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Eosinophil Ribonucleases and Their Cutaneous Lesion-Forming Activity

Douglas A. Plager,*2 Mark D. P. Davis,* Amy G. Andrews,† Michael J. Coenen,* Terry J. George,* Gerald J. Gleich,‡§ and Kristin M. Leiferman3*

Eosinophil granule proteins are deposited in cutaneous lesions in many human diseases, but how these proteins contribute to pathophysiology is obscure. We injected eosinophil cationic protein (ECP or RNase 3), eosinophil-derived neurotoxin (EDN or RNase 2), eosinophil peroxidase (EPO), and major basic protein-1 (MBP1) intradermally into guinea pig and rabbit skin. ECP and EDN each induced distinct skin lesions at ≈2.5 µM that began at 2 days, peaking at ≈7 days and persisting up to 6 wk. These lesions were ulcerated (ECP) or crusted (EDN) with marked cellular infiltration. EPO and MBP1 (10 µM) each produced perceptible induration and erythema with moderate cellular infiltration resolving within 2 wk. ECP and EDN localized to dermal cells within 2 days, whereas EPO and MBP1 remained extracellular. Overall, cellular localization and RNase activity of ECP and EDN were critical for lesion formation; differential glycosylation, net cationic charge, or RNase activity alone did not account for lesion formation. Ulcerated lesions from patients with the hypereosinophilic syndrome showed ECP and EDN deposition comparable to that in guinea pig skin. In conclusion, ECP and EDN disrupt skin integrity and cause inflammation. Their presence in ulcerative skin lesions may explain certain findings in human eosinophil-associated diseases. The Journal of Immunology, 2009, 183: 4013–4020.

Secondary eosinophil granules contain several highly cationic proteins, including eosinophil cationic protein (ECP,3 RNase 3)2 eosinophil-derived neurotoxin (EDN, RNase 2), eosinophil peroxidase (EPO), and major basic protein-1 (MBP1) (1). EDN, EPO, and MBP1 are among the most abundantly transcribed genes in developing eosinophils (2) and are correspondingly abundant in isolated peripheral blood eosinophils (3). These granule proteins are extensively deposited in skin in several dermatoses, such as atopic dermatitis, bullous pemphigoid, and urticaria, and in the hypereosinophilic syndrome (HES) (4). In atopic dermatitis, eosinophil granule proteins are deposited at relatively high local concentrations (>1 µM) (5) without apparent cutaneous eosinophila, evidently as a result of cytolytic degranulation (6–8).

Numerous in vitro studies have reported cytotoxic properties of eosinophil granule proteins against mammalian cells, as well as pathogenic organisms, such as helminthes, bacteria, and viruses (1, 9, 10). Part of this cytotoxicity appears attributable to the cationic nature of the granule proteins (pIs of ~11 for ECP, EPO, and MBP1 and a pI of 8.7 for EDN) (2, 11, 12). Additionally, ECP and EDN possess RNase activity, and EPO has peroxidase activity, whereas MBP1 is a C-type lectin (13). ECP and EDN are unique among the granule proteins in their ability to induce neurotoxicity, referred to as the Gordon phenomenon (14, 15), and their RNase activity appears critical for this ability (15). MBP1 activates several human cell types, such as mast cells, basophils, and eosinophils (16–18). Thus, eosinophil granule proteins have broad potential to impact cellular and tissue functions.

Few studies have examined the in vivo effects of eosinophil granule proteins in skin. They induce cutaneous wheal and flare reactions (19, 20) and increase cutaneous vasopermeability (5). Overall, the importance of eosinophils in the pathophysiology of atopic disease, including atopic dermatitis, is controversial (21–24). Nonetheless, in diseases with extensive cutaneous eosinophil granule protein deposition, such as in HES and severe atopic dermatitis, cutaneous lesions are common. To define the in vivo effects of eosinophil granule protein deposition, we injected them intradermally into guinea pig skin and monitored cutaneous lesion formation. Subsequently, we examined the mechanism(s) of lesion formation, particularly with ECP and EDN, and compared immunohistologic staining of eosinophil granule proteins to that observed in erosive and ulcerative lesions in the hypereosinophilic syndrome.

Materials and Methods

Human eosinophil granule proteins

After approval by the Mayo Clinic Institutional Review Board, eosinophils from patients with marked eosinophilia (up to 84%) were obtained by cytapheresis (25). The methods for purifying eosinophil granules and granule proteins have been described (14, 26–28). Distilled and deionized water was used throughout all eosinophil granule protein purifications. Briefly, after cell lysis and granule isolation, granules were solubilized in 0.01 M Na2HPO4,0.05 M KH2PO4 (pH 8.0) centrifuged, applied to a carboxymethyl (CM)-Sepharose column, and eluted with a linear NaCl gradient.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0900055
(0.15–1.5 M). Fractions rich in EPO activity were pooled and dialyzed against PBS (8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl (pH 7.40)) and concentrated. The concentrated solution was centrifuged, and the supernatant was removed and frozen at –70°C. All preparations of RNAse activity (absorbance at 412 nm/absorbance at 280 nm) between 0.95 and 1.04.

Fractions from the Sephadex G-50 column containing ECP and EDN were pooled, dialyzed against one-half concentration PBS (pH 7.4), concentrated, and fractionated on a heparin-Sepharose CL-6B column (1.2 × 8 cm) equilibrated with one-half concentration PBS. Proteins bound to the column were eluted with a linear NaCl gradient (0.07–1.5 M NaCl). EDN eluted at a low salt concentration (~0.2 M NaCl), whereas ECP eluted at ~0.4 M NaCl (14). Excretion coefficients at 280 nm of 1.64 (mg/ml⁻¹ cm⁻¹) for EDN and 1.55 (mg/ml⁻¹ cm⁻¹) for ECP were used to determine RNA activity concentrations (14). To obtain ECP1 and ECP2 (14), two forms of differentially glycosylated ECP. ECP fractions pooled from the initial heparin-Sepharose column were subjected to a second heparin-Sepharose chromatography. These latter fractions were analyzed by SDS-PAGE followed by colloidal Coomassie blue staining (GelCode Blue; Pierce), and ECP1 and ECP2 were pooled separately.

Fractions containing MBP1 (2, 29) from the Sephadex G-50 column were pooled and stored at ~70°C in 0.025 M sodium acetate, 0.15 M NaCl (pH 4.2) to inhibit polymerization. MBP1 concentrations were determined using an extinction coefficient of 277 nm of 2.63 (mg/ml⁻¹ cm⁻¹) (26). Use of a more recently determined extinction coefficient (3.67 (mg/ml⁻¹ cm⁻¹)) would have resulted in 28% lower calculated MBP1 concentrations (2).

The original calculations were used throughout the study for consistency.

Results

Indirect immunofluorescence and histologic staining

Human ECP, EDN, EPO, and MBP were detected in formalin-fixed, paraffin-embedded biopsy specimens of cutaneous injection sites by indirect immunofluorescence. Briefly, serial tissue sections (5 µm) were mounted on positively charged microscope slides, deparaffinized, and incubated in 0.1% trypsin for 1 h at 37°C. The slides were incubated overnight in 10% normal goat serum at 4°C. The next day, sections were washed and overlaid with either control rabbit Ab (normal rabbit IgG or rabbit preimmunization serum) or granule protein-specific Ab (affinity chromatography-purified rabbit anti-human EDN and MBP or rabbit antiserum against ECP and EPO). After incubation, sections were washed with 1% chromotrope 2R (J. T. Baker) to eliminate nonspecific eosinophil staining (30). Subsequently, slides were overlaid with FITC-conjugated goat anti-rabbit IgG (SouthernBiotech). After a final wash, sections were mounted in a glycerol solution containing p-phenylenediamine to prevent fading (31). Representative photomicrographs were taken with a Zeiss Axiohot microscope equipped with excitor and barrier filter set: blue BP450/490; LP520/560 (N 487910). Histologic examination with H&E stains was also performed to examine cellular infiltration.

RNAse activity measurement

Mouse liver RNA was isolated using RNA-STAT protocol (Tel-Test) and quantitated by absorbance at 260 nm. RNA was stored in 1× Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA (pH 8.3) in diethylpyrocarbonate-treated sterile H₂O). To assay for RNAse activity (32), stock YOYO-1 dye (1 mM; Invitrogen) was added to 10 µg/ml mouse liver RNA in Tris-acetate-EDTA buffer to a final YOYO-1 concentration of 2 µM, and this solution was heated at 65°C for 10 min. This RNA/YOYO-1 solution (100 µl) was added to the wells of a polystyrene flat-bottom 96-well plate and measured using a Cytofluor plate reader series 4000 (PerSeptive Biosystems) with excitation at 485 nm and emission at 530 nm. The RNA/YOYO-1 complex was allowed to equilibrate for 1 h before addition of protein (EDN, ECP, RNase A, or Ang) to duplicate wells, and fluorescence readouts were recorded. Initial RNAse velocity values were calculated from the change in arbitrary fluorescence units occurring from 2 to 4 min after the addition of protein and normalized per mass of protein added.

Results

Cutaneous lesion formation

Reducing SDS-PAGE was used to assess the purity of the eosinophil granule protein preparations (Fig. 1). Fig. 2 shows representative lesions following eosinophil granule protein injection, with robust lesions from ECP and EDN, and less intense or no lesions from EPO, MBP1, and bovine RNase A. In this experiment, granule proteins at 10 µM and 2.5 µM and their storage buffer solutions were injected intradermally, and injection sites were observed for up to 4 wk; Table I shows the lesion grade for each granule protein. Overall, ECP induced the most severe lesions, followed by EDN; both ECP and EDN had greater lesion-forming activity than did EPO or MBP1. ECP lesions were distinctly and consistently ulcerative, and EDN lesions typically showed white, dry crusts (Fig. 2). ECP and EDN lesion formation began within 2 days and peaked ~7 days after injection (Table I). Injection sites of identical samples on duplicate guinea pigs were similar in magnitude and appearance, while the overall severity of the 2.5 µM injection sites was diminished relative to the 10 µM sites (Table I). Bovine RNase A, sterile PBS, and phosphate buffer with 1 M NaCl

Intradermal injections and their assessment

The Institutional Animal Use and Care Committee approved the studies. Hartley guinea pigs, 400–600 g, were anesthetized with 100 mg/ml ketamine (Fort Dodge Laboratories) by i.m. injections of 0.5 ml per kilogram body mass. Fifty or 90 Hartley guinea pigs, 400–600 g, were anesthetized with 100 mg/ml ketamine (Nembutal), and biopsies were immediately obtained by gently lifting the skin and using curved scissors to cut around the injection sites. Twenty-two guinea pigs and two rabbits were used in the studies; not all were tested with the same injections at the same time. The number tested is listed in each table for the respective experiments.

Indirect immunofluorescence and histologic staining

Human ECP, EDN, EPO, and MBP were detected in formalin-fixed, paraffin-embedded biopsy specimens of cutaneous injection sites by indirect immunofluorescence. Briefly, serial tissue sections (5 µm) were mounted on positively charged microscope slides, deparaffinized, and incubated in 0.1% trypsin for 1 h at 37°C. The slides were incubated overnight in 10% normal goat serum at 4°C. The next day, sections were washed and overlaid with either control rabbit Ab (normal rabbit IgG or rabbit preimmunization serum) or granule protein-specific Ab (affinity chromatography-purified rabbit anti-human EDN and MBP or rabbit antiserum against ECP and EPO). After incubation, sections were washed with 1% chromotrope 2R (J. T. Baker) to eliminate nonspecific eosinophil staining (30). Subsequently, slides were overlaid with FITC-conjugated goat anti-rabbit IgG (SouthernBiotech). After a final wash, sections were mounted in a glycerol solution containing p-phenylenediamine to prevent fading (31). Representative photomicrographs were taken with a Zeiss Axiohot microscope equipped with excitor and barrier filter set: blue BP450/490; LP520/560 (N 487910). Histologic examination with H&E stains was also performed to examine cellular infiltration.

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injection sites did not cause lesions after injection (Fig. 2). Undiluted acetate buffer caused a trace lesion (0.5 score) that returned to normal after 1 wk. However, for control purposes, this was an extreme acetate buffer concentration because the stock MBP1 in acetate buffer was diluted >20-fold with sterile PBS before injection. Intradermal injections of the granule proteins at 25 μM were also performed in two rabbits. Similar lesion formation occurred except that lesions from EPO were relatively more intense and approached the severity of lesions from EDN (Table I).

Additional experiments were performed with a wider range of ECP and EDN concentrations. Lesions were consistently elicited with ≥2.5 μM ECP or EDN, and visible lesions frequently formed even at 1 μM ECP or EDN. Lesions remained detectable for >2 wk with ≥2.5 μM ECP or EDN (Table I). Alopecia persisted at least 4 wk at 10 EDN injection sites (two 9 μM and eight 10 μM) on four guinea pigs. The 10 μM EDN injection sites on one guinea pig were monitored up to 6 wk after injection and showed hair regrowth at this time. Alopecia and crusting persisted longer than 4 wk at three ECP injection sites (all 10 μM) on each of two guinea pigs.

To rule out a potential contribution from bacterial or LPS contamination in purified eosinophil granule protein preparations, three approaches were taken. First, purified LPS at concentrations up to 100 μg/ml was intradermally injected into guinea pigs. Lesion-forming activity by endotoxin was minimal, never exceeding a trace lesion (0.5 score), even at 100 μg/ml from 2 to 14 days after injection. Second, to avoid bacterial contamination of injected protein samples, all samples were centrifuged at 12,000 × g for 10 min before injection, and only the supernatants were injected. A supernatant sample of ECP was tested for bacterial contamination by culture at the Mayo Clinic Microbiology Laboratory, and no microorganisms were detected after 5 days of culture. Finally, to rule out a pathogenic bacterial infection, a representative lesion induced by ECP was biopsied, and a portion was cultured. Testing was performed on both agar plates and in liquid broth; coagulase-negative *Staphylococcus* and *Streptococcus viridans*, likely commensal organisms, were cultured from the biopsy.

**Mechanism of ECP- and EDN-induced cutaneous lesion formation**

**Cellular localization of ECP and EDN and cellular infiltration.** Within 24 h after ECP injection, heterophil and lymphomononuclear subdermal cell infiltrates were prominent in the dermis, including near cutaneous trunci muscles, and these infiltrates remained substantial 2, 3, and 4 days after injection (Figs. 3, A and B). Cellular infiltration increased in the upper dermis during this time and peaked at approximately day 7; cell infiltrates began to decrease at approximately day 14. Similar patterns of cellular infiltration occurred for the other three granule proteins, but with intensities proportional to the lesion-forming activities of the granule proteins (i.e., less cellular infiltration with decreased lesion formation). Likewise, intradermal injection of PBS and RNase A, neither of which induced a skin lesion, resulted in minimal or no detectable cellular infiltration during the first week.

Immunofluorescence staining showed that most of the intradermally injected ECP and EDN, diffusely distributed in tissues initially after injection (Fig. 3C), were associated with cells in the dermis within 48 h (Fig. 3D–F). In contrast, EPO and MBP1 remained primarily extracellular and appeared to bind to dermal matrix fibers (Fig. 3, G and H) (5).

**Differential glycosylation.** Glycosylation influences protein recognition and activity, and because of heterogeneous glycosylation, ECP exists in two predominant forms, that is, ECP1 and ECP2 (14). Therefore, a sample of ECP containing a mixture of ECP1 and ECP2 was subjected to a second heparin-Sepharose chromatographic separation with NaCl gradient elution. SDS-PAGE analyses showed fractions containing predominantly the higher molecular mass ECP1 or the lower molecular mass ECP2 that eluted

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**Table I. Cutaneous lesion severity grade and kinetics following intradermal injection of eosinophil granule proteins**

<table>
<thead>
<tr>
<th>Observation Time</th>
<th>ECP Guinea pigs</th>
<th>ECP Rabbits</th>
<th>EDN Guinea pigs</th>
<th>EDN Rabbits</th>
<th>EPO Guinea pigs</th>
<th>EPO Rabbits</th>
<th>MBP1 Guinea pigs</th>
<th>MBP1 Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>1</td>
<td>1</td>
<td>0.75</td>
<td>1</td>
<td>0</td>
<td>0.25</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Day 2</td>
<td>2</td>
<td>1.75</td>
<td>1.25</td>
<td>1.75</td>
<td>0</td>
<td>0.25</td>
<td>0.75</td>
<td>0.25</td>
</tr>
<tr>
<td>Day 3</td>
<td>3.5</td>
<td>3.5</td>
<td>2</td>
<td>2.5</td>
<td>0.25</td>
<td>0.25</td>
<td>2</td>
<td>0.25</td>
</tr>
<tr>
<td>Day 4</td>
<td>3</td>
<td>3.25</td>
<td>2</td>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Average lesion grade for single 50-μl intradermal injections of respective concentrations, 2.5 and 10 μM (into two guinea pigs for each concentration at each time point) and 25 μM (into two rabbits for each concentration at each time point); lesion grading scale from 0 to 4, see Materials and Methods for lesion score description.

* ND, not done.
from the heparin-Sepharose column at lower and higher salt concentrations, respectively, and that displayed an ~2 kDa difference in SDS-PAGE mobility. Intradermal injections of unseparated ECP, ECP1, and ECP2 at 10 and 2.5 μM resulted in lesions of similar severity among the 10 and 2.5 μM injections, respectively. These injection sites were biopsied 1 wk later and stained by immunofluorescence for ECP. For all three ECP preparations, specific staining was detected, and the majority of staining appeared localized to cells.

Net cationic charge. The limited lesion-forming activity of MBP1 (13.8 kDa, +16 net positive charge) suggested that strong cationic charge is not sufficient to induce cutaneous lesions as intense as those caused by ECP or EDN. Similarly, intradermally injected poly-L-arginine at concentrations up to 25 μM showed a maximum of a grade 1 lesion that subsided to a trace lesion after 1 wk. The influence of protein net cationic charge on lesion formation was also examined by injecting RNases of different charges. Ninety microliters of ECP (10 μM), EDN (10 μM), Ang (10 μM), and RNase A (15 μM) was each injected intradermally in duplicate into two guinea pigs. Table II shows the course of lesion formation; lesion scores for duplicate injections on the same guinea pig and on different guinea pigs, as graded by two investigators, varied by ≤0.5. Lesion severity was not directly proportional to the net cationic charge among these RNases because, although ECP (+14 charge) and RNase A (+4 charge) caused the most and least severe lesions, respectively, the more highly cationic Ang (+10 charge) did not induce more severe lesions than those of EDN (+7 charge).

RNase activity. To test the influence of RNase activity on ECP and EDN lesion formation, RNasin was mixed with ECP and EDN before intradermal injections of each mixture. For comparison, ECP or EDN mixed with sterile PBS was also injected. The ECP plus RNasin lesion was diminished in both size and intensity by 50–70%; however, the EDN plus RNasin lesion was unaffected. Immunofluorescence staining of sites biopsied 4 days after injection detected minimal ECP in the ECP plus RNasin injection site; in contrast, staining of ECP and EDN in the remaining three sites, that is, ECP without RNasin and EDN with and without RNasin, was comparable to untreated injection sites.

To address further the role of RNase activity in lesion formation, an active site histidine of ECP and EDN was carboxymethylated. We first confirmed the relative RNase activities of ECP, EDN, Ang, and RNase A (Fig. 4A). Following sham treatment or treatment with 75 mM iodoacetate, samples of the unmodified (sham-treated) and CM-ECP or EDN were reisolated by heparin-Sepharose chromatography. Peak fractions of unmodified-ECP, unmodified-EDN, CM-ECP, and CM-EDN were tested for RNase activity; carboxymethylation reduced the RNase activity of ECP and EDN by ~80 and 87% (Fig. 4B), respectively. Carboxymethylation of ECP and EDN strikingly reduced lesion-forming activities by two to three grades both on days 3 and 7 (Table III). Immunofluorescence staining for ECP at injection sites of unmodified- and CM-ECP at day 3 demonstrated that carboxymethylation did not alter the cellular localization or the overall immunofluorescence distribution of
the protein (Fig. 5); immunofluorescence staining patterns were similar for unmodified-EDN and CM-EDN.

Relationship to human skin disease

Morphologically, the lesions resulting from ECP and EDN injections into guinea pig skin showed similarity to erosive and ulcerative lesions in patients with HES (Fig. 6A–C) (33). Immunofluorescence staining of lesional tissue from four such patients demonstrated both cellular localization and extracellular deposition of ECP and EDN (Fig. 6, D and F), similar to that observed in guinea pig skin at different time points following intradermal protein injections (Fig. 3), with the exception that the human tissue showed staining of intact eosinophils. The MBP1 cellular staining, as represented by brightly fluorescent ovals, reflects the relative numbers of intact eosinophils (Fig. 6E). Both EDN (Fig. 6F) and especially ECP (Fig. 6D) staining shows more extensive cellular localization than accountable by MBP1-associated eosinophil staining, suggesting their localization to cells other than eosinophils.

Discussion

Eosinophil granule protein deposition occurs in a variety of dermatoses (4). Several of the granule proteins induce a cutaneous wheal-and-flare reaction and increase cutaneous vasopermeability in vivo (5, 19, 20). The in vitro cytotoxicity of eosinophil granule proteins is also well documented (1). Additionally, the hypersensitivity syndromes are often associated with cutaneous lesions (34), and atopic dermatitis shows strong association with eosinophil activity (35, 36). Even so, other reports suggest that eosinophils have a limited impact on asthma and atopic dermatitis inflammation (21–24). To help clarify these issues, we investigated the effects of eosinophil granule proteins on guinea pig and rabbit skin through injections into the dermis, a site to which the granule proteins localize in cutaneous diseases. Intradermal injection of micromolar concentrations of four eosinophil granule proteins, ECP, EDN, EPO, and MBP1, produced cutaneous lesions with cellular infiltration. The ECP- and EDN-induced lesions were substantially more pronounced than the EPO- and MBP1-induced lesions. This was unexpected for EDN because of its limited in vitro cytotoxicity toward K562 cells relative to that of ECP, EPO, and MBP1 (2). The lesion-forming activity of ECP and EDN was associated with their localization in dermal cells. Furthermore, RNase activity appears important to their lesion-forming activities, and their respective net positive charges also may have modulated lesion formation. However, neither RNase activity nor high net positive charge alone was sufficient to account for full lesion formation.

The marked lesion-forming activities of ECP and EDN focused our attention on these proteins (Fig. 2). An initial concern was whether a contaminant or an infectious component might have contributed to lesion formation. Because lesion-forming activity varied among the different granule proteins and was consistent for a given granule protein, we think that no systematic factor or contaminant was substantially contributing to EDN- and ECP-induced lesions. Several approaches, addressing potential endotoxin or bacterial contamination of the protein preparations and postinjection infection, showed that bacterial or endotoxin contamination did not explain EDN- and ECP-induced lesions. Finally, the ability of RNasin, a specific RNase inhibitor, to diminish an ECP-induced lesion by ~60% and the ability of carboxymethylation to diminish ECP- and EDN-induced lesions

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### Table II. Cutaneous lesion severity grade and kinetics following intradermal injection of various RNases

<table>
<thead>
<tr>
<th>Observation Time</th>
<th>ECP, +14† (10 μM)</th>
<th>EDN, +7 (10 μM)</th>
<th>Ang, +10 (10 μM)</th>
<th>RNase A, +4 (15 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Guinea pig 1</td>
<td>Guinea pig 2</td>
<td>Guinea pig 1</td>
<td>Guinea pig 2</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.75</td>
<td>1</td>
<td>1</td>
<td>0.75</td>
</tr>
<tr>
<td>Day 3</td>
<td>3</td>
<td>3.5</td>
<td>3</td>
<td>3.5</td>
</tr>
<tr>
<td>Day 7</td>
<td>3.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Day 14</td>
<td>3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

† Average of lesion grades for duplicate 90-μl injections on each of two guinea pigs; lesion grading scale from 0 to 4, see Materials and Methods for lesion score description.

‡ Excess number of basic amino acids for the given RNase.

§ ND, not done.

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![Figure 4](image_url)

**FIGURE 4.** RNase activity of unmodified and carboxymethylated RNases. A, RNase activity, reflected by a decrease in fluorescence, after 60 min equilibration, is shown for PBS (●), Ang (○), ECP (△), EDN (□), and bovine RNase A (■). Each protein was added at 1 μg, and data are plotted as the percentage of maximum fluorescence. B, An equal volume of the heparin-Sepharose CL-6B column buffer (●), the peak fraction of CM-EDN (absorbance at 280 nm of 0.205; ○), and the peak fraction of unmodified EDN (absorbance at 280 nm of 0.271; △) were each added to separate wells in duplicate after the 60-min equilibration. Initial RNase velocities (the change in fluorescence over the 2-min interval beginning 2 min after protein addition and normalized per unit of absorbance at 280 nm) for CM-EDN (911/2/0.205 = 2222) and for unmodified EDN (9121/20.275 = 16,584) indicated an 87% reduction of RNase activity following carboxymethylation of EDN.

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![Figure 6](image_url)

**FIGURE 6.** Guineapig skin lesions show strong association with eosinophil activity (35, 36). Even so, other reports suggest that eosinophils have a limited impact on asthma and atopic dermatitis inflammation (21–24). To help clarify these issues, we investigated the effects of eosinophil granule proteins on guinea pig and rabbit skin through injections into the dermis, a site to which the granule proteins localize in cutaneous diseases.
(Table III) support the specific lesion-forming activities of ECP and EDN. Notably, RNAsin (pI of 4.7) probably did not inhibit an EDN-induced lesion because of the relatively short half-life of the EDN-RNasin complex (EDN, +7 net charge) compared with that of angiogenin (+10 net charge) (15), and presumably to that of ECP (+14 net charge). A short EDN-RNasin half-life compared with that of ECP-RNasin is also consistent with the immunofluorescence detection of intradermally injected EDN, but not ECP, when mixed with RNasin. Overall, it is unlikely that the cutaneous lesions caused by intradermal injections of ECP or EDN are attributable to a contaminating substance or infectious component.

The ECP- and EDN-induced lesions developed over 1 wk, occurred at concentrations as low as 1 μM, and occurred in both guinea pig and rabbit skin (Fig. 2 and Table I). ECP and EDN concentrations >1 μM are likely deposited in human skin disease (5). Given the pronounced lesion-forming activities of ECP and EDN, we considered distinctive properties of ECP and EDN compared with the less active lesion-forming EPO and MBP1. Two such properties are neutrotoxicity and RNase activity. A third property, distinguishing ECP and EDN from EPO and MBP1, is cellular localization after intradermal injection (Fig. 3). Cellular localization of ECP and EDN appears consistent with previous observations for another RNase, oncosite RNasin (37), and with our staining for ECP and EDN compared with MBP1 in HES skin lesions (Fig. 6). Interestingly, cellular internalization can increase the neutrotoxicity of an RNase by 1000-fold (38). Thus, internalization of ECP and EDN, as suggested by the appearance of the immunofluorescent cells within the dermis containing ECP and EDN (Fig. 3), might account for the unexpectedly high lesion-forming activity of EDN. Unfortunately, due to the paucity of guinea pig-specific reagents, the identity of the cells accumulating ECP and EDN remains unknown. However, ECP has been observed in macrophage-like dermal cells after house dust mite patch testing of humans (39), and EDN has recently been reported as an endogenous molecule expression (43), and TNF-α induces release of inflammatory mediators, such as IL-8 ligation exists (41). Additionally, EDN is chemotactic for dendritic cells and stimulates release of angiogenin (40). The relative RNase activities for these four RNases against the RNA substrate used in this study (i.e., mouse liver total RNA) were as follows: RNase A was more active than EDN, and these were substantially more active than ECP, which was substantially more active than Ang (Fig. 4). These relative activities are expected from previous reports. However, the relative lesion-forming activities of ECP and EDN were substantially greater than that of Ang, which was substantially greater than that of RNase A (Table II). Therefore, lesion formation does not depend solely and directly on RNase activity. Second, we tested carboxymethylated ECP and EDN, and this treatment strikingly inhibited lesion formation (Table III). Thus, at least for ECP and EDN, RNase activity is required for maximal lesion formation. Because carboxymethylation inserts one negative charge per carboxymethyl group added, these changed molecular charges of the carboxymethylated ECP and EDN could be confounding factors (see below). Also, factors other than RNase activity appear to be involved, because while the RNase activity of ECP is substantially less than that of EDN, ECP produces a more prominent lesion.

Cationicity is often associated with cytotoxicity (45). Thus, net positive charge, as suggested by the cytotoxic activity of the non-RNase MBP1 (+16, molecular mass of 13.8 kDa) (2), may contribute to lesion formation. For example, a more highly cationic protein may have an increased partitioning to the cell’s surface or increased direct cytotoxicity or both. The protein cationicity of the RNases decreases in the following order: ECP, Ang, EDN, and RNase A (Table II). Because the more highly charged Ang does not produce a more pronounced lesion than that caused by EDN, nor does MBP1 or poly-l-arginine cause such pronounced lesions, a direct relationship between net positive charge and lesion-forming activity does not exist. Glycosylation can affect a protein’s net charge, and both ECP and EDN are glycosylated, while Ang and RNase A are not. A recent study showed that the N-linked carbohydrates on ECP are in part made up of sialic acid, galactose, and acetylgalactosamine (46). These individual carbohydrates are suggestive of “complex type” N-linked glycosylation, which typically involves noncharged carbohydrates and negatively charged sialic acid. This form of glycosylation would only diminish ECP’s cationicity and presumably any associated cation-dependent cytotoxicity. Alternatively, such glycosylation could confer other properties to ECP and EDN that contribute to their distinct lesion-forming capabilities compared with Ang and RNase A, even though differential glycosylation of ECP1 and ECP2 did not noticeably alter their lesion-forming activities.

Thirteen members of the human RNase family have been identified (47). Among these 13 genes, ECP and EDN form a distinct clad with substantial homology. Thus, unidentified molecular characteristics specific to ECP and EDN may be critical to their cutaneous lesion-forming activity. For instance, after intradermal injection, it is unknown whether Ang and RNase A localize to dermal cells like ECP and EDN. However, similar to their difference in lesion-forming potency reported herein, the alarmin adjuvant effect of EDN reported by Yang et al. was not recapitulated by human angiogenin (40). The
relatively specific lesion-inducing activities of ECP and EDN compared with other RNases are also reminiscent of their unique neurotoxic and antiviral activities (48).

Morphologically, the lesions resulting from ECP or EDN injection into guinea pig skin showed similarity to erosive and ulcerative lesions in an HES variant (Fig. 6A–C) that, until recently, was associated with a grave prognosis (33). HES with ulcerative lesions appears to be a presentation of myeloproliferative HES (associated with a deletion on chromosome 4 resulting in a fusion gene product, FIP1L1-PDGFRα, and yielding a novel kinase sensitive to imatinib mesylate therapy with long-term disease control) (49, 50). The biopsy specimens from 4 HES patients showed both eosinophil infiltration and eosinophil granule protein deposition; the staining of cell-localized ECP and EDN was extensive, out of proportion to the number of infiltrating eosinophils identified by MBP1 staining, and similar to that observed in guinea pig skin at later time points following injection of ECP and EDN. Ongoing deposition of ECP and EDN in human tissue, unlike single injections of ECP or EDN into guinea pig skin, likely contributed to the relatively greater extracellular staining of ECP and EDN in human tissue compared with later guinea pig intradermal injection sites (≥2 days postinjection). Because of ethical considerations, it is not possible directly to study the effects of eosinophil granule proteins injected into human skin; however, these analogous findings by formation of lesions and by immunostaining support the conclusion that ECP and EDN cause cutaneous lesions in human disease.

In summary, all four eosinophil granule proteins, ECP, EDN, EPO, and MBP1, induce a cutaneous lesion after intradermal injection into guinea pig and rabbit skin at pathophysiologically relevant concentrations. The ECP- and EDN-induced lesions are more intense than lesions induced by EPO and MBP1, and this difference appears to be closely associated with cellular internalization and RNase activity of ECP and EDN. Net cationic charge may also modulate lesion-forming activity. However, neither cationicity or RNase activity directly correlates with lesion-forming activity, suggesting the potential importance of other properties specific to ECP and EDN. Overall, these data provide further direct evidence that deposition of the eosinophil granule proteins, particularly ECP and EDN, at micromolar concentrations, can severely effect cutaneous structure and function in diseases in which granule proteins are deposited including in the hypereosinophilic syndrome associated with mucocutaneous ulcerations (Fig. 6) (33, 34, 50).

FIGURE 5. Immunofluorescence staining for intradermally injected unmodified-ECP and CM-ECP. Injection site biopsies were taken from one of three guinea pigs 3 days after injecting unmodified ECP (A) and CM-ECP (B). Polyclonal rabbit anti-ECP was used to stain for ECP in these specimens, and a representative area of the dermis in each shows comparable cellular and extracellular staining (magnification, ×160).

FIGURE 6. Immunohistologic staining similarities to ulcerated human tissue in HES lesion. A, Lesion on guinea pig flank skin from ECP injection shows similar appearance to eroded and ulcerated lesions in an HES patient. B, Ulcerations and erosions in patient with HES. C, Oral lesions in HES with central epithelial ulceration and surrounding erythema from which a biopsy specimen was obtained and stained for eosinophil granule proteins. Photomicrographs (magnifications, ×160) of serial sections of lesional mucosa (C) from a patient with HES stained with anti-ECP (D), anti-MBP1 (E), anti-EDN (F), and H&E (G).
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Disclosures

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References