The Late Phase of the Immediate Wheal and Flare Skin Reaction

ITS DEPENDENCE UPON IgE ANTIBODIES

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ABSTRACT IgE antibodies are usually thought to induce only immediate skin reactions. We have shown that the intradermal injection of a number of different allergens can produce a prolonged inflammatory reaction after the immediate wheal and flare in most sensitive subjects. This late inflammatory response occurs 6–12 h after challenge and is characterized by diffuse edema, erythema, pruritus, tenderness, and heat. Both immediate and late responses can also be seen after passive sensitization of skin sites in nonatopic subjects. That IgE is involved in inducing the reaction was shown by the abolition of both immediate and late responses by passive transfer tests in the following experiments: (*a*) heating atopic serum at 56°C for 4 h, (*b*) removing IgE from the atopic serum by a solid phase anti-IgE immunoabsorbent, and (*c*) competitively inhibiting the binding of IgE antibodies to cells by an IgE myeloma protein. In addition, both responses were induced by affinity chromatography-purified IgE antibody, followed by antigenic challenge. Very similar lesions could also be induced by intradermal injection of Compound 48/80, thus suggesting a central role in the reaction for the mast cell or basophil. Histologically, the late phase is characterized by edema and a mixed cellular infiltration, predominantly lymphocytic but also containing eosinophils, neutrophils, and basophils. Direct immunofluorescent staining did not show deposition of immunoglobulins or complement components, except IgM in 2 of 15 and C3 in 1 of 15 patients. This finding indicates that the late phase does not depend on the deposition of immune complexes. The results of the study suggest that IgE-allergen interaction on the surfaces of mast cells or on infiltrating basophils causes both immediate and late cutaneous responses.

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INTRODUCTION

The wheal and flare reaction is characteristic of the type I (1) IgE-mediated hypersensitivity reaction in human skin. This reaction develops rapidly after injection of antigen, peaks in 10–20 min, and then subsides within a few hours. Careful observation for longer periods, however, has shown that in many instances a late inflammatory response appears at the same site and is quite different in appearance from the initial reaction. Although such late reactions have been observed for many years (2, 3), their significance has been obscure. Interest has been recently revived in these late responses, largely through the emphasis on dual skin reactions by Pepys and his colleagues (4). Intradermal antigenic challenges of patients with allergic bronchopulmonary aspergillosis (5), for example, have elicited an initial wheal and flare, which usually resolves completely, only to be followed by a reaction at the same site, characterized by diffuse erythema and edema. This late response typically appears by 3–4 h after challenge, peaks at 6–12 h, gradually subsides, and resolves by 24 h. Histopathological and serological studies of these dual reactions have suggested that the late cutaneous response occurs as a result of an Arthus (type III) (1) reaction. In contrast, Dolovich et al. (6, 7) have demonstrated that late cutaneous responses can be induced by injection of a variety of antigens: *Bacillus subtilis*

enzyme preparations, ragweed pollen, and significantly, by a monospecific antiserum to IgE. They concluded that certain late cutaneous responses are dependent primarily for their induction on IgE and do not require other immunoglobulins or complement for their progression (7).

Initial observations in this laboratory confirmed that intradermal injections of ragweed pollen extract readily induce a late inflammatory response in sensitive individuals after the intial wheal and flare. In this study we have investigated the gross, microscopic, and immunofluorescent appearances of the late phase of the immediate skin reaction; in other words, the late-phase response (LPR).[1] We have shown that the LPR occurs after injection of a variety of antigens, is dependent on IgE antibodies, and histologically is characterized by a predominance of lymphocytes.

METHODS

Subjects. Skin reactions were induced in 23 adult subjects. 15 patients were atopic and were selected from laboratory personnel and at random from patients attending the Division of Allergic Diseases at the Mayo Clinic. 10 of these had a history of ragweed hay fever and positive immediate skin tests to short ragweed extract. Analyses of the sera of these patients showed that each had significant levels of IgE antibodies to short ragweed, as measured by the radioallergosorbent test (RAST) (8, 9). 5 of the 10 subjects in the ragweed group had received maintenance hyposensitization to ragweed pollen extract for at least the previous 12 mo. The remaining five atopic subjects also had allergic rhinitis but were sensitive to a variety of allergens: two to timothy grass pollen, two to guinea pig dander, and one to *Alternaria.* All had positive immediate skin tests to the respective antigens, and the grass pollen and *Alternaria*-sensitive subjects had significantly elevated levels of specific IgE antibody. The other eight subjects had no history of atopic disease, negative immediate and late skin tests to short ragweed pollen, and no increase in IgE antibodies to short ragweed, as measured by the RAST. Informed consent was obtained from all subjects.

Intracutaneous tests. For the passive transfer tests, 0.1 ml of serum was injected intradermally on the forearm, followed in 18–24 h by the intradermal injection of 0.05 ml of allergenic solution at the same site. Sensitive subjects were injected intradermally on the forearm with 0.05 ml of test solution. The sites were examined at 15 min and intervals up to 96 h. The diameters of the reactions were measured in two perpendicular directions and the characteristics at different times noted.

Test materials. Short ragweed pollen (*Ambrosia elatior* lot no. 18-52-72) was obtained from Greer Laboratories, Inc., Lenoir, N. C. 10 g of pollen were defatted with diethyl ether, dried, suspended in distilled water, and stirred for 48 h, and the supernate was lyophilized. For each experiment a fresh solution of ragweed extract was prepared by dissolving 90 mg of the lyophilized material in 3 ml of sterile, nonpyrogenic 0.9% sodium chloride USP (Travenol Laboratories, Inc., Deerfield, Ill.) and by sterilization through a 0.22 μm membrane (Millipore Corporation, Bedford, Mass.). As preservatives, penicillin G (Sigma Chemical Company, St. Louis, Mo.) and dihydrostreptomycin sulfate (Calbiochem, San Diego, Calif.) were added at concentrations of 100 U and 100 μg/ml, respectively. Intradermal injections of 0.1 ml of the diluent, plus penicillin and streptomycin in these concentrations, failed to elicit inflammatory responses in either the sensitive or passive transfer subjects. In a preliminary study, the ability of the ragweed extract to elicit a late reaction was tested in a normal subject, passively sensitized with serum from a sensitive subject. Late reactions were elicited by undiluted serum and by serial fivefold dilutions to 1:625. We elected to use an antigen dilution of 1:100 in the passive transfer tests. For direct skin testing of ragweed-allergic subjects, the initial dilution of extract used was 1:6,250.

The extracts of timothy grass, *Alternaria,* and guinea pig dander were all commercial preparations (Center Laboratories, Inc., Port Washington, N. Y.). Dilutions were made with phenol-saline diluent from Center Laboratories.

Sera for passive transfer tests were obtained from five subjects allergic to short ragweed pollen who had significant elevations of specific IgE antibodies to short ragweed (10), ranging from 668 to 3,041 ng/ml. None of these sera contained hepatitis B antigen by radioimmunoassay. Each serum was sterilized by passage through a 0.22 μm Millipore filter. A control nonatopic serum was similarly prepared.

Histamine phosphate injection USP, 1 mg base/ml (Eli Lilly and Co., Indianapolis, Ind.), was used in a 1:10 dilution. Bradykinin triacetate (Sigma Chemical Company) was used in a concentration of 0.15 mg/ml. Preliminary testing showed that 0.1 ml of these solutions resulted in a wheal of comparable diameter (15–20 mm) to that induced by the passive transfer tests with serum containing anti-ragweed IgE antibodies followed by challenge with ragweed antigen. Compound 48/80 (Sigma Chemical Company) was dissolved in saline and the pH adjusted to 7.3 by addition of 0.1 M phosphate buffer. Preliminary titration showed that the concentration of 5 mg/ml gave an initial wheal of 15–20 mm in diameter.

Inactivation of IgE protein was performed by heating two atopic sera from ragweed-sensitive donors at 56°C for 4 h. The IgE protein level (11) fell from 3,237 to 39 ng/ml in the first serum and from 3,204 to 113 ng/ml in the second, reductions of 98.8% and 96.5%, respectively.

IgE was also removed from an atopic serum by an anti-IgE immunoabsorbent. IgE protein was purified from myeloma serum P. S. as described elsewhere (11) and digested with papain to yield IgE(Fc) (12, 13). A burro was immunized with IgE(Fc) by subcutaneous injection of 2 mg in complete Freund's adjuvant at time 0, 1 mg in incomplete Freund's at 5 wk, and 4 mg in incomplete Freund's at 5 mo. The animal was bled after the second and third injections and the sera were pooled. 15 ml of clotted plasma from a human subject deficient in IgE was added to cyanogen bromide-activated sepharose 2B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) (14) and 1x borate (14) and rotated overnight at 4°C. It was then washed with 1x borate until the absorbence at 277 nm was less than 0.05. Burro anti-IgE was rendered specific by absorption in batches with the solid phase serum from the IgE-deficient patient. Ratios of packed volume of immunoabsorbent to volume of serum ranged from 1:2 to 1:4. The gamma globulin-containing fraction of the absorbed

[1] *Abbreviations used in this paper:* IF, immunofluorescence; LPR, late-phase response; NBS, normal burro serum; RAST, radioallergosorbent test.

TABLE I

Effect of Treatment of Atopic Serum with Solid Phase Immunoabsorbent

	IgG	IgM	IgA	IgE	IgE antibody
	mg/ml	*mg/ml*	*mg/ml*	*ng/ml*	*% counts bound**
Serum before treatment	7.64	0.36	0.65	10,153	36.11
Serum treated with solid phase anti-IgE (pool I)	6.85	0.27	0.28	28	0.39
Eluate from solid phase anti-IgE (pool II)	0.08	0	0	696	30.76
Serum treated with solid phase NBS gamma globulin (pool I)	6.66	0.30	0.43	5,988	38.87
Eluate from solid phase NBS gamma globulin (pool II)	0	0	0	13	1.34

The concentrations refer to 10-ml volumes in all cases except for the anti-IgE pool II (5 ml) and NBS gamma globulin pool II (2.2 ml).

* The percentages are directly related to the quantity of IgE antibody in the test solution (10). Normal serum (50 μl) yielded a value of 0.16% of counts bound. Thus, barely detectable amounts of ragweed antibody to ragweed remained in the serum treated with solid phase anti-IgE (slightly more than twice the value given by the normal serum control).

burro serum was obtained by precipitation with an equal volume of 28% Na_2SO_4, washed three times with 14% Na_2SO_4, dissolved in distilled water, and dialyzed against 0.15 M NaCl. The resulting solution contained 18.2 mg protein/ml by spectrophotometric analysis. Immunoelectrophoresis showed that the burro antibody migrated in the fast gamma region and gave a single band when reacted with IgE (P. S.) myeloma serum and no band with normal human serum. 300 mg of burro gamma globulin anti-IgE(Fc) was coupled to 30 ml of cyanogen bromide-activated Sepharose 2B, as previously described (14). As a control, 300 mg of gamma globulin prepared by Na_2SO_4 precipitation from normal burro serum (NBS) in an identical manner was coupled to 30 ml of cyanogen bromide-activated 2B. By spectrophotometric analysis, 98% of the anti-IgE(Fc) and 99% of gamma globulin from NBS were bound to the Sepharose. Each immunoabsorbent was poured into a 1.2 × 30-cm column with approximately 1 cm of Sephadex G-100 (Pharmacia) at the base to facilitate flow. 10 ml of an allergic serum known to contain 10,153 ng/ml of IgE was added to each column and the flow was stopped for 30 min after entry into the immunoabsorbent. The 1x borate buffer was then allowed to move down the column until just before elution began and the flow was stopped again for a further 30 min. Fractions were collected and absorbence was monitored at 277 nm with a Gilford spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). When the absorbence was less than 0.100, glycine HCl (0.05 M, pH 2.20) was applied to remove the bound IgE. The elution was discontinued when the absorption became less than 0.010. The fall-through peaks from each column were pooled (pool I) and ultrafiltered to 10 ml with a Diaflo UM-2 membrane (Amicon Corp., Lexington, Mass.). The glycine HCl peaks from each were also pooled (pool II) and ultrafiltered with a UM-2 membrane to 5.0 ml from the anti-IgE column and 2.2 ml from the NBS gamma globulin column. IgE levels from the pooled specimens and untreated serum were measured by radioimmunoassay (11). To verify the specificity of the immunoabsorption procedure, the levels of IgG, IgM, and IgA were measured by an automated immunoprecipitation method (15) and, where necessary, by low-level immunoglobulin radial immunodiffusion plates (Meloy Laboratories, Inc., Springfield, Va.). The results appear in Table I. In addition, this table shows that specific ragweed IgE antibodies, as determined by the RAST (8, 9), were removed by the immunoabsorption procedure by solid phase IgG anti-IgE, but not by solid phase NBS gamma globulin, and they were recovered in the glycine-HCl eluates from the anti-IgE column in appreciable amounts. For passive transfer testing, 0.1 ml of pools I and II from each column was used for sensitization.

Competitive inhibition of the allergen-specific IgE interaction was also performed by passive transfer testing. Skin sites were sensitized by intradermal injections of solutions of an allergic serum and an equal volume of IgE myeloma P. S. in varying dilutions, as done by Stanworth et al. (16). The IgE myeloma P. S. was purified (11) and the resulting solution contained 5 mg/ml of IgE. Table II indicates the composition of the various sensitizing solutions employed in this experiment.

Analyses of the inflammatory response

Skin biopsy. 4-mm punch biopsies from both allergic and passive transfer group were taken between 7 and 8 h after antigenic challenge. In addition, serial biopsies were performed on three subjects at 1, 4, and 8 h after provocation. Each tissue specimen was bisected; one half was fixed in 10% buffered formalin, and the other divided again and one piece placed in 5% glutaraldehyde and the other in glutaraldehyde-formaldehyde fixative of high osmolality, as described by Karnovsky (17) for electron microscopy. The specimens fixed in formalin were cut in 5–6 μm sections and stained with hematoxylin and eosin for routine his-

tological study, with chromatrope 2R for eosinophils (18) and acridine orange for basophilic cytoplasmic inclusions (19). In addition, 3-μm sections were stained with Giemsa stain, as recommended by Askenase (20). A quantitative analysis of the cellular infiltrate was performed on these sections by counting and classifying every leukocyte seen in the 4-mm biopsy specimen which was considered to be an infiltrating cell. The specimens fixed in glutaraldehyde and Karnovsky's fixative were prepared for electron microscopic study by washing them in phosphate buffer (0.01 M, pH 7.4), postfixing in 1% osmium, dehydrating in graded concentrations of ethanol, and embedding in Epon 812 (Shell Chemical Co., New York). Sections of 700 Å were cut with an LKB Ultratome III microtome (LKB Instruments, Inc., Rockville, Md.), then stained with Reynolds' lead citrate and uranyl acetate, and attached to grids of 300-mesh uncoated copper. A Philips 201 electron microscope (Philips Electronic Instruments, Mount Vernon, N. Y.) at 60 kV was used for examination of the sections. No difference was found between specimens fixed only with glutaraldehyde and those fixed with the Karnovsky's medium.

Skin windows. A modification of the method of Rebuck and Crowley (21) was employed. Nonallergic subjects were sensitized with atopic serum at two sites and challenged the following day with antigen as described above. After the development of the resulting wheal (within 15 min of the challenge), the skin overlying the wheal was scraped in an area of approximately 5 × 5 mm to a sufficient depth as to allow tissue fluid, but not blood, to accumulate at the site. Glass cover slips were applied and changed at 2-h intervals for 12 h. A control site into which 0.05 ml of short ragweed was injected intradermally was treated in the same way. Two allergic subjects were also studied by the same method, but without prior sensitization. As controls in these patients, 0.05 ml of diluent and of an antigen to which the subjects were not allergic (timothy grass) were injected intradermally.

Immunofluorescence

Some of the bisected 4-mm punch biopsy specimens were quick-frozen in liquid nitrogen and submitted for immunofluorescent (IF) staining and examination by the following method: Fluorescein isothiocyanate (FITC)-labeled antisera to human IgM, IgA, and C3, as well as FITC-labeled goat anti-rabbit and rabbit anti-goat IgG antisera, were purchased from Hyland Div., Travenol Laboratories, Inc., Cost Mesa, Calif. Antisera to human IgG and IgE were prepared by us and labeled with FITC by previously outlined methods (22). Rabbit antiserum to factor B was prepared after isolating and assaying factor B according to the method of Götze and Müller-Eberhard (23). Antiserum to human properdin was made in a goat after properdin was isolated and tested, according to Pensky et al. (24). Rabbit antisera to goat and human C1q and C4 were also purchased (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.). All antisera used in the IF procedures were checked for specificity and activity by double immunodiffusion (Ouchterlony) and by immunoelectrophoresis. Units of antiserum, antibody protein assays, fluorescein-protein ratios, and use dilutions conformed to previous standards (22). Direct IF staining of tissues was performed with labeled antisera to IgG, IgA, IgM, and IgE and C3 by established methods (22). A modified indirect IF method, recently described (25, 26), was employed to test the tissues for C1q, C4, factor B, and properdin. Initial treatments of tissues with rabbit antisera to C1q, C4, and factor B were followed by treatment with labeled goat anti-rabbit IgG. When goat antiproperdin was used, labeled rabbit anti-goat IgG was employed as the second step of the procedure.

RESULTS

Initial observations revealed that most patients with ragweed hay fever manifested a dual reaction after the intradermal injection of pollen extract. We then determined whether late reactions could be induced by a variety of allergens. The results presented in Fig. 1 illustrate that the LPR can be elicited by four different types of allergens and that the reactions are essentially identical, both in the degree of inflammation and in the sequence of events. The late phase generally begins to develop at the 4-h mark, reaches its peak between 8–12 h, and thereafter gradually subsides over 24 h or so. Subsequently, 15 atopic subjects were tested and 14 of these showed the LPR when challenged intradermally by the respective allergen. The exception was a ragweed-sensitive patient treated by long-term hyposensitization.

TABLE II

Competitive Inhibition of Immediate and Late-Phase Responses by IgE Myeloma P.S.

Solution number	Composition of sensitizing material				Average diameter of edema‡						
	IgE myeloma P.S.	M.C. serum*	0.9% NaCl	P.S.IgE:M.C.IgE	0	½ h	1 h	2½ h	5 h	8 h	12 h
		ml						*mm*			
1	0.1 (5 mg/ml)	0.1	—	1,000:1	8	7	—	—	—	—	—
2	0.1 (0.5 mg/ml)	0.1	—	100:1	8	12	10	—	—	—	—
3	0.1 (0.05 mg/ml)	0.1	—	10:1	8	15.5	11.5	39	30	44	40
4	0.1 (5 mg/ml)	—	0.1	—	8	—	—	—	—	—	—
5	—	0.1	0.1	—	8	20	19	33.5	37.5	50	54
6§	—	—	—	—	8	—	—	—	—	—	—

* Allergic serum from patient M.C., IgE protein, 5,000 ng/ml.
‡ After challenge of sensitized site with ragweed antigen.
§ Site not sensitized before allergenic challenge.

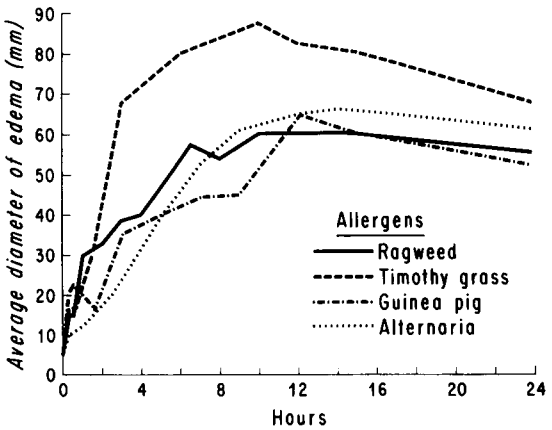

FIGURE 1 Evolution of the LPR in sensitive subjects. Each curve represents a single subject.

In these experiments, we found that the LPR could not be induced unless a fairly large (approximately 15–20 mm) initial wheal resulted. In each case this required a sufficiently high concentration of allergenic extract to elicit the LPR; for example, for timothy grass, 100 protein nitrogen units (PNU)/ml, and for *Alternaria,* 500 PNU of allergen/ml. These latter concentrations are regularly employed for diagnostic skin testing in our clinic and none provoked reactions in the skin of normal subjects.

To study the factors responsible for the LPR, we determined whether it could be produced after passive cutaneous (Prausnitz-Küstner) sensitization. We found that the LPR could be passively transferred in a reproducible manner. In all eight nonatopic subjects, the LPR was induced after passive transfer of each of five allergic sera from ragweed-sensitive subjects. Fig. 2 depicts the timed sequence of inflammatory edema in one subject sensitized by each of the five sera. The LPR's induced in the spontaneously sensitive patients and the normals by passive transfer of atopic sera were essentially identical in development and appearance. However, as a comparison of Figs. 1 and 2 indicates, the allergic subjects generally exhibited reactions which were larger, more persistent, and peaked later than the passively sensitized normals. In both groups, a pruritic wheal and flare reaction rapidly appeared after antigenic challenge and reached a peak between 15 and 30 min. (Fig. 3*a*). In the ensuing 60 min, the wheal became less distinct and gradually merged into the flare zone; thus, at 90 min (Fig. 3*b*) the lesion was diffusely edematous and erythematous, but asymptomatic. Over the next 2–3 h, the lesion remained quiescent; in no case did the inflammatory reaction disappear during this period. Then, at 4–5 h (Fig. 3*c*), mild pruritus heralded an exacerbation of inflammation, which peaked at 6–12 h. At the height of the response (Fig. 3*d*), the lesion was characterized by erythema, warmth, edema, pruritus, and/or tenderness, much more extensive in area and producing greater discomfort than the initial wheal and flare response. It was of great interest that in some subjects a "target lesion," remarkably similar to that seen in erythema multiforme, was noted at the peak of the response. After this period, the lesions gradually subsided, usually by 24 h, but some cases, especially in the allergic group, required up to 48 h to resolve completely. In addition, some of the passive transfer group demonstrated petechiae at the challenged sites 36–48 h later, which persisted for some days.

The finding that the LPR was present in patients sensitive to a variety of antigens and that the LPR could be passively transferred raised the question whether it was induced by IgE antibodies. To answer this, we tested (*a*) the effect of two procedures to remove IgE protein, and (*b*) the effects of competitive inhibition of the binding of allergic IgE antibodies to cells by an excess of IgE myeloma protein. In the first procedure IgE protein was inactivated by heating the atopic serum from ragweed-sensitive donors. As shown in Fig. 4, the immediate response and the LPR were abolished at the site sensitized by heated serum, but both activities were retained by unheataed serum. The same was found in all six subjects tested. In the second procedure an atopic serum was treated with the anti-IgE immunoabsorbent and with solid phase NBS gamma globulin as a control. As shown in Table I, the serum treated with solid phase anti-IgE (pool I) had all but 0.27% of its IgE protein removed (including almost all of its anti-ragweed IgE, as shown by the RAST values), while the levels of the other immunoglobulins showed little change. The results recorded in Fig. 5 indicate that removal of the IgE essentially abolishes both the initial wheal and flare and

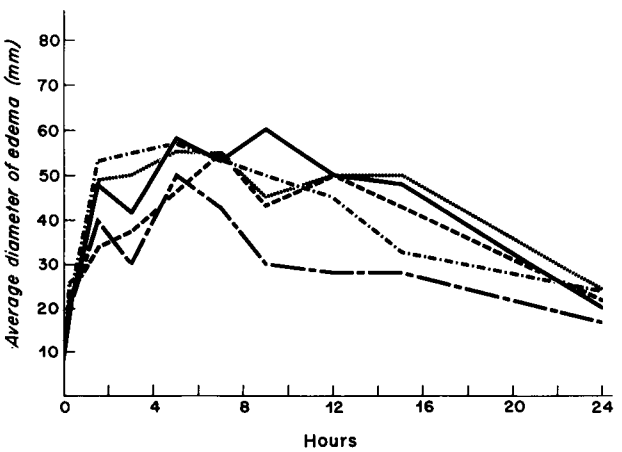

FIGURE 2 Evolution of the LPR after passive transfer of allergic sera. The responses in a single subject were elicited by sera from five donors.

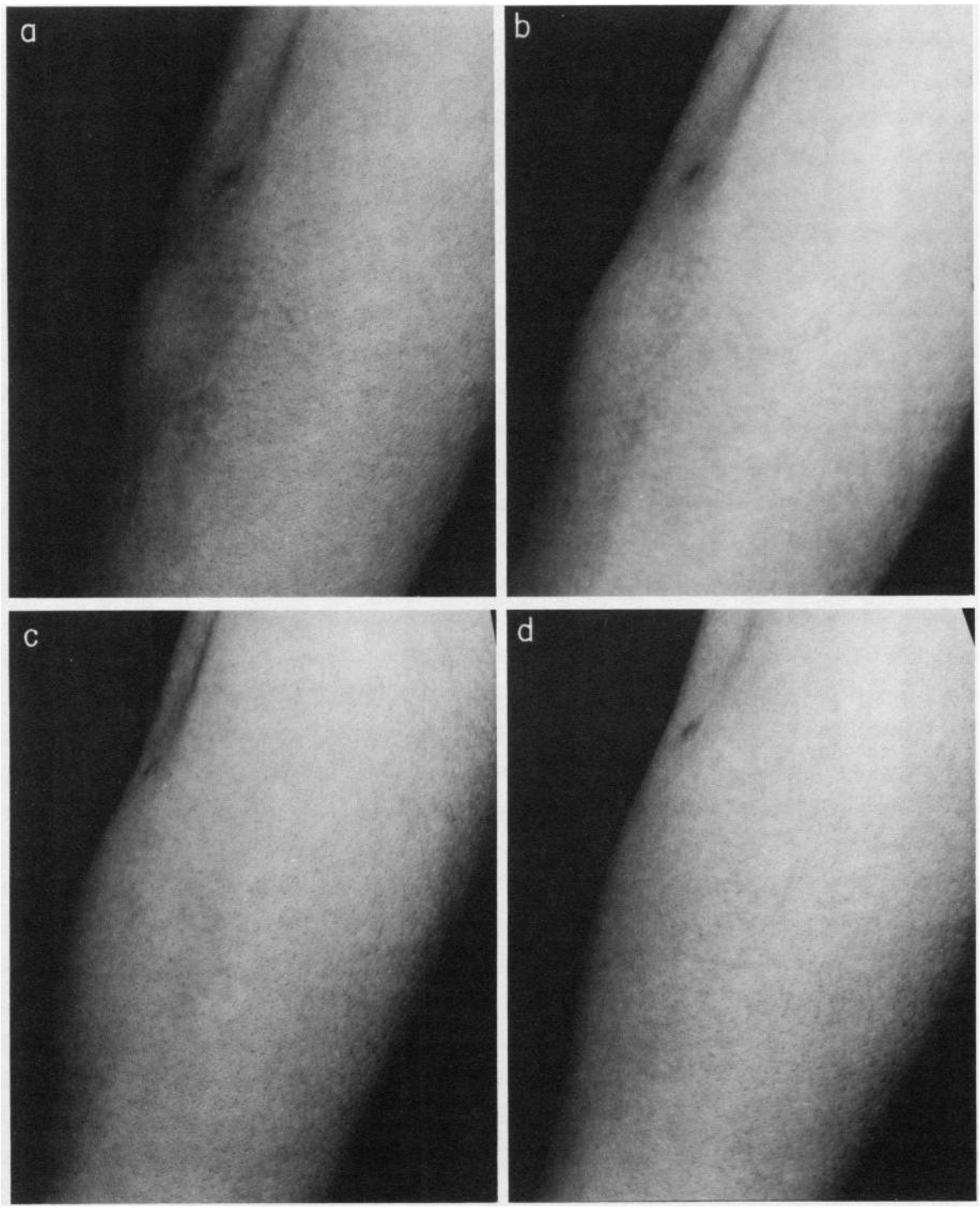

FIGURE 3 Appearance of the cutaneous response at timed intervals after challenge of a site passively sensitized. In the serial photographs, compare the position of the proximal skin scar. (*a*) 15 min. Typical wheal and flare. (*b*) 90 min. Diffuse edema. (*c*) 5 h. Increasing edema and erythema. (*d*) 8 h. The LPR at its peak. Extensive edema and erythema.

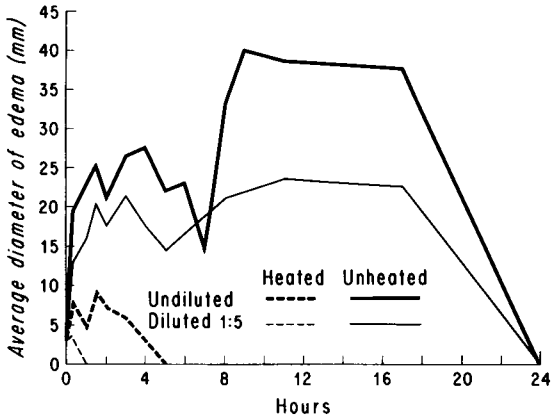

FIGURE 4 Abolition of the LPR by heating allergic serum at 56°C for 4 h.

the LPR, and that both of these reactions are produced by serum treated with the NBS gamma globulin immunoabsorbent (pool I). Furthermore, elution of the IgE from the anti-IgE immunoabsorbent (pool II) yielded a solution containing significant amounts of anti-ragweed IgE antibodies and virtually none of the other immunoglobulins (Table I). Subsequent passive transfer testing with this solution revealed a typical Type I immune response, followed by the LPR, as seen in Fig. 5. These experiments were repeated in two other subjects with the same result. This immunoabsorption experiment was repeated in part (with the equivalent sera to each pool I only) with serum from another ragweed-sensitive subject, with the same result described above.

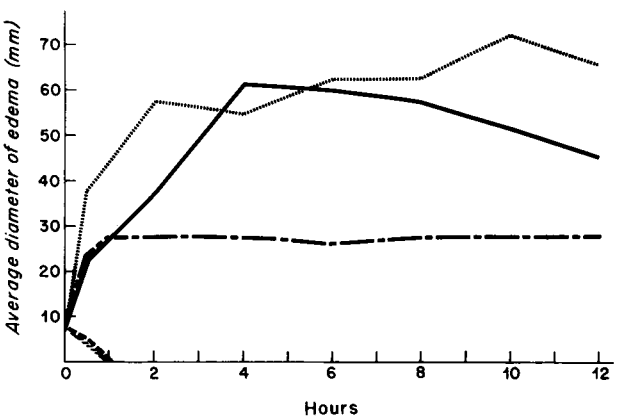

FIGURE 5 Dependence of the LPR on IgE. Retention of the response by sera containing IgE and abolition by sera from which IgE was specifically removed. (——) Atopic serum and ragweed; (· · ·) atopic serum treated with solid phase-NBS gamma globulin column (pool I) and ragweed; (– – –) atopic serum treated with anti-IgE immunoabsorbent (pool I) and ragweed; (— · —) eluate from the anti-IgE immunosorbent (pool II) and ragweed; (///) eluate from the solid phase NBS gamma globulin column (pool II) and ragweed.

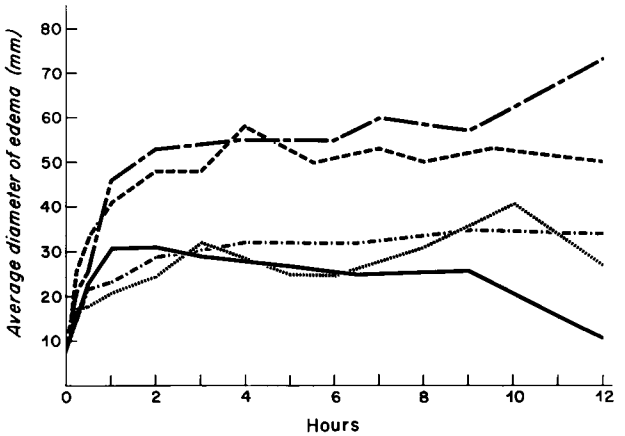

FIGURE 6 Inflammatory responses induced by Compound 48/80. The responses in five different subjects. (- - - -) Nonatopic; (· · · ·) atopic.

In the next series of experiments, excessive quantities of IgE myeloma protein were found to inhibit both the immediate and late phases of the allergic cutaneous response. Table II indicates that IgE myeloma protein, in concentrations of 1,000 and 100 times in excess of that of an allergic serum, effectively blocked the LPR. On the other hand, a 10-fold excess of IgE-P.S. failed to do so. Exactly similar results were found when another nonatopic subject was tested in like manner. The above studies thus furnish strong evidence for the crucial role of IgE in inducing a much more prolonged inflammatory response than the immediate wheal and flare reaction.

To pursue the possibility that IgG antibodies in the recipient may yet be playing a role in the induction of the LPR, we investigated the effect of producing a vasopermeability event in a nonatopic recipient, together with challenge of the site by allergen. Neither of the following experiments elicited the LPR: (*a*) injection of ragweed antigen and histamine, the latter in sufficient concentration to produce a wheal and flare comparable in size to that shown to lead to the LPR on regular passive transfer testing; and (*b*) injection of similar doses of ragweed antigen and histamine into a site previously sensitized by serum treated with the anti-IgE immunoabsorbent. This latter finding also argues against a role of passively transferred IgG in the induction of the LPR.

Because of the observation that IgE antibodies were responsible for the LPR, we sought to define the mediators of the LPR. In the first series of experiments, we found that the LPR could not be induced in nonatopic subjects by injection of histamine alone, bradykinin alone, or by a combination of the two. Next, five subjects were injected intradermally with Compound 48/80, a chemical substance known to release a variety of vasoactive amines from mast cells (27–33). Two (one atopic, the other nonatopic) of the five showed very similar

TABLE III
Histopathological Data

Subject*	Time of Biopsy h	Edema‡	Cellular infiltrate§	Vessel damage‖
Passive transfer¶				
T. P.	1	1+	1+	0
	4	2+	(1–2)+	(2–3)+
	8	3+	3+	3+
R. C.	1	1+	0	0
	4	(1–2)+	2+	2+
	8	2+	(2–3)+	(2–3)+
H. S.	1	1+	1+	1+
	4	1+	2+	2+
	8	(2–3)	3+	3+
K. L.	8	(2–3)+	(2–3)+	2+
G. L.	8	2+	(2–3)+	3+
M. L.	8	2+	2+	2+
P. B.	8	(2–3)+	(3–4)+	(3–4)+
B. A.	8	2+	(3–4)+	(3–4)+
Allergic	Allergen			
B. S.	Ragweed	2+	2+	2+
M. Ca.	Ragweed	2+	3+	3+
J. H.	Ragweed	2+	3+	(3–4)+
S. D.	Ragweed	2+	2+	2+
P. W.	Ragweed	3+	3+	(3–4)+
G. J.	Ragweed**	(2–3)+	2+	(3–4)+
O. P.	Ragweed**	(2–3)+	3+	(3–4)+
L. K.	Ragweed**	2+	2+	2+
D. Le.	Guinea pig	3+	3+	3+
D. Lo.	Guinea pig	1+	2+	(2–3)+
C. F.	Timothy grass	1+	(2–3)+	(2–3)+
Compound 48/80				
D. Lo. (6 h)		2+	2+	2+
K. C.		1+	1+	1+
Controls	Material injected			
J. S.	Ragweed only	±	1+	1+
D. E.	Heated serum + ragweed	±	1+	1+
A. P.	Heated serum + ragweed	0	(1–2)+	1+
B. S.	Timothy grass‡‡	0	1+	1+

* Biopsies taken at 7–8 h unless otherwise stated.
‡ 1+, significant separation of collagen; 2+, tendency to loss of rete ridges and papillary dermis; 3+, complete loss of rete ridges and papillary dermis; 4+, gross edema.
§ 1+, perivascular infiltrate; 2+, perivascular and collagenous infiltrate; 3+, <50 cells per high-power field through collagen; 4+, gross infiltrate.
‖ 1+, vasodilatation; 2+, perivascular infiltrate and vasodilatation; 3+, like 2+, with hyalinization; 4+, hemorrhage and/or necrosis.
¶ Serum used for passive transfer contained 3,041 ng/ml of IgE antibody to short ragweed.
** Patient receiving long-term hyposensitization to ragweed.
‡‡ Allergic to ragweed, but not to timothy grass.

reactions to the typical LPR induced by IgE-containing serum and antigen. The other three exhibited inflammatory responses of similar duration as the LPR but with more prolonged tenderness and less edema (Fig. 6). Thus, the LPR is not mediated solely by histamine or bradykinin, but can be mimicked quite well by the injection of Compound 48/80. These findings, together with the knowledge that IgE antibodies exert their role

TABLE IV

Differential Counts of Cellular Infiltrates in 8-h Lesions (3-μm Sections, Giemsa Stain)

Subject	Number of cells counted*	Basophil/mast		Eosinophils		Neutrophils		Mononuclear	
		no.	%	*no.*	%	*no.*	%	*no.*	%
Passive transfer									
T. P.	239	5	2	51	21	51	21	132	56
K. L.	156	6	4	11	7	37	24	102	65
H. S.	200	4	2	36	18	60	30	100	50
Allergic									
S. D.	309	13	4	87	28	80	26	129	42
J. H.	269	5	2	108	40	27	10	129	48
P. W.	1,000	8	1	117	12	612	61	263	26
D. Le.	304	9	3	82	27	74	25	139	45
O. P.	500	26	5	40	8	197	40	237	47
Compound 48/80									
D. Lo.	134	9	7	13	10	49	36	63	47
Control									
J. S.	18	4	22	2	11	1	6	11	61
A. P.	62	2	4	3	6	20	28	37	62
M. J.‖	53	3	6	0	0	18	34	32	60

* Counts comprised all the cells considered to be infiltrating the punch biopsy specimen, measuring 4 mm along the epidermal surface.

‡ Nonatopic; ragweed was injected but site not sensitized.

§ Nonatopic; site sensitized with heat serum and challenged with ragweed and histamine.

‖ Atopic, challenge with an extract (timothy grass) to which the subject was not sensitive.

TABLE V

*Analysis of Cellular Infiltrate by Skin Window Technique**

Solution	Time	Basophils	Eosinophils	Neutrophils	Mononuclear
	h	%	%	%	%
Ragweed‡	2	Inadequate cell number			
	4	—	—	92	7
	6	0.5	5.5	70	24
	8	4	7	69	20
	10	2	8	41	49
	12	8	14	46	32
Timothy grass‡	2	Inadequate cell number			
	4	—	—	97	3
	6	—	—	82	18
	8	—	—	79	21
	10	—	1.5	62	36.5
	12	—	1	64	35
Diluent	2	—	1.5	98.5	—
	4	—	4	94	2
	6	0.5	1.5	78	20
	8	0.5	1.5	68	30
	10	—	4	71	25
	12	0.5	2.5	78	19

* 500 cells counted in each specimen.

‡ The subject was allergic to ragweed but not to timothy grass pollen.

in the inflammatory response through binding to receptors on mast cells and basophils (34, 35), argue that in the LPR either or both of these two cell types must play an essential role.

To investigate the pathophysiology of the LPR further, we analyzed the histological characteristics of the lesions by a variety of methods. Table III lists the histopathological data from the skin biopsies. In serial biopsies of three subjects passively sensitized by serum, we observed an increasing degree of inflammation during the period of 1–8 h. Edema, cellular infiltration and vascular changes all increased during this time interval. Initially, the cellular infiltrate was entirely mononuclear and limited to the perivascular tissues, but with time, eosinophils, neutrophils, and mononuclear cells increased with invasion of the collagen as well. Biopsy of a 24-h lesion induced in a ragweed-allergic subject showed a marked (4+) cellular infiltration, almost entirely mononuclear. Because most of the LPR's elicited in these experiments were fully developed by approximately 8 h, this time period was selected to examine the histology of the LPR. The responses in spontaneously sensitive and passively sensitized subjects were essentially identical. All 8-h lesions revealed moderate edema and cellular infiltration, the latter mostly perivascular (Fig. 7). The vessel changes consisted of vasodilatation, perivascular cellular invasion, and endothelial hyalinization, with some vessels also showing hemorrhage and necrosis. In general, the mononuclear cell was the predominant infiltrating cell type, although neutrophils and eosinophils were also prominent. Biopsies of the lesions induced by Compound 48/80 were obtained in two subjects at 6 and 8 h, respectively. They exhibited edema, vessel change, and cellular infiltration, but of less intensity than the allergen-induced lesions. Three groups of control lesions were also biopsied: (*a*) challenge of a nonsensitive subject with ragweed without prior passive sensitization; (*b*) sensitization with heated serum followed by ragweed challenge; and (*c*) challenge of an allergic subject with an antigen to which he was not sensitive. All of these control lesions were essentially identical: minimal edema, a mixed perivascular cellular infiltrate (almost entirely mononuclear), and mild vasodilatation.

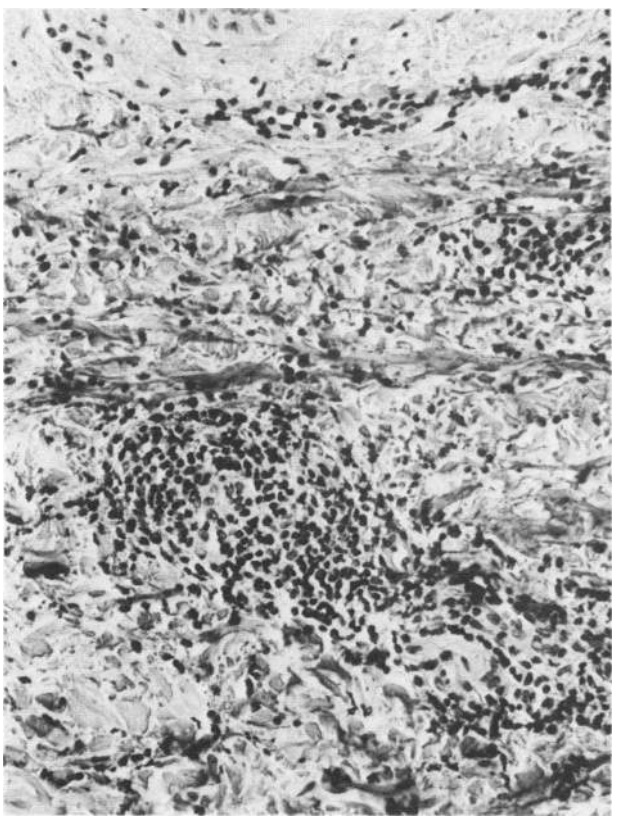

FIGURE 7 Histopathology of the LPR. Intense cellular infiltration in perivascular areas of the dermis and scattered throughout collagen. × 250.

A quantitative analysis of the infiltrating cells was performed on the biopsied specimens by employing a variety of histochemical stains and by the Rebuck skin window technique. Table IV shows the differential counts of these cells on 3-μm sections of 8-h lesions stained with Giemsa. In seven of eight lesions, the mononuclear cell was the most common, but one lesion did show a predominance of neutrophils. However, the infiltrate was generally mixed, with significant numbers of eosinophils and basophils as well. The presence of basophils and/or mast cells was confirmed by staining with acridine orange. That basophils are recruited to the area was shown by the skin window studies. Table V lists the cell differential counts of one of the four subjects tested in this manner. We observed in each of the late responses a significant infiltration of both basophils and eosinophils, particularly at the peak of the LPR: i.e., from 6 to 12 h. Each control site showed none, or at best no more than 1%, of either basophils or eosinophils during the 12-h observation.

Electron microscopic examination of the LPR (at 8 h) was performed specifically to define the nature of the mononuclear cellular infiltrate. A differential count was performed on 250 infiltrating cells in four specimens. Of these, 119 (48%) were lymphocytes, 18 (7%) monocytes or macrophages, 67 (27%) eosinophils, 23 (9%) neutrophils, 7 (3%) basophils (1 mast cell was also noted), 3 (1%) plasma cells, and 13 (5%) viable, but completely degranulated, cells. There did not appear to be increased numbers of platelets, but one noticeable feature was the presence of numerous free eosinophil granules throughout the tissue.

The LPR was studied by IF to determine whether

TABLE VI
Immunofluorescent Analysis of Biopsied Lesions

	Stage of lesions		
	1 h	4 h	8 h
IgG	0/3	0/3	0/15
IgM	0/3	1/3	2/15
IgA	0/3	0/3	0/15
C3	2/3	0/3	1/15
Fibrin	0/3	0/3	0/15
IgE	0/3	0/3	0/15

immunoglobulins or complement components were contributing to the reaction. These results are shown in Table VI. IgM was detected in only 3 of 15 examinations while IgG and IgA were not found at all. In addition, C3 was demonstrated infrequently (in two of the 1-h biopsies and in only one of the 15 biopsies taken at the 8-h mark). In none of the three subjects whose biopsies showed the deposition of C3 were C1q, C5, properdin, or factor B detected. IgE was not seen in the vessels or in other specific skin structures. Immunofluorescent studies were also done in two subjects who received heated serum, followed by ragweed and histamine. Neither immunoglobulins nor complement components were detected.

DISCUSSION

The results of these experiments indicate that the late phase of the immediate skin reaction is induced by the interaction of IgE antibodies with antigen. It would appear that any antigen able to stimulate the production of IgE antibodies is also capable of inducing the LPR. The LPR demonstrates all of the cardinal signs of inflammation and quantitatively it involves considerably greater area, and thus volume of tissue, than the immediate IgE allergic reaction. Clearly histamine alone is not the mediator of the LPR, but the ability of Compound 48/80 to induce lesions morphologically and histologically similar to the LPR suggests that mast cells and basophils could be involved in the development of the lesion. Remarkably, the lymphocyte is the most prevalent cell in the lesion, although eosinophils and neutrophils, as well as increased numbers of basophils, are also present.

That we were able to elicit the LPR in almost all allergic subjects suggests that the frequency of this response is much higher than previously appreciated. Thus our results confirm the work of Robertson et al. (36), who found that a dual skin reaction could be elicited in almost all ragweed-sensitive persons. Other studies with common allergens, however, have shown somewhat lower incidences (37–39), but such findings could be falsely low if a sufficiently large dose of antigen was not used. Although our lone negative reactor was receiving immunotherapy, this does not of itself appear to be a limiting factor, as is shown by the remaining subjects in this group as well as in the study by Taylor and Shivalkar (38).

Because the LPR can be induced by the intradermal challenge of various allergens in sensitive persons and by passive transfer of atopic serum, IgE is implicated as essential in its development. This conclusion is supported by the demonstration of Dolovich and his associates (7) that a late cutaneous allergic response, the description of which seems identical to the LPR, can be induced by the intradermal injection of antibodies to IgE. Our studies offer four additional lines of evidence for the essential role of IgE. Abolition of the LPR was demonstrated in three ways: (*a*) heating the allergic serum at 56°C for 4 h to denature the Fc piece of the IgE antibodies (40); (*b*) removing IgE from the serum by specific immunoabsorption; and (*c*) competitively inhibiting the interaction of specific IgE antibodies with the cell receptor sites by IgE myeloma protein. Fourthly, the LPR could be elicited by an affinity chromatography-purified IgE protein containing specific IgE antibodies to ragweed, followed by allergenic challenge.

The appearance and development of the LPR bear many similarities to the late component of the dual skin reactions demonstrated by Pepys et al. in their studies of patients with allergic bronchopulmonary aspergillosis and extrinsic allergic alveolitis (4). They concluded that such late cutaneous reactions occur as a result of an Arthus or type III immune response (1). Thus they found circulating precipitins specific for either *Aspergillus fumigatus* or avian antigens, significant edema, increased perivascular cellular infiltration (mostly mononuclear, but with neutrophils and eosinophils) on histological examination, and deposition of complement (β_1C/A) and immunoglobulins (IgM, IgG, IgA) in the majority of cases. Furthermore, Taylor and Shivalkar (38) found complement deposition in 6-h biopsies of subjects allergic to grass pollen. However, Dolovich et al. (7) could not confirm these findings by IF in 6-h biopsies of the late cutaneous allergic response induced in ragweed-sensitive subjects and by anti-IgE and *Bacillus subtilis* enzyme preparations in nonatopics. Our study supports the findings of Dolovich and colleagues; that is, we could not demonstrate the deposition of either immunoglobulins or complement to any consistent degree. As to the quality of the cellular infiltrate, the lymphocyte, rather than the neutrophil or the eosinophil, was predominant, with only one exception. Yet there were also significant accumulations of basophils and eosinophils, the basophils being particularly noticeable with skin window technique. Such cells are rarely detected with this method (21, 41) but it is of interest

that Felarca and Lowell (42) also observed basophils and eosinophils in 18 and 24-h allergic skin reactions. Thus, although the histological characteristics of the LPR bear many similarities to that induced by a type III reaction, we do not believe it results from an immune complex-complement activation phenomenon. Such a conclusion does not refute the findings of Pepys et al. (4), because we utilized different antigenic stimuli.

Although degranulation of mast cells and basophils appears to be the central mechanism in the development of the LPR, the precise sequence of chemical events remains obscure. In addition to our findings that histamine alone cannot induce the LPR, Greaves and Schuster (43) have shown that histamine and 5-hydroxytryptamine quickly induce tachyphylaxis and thus neither can prolong vascular permeability for 12 h or so. Perhaps other mediators released as a result of IgE-antigen interaction on the surface of the mast cell or basophil, such as slow-reacting substance (44), eosinophil chemotactic factor of anaphylaxis (31), or the enzyme described by Newball et al. (45), which possesses kallikrein-like activity, act as the prime vasopermeability agents, either alone or in sequence. In this regard, the intradermal injection of kallikrein has been shown to induce a prolonged, painful inflammatory response, similar in many respects to the LPR (46), and kallikrein also exhibits neutrophil, basophil, and mononuclear cell chemotactic activity (47–49).

What is the significance of the LPR? We have no evidence that it is responsible for certain dermatitides, although it may conceivably play a role in chronic angioedema or urticaria. Nonetheless, because the respiratory tract is another major target organ for allergic reactions, the LPR may be important in the pathophysiology of asthma. Indeed, late asthmatic responses can result from bronchial challenge by antigen and correspond in time to the LPR of skin. Such late asthmatic responses have been induced by inhalation of ragweed pollen (36), house dust (50), and the house dust mite, *Dermatophagoides pteronyssinus* (51). Finally, the LPR could be important in host defense against parasite infestation.

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