

Dose dependence and time course of the immunologic response to administration of standardized cat allergen extract

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Background: The immunologic response to allergen immunotherapy with 3 serial 5-fold doses of cat extract has been studied after approximately 5 weeks of immunotherapy. The highest dose containing 15 µg of Fel d 1 produced the most consistent and favorable response. It is unknown whether the comparative response on reaching a maintenance dose is maintained with long-term maintenance therapy.

Objective: The purpose of this investigation was to evaluate the immunologic responses with these 3 serial doses of cat hair and dander extract at baseline, after reaching the maintenance dose (approximately 5 weeks), and after 1 year of maintenance immunotherapy.

Methods: Twenty-eight patients with cat allergy randomized in a double-blind study were assigned to one of 4 treatment groups: placebo or cat hair and dander extract containing 0.6 µg of Fel d 1, 3 µg of Fel d 1, and 15 µg of Fel d 1 at maintenance. Studies included skin prick tests and late cutaneous reactions with cat hair and dander extract, titrated nasal challenges with the extract, serum cat allergen-specific IgG4 and IgE measurement, and flow cytometric and ELISA analysis of whole blood and intranasal cytokines (TGF-β, IL-10, IFN-γ, IL-4, and IL-5).

Results: Twenty-six subjects completed the study. After both 5 weeks and 1 year, significant and dose-dependent differences were seen with total symptom scores on nasal challenge ($P < .0001$), with titrated skin prick testing with cat dander extract at 5 weeks ($P = .014$) and 1 year ($P < .0001$), and with cat-specific IgG4 measurement at 5 weeks ($P = .004$) and 1 year ($P = .003$). At 1 year, neither flow cytometry of whole blood nor ELISA evaluation of nasal cytokines demonstrated any significant differences among the treatment groups.

Conclusion: The response to titrated nasal allergen challenge,

titrated skin prick testing, and allergen-specific IgG4 measurement to cat immunotherapy at 5 weeks is predictive of the response at 1 year. (*J Allergy Clin Immunol* 2004;114:1339-44.)

Key words: Immunotherapy, cat immunotherapy, cluster, dose response, cat extract, immunologic response

Allergen immunotherapy is an effective form of treatment for both allergic rhinitis and allergic bronchial asthma.¹ High doses of standardized extracts, expressed in major allergen content of the extract, have been proved effective in treating patients sensitive to ragweed, timothy grass, house dust mites, and cat.¹ There are only a few studies that address the effectiveness of lower compared with higher doses of extract.

Franklin and Lowell² reduced maintenance doses of ragweed by 95% with significant loss of effectiveness. Haugaard et al³ found a maintenance dose of *Der matophagoides pteronyssinus* extract containing 0.7 µg of Der p 1 less effective than one containing 7 µg at reducing bronchial responsiveness to inhalation of the mite extract. A number of studies at Johns Hopkins found ragweed extracts containing 0.6 to 2 µg of the major allergen Amb a 1 to be less consistently effective than those containing concentrations between 6 and 12.4 µg.⁴⁻⁶ Double-blind studies with standardized cat allergenic extracts have demonstrated that maintenance doses containing 11.3 µg of Fel d 1,⁷ 13.8 µg of Fel d 1,⁸ 15.0 µg of Fel d 1,⁹ and 17.3 µg of Fel d 1¹⁰ reduced symptoms resulting from cat exposure. Maintenance doses of cat extract of this magnitude are not frequently used in clinical practice in the United States.¹

An earlier study assessed whether doses of standardized cat extract containing lower concentrations of Fel d 1 were as effective as those containing 15 µg of Fel d 1 at the time maintenance doses were first reached.¹¹ An additional purpose of the study was to examine a variety of immunologic outcomes to determine which were most sensitive in reflecting the short-term response to allergen immunotherapy.¹¹ There was an apparent dose response for suppression of skin prick test responses and increases in cat-specific IgG4 levels.¹¹ For each outcome, the changes with maintenance doses of cat dander extract containing 0.6 µg of Fel d 1 did not differ significantly from placebo, whereas those containing both 3 and 15 µg showed

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Abbreviations used

CRU: Clinical Research Unit
NJMRC: National Jewish Medical and Research Center

significant changes compared with placebo but were not significantly different from each other.¹¹ On the other hand, only the highest dose of cat extract induced a significant decrease in the percentage of CD4⁺ IL-4⁺ cells in the peripheral circulation.¹¹ The highest dose alone produced a decrease in the IL-4⁺/IFN- γ ratio, although this did not quite reach a significant level ($P = .08$).¹¹

Both skin test response suppression and increases in IgG4 levels are characteristically seen with successful immunotherapy.¹² There is some suggestion that changes in titrated skin prick test responses might be reflected in decreased symptoms during natural pollen exposure.¹² Shifts in relative T_H2 and T_H1 cytokine expression are currently regarded as a possible mechanism of the response to allergen immunotherapy. Furthermore, changes in expression of mRNA for these cytokines have correlated with clinical improvement with immunotherapy.¹³ Therefore the fact that these immunologic changes were observed only with the 15- μ g dose of Fel d 1¹¹ suggests that, at least in the short-term response, this is the only dose consistently inducing immunologic changes clearly associated with clinical improvement.

Past studies have examined the role of suppressor cells in specific immunotherapy.¹⁴⁻¹⁶ A uniform phenotype of suppressor cells remains unknown, but studies point toward CD4⁺CD25⁺ T lymphocytes.^{17,18} Recently, suppressor cells have been defined as T cells that suppress immune responses through cell-cell interactions and expression of inhibitory cell-surface molecules, production of IL-10 and TGF- β , or both.^{17,19,20}

The purpose of the present study was to examine those parameters that showed significant changes in the previous cat immunotherapy study in a new cohort of subjects ($n = 28$) and follow them during immunotherapy for a longer period of time (12 months). This study addressed the question of whether the immunologic responses measured on first reaching maintenance are reflective of long-term efficacy.

METHODS

Study design

This was a randomized, double-blind, placebo-controlled trial. The study was conducted at the National Jewish Medical and Research Center (NJMRC) Clinical Research Unit (CRU) after approval from the institutional review board and individual consent by the participants. The study took place between November 2002 and April 2004. Subjects with cat allergy were recruited through the CRU database and by advertisement. A total of 28 subjects were recruited. Subjects were required to be older than 18 years, to have a history of allergic rhinitis with or without asthma symptoms on cat exposure, to have a 5-mm or larger wheal on skin prick testing to 10,000 BAU/mL

cat hair and dander extract, to have an unchanged exposure to cats throughout the study, and to be in general good health.

Subjects were excluded if they were pregnant or not using appropriate birth control throughout the study; if they were taking controller medications for asthma (inhaled corticosteroids, long-acting β -agonists, and leukotriene modifiers); if their FEV₁ was 80% or less of predicted value; if they had received immunotherapy to cat dander, house dust, or unknown extracts in the previous 5 years; if they were taking and were unable to discontinue daily antihistamines or nasal corticosteroid or decongestant sprays; or if they had other nasal diseases that might interfere with the nasal challenges (eg, upper respiratory tract infection within 2 weeks of challenge or significant nasal polyposis). Patients who were taking β -blockers or monoamine oxidase inhibitors were also excluded.

In addition to spirometry and skin prick testing to cat hair and dander, subjects were also skin tested to tree pollen if any of their nasal challenge visits would occur during tree season in Denver (mid-February through April). Patients were scheduled so that they would not be experiencing seasonal allergic rhinitis symptoms during the evaluation days. There were 3 evaluation days: (1) baseline, (2) after the maintenance dose was reached (approximately 5 weeks after baseline), and (3) 1 year after the initiation of immunotherapy. On each of these days, patients performed spirometry, underwent a physical examination, had blood drawn, and underwent titrated nasal challenges, titrated skin prick tests, and intradermal tests for evaluation of late cutaneous reactions. For women, a serum pregnancy test was performed at each of these visits. A diary was given to each subject so that they could record any late reactions or changes in their general health between visits.

Immunotherapy

The immunotherapy doses were randomly assigned by the NJMRC pharmacy. Subjects were assigned to receive cat hair and dander extract containing either a high dose (15 μ g of Fel d 1), a medium dose (3 μ g of Fel d 1), or a low dose (0.6 μ g of Fel d 1) at maintenance, or they received placebo (Table I). The extracts were prepared by a registered pharmacist at the NJMRC Pharmacy. Ten thousand bioequivalent allergy units per milliliter of lyophilized cat hair and dander extract was used for this study and was provided by ALK Laboratories (Austin, Tex). Caramelized sugar and histamine were added to the placebo and the 2 lowest doses of cat extract so that they simulated the most concentrated extract in both color and tendency to produce local reactions.

The immunotherapy was administered in the CRU on a cluster regimen by a registered nurse.²¹ This schedule achieved maintenance dosing in 8 visits over a period of 4 weeks. Subjects then received their maintenance dose weekly for 4 weeks, then every other week for 2 visits, and finally monthly until approximately 1 year after they reached their maintenance dose (mean, 11 months; range, 7-12 months). This schedule was altered, if necessary, depending on subject tolerance.

Subjects were provided with loratadine 10-mg tablets and zafirlukast 20-mg tablets to take 2 hours before reporting for injections. They were required to wait in the CRU under observation for at least 30 minutes after the final injection of each visit. Subjects with asthma were required to measure their peak flow before injections and before leaving the CRU. A record was kept of all adverse events. Subjects were also given a diary to record any late adverse events or changes in their general health between visits.

Titration nasal challenge

Nasal challenge with cat hair and dander extract was performed at baseline, after reaching the maintenance dose of immunotherapy, and after 1 year of maintenance immunotherapy. Before starting the allergen challenge, 3 nasal saline washes were performed. The

TABLE I. Vial allergen content (cat dander extract containing Fel d 1 in micrograms per milliliter)

	High dose	Medium dose	Low dose	Placebo
Vial 4	0.03	0.006	0.0012	0
Vial 3	0.3	0.06	0.012	0
Vial 2	3.0	0.6	0.12	0
Vial 1	30	6	1.2	0

prechallenge nasal specimens were then collected, and patients were asked to score their baseline symptoms by using a modification of the Bousquet scoring system.²² The cat allergen extract nasal challenges were conducted under an Investigational New Drug application issued by the US Food and Drug Administration. The nasal challenge solutions were made by diluting the extract with Human Serum Albumin Saline (0.9% NaCl, 0.03% human serum albumin, and 0.4% phenol; Hollister-Stier Laboratories, LLC, Spokane, Wash). Serial 10-fold dilutions were made from 10,000 BAU/mL and 3333.3 BAU/mL to yield a total of 10 one-half log concentrations of cat allergen extract.

The nasal challenges were performed by squirting one tenth of a milliliter of solution into both nostrils at 10-minute intervals (metered pump nasal spray bottles; PharmaSource International, Inc, Centennial, Colo). The first dose contained saline, and the subsequent doses contained cat allergen extract in increasing one-half log concentrations from 0.3 BAU/mL to 10,000 BAU/mL. Patients were asked to score their symptoms on the basis of the Bousquet scoring system 10 minutes after each dose until a score of 5 was reached.²² At that point, an additional dose, containing the equivalent of 2 doses above the end point, was administered in each nostril. Early symptom scores were recorded at this point. The patient was then asked to score his or her symptoms every hour for 6 hours (late-phase symptoms), at which point the postchallenge nasal secretion specimens were collected. On subsequent challenges, the same doses of cat allergen extract administered at baseline were given.

Titration skin prick tests

Titration skin prick tests were performed in duplicate on the patient's back by using the same dilutions of cat allergen as the nasal challenges. Testing was conducted with increasing concentrations until a mean wheal of 5 mm was achieved, with at least one dilution above and one below the dilution that produced the 5-mm wheal.

Late-phase cutaneous response

Intradermal tests with the cat allergen were performed by using dilutions of the same doses used for the nasal challenges, with the starting dose at a 1:1000 dilution of the dose that produced the 5-mm wheal on titration skin prick testing. The injected dose was increased until a wheal of 15 mm in diameter was achieved. The reaction was read at 20 minutes and then again after 6 hours. On subsequent evaluation days, the same dose was used that initially produced the 15-mm wheal.

Nasal cytokine, serum TGF- β , and cat dander-specific IgE and IgG4 levels

Nasal sponge collection of secretions for cytokine analysis was similar to methods described elsewhere.^{23,24} ELISA kits from BD Pharmingen (San Diego, Calif) and R&D Systems (Minneapolis, Minn) were used to measure each nasal cytokine (IL-4, IL-5, and IFN- γ) and serum TGF- β levels. Allergen-specific IgE and IgG4

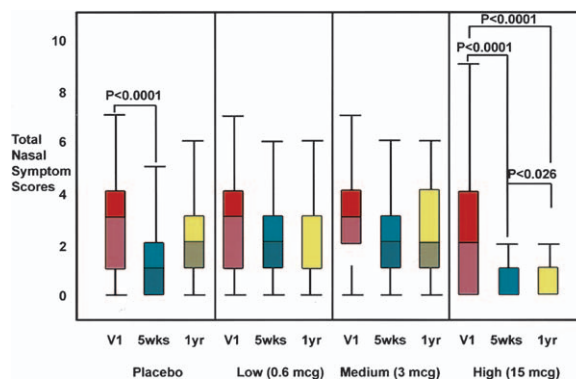


FIG 1. Total early symptom scores from all doses during titrated nasal challenge at baseline (V1), after 5 weeks, and after 1 year of immunotherapy. The bottom and top edges of the box are located at the 25th and 75th percentiles. The center horizontal line is the median. The bottom and top end of the whiskers show the 20th and 80th percentiles. A significant dose-response relationship exists between increasing dose and decreasing symptoms at 5 weeks ($P < .0001$) and 1 year ($P < .0001$).

were measured by Pharmacia CAP systems (Pharmacia Diagnostics, Uppsala, Sweden).

Analysis of CD4⁺ and CD4⁺/CD25⁺ cells

Intracellular detection of IL-4 and IFN- γ in CD4⁺ cells and IL-10 and TGF- β in CD4⁺CD25⁺ cells was performed by using flow cytometric analysis, as described elsewhere.^{25,26} Measurements were made with whole blood (incubated at both 4 hours and overnight), and the cells were not stimulated with antigen or mitogen.^{25,26}

Statistical analysis

The statistical package SAS version 8.2 (SAS Institute, Cary, NC) was used for all analyses. A nonparametric test, the Wilcoxon signed-rank test, was used to compare between visits for all the data. Adjustments for multiple comparisons were done by using the false discovery rate procedure. A linear regression model was used to test for dose-response relationships. A mixed effects linear regression model was used in which natural logarithm-transformed wheal size was used as the outcome variable and natural logarithm-transformed allergen concentration was used as the predictor variable to determine the allergen concentration that generated a wheal size of 5 mm. In this model measurements from the same subject were considered to be correlated, and measurements from different subjects were treated as independent. Statistical significance was claimed when a P value of .05 or less (2-tailed) was reached.

RESULTS

Demographics

Twenty-eight subjects were initially enrolled. One subject dropped out of the study, and one was lost to follow-up. Thus a total of 26 patients completed the study (6 in the placebo group, 7 in the low-dose group, 7 in the medium-dose group, and 6 in the high-dose group). Twenty-five of the subjects did not experience any systemic reactions and achieved maintenance therapy in 5 weeks. One subject had a systemic reaction consisting of

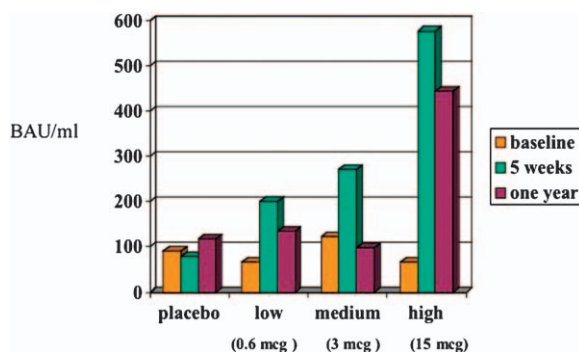


FIG 2. Titrated skin prick testing: mean cat allergen concentration (BAU/mL) at baseline, 5 weeks, and 1 year needed to induce a mean wheal size of 5 mm. The dose effect was significant after 5 weeks ($P = .014$) and after 1 year ($P < .0001$).

generalized pruritus after the administration of the first dose of vial 1. The reaction was relieved with oral diphenhydramine, and he achieved maintenance therapy in 6 weeks with no further reactions.

Titrated nasal challenge

At 5 weeks after the initiation of immunotherapy, both the placebo and high-dose groups had significant decreases in early symptom scores during nasal challenge with cat allergen extract ($P < .0001$, Fig 1). However, there was a significant dose-response relationship between dose and decreasing total symptom scores ($P < .0001$, Fig 1). Comparison of the response at 1 year revealed that only the high-dose group exhibited a significant decrease in total early symptom scores compared with baseline values ($P < .0001$, Fig 1) and compared with values at 5 weeks ($P = .026$, Fig 1). Again, there was a significant dose-response relationship ($P < .0001$, Fig 1). No significant changes in late-phase symptoms were seen (data not shown). It should be noted that only 17% of the subjects had a postchallenge 6-hour nasal symptom score of more than 2 out of a possible 18 points at baseline.

Titrated skin prick tests

After 5 weeks of immunotherapy, on the basis of linear regression, there was a statistically significant dose-response effect over all 4 treatment groups ($P = .014$), with the high-dose group exhibiting the largest increase in cat allergen concentration needed to produce a mean wheal of 5 mm (Fig 2). After 1 year, there was also a statistically significant dose-response relationship ($P < .0001$, Fig 2). Mean group responses were not significantly different from baseline. The change in the high-dose group did reach a P value of .063 at both 5 weeks and 1 year. To address the question of whether some individuals might not show a response at 5 weeks but would at 1 year, we examined the individual responses to the titrated skin prick tests. Of the 20 subjects who received active cat extract, the greatest increase in allergen dose required to produce a wheal of 5 mm was achieved after 5 weeks in 16 patients. In the 4 patients who showed a continued increase in

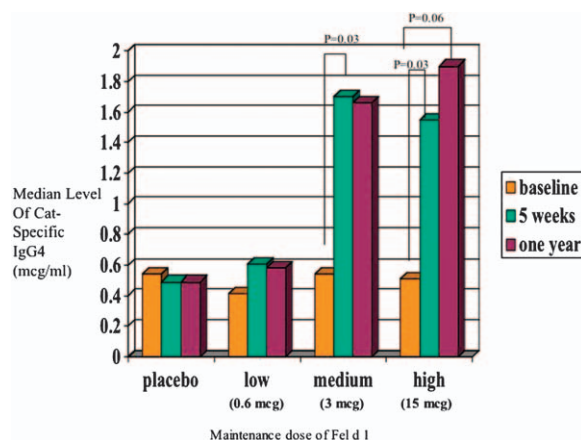


FIG 3. Median levels of cat-specific IgG4 ($\mu\text{g/mL}$) at baseline, after 5 weeks, and after 1 year of immunotherapy. The overall dose effect at 5 weeks is significant ($P = .004$), as are the changes with 3 and 15 μg ($P = .03$). After 1 year, the overall dose effect is significant ($P = .003$).

allergen dose at 1 year, all had between a 2.5- and 4.4-fold increase in allergen dose by 5 weeks, and only one (in the high dose group) had a greater than 2-fold further increase in allergen dose at 1 year.

Late-phase cutaneous response

There was a decreased late-phase cutaneous response in all 4 treatment groups at 5 weeks, with the placebo, low-dose, and medium-dose groups achieving statistical significance ($P = .02$, $P = .03$, and $P = .02$, respectively; data not shown). At 1 year of therapy, all groups demonstrated decreased late-phase responses; however, only the medium-dose group achieved statistical significance ($P = .031$, data not shown).

Serum antibodies

After 5 weeks of treatment, cat hair extract immunotherapy led to a significant dose-dependent increase in cat allergen-specific IgG4 levels ($P = .004$). The median IgG4 levels increased most significantly in the medium- and high-dose groups ($P = .03$, Fig 3). At 5 weeks, cat-specific IgE levels increased significantly in only the high-dose group ($P = .03$, data not shown). After 1 year of treatment, there was also a significant dose-dependent increase in allergen-specific IgG4 levels ($P = .003$, Fig 3). Although levels of cat-specific IgE decreased in the medium- and high-dose groups, there were no significant changes compared with baseline values (data not shown).

Nasal cytokines

After 5 weeks, there were no significant changes from baseline in IL-4, IL-5, and IFN- γ levels. However, there was a significant dose effect for an increased IFN- γ /IL-4 ratio ($P = .007$, data not shown). After 1 year, there were no significant changes in IL-4, IFN- γ , or IFN- γ /IL-4 values compared with baseline (data not shown). There was a decrease in IL-5 levels in the low- and medium-dose

groups after 1 year of therapy ($P = .016$ and $P = .031$, respectively; data not shown). IL-10 and TGF- β levels were less than the limits of detection in the nasal secretions.

Flow cytometry and serum TGF- β levels

After 5 weeks and 1 year, there was no significant increase in the relative number of CD4⁺/CD25⁺ cells or an increase in intracellular staining for TGF- β or IL-10 in these cells (data not shown). There were also no significant differences in serum TGF- β levels between the active treatment groups and the placebo group (data not shown) or consistent changes in the IFN- γ /IL-4 ratio in CD4⁺ cells.

DISCUSSION

The beneficial effects of immunotherapy could be the result of several possible mechanisms. One hypothesis is the induction of blocking antibodies, and IgG4 has been postulated to be such an antibody.²⁷ Our results showed a significant increase in IgG4 levels in the medium- and high-dose groups with a significant dose response. The significant dose response persisted after 1 year of therapy. In venom immunotherapy the development of venom-specific IgG antibody has been shown to correlate with clinical efficacy. This has not been the case with other forms of immunotherapy.²⁷ Increases in IgG4 levels have been found to be primarily an indication of stimulation of the immune system and generally have not been thought to correlate with symptomatic response. Another possible mechanism by which immunotherapy is effective is an overall decrease in allergen-specific IgE levels.²⁸ Our study demonstrated an increase of cat-specific IgE after 5 weeks of immunotherapy, and this agrees with previous studies showing an initial increase followed by a decrease in allergen-specific IgE levels after years of immunotherapy.²⁸ Bousquet et al²⁹ reported a correlation between the end point of titrated skin prick tests, titrated nasal challenges, and nasal symptoms during natural pollen exposure. In our study titrated skin prick test responses showed a significant dose-response effect both on reaching maintenance and after 1 year of immunotherapy. This correlated with the findings at 5 weeks in the previous study of immunotherapy with cat dander extract.¹¹

A more recent postulated mechanism for the response to immunotherapy is the induction of regulatory T cells, such as CD4⁺/CD25⁺ T cells. Previous studies, including those with grass pollen and bee venom, have shown up-regulation of these cells after immunotherapy, as well as increased levels of TGF- β , IL-10, or both.^{17,18} However, these studies used allergen-stimulated PBMCs. To our knowledge, our study is the first of its kind to use unstimulated whole blood. We wanted to look at CD4⁺/CD25⁺ cells directly *ex vivo* and evaluate the production of TGF- β and IL-10. We found that CD4⁺/CD25⁺ cells were not increased after 5 weeks or 1 year of immunotherapy. This is in agreement with others who studied

Fel d 1 peptide immunotherapy and showed no significant increase in CD4⁺/CD25⁺ cells.³⁰ In addition, we found no increase in intracellular staining of TGF- β or IL-10.

A shift from predominantly T_H2 cytokines (eg, IL-4 and IL-5) to T_H1 cytokines (eg, IFN- γ) during immunotherapy is another potential mechanism.³¹ A previous study of cat immunotherapy showed a significant decrease in the percentage of CD4⁺/IL-4⁺ cells in mitogen-stimulated PBMCs in the high-dose group.¹¹ We did not observe any consistent shift in CD4⁺ IFN- γ /IL-4 ratio. A limitation of our study is that we did not stimulate the blood cells with specific antigen, as has been done in most previous studies. There were no significant changes in nasal cytokine levels 6 hours after nasal allergen challenge, which is similar to the findings of the previous immunotherapy study with cat dander extract.¹¹ The failure to see significant changes in nasal cytokine levels might reflect the fact that the subjects did not demonstrate a significant late phase in nasal symptoms.

The results of this study show a significant clinical dose-response effect to cat hair and dander extract immunotherapy. The immunotherapy demonstrated an immunologic response in the medium- and high-dose groups. However, the high-dose group exhibited the most significantly and consistently improved response. This response was evident on reaching maintenance after 5 weeks and remained essentially unchanged after a year of maintenance immunotherapy, whether examining treatment group means or individual subject changes in titrated skin prick tests. Thus our study results are similar to the previous study of the dose response to cat immunotherapy in terms of antibody response and skin prick testing.¹¹ This suggests that the relative response to increasing doses of allergen extract is manifest on initially reaching maintenance therapy and that short-term studies might suffice to establish effective doses for other allergen extracts in the future. The results also suggest that titrated skin prick testing might be used in a clinical setting to assess adequate response to immunotherapy. The limiting factor for this use is the need for extracts of reproducible potency. In the short term this might be accomplished by making fresh dilutions of the maintenance extract. In the longer term it will depend on the availability of standardized extracts for those allergens.

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