

Cellular inflammatory responses during immediate, developing, and established late-phase allergic cutaneous reactions: Effects of cetirizine

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Background: In some previous studies, the antihistamine cetirizine has inhibited both developing (at 6 hours) and established (at 24 hours) gross late-phase skin reactions (LPR) to pollen antigens, possibly relevant to clinical drug effects. However, the effects of cetirizine at the histologic level require further definition.

Objective: To characterize cetirizine effects on gross and histologic inflammatory events from 20 minutes to 24 hours after intradermal antigen challenge in sensitive patients.

Methods: Gross and histologic responses to intradermal pollen antigen, codeine, histamine, and buffer diluent were assessed during randomized 7-day treatments with cetirizine and placebo. Accumulated neutrophils, eosinophils, activated (EG2⁺) eosinophils, and T lymphocytes were quantitated. The degrees of extracellular deposition of lactoferrin from neutrophils and eosinophilic cationic protein (ECP) from eosinophils were also assessed.

Results: During placebo treatment, wheal-and-flare responses were significantly greater to antigen at 20 minutes ($p < 0.01$) and induration at 6 hours ($p < 0.01$) at antigen challenge sites than at buffer diluent sites. During cetirizine treatment, these wheal-and-flare responses to antigen were inhibited significantly ($p < 0.01$) but gross LPRs were not affected. During placebo treatment, significantly more cells per high-power field were found in antigen sites than in buffer sites of neutrophils at 20 minutes ($p < 0.01$) and 24 hours; than in eosinophils at 20 minutes, 6 hours, and 24 hours ($p < 0.01$ for each); than in EG2⁺ cells at 20 minutes ($p = 0.004$), 6 hours ($p = 0.001$), and 24 hours ($p = 0.02$); and at T lymphocyte sites at 24 hours ($p = 0.001$). Extracellular deposition of lactoferrin and ECP was significantly greater at antigen sites than at buffer sites at 6 and 24 hours. Cetirizine treatment had no significant effect on these responses.

Conclusion: Neutrophils, eosinophils, and T lymphocytes were persistently more common at antigen sites than at

buffer sites through 24 hours. Many of these neutrophils and eosinophils were activated, releasing more lactoferrin and ECP into the extracellular dermis for at least 24 hours after antigen challenge. Cetirizine inhibited gross immediate responses to antigen, but not the gross LPR nor the cellular inflammatory responses seen in such LPR sites. (*J Allergy Clin Immunol* 1997;100:341-7.)

Key words: Late-phase response, cutaneous allergic response, neutrophils, eosinophils, lactoferrin, eosinophil cationic protein, cetirizine

Late-phase reactions (LPRs) in the skin after allergen challenge in sensitive subjects have received much investigation. The LPR is characterized on gross observation by erythema and induration and on histologic examination by a profound cellular inflammatory response consisting of basophils, neutrophils, eosinophils, and mononuclear leukocytes.¹ Events occurring within the first 4 to 12 hours are considered the "developing" LPR and those at 24 hours or later the "established" LPR.² The relationship between these two phases is not at all clear, with some investigators placing a primary role on T_{H2}-type lymphocytes as being responsible for the later appearance and activation of granulocytes (particularly eosinophils) seen in the established LPR.³ However, some preliminary findings have also shown persistent deposition of eosinophil and neutrophil granular proteins and accumulations of activated granulocytes from the early through the later phases of the LPR.^{2,4}

Cetirizine, an active metabolite of hydroxyzine, has been shown to inhibit cellular inflammatory responses occurring after diverse stimuli, including allergen challenge.⁵⁻¹⁰ In some models, cetirizine has been found to inhibit the LPR to allergen.¹¹⁻¹³ In a recent skin chamber study, we found no anti-inflammatory effects of cetirizine on the events during the developing LPR in the first 6 hours after challenge.¹⁴ However, in our hands, this skin chamber model is not ideal to study the LPR at 24 hours. Therefore, we used biopsy specimens from intradermal antigen injection sites to characterize the sequence of cellular inflammatory events during the immediate reaction, the LPR at 6 and 24 hours, and the effects of cetirizine on these cutaneous allergic reactions.

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Supported by National Institutes of Health Grant No. RO1 AI 14332 and Pfizer Central Research.

Submitted for publication Jan. 13, 1997; revised March 27, 1997; accepted for publication March 28, 1997.

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0091-6749/97 \$5.00 + 0 1/1/82539

Abbreviations used

ECP:	Eosinophil cationic protein
EG ₂ :	Monoclonal antibody against cleaved eosinophil cationic protein
HPF:	High-power field
LPR:	Late-phase reaction
PNU:	Protein nitrogen units
T _{H2} :	Type 2 T helper lymphocytes

METHODS**Subjects and protocol**

This study consisted of 15 subjects with previously demonstrated immediate responses and LPRs after intradermal injections of 10 and 100 protein nitrogen units (PNU) per milliliter of ragweed or grass extract. After giving informed consent, subjects received 20 mg/day cetirizine or placebo for 7 days, with a 14-day drug-free interval, in a randomized, double-blind, crossover design. Each subject underwent the procedures as listed below:

Days 6 and 27. Blood was obtained before the ingestion of cetirizine to determine plasma cetirizine levels by Pfizer Central Research (New York, N.Y.) for analysis by high-performance liquid chromatography. Wheal-and-flare responses were measured at 20 minutes and erythema and induration were determined at 6 hours after intradermal injection of phosphate-buffered saline, histamine (0.02 mg/ml), codeine (1 mg/ml), and antigen (serial 10-fold dilutions from 100 to 1 PNU/ml).

Days 7 and 28. Skin biopsies were performed on these days.

Skin biopsy studies

Tissues for punch biopsies (3 mm in diameter) were obtained from paired sites after the injection of antigen (0.02 ml 100 PNU/ml of grass or ragweed pollen antigen, Greer Corp, Lenoir, N.C.) or buffer. Tissues were sampled at 20 minutes (the immediate reaction), at 6 hours (the developing, "early" LPR), and at 24 hours (the established, "late" LPR).

Biopsy specimens were placed in an embedding medium for frozen sections and frozen immediately in liquid nitrogen. Replicate 6- μ sections were cut, fixed in acetone for 10 minutes, and underwent standard immunohistochemical staining procedures with the use of EG₁, EG₂ (Pharmacia, Inc., Piscataway, N.J.), and Leu 4 (Becton-Dickinson, Mountain View, Calif.) monoclonal antibodies in an alkaline phosphatase anti-alkaline phosphatase technique. Lactoferrin deposition was detected by use of a rabbit anti-lactoferrin antibody (Dako) in a peroxidase-anti-peroxidase technique as previously described.¹⁵ Quantitation of cells and deposition of lactoferrin and eosinophil cationic protein (ECP) were determined in duplicate by two independent observers reviewing coded biopsy slides. Cells were counted in all high-power fields (HPFs) starting from the dermoepidermal junction. Neutrophils were identified as cells staining positive for lactoferrin. Total eosinophils were considered to be the EG₁⁺ cells, with identity confirmed in a few cases by use of an antibody against eosinophil major basic protein (kindly provided by Dr. Gerald Gleich, Mayo Clinic, Rochester, Minn.). EG₂⁺ cells (monoclonal antibody against cleaved ECP) were considered to be activated eosinophils, as reported previously. T lymphocytes were defined by their surface expression of CD3 (Leu 4⁺).² Cell frequency was quantitated as cells/HPF in coded slides read by two observers. Extracellular deposition of lactoferrin and ECP (EG₂⁺) material was semiquantitated

on a 0 to 4⁺ scale, with good agreement in readings by two independent observers.

Statistical analysis

A two-way repeated-measures analysis of variance was performed for each of the parameters. Then, for findings that were distributed normally, results were analyzed by Student's *t* test for paired observations. Nonparametric findings were analyzed by the Wilcoxon rank sum test.

RESULTS**Plasma cetirizine levels**

Cetirizine levels (results available in 13 of 15 subjects) in plasma obtained just before drug or placebo dosing on day 6 of treatment were 139 ± 36 μ g/ml during cetirizine treatment. No cetirizine was detected in plasma obtained during placebo treatment. All findings reported below were obtained in all 15 individuals in the study group.

Skin test responses

Cetirizine treatment significantly inhibited the 20-minute wheal-and-flare responses. After intradermal injection of 100, 10, and 1 PNU/ml of antigen, cetirizine inhibited wheal responses by $32\% \pm 5\%$, $31\% \pm 4\%$, and $30\% \pm 4\%$, respectively, as compared with placebo ($p < 0.01$) (Fig. 1). Cetirizine also significantly inhibited the 20-minute flare response to 100, 10, and 1 PNU/ml doses of antigen ($45\% \pm 3\%$, $53\% \pm 4\%$, and $62\% \pm 4\%$ inhibition compared with placebo, respectively; $p < 0.001$) (Fig. 1). During cetirizine treatment there was also inhibition of the 20-minute wheal and flare responses to histamine ($40\% \pm 4\%$ and $69\% \pm 3\%$, respectively) and codeine ($42\% \pm 3\%$ and $58\% \pm 4\%$, respectively) ($p = 0.001$). Despite the impressive inhibition by cetirizine of early skin test responses, cetirizine had no significant effect on the late-phase induration or erythema responses 6 hours after injection of either the 100 or 10 PNU antigen doses (Fig. 2). LPRs were seen at the 1 PNU/ml injection site in too few subjects to assess cetirizine effects.

Skin biopsy tissue histologic patterns

Neutrophils. Neutrophils were increased at antigen challenge sites compared with buffer sites throughout the 24-hour period during both placebo and cetirizine treatment (Fig. 3). During placebo administration, accumulation of neutrophils was significantly greater at antigen sites (4.4 ± 0.5 /HPF) compared with buffer sites (2.6 ± 0.5 /HPF) at 20 minutes ($p = 0.004$). At the 6-hour period, neutrophils were more frequent at antigen sites than at buffer sites (9.7 ± 2.7 /HPF vs 7.3 ± 2.3 /HPF); however, this difference was not significant, due largely to a nonspecific accumulation of neutrophils at the buffer site in a few subjects. At 24 hours, neutrophil frequency in buffer sites declined slightly (6.4 ± 2.3 /HPF), while neutrophil accumulation at the antigen site continued to increase (11.3 ± 2.1 /HPF), so that the difference between antigen and buffer sites was again significant ($p < 0.05$).

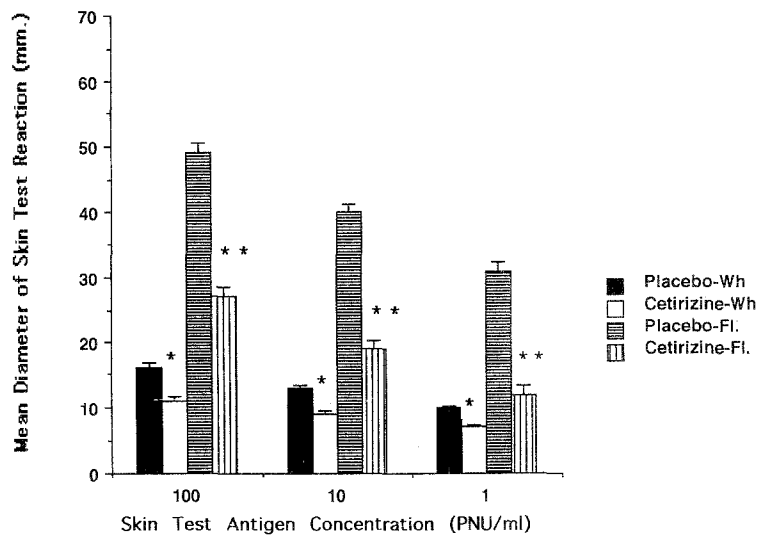


FIG. 1. Mean ($\pm$ SEM) of the mean diameters (in millimeters) of the wheal-and-flare responses 20 minutes after intradermal injection of ragweed or grass extract (100 PNU/ml, 10 PNU/ml) during cetirizine or placebo treatment. *Wh*, Wheal; *Fl*, flare. * $p < 0.01$, ** $p < 0.001$ vs placebo.

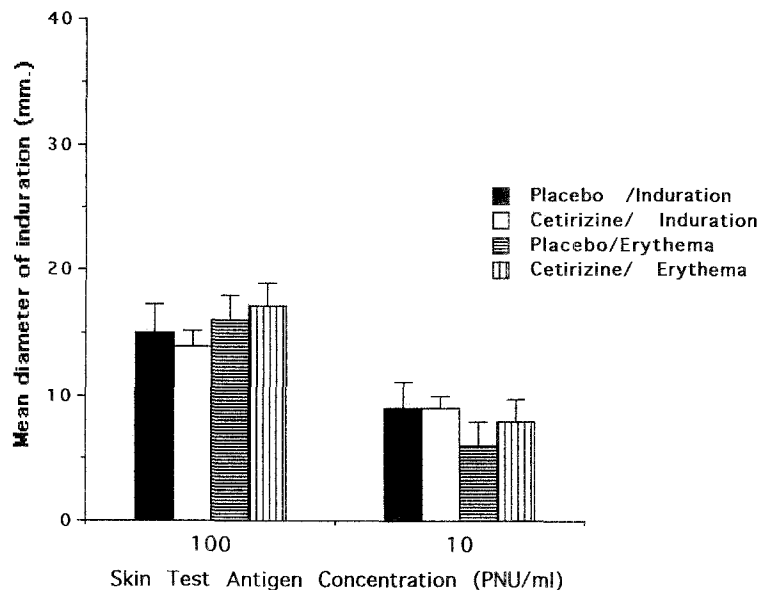


FIG. 2. Mean ($\pm$ SEM) of the mean diameter of induration and erythema (in millimeters) 6 hours after intradermal injection of ragweed or grass extract (100 PNU/ml, 10 PNU/ml) during cetirizine or placebo treatment.

During cetirizine administration, a similar pattern of neutrophil influx occurred. At 20 minutes significantly more neutrophils were present in antigen sites than in buffer sites ($4.9 \pm 0.9/\text{HPF}$ vs $1.9 \pm 0.4/\text{HPF}$; $p = 0.004$) (Fig. 3). At 6 hours, neutrophils were more frequent in antigen sites than in buffer sites ($14.7 \pm 0.3/\text{HPF}$ vs $10.4 \pm 2.2/\text{HPF}$). However, again, this difference between accumulation at antigen and buffer sites was not significant because of neutrophil accumulation in the buffer sites of several subjects. At 24 hours, neutrophils were significantly more common in antigen sites compared with buffer sites (11.6 ± 2.2 vs $6.7 \pm 1.6/\text{HPF}$; $p =$

0.008). Overall, there was no significant difference in neutrophil accumulation at any time period, at either antigen or buffer sites, when comparing the periods of cetirizine treatment and placebo treatment.

More striking, perhaps, was the greater extracellular deposition in antigen sites than in buffer sites of lactoferrin, a protein released from the secondary granules of activated neutrophils. During placebo treatment, the small amount of extracellular lactoferrin deposition (graded on a 0 to 4+ scale) in antigen sites at 20 minutes (0.8 ± 0.2) was significantly greater than in the paired buffer sites ($p < 0.001$) (Table I). Lactoferrin deposition

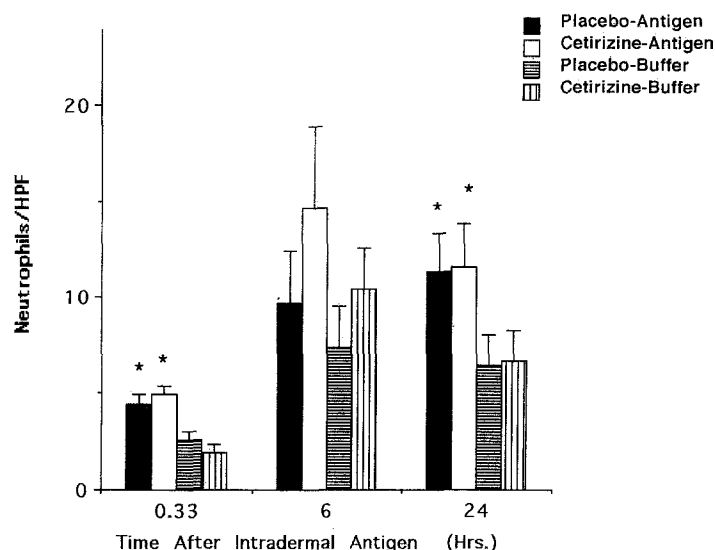


FIG. 3. Mean ($\pm$ SEM) number of neutrophils/HPF of dermis at 20 minutes, 6 hours, and 24 hours after intradermal injection of ragweed or grass extract (100 PNU/ml) during cetirizine or placebo treatment. * $p < 0.01$ compared with placebo.

TABLE I. Extracellular deposition of lactoferrin and eosinophil cationic protein in skin biopsy specimens

	Placebo		Cetirizine	
	Lactoferrin	Eosinophil cationic protein	Lactoferrin	Eosinophil cationic protein
20 minutes				
Antigen site	0.8 ± 0.2	0.6 ± 0.2	0.8 ± 0.3	0.5 ± 0.2
Buffer site	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
6 hours				
Antigen site	2.8 ± 0.2	2.1 ± 0.4	3.4 ± 0.2	2.6 ± 0.3
Buffer site	1.1 ± 0.4	0.1 ± 0.1	1.5 ± 0.3	0.4 ± 0.2
24 hours				
Antigen site	2.9 ± 0.3	2.9 ± 0.4	3.1 ± 0.2	3.1 ± 0.3
Buffer site	1.2 ± 0.3	0.4 ± 0.2	1.3 ± 0.3	0.3 ± 0.2

Data are mean $\pm$ SEM extracellular deposition (0-4⁺ scale).
Intradermal injection of pollen antigen, 100 PNU/ml.

was also significantly greater in antigen sites than in buffer sites at both 6 and 24 hours (2.8 ± 0.2 vs 1.1 ± 0.4 , $p < 0.0001$; and 2.9 ± 0.3 vs 1.2 ± 0.3 , $p = 0.001$, respectively). There was no difference between the cetirizine and placebo treatment periods in the degree of extracellular deposition of lactoferrin in antigen or buffer sites at any time period after challenge.

Eosinophils. During placebo treatment, the small number of eosinophils present at 20 minutes in the antigen sites (1.0 ± 0.2 /HPF) was significantly greater than the few eosinophils in buffer sites (0.2 ± 0.1 /HPF; $p = 0.002$) (Fig. 4). The frequency of eosinophils in the antigen sites was also significantly greater than in buffer sites at 6 hours (2.7 ± 0.9 vs 0.5 ± 0.2 /HPF; $p = 0.002$) and at 24 hours (2.9 ± 0.8 vs 0.7 ± 0.2 /HPF; $p = 0.004$).

A similar pattern of differences in eosinophil accumulation between antigen and buffer sites occurred during cetirizine treatment (Fig. 4). Eosinophils were signifi-

cantly more frequent in antigen sites than in buffer sites at 20 minutes (0.7 ± 0.2 vs 0.1 ± 0.03 /HPF; $p = 0.0001$), 6 hours (2.4 ± 0.5 vs 0.6 ± 0.3 /HPF; $p = 0.01$), and 24 hours (2.9 ± 0.7 vs 1.1 ± 0.2 /HPF; $p < 0.05$).

There was no significant difference during the placebo versus cetirizine treatment periods in the eosinophil accumulation at antigen or buffer sites at any time point measured.

A similar pattern of a progressive increase in the frequency of activated (EG_2^+) eosinophils was seen in antigen sites, which was significantly different from that seen in buffer sites, during both placebo and cetirizine treatment. During placebo administration, EG_2^+ cells were more frequent at antigen sites than at buffer sites at 20 minutes (0.6 ± 0.1 vs 0.2 ± 0.1 /HPF; $p = 0.004$), at 6 hours (1.4 ± 0.3 vs 0.6 ± 0.2 /HPF; $p = 0.01$), and at 24 hours (2.5 ± 0.7 vs 1.2 ± 0.6 /HPF; $p = 0.02$) (Fig. 5). Likewise, during cetirizine treatment, EG_2^+ cells were

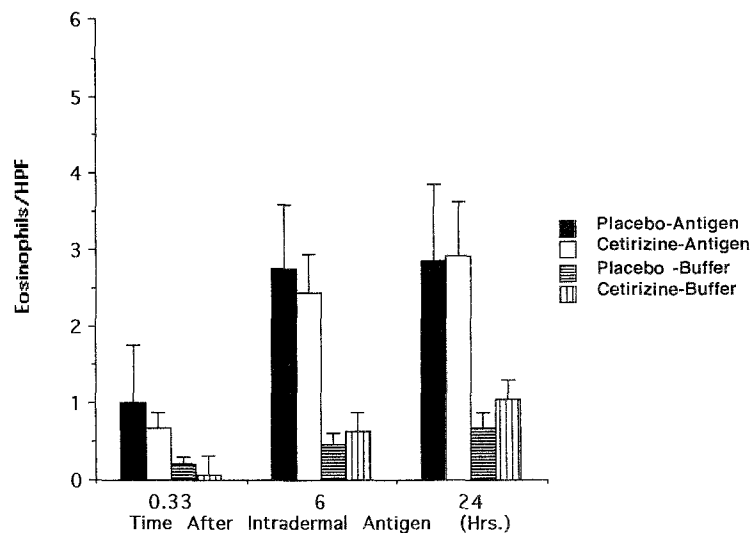


FIG. 4. Mean ($\pm$ SEM) number of eosinophils/HPF of dermis at 20 minutes, 6 hours, and 24 hours after intradermal injection of ragweed or grass extract (100 PNU/ml) during cetirizine or placebo treatment. All antigen sites differed significantly from placebo sites ($p < 0.01$).

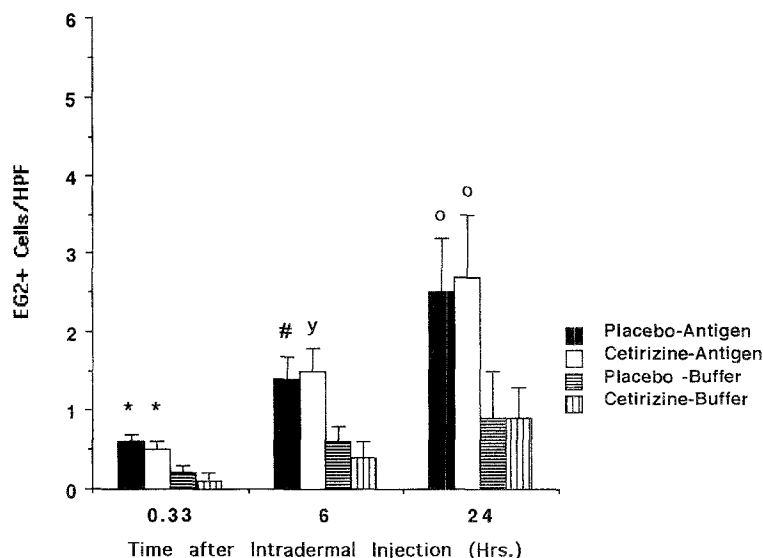


FIG. 5. Mean ($\pm$ SEM) number of EG₂⁺ cells/HPF of dermis at 20 minutes, 6 hours, and 24 hours after intradermal injection of ragweed or grass extract (100 PNU/ml) during cetirizine or placebo treatment. * $p = 0.004$, # $p = 0.01$, $y p = 0.001$; $o p = 0.02$ vs placebo.

more frequent in antigen sites than at buffer sites at 20 minutes (0.5 ± 0.1 vs 0.1 ± 0.1 /HPF; $p = 0.004$), 6 hours (1.5 ± 0.3 vs 0.4 ± 0.2 /HPF; $p = 0.001$), and 24 hours (2.7 ± 0.8 vs 0.9 ± 0.4 /HPF; $p = 0.02$) (Fig. 5).

When biopsy specimens obtained during placebo and cetirizine treatment were compared, the frequency of activated eosinophils seen at antigen or buffer sites did not differ at any period. A puzzling occasional finding was a greater frequency of EG₂⁺ cells than total eosinophils in the same biopsy specimen. This may be due to an observation that cells other than eosinophils may occasionally appear as EG₂⁺.²¹

In support of our finding of increased numbers of

EG₂⁺ cells at antigen sites compared with buffer sites, we also found increased extracellular deposition of cleaved ECP, a protein released from the granules of activated eosinophils (Table I). However, in contrast to lactoferrin deposition, extracellular ECP deposition in antigen sites did not plateau between 6 and 24 hours, but rather increased progressively throughout the 24-hour period. ECP deposition was significantly greater at antigen than at buffer sites at each time assessed (20 minutes: 0.6 ± 0.2 vs 0.2 ± 0.1 , $p = 0.02$; 6 hours: 2.1 ± 0.4 vs 0.1 ± 0.1 , $p < 0.001$; 24 hours: 2.9 ± 0.4 vs 0.4 ± 0.2 , $p < 0.0001$). When cetirizine and placebo treatments were compared, the degree of extracellular ECP

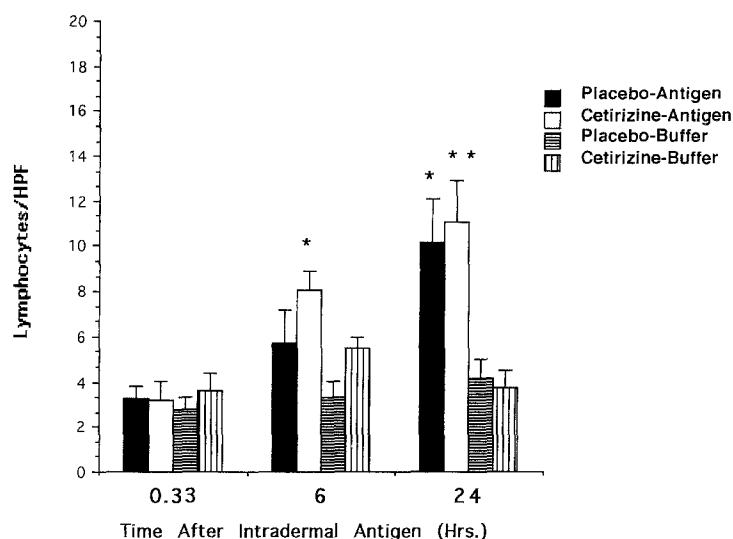


FIG. 6. Mean ($\pm$ SEM) number of T lymphocytes/HPF dermis at 20 minutes, 6 hours, and 24 hours after intradermal injection of ragweed or grass extract (100 PNU/ml) during cetirizine or placebo treatment. * $p = 0.004$, ** $p = 0.001$ vs. placebo.

deposition at any time after challenge did not differ at either antigen or buffer sites.

T lymphocytes. The mean frequency of lymphocytes in antigen challenge sites increased progressively throughout the 24-hour period. During placebo treatment, only at 24 hours was the number of lymphocytes significantly greater in antigen sites than in buffer sites (10.2 ± 2.0 vs 4.2 ± 0.9 /HPF; $p = 0.004$) (Fig. 6). During cetirizine administration, significantly more T cells were present at antigen than at buffer sites at both 6 and 24 hours (6.3 ± 0.9 vs 3.5 ± 0.5 /HPF, $p = 0.004$; 11.1 ± 1.9 vs 3.2 ± 0.8 /HPF, $p = 0.001$, respectively) (Fig. 6). The numbers of lymphocytes seen at antigen or buffer sites at any period did not differ significantly between cetirizine and placebo treatments.

DISCUSSION

We have determined the pattern of granulocyte and mononuclear cell accumulation and activation after intradermal injection of allergen, from the immediate through the "early" and "late" stages of LPR. We have found a significantly greater accumulation of both neutrophils and eosinophils at antigen than at buffer challenge sites throughout this period. We have shown that both neutrophils and eosinophils in LPR sites are activated and that the granular proteins they secrete persist in the dermis for at least 24 hours after intradermal antigen injection. T lymphocytes were also significantly increased at 24 hours at antigen compared with buffer sites. Cetirizine treatment resulted in therapeutic plasma levels and inhibition of immediate wheal-and-flare responses to antigen, histamine, and codeine. However, neither the gross LPR nor the cellular inflammatory responses or dermal deposition of lactoferrin or ECP in LPR sites was affected.

The findings reported here extend the findings of

some previous studies of cutaneous LPR. We previously reported a progressively greater accumulation of eosinophils over 4 hours after intradermal antigen challenge compared with buffer challenge in sensitive subjects. This accumulation could be inhibited by prior treatment with corticosteroids, but not with hydroxyzine.^{16, 17} Bierman et al.¹² later found an increase in eosinophils in the skin at sites of intradermal antigen injection 6 hours earlier, and these were not inhibited by another H1 antihistamine, astemizole.¹⁸

Frew and Kay³ reported sequential histologic responses in intense LPRs to intradermal antigen challenge. They found a prominent infiltration of neutrophils, eosinophils, and EG_2^+ cells peaking at 6 hours and persisting for 24 to 48 hours. Most interesting was the significant correlation between the numbers of EG_2^+ cells at 48 hours and the magnitude of the gross LPR response at 6 hours. In addition, the number of T cells at 24 hours correlated with the number of EG_2^+ cells at 24 but not at 48 hours.

In subsequent studies, these authors determined that the number of EG_2^+ cells in biopsy tissues during the LPR correlated with the frequency of cells (thought to be mainly T cells) expressing messenger ribonucleic acid for IL-4 and IL-5 but not for interferon- γ .¹⁹ Treatment with steroids, but not cetirizine, decreased both the extent of the gross induration at 6 and 24 hours and the numbers of eosinophils and neutrophils seen in biopsy specimens 24 hours after intradermal antigen administration.²⁰ More recently, these investigators reported that both EG_2^+ cells and neutrophils were increased in biopsy specimens from 1 to 96 hours after intradermal allergen injection resulting in LPR, but not in delayed hypersensitivity responses to partial protein derivative in the same individuals.²

In only one other study has the deposition of granular

proteins been documented in an ongoing skin LPR. In four patients, Leiferman et al.⁴ reported an increase in extracellular deposition of neutrophil elastase, eosinophil-derived neurotoxin (another cationic granule protein), and major basic protein as early as 15 minutes after intradermal allergen challenge and persisting for up to 96 hours in some specimens.

These previous reports and ours agree that the LPR involves a very rapid granulocyte accumulation, activation, and release of granule constituents that increases through the first 6 hours and may persist for up to 24 hours after intradermal allergen injection. We suggest that both eosinophils and neutrophils likely have pathogenic roles in the "early" and "late" components of the skin LPR.

In our studies reported here, cetirizine did not affect the leukocyte accumulation or deposition of granule proteins seen during the cutaneous LPRs to intradermal antigen. These findings of a lack of an anti-inflammatory effect of cetirizine in the skin reported here and by Varney et al.²⁰ are in contrast with the inhibition of inflammatory responses in the skin by cetirizine reported by other groups.^{5, 7-9} These latter observations were generally made with the use of skin window or skin chamber approaches, raising the possibility that cetirizine may have a preferential inhibitory effect on granulocytes that exude from the dermis onto overlying cover glasses or into overlying chamber fluids. However, in a recent study we found no inhibitory effect of cetirizine on the exudation of granulocytes into skin chambers overlying a developing LPR during the first 6 hours of antigen challenge.¹⁴ It may be that any inhibitory effect of cetirizine on skin chamber inflammatory responses is manifest well after the first development of the LPR during 6 hours after intradermal allergen, as observed by Charlesworth et al.⁷ The in vivo significance of the latter finding is uncertain because we and Varney et al.²⁰ found no effects on gross LPR expression at any time after challenge. Therefore, we must conclude at this time that the predominant, if not the only, effect of cetirizine is on the immediate phase of IgE-mediated reactions.

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