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Eosinophil cationic protein/RNase 3 is another RNase A-family ribonuclease with direct antiviral activity

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ABSTRACT

Eosinophil cationic protein (ECP) is one of two RNase A-superfamily ribonucleases found in secretory granules of human eosinophilic leukocytes. Although the physiologic function of eosinophils [and thus of the two eosinophil ribonucleases, ECP and eosinophil-derived neurotoxin (EDN)] remains controversial, we have recently shown that isolated human eosinophils promote ribonuclease-dependent toxicity toward extracellular virions of the single-stranded RNA virus, respiratory syncytial virus, group B (RSV-B). We have also shown that recombinant human EDN (rhEDN) can act alone as a ribonuclease-dependent antiviral agent. In this work, we provide a biochemical characterization of recombinant human ECP (rhECP) prepared in baculovirus, and demonstrate that rhECP also promotes ribonuclease-dependent antiviral activity. The rhECP described here is N-glycosylated, as is native ECP, and has ~100-fold more ribonuclease activity than non-glycosylated rhECP prepared in bacteria. The enzymatic activity of rhECP was sensitive to inhibition by placental ribonuclease inhibitor (RI). Although rhECP was not as effective as rhEDN at reducing viral infectivity (500 nM rhECP reduced infectivity of RSV-B ~6 fold; 500 nM rhEDN, >50 fold), the antiviral activity appears to be unique to the eosinophil ribonucleases; no reduction in infectivity was promoted by bovine RNase A, by the amphibian ribonuclease, onconase, nor by the closely-related human ribonuclease, RNase k6. Interestingly, combinations of rhEDN and rhECP did not result in either a synergistic or even an additive antiviral effect. Taken together, these results suggest that that the interaction between the eosinophil ribonucleases and the extracellular virions of RSV-B may be specific and saturable.

INTRODUCTION

Eosinophil cationic protein (ECP) is a major component of the large secretory granules of human eosinophilic leukocytes. ECP is expressed uniquely in eosinophils, and although it has been characterized as a cytotoxin, a helminthotoxin and as a bactericidal agent in numerous assays performed *in vitro* (1,2), the physiologic role of ECP (and of eosinophils) remains unclear. Gleich and colleagues (3) were the first to note that the N-terminal sequences of both ECP and of its counterpart, the eosinophil-derived neurotoxin (EDN), showed distinct similarities to RNase A, a bovine ribonuclease that has since been identified as the prototype of an extensive gene superfamily (4). Molecular cloning confirmed that both ECP and EDN (67% amino acid sequence similarity) were members of the RNase A superfamily (5–8), and both have since been shown to be active ribonucleases (9–12). Interestingly, neither ECP's helminthotoxicity (13) nor its bactericidal activity (11) were found to require ribonuclease activity, findings that were both unexpected and counterintuitive from an evolutionary perspective. These unlikely results take on particular significance in light of the findings of Rosenberg and colleagues (14) showing that, despite the extraordinarily rapid rate at which primate ECP (and EDN) coding sequences are incorporating mutations, all evolutionary variants include the eight structural cysteines and histidine and lysine catalytic residues that are necessary to maintain ribonuclease activity.

As part of our ongoing effort to identify a function (or functions) for ECP and EDN that take ribonuclease activity into account, we have begun to explore the relationships linking eosinophils, eosinophil granule proteins and respiratory disease caused by the single-stranded RNA virus, respiratory syncytial virus (RSV; family Paramyxoviridae; 15–23). In response to infection with RSV, eosinophils are recruited to and degranulate into the lung parenchyma (15,16), and the wheezing characteristic of RSV infection has been associated with increased concentrations of ECP in respiratory secretions (17). At the cellular level, Stark and colleagues (19) have shown that cultured respiratory

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epithelial cells support increased adherence of activated eosinophils, and Kimpen and colleagues have demonstrated direct interaction with (21) and activation of (20) eosinophils by RSV. Several groups have reported production of various eosinophil chemoattractants by RSV-infected pulmonary epithelial cells (24,25; A.M.Harrison *et al*., manuscript in review)

While eosinophils are generally perceived as the villains of RSV infection, promoting both tissue damage and bronchospasm, we have begun to consider whether eosinophils, and specifically eosinophil ribonucleases, play some role in host defense against an RNA virus such as RSV. This 'double-edged sword' hypothesis is similar to that proposed to explain the intermingling positive and negative proinflammatory roles of neutrophils (26). To this end, we have recently shown that eosinophils have inherent antiviral activity against isolated virions of RSV *in vitro*, and that EDN can act alone in mediating this antiviral effect (27). In this work, we provide a biochemical characterization of a novel formulation of recombinant human ECP (rhECP), and demonstrate that rhECP also has direct activity against extracellular forms of RSV. Interestingly, this antiviral activity that is not shared with other RNase A-family ribonucleases. This specificity for EDN and ECP, along with the observation that EDN/ECP combinations did not enhance antiviral activity above that measured for EDN alone, has permitted us to speculate on a possible ribonuclease-mediated mechanism of antiviral action.

MATERIALS AND METHODS

Production of recombinant protein

Recombinant baculoviral vectors were constructed by inserting the full-length coding sequence of human ECP (DDBJ/EMBL/GenBank accession no. X15161, nucleotides 55–537) in-frame with a C-terminal 'Flag' octapeptide (International Biotechnologies, Inc., New Haven, CT) into the *Bam*HI/*Xba*I sites of the pVL1393 transfer vector (Invitrogen, San Diego, CA). Three micrograms of recombinant vector and 0.5 μg of linear wild type baculovirus AcNPV (Pharmingen, San Diego, CA) were used to cotransfect *Spodoptera frugiperda* (Sf9) cells by the lipofectin method (Gibco BRL, Gaithersburg, MD). Polyhedrin-deficient recombinant viruses were selected and cloned by standard plaque assay and the presence of the protein product was confirmed by immunoblotting with both the M2 monoclonal anti-FLAG antibody (11,12) and polyclonal rabbit anti-ECP antiserum. Large scale production of rhECP was achieved by infecting 100 ml suspension cultures of Sf9 cells (2×10^6/ml) with recombinant virions. Supernatants containing rhECP secreted from the infected cells were harvested 72–96 h post-infection. Recombinant vectors encoding two additional RNase A-family ribonucleases, human EDN (rhEDN; DDBJ/EMBL/GenBank accession no. M24157) and human RNase k6 (rhRK6; DDBJ/EMBL/GenBank accession no. U64998) were prepared in a similar fashion and were used to transfect Sf9 cells, and supernatants containing secreted recombinant protein were likewise harvested at 72–96 h post-infection.

Protein purification

Supernatants harvested from 100 ml infected cultures were dialyzed (4°C) against 50 mM Tris pH 8.0 + 1 mM NaCl. Recombinant ECP was intially concentrated by heparin–Sepharose column chromatography (Pharmacia Biotech, Piscataway, NJ) and eluted via a salt-gradient (1–500 mM NaCl in 50 mM Tris, pH 8.0). The fractions containing recombinant protein were concentrated (Centricon 10 concentrator, Amicon, Beverly, MA) and subjected to size-fractionation (Superdex 200, Pharmacia Biotech), resulting in purified protein, with typical yields of 1–2 μg rhECP per ml infected Sf9 supernatant. Protein concentration was determined by BCA assay (Pierce, Rockford, IL) against bovine serum albumin standards. Recombinant proteins rhEDN and rhRK6 were purified in an analogous fashion.

Protein deglycosylation and western blotting

Aliquots of purified rhECP were heat/detergent denatured prior to attempted deglycosylation with endoglycosidase H (endo H) or with peptide N-glycosidase F (PNGase F; New England Biolabs, Beverly, MA) as per the manufacturer's instructions. Negative controls were treated identically, save for the addition of distilled water in place of enzyme. Western blots were performed as previously described (11,12); primary antibodies included a 1:200 dilution of M2 anti-Flag mAb and a 1:300 dilution of rabbit polyclonal anti-ECP (28) with secondary antibodies including alkaline-phosphatase conjugated goat anti-mouse or anti-rabbit IgG, respectively (BioRad, Richmond, CA), and NBT and BCIP (BioRad) as developing reagents. Native ECP was detected in a 1% triton-extract from peripheral blood leukocytes, also as previously described (29).

Ribonuclease assay

The ribonuclease assay was as described previously in detail (11,12,30). Briefly, the concentration of perchloric acid soluble ribonucleotides generated from acid-precipitable yeast tRNA (Sigma, St Louis, MO) in 40 mM sodium phosphate, pH 7.5 by a given quantity of ribonuclease (RNase A, rhEDN, rhECP or rhRK6) was measured spectrophotometrically at 260 nm. Each point shown on the double reciprocal plot and on the inhibition plot represents an intial rate of reaction determined at a given enzyme and substrate concentrations from at least four sequential time points, each measured in triplicate. Ribonuclease inhibitor (RI; 40 U/μl) was purchased from Boehringer Mannheim (Indianapolis, IN). The assays measuring ribonuclease activity in Tables 1 and 2 were performed using 10 μl of remaining viral stock to which ribonuclease has been added to the final concentration indicated (see antiviral assay below) and 10 μl of 4 mg/ml yeast tRNA in an 0.8 ml reaction volume. Initial rates were determined from two sequential time points, each measured in triplicate.

Antiviral activity assay

Direct antiviral activity of rhECP and other ribonucleases was performed by the quantitative shell vial amplification technique (27,31). The major advantage of this technique is that changes in viral infectivity can be measured more rapidly than can be achieved with the standard plaque assay (16 h versus 8 days). Recombinant proteins (rhECP, rhEDN, rhRK6), native proteins (RNase A, onconase) or equivalent volumes of buffer control (50 mM Tris pH 8.0 + 150 mM NaCl) were added to viral stocks of RSV prepared as described (27). After 2 h gentle rotation at room temperature, the treated viral stocks (200 μl) were used to infect target HEp-2 cells (human pulmonary epithelial/laryngeal carcinoma cells) present in confluent monolayers ($3–4 \times 10^5$ cells/monolayer) on coverslips in one dram shell vials (Viromed, Minneapolis, MN). Inoculated shell vials were centrifuged at

700 *g* at 22°C for 1 h to amplify the infection. One ml maintenance medium [Eagle's Minimal Essential Medium (EMEM) + 10% heat-inactivated fetal bovine serum + 2 mM glutamine] was added, and the shell vials were incubated at 37°C, 5% CO_2 for 16 h, conditions permitting only a single round of RSV infection. After incubation, the monolayers were washed and fixed with cold acetone. Immunofluorescence staining for primary RSV-infected cells was performed with mouse anti-RSV blend FITC-labelled monoclonal antibody (Chemicon International, Temecula, CA). Each coverslip was observed under fluorescence microscopy and the number of fluorescent cells per coverslip determined; each condition was assayed in triplicate. Data is expressed as infectious units/ml ± SD; an infectious unit is defined as the component within the viral suspension that results in the detectable infection of a single cell in the confluent monolayer. We have shown previously that infectious units are linear with respect to viral dilution over the range utilized in these experiments (r^2 = 0.987; ref. 27) and have been shown to correlate with plaque-forming units over a wide range of viral concentrations (31). No toxicity to the HEp-2 monolayer was observed at any of the ribonuclease concentrations indicated.

Table 1. Dose-dependent reduction in RSV-B infectivity (infectious U/ml) in response to rhECP alone

rhECP (nM)	Infectious U/ml	RNase activity (pmol/min)	Fold reduction viral titer
0	4183 ± 180	0.8	–
62.5	4338 ± 166	0.8	–
125	3145 ± 106*	1.2	1.3
250	1983 ± 103**	1.9	2.1
500	660 ± 28**	3.6	6.3
500 + 400 U RI[a]	1687 ± 120	2.0	2.5

RSV-B infectivity was determined by quantitative shell vial assay as described (27,31); values shown are averages of duplicate samples ± SD. Ribonuclease activity (pmol acid-soluble ribonucleotides generated per min was determined using 10 µl aliquots of viral supernatant to which rhECP was added to the concentrations indicated, with time points assayed in triplicate.
[a]Abbreviation RI refers to placental ribonuclease inhibitor. Values shown differ significantly from baseline ([rhECP] = 0) at the **P* < 0.05 and ***P* < 0.01 levels.

Table 2. Reduction in RSV-B infectivity (infectious U/ml) in response to rhEDN alone and in combination with rhECP

rhEDN (nM)	rhECP (nM)	Infectious U/ml	RNase activity (pmol/min)	Fold reduction viral titer
0	0	4183 ± 180	0.8	–
500	0	78 ± 25**	201	54
500	62.5	100 ± 28**	185	42
500	125	125 ± 0**	206	33
500	250	138 ± 11**	217	30
500	500	105 ± 21**	229	39

Measurements of both infectivity and ribonuclease activity (pmol/min) are as described in the legend to Table 1. Values shown differ significantly from baseline ([rhEDN] = [rhECP] = 0) at the (**)*P* < 0.01 level.

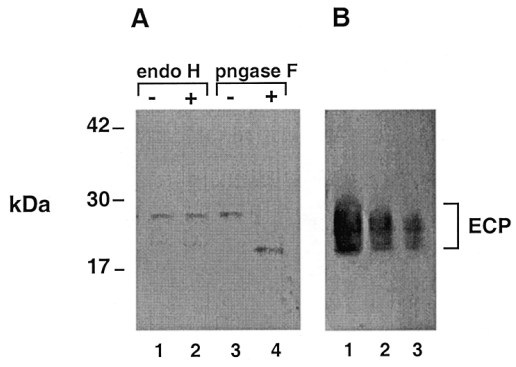

Figure 1. (**A**) Western blot probed with M2 mAb demonstrating electrophoretic mobility and glycosylation of recombinant human ECP (rhECP) isolated from supernatants from baculovirus-infected Sf9 cells. Purified protein in lanes 2 and 4 were subjected to deglycosylation with endoglycosidase H (endo H) and endoglycosidase F (pngase F), respectively. (**B**) Western blot probed with polyclonal anti-ECP antiserum demonstrating electrophoretic mobility of native ECP detected in an extract from human peripheral blood eosinophils.

RESULTS

Electrophoretic mobility and glycosylation of rhECP

The electrophoretic mobility of rhECP prepared using the baculovirus expression system is shown in Figure 1A. RhECP migrates as a single species of molecular mass of 22 kDa, similar to the most extensively glycosylated form of the native immunoreactive protein (Fig. 1B; 29). Upon treatment with PNGase F (but not endo H), rhECP is deglycosylated to a single species of molecular mass ~18 kDa, suggesting substitution with complex carbohydrates at one or more of its three potential N-glycosylation sites (5–8). The activity of endo H was confirmed by digestion of immature forms of the recombinant protein identified in lysates of infected Sf9 cells (data not shown).

Ribonuclease activity of rhECP

Shown in Figure 2A is a double reciprocal (Lineweaver–Burk) plot comprised of initial rates of reaction (1/V) in which acid-soluble ribonucleotides generated per unit time (pmol/s) at the given enzyme concentration [E] were measured at varying substrate concentrations (1/[S]). The catalytic constants for baculovirus-derived rhECP include k_{cat} = 0.100 s^{-1}, K_m = 1.9 µM and the catalytic efficiency $k_{cat}/K_m = 4.9 \times 10^4$ $M^{-1}s^{-1}$. Surprisingly, the values obtained for k_{cat}, and thus for catalytic efficiency k_{cat}/K_m, for the baculovirus-derived form of rhECP differed dramatically from those obtained previously for rhECP prepared in bacteria (k_{cat} = 0.0024 s^{-1}, K_m = 4.1 µM, $k_{cat}/K_m = 0.59 \times 10^3$ $M^{-1}s^{-1}$; ref. 30). The relationship between glycosylation state and catalytic activity will be considered further in the Discussion.

RI is a cytosolic protein with high affinity for many RNase A-family ribonucleases (32), with particularly strong interactions reported between RI and RNase A (33,34) and RI and EDN/RNase 2 (35). Shown in Figure 2B is a bar graph demonstrating initial rates of reaction fixed enzyme (rhECP) and substrate (tRNA) concentrations in the presence of increasing concentrations of a

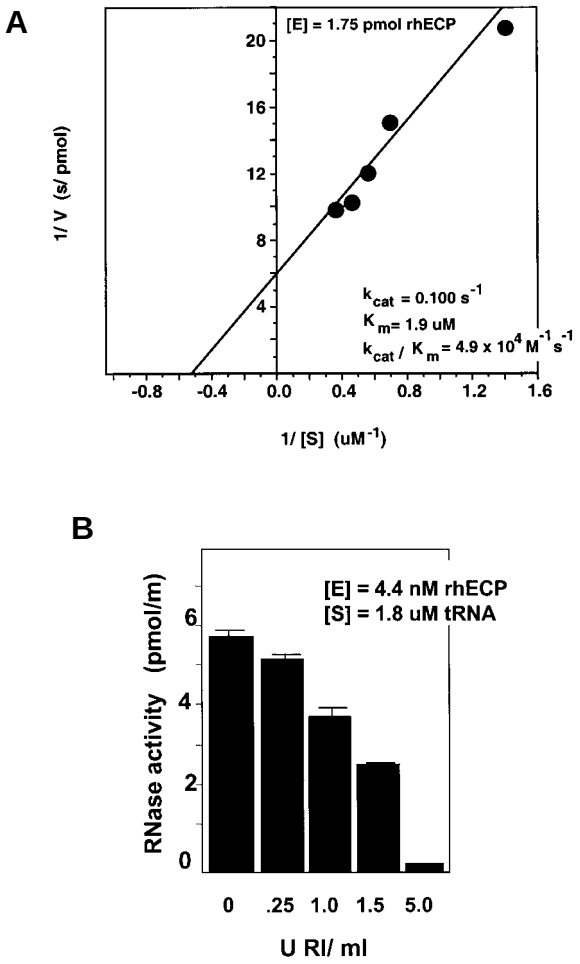

Figure 2. (**A**) Double reciprocal (Lineweaver–Burk) plots depicting rates of ribonuclease activity (pmol soluble ribonucleotides generated/s) at varying substrate concentrations (μM yeast tRNA) determined for baculovirus-derived rhECP. Catalytic constants K_m and k_{cat} were determined from *x* and *y* intercepts as previously described (12); $r^2 = 0.97$. (**B**) Ribonuclease activity (pmol/min) at single enzyme/substrate concentrations in the presence of increasing concentrations of placental RI.

commercial preparation of placental RI. These results demonstrate that rhECP is sensitive to the inhibitory effects of RI.

Antiviral activity of rhECP

We have shown previously that rhEDN promotes a dose-dependent, ribonuclease-dependent decrease in RSV-B infectivity when introduced directly into viral suspensions. In Tables 1 and 2, the antiviral activities of rhECP and rhEDN, separately and in combination, are presented. Despite the fact that ECP is more cationic, and generally more toxic than EDN (1,2), we found that rhECP is nearly 10-fold less effective than rhEDN in reducing the infectivity of RSV-B on a molar basis, with 500 nM rhECP reducing infectivity ~6-fold, as compared to the 54-fold observed with 500 nM rhEDN. The antiviral activity of rhECP is reduced in the presence of RI (Table 1); the concentration of RI used (0.8 U/pmol enzyme) was relatively low, enough so that a complete inhibition of rhECP woud not be expected. Interestingly, the combinations of rhECP (62.5–500 nM) with 500 nM rhEDN displayed no synergistic nor any additive antiviral effect (Table 2; see Discussion).

While the data in Tables 1 and 2 would suggest that antiviral activity correlates with increased ribonuclease activity (3.6 pmol/min from 500 nM rhECP versus 201 pmol/min from 500 nM rhEDN), previous work (27) as well as the data presented in Table 3 indicate that ribonuclease activity is essential but not sufficient to explain the overall effectiveness of EDN and ECP. RNase A (bovine RNase 1), the prototype of this family, is a potent ribonuclease with no apparent antiviral activity even at concentrations as high as 4000 nM. Onconase, an amphibian ribonuclease that has low catalytic activity but which has antitumor and antiviral activity against intracellular forms of HIV (36) also has no direct antiviral activity against RSV-B in this assay. Human RNase k6, a ribonuclease with moderate catalytic activity that is closely related to EDN and ECP (50% amino acid sequence similarity; 37) likewise promotes no loss of RSV-B infectivity.

DISCUSSION

In this work, we present a biochemical and enzymatic characterization of rhECP/RNase 3 prepared in Sf9 cells, and present evidence demonstrating that this protein has antiviral activity against RSV-B *in vitro*. Although ribonuclease activity appears to be crucial to the antiviral activity promoted by both rhECP and rhEDN, we have found that other members of the RNase A ribonuclease family, including the closely-related human RNase k6, have no direct antiviral activity in this assay. Taken together, these results suggest that EDN and ECP, the two most rapidly evolving coding sequences known among primates (14), may be responding to constraints promoting increased antiviral activity specifically within this lineage.

Table 3. Dose-dependent reduction in RSV-B infectivity (infectious U/ml; triplicates ± SD) in response to rhRNase k6, RNase A and onconase

Agent added (nM)	Infectious U/ml	RNase activity (pmol/min)
rhRNase k6		
0	2452 ± 108	0.7
150	2405 ± 359	21.2
300	2562 ± 115	59.2
500	2500 ± 133	69.5
RNase A[a]		
0	4360 ± 252	0.78
400	4020 ± 342	1450
4000	4380 ± 284	17840
Onconase		
0	3273 ± 240	0.62
40	3203 ± 240	0.70
400	2995 ± 131	0.68
4000	3060 ± 143	1.0

Measurements of RSV-B infectivity and ribonuclease assays are as described in the legend to Table 1.

[a]Values reported previously (27), included here for comparison.

Prior to this time, we have worked extensively with rhECP prepared as a recombinant secretory protein in bacteria (11,30). This non-glycosylated form of rhECP was both toxic and enzymatically active with a catalytic efficiency (K_m/k_{cat}) measured at 0.59×10^3 $M^{-1}s^{-1}$ (30). The rhECP prepared as a baculovirus-derived secretory protein is N-glycosylated, with electrophoretic mobility similar to that of the most extensively glycosylated form of the native protein (29). The ribonucleolytic activity of this glycosylated form of rhECP is nearly 100-fold greater than that prepared in bacteria ($K_m/k_{cat} = 4.9 \times 10^4$ $M^{-1}s^{-1}$). This finding itself is not unusual, as there are a number of proteins (both recombinant and native) in which biological activity has been shown to be enhanced by N-glycosylation (38–45). ECP has three potential sites for N-linked glycosylation; the observation that rhECP migrates as a single band by polyacrylamide gel electrophoresis suggests a homogeneous pattern of glycosylation, but this has not been established with certainty. Similarly, the molecular basis for enhanced ribonuclease activity has not yet been clarified, but the presence of negatively-charged glycosyl groups may alter the pattern of intracellular protein folding, and will also effectively reduce the net positive charge characteristic of the mature ECP polypeptide (calculated pI = 11.8) which may in turn alter the way in which rhECP interacts with its negatively-charged polyribonucleotide substrate. We have also examined the interaction of rhECP with placental RI, a ubiquitous cytoplasmic protein with high affinity (K_is from ~10^{-14} to 10^{-16}) for several of the RNase A family ribonucleases (32). Although its biological role has not been clarified, RI may serve to protect intracellular RNA from degradation by secretory ribonucleases such as ECP that found their way into the cell cytoplasm (32).

As noted earlier, we have recently begun to explore the relationships that connect eosinophils, eosinophil ribonucleases and respiratory viral pathogens associated with pulmonary eosinophilia, such as RSV. Specifically, we have begun to consider whether eosinophils, with well-characterized destructive pro-inflammatory features, might also provide some degree of innate host defense against these viruses. To this end, we have shown in our previous studies that eosinophils promote a dose-dependent reduction in RSV-B infectivity, an effect directly dependent on the actions of the eosinophil secretory ribonucleases. We have also shown that rhEDN can act alone as an antiviral agent, promoting ribonuclease-dependent destruction of the viral genome within the extracellular milieu (27). In this work we demonstrate that rhECP also has direct antiviral activity, activity that is at least in part also dependent on ribonuclease activity. Interestingly, this direct antiviral activity appears to be a unique feature of the eosinophil ribonucleases. The prototype of this family, RNase A, is a potent ribonuclease with no direct antiviral activity in this assay, and onconase, an amphibian ribonuclease that has been shown to mediate antiviral activity against intracellular forms of HIV (36) was similarly ineffective. Perhaps most intriguing was the observation that human RNase k6, a moderately powerful ribonuclease with 50% amino acid sequence similarity to EDN/ECP, prepared in an identical fashion from supernatants from baculovirus-infected Sf9 cells, also proved to be unable to reduce infectivity of RSV-B. As all of these ribonucleases are capable of generalized RNA degradation, it would seem as though the eosinophil ribonucleases must possess some unique, specific features apart from ribonuclease activity that permit them to penetrate the viral capsid to gain access to the viral RNA genome. The results of the EDN/ECP combination experiments permit some speculation as to how this might occur. We were initially surprised to find that the EDN/ECP combinations resulted in no synergy nor any observable additivity. Upon further consideration we realized that these results suggested that EDN might be binding to a specific, saturable target molecule, most likely on the viral surface. While ECP can promote some antiviral activity on its own, its reduced antiviral toxicity may be due to lower affinity for this specific target. When presented in the combinations shown, the entire antiviral effect observed is that promoted by rhEDN, the higher-affinity ribonuclease. While this mechanism remains speculative, we have obtained preliminary data suggesting that the ribonucleolytically inactivated form of rhEDN, rhEDNdK[38], previously shown to have no activity as an antiviral agent (27), is capable of blocking the antiviral activity of ribonucleolytically active rhEDN when presented in equimolar amounts (J.B.Domachowske and H.F.Rosenberg, manuscript in review). While these results provide evidence in support of a specific, saturable interaction between rhEDN and an as yet unidentified target molecule, the nature of this entity and the specifics of the proposed interaction both remain to be clarified.

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