

IDENTIFICATION OF HISTAMINE RELEASING FACTOR(S) IN THE LATE PHASE OF CUTANEOUS IgE-MEDIATED REACTIONS¹

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We have shown that fluids collected from antigen-challenged skin blisters during the late phase reaction cause the release of substantial amounts of histamine ($\bar{x}$ = 42%, n = 14) from human basophils *in vitro*. Control fluids collected either during the immediate phase or from an unchallenged blister released $\leq 10\%$ histamine from both basophils and lung mast cells. Late phase blister fluids induced low levels of histamine release from human lung cells ($\bar{x}$ = 11%, n = 4) that were slightly but not significantly greater than levels induced by control blister fluids. The characteristics of basophil release were similar to IgE-mediated stimuli in dose dependence, calcium and temperature requirements, and kinetics. The IgE dependence of the late phase blister fluid was demonstrated by desensitization of the basophils to anti-IgE, which obviated the response to anti-IgE and blister fluid but did not affect a non-IgE-mediated stimulus. Removal of the cell surface IgE with lactic acid also abolished the response to both anti-IgE and late phase blister fluid. Incubation of the "stripped" cells with serum containing IgE myeloma restored the response to anti-IgE but failed to affect response to late phase blister fluid. The characteristics of release obtained with this factor closely resemble those of an IgE-dependent histamine releasing factor from cultured macrophages previously described by our group.

When an allergic individual is challenged with an appropriate antigen, immediate signs of an allergic response, apparently due to release of mast cell mediators, appear within 5 to 15 min. The external signs of the response then subside, but in the majority of individuals exposed to high doses of antigen, a late phase response begins 3 to 11 hr after antigen challenge (1). The late phase reaction (LPR)⁴ in the skin is characterized by edema and erythema (2), and in the nose and lungs, by increased resistance to airflow (3). The LPR is particu-

larly interesting because microscopic evaluation of the lesion reveals inflammatory cells similar to those found in prolonged inflammatory reactions (4, 5).

Although the timing of the LPR is suggestive of IgE-mediated inflammation, both Dolovich et al. (1) and Solley et al. (2) demonstrated that antigen-induced LPR were IgE antibody dependent. Because compound 48/80 is also reported to induce a LPR (6), it is likely that the initial event is mast cell degranulation and the release of mediators. However, the relationship between mast cell mediators and the LPR has not been defined, although Tanenbaum et al. (7) have found substances in rat mast cell granules that induce *in vivo* LPR. Some insight into the pathogenesis of the LPR in the skin can be derived from histologic studies, which demonstrate eosinophils, neutrophils, and variable numbers of basophils at the site of the inflammatory lesion (5, 8).

Perhaps the most important information about the pathogenesis of the LPR in humans comes from the demonstration that basophils and/or mast cells are activated *in vivo* during the LPR (9). Thus, elevated levels of histamine and high m.w. neutrophil chemotactic factor (10, 11) (basophil/mast cell mediators) are found in the blood during LPR in the lung 4 to 12 hr after bronchial challenge with antigen. Recently, using an *in vivo* model of intranasal antigen challenge, we have observed that histamine, leukotrienes, kinin levels, and tosyl arginine methyl ester-esterase activity are elevated in nasal washes concomitantly with symptoms in those individuals experiencing a LPR (9, 12, 13).

To analyze mediator release in LPR, it is necessary to determine the cell of origin of the mediators and the activating stimulus for this cell. We have obtained circumstantial evidence that the basophil, not the mast cell (13), is the source of the mediators found in nasal lavages during LPR, because prostaglandin D₂ (PGD₂; an arachidonate metabolite produced by mast cells but not basophils) is not found during the LPR. To address the latter question, we investigated the possibility that a histamine releasing factor was present in skin blister fluids during the LPR. We speculated that the factor was capable of inducing *in vivo* wheal and flare reactions in the skin and also causing *in vitro* histamine release from basophils. Equivocal results were obtained with the *in vivo* experiments due to patient variability in responses. However, the *in vitro* experiments demonstrated potent histamine releasing activity that is present in blister fluids 8 hr, but not 30 min, after antigen challenge.

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⁴ Abbreviations used in this paper: PGD₂, prostaglandin D₂; LPR, late phase reaction; PNU, protein nitrogen units.

MATERIALS AND METHODS

Materials. Goat anti-human IgE-Fc antibody was the generous gift of Dr. Kimishige Ishizaka, Johns Hopkins University School of Medicine. Serum from a patient with IgE myeloma (H.L.) was obtained from Dr. Henry Gewurz, Rush Medical College, Chicago. Ragweed extract was short ragweed pollen from Center Laboratories, Port Washington, NY (lot 8221921) containing 7.4 μ g ragweed antigen E per ml of concentrate (20,000 protein nitrogen units (PNU)/ml). Serum from a hyperimmunized patient with high titer antigen E-specific IgG antibody was obtained from Dr. Anne Kagey-Sobotka, Johns Hopkins University School of Medicine. Other supplies were purchased: piperazine-N,N'-bis-2-ethane sulfonic acid (PIPES) (Sigma Chemical Co., St. Louis, MO), EDTA (Sigma), lactic acid (Sigma), and human serum albumin (Miles Laboratories, Elkhart, IN).

Buffers. PIPES-albumin buffer (PA) consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, and 0.003% human serum albumin, adjusted to pH 7.4. PA containing 1.0 mM CaCl_2 and 1.0 mM MgCl_2 (PACM) was used in the release experiments. PA with 5 mM EDTA (PA-EDTA) was used during desensitization and lactic acid treatment.

Patients and leukocyte donors. Informed consent was obtained from patients and the studies were approved by the Johns Hopkins University Joint Committee on Clinical Investigations. Twelve ragweed-sensitive patients, previously shown to have ragweed-positive skin tests and late phase reactions between 4 and 8 hr later, were utilized for bullae generation. Fourteen leukocyte donors, both atopic and nonatopic, were tested for the capacity of their leukocytes to release histamine in response to bullous fluids. Eight "responders" were identified, of whom six released substantial ($>20\%$) amounts of histamine. These six basophil donors were used in all subsequent experiments.

Lung mast cells. Lung mast cells were obtained by the method described by Schulman et al. (14). Both pure and impure cells were challenged with late phase blister fluids and histamine release was examined.

Bullae formation. The skin bullae were made on the volar surface of the forearm by using a described heat and suction device (15). The bullae were 5 to 7 mm in diameter and contained 100 to 150 μ l of fluid. The bullae were secured with padding and left overnight for the thermo-mechanical injury to resolve. The following day, the bulla base on the right arm was injected with 0.05 ml of ragweed extract at a concentration (usually 20 PNU or 200 PNU/ml) previously shown to induce LPR. The bulla on the left arm was injected with the same volume of the saline diluent. At various times, the size of the wheal under the bullae was measured and the bullous fluid was aspirated through the bullae base. The volume of the recovered fluid was measured, and (except for some experiments described in Results) the fluid was diluted immediately in 1.0 ml cold PA, was centrifuged at 2000 rpm for 5 min to remove debris, and was aliquoted into separate tubes for additional analysis.

Bullous fluid transfer reaction. In 10 experiments, 0.05 ml of undiluted bullous fluid was injected intradermally into the arm of the same individual. The diameter of the wheal and erythema and the erythematous/edematous lesion of the transfer reaction were measured at 15 min.

Leukocyte histamine release. Venous blood was obtained from volunteers who had given informed consent, as described earlier. Mixed leukocytes were isolated by dextran-EDTA sedimentation (16, 17), were washed twice in PA, were resuspended in PACM, and were incubated with or without the stimuli at 37°C in a final vol of 0.1 ml. The reaction was terminated after 45 min by the addition of 0.9 ml of cold PA, the supernatants were recovered after centrifugation, (100 $\times$ G, 2 min, 20°C), and their histamine content was assayed by the automated fluorometric method of Siraganian (18). The total histamine content was estimated by lysis with 1.6% perchloric acid, and the percent histamine release was calculated as a percentage of the total after subtraction of spontaneous release (usually $<5\%$).

Desensitization. In the desensitization experiments, the washed cells were divided into two groups. The first group was incubated at 37°C with 0.1 μ g/ml anti-IgE in PA-EDTA for 2 hr. The second group (control) was exposed to PA-EDTA alone under the same conditions. The cells were then washed four times in PA and were resuspended in the required volume of PACM, and the release reaction carried out.

Use of IgG blocking antibody. Serum containing antigen E-specific IgG was used to show that the histamine release induced by the blister fluids as not due to contaminating ragweed antigens. Briefly, serum containing IgG was preincubated with ragweed extract or blister fluid at 37°C for 30 min, the basophils of a ragweed-sensitive donor were then added, and the incubation was allowed to proceed for 45 min at 37°C. As controls, ragweed extract and blister fluid

were preincubated with an equal amount of normal serum.

Removal of cell surface IgE. Experiments to remove the surface IgE were carried out by using the method described by Pruzansky et al. (19). Briefly, the washed leukocytes were divided into three groups. Two groups (B and C) were treated with lactic acid, pH 3.9, for 10 min at room temperature, and the cells were then washed four times in PA. In an effort to restore IgE to the cell surface, the cells in group C were incubated with IgE myeloma (50 μ g/ml) for 20 min at 37°C in the presence of 4 mM EDTA. A control group (A) was not exposed to lactic acid or IgE myeloma. The cells were then washed four times in PA, were resuspended in PACM, and the release experiments were carried out.

RESULTS

The fluid was collected from blisters of atopic individuals 8 hr after challenge with ragweed antigen, and the undiluted fluid was injected into the skin of the same individual, where it produced an immediate wheal and flare. The size of the wheal induced by fluid from ragweed challenged blisters was the same as that induced by control fluids at 30 min; at 8 hr the response induced by the ragweed challenged blister fluids appeared to be larger than that found with the control fluid. Due to patient variability, however, this difference was not statistically significant. It became obvious that the *in vivo* approach would yield only semiquantitative data, and therefore we set out to determine whether blister fluids would induce histamine release *in vitro*.

Nine sets of control and ragweed challenged blister fluids were obtained. All of the fluids collected from the control blisters at both 30 min and at 8 hr caused less than 10% histamine release. Fluids collected from the ragweed-challenged blisters at 30 min also produced only negligible histamine release. However, six of the nine fluids collected at 8 hr from the ragweed-challenged blisters caused substantial ($\geq 20\%$) histamine release. One fluid produced histamine release that was greater than control ($>10\%$), and the remaining two fluids were inactive. Each fluid was tested on the basophils of at least three different basophil donors.

Subsequent experiments were carried out with the six donors who released more than 20% of their basophil histamine in response to blister fluids, and with the six fluids that induced this level of release. Figure 1 records the basophil histamine release observed with blister fluids obtained at 30 min and 8 hr from the control and ragweed-challenged blister fluids. As noted, less than 10% histamine release was observed with the 30 min ragweed-challenged or control fluids. The blister fluids obtained 8 hr after ragweed challenge caused 42% his-

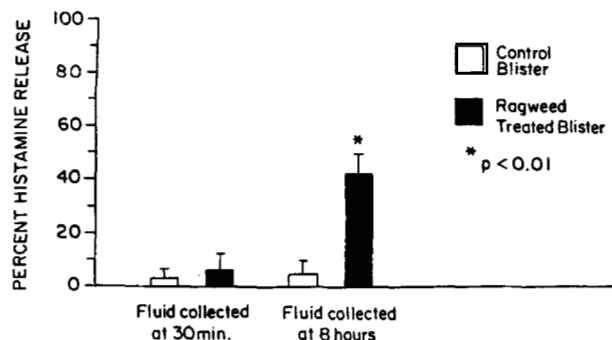


Figure 1. Basophil histamine release: cells were challenged with fluids collected from antigen-treated blisters and control blisters at both 30 min and 8 hr. The results from six (of nine) sets of blisters are shown (see text). *, $p < 0.01$.

tamine release, whereas release induced in the control 8 hr fluid was minimal.

The six "active" late phase blister fluids were tested on four different lung mast cell preparations. Histamine release ranged from 8 to 17% ($\bar{x} = 11.3 \pm 3.9$) (data not shown) and was slightly but not significantly higher than that induced by the control fluids.

The amount of histamine released in these experiments varied considerably; however, we were able to show that in the more potent fluids there was dose-dependent release of histamine (Fig. 2A). The release was in excess of 70% when the fluid was utilized at the concentrations normally employed, and fell to 20% when the fluid was diluted 10-fold. The histamine release varied considerably from preparation to preparation. Figure 2B shows the kinetics of blister fluid-induced histamine release. These kinetics, with half-maximal release at approximately 10 min, are similar to those found with IgE-dependent stimuli and are quite different from the rates of release seen with the peptide-releasing agents, such as f-met peptide or C5a, which are much faster (20, 21).

Both the temperature dependence and calcium dependence of the release caused by late phase blister fluid were similar to those found with an IgE-mediated stimulus. In a series of four experiments, blister fluid-induced release in a buffer containing 1 mM calcium was $72.5 \pm 1.6\%$, whereas in the absence of calcium or in the presence of EDTA (4 mM), histamine release was $6.8 \pm 1.3\%$ and $6.6 \pm 0.8\%$ respectively, and did not differ from the spontaneous release. These results are identical to anti-IgE-induced release and quite different from release caused by agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA) or hyperosmolarity, which are not absolutely dependent on extracellular calcium (22, 23). In a series of four experiments, blister fluid-induced release at 37°C was $22 \pm 9\%$, whereas the release at 22°C and 0°C did not differ significantly from the control. IgE-mediated release exhibits a similar temperature dependence that is not always found when other stimuli are used.

The experiments shown in Figure 3 indicate that low levels of antigen-induced release and late phase blister fluid-induced release are synergistic; that is, instead of the expected summation of the release caused by fluid and antigen alone, twice that amount was obtained when both were added to the cells to stimulate histamine release ($p < 0.01$). The release induced by blister fluid

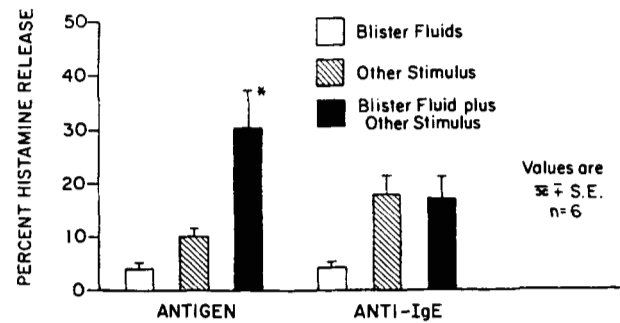


Figure 3. Effect of suboptimal levels of anti-IgE ($0.01 \mu\text{g/ml}$) and allergen on release due to late phase blister fluid. All stimuli were added simultaneously to leukocytes, and the mixtures were incubated for 45 min. Antigen and blister fluid cause significantly ($p < 0.01$) greater release than expected, assuming additivity.

was not, however, synergistic with that induced by anti-IgE; the response noted was either additive or less than additive, and was not statistically different from that anticipated assuming additivity.

Having determined the optimum conditions for basophil histamine release, we turned to the mechanism by which release occurred. We felt it was unlikely that the ragweed extract was carried over into the late phase blister fluids, but to eliminate that possibility we used human serum containing high titer IgG antibodies against ragweed antigen E. The ragweed extract (0.36 PNU/ml) and an appropriate dilution of a late phase blister fluid (which, even if all the injected ragweed were in the fluid, contained 0.36 PNU/ml) were treated with the antibodies and then used to challenge leukocytes from the ragweed-sensitive donors used in this study. In each case, pretreatment with the antibodies abolished the histamine release caused by the ragweed extract, but failed to affect the release caused by the blister fluid (data not shown). These data, together with the fact that only one-third of the basophil donors showed any sensitivity to ragweed, led us to the conclusion that ragweed contamination of the late phase blister fluids did not cause blister fluid-induced histamine release.

It is well known that when basophils are exposed to anti-IgE in the absence of exogenous calcium they lose their ability to respond to a subsequent IgE-mediated challenge in the presence of calcium. This desensitization is "specific", affecting only IgE-mediated stimuli; secretagogues that bypass the crosslinking of IgE are unaffected (24, 25). Because several characteristics of the release caused by the late phase blister fluids indicated it may be IgE-mediated, we studied the effect of anti-IgE desensitization on blister fluids to ascertain whether these stimuli cross-desensitize with anti-IgE-induced histamine release. Figure 4 shows the results of these experiments. Histamine release to anti-IgE was 36% in control cells and fell to less than 5% in the desensitized cells. Blister fluids induced histamine release of 30% in control basophils; after anti-IgE desensitization the release fell to only 3%. In the same experiment, the release caused by an IgE-independent stimulus such as f-met peptide was completely unaffected by desensitization with anti-IgE.

In a final series of experiments, we determined whether release induced by blister fluid was dependent on cell surface IgE. To remove the IgE, we treated the cells with lactic acid (15); this dissociates IgE from its receptors and

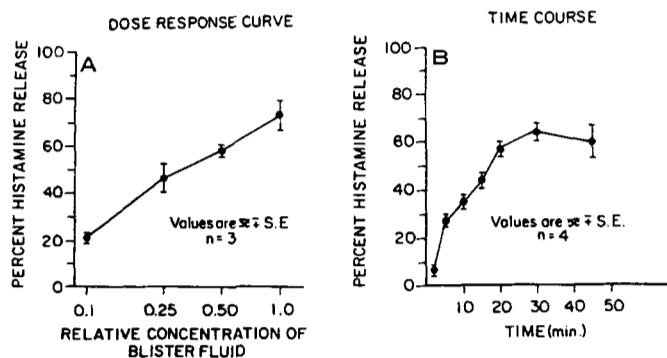


Figure 2. A. Dose dependence: basophils challenged with late phase blister fluid show a dose-dependent release of histamine. The results represent one donor tested with two different fluids, and a second donor tested with one of these fluids. B. Kinetics: the kinetics of release are slow, with half-maximal release at 10 to 15 min. The results represent the mean of two fluids tested on two different donors.

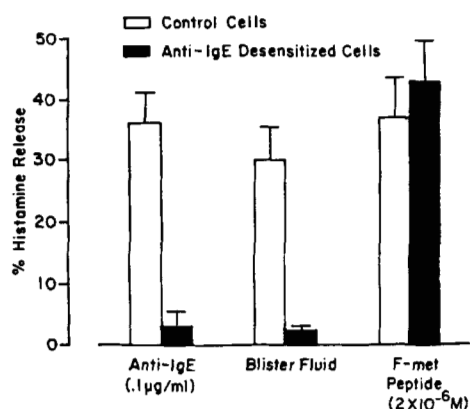


Figure 4. Desensitization: cells desensitized to anti-IgE fail to respond to either anti-IgE or late phase blister fluid, whereas f-met-peptide-induced release is unaffected. Control cells release to all stimuli.

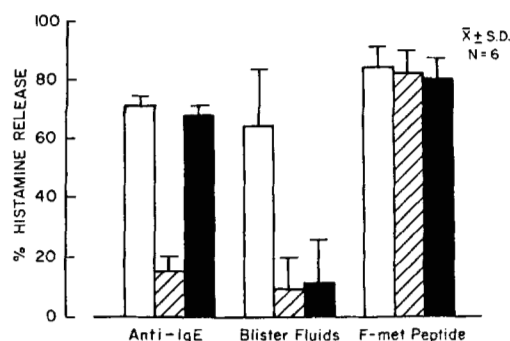


Figure 5. Lactic acid treatment: control cells (white bars), lactic acid-treated cells (hatched bars), and basophils resensitized with IgE myeloma (black bars) were challenged with anti-IgE (0.1 µg/ml), late phase blister fluid, and f-met-peptide (10⁻⁶ M).

thus markedly reduces the cell surface IgE concentration. We also restored IgE to the cell surface of the lactic acid-treated basophils by incubating these cells with IgE myeloma (Fig. 5). With anti-IgE as the stimulus, histamine release fell from 70% to about 15% in the lactic acid-treated cells; release was completely restored when these lactic acid-treated cells were treated with IgE myeloma. With f-met-peptide as the releasing agent, neither lactic acid treatment nor a subsequent incubation with IgE myeloma affected histamine release. Blister fluids induced 64% histamine release from untreated basophils, but only 10% release from lactic acid-treated basophils. However, in this instance, IgE myeloma incubation did not restore the ability of the cells to respond to the blister fluid.

DISCUSSION

These experiments demonstrate that during the late phase reaction in the skin, factor(s) appear in blister fluids that induce basophil histamine release but have minimal effect on human lung mast cells. There may be low levels of releasing activity (i.e., inducing ≤10% histamine release) for both basophils and lung mast cells in the control blisters. We have focused on the late phase blisters because these blisters contain considerably greater levels of releasing activity than the control blisters (Fig. 1).

The kinetics of this release and its dependence on both calcium and temperature parallels that caused by IgE-mediated stimuli. The experiments shown in Figure 3

also suggest an IgE-dependent mechanism, i.e., synergy occurs between antigen-induced basophil histamine release and that caused by the blister fluids, whereas this is not the case with anti-IgE. These results are difficult to explain. Although we have shown that anti-IgE and antigen represent very different signals for release (26), we are unable to explain the synergy between antigen and late phase blister fluid.

The supposition that the late phase factor(s) caused release by crosslinking IgE is supported by two types of experiments. It has been shown repeatedly that desensitization with anti-IgE blocks the mediator release caused by other IgE-dependent stimuli (21, 22). Release caused by a wide variety of other signals such as anaphylatoxins, f-met peptides, hyperosmolarity, calcium ionophore, TPA, etc. occurs perfectly well from anti-IgE-desensitized cells (22, 23). Thus, in the context of considerable previous experimentation, the desensitization results strongly suggest that the blister fluids are interacting with cell surface IgE.

The interpretation of the results obtained after removing the IgE from the basophil cell surface with lactic acid is more complex. The fact that the acid treatment obviates release by blister factor(s) and anti-IgE, while not affecting release by f-met peptides, suggests that IgE must be present on the surface of the cells for the late phase factor to induce release, and that blister fluid factor(s) binds to cell surface IgE. On the other hand, the response to anti-IgE is restored by subsequently treating the cells with IgE myeloma, but the response to blister fluid is not similarly reconstituted. Because lactic acid treatment of basophils results in cell-bound IgE levels of 2 to 20% of controls, and IgE myeloma incubation of lactic acid-treated basophils results in cell-bound IgE levels of 20 to 100% of controls (27), the experimental maneuvers do in fact remove and restore cell surface IgE molecules. Thus, the results suggest that the IgE binding factor(s) contained in the blister fluid are much weaker stimuli than anti-IgE, and that the partial restoration of cell surface IgE by IgE myeloma is insufficient for restoration of the response. It is more likely, however, that this result is due to as yet unappreciated characteristics of the releasing factor(s).

Our approach in these experiments was dictated to some extent by other observations recently made in this laboratory. We have described factors derived from cultured human alveolar macrophages that cause basophil, and to a lesser extent, mast cell histamine release (28); the release reaction is calcium and temperature dependent and with slow kinetics (27, 28), and the mechanism of release appears to be due to the crosslinking of cell-bound IgE (27). Thus, cells desensitized to anti-IgE fail to respond to macrophage factors. Experiments aimed at absorbing the factors with IgE-coated gels or inhibiting the ability to cause release with fluid phase IgE myeloma generally reinforce this concept, but the data are not nearly as unequivocal as those obtained if anti-IgE is the releasing stimulus. This suggested to us that macrophage factors had considerably lower avidity for IgE than anti-IgE. The macrophage-derived factors and late phase blister fluids are similar in that release by both stimuli is regularly obviated by stripping basophils of their IgE. However, the activity of macrophage-derived factors is partially restored when the basophils are incubated with

IgE myeloma, which is not the case with late phase blister fluids. These results are compatible with a signal that is weaker than anti-IgE. The last observation also suggests that blister fluid factors have a lower avidity for IgE myeloma than most macrophage-derived factors. Finally, the late phase blister fluids do not release histamine from the cells of all individuals, and macrophage-derived releasing factors have the same characteristics.

The next logical step in this experimentation would be to characterize the releasing factor(s) obtained from the late-phase blister fluids. However, as noted, this material is active only at relatively high concentrations, and less than 0.1 ml is obtained during an 8-hr experiment. We have therefore decided to first characterize the macrophage-derived factors, to prepare antibodies to them, and then to ascertain whether they are similar or identical to the factors in blister fluids.

In sum, we have described factors that appear in the late but not the early phase of IgE-mediated reactions and which cause mediator release from human basophils. It is highly unlikely that these factors induce release by crosslinking cell surface IgE molecules on the basophil, but this has not been unequivocally demonstrated. We believe that these factors are similar to those obtained from cultured human macrophages, but we are unable at this time to do gel chromatography or similar characterization of blister fluid factors. If we can strengthen the evidence for similarities between blister fluid- and macrophage-derived factors, we will have the basis for a rather unique insight into the pathogenesis of basophil/mast cell mediator release during the late phase of IgE-mediated reactions in man.

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