

A double-blind, placebo-controlled immunotherapy dose-response study with standardized cat extract

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Background: Allergen immunotherapy with doses of cat extract containing approximately 15 µg of the major allergen, Fel d 1, have been proved clinically effective in several double-blind, placebo-controlled studies. However, the maintenance doses used in allergy practice in the United States are often considerably less than this proven dose.

Objective: The purpose of this investigation was to determine whether maintenance immunotherapy with cat dander extract containing 0.6 µg or 3.0 µg of Fel d 1 was more effective than placebo and similar in efficacy to treatment with extracts containing 15.0 µg Fel d 1, immunologic parameters being used as the outcome.

Methods: Twenty-eight cat-allergic patients were randomly entered, 7 in each group, into a double-blind, placebo-controlled comparison of the immunologic response to treatment with placebo or cat dander extract containing 0.6 µg, 3.0 µg, or 15.0 µg of Fel d 1. Maintenance doses were achieved in 8 visits over a period of 4 weeks through use of a cluster regimen; each subject then received 1 weekly maintenance injection before posttreatment measurements were made. The response to immunotherapy was assessed before immunotherapy and after the first weekly maintenance injection. Studies included responses to titrated skin prick tests to cat extract and an unrelated allergen and serum allergen-specific IgE and IgG₄. Titrated nasal challenges were performed with cat extract; measurement of mRNA and secreted cytokines (IL-4, IL-5, and IFN-γ) was done at 6 hours. Serum cytokines (IL-4, IL-5, IFN-γ) were measured, and flow cytometric analysis of intracellular cytokines (IL-4, IL-5, IFN-γ) was performed. Cat allergen-stimulated lymphocyte proliferation was performed with measurement of cytokines in the supernatant (IL-4, IL-5, IFN-γ).

Results: All 28 subjects completed the study. Significant and dose-dependent differences were encountered in the titrated skin prick tests ($P = .008$), the cat-specific IgG₄ ($P = .01$), and the reduction in CD4⁺/IL-4⁺ PBMCs on flow cytometry ($P = .03$). There were no significant differences between placebo and cat dander extract containing Fel d 1 0.6 µg. Both extracts containing 3.0 µg and 15.0 µg produced significant decreases in skin prick test sensitivity ($P = .02$ and $P = .002$, respectively). The extracts containing 3.0 µg and 15.0 µg produced significant

increases in cat-specific IgG₄ ($P = .01$ and $P = .006$, respectively). Only the 15.0-µg-per-dose extract produced a significant reduction in the percent of CD4⁺/IL-4⁺ PBMCs ($P = .003$).

Conclusion: In this double-blind, placebo-controlled study, a maintenance dose of cat dander extract containing 15.0 µg Fel d 1 produced the most consistent immunologic response. A dose of 3.0 µg reduced skin prick test sensitivity and increased cat-specific IgG₄ but did not reduce the circulating CD4⁺/IL-4⁺ PBMCs, a change that is likely related to the clinically significant response to allergen immunotherapy. These findings suggest that a maintenance dose of 15.0 µg of Fel d 1 is most apt to be clinically effective for allergen immunotherapy. (*J Allergy Clin Immunol* 2003;111:155-61.)

Key words: 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 extracts, have been proved effective in treating patients sensitive to ragweed, timothy, house dust mites, and cat.¹ However, many certified allergists practicing in the United States do not prescribe regimens that contain the amounts of major allergen that have been identified as being effective.¹ Only a few studies have addressed the effectiveness of various doses of extract. Franklin and Lowell² reduced maintenance doses of ragweed by 95%, with significant loss of effectiveness. Hauge et al³ found a maintenance dose of *Der-matophagoides pteronyssinus* extract containing 0.7 µg Der p 1 to be less effective at reducing bronchial responsiveness to inhalation of the mite extract than a dose containing 7.0 µg. A number of studies at Johns Hopkins found ragweed extracts containing 0.6 to 2.0 µg of the major allergen Amb a 1 to be less consistently effective than extracts containing concentrations between 6.0 and 12.4 µg Amb a 1.⁴⁻⁶

Previous double-blind studies with standardized cat allergenic extracts have demonstrated that maintenance doses containing 11.3 µg Fel d 1,⁷ 13.8 µg Fel d,⁸ 15.0 µg Fel d 1,⁹ and 17.3 µg Fel d 1¹⁰ reduced symptoms related to cat exposure. Maintenance doses of this magnitude are not frequently prescribed in clinical practice in the United States.¹

In this study, we assessed whether maintenance doses of standardized cat extract containing lower concentrations of Fel d 1 were equally effective. We also examined a variety of immunologic outcomes to determine which were most sensitive in reflecting the short-term response to allergen immunotherapy.

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TABLE I. Vial allergen content

Vial no.	Fel d 1 ($\mu\text{g/mL}$)			Placebo
	High dose	Medium dose	Low dose	
4	0.03	0.006	0.0012	0
3	0.3	0.06	0.012	0
2	3.0	0.6	0.12	0
1	30	6	1.2	0

TABLE II. Immunotherapy dosing schedule

Visit and dose (mL)	Vial no.
1	
0.10	4
0.40	4
0.10	3
2	
0.20	3
0.40	3
0.07	2
3	
0.10	2
0.15	2
0.25	2
4	
0.35	2
0.50	2
5	
0.07	1
0.10	1
6	
0.15	1
0.20	1
7	
0.30	1
0.40	1
8	
0.50	1
9	
0.50	1

Abbreviation used

BAU: Bioequivalent allergy units

METHODS**Subjects**

Adult subjects with allergic rhinitis and positive skin prick test results to cat hair extract were enrolled in the study. Each of the subjects had a history of allergic rhinitis with or without asthma symptoms on exposure to cats or had perennial symptoms and close exposure to cats. Skin prick tests, performed with a small pox needle (Hollister, Stier, Spokane, Wash), were ≥ 5 mm in diameter with cat extract 10,000 bioequivalent allergy units (BAU) per milliliter (Hollister Stier). Each subject had an FEV₁ of $\geq 80\%$ predicted, and no subject had a history of persistent asthma or regular control medication for asthma. Each subject's level of cat exposure was constant for 1 month before enrollment in the study and throughout the study duration. No subject had received immunotherapy with cat extract, house dust extract, or unknown allergens during the 5 years prior to

the study. Antihistamines were prohibited 7 days before skin testing or nasal challenge studies, and nasal sprays (steroids, decongestants) were prohibited 30 days before and throughout the study.

The Institutional Review Board of National Jewish Medical and Research Center approved the study. All subjects signed approved consent forms before participating.

Study design

Subjects were randomly assigned, 7 to each group, to receive 1 of 3 extracts containing cat hair and dander extract or matching placebo. At the maintenance injection of 0.5 mL of the active extracts, each subject received a dose of Fel d 1 of 0.6 μg , 3.0 μg , or 15.0 μg as a dilution in albumin saline solution of standardized cat hair extract 10,000 BAU/mL (ALK-Abelló, Wallingford, Conn). Fel d 1 content was obtained from the manufacturer. The 10,000-BAU/mL extract used in the study contained 64 μg of Fel d 1/mL; therefore, the highest dose (15 μg) was equivalent to 2,344 BAU of this extract.

The concentrations of extract in the 3 active treatment groups are shown in Table I. Placebo and 0.6 μg and 3.0 μg maintenance extracts were colored with caramelized sugar (prepared by the National Jewish Medical and Research Center Pharmacy) and contained small amounts of histamine to mimic the color and reactions of the concentrated extract. Each subject received 0.5 mL as a maintenance injection from vial 1.

Injections were administered by a cluster protocol over a period of 5 weeks (Table II). Injections were administered twice weekly for 8 visits; these were followed by an additional injection 1 week later. Progression was accomplished with 3 injections at 30-minute intervals for the first 3 visits, 2 injections at 30-minute intervals for the next 4 visits, and a single injection at the eighth visit.

Subjects received loratadine 10 mg approximately 2 hours before each injection visit to reduce the risk of local and systemic reactions.¹¹

Immunologic testing

Each subject underwent immunologic testing during the 7 days before the initiation of immunotherapy and again within 7 days after the ninth visit. All injections and testing were performed double-blind.

Skin prick testing

Endpoint titration for skin prick testing was carried out for cat hair extract and another allergen to which the subject reacted (either standardized short ragweed 1:10 w/v or timothy 100,000 BAU/mL). Twelve serial, approximately half-log dilutions were used: cat hair and dander, dilution 1 containing 0.01 BAU/mL and dilution 12 containing 3,000 BAU/mL; short ragweed, from 1:300,000 w/v to 1:10 w/v; and timothy, from 3 to 100,000 BAU/mL. Mean wheal diameters (each the average of the largest diameter and the corresponding midpoint perpendicular diameter) were recorded. The endpoint was the dilution that produced a 5-mm mean wheal diameter, as determined by extrapolation on a plot of dilution versus mean wheal diameter. All negative controls were <3 mm in mean diameter.

Nasal challenges

Nasal provocation was performed through use of the scoring system of Bousquet et al¹² and Lack et al¹³ on the basis of symptom scores for nasal blockage, pruritus, sneezing, rhinorrhea, and conjunctivitis. Tenfold dilutions of lyophilized cat hair extract (ALK-Abelló) were instilled into both nostrils at 15-minute intervals until a symptom score of 5 was obtained.

Nasal cytokines

Specimens were obtained at baseline and 6 hours after nasal allergen challenge with cat extract. At the designated time, the patient blew his nose; 3 filter papers (Whatman No. 42; 7 $\times$ 30 mm) were

TABLE III. Patient characteristics at baseline

	Placebo	Fel d 1 (μg)			P value
		0.06	3.0	15	
Age (y): mean ± SD	34.3 ± 12.2	35.0 ± 6.5	35.1 ± 7.9	37.3 ± 8.8	NS
No. of patients					
Sex: M/F	2/5	2/5	1/6	1/6	NS
Current smoking	1	0	0	0	NS
Cat in home	2	1	2	2	NS
Asthma (no. of patients with data available)	2 (5)	3 (7)	6 (7)	5 (6)	NS
Cat SPT (threshold dilution): median (interquartile range)	10 (1)	12 (2)	11 (1)	11 (1)	NS
Cat IgE (kU _a /L): median (interquartile range)	2.66 (15.15)	1.19 (1.19)	3.67 (8.00)	3.52 (5.05)	NS
Cat IgG ₄ (mg/L): mean ± SD	794 ± 398	642 ± 620	358 ± 154	473 ± 288	NS
CD4 ⁺ /IL-4 ⁺ (percent of CD4 ⁺): median (interquartile range)	21.6 (15.3)	14.9 (10.4)	13.6 (7.1)	34.0 (21.4)	.009*

NS, Not significant; M, male; F, female; SPT, skin prick test.

*Among groups (Kruskal-Wallis).

then placed in the anterior portion of each nostril, as described by Alam et al¹⁴ and Sim et al.¹⁶ These papers remained in place until wet (usually <5 minutes). The filter papers were then air-dried, sealed in a 2-mL microfuge tube, and frozen at -80°C for up to 2 months.

Nasal scraping were obtained through use of Rhino-probes (Arlington Scientific, Springville, Utah) and immediately immersed in RNAlater (Ambion, Austin, Tex); they were then stored at 4°C for <1 month.

Cytokine protein levels were determined by eluting the filter papers into a total of 1200 μL of buffer. The elution buffer was as described by Alam et al¹⁴ and Sim et al.¹⁶ Specimens were rocked overnight at 4°C. Levels of IL-5 and IFN-γ were determined through use of commercially available kits from BD PharMingen (San Diego, Calif). IL-4 was determined through use of the high-sensitivity immunoassay kit from R&D Systems (Minneapolis, Minn).

Total RNA was isolated from homogenized samples with the RNeasy kit (Qiagen, Valencia, Calif) and treated with DNase (Qiagen). Reverse transcription with random hexamers was carried out within 1 week, as previously described, and relative amounts of mRNA for IL-4, IL-5, and IFN-γ were determined through use of Pre-Developed Assay Reagents (Applied Biosystems, Foster City, Calif).^{17,18}

Cat-specific immunoglobulin measurements

Serum was obtained before and after completion of immunotherapy. Undiluted samples were analyzed for allergen-specific IgE by means of the Pharmacia CAP system (Pharmacia Diagnostics, Uppsala, Sweden). Cat-specific IgG₄ was assayed through use of diluted 1:1000 serum with Pharmacia CAP system-specific IgG₄ FEIA (Pharmacia Diagnostics).

Lymphocyte proliferation assay

PBMCs were isolated and cultured as previously described.¹⁹ Briefly, PBMCs were isolated by density centrifugation through use of Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, NJ). The cells were resuspended at 1 × 10⁶ cells/mL in RPMI 1640 (Bioproducts, Walkersville, Md) supplemented with 10% FBS (Gemini Bioproducts), L-glutamine (200 mmol/L), penicillin (10,000 U/mL), and streptomycin (10,000 μg/mL). Cells were cultured for 7 days at 37°C in 5% CO₂ in the presence and in the absence of tetanus (250 ng/mL), increasing concentrations of ALK-Abelló lyophilized cat extract (1, 10, 100, 1000, or 5000 BAU/mL), and diluent controls. Cultures were then pulse-labeled with tritiated thymidine for 6 hours and harvested onto glass fiber disks to evaluate [3H]-thymidine incorporation in counts per minute. Stimulation indices were calculated. Each result was expressed as the change in cat extract dilution

provoking maximal stimulation index. Aliquots of cell culture supernatants were stored at -20°C until further analysis was done.

Serum and PBMC culture supernatant cytokine assays

Cell culture supernatants were assayed through use of sandwich enzyme immunoassay kits for the following cytokines: IL-4 (R&D Systems; detection limit, 0.24 pg/mL) and IL-5 and IFN-γ (both Opteia-Pharmingen, Los Angeles, Calif; detection limits, 7.8 pg/mL). Sera were analyzed for TGF-β (R&D Systems) as well as IL-4 and IFN-γ.

Flow cytometric analysis

PBMCs were stimulated with phorbol myristate acetate 25 ng/mL and ionomycin 1 μg/mL. Cells were permeabilized. Staining for surface markers and intracellular cytokines was performed through use of standard Becton Dickinson (Franklin Lakes, NJ) protocol and reagents. Mouse monoclonal antibodies included anti-IFN-γ conjugated to fluorescein isothiocyanate, anti-IL-4 conjugated to phycoerythrin, and anti-CD4 conjugated to peridin chlorophyll protein. Cells were analyzed by flow cytometry (FAC-Scan), and the frequency of cytokine-producing cells in subpopulations was calculated.

Statistical analysis

Dose-related responses in flow cytometry, lymphocyte proliferation, and skin prick tests were evaluated by 1-way ANOVA. Each dose-related response was compared with the placebo response through use of a 2-sample *t* test.²⁰ Dose-related responses in serum cat IgG₄ were evaluated through use of the Kruskal-Wallis test; each dose-related response was compared with the placebo response through use of the Wilcoxon rank-sum test.²¹ Multiple comparisons were controlled for through use of the false-discovery rate procedure.²² In each analysis, achieved significance (*P*) levels less than .05 were considered to be statistically significant.

RESULTS

Demographics

Twenty-eight subjects were enrolled, and all of them completed the study. Their demographic information and baseline values are given in Table III. With the exception of the percent of CD3⁺ PBMCs staining positive for IL-4, there were no significant differences among the 4 treatment groups at baseline.

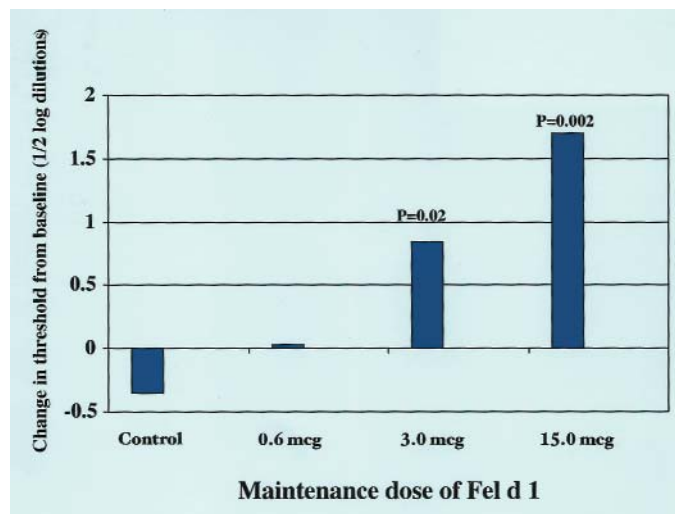


FIG 1. Changes in titrated skin prick test results. Shown are the changes in threshold of titrated skin prick tests to cat allergen extract from before immunotherapy to after the first maintenance injection. The overall dose effect is significant ($P = .008$), as are the changes with 3.0 μg ($P = .02$) and 15.0 μg ($P = .002$).

TABLE IV. Immunologic responses to cat immunotherapy

Immunotherapy dose	Titrated skin prick tests		Flow cytometry		Cat-specific IgG ₄	
	Mean change (half-log dilutions)	SD	Mean decrease in CD4 ⁺ /IL-4 ⁺ PBMCs	SD	Median change (mg/L)	Interquartile range
Placebo	-0.35	0.69	-4.2%	13.5	-4	5
0.6 μg	0.03	0.94	1.86%	5.25	144	240
3.0 μg	0.84 ($P = .02$)	0.86	2.92%	6.00	779 ($P = .01$)	1555
15.0 μg	1.70 ($P = .002$)	0.92	-11.4% ($P = .003$)	8.00	729 ($P = .006$)	2143

Immunotherapy injections

All subjects completed the projected 9 visits, and no modifications were required in the projected injection schedules. There were no systemic reactions, and only 1 patient experienced repeated large local reactions.

Skin prick testing

Endpoint skin prick titration to cat and either short ragweed or timothy was carried out before and after immunotherapy. Changes in dilution (the higher numeral, the higher the concentration) of cat hair extract eliciting positive test results in treatment groups were compared with those in the placebo group (Table IV and Fig 1). A significant dose-dependent decrease in skin reactivity was observed across all groups ($P = .008$). Statistically significant decreases in skin prick test sensitivity to cat were achieved only in the 3.0- μg ($P = .02$) and 15.0- μg ($P = .002$) Fel d 1 groups. Mean changes were an increase of 0.84 dilutions and an increase of 1.70 dilutions, respectively. There were no significant changes in endpoint skin titration to short ragweed or timothy in any group.

Serum antibody test

Cat hair extract immunotherapy led to a dose-dependent trend for increased allergen-specific IgG₄ ($P = .01$; Table IV and Fig 2). The mean change in comparison with placebo was statistically significant in the 3.0- μg ($P = .01$) and 15.0- μg ($P = .006$) Fel d 1 groups. No significant changes were found in allergen-specific IgE levels.

Flow cytometric analysis

At baseline, CD4⁺ PBMCs ranged from 42.3% to 45.73% in the 4 groups, the differences being nonsignificant. However, the percentage of CD4⁺ PBMCs also staining for IL-4 did vary significantly, the highest percent being seen in subjects in the 15- μg group (Table III). Cat hair extract immunotherapy led to a dose-dependent trend for a decrease in the percent of CD4⁺/IL-4⁺ cells ($P = .03$). A statistically significant decrease in IL-4-producing CD4⁺ cells was found only in the 15.0- μg treatment group (mean decrease, 11.4%; $P = .003$; Table IV and Fig 3). There was no statistically significant change in the proportion of CD4⁺ cells with IFN- γ or in IL-4/IFN- γ .

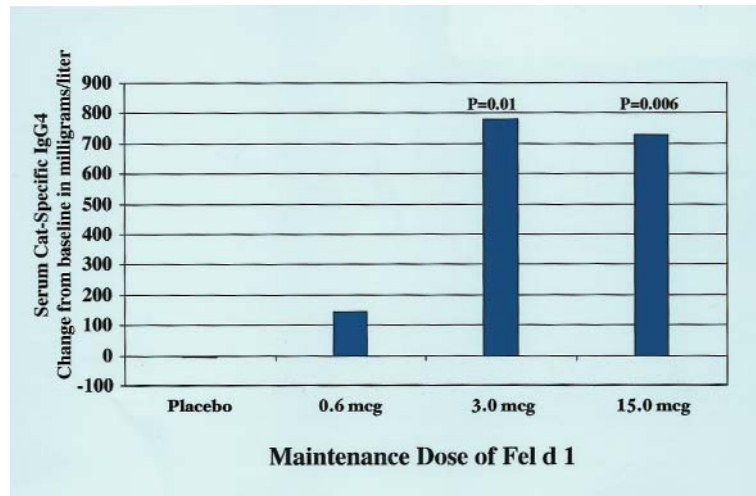


FIG 2. Increases from baseline to after the first maintenance injection in serum cat allergen-specific IgG₄. The overall dose effect is significant ($P = .01$), as are the changes with 3.0 μ g ($P = .01$) and 15.0 μ g ($P = .006$).

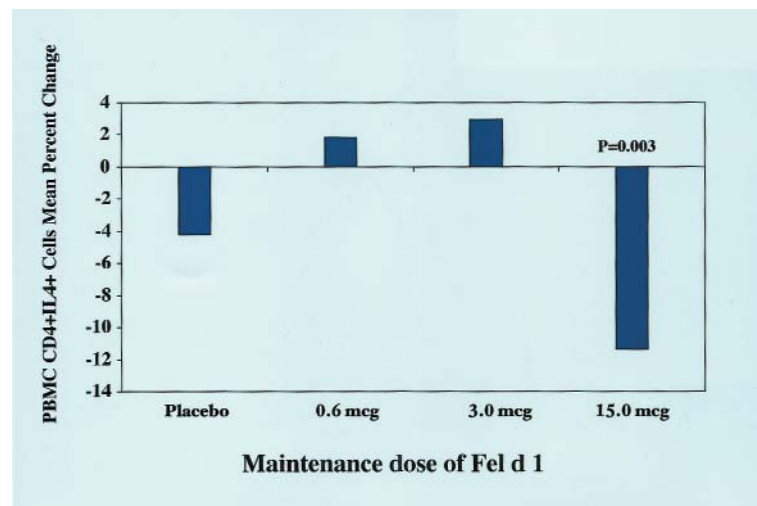


FIG 3. Changes from baseline to after the first maintenance injection in the percent of PBMCs positive for CD4 and IL-4 (by flow cytometry). The overall dose effect is significant ($P = .03$), as is the result with 15.0 μ g ($P = .003$).

Other results

There were no significant differences in the threshold values for nasal allergen challenges or PBMC proliferation assays. Statistical significance was not achieved in the cytokines that were assayed. Most levels of IL-4 and IFN- γ in serum samples were below the limits of detection, as were most levels of IL-4 and IL-5 in lymphocyte culture supernatants.

DISCUSSION

This is, to our knowledge, the first assessment of a dose-response to immunotherapy with standardized cat hair and dander extract. The dual purpose of the study was (1) to determine whether maintenance doses of extract containing less than 15.0 μ g Fel d 1, which are

often used in clinical practice, induce immunologic responses similar to those seen with the higher dose and (2) to determine what outcome parameters would be most effective in monitoring the short-term immunologic response to immunotherapy. The results clearly showed a difference in the level of response depending on the dosage of cat allergen extract administered.

Three of the immunologic assessments demonstrated an overall dose effect over the 4 treatment groups. There was an overall suppression of skin prick test reactivity to cat, increasing with increasing doses of cat extract ($P = .008$); there was an overall increase in cat-specific IgG₄ with increasing doses of cat extract ($P = .01$); and there was a dose-effect suppression of peripheral blood CD4⁺/IL-4⁺ T cells ($P = .03$).

Suppression of the immediate cutaneous reaction to allergen extract is a well-recognized response to allergy

immunotherapy.^{13,23,24} It has been reported after administration of immunotherapy by conventional,²³ semi-rush,²⁴ and rush¹³ schedules. The endpoint of titrated skin prick tests performed after immunotherapy has been shown to correlate with both the threshold of titrated nasal challenge²⁴ and the nasal symptoms reported by the patient during the corresponding pollen season²⁴ and to be a predictor of the duration of clinical remission after discontinuation of allergen immunotherapy.²⁵ In our study there was a significant overall effect of increasing doses of cat extract to produce increasing skin prick test suppression. The changes with 0.6 µg Fel d 1 did not differ significantly from what was seen with placebo, whereas both the 3.0-µg and 15.0-µg groups showed significant changes from placebo and were not significantly different from one other.

Increases in the serum levels of allergen-specific IgG₄ are regularly reported with successful immunotherapy.²⁶⁻²⁸ The significance of these increases in relation to the clinical response is less clear. Nakagawa et al²⁷ reported that patients with perennial rhinitis who responded to immunotherapy with house dust mites showed a statistically significant increase of IgG₄ in comparison with patients who did not respond. They also reported a weak ($r = 0.31$) but significant correlation between the increase in IgG₄ antibodies and clinical improvement. Gehlhar et al²⁶ did not observe any correlation between the amount of IgG₄ antibodies and reduction of symptoms to grass pollen; they did, however, find that the ratio of allergen-specific IgG₄ to IgG₁ significantly correlated with symptomatic improvement. Djurup and Osterballe²⁸ measured specific IgG₄ during the course of immunotherapy with grass pollen extract; they also reported that seasonal symptom scores were not related to preseasonal levels of specific IgG₄. Indeed, they reported that an early rise in IgG₄ above 8.0 U/mL was predictive of a poor clinical result at the end of immunotherapy. In our study, there was an overall significant increase in cat-specific IgG₄ antibodies, with significant increases in the 3.0-µg and 15-µg dosing groups.

Recently there has been interest in the effect of immunotherapy on the balance between T_H1 and T_H2 responses.²⁹ In a long-term study of immunotherapy in grass-allergic patients, the late response to antigen stimulation showed an increase in cells expressing mRNA for IFN-γ and a decrease in those expressing message for IL-4.²⁹ Flow cytometry conducted on mitogen-stimulated PBMCs has demonstrated shifts of a similar nature.^{13,30} After rush immunotherapy, there was a marked increase in the percentage of IFN-γ-producing CD4⁺ peripheral blood lymphocytes.¹³ The increase in IFN-γ-containing CD4⁺ cells correlated with suppression of skin prick test results but not with specific IgG₄ levels. Immunotherapy administered according to a conventional schedule was reported to produce a decrease in the ratio of PBMCs staining for IL-4 in comparison with those staining for IFN-γ.³⁰ The reduction in ratio was significant at the time that maintenance doses were achieved, and it increased further after 1 year; this was in part due to progressive decrease in the percent of PBMCs with positive intracel-

lular staining for IL-4. In our study, only the highest dose of cat extract (15.0 µg) induced a significant decrease in the percentage of CD4⁺/IL-4⁺ cells in the peripheral circulation. Because the percentage of IL-4-staining cells was higher at baseline in the 15.0-µg dose group, a regression to the mean is a possible explanation for our findings. However, the change was highly significant and is consistent with the findings of others, suggesting that it might have represented a true and significant response to high-dose immunotherapy.

The limitation of this study should be acknowledged. First, the number of subjects was small. (Nevertheless, significant alterations were demonstrated in several immunologic responses in both the medium-dose and the high-dose groups.) Second, the duration of treatment was short. (Yet each of the subjects reached the projected maintenance dose and received at least 1 additional maintenance injection before follow-up assessment.) Third, it must be acknowledged that there might have been further development of significant responses with longer maintenance therapy. Fourth, no assessment of clinical response was attempted, and group differences in the response to nasal challenge were not detected.

It is clear that the results of this study should be confirmed and extended both in duration of treatment and in assessment of the correlation between the immunologic parameters and symptomatic improvement.

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