Immunologic response to administration of standardized dog allergen extract at differing doses

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Background: The immunologic response to immunotherapy with dog extract is not well characterized.
Objective: The purpose of this study was to examine the immunologic response to 3 doses of dog extract expressed as their Can f 1 content.
Methods: Cluster immunotherapy was administered to 28 patients with dog allergy who were randomly assigned to 1 of 4 treatment arms: placebo or acetone-precipitated extract containing 0.6 μg, 3.0 μg, or 15.0 μg Can f 1 per 0.5 mL maintenance dose. Studies included titrated skin prick tests, the late cutaneous response, titrated nasal challenge with dog extract, and serum allergen-specific IgE and IgG_4. Dog allergen-stimulated lymphocyte proliferation was performed with measurement of secreted cytokines by ELISA and of intracellular cytokines by flow cytometry.
Results: There was a significant dose-dependent response in suppression of titrated skin prick tests and suppression of the late cutaneous response. There was a significant increase from baseline in dog-specific IgG_4 in both the high-dose and low-dose groups and a dose-dependent suppression of secreted TNF-α and increase in secreted TGF-β. There was a dose-dependent trend in suppression of secreted IL-4 with a significant decrease from baseline in the high-dose group. There were no significant changes in symptom scores; lymphocyte proliferation; secreted IFN-γ, IL-10, or IL-5; or intracellular cytokine production.
Conclusion: The dose-response in immunologic parameters after immunotherapy with dog extract is similar to that previously demonstrated with cat extract.
Clinical implications: The greatest and most consistent response is seen with a dose containing 15 μg Can f 1.
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Key words: *Immunotherapy, acetone-precipitated dog extract, Can f 1, cytokine, allergen-specific IgE, allergen-specific IgG_4, skin prick test, late cutaneous response*

Allergen immunotherapy is an effective form of treatment for both allergic rhinitis and allergic bronchial asthma.[1] High doses of standardized extracts have been proven effective in treating patients with sensitivity to ragweed, timothy grass, house dust mites, and cat.[1] In 2 previous studies examining the immunologic response to maintenance doses after 5 weeks and after 1 year and 5 weeks of immunotherapy using cat extract, the dose of cat dander extract containing 15 μg Fel d 1 was shown to be most effective.[2,3] The second study demonstrated that the dose-response at 5 weeks is the same as that observed after 5 weeks and 1 year of maintenance immunotherapy, thus providing rationale for a 5-week study examining the immunologic response to dog extract.[3]

To date, few studies have examined the effectiveness of immunotherapy with dog extract. Although dog extracts are not standardized in the United States, the major dog allergen, Can f 1, has been isolated, purified, and expressed.[4] It is therefore now possible to examine the dose-response to dog allergen extracts expressed as potency in content of major allergen despite lack of standardization. Most dog allergen extracts are reported to contain roughly 5 μg Can f 1 per milliliter concentrated extract.[1] However, the acetone-precipitated (AP) dog extract 1:100 wt/vol produced by Hollister-Stier Laboratories (Spokane, Wash) contains more than 100 μg Can f 1 per mL and has been reported to contain as high as 165 μg/mL according the manufacturer. A recent study at National Jewish Medical and Research Center confirmed the greater potency of this extract, comparing it to conventional dog extracts by skin testing.[5] The purpose of this study was to examine those parameters that showed significant changes in the previous cat immunotherapy studies in a cohort of subjects receiving cluster immunotherapy with the AP dog extract. This study addresses the question whether immunotherapy with dog extract follows the same dose-response pattern previously observed with immunotherapy with cat extract.

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Abbreviations used
AP: Acetone-precipitated
cpm: Counts per minute
SPT: Skin prick test

METHODS

Subjects

Adult subjects sensitized to dogs were recruited. Each of the subjects had a history of rhinitis symptoms with or without asthma symptoms on exposure to dogs or had perennial rhinitis symptoms and close exposure to dogs. Skin tests were performed using the prick method with a DuoTip (Lincoln Diagnostics, Decatur, Ill) using AP dog extract 1:100 wt/vol (Hollister-Stier Laboratories). Skin wheals were required to be ≥5 mm in diameter. Each subject had an FEV_1 ≥ 80% predicted, and no subject had a history of persistent asthma or regular use of control medication for asthma. No subject had received immunotherapy with dog or other allergen extracts during the 5 years before the study. Antihistamines were withheld 7 days before skin testing or nasal challenge studies. Corticosteroid nasal sprays were withheld 30 days before and throughout the study. Subjects were excluded if they were pregnant or not using appropriate birth control or if they were taking β-blockers or monoamine oxidase inhibitors. The Institutional Review Board of National Jewish Medical and Research Center approved the study. All subjects signed approved consent forms before participating.

Study design

Twenty-eight subjects were randomly assigned to 1 of 4 treatment arms: placebo or 1 of 3 extracts containing dog antigen prepared from the 1:100 wt/vol extract of AP dog (Hollister-Stier Laboratories), which contained approximately 161 μg/mL Can f 1 (information provided by the extract manufacturer). At the maintenance injection of 0.5 mL of the active extract, subjects received a dose of dog extract containing Can f 1 0.6 μg, 3.0 μg, or 15 μg diluted in albumin saline solution (Hollister-Stier Laboratories). The concentrations of extract in the 3 active treatment groups are shown in Table I. All placebo, low-dose, and medium-dose vials 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 reaction of the equivalent high-dose vial. Each subject received 0.5 mL as a maintenance injection from vial 1.

Injections were administered by a cluster protocol over a period of 4 weeks. Injections were administered twice weekly for 8 visits. 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 last visit. An additional maintenance injection was given 1 week later (Table II). Subjects remained in the area for 60 minutes after the last of multiple injections and 30 minutes after single injections. This schedule was altered, if necessary, depending on subject tolerance. All immunotherapy was administered in the Clinical Research Unit by a registered nurse or medical doctor. Subjects received fexofenadine 180 mg and zafirlukast 20 mg approximately 2 hours before each injection visit to reduce the risk of local and systemic reaction. All injections were performed in a double-blind fashion. Each subject underwent immunologic testing including nasal challenge, skin testing, and laboratory analysis before immunotherapy and again within 7 days after receiving the first weekly maintenance injection.

TABLE I. Immunotherapy dosing schedule (μg Can f 1/mL)

Vial #	High dose	Medium dose	Low dose	Placebo
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

Titrated nasal challenge

Nasal challenge with dog allergen extract was performed before and after 5 weeks of cluster immunotherapy with AP dog extract (Hollister-Stier Laboratories) using the method previously used.[3] Before starting the allergen challenge, 3 saline lavages were performed to remove accumulated secretions. The nasal challenges were performed by spraying .1 mL solution into both nostrils at 10-minute intervals (metered pump nasal spray bottles; PharmaSource International, Inc, Centennial, Colo). The first dose contained saline, and subsequent doses contained AP dog extract in increasing ½ log concentrations from 1:3,000,000 to 1:100 wt/vol. Patients were asked to score their symptoms on the basis of the scoring system by Bousquet et al[6] 10 minutes after each dose until a score of 5 was reached. Briefly, symptoms such as sneezing, rhinorrhea, nasal congestion, pruritus, and conjunctivitis were assigned a number between 0 and 3. Patients were to be asked to rate their symptoms at each incremental dose until a noncumulative score of 5 was achieved. The same dose at which a score of 5 was produced before immunotherapy was to be administered 1 week after the completion of immunotherapy.

Titrated skin prick tests

Titrated skin prick tests (SPTs) were performed in duplicate on the patient's back using the same dilutions of dog allergen extract as used in the nasal challenges. Testing was conducted with increasing concentrations until a mean wheal of 5 mm was achieved, with at least 1 dilution above and 1 below the dilution that produced the 5-mm wheal.

Late-phase cutaneous response

Intradermal tests with the dog allergen were performed using dilutions of the dog extract used for the nasal challenges, with the starting dose a 1:10 dilution of the dose that produced the 5-mm wheal on skin prick testing. The injected dose was increased until a wheal of ≥15 mm in diameter was achieved. The late cutaneous reaction was read after 6 hours. On subsequent evaluation, the same dose of extract was used that initially produced the 15-mm wheal.

Dog-specific immunoglobulin measurements

Serum was obtained before and after completion of immunotherapy. Undiluted samples were analyzed for allergen-specific IgE by means of Pharmacia CAP system (Pharmacia Diagnostics, Uppsala, Sweden). Dog-specific IgG_4 was assayed via the Pharmacia CAP system-specific IgG_4 FEIA (Pharmacia Diagnostics) using serum diluted 1:1000.

Proliferation assay

PBMCs were isolated by means of Ficoll-Hypaque density gradient centrifugation from heparinized venous blood. Cells were washed and resuspended at 1×10^6 cells/mL in RPMI supplemented (Cellgro, Herndon, Va) with 5% AB human sera, penicillin-streptomycin, and L-glutamine. Triplicate wells containing 1×10^5 PBMCs were incubated with dog extract containing 1, 5, or 10 μg/mL Can f 1 in a 37°C, 5% CO_2 incubator for 6 days. Cultures were then pulsed with tritiated thymidine for 6 hours and harvested onto glass fiber disks. The mean counts per minute (cpm) were determined. Stimulation indices (mean cpm Can f 1/mean cpm media alone) were calculated.

Cytokine induction

PBMCs at 1×10^6/mL were cultured with media only, or media containing dog extract with 1 and 10 μg/mL Can f 1 for 5 days at 37°C in a CO_2 incubator. Supernatants were collected and frozen at −70°C until analyzed.

ELISA for TGF-β

Supernatants from the cytokine induction assay were thawed and tested for TGF-β using a TGF-β ELISA kit following the manufacturer's instructions (BioSource, Camarillo, Calif).

IL-4, IL-5, IL-10, IFN-γ, and TNF-α measurement

The LINCOplex assay was conducted as per the manufacturer's instructions (Linco Research, Inc, St Charles, Mo). In brief, the assay is based on conventional sandwich assay technology. The antibody-specific cytokine is covalently coupled to microspheres (Luminex Corp, Austin, Tex), with each antibody coupled to a different microsphere uniquely labeled with a fluorescent dye. The microspheres were incubated with 25-μL standards, controls, and samples in a 96-well microtiter filter plate for 1 hour at room temperature. After incubation, the plate was washed to remove excess reagents, and detection antibody, in the form of a mixture containing each of the 5 antibodies, was added. After a 30-minute incubation at room temperature, streptavidin-phycoerythrin was added for an additional 30 minutes. After a final wash step, the beads were resuspended in buffer and read on the Luminex^{100}) instrument to determine the concentration of the cytokines.

Intracellular cytokine analysis

Cells were stimulated with 5 μL CD28/49d. Cells were then stimulated with dog extract containing either 1, 5, or 10 μg Can f 1 or with staphylococcal enterotoxin B. Cells were subsequently incubated for 6 hours at 37°C. Brefeldin A (10 μg/mL) was added for the final 4 hours of incubation. Cells were then washed and frozen at −70°C for as long as 1 month. At a later date, cells were thawed and prepared for staining. Intracellular detection of IL-4 and IFN-γ in $CD4^+$ cells and IL-10 in $CD4^+CD25^+$ cells was performed by using flow-cytometric analysis as described elsewhere.[7,8]

Statistical analysis

Outcome variables were analyzed as absolute change from baseline (post − pre, for SPT and secreted IL-4, TGF-β, and TNF-α) or relative percentage change from baseline (100 × [post − pre]/pre, for late cutaneous response and serum IgG_4). Outcome variables were compared between groups using the Kruskal-Wallis test; pairwise group comparisons were made using the Wilcoxon rank-sum test. To examine further the dose-response relationship between dog extract dose and outcomes, linear mixed models were used, with quantitative dose and dilution as predictor variables.[9] For certain variables, pre and post scores were log-transformed before analysis (IgG_4 and secreted IL-4 and IFN-γ). A random intercept term was used to account for repeated measures within subjects (across dilution treatments). Statistical significance was defined as a P value of .05 or less, and all reported P values are based on 2-sided tests.

RESULTS

Twenty-eight subjects were initially enrolled in the study. One subject dropped out of the study because of scheduling issues. Thus, a total of 27 subjects completed the study (7 in the placebo group, 6 in the low-dose group, 7 in the medium-dose group, and 7 in the high-dose group). Six individuals in the high-dose group, 7 in the medium-dose group, 4 in the low-dose group, and 3 in the placebo group had regular exposure to at least 1 dog. No subject experienced a systemic reaction to the immunotherapy. All subjects achieved maintenance therapy in 4 weeks.

TABLE II. Immunotherapy dosing schedule

Visit no.	Dose	Dilution
1	0.10 mL	Vial #4
	0.40 mL	Vial #4
	0.10 mL	Vial #3
2	0.20 mL	Vial #3
	0.40 mL	Vial #3
	0.07 mL	Vial #2
3	0.10 mL	Vial #2
	0.15 mL	Vial #2
	0.25 mL	Vial #2
4	0.35 mL	Vial #2
	0.50 mL	Vial #2
5	0.07 mL	Vial #1
	0.10 mL	Vial #1
6	0.15 mL	Vial #1
	0.20 mL	Vial #1
7	0.30 mL	Vial #1
	0.40 mL	Vial #1
8	0.50 mL	Vial #1
9	0.50 mL	Vial #1

Titrated SPTs

Results were expressed as absolute change from baseline (post − pre, in millimeters) using the change in mean wheal size at the dose producing a 5-mm wheal preimmunotherapy. After 5 weeks of immunotherapy, there was a statistically significant dose-dependent suppression over all 4 treatment groups ($P = .0003$) with the high-dose group exhibiting the largest change from baseline (Fig 1). There was a statistically significant absolute decrease from baseline in both the medium and high-dose groups ($P = .002$, $P < .0001$, respectively) with no significant change from baseline in the low- dose or placebo group. There were statistically significant differences when the high-dose group was compared with the placebo ($P = .0003$), when the medium-dose group was compared with the placebo ($P = .006$), and when the high-dose group was compared with the low-dose group ($P = .05$).

Late cutaneous response

Results were expressed as relative percent change from baseline using the change in mean wheal size at 6 hours with the dose resulting in a 15-mm wheal preimmunotherapy. There was an overall dose-response in suppression of late cutaneous response ($P = .03$) and a statistically significant difference when the high-dose group was compared with the placebo group ($P = .05$). In addition, there was a statistically significant suppression from baseline in both the high-dose and medium-dose groups ($P = .03$, $P = .03$, respectively; Fig 2), with no significant change noted in the low-dose or placebo group.

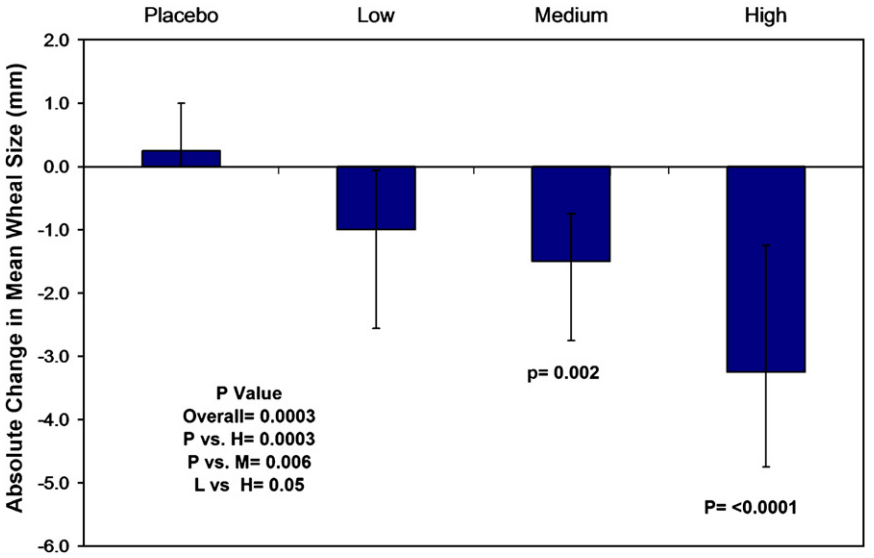

FIG 1. Titrated SPTs. There is an overall significant dose-response for all treatment groups ($P = .0003$), a significant difference from baseline when the high-dose group is compared with the placebo and with the low-dose group ($P = .0003$, $P = .05$, respectively) and when the medium-dose group is compared with the placebo group ($P = .006$). *Bars* represent 25% to 75% interquartile range. *P,* Placebo; *H,* high dose; *M,* medium dose; *L,* low dose.

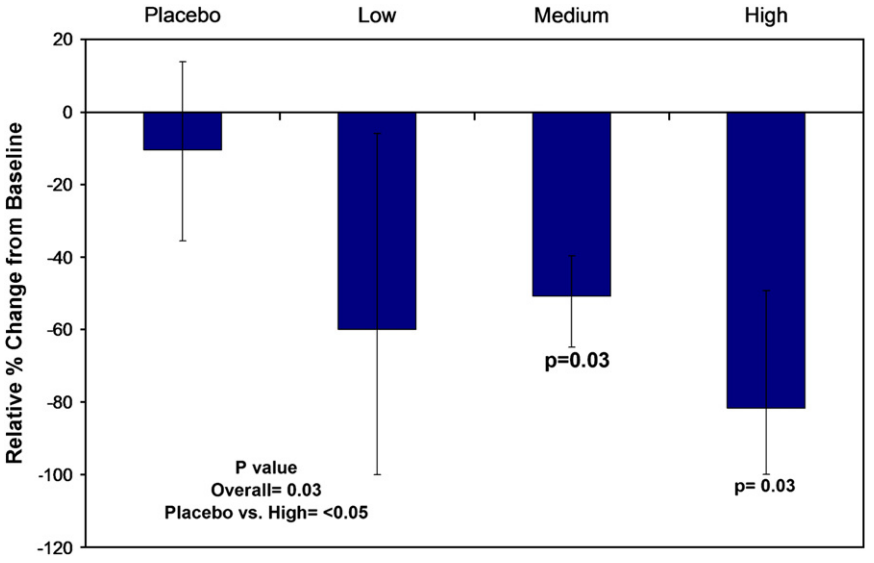

FIG 2. Late cutaneous response. There is an overall significant dose-response for all 4 treatment groups ($P = .03$). There is a significant difference from baseline when the high-dose group is compared with the placebo ($P \leq .05$). *Bars* represent 25% to 75% interquartile range.

Serum antibodies

Results were expressed as relative percent change from baseline. There was an overall dose-dependent trend for an increase in dog-specific IgG_4 levels, although this was not statistically significant. There was a statistically significant increase from baseline in both the high-dose and low-dose groups ($P = .02$, $P = .03$, respectively; Fig 3). There were no significant changes in dog-specific IgE in the 4 groups.

Secreted cytokines from stimulated PBMCs

Results were expressed as absolute change from baseline. There was an overall dose-response suppression of secreted TNF-α (average decrease of 13 pg/mL per unit increase in dose; $P = .02$; Fig 4). In addition, there was a dose-response increase in secreted TGF-β (average increase of 13 ng/mL per unit increase in dose; $P = .03$; Fig 5). Finally, there was a significant absolute decrease from baseline in secreted IL-4 in the high-dose group

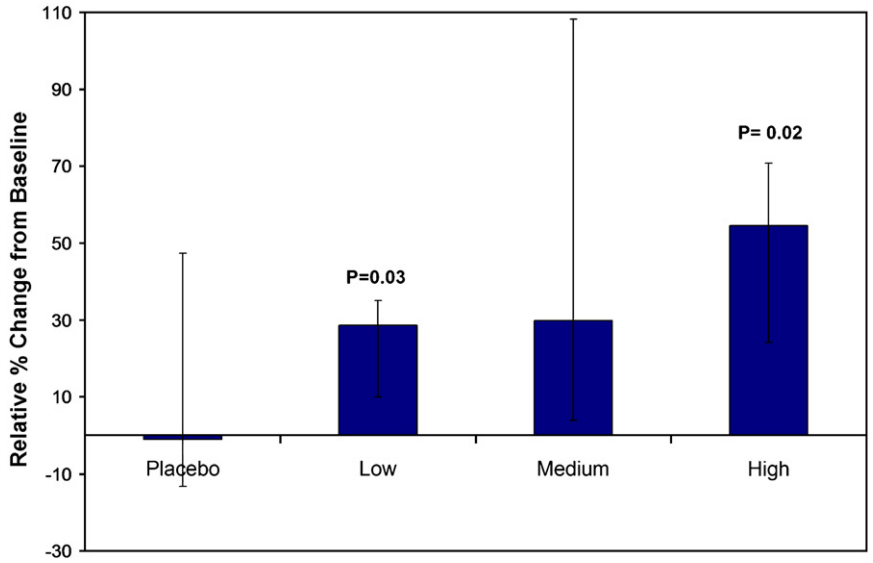

FIG 3. Serum IgG_4. There is an overall trend for a dose-response increase in dog-specific IgG_4, although this is not statistically significant. There is a significant increase from baseline in both the high-dose and the low-dose groups ($P = .03$, $P = .02$, respectively). *Bars* represent 25% to 75% interquartile range.

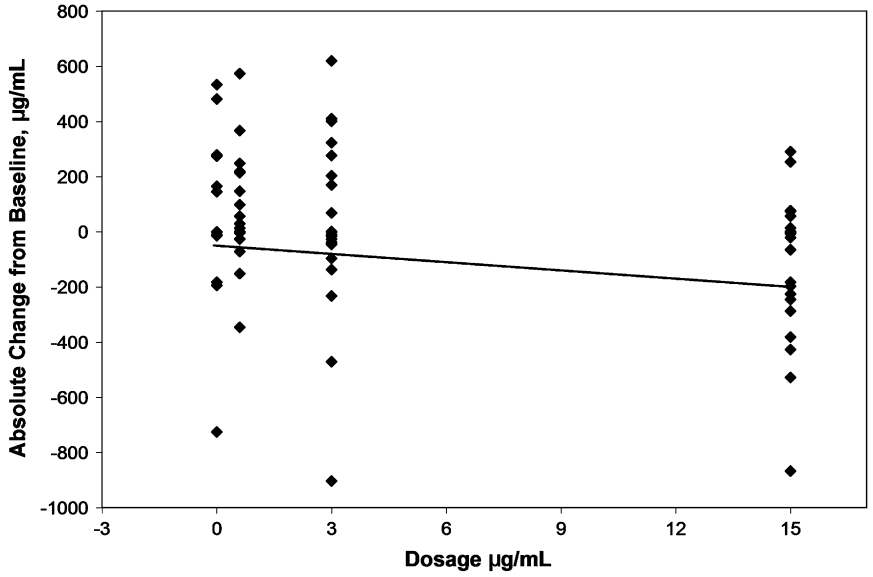

FIG 4. TNF-α secreted by dog allergen–stimulated PBMCs. Using the regression model, there is an overall dose-response in suppression of secreted TNF-α ($P = .02$).

($P = .05$; Fig 6). There were no significant changes from baseline in secreted IFN-δ, IL-5, or IL-10.

Other outcomes

After 5 weeks of immunotherapy, there was no significant change in symptom scores on nasal challenge when the same dose was delivered as at baseline. However, a misunderstanding resulted in cumulative scoring rather than noncumulative scoring. This resulted in premature achievement of a Bousquet score of 5 and thus did not accurately reflect the threshold dose of intranasal Can f 1 tolerated by the patient.

There was no statistically significant difference in lymphocyte proliferation after stimulation with dog extract containing 1, 5, or 10 μg Can f 1 for 1 or 6 days. There were no statistically significant differences in secreted IFN-γ, IL-10, or IL-5 or in intracellular production of IFN-γ, IL-4, or IL-5.

DISCUSSION

The current study demonstrated a dose-dependent response to dog extract. There was a dose-dependent

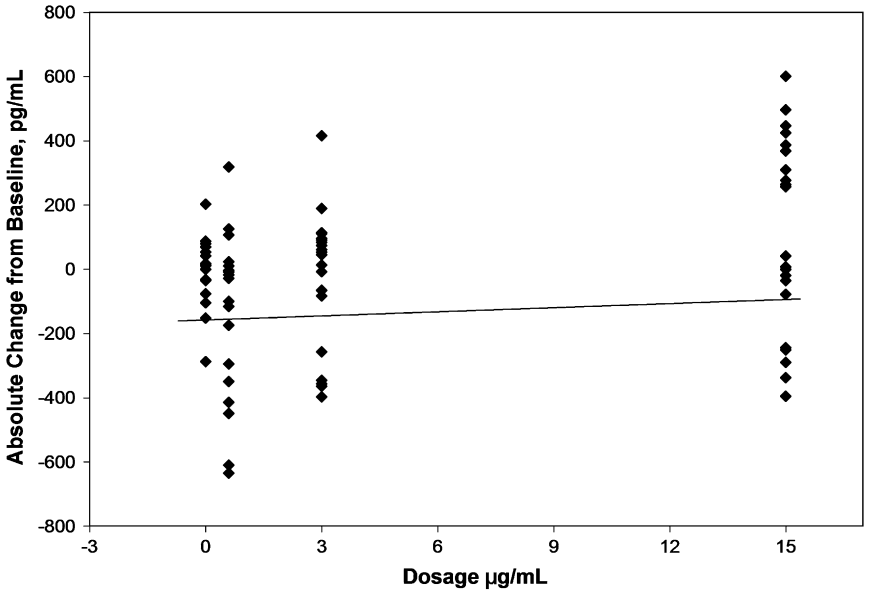

FIG 5. TGF-β secreted by dog allergen–stimulated PBMCs. Using the regression model, there is an overall dose-response in the increase in secreted TGF-β ($P = .03$).

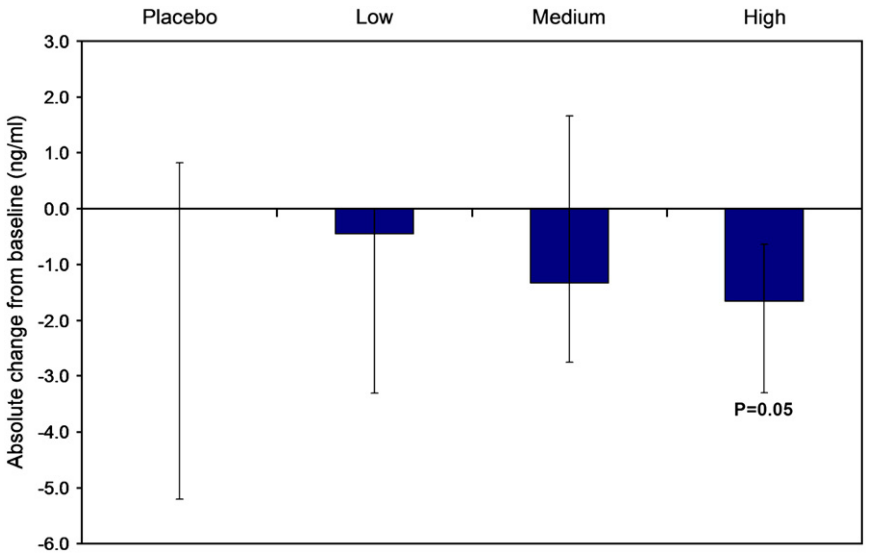

FIG 6. IL-4 secreted by dog allergen–stimulated PBMCs. There is significant decrease from baseline in the high-dose group ($P = .05$). *Bars* represent 25% to 75% interquartile range.

suppression of titrated SPTs and of the late cutaneous response. In addition, there was an increase in IgG_4 from baseline in both the high-does and low-dose groups, an overall dose-response increase in secreted TGF-β and reduction in secreted TNF-α, and a suppression of secreted IL-4 in the high-dose group.

Earlier reports suggested that dog extract was less effective than cat in reducing bronchial response but also caused fewer reactions.[10] However, most dog allergen extracts are reported to contain roughly 5 μg Can f 1 per mL concentrated extract.[1] On the other hand, the AP dog extract produced by Hollister-Stier Laboratories reportedly contains more than 100 μg Can f 1 per mL (1:100 wt/vol). In this study, subjects receiving immunotherapy with AP dog extract failed to demonstrate suppression of symptom scores on nasal allergen challenge. This was thought to be a result of a misunderstanding in the conduct of the nasal challenge that resulted in suboptimal dosing. Because many of the immunologic parameters that have been associated with successful immunotherapy that were observed in previous studies with cat extract were also seen in this study with immunotherapy with dog extract, it is reasonable to assume that both extracts would have similar clinical efficacy.

It is not known whether acetone precipitation destroys any clinically relevant epitopes on the dog allergen. However, a previous study confirmed the greater potency of the AP dog extract compared with conventional dog extract by skin testing.[5] To date, few studies have examined the clinical and immunologic effectiveness of dog immunotherapy. In 1995, Hedlin et al[10] reported increased tolerance on exposure to dogs or cats 5 years after stopping immunotherapy. This is, to our knowledge, the first dose-response immunotherapy study with dog extract in which dosing was expressed by major allergen content. It is also the first dose-response assessment of immunotherapy using dog extract. The purpose of this study was (1) to demonstrate the immunologic response to dog extract and (2) to compare the dose-response as expressed in content of the major allergy to that observed in previous studies using standardized cat extract. Previous cat studies have demonstrated that after 5 weeks of cluster immunotherapy with standardized cat extract, there was a dose-dependent suppression of SPTs and increases in cat-specific IgG_4 levels.[2] For each outcome, the changes with maintenance doses of cat dander extract containing 0.6 μg Fel d 1 did not differ significantly from placebo, whereas those containing 3 and 15 μg showed significant changes compared with placebo but were not significantly different from each other. However, only the 15-μg maintenance dose induced a significant decrease in the percentage of $CD4^+$ IL-4 cells in the peripheral blood. After 1 year of immunotherapy with the standardized cat extract, there was dose-dependent suppression of titrated SPTs and of symptom scores with nasal challenge, with only the highest dose group demonstrating significant change from baseline. In addition, there was a dose-dependent increase in cat-specific IgG_4 after 1 year of therapy.[3] Overall, there is little, if any, immunologic difference in the results after 5 weeks or after 5 weeks and 1 year of immunotherapy with cat extract.[2,3] It is likely that a similar conclusion would be drawn after immunotherapy with dog extract.

Suppression of the immediate cutaneous reaction to allergen extract is a well recognized response to allergy immunotherapy.[11-13] This response has been reported after conventional,[12] cluster,[13] and rush[11] immunotherapy protocols. The endpoint of titrated SPTs performed after immunotherapy has been shown to correlate with both the threshold of titrated nasal challenge and the nasal symptoms reported by the patient during the corresponding pollen season.[13] In addition, this endpoint has been shown to be a predictor of persisting clinical remission after discontinuation of allergen immunotherapy.[14] This study clearly demonstrated an overall dose-dependent suppression of titrated SPTs, with the extract containing 15 μg Can f 1 exhibiting the largest change from baseline. The late cutaneous response has also been shown to correlate with response to immunotherapy.[12] This study demonstrated a suppression of the late cutaneous response in both the high-dose and the medium-dose groups.

Recent studies indicate that the rise in allergen-specific IgG_4 represents a switch from IgE-induced by IL-10 secreting regulatory T cells, and increases in the level of allergen-specific IgG_4 have been reported with successful immunotherapy.[15,16] However, the significance of this increase is unclear. Nakagawa et al[16] reported that patients with perennial rhinitis who responded to immunotherapy with house dust mites showed a statistically significant increase in IgG_4 compared with patients who did not respond. The authors also reported a weak yet significant correlation between the increase in IgG_4 antibodies and clinical improvement. In our study, there was a significant increase from baseline in IgG_4 in both the high-dose and low-dose groups.

Past studies have examined the role of suppressor T cells in immunotherapy.[17,18] Recently, suppressor cells have been defined as T cells that suppress immune responses via cell/cell interactions, express inhibitory cell-surface molecules, and produce IL-10 and TGF-β.[6,19,20] The findings of Jutel et al[21] further support this theory, demonstrating an increase in secreted IL-10 and TGF-β in allergen-specific T cells. A uniform phenotype of suppressor cells remains unknown, but studies point toward the $CD4^+/CD25^+$ T lymphocyte.[6,20,22] This study did not demonstrate any changes in IL-10 postimmunotherapy. However, there was a significant dose-response increase in secreted TGF-β. A shift in predominantly T_H2 cytokines (eg, TNF-α, IL-4) to T_H1 cytokines (eg, IFN-δ) during immunotherapy is also a potential mechanism of immunotherapy.[23] 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.[2] In this study, there was a significant dose-response suppression of secreted TNF-α. In addition, there was a significant reduction in IL-4 secreted by stimulated PBMCs in the high-dose group.

The results of this study show a significant immunologic response to dog extract. Although some significant responses were demonstrated in the both the medium-dose and low-dose groups, the high-dose group containing 15 μg Can f 1 per maintenance injection demonstrated the most significant and consistent responses. These results are consistent with the previous cat studies that demonstrated the most consistent response with the dose of cat extract containing 15 μg Fel d 1.

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