Recombinant Human Eosinophil-Derived Neurotoxin/RNase 2 Functions as an Effective Antiviral Agent against Respiratory Syncytial Virus

Joseph B. Domachowske, Kimberly D. Dyer, Cynthia A. Bonville, and Helene F. Rosenberg

A dose-dependent decrease in infectivity was observed on introduction of eosinophils into suspensions of respiratory syncytial virus group B (RSV-B). This antiviral effect was reversed by ribonuclease inhibitor, suggesting a role for the eosinophil secretory ribonucleases. Recombinant eosinophil-derived neurotoxin (rhEDN), the major eosinophil ribonuclease, promoted a dose-dependent decrease in RSV-B infectivity, with a 40-fold reduction observed in response to 50 nM rhEDN. Ribonuclease inhibitor (rhEDN) had no antiviral activity. Semiquantitative reverse transcriptase–polymerase chain reaction demonstrated loss of viral genomic RNA in response to rhEDN, suggesting that this protein promotes the direct ribonucleolytic destruction of extracellular virions. Ribonuclease A had no antiviral activity even at ~1000-fold higher concentrations, suggesting that rhEDN has unique features other than ribonuclease activity that are crucial to its effectiveness. These results suggest that rhEDN may have potential as a therapeutic agent for prevention or treatment of disease caused by RSV.

Although the involvement of eosinophils in the pathophysiology of allergic diseases and asthma has been studied extensively, the potential beneficial roles played by these cells remain poorly understood [1–6]. Human eosinophils contain a number of distinct secretory effectors, including eosinophil peroxidase, major basic protein, eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP) [6, 7]. EDN and ECP are closely related proteins that have ribonuclease activity [8, 9] and that are members of the RNase A superfamily [10–15]. EDN/RNase 2, the major eosinophil ribonuclease, is 100-fold more ribonuclease activity than ECP/RNase 3 [8], and although EDN displays specific neurotoxic activity when injected directly into the central nervous systems of experimental animals [16–19], it has no defined physiologic role. A number of recent studies have suggested that EDN’s ribonuclease activity is likely to be of primary importance to its as-yet-undetermined function [15, 20, 21]. These findings, together with others relating to ribonucleases and viral infection [22–25], have prompted us to consider the possibility that eosinophil secretory ribonucleases could promote the destruction of single-stranded RNA viruses.

In an initial investigation of this hypothesis, we demonstrated that eosinophils can inhibit retroviral transduction of human target cells by a ribonuclease-dependent mechanism [26]. In this work, we have focused on respiratory syncytial virus (RSV), a major pathogen of the family Paramyxoviridae. There are a number of intriguing associations that link eosinophils, eosinophil granule proteins, asthma and allergic bronchospasm, and respiratory disease caused by RSV [27–38]. For example, several groups have shown that during RSV infection, eosinophils are recruited to and degranulate into the lung parenchyma [31–34], and wheezing during RSV infection is associated with increased concentrations of ECP in respiratory secretions [31]. Most dramatically, children previously vaccinated with a formalin-inactivated RSV vaccine who subsequently developed natural RSV infection had increased blood eosinophil counts [39], and massive eosinophil infiltrates were observed in post-mortem specimens of vaccinated children who died of RSV pneumonia [40]. While the role of eosinophils in host defense against RSV remains speculative, these studies demonstrate that recruitment of eosinophils to the respiratory tract can and does occur in response to RSV infection and, when exaggerated, may lead to a more severe form of RSV disease.

In this work, we investigated whether the inherent antiviral activity of eosinophils was due to the actions of their secretory ribonucleases, particularly the major eosinophil ribonuclease, EDN.

Materials and Methods

Preparation of virus stocks. RSV-B (ATCC VR-1401) was obtained from American Type Culture Collection (Rockville, MD). Clinical isolates of RSV-A and parainfluenza virus (PIV) types 1, 2, and 3 were gifts from Leonard B. Weiner (SUNY Health Science Center at Syracuse). HEp-2 cells (human laryngeal carcinoma), maintained in Eagle MEM + 10% heat-inactivated fetal bovine...
serum and 2 mM glutamine, and RhMk (rhesus monkey kidney) cells, maintained in Eagle MEM + 10% fetal bovine serum, were obtained from Viromed (Minneapolis). RSV-A and RSV-B were used to inoculate 180-cm² flasks containing semiconfluent monolayers of HEp-2 cells, and PIV-1, -2, and -3 were used to inoculate monolayers of RhMk cells, each in 50 mL of maintenance medium. When cytopathic effect reached ~80%, the supernatants were harvested, centrifuged at 2000 rpm to remove cellular debris, and flash-frozen in 1-mL aliquots (virus suspension).

Quantitative shell vial assay of virus infectivity. We have adapted shell vial technology, more commonly used in clinical diagnostic laboratories, for the experiments described here. Our assay permits more rapid evaluation of changes in virus infectivity than can be achieved with the traditional plaque assay (24 h vs. 8 days); the dose-response curve is discussed below and also in figure 1. Shell vials (4 mL) containing round coverslips with confluent monolayers of the appropriate target cell line (HEp-2 for RSV, RhMk for PIV) were obtained from Viromed. Shell vials were inoculated with 200 µL of virus suspension from each experimental condition, then centrifuged at 700 g at 22°C for 1 h to amplify the infection. One milliliter of maintenance medium was added, and the shell vials were incubated at 37°C for 16 h. Monolayers were washed and fixed with cold acetone for 20 min. Immunofluorescence staining was done with mouse anti-RSV blend fluorescein isothiocyanate–labeled monoclonal antibodies (Chemicon International, Temecula, CA). Stained coverslips were mounted onto slides with FA mounting fluid, pH 7.2 (Difco Laboratories, Detroit). Each coverslip was observed under fluorescence microscopy, and the number of fluorescent cells per coverslip was determined; each condition was assayed in triplicate. Data are expressed in terms of infectious units per milliliter; we have defined an infectious unit as the component within the virus suspension that results in the detectable infection of a single cell in the confluent monolayer. In figure 1 we demonstrate that there is a linear relationship ($r^2 = 0.987$) between the number of infected HEp-2 cells in a confluent monolayer (3–4 × 10⁵ cells/monolayer) and the dilution of virus suspension within the range used for our experiments. We have compared our measure of virus infectivity with that obtained from the traditional plaque assay [41] and found a close correlation between the number of plaque-forming units and number of infectious units as defined here in a given volume of virus suspension (e.g., 1.8 × 10⁵ pfu/mL by standard plaque assay vs. 1.9 × 10⁵ pfu/mL by quantitative shell vial assay). The correlation has been confirmed over a broad range of concentrations.

Eosinophil isolation. Peripheral blood eosinophils were freshly isolated from normal volunteers by CD16 negative selection as described [42, 43]. Briefly, 60 mL of peripheral blood per isolation was subjected to ficoll-hypaque density centrifugation (Organon Teknika, Durham, NC), and the erythrocytes comigrating with the granulocyte pellet were lysed with ACK lysing buffer (BioWhittaker, Walkersville, MD). Granulocytes washed twice with PBS + 0.5% bovine serum albumin + 1 mM EDTA (PBE) were incubated with anti-CD16–conjugated magnetic beads and isolated by magnetic activated cell sorting according to the manufacturer’s instructions (Miltenyi Biotec, Sunnyvale, CA). Eosinophils isolated by this method were 94%–97% pure as determined by Diff-Quik staining (Fisher Scientific, Pittsburgh), and >95% viable by trypan blue exclusion.

Neutrophil and mononuclear cell isolation. Peripheral blood neutrophils and mononuclear cell fractions were freshly isolated from normal volunteers by ficoll-hypaque density centrifugation (Organon Teknika). The mononuclear cell fraction was harvested, and the erythrocytes comigrating with the granulocyte pellet were lysed with ACK lysing buffer (BioWhittaker). The two different leukocyte fractions were washed twice with and resuspended in PBE as described for eosinophils. The neutrophil fractions contained <2% eosinophils as determined by Diff-Quik staining (Fisher Scientific).

Preparation of recombinant human (rh) proteins rhEDN and ribonucleolytically inactivated rhEDN (rhEDNdK38). Creation of the recombinant plasmid constructs (pFLAG-CTS; International Biotechnologies, New Haven, CT) and preparation of the recombinant protein was as previously described [20]. Briefly, recombinant proteins were isolated from 2–4 L of bacterial culture after a 1-h induction with isopropyl-1-thio-β-D-galactoside. After harvest and sucrose lysis, recombinant proteins were concentrated and isolated by M2 monoclonal antibody–agarose affinity chromatography (International Biotechnologies). The concentration of recombinant proteins was determined by comparison with serial dilutions of a known concentration of FLAG-conjugated standard as described [20]. Some of the rhEDN used was prepared in SP9 cells via inserting the full-length EDN cDNA [12] and C-terminal FLAG into the pVL1393 expression vector (InVitrogen, San Diego), which was used together with the AcNPV wild type baculovirus DNA to cotransfect as described [44].

Treatment of virus stocks with isolated human leukocytes or recombinant proteins. Eosinophils isolated as described were resuspended at 10⁵–10⁶ cells/mL in PBE and introduced into virus suspensions (1:300 dilution) at concentrations varying from...
Table 1. Dose-dependent reduction in RSV-B titer in response to treatment with human eosinophils (0–4 × 10^6/mL) and ribonuclease activities measured in virus suspensions to which eosinophils were added and subsequently removed.

<table>
<thead>
<tr>
<th>Eosinophils (×10^6/mL)</th>
<th>Infectivity (infectious units/mL ± SD)</th>
<th>Fold reduction</th>
<th>RNase activity (pmol/mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2423 ± 81</td>
<td>—</td>
<td>0.94</td>
</tr>
<tr>
<td>0.1</td>
<td>1855 ± 93</td>
<td>—</td>
<td>1.2</td>
</tr>
<tr>
<td>0.2</td>
<td>1500 ± 131*</td>
<td>1.6</td>
<td>3.6</td>
</tr>
<tr>
<td>0.4</td>
<td>675 ± 44</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>547 ± 17†</td>
<td>4.4</td>
<td>8.2</td>
</tr>
<tr>
<td>1.3</td>
<td>354 ± 52</td>
<td>6.8</td>
<td>30</td>
</tr>
<tr>
<td>4.0</td>
<td>133 ± 51†</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Infectivity is expressed as infectious units/mL ± SD. Initial rates (RNase activity) were calculated from 3 or 4 consecutive time points determined in triplicate, with r^2 ≥ .90 for all plots.

* P < .05
† P < .01

0 to 4 × 10^6 cells/mL, as indicated in each experiment, with PBE buffer added as needed for equivalent volumes. After 2 h of gentle rotation at room temperature, eosinophils were removed by centrifugation, and the treated virus suspension was used to infect target cells in the cell vial assay. We were unable to extend the time of exposure (tested at 4, 6, 8, and 24 h), as the virus suspensions (both control and experimental) all experienced dramatic nonspecific drops in titer, confounding the analysis of the results. In experiments indicated, placental ribonuclease inhibitor (200 U/mL final concentration; Boehringer Mannheim, Indianapolis) was added to the virus stocks just before addition of eosinophils. Recombinant proteins (rhEDN, rhEDNdK38) prepared as described were added to the virus stocks with buffer added as needed for equivalent volumes, and 10× PBS was added to maintain isotonicity.

Ribonuclease assay. Ribonuclease assay was done as described in detail [20]. Reactions were initiated with 40 μg of yeast tRNA as substrate (Sigma, St. Louis) added to 0.8-mL reactions containing 40 mM sodium phosphate, pH 7.0, and 10 μL of virus stocks treated with eosinophils, rhEDN, rhEDNdK38, or buffer controls. Reactions were stopped at given time points by the addition of ice-cold 3% perchloric acid with 40 nM lanthanum nitrate, and acid-soluble ribonucleotides remaining in the supernatant fraction after centrifugation were quantitated spectrophotometrically at 260 nM. All time points were tested in triplicate. Calculations included the following approximations: the average molecular weight (M_m) of tRNA as M_m = 28,100 (75–90 ribonucleotides/ribonucleotide), with absorbance at 260 nm of 1.0 corresponding to 40 μg of RNA [20]. Ribonuclease inhibitor was purchased from Boehringer Mannheim. Slopes and correlation coefficients (r^2) were determined with the assistance of Microsoft Excel software online at the National Institutes of Health.

Reverse transcriptase–polymerase chain reaction (RT-PCR). RNA was isolated from 1-mL volumes of virus stocks treated with 100 nM rhEDN or 100 nM rhEDNdK38 using RNAzol B (TelTest, Friendswood, TX) as per manufacturer’s instructions. Complementary DNA (cDNA) was prepared from resuspended RNA by use of an RT-PCR first-strand synthesis kit (Boehringer Mannheim) with random hexamer priming. Forty-cycle PCR was done on serial dilutions of cDNA with RSV G protein–specific 5′ and 3′ primers as described [45]. The PCR products were separated by 2.5% agarose gel electrophoresis, and the single amplification product of appropriate size was identified.

Statistical analysis. For each experiment, triplicate assays of each condition were done and SDs were calculated. Two-way analysis of variance was done to determine levels of significance by use of Microsoft Excel 5.0 software.

Results

For these studies, we used a modified version of the shell vial assay, as this has enabled us to determine changes in virus infectivity much more rapidly than would be possible using the traditional plaque assay (24 h vs. 8 days). The data in figure 1 demonstrate the linear dose response (r^2 = .987) in terms of infectious units per milliliter versus dilution of virus suspension. Infectious units are comparable to plaque-forming units over a broad range of concentrations (Domachowske JB, Bonville CA, unpublished data).

The data in table 1 demonstrate that exposure to isolated human eosinophils markedly reduces the infectivity of RSV-B and that the degree of impairment corresponds to the concentration of eosinophils within the given range (0–4 × 10^6/mL). At the highest eosinophil concentration (4 × 10^6/mL), we observed an 18-fold reduction in infectivity compared with the buffer alone (no eosinophils) control. The ribonuclease activities detected in eosinophil-treated virus suspensions represent the enzymatic activities of the eosinophil granule ribonucleases EDN and ECP, which are released under these experimental conditions [26].

The data in table 2 demonstrate that the ~4-fold reduction in virus infectivity resulting from the introduction of 0.4 × 10^6 eosinophils/mL into virus stocks of RSV-B was eliminated when placental ribonuclease inhibitor was included (P < .01).

Table 2. RSV-B remaining after treatment of virus suspensions with isolated human eosinophils, neutrophils, or mononuclear cells (at 4 × 10^6/mL), and buffer control with or without ribonuclease inhibitor (RI) at 200 U/mL.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infectivity (infectious units/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophils</td>
<td>548 ± 11*</td>
</tr>
<tr>
<td>Eosinophils + RI</td>
<td>2113 ± 172</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1899 ± 184†</td>
</tr>
<tr>
<td>Neutrophils + RI</td>
<td>1868 ± 251†</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td>2645 ± 210</td>
</tr>
<tr>
<td>Mononuclear cells + RI</td>
<td>2818 ± 106</td>
</tr>
<tr>
<td>Buffer</td>
<td>2423 ± 81</td>
</tr>
<tr>
<td>Buffer + RI</td>
<td>2248 ± 94</td>
</tr>
</tbody>
</table>

NOTE. Data are averages of triplicate samples ± SD.

* P < .01 vs. baseline (buffer).
† P < .05 vs. baseline (buffer).
Not only does this observation suggest that the ribonuclease activity released by the eosinophils is crucial to the antiviral effect, the fact that ribonuclease inhibitor alone can eliminate the antiviral effect effectively rules out other mechanisms that might explain this phenomenon, such as nonspecific virus binding or incorporation by eosinophils. In addition, we determined that eosinophils themselves were not infected by RSV-B, as demonstrated by immunofluorescence staining after overnight incubation (data not shown). Isolated human neutrophils (0.4 \times 10^6/mL) have a slight but statistically significant ability to impair RSV-B infectivity compared with control (P < .05); however, the neutrophil-mediated reduction in infectivity was not reversed by ribonuclease inhibitor, suggesting a mechanism distinct from that promoted by eosinophils. Mononuclear cells had no effect on RSV-B infectivity; similarly, no loss of RSV-B infectivity was observed when virus stocks were pretreated with cells of the K562 (human erythroleukemia) or AML 3D10 (human promyelocytic leukemia [46]) cell lines (data not shown).

### Table 3. Reduction in infectivity of RSV-A and parainfluenza viruses (PIV) types 1, 2, and 3 in response to human eosinophils (0.4 \times 10^6/mL), with or without ribonuclease inhibitor (RI, 200 U/mL).

<table>
<thead>
<tr>
<th>Agent</th>
<th>RSV-A</th>
<th>PIV-1</th>
<th>PIV-2</th>
<th>PIV-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosinophils</td>
<td>375 ± 95*</td>
<td>2590 ± 315*</td>
<td>2092 ± 127*</td>
<td>2113 ± 150*</td>
</tr>
<tr>
<td>Eosinophils + RI</td>
<td>1885 ± 185</td>
<td>4585 ± 260</td>
<td>3653 ± 150</td>
<td>3752 ± 122</td>
</tr>
<tr>
<td>Buffer</td>
<td>2395 ± 155</td>
<td>5820 ± 320</td>
<td>5362 ± 142</td>
<td>4955 ± 198</td>
</tr>
<tr>
<td>Buffer + RI</td>
<td>2120 ± 95</td>
<td>5115 ± 136</td>
<td>4593 ± 350</td>
<td>5175 ± 300</td>
</tr>
<tr>
<td>Fold reduction</td>
<td>6.4</td>
<td>2.2</td>
<td>2.6</td>
<td>2.3</td>
</tr>
</tbody>
</table>

NOTE. Data are infectious units/mL and are averages of triplicate samples ± SD. Fold reduction = eosinophils/buffer control.

* P < .05 vs. baseline (buffer).

We have extended our observations on eosinophil-mediated antiviral activity to include clinical isolates of RSV-A and PIV-1, -2, and -3 (table 3). As anticipated, virions of these paramyxoviruses display similar (although not identical) ribonuclease-dependent sensitivity to isolated human eosinophils; the PIVs display somewhat less sensitivity than either of the RSV isolates.

The data in table 4 demonstrate that EDN, the major eosinophil ribonuclease, can act alone as an antiviral agent and that loss of virus infectivity (up to 40-fold) increases in direct proportion to its concentration (0–50 nM rhEDN). The ribonucleolytically inactivated rhEDNdK38, in which the active-site lysine (K38) of rhEDN was converted by site-directed mutagenesis to a catalytically inactive arginine residue [20], displayed no antiviral activity in this assay. This result indicates, as anticipated from our previous results with isolated eosinophils, that the capacity for ribonuclease activity is crucial to antiviral activity. This observation is supported by the data in figure 2, in which loss of viral genomic RNA is observed in response to rhEDN but not in response to rhEDNdK38.

Interestingly, no antiviral activity was observed in virus suspensions treated with RNase A (bovine pancreatic ribonuclease) at concentrations ranging from 40 to 4000 nM (table 5). The ribonuclease activities measured in virus suspensions containing RNase A range from 203 to 22,300 pmol of ribonucleotides/mL/min, representing ~20- to 2000-fold more enzymatic activity than was measured in virus suspensions to which

### Table 4. Dose-dependent reduction in RSV-B infectivity in response to rhEDN (0–50 nM) and to ribonucleolytically inactivated rhEDNdK38 (36 nM) and in parainfluenza virus (PIV)-3 infectivity in response to 40 nM rhEDN.

<table>
<thead>
<tr>
<th></th>
<th>Infectious units/mL</th>
<th>Fold reduction</th>
<th>RNase activity (pmol/mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 nM rhEDN</td>
<td>5585 ± 266</td>
<td>—</td>
<td>0.98</td>
</tr>
<tr>
<td>0.29 nM rhEDN</td>
<td>2315 ± 32*</td>
<td>2.4</td>
<td>1.1</td>
</tr>
<tr>
<td>1.4 nM rhEDN</td>
<td>760 ± 48</td>
<td>7.3</td>
<td>1.8</td>
</tr>
<tr>
<td>7.2 nM rhEDN</td>
<td>350 ± 21*</td>
<td>16</td>
<td>3.8</td>
</tr>
<tr>
<td>50 nM rhEDN</td>
<td>140 ± 10*</td>
<td>40</td>
<td>10.1</td>
</tr>
<tr>
<td>36 nM rhEDNdK38</td>
<td>5400 ± 175</td>
<td>—</td>
<td>0.97</td>
</tr>
<tr>
<td>PIV-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 nM rhEDN</td>
<td>7730</td>
<td>—</td>
<td>0.97</td>
</tr>
<tr>
<td>40 nM rhEDN</td>
<td>1881 ± 95*</td>
<td>4.1</td>
<td>9.4</td>
</tr>
</tbody>
</table>

NOTE. Data are averages of triplicate readings ± SD. Initial rates (RNase activity) were calculated from 4 consecutive time points determined in triplicate, with r^2 \approx 9.0 for all plots.

* P < .05 vs. baseline (0 nM).

† P < .01 vs. baseline (0 nM).

Single point reading only.

Figure 2. Reverse transcriptase–polymerase chain reaction detection of 217-bp segment of RSV G protein gene. With serial 10-fold dilutions (n, undiluted; −1, 10^{-1} dilution; etc.) of cDNAs prepared from RNA isolated from virions treated with 100 nM rhEDN or 100 nM rhEDNdK38 used as templates, results suggest ribonucleolytic destruction of RNA viral genome.
Table 5. Dose-dependent reduction in RSV-B infectivity in response to RNase A (0–4000 nM).

<table>
<thead>
<tr>
<th>RNase A</th>
<th>Infectious units/mL</th>
<th>Fold reduction</th>
<th>RNase activity (pmol/mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 nM</td>
<td>4360 ± 252</td>
<td>—</td>
<td>0.97</td>
</tr>
<tr>
<td>40 nM</td>
<td>3720 ± 342</td>
<td>—</td>
<td>203</td>
</tr>
<tr>
<td>400 nM</td>
<td>4020 ± 225</td>
<td>—</td>
<td>1812</td>
</tr>
<tr>
<td>4000 nM</td>
<td>4380 ± 284</td>
<td>—</td>
<td>22,300</td>
</tr>
</tbody>
</table>

NOTE. Data are averages of triplicate samples ± SD. Initial rates (RNase activity) were calculated from 4 consecutive time points determined in triplicate, with \( r^2 \approx 9.0 \) for all plots.

50 nM rhEDN had been added (table 4). These results indicate that while ribonuclease activity may be crucial to the antiviral activity, other properties that are unique to rhEDN clearly contribute to its overall effectiveness. An alignment of the amino acid sequences encoding human EDN and bovine RNase A is shown in figure 3. Although both contain the characteristic eight structural cysteines and the histidine and lysine active site components, and both are potent ribonuclease [47], there is significant divergence in primary structure.

Discussion

In this work, we have demonstrated that isolated human eosinophils are active against virions of RSV-A and -B and PIV-1, -2, and -3 via the actions of their secreted ribonucleases. We have also shown that the major eosinophil ribonuclease, EDN, can function alone as an effective antiviral agent in this experimental system. The rhEDN-mediated antiviral effect is dose-dependent in the nanomolar range and could not be replicated by a ribonucleolytically inactivated form of the recombinant protein. Furthermore, we have shown that the related ribonuclease, RNase A, did not reduce virus infectivity even at 1000-fold-higher concentrations, suggesting that while ribonuclease activity may be crucial to antiviral activity, other unique features of rhEDN are clearly necessary for this effect.

As the role of eosinophils in host defense against RSV remains controversial, it is premature to conclude that EDN functions physiologically as an antiviral agent. Despite much research, the physiologic function of EDN remains unknown [6, 7]. Although EDN was shown to be similar first in amino acid content [10] and later in primary structure [11–14] to the eosinophil cationic toxin, ECP, EDN shared little to none of ECP’s helminthotoxic [48, 49], bactericidal [50, 51], or cytotoxic [52, 53] activities. The observation that the primary structure of EDN was similar to that of RNase A [10, 11, 13] and that EDN was a potent ribonuclease against standard substrates [47] suggested that the physiologic function of EDN might encompass this enzymatic activity; our recent study of the evolutionary history of EDN has provided additional support for this hypothesis [15, 20, 21]. In EDN’s role as an antiviral agent, its ribonuclease activity has been shown to be a crucial feature; confirmation of antiviral activity as EDN’s true physiologic role awaits further clarification of the role of eosinophils in host defense against RSV and similar viral pathogens [26, 54].

Although we have shown that rhEDN can work alone to reduce virus infectivity, this does not necessarily imply that EDN does work alone in the context of an eosinophil; in earlier work, Klebanoff and Coombs [54] have shown that the granule protein eosinophil peroxidase also has isolated activity against clinical strains of human immunodeficiency virus (HIV)-1. In addition, the recombinant protein differs substantially from native EDN, most notably in that it lacks the extensive glycosylation characteristic of EDN both within granules and released from activated eosinophils [55, 56]. The combined ribonucleolytic, membrane-lytic, and oxidative properties of the four major eosinophil granule proteins may each contribute to the antiviral activity observed with human eosinophils.

Our observation that RNase A, even at the highest concentrations, promotes no loss in virus infectivity suggests that other features of EDN apart from its ribonuclease activity are crucial to its antiviral activity. Both EDN and RNase A are highly active generalized ribonucleases, and both can readily digest RNAs extracted from multiple sources [57, 58]. However, EDN’s ability to inactivate extracellular virions depends not only on its ribonuclease activity but also on its ability to penetrate the viral capsid and gain access to the viral RNA genome. The mechanism of this penetration is not readily apparent.

Figure 3. Alignment of amino acid sequences of human EDN and bovine RNase A. Conserved cysteines are enclosed in shaded boxes, and catalytic histidines 15 and 129 and lysine 38 in open boxes. 9-residue loop (distinguishing feature of EDN) is denoted by overhead bar marked “a.” Human EDN and bovine RNase A are highly divergent members of RNase A gene superfamily [47].
However, one major distinguishing feature of EDN is a nine-residue carboxy-terminal loop that is not present in the primary structure of RNase A. This loop is highly charged, with an independent isoelectric point calculated at 10.3. Many cationic proteins and peptides, including ECP/RNase 3, eosinophil major basic protein, and the neutrophil defensins, have membrane-disruptive properties [50, 53, 59, 60]. This loop is located on the outer surface of the protein, as determined by the recent crystallographic analysis of rhEDN by Mosimann et al. [61], and may contribute to EDN’s antiviral potency.

As most known antiviral agents function by blocking ongoing viral replication in infected cells, EDN’s ability to inactive extracellular virions is unique and unexpected and represents a completely unexplored mechanism of antiviral activity. EDN may ultimately find a role as a synergistic antiviral agent, working in concert with a replication inhibitor such as ribavirin [62, 63]. This approach would be analogous to that in current use against HIV infection, in which multiple agents are directed against different phases of the virus’s life cycle [64–66]. In its favor, EDN is a relatively soluble and thermostable protein, clearly quite active at low concentrations, with no direct toxicity to bronchial epithelial cells [67]. Toward this end, the antiviral activity of rhEDN needs to be studied in appropriate cell culture and animal models of RSV disease [68, 69] to evaluate its therapeutic efficacy and potential toxicity.

Acknowledgments

We thank John I. Gallin, Harry L. Malech, and Leonard B. Weiner for helpful discussions and for their ongoing support of this work.

References

17. Sorrentino S, Glitz DG, Hamann KJ, Loegering DA, Checkel JL, Gleich GJ. Eosinophil cationic protein and eosinophil-de-