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Hymenoptera venom immunotherapy

I. Induction of T cell-mediated immunity by honeybee venom immunotherapy: Relationships with specific antibody responses

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The specific cell-mediated and humoral immune responses of 14 children allergic to honeybee venom were studied. An 8-day rush venom immunotherapy induced an increase in T proliferative ($p < 0.04$) and T suppressive ($p < 0.003$) cell-specific activities. Antibody variations, an increase in specific IgG4 ($p \neq 0.05$), and a decrease in specific IgE ($p < 0.01$) were observed 1 year later. Initial high T suppressive cell activity prevents T proliferative cell increase during rush venom immunotherapy. High initial levels of specific IgG1 and specific IgG4 have opposing effects on the increase in T suppressive cell activity, the former being positively correlated with intensive increase ($r = 0.840$; $p < 0.005$), the latter negatively with T suppressive cell increase ($r = -0.709$; $p < 0.001$). These data indicate that there are interrelationships between the cell-mediated immunity and the antibody responses in honeybee allergy. (J ALLERGY CLIN IMMUNOL 1989;83:563-71.)

The generalized reactions caused by honeybee stings were recognized to be anaphylactic reactions as early as 1925.¹ Hymenoptera-sting sensitivity is a relatively common cause of allergic reactions, affecting at least 0.4% to 0.8%²⁻⁴ and perhaps up to

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Abbreviations used

RIT:	Rush venom immunotherapy
BV:	Honeybee venom
JV:	Yellow jacket venom
MNC:	Mononuclear cell
BV TS:	Bee venom T suppressive cell activity
BV TP:	Bee venom T proliferative cell activity
MLR:	Mixed lymphocyte reaction
sIgG1:	Specific IgG1
sIgG4:	Specific IgG4
sIgE:	Specific IgE
FCS:	Fetal calf serum
TH:	T helper cell activity

4%⁵ of the population. The patients are at life-threatening risk, and this sensitivity can persist for several years.^{5,6}

Immunotherapy with BV has been proved to provide significant protection against such stings.⁷ The hyposensitizing treatment schedules currently used for venom immunotherapy are short, from a few days (RIT) to a few weeks,⁶ but maintenance doses must be continued for several years. The life-threatening nature of venom reactions elicited the rationale for the use of RIT, which has now been demonstrated to reduce adverse reactions and to provide a faster rise in sIgG.⁸ Venom immunotherapy with BV induces sIgG blocking antibody and, at the same time, reduces sIgE. However, there may be a transient rise in sIgE in some individuals,⁹⁻²² which was not observed when venom immunotherapy was performed by modified antigens.^{19,20} Both phenomena (sIgG increase and sIgE decrease) are considered to be important in assessing venom immunotherapy efficacy,^{11,22-24} even though considerable individual variations in the antibody responses to RIT were reported.²⁵

The development of monoclonal antibodies has made it possible to investigate IgG subclasses. RIT had been demonstrated to induce increases in both IgG1 and IgG4²⁶⁻²⁸ but not in IgG2 and IgG3.^{16,27} The efficacy of RIT was related to the increase in sIgG4¹⁶ and a recent study has demonstrated an inverse relationship between sIgE and sIgG4 with sIgE decreasing and sIgG4 increasing.²⁶

The IgE and IgG responses are both under the control of T-lymphocytes. They depend on TH functions for induction and are under the control of TS regulatory mechanisms.²⁹ Surprisingly, although numerous studies have been conducted to evaluate antibody variations in honeybee allergies, very little has been done to investigate T cell responses. Specific T-lymphocyte proliferation was found to be increased in honeybee-allergic patients but not in beekeepers who failed to release interleukin-2.³⁰ Lymphocyte proliferation was reported in another study³¹ to be very low and nonspecific. Injection of BV induces a specific TH response in both humans³¹ and rats.³²

However, none of the studies published to date has simultaneously investigated both the specific humoral and the cell-mediated immunities in honeybee allergies, and there has been no study of the time course of cell-mediated immunity in such patients.

The present study was therefore designed to analyze the variations in specific TH and TS responses and to quantify the specific antibody responses (sIgE, sIgG1, sIgG4) in a group of honeybee-allergic children treated by RIT. Analysis of the data on the cellular and humoral responses indicates that RIT induces

rapid increases in specific TH and TS activities and that the later antibody responses observed are clearly related to the early changes in T-lymphocyte activities.

MATERIAL AND METHODS

Study population

Fourteen children (four girls and 10 boys) aged from 4 to 14 years were selected for the study. All parents and children agreed to participate in the study and gave informed consent.

All children had systemic allergic reactions after honeybee stings (between 3 to 6 months before treatment) and positive skin tests to BV (always grade ≥ 2 with a mean of 3.14 ± 0.90 SE).

Skin tests were performed by intradermal injection of 0.03 ml of antigen solution with the same antigens as previously described.³⁴ Intradermal reactivity was classified from 4 (wheal ≥ 5 mm to 10^{-4} μ g of venom per milliliter) to 1 (wheal ≥ 5 mm to 0.1 μ g of venom per milliliter); class 0 was reserved for negative responders at the highest antigen concentration.

After previous publications,^{35,36} RAST classes were as follows: class 0, from 0.07 to 0.1 IU/ml; class 1, for patient serum, <0.3 IU/ml; class 2, levels <1 IU/ml; class 3, levels <4 IU/ml, and class 4, upper levels); 10 patients were retested a year later.

Immunotherapy protocol

Previous experience had demonstrated that an 8-day period for hyposensitization was well accepted and tolerated by children to the number of daily injections and the duration of hospitalization.

Therefore, the patients were treated according to the following protocol with BV (lot no. Y 34236, Stallergènes Laboratoires, Fresnes, France): Each patient was initially administered a dose of venom of 0.00001 μ g. The dose was doubled and repeated at 2-hour intervals to a maximum of 0.0008 μ g on the first day. On the second day, the first dose was 0.001 μ g, and doubling doses were administered every 2 hours to a maximum dose of 0.08 μ g. Thereafter, injections were administered every 4 hours with doubling doses from 0.1 to 0.8 μ g on the third day, from 1 to 8 μ g on the fourth day, and from 10 to 50 μ g on the fifth day. The RIT involved two injections per day: 60 and 80 μ g on the sixth day, two 90 μ g injections on the seventh day, and 100 μ g on the last day.

Maintenance dose (100 μ g per injection) was administered on days 14 and 28, and once a month thereafter for the following years.

The same lot of BV antigen was used for in vivo injection (skin test or hyposensitization) and in vitro testing (specific humoral skin and cellular immunity quantification) during the entire study.

MNC isolation

MNC suspensions were isolated by lymphoprep (Flobio, Courbevoie, France)-gradient centrifugation of heparinized

μ l of 10% FCS RPMI 1640 and 100 μ l of 10% FCS RPMI 1640 containing the same concentrations of BV antigen (responding TP cell cultures) or without antigen (control TP cell cultures) to quantify specific TP cell activity.

A third row of the tissue-culture plates was filled with 50 μ l of BV TS, 50 μ l of 10% FCS RPMI 1640, and 100 μ l of 10% FCS RPMI 1640 with or without antigens to evaluate the efficacy of mitomycin C treatment on BV TS cells. No mitomycin C-treated culture expressed >500 cpm.

All samples, on a single multiwell plate, were incubated as above for 6 days. On the morning of the sixth day, 1 μ Ci of ^{3} H-thymidin (CEA Gif/Yvette, France) was added to each well, and the cells were incubated for an additional 4 hours. Harvesting was performed with a MASH II cell harvester (OSI, Paris, France). Mitogenic ability was assessed as the amount of radioactivity incorporated by the cells, measured by a liquid scintillation β -counter (LKB, Paris, France).

The results are expressed with the following sampled quantifications: TP ag, counts per minute of BV TP cultured in the presence of the BV antigen; TPc, counts per minute of BV TP cultured without antigen; TS ag, counts per minute of BV TS cultured in the presence of BV antigen; TSc, counts per minute of BV TS cultured without antigen; Coc ag, counts per minute of cocultures of BV TS and BV TP in the presence of BV antigen; and Coc c, counts per minute of the same cocultures without antigen.

Specific TP activity is expressed with the following index:

$$\text{TP index} = \frac{\text{TP ag} - \text{TPc}}{\text{TPc}}$$

Specific TS activity is expressed with the following index:

$$\text{TS activity} = \frac{(\text{TP ag} - \text{TPc}) - [(\text{Coc ag} - \text{TS ag}) - (\text{Coc c} - \text{TS c})]}{(\text{TP ag} - \text{TPc})}$$

$$\text{TS index (\% of suppression)} = 1 - (\text{TS activity}) \times 100$$

This complicated index of TS activity was necessary to take account of a possible auto-mixed lymphocyte reaction between TP and TS MNCs. Such auto-mixed lymphocyte reaction was observed for one patient in the study; the co-culture response without antigen was 17.200 cpm in this case (<1000 cpm for all other patients).

Specificity of TP and TS activity

The specificity of TP and TS activities was assessed in two subjects who were only allergic to honeybee and in two other subjects only allergic to *Vespula* venom. For those patients we simultaneously generated TS cells for 2 days in the presence of honeybee antigen and of JV antigen (*Apis mellifera*, lot no. Y 34236; *Vespula*, lot no. Y 34250, 50 μ g/10⁶ cells, Stallergènes). Cultures were performed 2 days later with the previously described protocol but in the presence of either BV antigen or JV antigen in a double-cross fashion.

Total IgE measurement

Total IgE was quantified with PRIST (Pharmacia Diagnostics AB, Uppsala, Sweden).^{37, 38}

Measurement of specific antibodies (sIgE, sIgG1, sIgG4)

Specific antibody levels were determined by the enzyme-linked immunofluorescent (ELIFA) technique described in part II of this study, pages 572-80.

Results are expressed in units per milliliter, the units being different for each antibody, as reported in part II.

Study protocol

Children selected to take part in this study were tested according to the following schedule. On the first day of hospitalization, before any treatment, a 25 ml blood sample was obtained (20 ml of lithium heparinate for MNC isolation and 5 ml for sera collection). Similar blood samples were obtained on the last day of hospitalization (2 hours after the last injection of RIT) and 1 year later.

TP and TS assays were run on the day of blood collection. Sera were aliquoted, randomized, and frozen the same day. Samples were tested blindly; all samples were thawed and quantified on the same day.

Statistical analysis

Statistical differences between the delta of each parameter measured at different times were calculated by Student's *t* test for paired experiments.

Evolution of the immunologic parameters during the RIT were correlated with Pearson's ranking test. Similar analysis was performed to compare evolution of the same parameters to initial values.

RESULTS

Before treatment

Cell-mediated immunity. The values before treatment are presented in Table II. TP activity was very low; only two individuals exhibited TP index values ≥ 2.0 for the smallest concentration and seven individuals for the highest. TS activity ($\geq 20\%$ of suppression) could be detected in three individuals at only one antigenic concentration.

Humoral immunity. IgE levels were slightly elevated. Seven of 14 children had total IgE levels >170 IU/ml. Seven of the 14 children had sIgE levels, quantified by enzyme-linked immunofluorescent assay >10 U/ml (equivalent of RAST class 3 or 4). sIgG4 ranged from 0.04 to 8.4 U/ml; the patients with the highest values (>3.5 U/ml) were those with a history of multiple honeybee stings. They also exhibited measurable TS activity before RIT ($\geq 19.8\%$ of suppression). In contrast, sIgG1 level was always low except for one individual (12.8 U/ml) who had been stung just before therapy (Table II).

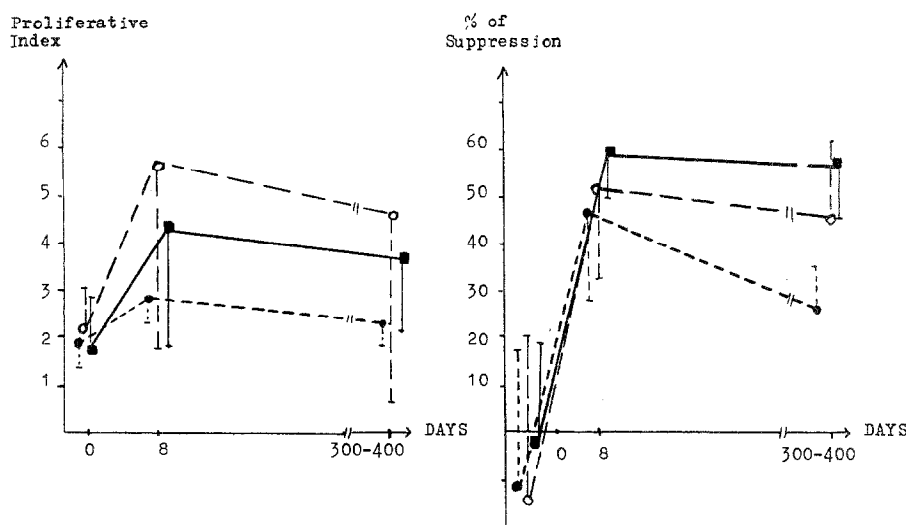


FIG. 2. Evolution of TP- and TS-specific activities during BV immunotherapy. TP and TS activities to BV antigen were quantified during RIT and 1 year later in 10 children allergic to BV. The curves represent the mean ($\pm$ SE) variations of the three BV antigen-tested concentrations for TP and TS (percent of suppression) indexes; 0.1 $\mu\text{g}/10^6$ cells ($\bullet$ --- $\bullet$); 1 $\mu\text{g}/10^6$ cells ($\circ$ --- $\circ$); 10 $\mu\text{g}/10^6$ cells ($\blacksquare$ — $\blacksquare$).

TABLE II. Evolution of immunologic parameters during RIT

	Before RIT	After RIT
TP activity (TP indexes)		
(μg BV/ 10^6 cells)		
0.1	1.5 ± 0.6	$2.6 \pm 2.1^*$
1	1.9 ± 0.8	$5.0 \pm 4.3^*$
10	2.1 ± 0.8	$4.4 \pm 2.6^\dagger$
TS activity (% of suppression)		
(μg BV/ 10^6 cells)		
0.1	-2.8 ± 32.5	$41.0 \pm 35.0^\ddagger$
1	-2.1 ± 35.0	$51.6 \pm 21.0^\ddagger$
10	-1.1 ± 17.4	$49.8 \pm 22.5^\ddagger$
Total IgE (IU/ml)	162.1 ± 75.1	148.6 ± 60.3
IgE (IU/ml)	15.3 ± 6.8	10.4 ± 8.9
IgG1 (IU/ml)	2.4 ± 3.9	4.2 ± 8.1
IgG4 (IU/ml)	4.6 ± 2.0	5.3 ± 2.0

All parameters were quantified just before and immediately after RIT for 14 tested children. Values represent mean $\pm$ SE.

Statistical differences between the delta of each parameter were evaluated by Student's *t* test for paired experiments.

Significant differences are indicated.

* $p < 0.05$.

† $p < 0.01$.

‡ $p < 0.001$.

Evolution of TP and TS activities during immunotherapy

During rush treatment. Both TP and TS activities increased during the 8 days of RIT (Table II; Fig. 2). These increases were highly significant for TP indexes ($p < 0.04$ for 0.1 μg , $p < 0.02$ for 1 μg , $p < 0.01$

for 10 μg) and for TS indexes ($p < 0.003$ for 0.1 μg , $p < 0.003$ for 1 μg , and $p < 0.001$ for 10 μg).

During the follow-up year. Both activities remained level 1 year later (Fig. 2). The apparent decrease in the TS index for 0.1 μg or in the TP indexes are far from significant ($p \neq 0.15$).

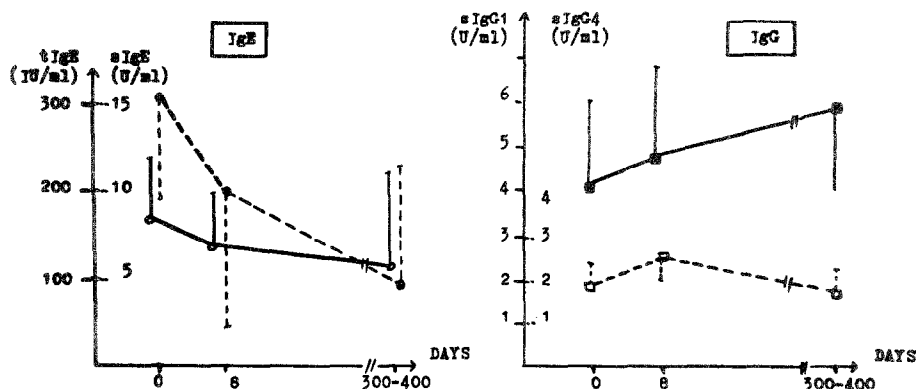


FIG. 3. Evolution of humoral immunity during BV immunotherapy. Total IgE (○—○), sIgE (●—●), sIgG1 (□—□), and sIgG4 (■—■) were measured during RIT and a year later in 10 children allergic to BV. The curves represent the mean evolution ($\pm$ SE) during the year of follow-up.

Remarks. The increase in TP index was negatively correlated with the initial level of TS index (at least $r = -0.518$; $p < 0.05$), indicating that children who exhibited TS activity before treatment did not increase TP activity during RIT.

The increase in TS index was negatively correlated with the initial level of TS activity (at least $r = -0.576$; $p < 0.02$) and with the initial level of sIgG4 (at least $r = -0.709$; $p < 0.001$). This indicates that TS activity generated by RIT was greater in patients with no regulatory TS activity or sIgG4 level before treatment. There was a positive correlation between the previous level of sIgG1 and the increase of TS indexes (at least $r = 0.840$; $p < 0.005$). Therefore, the pretreatment levels of sIgG1 and sIgG4 had opposite effects on the generation of TS activity during RIT.

There was no correlation between the pretreatment level of IgE (total or specific) and variations in TS activity induced by RIT.

Evolution of antibody levels during immunotherapy

During rush treatment. None of the variations observed for any of the antibodies measured were significant during the 8 days of RIT, including that of sIgE that decreased noticeably (32.0%) (Fig. 3). This absence of significant variations is certainly related to the wide range of values observed in the children tested.

During the follow-up year. IgE levels continued to decrease during the following year (Fig. 3); therefore, the levels reached a year after therapy were marginally lower for total IgE ($= 1.796$; $p < 0.08$) and significantly lower for sIgE ($= 4.005$; $p < 0.005$); sIgG4 increased ($= 2.042$; $p < 0.052$), whereas sIgG1, after a nonsignificant rise, returned to the initial level 1 year after treatment.

The relatively rapid decrease of sIgE that occurred in our patients may be related to their fairly high levels of sIgG4 before treatment ($r = 0.664$; $p < 0.01$) and is related to sIgG4 increase ($r = 0.736$; $p < 0.01$), but a study of a larger group of subjects will be required to verify this relationship.

Specificity of the measured TP or TS cell activity

Antigen specificity was tested for TP activity as well as for in vitro-generated TS activity in four monoallergic patients. This cross assay was performed after RIT. The results (Table III) indicate that, after treatment, a high level of TP activity (TP index > 1.5) could only be detected for honeybee culture in honeybee-allergic patients and for *Vespula* cultures in yellow jacket-allergic patients.

The in vitro generated suppressive activities were also antigen specific. BV suppressive activity could only be generated in honeybee-allergic patients and *Vespula* suppressive activity in yellow jacket-allergic patients. Those data provide evidence that antigen cross-reactivity detected between those two venoms³⁵ is minor and could not be detected in such assays quantifying cell-mediated immune functions.

DISCUSSION

The specific antibody and cell-mediated responses of a group of allergic children to BV have been used to analyze the development of these responses and the way in which they relate to one another. Although each of these responses has been examined in isolation,^{9-28, 30, 31} this is, to our knowledge, the first time that they have both been followed in a single group of subjects.

RIT was selected as the means of initiating the immune responses because this treatment is generally considered to be efficient^{6, 39, 40} and may, therefore,

TABLE III. Specificity of generated TP and TS activities in monoallergic children

Patient	TP activity (in TP index)				TS activity (% of suppression)							
					Generated with BV antigen				Generated with JV antigen			
	Tested on BV		Tested on JV		Tested on BV		Tested on JV		Tested on BV		Tested on JV	
	0.1	1	0.1	1	0.1	1	0.1	1	0.1	1	0.1	1
1	1.9	3.1*	0.8	0.9	-23.7*	-54.4*	-2.4	-6.4	-3.4	-2.8	+10.6	+6.4
2	1.7	4.2*	1.2	1.1	-21.8*	-28.4*	+1.3	-1.7	+1.2	+2.4	+1.2	-3.8
3	1.2	1.4	1.8	3.4*	+2.4	+5.5	+1.8	-0.9	-2.4	-4.7	-24.9*	-53.9*
4	0.5	0.7	1.9	5.3*	+1.2	+2.4	-1.3	-10.4	-4.2	-3.8	-18.9*	-47.3*

Patients allergic to honeybee (1 and 2) or to yellow jacket (3 and 4) were tested after rush venom immunotherapy for antigen specificity.

TP responses were measured in the presence of BV or JV antigen at 0.1 and 1 µg/ml.

Suppressor cells were generated by culture for 2 days in the presence of BV or JV antigen and tested in a coculture system in the presence of either BV or JV antigen. Responses are expressed as proliferative or suppressive indexes.

*Positive responses as compared to negative control responses.

provide a model system for evaluation of the major parameters of clinical efficacy. Thus, the study should lead to a better understanding of the mechanisms underlying RIT. Allergy to the venom used, BV, has been extensively studied, RIT has been demonstrated to induce specific and opposing changes in sIgE and sIgG antibodies,^{9,24} and these changes have been detailed for the IgG subclasses.^{16, 26-28} There is also clear evidence that changes in cell-mediated immunity occur in this allergy.^{30, 31}

BV-allergic children had little specific TP activity and almost no specific TS activity before treatment. These data indicate that the TP indexes of allergic children before treatment are lower than indexes of adults,³⁰ even when our index minimizes the responses. The children who had been stung several times, and who had high sIgG1 and sIgG4 levels, had some TS and TP activities before treatment but at a low level.

The cell-mediated immunity responses that were quantified in this study were antigen specific.³¹ Indeed, TS and TP indexes only increased for the antigen used in the hyposensitization and not for the control, JV, antigen (Table III), providing further evidence of antigen specificity.

The TP and TS responses to RIT were large. Similar response increases have been reported in rats injected with BV.³² It is clear that, in both humans (this study) and rats,³² this increase in cell-mediated immunity does not occur before repeated venom injections. The lack of specific TS activity before challenge, coupled with an increase in activity shortly after challenge, has been observed in humans for tetanus⁴¹ and ragweed⁴² antigens.

These data suggest that cell-mediated responses

reach low levels only in children who had been stung infrequently during a long period of time (once or twice a year). In fact, both the BV RIT used in this study and the rat immunization protocol used numerous injections during a short time period.

The induction of TS activity by immunotherapy was first observed in 1980 in patients suffering from ragweed allergy.³³ Several studies subsequently confirmed that immunotherapy induced specific TS activity to grass pollens.^{42, 44, 45} The specific suppressor cells that are generated can inhibit several in vitro systems, including lymphocyte proliferation,^{33, 46} antibody synthesis,⁴² antigen-induced histamine release,²⁸ and lymphocyte CD8 marker expression.^{42, 44} In all these studies, only repeated administration was found to induce specific TS activity.

The humoral response to BV RIT was slower to develop. The two major parameters of this response, a decrease in sIgE and an increase in sIgG (especially IgG4)^{13, 38, 39, 47} were not observed until 1 year after therapy. sIgE may increase during treatment, as suggested by several studies.^{15, 25, 28} In our study, sIgE remained unchanged. This is in agreement with other publications.^{9, 18, 21, 25, 27} Changes may have occurred between the end of RIT and the 1 year follow-up, but no samples have been tested during this period.

The decrease in sIgE and the increase in sIgG4 that took place during the year after RIT are in good agreement with the changes reported by Cheung et al.²⁶ A decrease in sIgE has generally not been detected until several years after treatment in most studies on either children¹⁵ or adults.^{22, 28} However, some recent studies in which modified antigen was used indicate that this may occur earlier.^{19, 20}

Early increases in sIgG4 after RIT have been re-

ported.²⁶ Müller et al.²⁷ observed such an increase within a few days after the start of treatment. The rapid increase in sIgG4 observed in our subjects (within 8 days) may be the result of prestimulation of sIgG4 synthesis, as indicated by the high levels of this antibody before treatment.

Although there was a small, nonsignificant increase in sIgG1 on day 8 of RIT in our patients, the level of this antibody was unchanged 1 year after therapy. There have been studies^{27, 28} of a significant increase in IgG1, but, since no measurements were made in this study between the termination of RIT and the 1-year follow-up, the changes observed 2 to 3 months after treatment²⁸ would not have been detected.

The experimental approach used in this study in which both humoral and cellular-immune responses to RIT were analyzed provides a unique opportunity to examine the respective roles of each of these components of the immune system in the response of allergic patients. The results indicate that both components are involved in the response to BV. The initial response of the cell-mediated system entails increases in both the opposing TP and TS activities, but the negative correlation observed between TP increase and initial TS activity suggests that high TS activity blocks the generation of TP cells or TP activity during RIT. This induced TS response may be influenced by the humoral system. The high initial sIgG4 level may tend to inhibit the TS increase, whereas the high level of sIgG1 is correlated with a large increase in TS activity. Thus, sIgG1 and sIgG4 may have opposing modulatory actions on the production of TS cell activity.

Suppressor cells have been reported to have Fc receptor.⁴⁸ Numbers of T γ (+) cells have been reported to be decreased in atopic patients⁴⁹⁻⁵¹ and to increase after immunotherapy.⁴⁹⁻⁵¹ Could those T γ suppressor cells be stimulated in an opposite way by two different signals, IgG1 and IgG4, the former inducing suppressive activity and the latter blocking this activity? Further studies involving T cell subsets are needed.

The present study demonstrates that honeybee RIT induces TP and TS activities and that these cellular changes occur much more rapidly than the well-documented humoral responses. They also provide evidence for an interrelationship between the RIT-induced T cell-mediated immunity and the slower induction of specific antibodies. Thus, the changes in specific antibodies induced by RIT may be directly related to the response of specific T cells to RIT. Consequently, the measurement of specific T cell functions could be an appropriate route toward understanding the immune changes caused by RIT. If this is true, then measurement of the early changes in

specific T cells may be used to predict later humoral changes resulting from the treatment, and hence provide an accurate indication of the effectiveness of RIT. If this is true, the clinical importance of such immune changes should be investigated.

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