

Activation of the coagulation pathway during ongoing allergic cutaneous reactions in humans

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The levels of histamine, fibrinopeptide A (FPA), and IgG were determined in chamber fluids overlying sites of antigen versus buffer incubation for up to 7 hours in seven atopic and four antigen-nonreactive subjects. Significant increases in histamine were observed at antigen versus buffer sites in the atopic subjects throughout the 7-hour period. FPA and IgG levels were higher in antigen than in buffer sites from 0 to 5 hours in the atopic subjects. Furthermore, FPA levels correlated with the magnitude of induration at 6 hours after antigen injection in atopic subjects. There were no differences in the levels of histamine, FPA, or IgG at antigen versus buffer sites in the skin test-negative subjects. We suggest that the combination of vascular leakage of proteins, induced by vasoactive mediator release, and activation of these proteins during ongoing cutaneous reactions is responsible for fibrin formation that contributes to the pathophysiology of late-phase allergic responses in the skin. (J ALLERGY CLIN IMMUNOL 1992;89:552-9.)

Key words: Fibrin, cutaneous allergic reactions, late-phase responses

Cutaneous responses after intradermal administration of pollen to pollen-sensitive subjects are characterized by an immediate (10 to 20 minutes) wheal-and-flare response followed by a more prolonged erythematous, indurated response (1 to 12 hours) that becomes maximal between the sixth and eighth hours after antigen injection.¹⁻³ This "late-phase" edematous, indurated, cutaneous LPR occurs in 60% of pollen-sensitive subjects² with the intensity directly related to the dose of antigen injected and the serum level of antigen-specific IgE antibody in the subject.^{2,3} Although the mediators responsible for this response are not known, histopathologic studies have demonstrated a mixed cellular infiltrate and evidence of fibrin deposition that has been believed to be characteristic of the reaction.^{2,4}

Abbreviations used

FPA: Fibrinopeptide A
LPR: Late-phase response
PNU: Protein nitrogen unit
LT: Leukotriene

With skin chambers placed over denuded skin-blister sites, we have been able to measure the accumulation of cells and mediators during ongoing cutaneous allergic reactions in humans.⁵ We have previously demonstrated the prolonged release of histamine and LTB₄ and the late appearance of platelet-activating factor at these sites.^{6,7} In addition, we have found increased activation of the intrinsic coagulation/kinin pathway from the third to fifth hours in chamber fluids overlying sites of continuous exposure to antigen as compared to that of sites exposed to buffer diluent.⁸ This was inferred based on the formation of C1-inactivator complexes with activated Hageman factor and kallikrein during this time period. We reasoned that such activation could affect plasma proteins exuding into these inflammatory sites, leading to increased coagulation and fibrin formation. The present studies were initiated to determine whether there was

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evidence of fibrin formation in ongoing cutaneous allergic reactions.

METHODS

Subjects

Eleven subjects, aged 25 to 35 years, were selected for the study after they had given their informed consent. Seven (three male and four female subjects) were atopic, as evidenced by the manifestation of ocular and respiratory symptoms during the grass- or ragweed-pollinating season, as well as intradermal skin test reactivity of ≤ 10 PNU of grass or ragweed pollen. In screening studies, all subjects exhibited an indurated LPR (3 to 60 mm in diameter) at 6 hours after intradermal injection of 0.02 ml of 100 PNU/ml of grass- or ragweed-pollen extract. Four subjects (two male and two female subjects) were not reactive to grass or ragweed pollen as evidenced by (1) no development of respiratory or ocular symptoms during either the grass- or ragweed-pollinating season, or (2) no immediate wheal or edematous LPR after intradermal injection of 0.02 cc of 1000 PNU/ml of grass- or ragweed-pollen antigen.

Skin-chamber study

Four skin blisters of 10 mm diameter were produced by gentle heat and suction, the epidermis was denuded, and a collection chamber was appended over the sites, as previously reported.⁵ Two chambers were filled with 0.3 ml of antigen (ragweed- or grass-pollen extract, Greer Laboratories, Lenoir, N.C.), and two chambers were filled with 0.3 ml of buffer (phosphate-buffered saline). Unlike our previous protocols, heparin was not added to any of the chamber fluids. The fluids were collected, the chambers were removed, and sites were replaced with fluids at 1, 3, 5, and 7 hours. Fluids were stored for determination of the concentrations of histamine, FPA, and IgG, as described below. In some experiments, antigen- and buffer-site chamber fluids were incubated *in vitro* at 37°C for 2 hours to determine if *in vitro* generation of FPA would occur. FPA levels after incubation were compared to levels measured from aliquots taken immediately before incubation and kept at 4°C.

Assay of skin-chamber fluids

Histamine was measured by radioenzymatic assay, as previously described and could detect concentrations as low as 1 to 2 ng/ml in the chamber fluids.⁹

FPA levels were determined by an RIA with kits (Altane Corp., Manhasset, N.Y.) that detected concentrations as low as 0.1 ng/ml.¹⁰⁻¹² In brief, samples were depleted of cross-reacting fibrinogen by bentonite precipitation, and the FPA levels were determined by a competitive inhibition assay between radiolabeled ¹²⁵I FPA and the FPA in the samples and specific rabbit antibody to FPA. Any FPA in the supernatant fluid competed with a measured amount of ¹²⁵I FPA for binding to the rabbit anti-FPA antibody. The labeled FPA bound to the antibody is then precipitated by addition of a goat antirabbit gammaglobulin in polyethylene glycol. The amount of radioactivity in the precipitate is inversely

proportional to the amount of FPA in the original sample and can be calculated by comparison to standard concentrations of FPA. In preliminary experiments we determined that (1) there was no interference in the assay by either ragweed- or grass-pollen extracts or phosphate-buffered saline, (2) there was < 500 ng/ml of FPA in plasma samples drawn from the 11 subjects just before skin-chamber challenge, (3) the optimal dilution of chamber fluid for FPA analysis was 1:100 to 1:200 (vol/vol); therefore, all samples were assayed at both 1:100 and 1:200 dilutions, and (4) synthetic FPA added to saline-, buffer-, or antigen-site chamber fluids resulted in a linear dose-response curve that was parallel to the curves with the standards and diluents provided in the commercial kit.

IgG concentrations were determined by an ELISA assay with a rabbit antihuman gammaglobulin antibody (Tago, Inc., Burlingame, Calif.) and peroxidase-conjugated antibody (Sigma Chemical Co., St. Louis, Mo.). This assay could detect IgG concentrations as low as 20 ng/ml as previously reported.^{8, 13}

Statistics

All data are presented as the mean $\pm$ SEM unless it is otherwise stated. The values were normally distributed (Kolmogorov-Smirnov) analysis; therefore, these data were analyzed first by two-way analysis of variance with time and challenge (buffer versus antigen) as the dependent factors. If a significant relationship was detected, these data were analyzed by Student's *t* test or *t* test for paired values and by linear regression analysis to determine statistical significance.

RESULTS

The concentrations of histamine in chamber fluids overlying antigen and buffer sites throughout the 7-hour incubation in the seven atopic, skin-sensitive subjects are depicted in Fig. 1. Histamine levels (nanograms per milliliter) in the fluids overlying the antigen sites peaked in the first hour (56 ± 11) and remained elevated (25 ± 3 to 19 ± 3) throughout the 7-hour antigen exposure. These histamine concentrations were significantly ($p < 0.001$) greater than in fluids overlying the buffer-site incubations (2 ± 1 to 3 ± 1) throughout the 7-hour period.

The concentrations of FPA and IgG in chamber fluids overlying antigen and buffer sites in the seven atopic subjects are depicted in Fig. 2. FPA levels (nanograms per milliliter) in antigen-site chamber fluids were 1245 ± 157 in the first hour, 3434 ± 647 in the 1- to 3-hour fluids, 2170 ± 345 in the 3- to 5-hour fluids, and 1897 ± 449 in the 5- to 7-hour fluids. The FPA levels at these antigen sites were significantly ($p < 0.01$ to 0.02) elevated compared to the analogous buffer sites (958 ± 137 to 1144 ± 229) throughout the first 5 hours of incubation.

To estimate the degree of plasma leakage into the

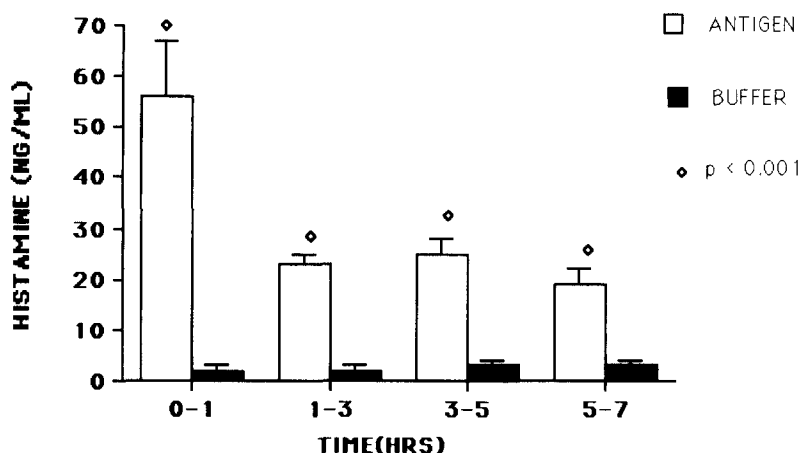


FIG. 1. Concentrations of histamine from denuded sites exposed to grass or ragweed-pollen antigen and buffer in seven atopic, antigen-sensitive subjects.

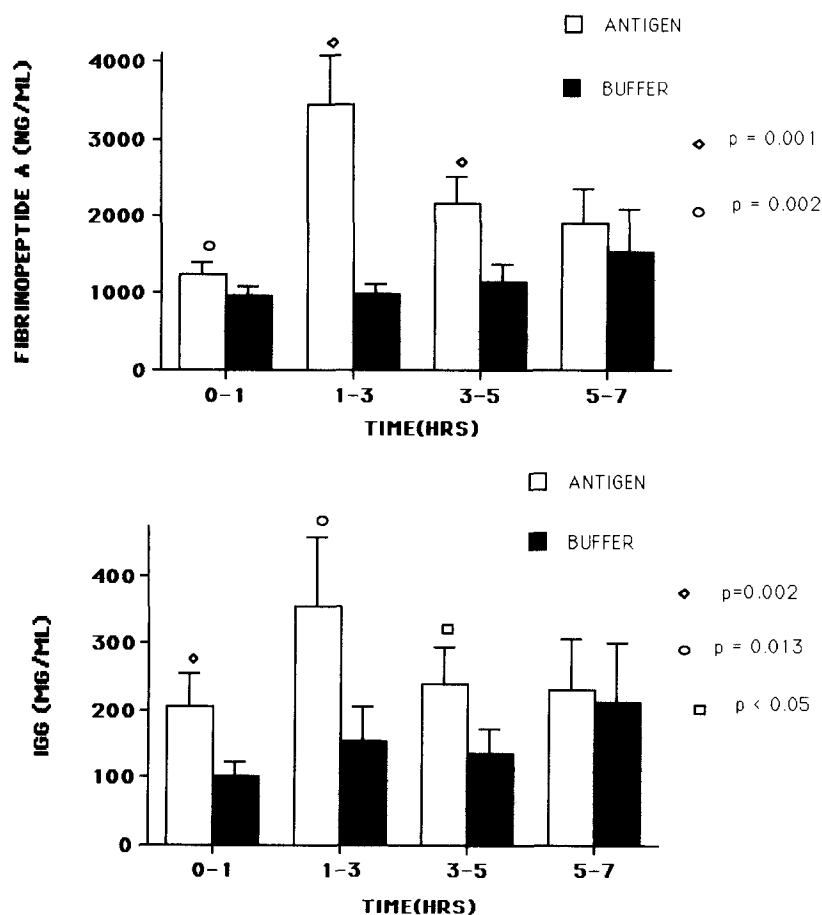


FIG. 2. Concentrations of FPA (top) and IgG (bottom) in chamber fluids from denuded sites exposed to grass- or ragweed-pollen antigen and buffer in seven subjects sensitive to grass- or ragweed-pollen antigen.

denuded skin-blister sites, we also analyzed IgG content in the overlying chamber fluids. IgG levels (milligrams per milliliter) in the fluids overlying the antigen sites were 204 ± 490 in the first hour,

356 ± 105 from the 1- to 3-hour fluids, falling to 239 ± 56 from 3- to 5-hour fluids, and 231 ± 75 by the 5- to 7-hour fluids. The IgG levels through the first 5 hours were significantly ($p < 0.01$ to 0.05)

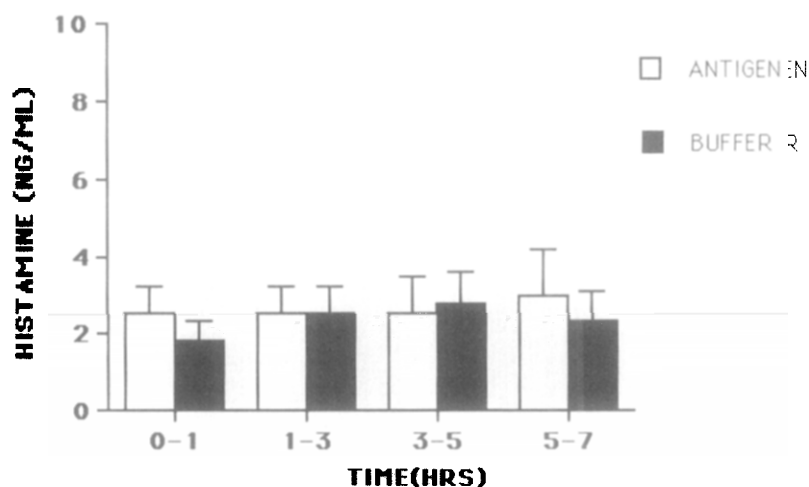


FIG. 3. Concentrations of histamine from denuded sites exposed to grass- or ragweed-pollen antigen and buffer in four subjects not sensitive to grass- or ragweed-pollen antigen.

elevated compared to analogous buffer-site levels (101 ± 23 to 153 ± 51).

The concentrations of histamine in chamber fluids overlying antigen- and buffer-site incubations in the four skin test-negative subjects are depicted in Fig. 3. In contrast to the situation observed in the atopic, skin test-positive subjects, there was no difference between the very low histamine levels in the chamber fluids at antigen or buffer sites throughout the 7-hour exposure. Likewise, as depicted in Fig. 5, there were no significant differences in chamber fluids at antigen-versus buffer-challenge sites in levels of FPA or IgG at any time in these skin test-negative subjects.

Comparisons of the net (antigen site minus buffer site) skin-chamber fluid levels of the mediators/proteins in atopic, skin-positive subjects compared to the skin test-negative subjects are depicted in Figs. 4 and 5. Net histamine concentrations in the atopic, skin test-positive subjects (53 ± 11 to 16 ± 2) were significantly ($p = 0.001$) higher than in the skin test-negative subjects (0.25 ± 0.25 to 0.75 ± 0.48) throughout the 7-hour time period (Fig. 5). Net FPA concentrations in the antigen-reactive subjects (287 ± 55 to 2480 ± 743) were significantly ($p = 0.03$ to 0.05) higher than in the skin test-negative subjects (0 ± 0 to 150 ± 150) throughout the first 5 hours (Fig. 6). Finally, net IgG levels in the atopic, skin test-positive subjects (93 ± 49 to 218 ± 59) were significantly higher ($p = 0.005$) than in the skin test-negative subjects (5 ± 5 to 54 ± 20) during the first 3 hours of antigen and buffer incubations (Fig. 6).

To determine if FPA could be generated in vitro during our storage and handling of the chamber fluids, we conducted the following additional experiment.

We have taken 0- to 1-, 1- to 3- and 3- to 5-hour antigen-site chamber fluids, and 0- to 1- and 1- to 3-hour buffer-site fluids and allowed them to incubate at 37°C for 2 hours. We then compared the FPA levels after incubation to levels measured in aliquots taken immediately before incubation and kept at 4°C (Table I). There was $<10\%$ difference in FPA before and after such incubations at antigen-site fluids without any greater increase in the 0- to 1-hour fluids than in the fluids from later time periods. If in vitro generation were playing a role, the 0- to 1-hour fluids (which had less exposure to the skin surface) would be expected to generate more FPA than the 1- to 3- or 3- to 5-hour fluids. Since very little FPA was generated under these conditions, in either antigen- or buffer-site fluids, in vitro generation cannot account for our findings.

All seven of the atopic skin test-reactive subjects exhibited an indurated reaction at 6 hours after intradermal injection of antigen. The size of the indurated reaction (mean diameter) correlated directly with the net concentrations of FPA in the 3- to 5-hour chamber-fluid site ($r = 0.72$; $p = 0.05$) (Fig. 7). There were no significant correlations between IgG and FPA levels at individual antigen sites. There was no reaction 6 hours after intradermal injection of 0.02 ml of 1000 PNU/ml of pollen antigen in the four skin test-negative subjects.

DISCUSSION

In this study we have found increased levels of FPA in ongoing allergic reactions at pollen-antigen-challenged skin sites of sensitive subjects. In contrast, levels of FPA were no different in antigen- than in buffer-control challenged sites in subjects not sensi-

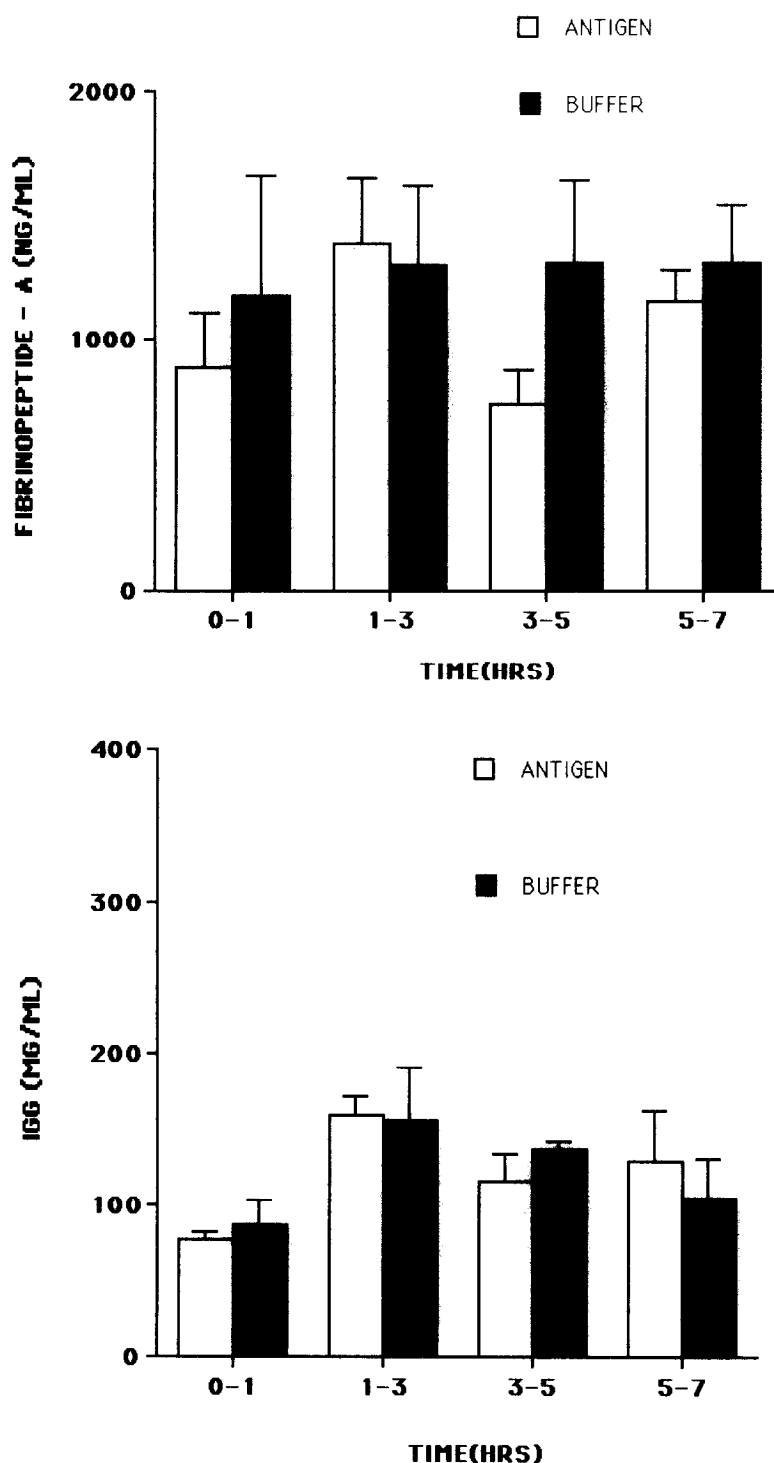


FIG. 4. Concentrations of FPA (top) and IgG (bottom) in chamber fluids from denuded sites exposed to grass- or ragweed-pollen antigen and buffer in four subjects not sensitive to grass- or ragweed-pollen antigen.

tive to these antigens. Further evidence of the ongoing allergic reactions in the sensitive subjects was the impressive prolonged histamine release in such sites.

FPA is a 16-amino acid peptide with a molecular

weight of 1535 daltons cleaved from the A α chains of fibrinogen by thrombin. The action of thrombin produces a fibrin monomer and two molecules each of FPA and B. The concentrations of FPA can thus

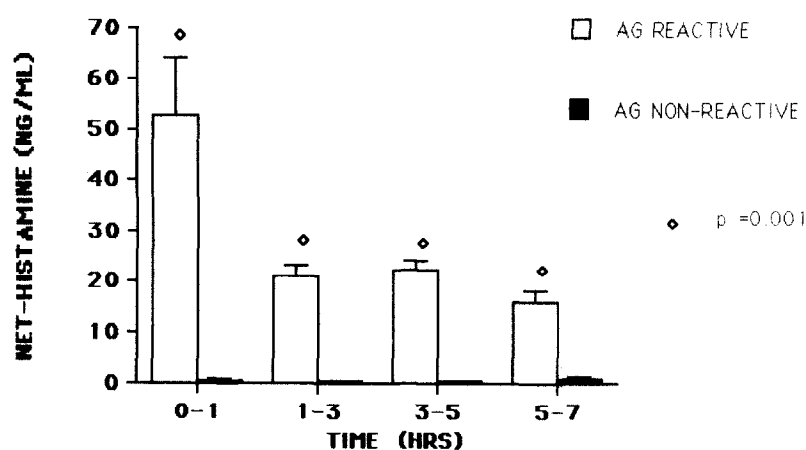


FIG. 5. Net concentration (histamine concentration at antigen-site minus histamine concentration at buffer-site chamber fluids) in individual subjects. Mean $\pm$ SEM for antigen (Ag)-reactive and Ag-nonreactive subjects.

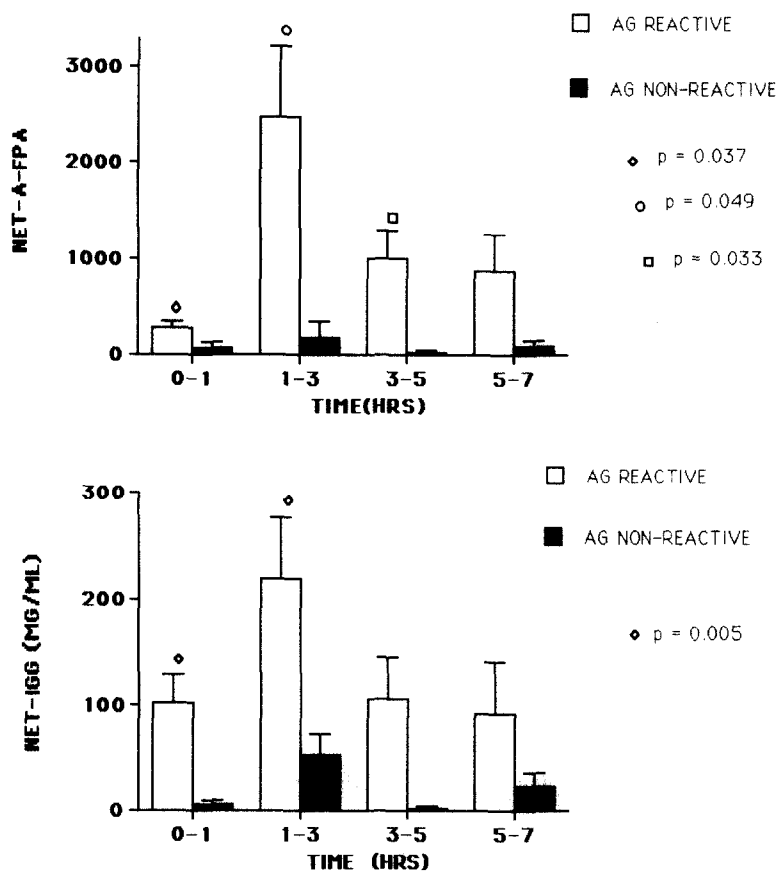


FIG. 6. Net concentrations of FPA and IgG in skin-chamber fluids from antigen (Ag)-reactive and Ag-nonreactive subjects. See Fig. 5 legend for definition of "net" concentration.

be directly related to the activation of fibrinogen and the amount of fibrin monomer produced.

The prominent fibrin deposition we observed may be related to the amount of fibrinogen in the plasma

extravasating into the allergic reaction sites. The modest trauma involved in the induction of the skin blister does lead to a moderate presence of plasma proteins (IgG levels of 100 to 200 mg/ml, about 10% to 15%

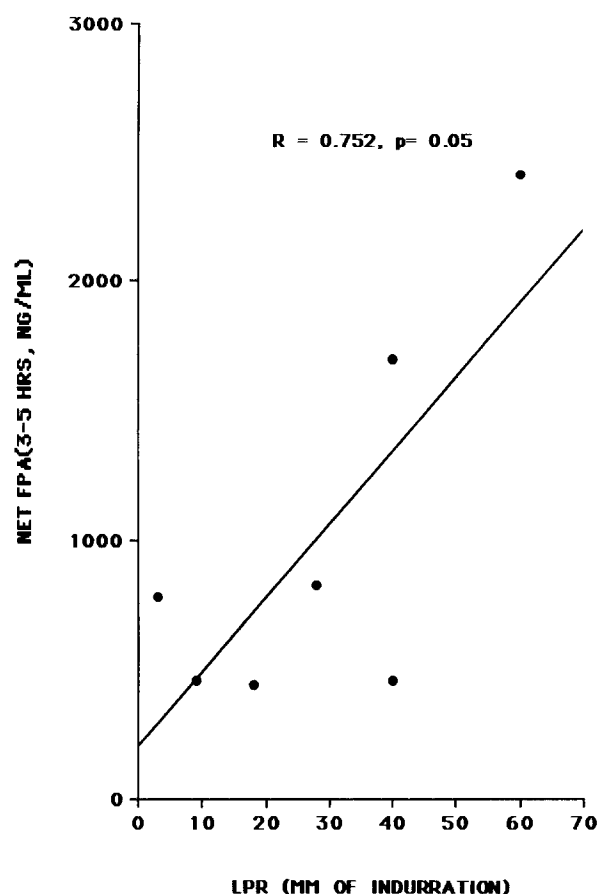


FIG. 7. LPR size after intradermal antigen injection is plotted on the *abscissa*; net (antigen minus buffer sites) FPA (nanograms per milliliter) from the 3- to 5-hour site in the atopic subjects is plotted on the *ordinate*.

of the concomitant plasma level) in the overlying control-chamber fluids containing buffer solutions. The IgG levels in fluids overlying the allergic-reaction sites were about twice as much (200 to 350 mg/ml during the 1- to 5-hour period). There was little difference in IgG levels at antigen and control sites (231 versus 212, respectively) during the 5- to 7-hour challenge period. The relevance of these findings is suggested by our observations that the levels of both FPA and IgG at antigen sites occurred in a similar temporal pattern, with peak levels during the 1- to 3-hour measurement period and gradually decreasing thereafter. The increased plasma leakage at these times is likely due to the effects of one or more vasoactive mediators that we have found to be increased in these sites, that is, histamine,⁵ LTC₄,⁶ and PAF.⁷

However, the increased amount of fibrin formation in these allergic reactions does not appear to be due just to increased local presence of plasma components. The levels of FPA did not correlate well with the

amount of IgG (as a marker of plasma leakage) in individual chamber fluids. This finding raises the question of whether there is an increased fibrinogen activation process underway in these ongoing allergic reactions. Previous *in vitro* studies have demonstrated that antigen challenge of sensitized basophils, human lung fragments, or isolated lung mast cells induces the release of proteases that can activate the Hageman factor pathway.¹⁴⁻¹⁷ As noted previously, we have found evidence of increased activation of this pathway in chamber fluids overlying allergic-reaction sites.⁸ In contrast, plasma leakage caused by mast cell-derived vasoactive molecules would provide these proteins, and contact activation can then be initiated by release of mast cell heparin¹⁸ or contact with connective tissue proteoglycans.¹⁹

More recently, we have found several lines of evidence suggesting that basophils may accumulate in these skin-allergic reaction sites; that is, the prolonged histamine release (noted previously and in the current study) is not accompanied by concomitant prolonged local accumulation of tryptase and prostaglandin D₂, components made in mast cells but not basophils.²⁰ Furthermore, in subjects pretreated with corticosteroids that should markedly inhibit entry of basophils into these allergic reaction sites, the prolonged release of histamine, but not the initial 1-hour peak release of histamine and tryptase, was inhibited.²⁰ Therefore, we postulate that activation of both mast cells and basophils may be playing a role in the activation of coagulation pathways, likely through the activation of the Hageman factor pathway previously observed by us.⁸

The findings reported here of increased fibrin formation during the development of allergic skin LPRs may be of pathophysiologic importance. As noted previously, at least one group of investigators has found increased deposition of fibrin in histologic studies of LPR, but not in histamine or control injection sites.⁴ We have also been impressed with the amount of fibrin observed on the blister base during the later phases of our skin chamber challenge. The findings in the present study of a significant correlation between the net FPA levels at antigen sites and the size of the LPR after intradermal antigen injection in individual allergic subjects suggest a relationship between these two events.

Based on these findings, we postulate that prolonged local antigen exposure induces activation of mast cells and then basophils. The resultant release of vasoactive mediators leads to increased extravasation of plasma components, which include components of the intrinsic coagulation pathway. Through both contact activation with tissue substrates and the

TABLE I. Effects of in vitro incubations of antigen- and buffer-site chamber fluids on FPA (nanograms per milliliter) levels

Time (hr)	Antigen-site incubation		Buffer-site incubation	
	None	2 hr	None	2 hr
0-1	330	360	330	260
1-3	680	720	230	280
3-5	670	680	—	—

action of proteases released by activated basophils and mast cells in these sites, activation of Hageman factor can occur leading to fibrin formation that absorbs and retains locally extravasated fluid. We have previously demonstrated increased accumulation and activation of inflammatory cells at these sites of ongoing allergic reactions. These activated cells can release mediators such as LTC₄, LTB₄, and platelet-activating factor⁷ that can themselves induce delayed-appearing inflammatory reactions. Together, these processes lead to the erythematous, indurated, painful swelling we call "LPRs."

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