

Role of cell-mediated immunity in Hymenoptera allergy

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*Currently there is little information regarding the immunologic mechanisms responsible for large local reactions (LLR) after Hymenoptera stings. To investigate this question, we measured in vitro lymphocyte proliferation and delayed skin reactivity to venom antigens in 10 subjects with LLR (six with LLR only and four with LLR and systemic reactions), seven subjects with systemic reactions, and eight nonallergic controls. The lymphocyte response to venoms in the LLR group was greater than that in either the systemic reactor group ($p < 0.05$) or the control group ($p < 0.001$). In contrast, lymphoproliferative responses to *Candida albicans*, streptokinase-streptodornase, and phytohemagglutinin were comparable in the three groups. Forty percent of the LLR group had positive delayed skin tests to venom antigens, and none of the patients in the systemic reactor group had such responses. These findings suggest that cellular immune mechanisms play a role in the pathogenesis of LLR after Hymenoptera stings. (J ALLERGY CLIN IMMUNOL 68:399, 1981.)*

Most interest in stinging insect allergy has focused on systemic reactions. However, many patients develop large local and often persistent reactions at the site of Hymenoptera stings. Although a number of reports have shown that approximately 50% of patients with LLR have IgE antibodies to Hymenoptera venoms,^{1, 2} the clinical characteristics of these reactions suggest that delayed immune mechanisms may play a role in their pathogenesis. Accordingly, the present study was initiated to measure delayed skin reactivity and in vitro lymphocyte responsiveness to Hymenoptera venoms in patients with histories of large local and systemic Hymenoptera reactions. Our findings suggest that cellular immune mechanisms are, at least partially, responsible for mediating large local responses after Hymenoptera stings.

Abbreviations used

LLR:	Large local reactions
SKSD:	Streptokinase-streptodornase
PHA:	Phytohemagglutinin
HSA:	Human serum albumin

MATERIALS AND METHODS

Patients

Seventeen patients with histories of Hymenoptera sensitivity were chosen for the study. Six had experienced LLR (defined as swelling at the sting site of 6 inches in diameter or greater, lasting for 24 hr or longer), four had had LLR and systemic reactions, and seven had experienced only systemic reactions. The patients with systemic reactions had had one or more of the following signs or symptoms: generalized pruritus and hives, wheezing, shortness of breath, or shock requiring emergency treatment. All but two patients had been stung in the 9-mo period prior to the investigation. The other two were placed one each in the LLR and systemic reaction groups, respectively. Eight control subjects who had never experienced a large local or systemic reaction were also studied. At the time of the investigation, none of the patients was receiving venom immunotherapy; however, two patients of the systemic reaction group had received prior whole-body Hymenoptera extract immunotherapy. All subjects provided written informed consent.

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TABLE I. Effect of various diluents on lymphocyte transformation*

Experiment	Commercial HSA diluent			RPMI-1640		
	Venom	<i>C. albicans</i>	SKSD	Venom	<i>C. albicans</i>	SKSD
1	0	2,827	4,851	2,724	60,994	55,131
2	0	4,442	15,430	34,942	41,090	9,435
3	250	4,078	0	42,944	121,281	78,666
4	0	0	0	3,813	2,179	8,843
5	0	3,796	17,991	19,637	51,719	18,957
6	0	764	0	12,545	58,380	25,824
7	0	306	0	4,499	6,141	10,794
8	0	0	0	15,022	7,886	43,955
Mean	31	2,027	4,784	17,016	43,709	31,451
SEM	31	689	2,680	5,277	14,029	9,020

*Expressed as cpm. Venom response, HSA vs RPMI-1640 $p < 0.01$; *Candida* response, HSA vs RPMI-1640 $p < 0.01$; SKSD response, HSA vs RPMI-1640 $p < 0.02$.

TABLE II. Lymphocyte transformation* and delayed skin-test results in patients with LLR or LLR and systemic reactions

Patient	Venom	<i>C. albicans</i>	SKSD	PHA	Delayed skin test	SR
1	44,803	41,090	9,435	18,071	+	—
2	13,069	58,380	25,824	15,496	+	—
3	15,022	10,406	43,955	11,955	+	—
4	19,637	51,719	18,956	33,736	—	—
5	4,499	6,141	10,794	21,679	—	—
6	3,813	2,179	8,843	19,092	—	—
7	42,944	121,281	78,666	48,478	—	+
8	8,251	60,994	55,131	33,216	—	+
9	3,013	1,462	3,418	12,807	+	+
10	128	3,659	63,618	100,752	—	+
Mean	15,518	35,731	31,864	31,528	4/10	4/10
SEM	5,096	12,293	8,417	8,505		

SR = systemic reaction.

*Expressed as cpm.

Skin testing

Hymenoptera venoms (Pharmalgen; Pharmacia Diagnostics, Piscataway, N. J.) from honeybee, yellow jacket, yellow hornet, white-faced hornet, and wasp were used for immediate and delayed skin tests. Immediate skin tests were performed with puncture and intradermal techniques. Individual venoms at 0.01 $\mu\text{g/ml}$ were used for puncture tests. Intradermal tests were performed by injection of approximately 0.02 ml of serially diluted venoms between 0.001 and 1.0 $\mu\text{g/ml}$. Positive responses were defined as the development of a 5-mm wheal or greater within 15 min. Delayed hypersensitivity skin tests were performed by intradermal injection of 0.1 ml of the same concentration of

specific venom that had produced a positive immediate skin test. These tests were examined at 6 to 8, 24, and 48 hr. Delayed skin tests were considered positive if induration of 5 mm or greater was present at 48 hr.

Lymphocyte transformation techniques

Lymphocyte transformation was performed with conventional microtiter techniques.³ Lymphocytes were separated from heparinized blood by centrifugation on Ficoll-Hypaque gradients and washed twice with phosphate-buffered saline. The cells were delivered in 200- μl aliquots into flat-bottomed tissue culture trays (Costar, Cambridge, Mass.) and cultured for various periods. One microcurie of

TABLE III. Lymphocyte transformation* and delayed skin-test results in patients with systemic reactions

Patient	Venom	<i>C. albicans</i>	SKSD	PHA	Delayed skin test
1	998	31,646	31,588	115,948	—
2	668	7,869	531	16,561	—
3	1,206	49,516	99,001	22,696	—
4	6,977	3,782	1,203	68,055	—
5	0	3,840	3,100	16,089	n.d.
6	225	n.d.	n.d.	37,545	—
7	0	n.d.	n.d.	20,874	—
Mean	1,439	19,331	27,085	42,538	0/6
SEM	940	9,154	18,898	14,060	

n.d. = not determined.

*Expressed as cpm.

TABLE IV. Lymphocyte transformation* to venom antigens in nonallergic controls

Subject	Honeybee	Yellow jacket	Yellow hornet	White-faced hornet	Wasp
1	654	0	n.d.	n.d.	115
2	18	n.d.	72	0	0
3	0	329	0	0	0
4	299	0	n.d.	n.d.	0
5	652	0	n.d.	728	0
6	3,094	0	n.d.	n.d.	3,097
7	4,260	577	n.d.	n.d.	683
8	588	216	0	3,044	876
Mean $\pm$ SEM			643 $\pm$ 208		

n.d. = not determined.

*Expressed as cpm.

TABLE V. Lymphocyte transformation* to nonvenom stimulants in nonallergic controls

Subject	<i>C. albicans</i>	SKSD	PHA
1	3,992	4,272	17,671
2	7,640	n.d.	47,900
3	82,307	24,929	44,555
4	12,975	18,318	10,485
5	2,715	7,894	80,284
6	33,915	25,762	17,994
7	47,215	95,504	34,153
8	2,306	6,163	18,234
Mean	24,133	26,120	33,910
SEM	10,133	12,036	8,206

n.d. = not determined.

*Expressed as cpm.

tritiated thymidine (^3H -TdR) was added to each well 18 to 24 hr before harvesting, and the cells were then collected on glass fiber filters with an automatic harvester. Radioisotope incorporation was determined in a Packard Tricarb liquid scintillation counter.

PHA (1 $\mu\text{g}/\text{ml}$; Burroughs Wellcome Co., Research Triangle Park, N. C.), *Candida albicans* (1:1000 dilution of

stock; Hollister-Stier, Spokane, Wash.), SKSD (400 units SK/100 SD/ml; Lederle Laboratories, Pearl River, N. Y.), and serial dilutions between 0.001 and 10 $\mu\text{g}/\text{ml}$ of specific Hymenoptera venoms were used as lymphocyte stimulants. In general, patients' cells were cultured with at least two and often three venoms; however, the values reported are those to which each patient was most allergic as determined by serial dilution skin testing. In all patients, blood for the lymphocyte transformation studies was taken before performing delayed skin tests in order to avoid the possibility of de novo sensitization. Lymphocytes from nonallergic controls were cultured with a minimum of three Hymenoptera antigens—honeybee and wasp venoms and at least one vespid venom. All assays were performed in quadruplicate. Results are expressed as counts per minute (cpm) in antigen- or mitogen-stimulated cultures minus counts per minute in control cultures. Statistical analysis was performed by Student's *t* test.

RESULTS

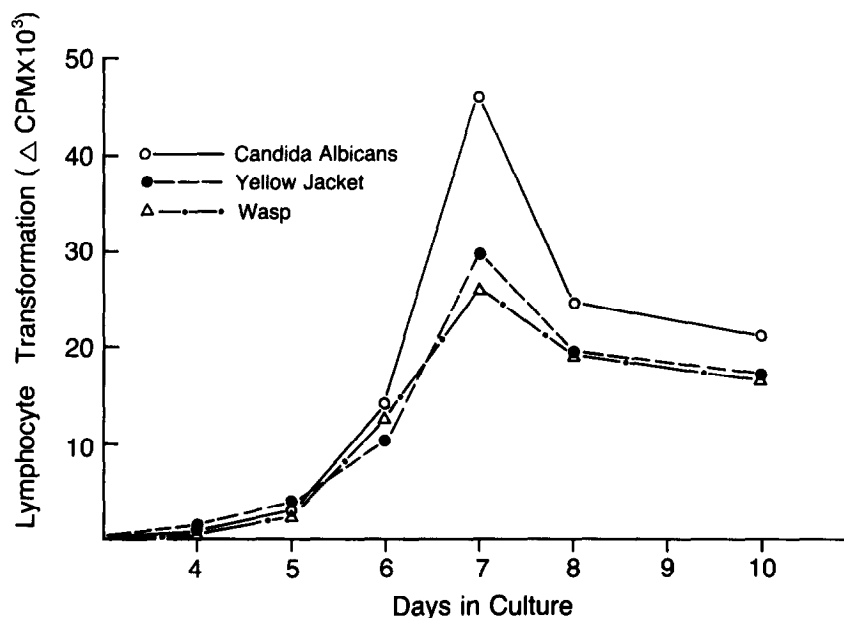
Determination of optimal in vitro lymphocyte culture conditions

Preliminary studies were performed to determine optimal culture conditions with venom antigens. The following variables were examined: (1) type of venom

TABLE VI. Summary of lymphocyte transformation studies*

Group	Venom	p value	<i>C. albicans</i>
LLR or LLR plus systemic reactions	15,518 $\pm$ 5,096	—	35,731 $\pm$ 12,293
Systemic reactions only	1,439 $\pm$ 940	<0.05	19,331 $\pm$ 9,154
Nonallergic controls	643 $\pm$ 208	<0.001	24,133 $\pm$ 10,133

n.s. = not significant.

*Expressed as mean cpm $\pm$ 1 SEM.**FIG. 1.** Kinetic study of lymphocyte transformation to *Candida albicans*, yellow jacket, and wasp venoms in one representative patient.

diluent, (2) optimal length of incubation and cell density, (3) best source and concentration of serum, and (4) shape of tissue culture wells.

To examine the effect of the diluent used in antigen preparation, the Hymenoptera venoms, *C. albicans*, and SKSD stimulants were diluted in commercial venom diluent containing HSA (Pharmalgen; Pharmacia) and culture medium RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) Both diluents were supplemented with L-glutamine, penicillin, streptomycin, and 5% serum. As shown in Table I, the commercial venom diluent suppressed lymphoproliferative responses to all three antigens. Although the reason for this suppression is uncertain, it may reflect the potential toxicity of the phenol in the diluent or the binding of the antigens by the albumin. In any event, RPMI-1640 was used for antigen and mitogen preparation in all subsequent studies.

To determine the optimal duration of in vitro incu-

bation of lymphocytes with venom antigens, proliferative responses were measured after 4, 5, 6, 7, 8, and 10 days of culture. A representative kinetic study from one patient is shown in Fig. 1. As can be seen, day 7 was optimal for venom- and *C. albicans*-induced stimulation. In contrast, PHA-stimulated proliferation was maximal at day 4 (data not shown); however, the stimulatory effect of this mitogen was still readily measurable at day 7, permitting all cultures to be harvested after 7 days of culture.

In other preliminary studies, the effect on lymphocyte transformation of different cell concentrations (1 , 2 , and 4×10^6 cells/ml), sera (human AB both dialyzed and nondialyzed and fetal calf serum), and culture-well geometry (flat- vs round-bottom wells) were examined. These studies showed that 1×10^6 cells/ml, 5% nondialyzed AB serum, and flat-bottomed wells produced optimal results. In all subsequent studies, these conditions were used. In addi-

p value	SKSD	p value	PHA	p value
—	31,864 ± 8,417	—	31,528 ± 8,505	—
n.s.	27,085 ± 18,898	n.s.	42,538 ± 14,060	n.s.
n.s.	26,120 ± 12,036	n.s.	33,910 ± 8,206	n.s.

tion, the serum employed in all experiments was from a single AB-negative donor who was not allergic to Hymenoptera venoms.

Lymphocyte proliferation and skin test results in Hymenoptera-allergic individuals

Table II shows lymphoproliferative responses to venom antigens, *C. albicans*, SKSD, and PHA, and delayed skin-test results in 10 patients with histories of LLR after Hymenoptera stings. As indicated, six of these individuals had experienced only LLR and four had had concomitant systemic reactions. Tables III, IV, and V present similar data for patients with histories of systemic reactions and for nonallergic controls. In Table VI the data have been summarized and statistically analyzed.

As a group, patients with LLR had significantly greater lymphoproliferative responses to venom antigens than did either nonallergic controls ($p < 0.001$) or patients who had had only systemic reactions ($p < 0.05$). In contrast, lymphocytes from patients with LLR, those with systemic reactions, and nonallergic controls responded comparably to *C. albicans*, SKSD, and PHA.

Delayed (48 hr) skin tests to venom antigens were positive in four of 10 LLR patients and negative in six of six systemic reaction patients in whom the test was performed. All patients with negative 48-hr skin tests were also negative at 6 to 8 and 24 hr. In contrast, the skin-test sites in the four patients with positive delayed reactions were also positive at 6 to 8 and 24 hr.

Immediate skin tests were positive in five of six patients who had experienced only LLR and in all 11 patients who had histories of systemic Hymenoptera allergy (seven with systemic reactions only and four with LLR and concomitant systemic reactions). In contrast, immediate skin tests were negative in three control subjects who permitted the tests to be performed.

DISCUSSION

This study demonstrates that patients with LLR after Hymenoptera stings had greater in vitro lymphocyte transformation responses to venom antigens than those of either nonallergic controls or patients who

had experienced systemic reactions. In contrast, lymphoproliferative responses to SKSD, *C. albicans*, and PHA were similar in these three groups. Furthermore, 40% of LLR patients had positive delayed skin tests to venom antigens, whereas delayed skin tests were negative in all patients with systemic reactions. These findings suggest that cellular immune mechanisms play a role in the pathogenesis of LLR after Hymenoptera stings. Further support for this conclusion comes from a more detailed look at the history and immunologic findings in one patient within our LLR group (patient 2, Table II). This individual had experienced an LLR with no associated systemic symptoms after a honeybee sting but showed no reaction to immediate venom skin tests. In contrast, she demonstrated a positive delayed venom skin test and her lymphocytes were reactive in vitro to honey bee venom. By conventional diagnostic criteria, such a patient would be classified as nonallergic. However, the use of lymphocyte transformation and delayed skin testing demonstrated that there was an immunologic basis for her reaction.

One point that requires additional discussion is why only 40% of our patients with LLR had positive delayed skin tests. One might expect that all patients who developed delayed local swelling should manifest a similar response after experimental challenge. There are at least three possible explanations for the discrepancy. First, we may have skin tested with insufficient venom to trigger a reaction. Indeed, in actual stings approximately 50 μ g of venom are injected,⁴ whereas the largest amount of venom we used for delayed skin testing was 0.1 μ g. Second, it is possible that not all LLR patients retained active cellular immunity to venom antigens at the time of the study. Clearly, patient 10 in the LLR group (Table II) did not have lymphocytes that recognized venom antigens. Third, it is possible that the immediate skin-test reactions provoked by venom skin testing blunted the development of later delayed skin responses. In this regard, Rocklin⁵ has demonstrated that histamine, one of the primary mediators released during immediate wheal and flare reactions, can suppress the in vivo and in vitro expression of cellular immunity. In his experiments, Rocklin showed that histamine re-

duced by half the size of experimental delayed skin reactions and also inhibited in vitro macrophage migration inhibitory factor production and antigen-induced lymphocyte proliferation. These effects were all blocked by histamine H₂ antagonists. Thus local histamine release occurring at immediate skin-test sites may suppress the development of subsequent delayed skin reactions at the same site. Since all but one of our LLR patients had positive immediate venom skin tests, it is possible that we failed to observe delayed venom skin responses in some patients because of the prior expression of immediate reactivity at the same skin site.

The problem of LLR to Hymenoptera stings has been addressed by relatively few investigators. Reisman et al.¹ and Green et al.² have reported that approximately 50% of patients with LLR have venom-specific IgE antibodies, as determined by immediate skin tests and radioallergosorbent assays. In the present study, 83% of patients with LLR demonstrated venom-specific IgE antibodies as measured by immediate skin tests. Collectively, these data clearly indicate that IgE anti-venom antibodies are present in many patients with LLR; however, this does not prove that IgE antibodies are involved in the pathogenesis of these responses. Indeed, in considering the potential immunopathologic mechanism(s) responsible for LLR, at least three possibilities exist: (1) they are type IV or cell-mediated immune reactions, (2) they are type III or Arthus-type responses, or (3) they represent the late phase of IgE-mediated (type I) reactions. To our knowledge, the present study provides the only information suggesting that type IV reactivity plays a role in mediating LLR to Hymenoptera stings. Similarly, the role of IgG antibodies in LLR has received little attention. Green et al.² found that only three of 19 patients with LLR had anti-venom IgG antibodies. In contrast, Hoffmann⁶ found IgG antibodies as well as IgE antibodies to bee venom allergens in eight of eight patients with recent large local bee sting reactions. However, he also found similar antibody profiles in a group of 13 patients with recent systemic reactions, leading him to conclude that the levels of IgE and IgG antibodies to bee venom allergens did not determine whether a sting reaction would be systemic or local. These findings, in conjunction with the knowledge that high levels of venom-specific IgG antibodies are protective against systemic Hymenoptera reactions,⁷ suggest that venom specific IgG antibodies probably do not play a major role in mediating LLR after Hymenoptera stings. Lastly, it is possible that late-phase skin reactions are responsible for LLR to Hymenoptera stings.⁸⁻¹⁰ These reactions are indurated, erythematous skin re-

sponses that develop after immediate wheal and flare reactions. Typically they appear 2 to 4 hr after antigen injection, peak within 6 to 12 hr, and usually resolve within 24 hr. Previous studies have clearly demonstrated that late-phase reactions, also called late cutaneous allergic responses, are IgE dependent.^{10, 11} This has been perhaps best proven by studies showing that intradermal injection of anti-IgE elicits a late-phase allergic reaction as well as the expected immediate response.¹⁰ Although large local Hymenoptera reactions are clinically similar to late-phase reactions, it seems unlikely that IgE antibodies are exclusively responsible for their development for at least two reasons. First, not all patients with LLR have demonstrable anti-venom IgE antibodies, and second, most patients who have venom-specific IgE antibodies do not develop LLR. Although our data do not directly address the role of Arthus or late-phase IgE reactions in large local Hymenoptera reactions, it is interesting to note that all four patients with positive delayed (48 hr) venom skin tests also had positive responses at 6 to 8 and 24 hr. These earlier reactions were probably expressions of late-phase IgE immunity, although skin biopsies would be required to verify this hypothesis.

In summary, it is probable that LLR are mediated by a combination of immune mechanisms. The erythema and swelling that often occur between 2 and 24 hr after a sting are probably the results of a late-phase IgE reaction and perhaps IgG-mediated events, whereas the induration that remains beyond 24 hr and in some patients persists for as long as 7 days is probably cell-mediated in etiology. We suggest that patients with histories of LLR have delayed skin tests performed with venom antigens as part of their diagnostic evaluations.

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