven patients had a CD-sens lower than that on treatment and six of them had nonreactive basophils. The median CD-sens of all 15 cat IgE-sensitized patients was significantly lower at 12–14 months than that of cat allergic patients, in general.

The plasma ABA decreased slightly during the follow up as did serum levels of IgG4 antibodies to cat allergen.

At 12–14 months, the median levels of ABA and IgG4 antibodies were lower than those of cat allergies. Thus, even if IgG4 antibodies could play a part in the decrease of sensitivity, as had been seen during ASIT (17), they may not be the most important factor in these patients. This is further supported by the finding that the serum levels of IgG4 antibodies to cat did not change much from pretreatment in 1998 to the last withdrawal sample despite a more generous attitude to cat contacts. It is possible that the cat IgE antibodies in the omalizumab–IgE immune complexes can also play a part in ABA.

The clinical evaluation was based on a physical examination consisting of spirometry with particular emphasis on FEV₁ and PEF values. Thus, FEV₁ was measured at each visit during the withdrawal and no decrease over time was seen in any but one patient.

At the final visit a year after the end of ESIT, the patients were asked to fill in a simplified symptom questionnaire with some of the questions used in the primary study during 1999. Of the 18 cat or mite allergic patients, 12 reported that their asthma had improved or not changed during the 12–14 months they had been without Xolair compared with when they were taking the drug. A CD-sens below the low quartile of the controls was found in eight of the 15 cat allergic patients. Seven of those eight reported less or the same asthma symptoms and seven less or unchanged nightly attacks and were taking less or the same medication. Of the eight patients reporting of asthma symptoms today compared with before treatment, i.e. 7 years ago, six were of the opinion that their symptoms now were less pronounced. Interestingly, three of the five patients reporting deterioration of their asthma had 1 year after the last injection improved

in FEV₁ and had nonresponsive basophils. Thus, other factors such as no longer being taken care of at the allergy clinic visits every 2–4 weeks, might influence the patient's experience of their symptoms and well-being.

Before the trial in 1998, 18 of 22 patients had detectable serum levels of IgE antibodies to cat. Interestingly, the concentration of IgE antibodies expressed in percentage of IgE was the same both before treatment and during treatment indicating that the IgE antibodies in the IgE–omalizumab complexes are accessible and that serum IgE antibody levels measured during Xolair treatment could be a clinically relevant expression of allergen exposure.

In conclusion, in this open long-term study, treatment of allergic asthma with Xolair allowed a significant proportion of patients, more than half of them, to stabilize their asthma with unchanged lung function, even 1 year after Xolair therapy was stopped. The mechanism seems to be mostly a downregulation of the basophil, and presumably mast cell reactivity perhaps combined with allergen-specific IgG4 antibodies. These most interesting findings need to be further studied. Allergy is mainly a disease of young people and the long-term effect of Xolair should be monitored in that group where an even better promotion of the natural disappearance of an IgE-mediated allergy can be expected.

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