

Release of eosinophil granule proteins during IgE-mediated allergic skin reactions

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To determine whether the eosinophil (EOS), a prominent component of human allergic skin reactions, releases its potentially pathogenic components in vivo, we appended collection chambers to the bases of unroofed skin blisters and challenged the sites for varying time periods with either pollen antigen (Ag) or buffer (B)-control solutions. In seven sensitive subjects, continuous challenge with pollen Ag consistently induced release of more major basic protein (MBP) and eosinophil-derived neutrophil (EDN) than did B solution. Low levels of both MBP and EDN were observed during the first hour with increased accumulation during the second to fifth hour. Comparison of Ag- versus B-challenged site responses in individual subjects demonstrated significantly higher levels of both MBP and EDN at Ag than at B sites during the second to fifth hour. Levels of both MBP and EDN in the second to fifth hour correlated significantly with histamine release in the same sites in the first hour ($r = 0.66$ and 0.83 , respectively). Imprints of the skin bases of the chambers after 5 hours demonstrated variable numbers of EOS at the Ag-challenged sites and only occasional EOS at the B-challenged sites; most cells on the skin bases were neutrophils. However, immunofluorescence localization of MBP in biopsy specimens of the blister bases revealed striking extra cellular MBP deposition. These findings indicate that EOS components accumulate in vivo in IgE-mediated human skin reactions, even when prominent EOS accumulation is not visualized, possibly because the EOS are degranulated in the allergic-reaction site. Release of EOS components in these reactions may be linked to earlier mast cell activation. Because cationic EOS granule proteins are toxic for tissues, they could play a pathogenic role in late-phase allergic reactions. (J ALLERGY CLIN IMMUNOL 1991;87:984-92.)

Prominent accumulation of EOS occurs frequently in the blood and involved tissues of symptomatic atopic humans.^{1,2} Although some original studies suggested that certain EOS components could down modulate allergic reactions by inactivating allergic mediators,³ the bulk of recent evidence indirectly and directly suggest a pathogenic role of EOS in human allergic reactions.^{1,4,5} Using skin biopsy and skin-window techniques, we and other investigators have found increased accumulation of EOS in the dermis several hours after the initial wheal-and-flare, IgE-

Abbreviations used

Ag:	Antigen
B:	Buffer
EOS:	Eosinophil(s)
EDN:	Eosinophil-derived neutrophil
LPR:	Late-phase reaction
MBP:	Major basic protein
Ab:	Antibody

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mediated reaction.⁶ There is evidence suggesting that EOS components are released in human allergic skin reactions. For example, extracellular deposition of EOS MBP has been found by immunohistochemical techniques in biopsy specimens of the extracellular portion of the dermis at the time skin LPRs appear at the site of injection of relevant Ags in sensitive subjects.⁷ Extracellular MBP is also increased in biopsy specimens of active atopic dermatitis lesions.⁸ However, it is not yet known when and how much of the EOS components are released after initiation of an IgE-mediated reaction.

These questions could be answered with a skin-chamber approach used extensively by us to investigate other humoral and cellular events in human allergic skin reactions.^{9,10} Using this approach, we have found that continuous pollen Ag challenge of denuded blister bases of sensitive humans lead to an initial peak and then a constant low-level plateau of histamine release for at least 9 hours.¹¹ Starting about 1 to 2 hours after initiation of Ag challenge, there was a progressive accumulation of granulocytes during the next several hours.¹² This was accompanied by increased levels in the overlying chamber fluids of the neutrophil components, leukotriene B₄, lactoferrin, and elastase.^{10,13} Levels of platelet-activating factor were increased somewhat later.¹⁰ Leukotriene C₄ levels were also increased in these chamber fluids starting several hours after Ag challenge; however, it is not known whether this leukotriene C₄ was released from EOS or other cells accumulating in the site or from continuously activated mast cells.⁹ These events begin before the peak of a gross LPR observed in the Ag-challenged sites (generally 6 to 8 hours). Therefore, it is possible that granulocyte activation with release of potentially toxic components could be pathogenic in such LPRs.

To determine whether EOS granule proteins are released in ongoing allergic skin reactions in humans, we have used our skin-chamber model to compare the temporal patterns of the accumulation of the EOS granule proteins MBP and EDN with that of histamine in Ag-challenged and buffer control-challenged sites in sensitive subjects. We compared such accumulation with the degree of EOS exudation and MBP deposition in the base of these skin reactions.

MATERIAL AND METHODS

Subjects

Seven volunteer atopic subjects (aged 21 to 32 years), each skin test reactive to ≤ 10 PNU/ml of ragweed and/or grass-pollen extract (Greer Laboratories, Lenoir, N.C.) were studied after informed consent was obtained. All subjects had a history of typical seasonal allergic rhinitis and were studied during an asymptomatic period of the year. No subject was receiving any medication for at least 2 weeks before this study. None had ever received allergy injection therapy.

Skin chambers and Ag challenge

Skin-chamber studies were performed as previously described by us.^{9,10} In brief, two blisters were induced on each forearm by heat/suction and unroofed aseptically, leaving a blister base at the dermal-epidermal junction. Collection chambers were then taped into each place over each denuded blister base. To wash away any mediators or tissue components released by induction of the blister itself, the chambers were filled with sterile isotonic phosphate-buffered saline (B) that was removed after 5 minutes and discarded.

Two of the chambers were filled with grass- or ragweed-pollen extract (100 PNU/ml in B containing 10 U/ml of heparin). The other two chambers were filled with the same B diluent containing heparin but no Ag. After 1 hour of incubation, the chamber fluids were collected, the blister bases were washed thoroughly, and the collection chambers were filled with fresh solutions of pollen extract or B, respectively. These sites were incubated for an additional 4 hours, and the fluids were collected. For each timed collection period, the fluids from the two Ag-challenged sites were pooled, as were the two B-challenged site fluids. An aliquot of the fluids was removed for differential leukocyte counts. The remainder of the fluids was immediately centrifuged (1000 rpm for 10 minutes at 37° C), and the supernatant was stored at -70° C for future analysis.

Blister-base imprints

After removal of the overlying collection chambers from each site at the fifth hour, cover glasses of 10 mm diameter were appended firmly to each site for 10 minutes. Each cover glass was air-dried, fixed, stained with Wright-Giemsa stain (Dif-Quik, American Scientific Products, McGraw Park, Ill.), and affixed to a coded slide for differential leukocyte counts. In some cases, one of the cover glasses from duplicate challenged sites was stained by Hansel's stain to confirm the presence of EOS and their granules.

Mediator analyses

Histamine levels in the skin-chamber fluids, obtained as described above, were analyzed by a radioenzymatic method that was used extensively by us in the past.⁹⁻¹¹ With this method, levels as low as 2 ng/ml can be assayed reliably.

Levels of EOS MBP and EDN in the skin-chamber fluids were assessed by radioimmunoassays, as previously reported.^{14,15} There was no evidence of interference in these assays by the Ag or B diluent placed initially in the skin chambers.

MBP deposition in the underlying dermis

The anti-MBP Ab used in these immunoassays was also used for a semiquantitative assessment of the deposition of MBP in 3 mm punch biopsy specimens obtained at the center of the blister base in two of the subjects after the 5 hours of local challenge with either the Ag or B solutions. Coded frozen sections of these biopsy specimens were fixed in buffered formalin and then processed for detection of MBP by immunofluorescence techniques, as previously described.¹⁶

Statistical analysis

In preliminary analysis (Kolmogorov-Smirnov test), it was found that the findings were normally distributed and therefore were analyzed by the Students' *t* test for paired observations. Correlations were assessed by linear-regression analysis. All statistical analyses were performed on a Macintosh computer (Apple Computer, Inc., Cupertino, Calif.) with a Statworks statistical software program (Cricket Software, Malvern, Pa.). All findings are expressed as the mean $\pm$ SEM unless it is otherwise stated.

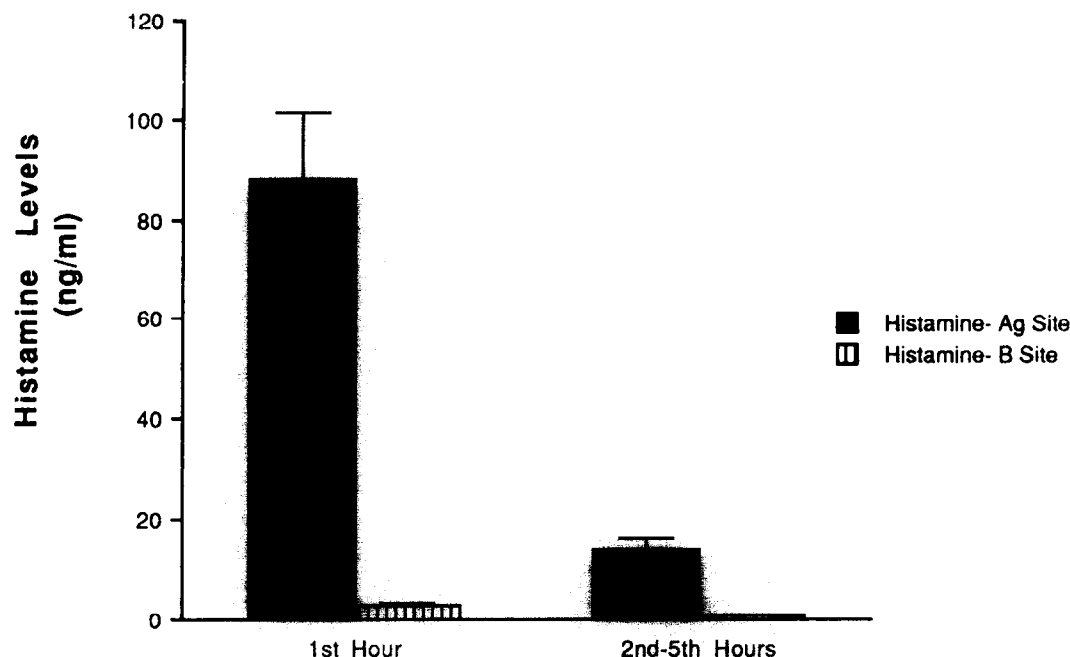


FIG. 1. Histamine levels (nanograms per milliliter) in chamber fluids obtained during the first hour and the second to fifth hour of challenge with Ag or B-control solution.

RESULTS

Histamine release (Fig. 1)

Histamine levels were markedly elevated in chamber fluids during the first hour at Ag-challenged sites when levels were compared to levels in fluids at the same time at the B-challenged sites (mean $\pm$ SEM, 88 ± 17 versus 3 ± 1 ng/ml; $p < 0.001$). Histamine levels in chamber fluids incubated during the second to fifth hour at Ag-challenged sites (18 ± 4 ng/ml) were significantly lower than in the first hour Ag-site fluids but were still significantly higher than in the second- to fifth-hour chamber fluids at the B sites (4 ± 2 ; $p < 0.01$). These findings were similar to findings reported previously by us in studies of other subjects.¹¹

MBP and EDN release

As illustrated in Fig. 2, MBP levels in the first-hour chamber fluids were higher at Ag-challenged sites than at B-challenged sites (85 ± 13 versus 40 ± 3 ng/ml; $p < 0.05$). MBP levels were higher in the second- to fifth-hour fluids than in the first-hour fluids at both Ag and B sites. However, MBP levels in these second- to fifth-hour fluids were significantly higher at the Ag-challenged than at the B-challenged sites (130 ± 13 versus 75 ± 10 ; $p < 0.01$).

As illustrated in Fig. 3, EDN levels were not significantly different in the first-hour chamber fluids

obtained at Ag- and B-challenged sites (19 ± 4 versus 13 ± 2 ng/ml; $p > 0.1$). During the second to fifth hour of incubation with Ag, the chamber fluid EDN levels (144 ± 21 ng/ml) were significantly higher than levels observed at both the second- to fifth-hour B-site fluids (72 ± 25 ; $p < 0.01$) and the first-hour Ag-site fluids (19 ± 4 ; $p < 0.001$).

To investigate whether the increased release of MBP and EDN at Ag-challenged sites during the second to fifth hour was related to earlier mast cell activation in such sites, we compared, in individual subjects, the histamine levels in the first-hour fluids with levels of both MBP and EDN in the second- to fifth-hour fluids at the same sites. In regression analysis, there was a moderate, statistically significant correlation between histamine and subsequent MBP levels ($r = 0.66$; $p < 0.05$) and a stronger correlation between histamine and subsequent EDN levels ($r = 0.83$; $p < 0.01$); Fig. 4.

Cytologic studies

More than 95% of cells in the chamber fluids were neutrophils with a small percentage of mononuclear cells. Only very occasionally were EOS present. Based on an average total leukocyte count of 1.2×10^6 in the Ag-site chamber fluids, we estimated that $<10,000$ EOS had exuded into such fluids at individual Ag-challenged sites.

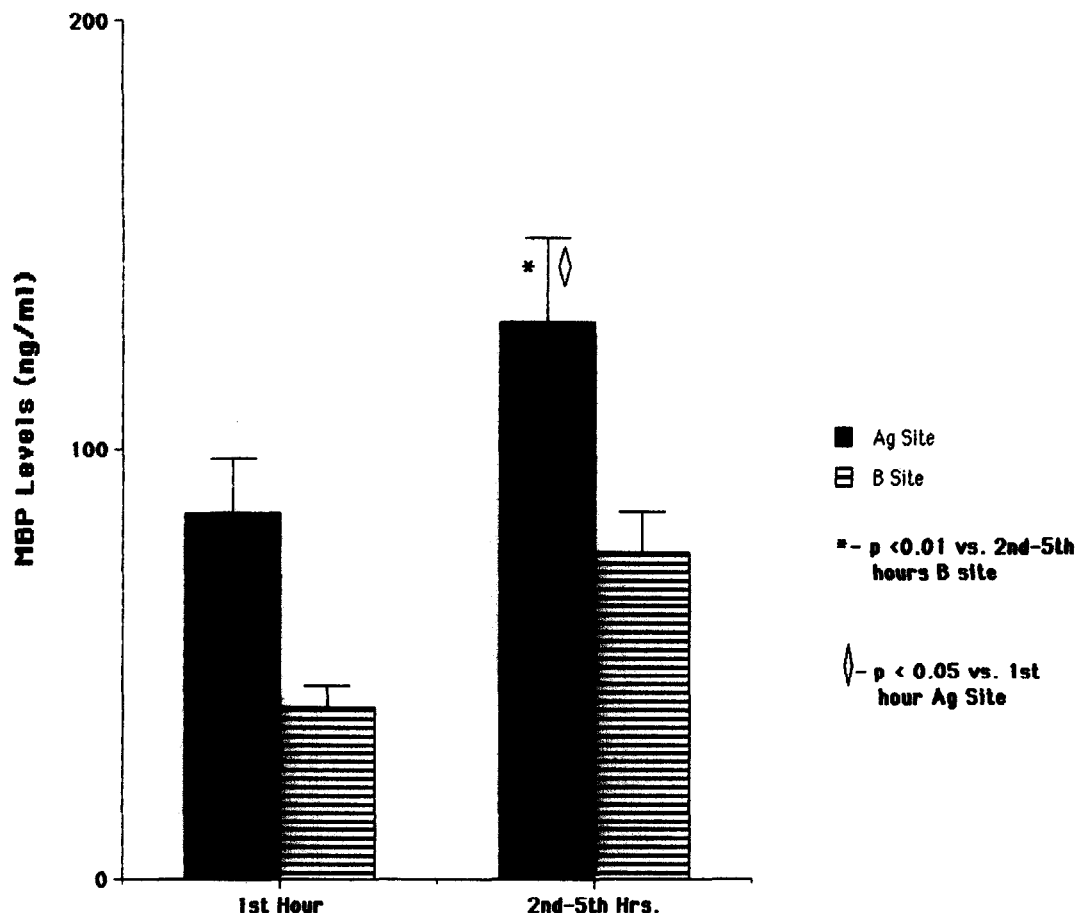


FIG. 2. MBP levels (nanograms per milliliter) in chamber fluid obtained during the first and the second to fifth hour of challenge with Ag or B solution.

Most cells adhering to cover glasses appended to the blister bases previously exposed to Ag or B solutions for the 5-hour challenge period were neutrophils ($93\% \pm 4\%$). EOS comprised a variable, small percentage of the inflammatory cell exudate at Ag-challenged sites (mean $\pm$ SEM, $6.4\% \pm 1.1\%$). Yet, this finding was significantly greater than that observed on the cover glasses appended to the sites previously exposed to B for 5 hours ($1.3\% \pm 0.4\%$; $p < 0.001$).

To determine whether there was any quantitative relationship between the levels of MBP or EDN at Ag-challenged sites and the EOS exudation present at the end of the challenge period, we compared at individual sites the levels of each of these EOS components after the second- to fifth-hour of incubation with the percentage of EOS per high-power field on cover glasses appended to the site right after that incubation. There was a moderate, statistically significant correlation between the EDN levels and percentage of EOS at these sites ($r = 0.70$; $p < 0.05$).

There was no correlation between the percentage of EOS and MBP levels ($r = 0.31$; p value, not significant).

MBP deposition in the underlying dermis

Replicate sections were obtained from biopsy specimens of the dermis underlying the skin-chamber sites after 5 hours in two subjects. In sections from the Ag-challenged sites incubated with rabbit anti-MBP serum and fluorescein isothiocyanate-conjugated goat antirabbit IgG, there was intense extracellular fluorescence in much of the dermis (Fig. 5, A). Most of this fluorescence was in an extracellular distribution, some corresponding to the scattered EOS granules we sometimes observed in appropriately stained sections of these sites. A minority of the MBP appeared to be localized within EOS also present within these sites. In sections of the dermis at the B-challenged sites, similarly incubated, there were only scattered areas of modest fluorescence (Fig. 5, B); only rarely were EOS present in such sites. There was no fluorescence

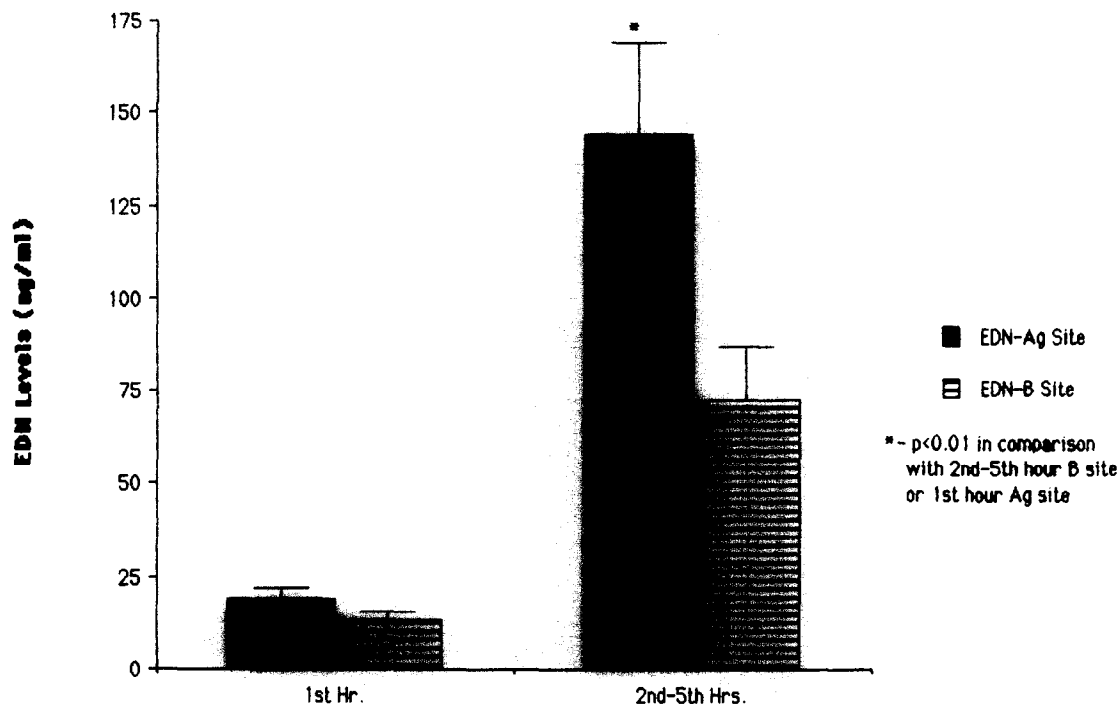


FIG. 3. EDN levels in chamber fluid obtained during the first and the second to fifth hour of challenge with Ag (solid bars) or B (striped bars) solution.

observed in replicate sections of the dermis at either Ag- or B-challenged sites that had been incubated with normal rabbit serum (instead of anti-MBP antiserum) and then fluorescein isothiocyanate-conjugated anti-rabbit IgG.

DISCUSSION

The skin-chamber findings reported in this study point to the *in vivo* accumulation of the EOS granule proteins MBP and EDN in skin sites of sensitive subjects. We conclude that the levels of these proteins in the overlying skin-chamber fluids reflect predominantly their *in vivo* release in the underlying dermis during the challenge periods for at least two reasons: (1) The fluids were gently centrifuged to obtain a cell-free supernatant for analysis. (2) There was a very small number ($<1\%$) of EOS in these chamber fluids. Therefore, any MBP or EDN secreted by such cells during the skin-chamber incubations on the blister bases could account for only a minority of these proteins found in the fluids. Both MBP and EDN were present in low levels in fluids obtained at the B (control)-challenged sites during the first hour, increasing in fluids obtained after an additional 4-hour incubation of the same challenge sites with fresh B solution. The MBP accumulation may be due, in part,

to MBP present in plasma exuding into these sites during the 4-hour incubation period.

However, there was definitely more accumulation of both MBP and EDN at the Ag-challenged than at the B-challenged sites. These differences were modestly significant during the first hour for MBP ($p < 0.05$) but not for EDN. During the second- to fifth-hour collection, levels of both MBP and EDN were both impressively higher at the Ag-challenged sites. The differences in EDN levels cannot be explained by a greater exudation of plasma at the Ag-challenged sites than at the B-challenged sites because the plasma EDN levels in asymptomatic allergic patients are too low (approximately 15 ng/ml^{15}) to account for the higher EDN levels found in the Ag-challenged site chamber fluids. The source of the EDN found in the second- to fifth-hour B-site chamber fluids is less clear. As noted by us previously,¹² there is a variable modest inflammatory cell accumulation at buffer sites likely caused by the minor trauma involved in the induction of the blister itself. Although only 1.3% of the inflammatory cells on the B-site blister bases were EOS, it is conceivable that EDN secreted by these cells and scattered EOS in the underlying dermis were detected in the overlying fluid. MBP levels in the chamber fluid could have been affected

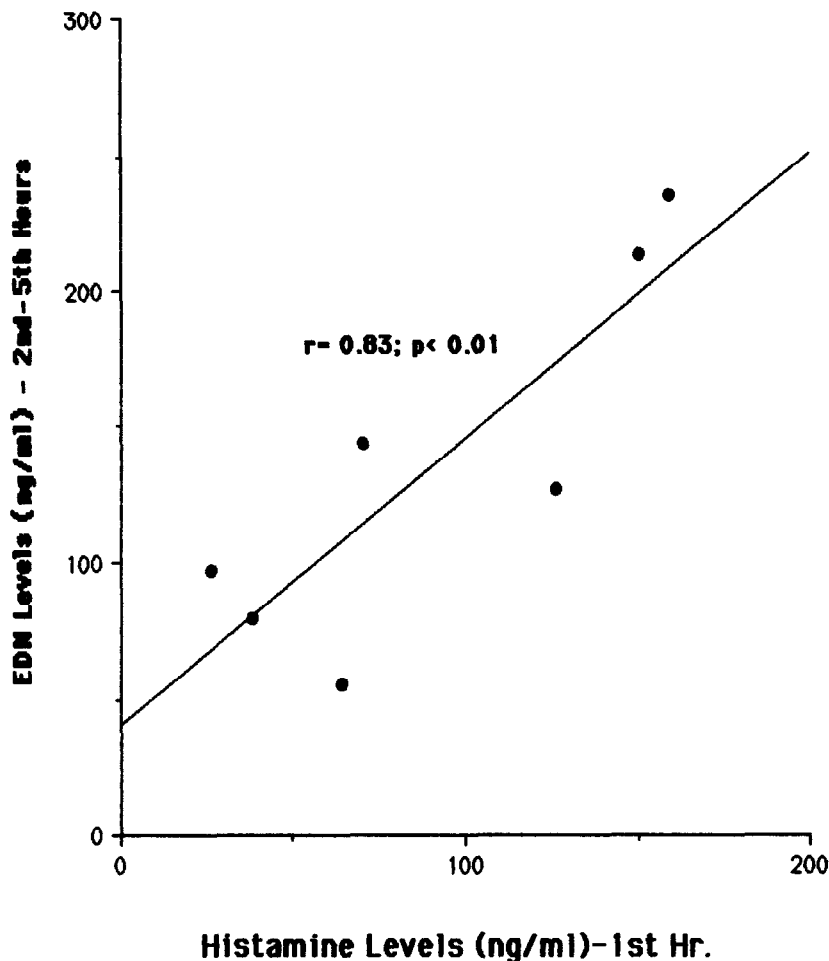


FIG. 4. Correlation between chamber-fluid histamine level in the first hour of Ag challenge and the EDN level during the second to fifth hour of Ag challenge at individual sites.

by a greater plasma exudation at Ag-challenge sites.

However, a greater local release of MBP in the Ag-challenged sites is suggested by our immunofluorescence studies of biopsy specimens obtained after the fifth hour from the dermis underlying the skin chambers. In sections of these biopsy specimens, carefully washed to remove adherent fluids, there was a prominent, diffuse deposition of MBP in the dermis of the Ag-challenged sites, mostly in an extracellular distribution. In comparison, the biopsy specimen sections from the B sites contained only a small, scattered amount of MBP. In previous quality-control studies, we found that the amount of MBP that could be present in the plasma exuding into such allergic reaction sites (about 350 ng/ml) would not be detectable by these immunofluorescence techniques. Therefore, the prominent MBP deposition in the dermis of Ag-challenged sites is most likely locally released. It is conceivable that the MBP in the B-site skin-chamber

fluids without impressive MBP deposition in the underlying dermis might reflect MBP in plasma that exudes into the chamber fluids but not present in sufficient amounts to be detected in the dermis.

These findings provide additional evidence that granulocytes not only accumulate in the site of ongoing allergic skin reactions but also release potentially pathogenic components as well. We have also found evidence that the neutrophils in the chamber fluid are activated and release some of their components.^{13, 27} There are increased levels at Ag-challenged sites of the neutrophil components, lactoferrin and elastase, during the same second- to fifth-hour period in which we report increased MBP and EDN levels in the current study.¹³ The release of these components is not just a nonselective loss of intracellular contents from dead or dying granulocytes. (1) These granulocytes in the chamber fluids are >98% viable as assessed by trypan blue staining.²⁷ (2) There is no

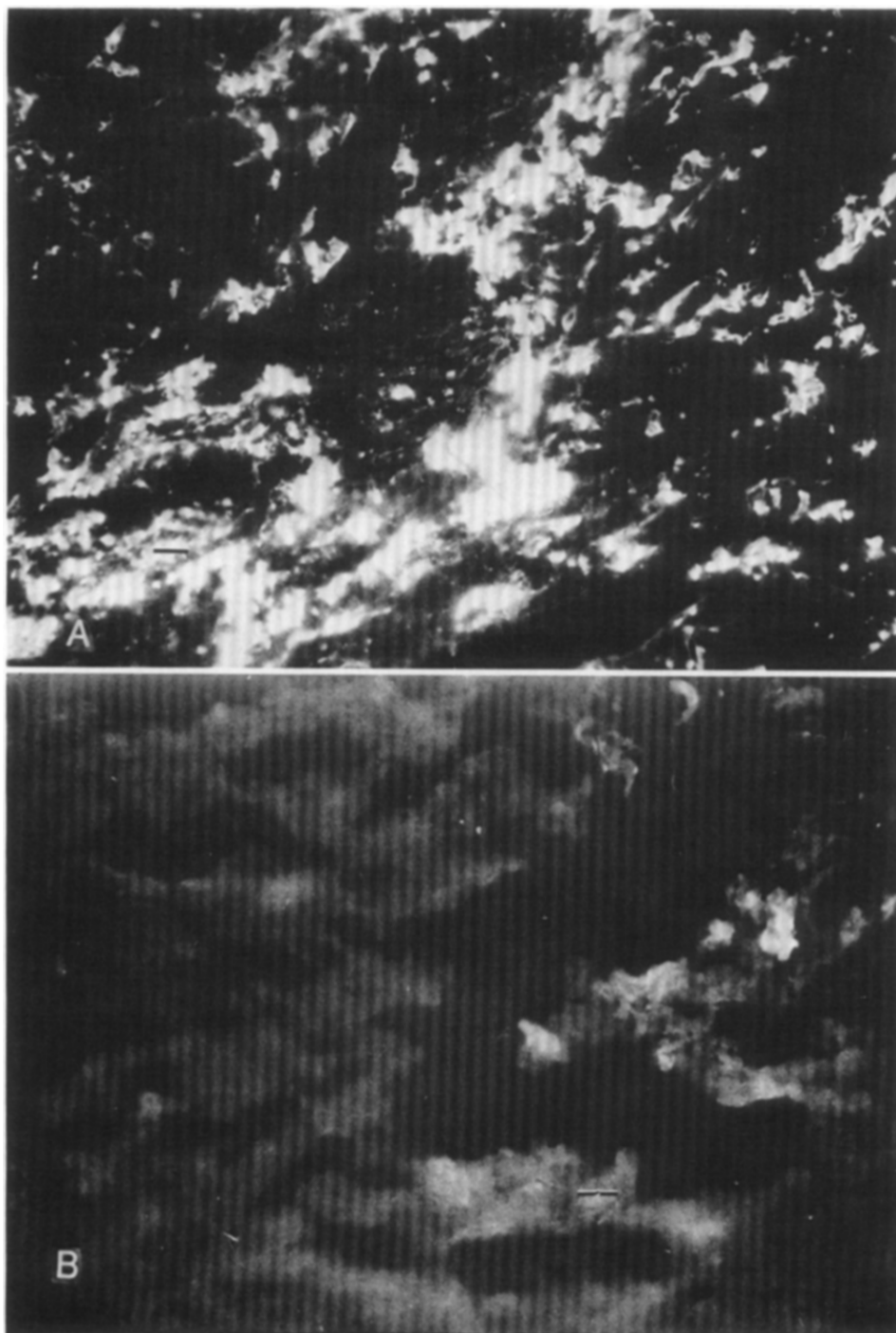


FIG. 5. Immunofluorescence study for MBP in the dermis underlying skin chambers after 5 hours of challenge. (Original magnification $\times 600$.) **A**, There is marked deposition of MBP throughout the dermis underlying Ag-challenged sites. **B**, In contrast, only scattered MBP deposition is apparent in the dermis at B-control challenge sites. Bar marker indicates a 10 μm diameter.

increased release at Ag-challenged sites of myeloperoxidase or lactic dehydrogenase, components of azurophilic granules and cytosol, respectively.¹³

There appears to be a relation between the release of MBP and EDN reported here and earlier mast cell

activation at the same allergic-reaction sites. The levels of histamine released in the first hour correlated very strongly with EDN levels in the second- to fifth-hour fluids at individual Ag-challenged sites. Our previous findings indicate that the histamine released dur-

ing the first hour comes from mast cells.¹¹ However, there are likely other factors playing a role in MBP and EDN accumulation here, since levels of both these proteins increased at the B-challenged sites (albeit to a lesser degree) during the second to fifth hour of incubation, even though little histamine was released at such sites during the first hour. Additional investigation will be required to determine whether there is a causal relationship between the early mast cell activation and subsequent EOS accumulation and degranulation.

The release of components like MBP and EDN into surrounding tissues could play a pathogenic role in human allergic reactions. Immunohistochemical studies have demonstrated increased deposition of extracellular MBP as well as MBP present in accumulated EOS in the lamina propria of the bronchial wall of many patients with severe asthma, but not other pulmonary conditions.¹ Some of these EOS were intact, whereas other EOS were in various stages of degranulation, often surrounded by their extruded granules. The usual staining of these tissues revealed thickened basement membrane zones, goblet cell hyperplasia, and pronounced MBP deposition in large patches of amorphous eosinophilic infiltrates in the lamina propria region.¹ Disruption of the epithelial layer was also commonly found in such tissues. This is of particular interest because of the finding that guinea pig respiratory epithelial cells are damaged after *in vitro* incubation with MBP or EOS cationic protein.^{4,5} EOS components are also shed into the bronchial lumen in asthma. Sputum MBP levels are increased in many patients with symptomatic asthma.^{17,18} MBP has also been found in Creola bodies, clusters of epithelial cells shed into the sputum of many patients with asthma.⁴ MBP also reduces respiratory ciliary activity,¹⁹ alters epithelial ion transport,²⁰ and increases the sensitivity of respiratory smooth muscle to cholinergic agonists.^{21,22} Increased serum ECP levels have been found during bronchoconstrictive responses to inhaled allergens.²³

Extracellular MBP has also been found in areas of inflammatory cell responses and tissue damage in sinusitis found in subjects with asthma.²⁴ Of note is the histopathology of the paranasal sinus epithelium that appeared similar to the bronchial epithelial changes observed in asthma.¹ Extracellular MBP has also been found in lesions of atopic dermatitis, even when few intact EOS were present, suggesting an extent of degranulation disproportionate to the extent of EOS accumulation in these lesions.⁸ Immunohistochemical studies have found that both EOS and extracellular MBP accumulate in the dermis during developing allergic LPRs.⁷ This deposition begins about 1 hour after intradermal Ag injection and often persists for at least

72 hours, even when the LPRs are no longer grossly visible. Of particular interest is that the MBP deposition was first noted in skin biopsy specimens at the same time we found increased release of MBP and EDN into overlying skin chambers. Of note is our finding that MBP and EDN levels were increased in chamber fluids, even when there were extremely few EOS in the fluids themselves (<1%). There was a higher percentage of EOS in imprints of the blister base at the Ag-challenged sites, but still <10% of the exudate. Moreover, there was not a strong correlation between the percentage of EOS in these imprints and the levels of MBP or EDN at the same sites. Charlesworth et al.,²⁵ using a similar skin-chamber model, have reported increased frequency of EOS in chamber fluids starting about 6 hours after Ag challenge; they did not report levels of EOS components in such fluids.

Other EOS components may also be toxic to tissues. EOS cationic protein (referred to above) and EDN are both toxic to nervous-system cells and may alter the function of peripheral nerves in the airways and skin with pathophysiologic consequences.^{1,2} EOS peroxidase can induce mast cell degranulation and damage pneumocytes.^{1,26}

Therefore, the findings reported in this study may have important relevance to pathophysiologic mechanisms in human allergic reactions. The approaches used will also provide a way in which we can assess quantitatively the effect of therapeutic approaches on the release of EOS components in comparison with clinical effects.

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