

Comparison of inflammatory events in skin sites with and without cutaneous late-phase reactions after prominent immediate IgE-mediated responses

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Background: A number of inflammatory events have been detected in skin chambers overlying sites of developing late-phase reactions (LPR) to pollen antigens in sensitive subjects. However, the pathogenic significance of such events is still unclear.

Objective: We sought to compare inflammatory responses and cytokine levels in skin chambers that overlie sites of antigen challenge in individuals with and individuals without LPRs after immediate wheal responses of similar intensity. **Results:** Early histamine releases at antigen sites were similar in eight subjects with LPR (+/+ group) and eight subjects without LPR (+/- group). However, histamine releases during hours 2 through 5 of antigen challenge were significantly greater in the +/+ subjects than in the +/- subjects. Total exuding leukocytes; percent eosinophils; and levels of eosinophil cationic protein, lactoferrin, and IL-8 were significantly greater at antigen versus buffer control challenge sites in both the +/+ and +/- groups, with no significant differences between the groups. IL-1 and IL-6 levels were not greater at antigen sites than at buffer sites.

Conclusions: The only significantly greater antigen-induced response detected in +/+ subjects than in +/- subjects was in later histamine release, which is possibly a marker of other inflammatory responses because histamine itself does not induce LPRs. Other inflammatory events assessed may be somewhat greater in +/+ subjects, but not significantly so. (*J Allergy Clin Immunol* 1998;101:110-5.)

Key words: Late-phase reactions, IgE-mediated responses, cytokines, inflammatory cells

It is generally agreed that late-phase reactions (LPRs), which may follow immediate IgE-mediated responses, could play an important role in the pathogenesis of human allergic disease. However, despite intensive investigation, the mechanisms underlying development of such LPRs in the skin and airways are still not well defined. Using skin biopsy and skin-chamber approaches, we and other groups¹⁻⁴ have documented a number of inflammatory events during developing and expressed skin LPRs. However, the pathogenic signifi-

Abbreviations used

BD:	Buffer diluent
ECP:	Eosinophil cationic protein
LPR:	Late-phase reactions
MCP-1:	Monocyte chemotactic protein-1
PBS:	Phosphate-buffered saline
PNU:	Protein nitrogen units
RANTES:	Regulated upon activation normal T cell expressed and presumably secreted
+/+:	Subjects with immediate and subsequent late-phase reactions
+/-:	Subjects with immediate but no subsequent late-phase reactions

cance of such events is still unclear. Comparisons of sequential events in the skin in subjects with and without LPRs after equally prominent immediate wheal and flare reactions should provide important clues to the pathogenesis of LPRs. This report compares skin-chamber findings in these two populations of subjects with similar immediate IgE-mediated responses. We compared levels of histamine, inflammatory cell accumulation, lactoferrin, and eosinophil cationic protein (ECP) released from locally accumulating neutrophils and eosinophils, respectively, because we have previously found these to be elevated in fluids overlying developing LPRs.¹

METHODS

Subjects and skin testing

In screening studies, we identified eight atopic subjects with strong (≥ 12 mm diameter) wheal reactions to grass or ragweed pollen extract (100 protein nitrogen units [PNU]/ml; Greer Laboratories, Lenoir, N.C.) but little or no induration (≤ 2 mm diameter) at these sites at 6 hours. This group was hereafter called the +/- group. For comparison, we chose another group of eight atopic subjects with similar strong wheal responses to grass or ragweed extract (100 protein nitrogen units [PNU]/ml) and subsequent prominent induration (≥ 8 mm diameter) at these sites at 6 hours. This second group was called the +/+ group.

In all subjects from both groups, skin tests with serial 10-fold dilutions of the relevant pollen antigen extracts were performed. Skin tests with the buffer diluent (BD) were also done at the same time.

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Skin-chamber studies

Skin-chamber studies were performed as previously described.⁵ In brief, four blisters were induced on the volar surface of the forearms by gentle heat and suction. The epidermal blister roofs were removed from the blister bases at the dermal-epidermal junction, and the collection chambers were appended. After irrigating the blister bases with BD (phosphate-buffered saline [PBS]) twice for 5 minutes each, 0.3 ml of grass or ragweed pollen antigens (Greer Labs., Lenoir, N.C.) diluted to 100 PNU/ml in PBS containing 10 U/ml heparin was placed in chambers at two sites. As a control, PBS containing 10 U/ml heparin (BD) was placed in chambers at the other two sites. After 1 hour, the fluids were removed, and the fluids from duplicate chambers were pooled. They were then assayed for histamine levels by an ELISA (Immunotech) sensitive to 0.1 ng/ml.

The chamber bases were then irrigated with PBS. Fresh antigen and BD solutions containing 10 U/ml heparin were placed in the chambers previously containing antigen and BD solutions, respectively, and allowed to remain for an additional 5 hours. Fluids from the four challenge chambers were removed, fluids from the duplicate challenge sites were pooled, and total cell counts were obtained. The two pooled fluids were then centrifuged and supernatants were analyzed for levels of the following: (1) histamine; (2) ECP (radioimmunoassay [Pharmacia]; sensitivity, 2 ng/ml); (3) lactoferrin (ELISA, sensitivity, 2 µg/ml), as described by us⁶; (4) tumor necrosis factor-α (ELISA [R&D, Minneapolis, Minn.]; sensitivity, 15 pg/ml); (5) IL-1B (ELISA [R&D]; sensitivity, 4 pg/ml); and (6) IL-8 (ELISA [R&D]; sensitivity, 31 pg/ml). The cell pellets were resuspended in PBS containing heparin. Aliquots (100 µl) were counted for total cells (Coulter) and centrifuged (Shandon) for subsequent differential counting by Wright's staining (Dif-Quick).

Statistical analysis

Levels of cytokines and inflammatory mediators in skin-chamber fluids at sites were compared with levels in concomitant BD challenge sites in the same individuals by a paired students' *t* test when findings were normally distributed and the Wilcoxon rank sum test when nonparametric statistics were required. Findings at the antigen challenge sites were compared in the +/+ and +/- groups by unpaired *t* tests for normally distributed findings and the Mann-Whitney U test for nonparametric analysis.

Correlations between the mean diameter of the gross skin LPRs and the levels of individual inflammatory manifestations seen in the chamber fluids of the same +/+ subjects were attempted by using Spearman's rank analysis.

RESULTS

Gross skin-test reactions

We confirmed the findings of screening studies that eight subjects (the +/- group) exhibited little or no LPRs (mean ± SEM diameter of induration 6 hours after intradermal injection of 100 PNU/ml pollen antigen = 1 ± 1 mm). In the other eight subjects (the +/+ group) there were strong LPRs (mean diameter of induration at 6 hours = 12 ± 3 mm). However, the preceding wheal responses 20 minutes after administration of intradermal antigen were quite similar (17 ± 2 mm vs 18 ± 3 mm) in the +/+ and +/- groups, respectively (Table I). Further evidence of the similarity

TABLE I. Gross skin-test reactions

	+/- Group*	+/+ Group*
Mean (± SEM) wheal diameter at 20 min (mm)	17 ± 2	18 ± 3
Sensitivity† (PNU/ml)	1, 1, 1, 1, 1, 0.1, 0.1, 0.1	1, 1, 1, 1, 0.1, 0.1, 0.1, 0.1
LPRs (mm diameter of induration at 6 hours)	1 ± 1	12 ± 3

* +/- Group, Little or no induration; +/+ Group, prominent induration.

†Sensitivity = lowest concentration of pollen extract eliciting a wheal response twice the diameter or greater of the concomitant wheal response to BD at 20 minutes.

of the immediate wheal responses in the +/+ and +/- groups was the pattern of responses to intradermal injections of serial dilutions of the 100 PNU/ml antigen solution. The dilutions eliciting a minimal positive wheal response (greater than twice the diameter of the wheal response to the BD) was either 1 PNU/ml or 0.1 PNU/ml in the members of each group (Table I). No LPRs were seen at 6 hours at the sites of injection of the BD.

Skin-chamber findings

Histamine levels in skin chambers obtained after the first hour of antigen challenge were significantly higher than at companion BD challenge sites in both the +/+ (50 ± 7 vs 1.4 ± 0.5 ng/ml; *p* = 0.005) and the +/- (43 ± 15 vs 2 ± 0.8 ng/ml; *p* = 0.01) groups (Table II). However, the antigen site histamine levels in the +/+ and +/- groups were not significantly different from one another.

Histamine levels in chamber fluids obtained after hours 2 through 5 of antigen challenge were higher than at the companion BD challenge sites (Table II). However, these antigen site histamine levels were significantly higher in the +/+ group than in the +/- group (43 ± 15 vs 12 ± 5 ng/ml, respectively; *p* = 0.04). Indeed, there was sufficient variation in the antigen site histamine levels in the +/- group that the differences between antigen and BD sites were not significant in this group.

Leukocyte exudative responses

The total number of leukocytes (×10⁵) found in skin-chamber fluids after 5 hours of antigen challenge was significantly greater than that found in fluids at companion BD site challenges in both the +/+ (17 ± 5 vs 3 ± 1; *p* = 0.02) and +/- (13 ± 3 vs 3 ± 2; *p* = 0.05) groups (Table III). As noted, such responses were only slightly greater at the antigen sites of the +/+ subjects than at the antigen sites of the +/- subjects, but not significantly so.

Wright's stained cytospin preparation of these cells showed that a mean of 95% of the cells were granulocytes at the antigen sites. There was a somewhat higher percentage of eosinophils in such exudates found in the

TABLE II. Skin chamber histamine levels

	+/- Group		+/+ Group	
	Antigen sites	BD sites	Antigen sites	BD sites
Histamine levels after first hour of challenge (ng/ml)	43 ± 15*	2 ± 0.8	50 ± 7*	1 ± 0.5
Histamine levels after hours 2 through 5 of challenge (ng/ml)	12 ± 5	1 ± 0.2	43 ± 15*†	2 ± 1

*Greater than companion buffer sites, $p = 0.005$.†Greater than histamine levels in antigen sites of +/- subjects after hours 2 through 5 of challenge, $p = 0.04$.**TABLE III.** Skin chamber leukocytes

	+/- Group		+/+ Group	
	Antigen sites	BD sites	Antigen sites	BD sites
Total leukocytes ($\times 10^5$)	13 ± 3	3 ± 2	17 ± 56	3 ± 1
Eosinophils (%)	4.9 ± 1.7	1.2 ± 1.0	6.2 ± 1.7	2.0 ± 1.2

TABLE IV. Skin chamber levels of ECP and Lactoferrin

	+/- Group		+/+ Group	
	Antigen sites	BD sites	Antigen sites	BD sites
ECP levels (ng/ml)	53 ± 17*	6 ± 2	102 ± 16†	15 ± 3
Lactoferrin levels (μ g/ml)	12 ± 2†	4 ± 2	17 ± 2‡	5 ± 2

*Greater than at companion BD sites; $p = 0.02$.†Greater than at companion BD sites; $p = 0.01$.‡Greater than at companion BD sites; $p = 0.001$.

+/+ subjects than was found in the +/- subjects, but not significantly so ($6.2\% \pm 1.7\%$ vs $4.9\% \pm 1.8\%$; $p =$ not significant). Basophils were very infrequent in cells present in Alcian Blue-stained imprints on cover glasses that had been appended to blister bases for 15 minutes after removal of the overlying skin chambers after 5 hours of antigen challenge.

ECP levels were significantly higher at antigen than at BD challenge sites in fluids removed after hours 2 through 5 of challenge in the +/+ group (102 ± 16 ng/ml vs 15 ± 3 ng/ml; $p = 0.01$) (Table IV). ECP levels were also greater at antigen than at BD sites in the +/- group (53 ± 17 ng/ml vs 6 ± 2 ng/ml; $p = 0.02$) (Table IV), although not as great as in the +/+ group. However, these responses at the antigen challenge sites were not significantly different in the +/+ and +/- groups ($p = 0.4$).

Lactoferrin levels were significantly higher at antigen than at BD challenge sites in chamber fluids removed after hours 2 through 5 of challenge in both the +/+ group (17 ± 2 μ g/ml vs 5 ± 2 μ g/ml; $p = 0.001$) and the +/- group (12 ± 2 μ g/ml vs 4 ± 2 μ g/ml; $p = 0.01$) (Table IV). The lactoferrin levels in fluids overlying the antigen challenge sites were not significantly different in the +/+ and the +/- groups ($p = 0.6$).

Cytokine levels

Levels of IL-1, IL-6, and TNF- α in chamber fluids obtained after hours 2 through 5 of challenge were not significantly greater at antigen sites than BD sites in either the +/+ (59 ± 10 ng/ml vs 62 ± 18 ng/ml) or the +/- (47 ± 16 ng/ml vs 47 ± 19 ng/ml) groups. IL-6 levels were also not significantly greater at antigen than at BD sites in the +/+ (4769 ± 1017 ng/ml vs 5446 ± 1282 ng/ml; $p = 0.39$) or +/- (8440 ± 2201 vs 5730 ± 2267 ng/ml; $p = 0.32$) groups. The IL-6 levels at the antigen sites were actually somewhat higher in the +/- group, but not significantly so.

IL-8 levels were significantly higher at antigen sites than at BD sites after hours 2 through 5 of challenge in the +/+ (26 ± 4.7 μ g/ml vs 7.7 ± 1.9 μ g/ml; $p = 0.01$) and +/- (17 ± 5.3 μ g/ml vs 5 ± 3.4 μ g/ml; $p = 0.004$) groups. IL-8 levels at the antigen sites were higher in the +/+ than in the +/- subjects, but not significantly so ($p = 0.30$).

Correlations

The size of the individual +/+ subjects' gross skin LPRs (mean diameter) correlated significantly with lactoferrin levels ($p = 0.02$) and total inflammatory cell accumulation ($p = 0.04$) in the antigen site chamber fluids as determined by Spearman's rank analysis. How-

ever, the size of the gross skin LPRs did not correlate with the other inflammatory manifestations or cytokine levels found in these antigen site chamber fluids.

DISCUSSION

The findings in this study confirm some observations made by our group and others by use of skin-chamber approaches to investigate pathogenic mechanisms in LPRs. At the same time, this study resulted in new and somewhat surprising findings as well.

As noted previously by us,⁵ continued challenge of sensitive subjects with pollen antigen in this study resulted in an early peak of histamine release within the first hour. This resulted in similar histamine levels in both the $+/+$ and the $+/-$ groups that we compared in this study. This finding was not surprising, considering that the degree of immediate reactivity, as defined by the extent of gross wheal reactions, was quite similar in the two groups. The histamine release during the next 4 hours of challenge was also greater at antigen than at BD challenge sites in both the $+/+$ and the $+/-$ groups; however, here the antigen site histamine levels were significantly greater in the $+/+$ than in the $+/-$ subjects. Indirect evidence in our previous studies⁷ suggested that such histamine release after the first hour of antigen challenge came predominantly from ingressing basophils. However, we have been unable to find an increased frequency of such cells in the exudate at antigen sites by Alcian Blue staining. It is conceivable that the activation of such ingressing basophils leading to continued histamine release may render the cells poorly stainable by agents such as Alcian Blue. Others have reported a later (after 6 hours) increased basophil frequency in skin chambers challenged only once, at time zero.⁸ The latter protocol differs from the continuous antigen challenge employed by us.⁵

In this study we also confirmed earlier observations of significantly increased total granulocyte and eosinophil exudation in skin chambers at antigen rather than at BD challenge sites.^{6,9} Surprisingly, however, this granulocyte response was only slightly greater in the $+/+$ than in the $+/-$ subjects. Likewise, the skin-chamber levels of ECP and lactoferrin at antigen sites (released locally by exuding eosinophils and neutrophils, respectively) were also only modestly higher in the $+/+$ than the $+/-$ subjects. It has been postulated that the granulocyte inflammatory response, particularly that of eosinophils, plays a key part in the development and expression of gross LPRs. Some studies, by using experimental models of airway sensitivity to inhaled allergens, find decreased bronchial LPRs in animals treated previously to inhibit eosinophil responses.¹⁰ Although lymphocytes may have an important role in the synthesis and release of certain cytokines in sites of LPRs, the number of such cells are often not increased until after the first 6 hours of antigen challenge.¹¹ Indeed, there was no difference in the frequency of lymphocytes found in chamber fluids at antigen sites of $+/+$ and $+/-$ subjects in this study. However, because relatively few lymphocytes exude the

dermis into the overlying chamber fluid, the skin-chamber model may not be the best approach for studying the role of lymphocytes in LPRs.

Previous reports by other groups^{12,13} described increased levels of the cytokines IL-1 and IL-6 in overlying skin-chamber fluids at varying times after antigen challenge of sensitive subjects. In this study we found modest levels of IL-1 and sizable levels of IL-6 in antigen site chamber fluids, but not significantly more than at BD sites in both the $+/+$ and $+/-$ subject groups. The latter findings are similar to that recently reported by us in a larger cohort of $+/+$ subjects.⁴

We have previously found greater levels of the chemokine IL-8, a potent attractor and activator of neutrophils, at antigen sites than at BD sites in $+/+$ subjects.⁴ Therefore it was of particular interest that IL-8 levels were impressively higher at antigen sites than at BD sites in both the $+/+$ and $+/-$ subjects in this study. Although such IL-8 levels in antigen challenge sites were overall somewhat higher in the $+/+$ than in the $+/-$ subjects, the differences were not striking. Unfortunately, there was insufficient chamber fluid left from these specimens to assay them for levels of RANTES and MCP-1, two other chemokines with potentially important roles in allergic inflammation. We have recently found levels of both RANTES and MCP-1 to also be significantly higher in chamber fluids at antigen rather than at BD challenge sites in $+/+$ subjects.⁴

Several possible conclusions can be drawn from these findings of only a modestly greater granulocyte inflammatory response and possibly related IL-8 synthesis/release in the antigen challenge sites of those with prominent gross skin LPRs when compared with those without such LPRs after similar immediate reactions to antigen.

First, although it is unlikely that any one granulocyte inflammatory manifestation assessed in this study was the determining factor in whether a LPR would develop, perhaps the summation of modestly greater degrees of several concurrent inflammatory responses seen here result in LPR development. This possibility is suggested by our finding that the size of gross LPRs at 6 hours to intradermal antigen injection in the individual $+/+$ subjects correlated modestly with the degree of inflammatory cell exudation and levels of lactoferrin (but not ECP) in the skin chambers overlying antigen challenge sites. In our calculations, the individual inflammatory events (other than histamine levels at hours 2 through 5) would still not be significantly greater in the $+/+$ than $+/-$ groups if there had been 12 subjects in each subgroup.

Second, another inflammatory component that was not measured here could be responsible for LPRs. We have previously found increased levels of sulfido leukotrienes,¹⁴ leukotriene B₄,¹⁵ prostaglandin E,¹⁶ and metalloproteinases¹⁷ during the same time period of 5 to 6 hours of antigen challenge when skin LPRs typically develop. Unfortunately, there was insufficient chamber fluid available to assay for those proinflammatory medi-

ators in this study. We have also found indirect evidence of increased fibrin formation in skin chambers at antigen challenge sites in $+/+$ subjects,¹⁸ confirming one previous immunohistochemical study reporting increasing fibrin deposition in LPR sites.¹⁹ However, a comparative skin-chamber study of fibrin formation in $+/+$ and $+/-$ subjects would require a different approach, avoiding the use of heparin in the solutions placed in the chambers. We have found that low concentrations of heparin in such fluids are necessary to be able to retrieve the exuding inflammatory cells (assessed in this study) but may inhibit local fibrin formation.

The significantly higher levels of histamine in antigen sites rather than in BD sites after hours 2 through 5 of challenge may offer a clue as to other possible causal factors in LPR development. Histamine by itself does not induce a LPR.²⁰ However, if the histamine released after the first hour does come mainly from ingressing basophils,⁷ it is conceivable that the prolonged histamine release could be a marker for the release of a leukotriene or cytokine from these activated cells.²¹ The latter mediators could play causal roles in LPRs.

Third, a process completely distinct from the inflammatory events described above could be the major factor in the pathogenesis of LPRs. The gross appearance of these reactions certainly looks inflammatory. It is conceivable that differences in local T-lymphocyte function determines whether LPRs will develop.¹¹ However, we have observed that LPRs (unlike true delayed hypersensitivity reactions) are often less prominent at 24 hours than at 6 hours after intradermal injection of moderate allergen doses, even though the frequency of T lymphocytes is often greater at 24 hours than at 6 hours.^{22, 23} The kinetics of skin LPRs may be dose dependent, with higher concentrations inducing more persistent reactions. However, even after intradermal injection of high allergen doses, the mean diameter of LPR induration was higher at 6 than at 24 hours.²² It is conceivable that the profile of functional lymphocyte populations is different at 6 and 24 hours. However, the *in situ* hybridization studies of Kay's group²⁴ suggests more prominent synthesis of proinflammatory cytokines of the T_H2 -type at 24 hours.²²

In conclusion, one can only postulate, without definitive evidence, on the possible pathogenic mechanisms in skin LPRs. We believe that the pathogenesis of these reactions is likely quite complex, involving the prior interaction of several humoral and cellular components. Mast-cell activation is likely necessary, but not sufficient, because prominent immediate mast-cell activation after intradermal injection of opiates is not followed by development of a LPR.¹ The impressive inhibition of LPR development by systemic corticosteroid therapy² does suggest a pathogenic role for inflammatory leukocytes; however, corticosteroids modulate other biologic effects that may be related to the pathogenesis of LPRs. Thus further investigation will be required to clarify the pathogenic mechanisms involved in the development of

LPRs. The information obtained may allow more successful therapeutic approaches to LPRs in clinical settings.

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