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Suppression of the late cutaneous response by immunotherapy

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In a prospective, double-blind, placebo-controlled study, we examined the effect of mountain cedar (MC) immunotherapy on the MC-induced late cutaneous response (LCR). Fourteen MC-sensitive patients were intradermally skin tested before and after immunotherapy with MC extract. We measured the size of the wheal at 15 minutes and the area of tissue swelling at 6 hours. Patients were matched by the size of the LCR and started receiving either MC immunotherapy or placebo immunotherapy. MC-specific immunoglobulins (MC sIgG, MC sIgG1, MC sIgG4, and MC sIgE) were measured by ELISA. Symptom-medication scores (SMSs) were recorded on a daily basis during the MC season and tabulated at the end of the study. Comparison of the 14 paired patients revealed no significant differences between MC-treated and placebo-treated groups in preimmunotherapy MC sIgG1 and sIgG4. However, when MC immunotherapy was compared to placebo immunotherapy, patients receiving MC immunotherapy developed significantly higher MC sIgG1 ($p < 0.04$) and MC sIgG4 ($p < 0.01$) after immunotherapy. Patients receiving MC immunotherapy also demonstrated significantly greater suppression of the LCR after immunotherapy ($p < 0.005$) with the postimmunotherapy LCR correlating significantly with both MC sIgG4 ($r_s = 0.715$; $p = 0.008$) and cumulative dose of MC received ($r_s = 0.808$; $p = 0.004$). MC sIgE was similar in both groups after immunotherapy. The reduction in SMSs in the MC-treated group did not reach significance, nor was there a correlation of SMSs with MC sIgE, sIgG, sIgG1, or sIgG4. In conclusion, this prospective study demonstrated that the LCR in MC-sensitive patients was suppressed by MC immunotherapy and that this suppression was strongly correlated with both MC sIgG4 and cumulative dose of MC administered. (J ALLERGY CLIN IMMUNOL 1989;83:101-9.)

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Abbreviations used

FBS:	Fetal bovine serum
HSA:	Human serum albumin
ICR:	Immediate cutaneous reaction
LCR:	Late cutaneous reaction
MC:	Mountain cedar
MC sIgE:	Mountain cedar-specific IgE
MC sIgG:	Mountain cedar-specific IgG
MC sIgG1:	Mountain cedar-specific IgG1
MC sIgG4:	Mountain cedar-specific IgG4
OPD:	o-phenylenediamine
PBS:	Phosphate-buffered saline
SMS:	Symptom-medication score
OD:	Optical density

The effectiveness of immunotherapy for the treatment of pollen-induced rhinitis has been documented by numerous studies.¹⁻³ However, the mechanism by which immunotherapy alters symptoms secondary to pollen exposure has not been fully elucidated. Several immunologic changes are associated with antigen-specific immunotherapy. These changes include (1) blunting of the seasonal rise in antigen-specific IgE, (2) production of antigen-specific IgG antibody, (3) decrease in leukocyte histamine release in some patients, (4) induction of antigen-specific suppressor T-lymphocytes, and (5) increase in antigen-specific IgA and IgG in nasal secretions.⁴ Despite these observations, there has been inconsistent correlation between these immunologic changes and symptom amelioration, although a good correlation was observed with *in vitro* basophil histamine release and symptom improvement.⁵

It has been argued that the late-phase reaction may be a more useful model for allergic disease than the immediate-phase reaction.⁶ To examine the effect of immunotherapy on the LCR and to determine if there is a link with symptom improvement, we performed a prospective, double-blind, placebo-controlled study and found that immunotherapy induces production of allergen-specific IgG1 and IgG4 antibodies and that the level of allergen-specific IgG4 correlates strongly with suppression of the allergen-induced LCR.

MATERIAL AND METHODS

Patient selection

Only patients between the ages of 18 and 70 years, with both a typical history of rhinoconjunctivitis during the MC-pollen season and a 4+ prick skin test to MC were eligible for the study. None of the patients had previously received immunotherapy. The voluntary fully informed consent of the subjects used in this research was obtained as required by Air Force Regulation 169-9. We chose MC because it is a major pollen producer in southern Texas during the winter months, causing significant respiratory allergy.⁷

LCR

An LCR was elicited in all patients before immunotherapy and at the end of the study. MC extract (Holister-Stier Laboratories, Spokane, Wash., lot No. C7613313201) was used for all *in vitro* and *in vivo* assays and for immunotherapy. The extract was demonstrated to contain 1.2 mg of protein per milliliter, according to the method of Lowry et al.⁸ The stock extract was diluted with saline solution containing 0.03% HSA and injected at a dose of 0.02 ml of a 1:1000 dilution. The size of the induced wheal was then recorded at 15 minutes, and the area of tissue swelling was measured at 6 hours and circumscribed with a pen. The image was transferred with tape onto paper, the area was then measured with a zero-setting compensating planimeter

(Alvin Instruments, Seishi, Japan), and the results were recorded in square centimeters.

Matched subjects

A total of 24 patients were matched by the size of the LCR and assigned in a double-blind fashion to one of two groups (Fig. 1). One group received conventional immunotherapy with MC antigen beginning at a 1:100,000 wt/vol dilution and progressing by 0.05 ml increments until 0.5 ml was achieved. Then, a tenfold higher concentration was administered in the same dosing increments until maintenance was reached, which consisted of 0.5 ml of 1:100 wt/vol or the highest tolerated dose. The second group received a placebo injection consisting of 0.5 ml of caramelized glucose, 4.5 ml of 0.03% HSA, and 0.6 mg of histamine phosphate as the final concentration from which dilutions were made so that a graded increase in local reactions was attained as the dosage was increased. All patients received injections three to four times per week until maintenance was achieved and were then maintained on a once-a-week schedule. None of the patients had previously received immunotherapy.

SMSs

All patients recorded SMSs on a daily basis during the MC-pollen season. Patients recorded both nasal symptoms (itchy nose, congestion, sneezing, nose blowing, itchy eyes, or throat) and chest symptoms (wheezing, shortness of breath, chest tightness, or cough). All symptoms and dosages of medication were graded on a scale of 1 to 5, depending on severity. Medications were scored as 1 point per standard dose. All scores during the MC-pollen season were added to reach a total SMS for the season.

MC sIgE, sIgG, sIgG1, and sIgG4

Blood was obtained from subjects before institution of immunotherapy (September 1986), after maintenance, and at the peak of the MC season (January 1987).

MC sIgG antibodies. MC sIgG was measured quantitatively with a polyclonal antibody-based ELISA that has been previously described.⁹ In brief, Immulon II 96-well microtiter plates (Dynatech Industries, Alexandria, Va.) were coated with 57 µg of protein from crude MC extract in 100 µl of PBS, pH 7.2, and incubated at 37° C for 1 hour. The antigen was aspirated, and 200 µl of 0.1% HSA in PBS was placed in each well and incubated for 1 hour at 37° C. Two pairs of wells were coated with MC antigen with two pairs being left antigen free. The wells were washed three times with PBS with 0.05% Tween 20, aspirated, and dried; 100 µl of unknown sera was diluted 1:100 in 10% FBS in PBS and incubated for 2 hours at 37° C. The sera were then aspirated, and the wells were washed three times and dried; 100 µl of a 1:8000 dilution of goat antihuman IgG peroxidase (Tago, Inc., Burlingame, Calif.) in 10% FBS in PBS was added to all wells and incubated overnight at 4° C. The wells were then aspirated, washed three times, and dried; 150 µl of 0.5 mg o-phenylenediamine per milliliter of citrate buffer was added to each well. After 30

minutes at room temperature, the color reaction was stopped with 50 μ l of 5N H_2SO_4 and read at 492 nm. ODs of the antigen-free wells were subtracted from the ODs of the antigen-coated pairs of wells. The MC sIgG was quantitated into arbitrary units by interpolation from a reference curve developed from a high-titer MC sIgG serum pool.

MC sIgE antibodies. The MC sIgE assay was performed by a monoclonal antibody-based ELISA-transfer method, as previously reported.¹⁰ Briefly, the assay was run as above, except that antigen-free control wells were not used because of the low background of the monoclonal system. Antigen-coated wells were prepared, washed, and dried as above, and 100 μ l of unknown serum diluted 1:5 in 10% FBS in PBS was added to each well and incubated overnight at 4° C. After transferring to a second plate with wells coated with MC antigen and incubated at 4° C, the wells were aspirated, washed three times, and dried in the same manner. Sera were transferred to a third plate late in the afternoon. This procedure was repeated until a total of four transfers with five plates were completed. After the plates from the last transfer were washed, 100 μ l of a 2.5 μ g/ml of dilution of mouse monoclonal antihuman IgE antibody (Boehringer Mannheim, Indianapolis, Ind.) in 10% FBS in PBS was added to each well and incubated at room temperature for 3 hours. All the wells were then aspirated, washed three times, and dried; 100 μ l of a 1:5000 dilution of goat antimouse IgG peroxidase (Boehringer Mannheim, Indianapolis, Ind.) was added to all wells and incubated overnight at 4° C. The wells were aspirated, washed, and dried; 150 μ l of 0.5 mg o-phenylenediamine per milliliter of citrate buffer was added to each well. The wells were incubated for 30 minutes at room temperature and read as previously described.

The MC-sIgE concentration was expressed in international units per milliliter by interpolation from a reference curve analyzed in parallel with the unknowns. The reference curve was developed from a pool of high-titer sera whose content of MC sIgE was determined as previously described.¹⁰

MC-sIgG subclass antibodies, IgG1 and IgG4. Determinations of MC sIgG1 and sIgG4 were performed as described above for IgG antibodies with the following exceptions:

1. Serum samples were diluted 1:10 in 10% FBS in PBS.
2. Murine monoclonal antihuman IgG1 and IgG4 were used. These were all IgG1 isotypes and were demonstrated to be human immunoglobulin subclass-specific by direct binding and competitive inhibition assays as previously described.¹¹ The anti-IgG1 was catalog No. 912, Boehringer Mannheim, and the anti-IgG4 was clone SK-44, Miles Scientific, Naperville, Ill. Monoclonal antihuman IgG subclass antibodies were added after incubation for 2 hours at room temperature with serum. Samples were aspirated, and wells were washed. The monoclonal antibodies were incubated overnight at room temperature at equivalent antigen-binding dilutions according to the method of Djurup et al.¹² For anti-IgG1, the dilution used was 1:280, and for anti-IgG4, 1:75,000 in 10% FBS in PBS.

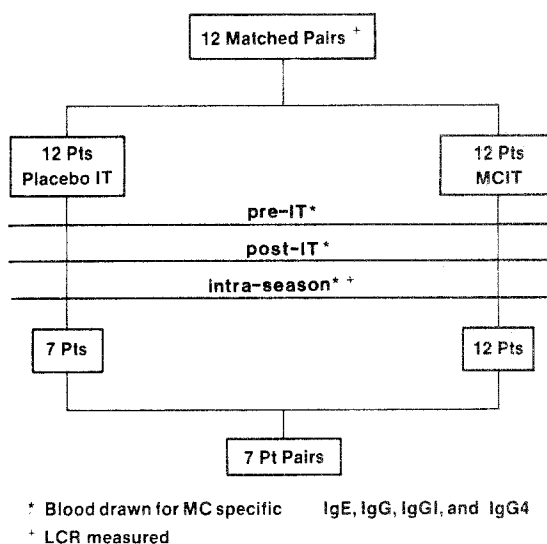


FIG. 1. Study protocol. Twelve matched pairs of patients began the study. Blood was analyzed for MC-specific antibodies before onset of immunotherapy (*pre-IT*), before the onset of the MC season but receiving maintenance immunotherapy (*post-IT*), and during the peak of the MC season (*intra-season*). Seven matched pairs of patients completed the study. LCR was measured before and at the conclusion of the study.

3. After wells were washed and dried, 100 μ l per well of a 1:1000 dilution of goat antimouse IgG peroxidase conjugate in 10% FBS in PBS was incubated for 1 hour at 37° C. After the wells were again aspirated and washed, substrate was added, and plates were developed as described above for IgG antibodies. Although IgG-subclass assay standardization could be achieved semiquantitatively with myelomas and subclass antibodies in isotopic dilution curves, such an assay could not be considered quantitative because of variable binding of myelomas with immunosorbents and variable primary antibody affinities for homologous myeloma antigens. Results are therefore expressed as net absorbances, yielding semiquantitative comparative data.

The specificities of all assays were proved by appropriate preincubation and direct binding with relevant and irrelevant antigens or myeloma immunoglobulins, as previously reported.¹¹ The lower limits of the assays were 2 SD above the mean level of a nonhuman antibody containing serum assayed 10 times.

Pollen counts

Pollen counts were performed at 1- to 2-day intervals during the MC-pollen season. Counts were done on a rotorod (Ted Brown Associates, Los Altos, Calif.) located on the roof of Wilford Hall Medical Center.

Statistical analysis

Mean antibody levels and SMSs between groups were compared by the two-tailed Wilcoxon signed-rank test. Correlations between sample data were tested by Spearman's

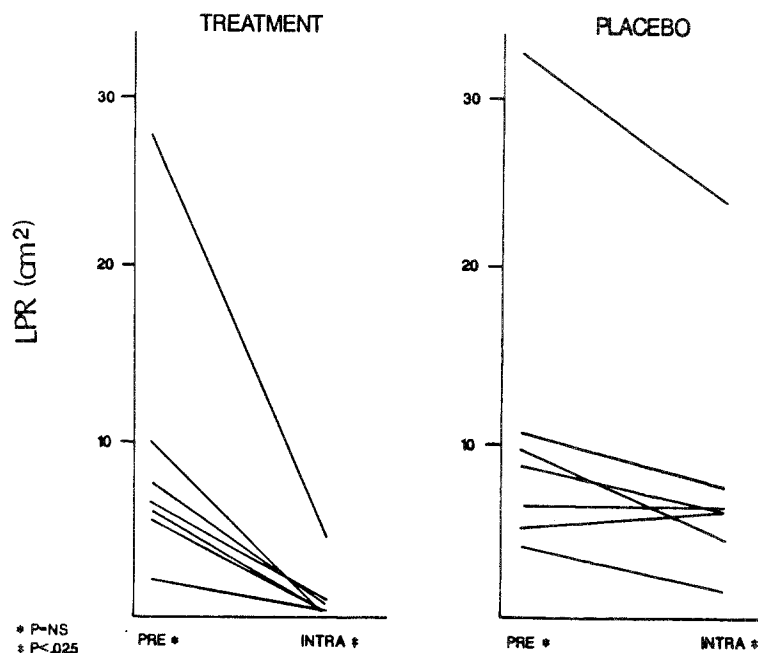


FIG. 2. Sizes of the LCR on the seven matched pairs of subjects before immunotherapy (*pre*) and at the end of the study (*Intra*); $\bar{x}$ (SD) for the treatment group versus the placebo-treated group was 9.36 cm² (8.38) versus 11.02 cm² (9.82) *pre* and 1.16 cm² (1.50) versus 7.87 cm² (7.22) *intra*. There was a significant difference in the size of the LCR between the treatment group and the placebo-treated group, $p < 0.025$, at the end of the study.

TABLE I. Patient characteristics

Treatment group				Placebo group			
Patient	Age (yr)	Sex	LCR (cm ²)	Patient	Age (yr)	Sex	LCR (cm ²)
J. H.	57	M	6.05	G. S.	48	M	5.23
M. T.	42	F	7.48	L. B.	44	F	10.55
F. R.	64	F	2.07	W. H.	58	M	4.17
C. T.	50	F	6.56	T. S.	38	M	9.70
P. M.	40	F	27.60	M. H.	37	F	32.64
J. L.	44	M	5.73	M. P.	44	F	6.28
M. S.	60	F	10.00	B. W.	52	F	8.55
$\bar{x}$	51			$\bar{x}$	46		
SD	9.47			SD	7.49		

rank correlation coefficients. Immediate and late skin reaction measurements were analyzed with the two-tailed Student's paired t test. Statistics were computed by use of the Statview 512+ software program (BrainPower, Inc., Calabasas, Calif.) on a Macintosh 512k microprocessor (Apple Computer, Inc., Cupertino, Calif.).

RESULTS

Patients

A total of 19 patients completed the study. The average age of the patients (six male and 13 female patients) was 47.4 years. Five patients were unable

to complete the study because of difficulty complying with the immunotherapy schedule. All patients except one were able to tolerate either 0.5 ml of a 1:100 concentration (wt/vol) of MC extract or full-dose placebo. One patient receiving MC extract was treated twice for anaphylaxis during the study, and one patient receiving placebo was treated for "anaphylaxis." After completion of the study, the code was broken, and a total of 12 patients were receiving the active treatment and seven patients were receiving placebo therapy. We then compared the seven matched pairs whose data are listed in Table I.

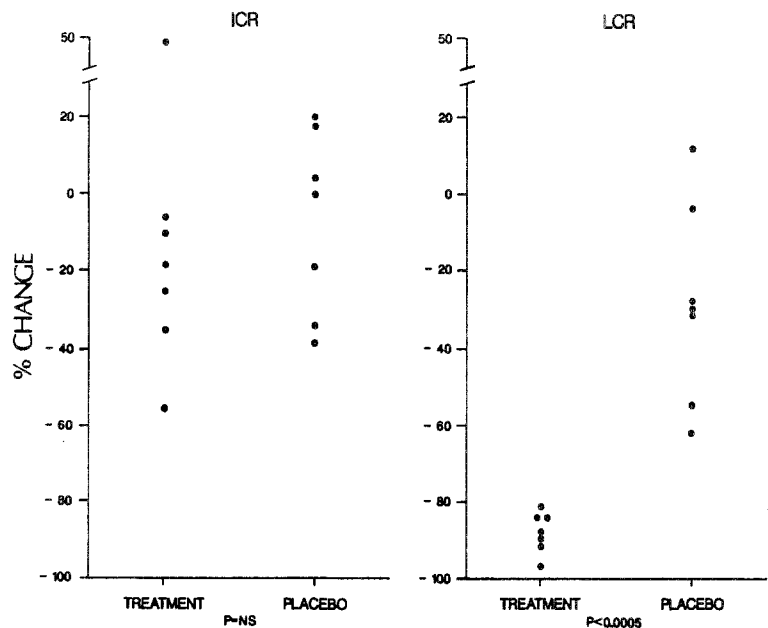


FIG. 3. Percent change of the ICR and LCR of the treatment group and placebo-treated subjects before immunotherapy and at the end of the study; $\bar{x}$ (SD) for the treatment group versus the placebo-treated group was -14.1% (3.24%) versus -5.4% (14.36%) for the ICR and -88.1% (5.5%) versus -27.4% (27.3%) for the LCR. There was significantly more change in the active-treatment group than in the placebo-treated group, $p < 0.0005$, for the LCR only.

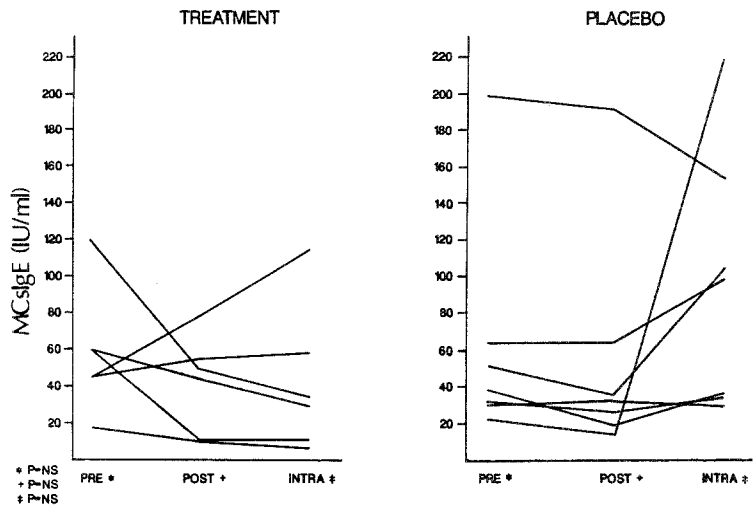


FIG. 4. Levels of MC sIgE antibodies in the sera of the study patients before immunotherapy (*pre*), at the start of the MC-pollen season (*post*), and at the end of the study (*intra*).

There was no significant difference in sex distribution or size of LCR, but the treated group was significantly older than the placebo-treated group (51 versus 46 years; $p = 0.036$). We then correlated age versus percent suppression of ICR and LCR, serum concentration of MC sIgG, sIgE, sIgG1, sIgG4, percent change of all antibody levels, and SMSs to determine if age affected any of these variables. None of the correlations was significant.

Size of the LCR

The 14 paired patients were matched according to the size of the LCR to an intradermal injection of 0.02 ml of 1:1000 dilution of MC extract. As previously stated, there was no significant difference in the size of the LCR before treatment in the two groups. However, there was a significant difference in the size of the LCR between the active-therapy group and the group receiving placebo injection, $p < 0.025$ (Fig.

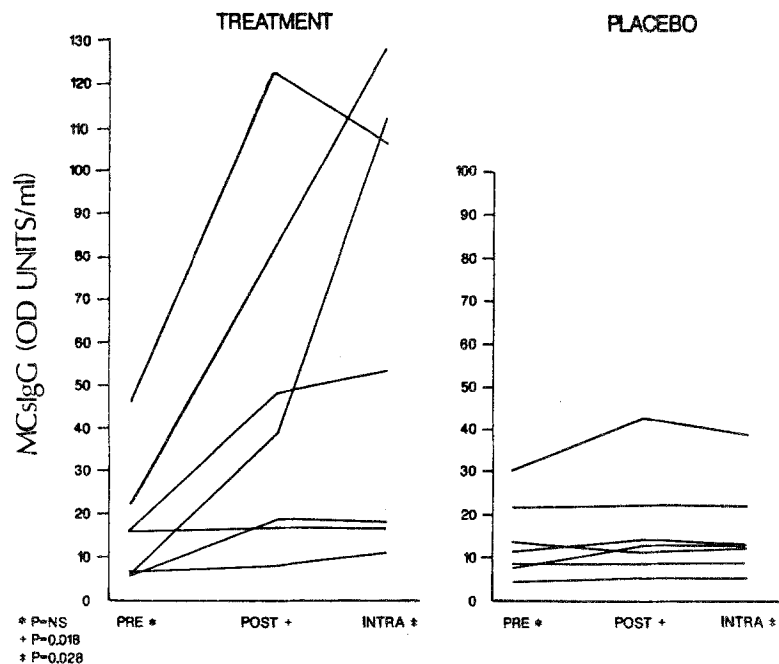


FIG. 5. Levels of MC sIgG antibodies in the sera of the study patients before immunotherapy (*pre*), at the start of the MC-pollen season (*post*), and at the end of the study (*intra*).

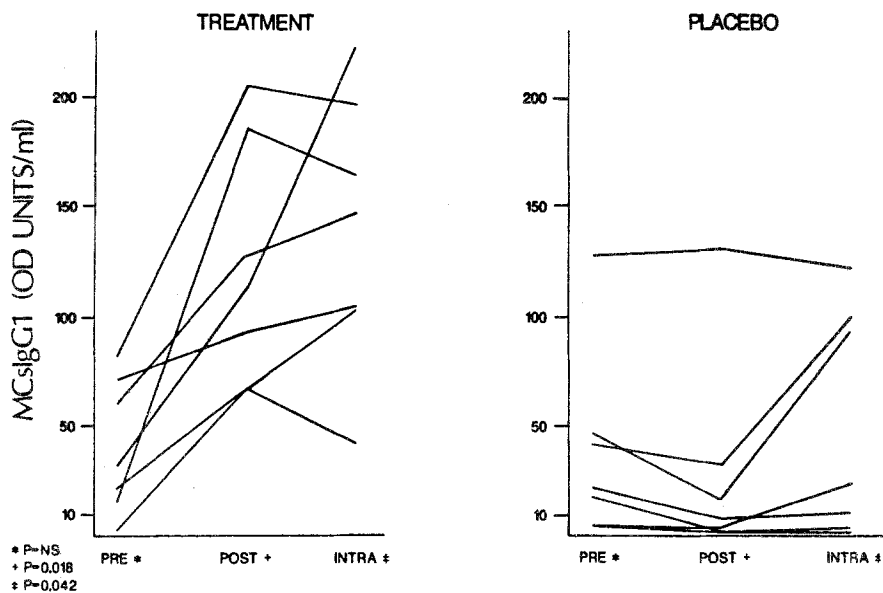


FIG. 6. Levels of MC sIgG1 antibodies in the sera of the study patients before immunotherapy (*pre*), at the start of the MC-pollen season (*post*), and at the end of the study (*intra*).

2). When the percent change in the LCR from baseline to intraseason was compared, the difference became even more apparent with a $p < 0.0005$ (Fig. 3). Interestingly, there was a significant decrease not only in the MC-treated group ($p = 0.018$) but also in the placebo-treated group, $p = 0.043$.

Size of the ICR

There was a significant difference in size of the immediate skin test reaction between the seven matched patient pairs before the institution of immunotherapy with larger ICRs in the placebo-treated group, $p < 0.005$. However, there was no significant

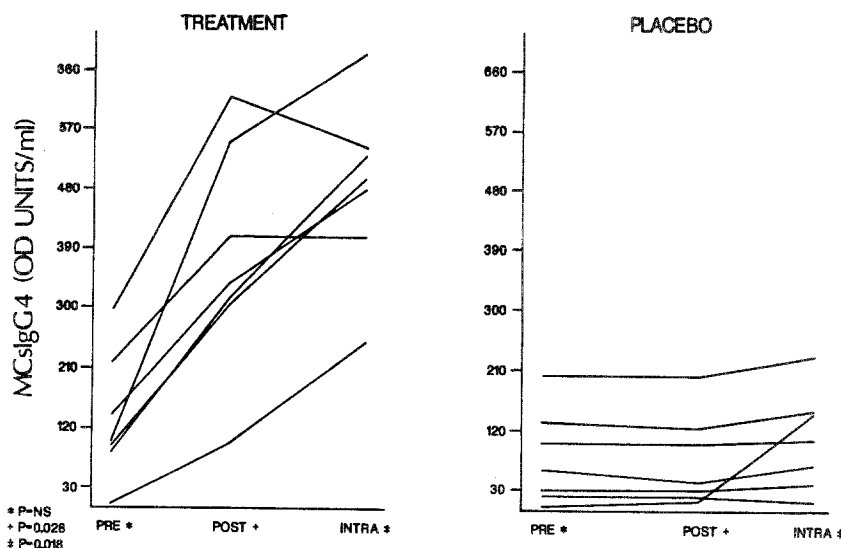


FIG. 7. Levels of MC sIgG4 antibodies in the sera of the study patients before immunotherapy (*pre*), at the start of the MC-pollen season (*post*), and at the end of the study (*intra*).

difference in the size of the ICR between the treated patients and patients receiving placebo at the end of the study. Also, the percent change in the ICR from baseline to intraseason was similar for both groups (Fig. 3).

MC-specific antibody levels

All patients had IgE, IgG, IgG1, and IgG4 levels drawn before the onset of immunotherapy, preseasonally, and intraseasonally. MC sIgE levels were comparable between the actively treated and placebo-treated groups at all times during the study. The diminished intraseasonal MC sIgE in the actively treated group was not significant (Fig. 4).

There were no significant differences in the levels of MC sIgG before immunotherapy. As expected, a significant difference was noted before the season and intraseasonally, with the active-treatment group having greater amounts of sIgG compared to the placebo-treated group, $p = 0.018$ and 0.028 , respectively (Fig. 5).

No significant differences in MC sIgG1 (Fig. 6) or MC sIgG4 levels (Fig. 7) were present between the treated patients and those receiving placebo before the onset of immunotherapy, but both MC sIgG1 and MC sIgG4 were significantly higher in the treated group before the season and intraseasonally.

LCR versus MC-specific antibodies

The relationship between MC-induced LCR and intraseasonal levels of MC sIgE, sIgG, sIgG1, and sIgG4 is presented in Table II. A significant correlation existed between intraseasonal levels of

MC sIgG4 and size of the LCR, $r_s = 0.701$ and $p = 0.011$, as well as with intraseasonal levels of MC sIgG and percent change of LCR, $r_s = 0.715$ and $p = 0.008$. As presented in Table II, the correlations between either the absolute size of the intraseasonal LCR or percent change of the LCR and MC sIgG, MC sIgG1, and MC sIgE were not significant.

There was no significant difference between SMSs of the active-treatment group and the placebo-treated group. However, the pollen counts for MC during the 1987 season were quite low, with the highest level being 1592 pollen grains per cubic meter recorded on January 15, 1987. Average grains during January were generally <200 pollen counts per cubic meter. In the 1985 and 1986 seasons, counts were on an average fourfold higher.

DISCUSSION

This study demonstrated that MC-specific immunotherapy led to a rise in MC sIgG. Elevation of sIgG with immunotherapy has been documented by numerous investigators, and several of these earlier studies revealed a correlation between increases in sIgG and clinical improvement.^{13, 14} However, the lack of predictive accuracy between blocking antibody level and symptomatic outcome in a given individual has led many to question a causal relationship between the two.^{13, 14} Indeed, in this study, immunotherapy led to a rise in levels of MC sIgG that was statistically significant when levels were compared to levels with placebo therapy. However, we found no relationship between level of MC sIgG and SMS.

Other studies have demonstrated that immunother-

TABLE II. Correlation coefficients between LCR and MC-specific antibody responses

LCR	r_s	p
Intra		
IgG	-0.530	0.056
IgG1	-0.324	0.242
IgG4	-0.701	0.011*
IgE	+0.110	0.110
Intra % change		
IgG	0.434	0.118
IgG1	0.407	0.143
IgG4	0.715	0.008*
IgE	0.300	0.280

Intra, during the study.

*Significant correlation.

apy not only causes a rise in sIgG antibody but also that the IgG isotypic response is restricted to IgG1 and IgG4.^{17, 18} Our previous work with MC immunotherapy documented a rise in both MC sIgG1 and MC sIgG4 with no rise of MC sIgG2 or MC sIgG3.¹⁵ Therefore, we only measured MC sIgG, sIgG1, and sIgG4. Other investigators have suggested that sIgG4 is the predominant sIgG antibody isotype stimulated by immunotherapy,¹⁶⁻¹⁸ and our results would confirm these observations with MC immunotherapy. Indeed, we found a statistically significant rise in MC sIgG4 in our active immunotherapy-treated group, but again this increase did not correlate with clinical improvement.

Djurup and Østerballe,¹⁶ studying the IgG subclass response to timothy grass pollen-extract immunotherapy, observed that patients had a poor long-term outcome with an early rise in allergen-specific IgG4 or an allergen-specific IgG4/IgG1 ratio of >1. More recently, Moss et al.¹¹ reported that the blocking antibody response to ryegrass immunotherapy was also restricted to IgG1 and IgG4 and found a strong inverse correlation between sIgG1 antibody levels and SMSs for rhinitis. Although our current study measured sIgG1 to MC and found a significant increase in MC sIgG1 in the active-treatment group compared to the placebo-treated group, we found no correlation between levels of MC sIgG1 and reduction in MC LCR or in SMSs.

Our data demonstrated that MC-specific immunotherapy suppressed the MC-induced LCR and that this suppression was highly correlated with the development of MC sIgG4. These findings are in general agreement with other investigators stressing the potential importance of blocking antibodies in the late-phase allergic response. Zetterstrom¹⁹ reported that

passive transfer of antigen-specific IgG antibody followed by challenge with specific antigen did not lead to an LCR, whereas transfer of antigen-specific IgE did. Warner et al.²⁰ noted that approximately 50% of children with late-phase reactions after bronchial provocation to *Dermatophagoides pteronyssinus* lost late asthmatic response after 1 year of immunotherapy with this antigen. Patients losing the late reaction also demonstrated the greatest improvement in symptoms. Metzger et al.²¹ noted that adult patients with late bronchial responses to *Alternaria tenuis* had preferential suppression of the late over the immediate asthmatic response after immunotherapy. Pienkowski et al.,²² in a retrospective analysis of patients receiving ragweed immunotherapy, noted that immunotherapy was associated with suppression of the LCR and that the size of the LCR correlated inversely with the level of IgG to ragweed antigen E. In our controlled prospective study, we confirmed this finding in that immunotherapy was associated with suppression of the LCR and that this suppression was correlated with production of MC sIgG4. Although correlation does not prove causality, since both correlating variables may be influenced by a third variable, the fact that MC sIgG4 is the only antibody to correlate significantly with reduction in MC LCR is evidence for a cause-and-effect relationship.

Despite the excellent correlation of suppression of the LCR with development of MC sIgG4, we found no correlation between the level of MC sIgG4 nor degree of suppression of the LCR with SMSs. Indeed, there was no significant difference in SMSs between either the active-treatment group or the group receiving placebo. There are several possible explanations for this apparent lack of symptomatic efficacy of our immunotherapy. As noted, the 1987 MC-pollen season was mild with generally light pollen counts. The lack of seasonal rise in MC sIgE or MC sIgG1 probably reflected a lack of significant natural exposure to MC pollen during the season, since we have previously demonstrated both MC sIgE and MC sIgG1 rise from natural exposure.¹⁵ Also, our total of only seven matched pairs of patients who successfully completed the study may not have been a large enough group to demonstrate statistically significant changes in SMSs. In addition, it should be noted that our immunotherapy was rush, and extrapolation of our results to a more standard protocol should be done cautiously. Finally, the lack of correlation between the various immunologic parameters and SMSs, despite a good correlation with suppression of the LCR, may indicate that the LCR may not be a good model for the allergic rhinoconjunctivitis caused by MC pollen. The pathogenic late-phase response may be different in various target

organs.²³ Indeed, the nasal model appears to be histologically different from the cutaneous model. Different immunologic regulatory pathways may be affected by immunotherapy in these systems or mast cell heterogeneity may account for different responses. Our data also suggest the possibility that suppression of the LCR may precede clinical changes in rhinoconjunctivitis.

In summary, this double-blind, placebo-controlled, prospective study demonstrated that the LCR in MC rhinoconjunctivitis was suppressed by antigen-specific immunotherapy and that this suppression was strongly correlated with MC sIgG4 antibody response, providing evidence that MC sIgG4 may be responsible for this suppression.

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