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Eosinophils, Ribonucleases and Host Defense: Solving the Puzzle

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Abstract

The eosinophil ribonucleases eosinophil-derived neurotoxin (EDN/RNase 2) and eosinophil cationic protein (ECP/RNase 3) are among the major secretory effector proteins of human eosinophilic leukocytes, cells whose role in host defense remains controversial and poorly understood. We have recently described the unusual manner in which this ribonuclease lineage has evolved, with extraordinary diversification observed in primate as well as in rodent EDNs and ECPs. The results of our evolutionary studies suggest that the EDN/ECP ribonucleases are in the process of being tailored for a specific, ribonuclease-related goal. With this in mind, we have begun to look carefully at some of the intriguing associations that link eosinophils and their ribonucleases to disease caused by the single-stranded RNA viral pathogen, respiratory syncytial virus (RSV). Recent work in our laboratory has demonstrated that eosinophils can mediate a direct, ribonuclease-dependent reduction in infectivity of RSV in vitro, and that EDN can function alone as an independent antiviral agent. The results of this work have led us to consider the possibility that the EDN/ECP ribonucleases represent a heretofore unrecognized element of innate and specific antiviral host defense.

Key Words

Eosinophilis
Ribonucleases
Antiviral agents
Host defense

Introduction

Eosinophils, ribonucleases, and host defense, at first glance, seem to be an unlikely trio. Eosinophils are studied primarily by allergists and are best known for their role in promoting the tissue damage and bronchospasm characteristic of respiratory allergies and asthma. Ribonucleases, on the other hand, have up until recently been the property of chemists, whose interests were focused on folding pathways and catalysis. Host defense, a broad-based area of immunology, has concentrated on the func-

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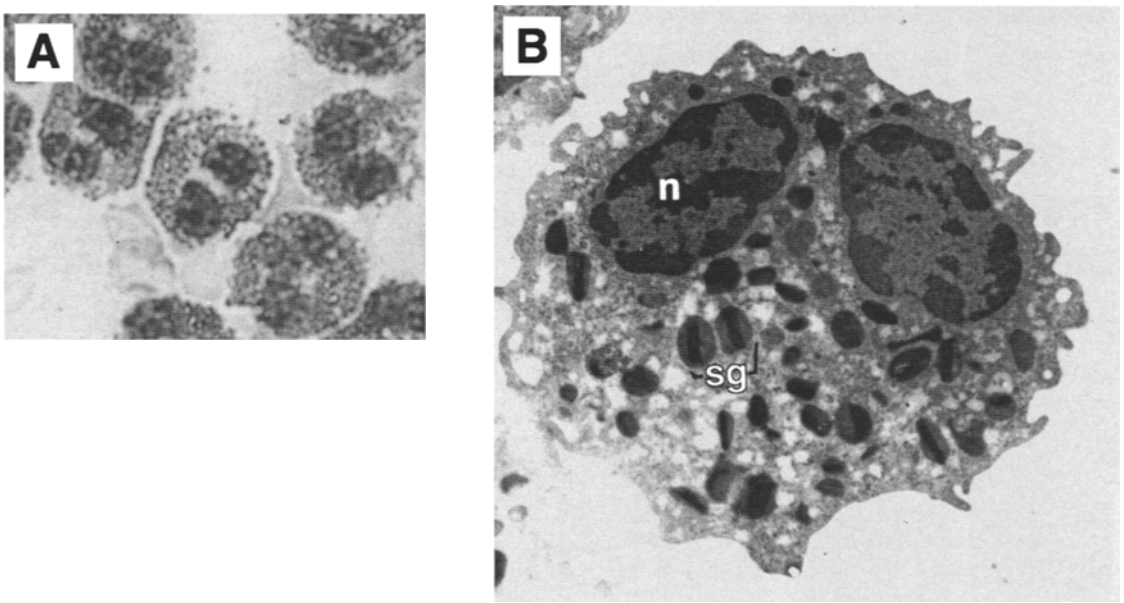

Fig. 1. **(A)** Light microscopic view of human eosinophilic leukocytes isolated from peripheral blood by anti-CD16 negative selection. **(B)** Electron microscopic view of a single human eosinophil. The characteristic bilobed nucleus and the large specific granules with electron dense cores are prominent. Photograph courtesy of Dr. Arne Egesten, University of Lund, Malmo, Sweden. Photographs reprinted with permission from Rosenberg HF, Chapter 5: Eosinophils, in Inflammation: basic principles and clinical correlates, Gallin JI, Snyderman, R, eds., Lippincott Williams & Wilkins, Philadelphia, PA, 1999, pp. 61–76.

tion of the better-known cellular components of the immune response, including lymphocytes, neutrophils, and macrophages. What we intend to do here is to begin by discussing the history of eosinophils and their role in host defense, and then go on to highlight the findings that have linked ribonucleases to eosinophil physiology. We will include our studies on the unusual pattern of evolution of these eosinophil ribonucleases and demonstrate how these molecular evolutionary studies have led us to consider several novel hypotheses regarding the role of eosinophils and their ribonucleases in host defense against a previously unrecognized group of target pathogens, specifically, single-stranded RNA viruses.

The Enigmatic Eosinophil

The human eosinophilic leukocyte, both light and electron microscopic views, is shown in Fig. 1. The eosinophil is a cell of the granulocyte lineage known primarily for its unique and distinctive morphology. Evident in the images shown is its characteristic bilobed nucleus and large refractile cytoplasmic secretory granules. Similar to other granulocytes, eosinophils are produced in the bone marrow from pluripotent stem cells and are released into the peripheral blood where they persist in circulation for several days. Thereafter, eosinophils migrate into the tissues—primarily of the pulmonary, gastrointestinal, and genitourinary tracts—where they survive for about a month. Paul Ehrlich was the first to describe eosinophils in print as "cells...so richly endowed with granules that their entire protoplasm stained violet" *(1)*. Interestingly, even today, little else about eosinophils can be said with such assurance. Part of the enigma of the eosinophil stems from the fact that, as noted

Table 1. Diseases Associated with Peripheral Blood and/or Tissue Eosinophilia

Parasitic diseases	**Gastrointestinal diseases**
Helminth infections	Inflammatory bowel disease
Visceral larval migrans	Eosinophilic gastroenteritis
Tropical eosinophilia	**Respiratory diseases**
Dermatologic diseases	Asthma
Atopic dermatitis	Allergic rhinitis
Urticaria	Eosinophilic pneumonia
Immunodeficiencies	Loeffler's syndrome
Hyper-IgE (Jobs') syndrome	Aspergillosis
Wiskott-Aldrich syndrome	**Viral disease**
Rheumatologic diseases	Human immunodeficiency virus-1
Hypersensitivity vasculitis	Respiratory syncytial virus
Eosinophilic fasciitis	**Graft-vs-host disease**
Myeloproliferative and neoplastic diseases	**Drug/Toxin reactions**
Idiopathic hypereosinophilic syndrome (IHES)	Cytokine therapies (IL-2, GM-CSF)
Kimura's disease	Toxic oil syndrome
Eosinophil leukemia	Eosinophilia-myalgia syndrome
Hodgkins' disease	

above, most of the research on these cells has focused on their detrimental features *(2)*. Table 1 is a list of conditions, syndromes, and diseases associated with blood and/or tissue eosinophilia; in nearly every case, the eosinophil plays the role of the villain. Meanwhile, the role of eosinophils in promoting host defense remains a subject of great controversy. Perhaps the most dramatic illustration of this point is the findings of Sher and colleagues *(3)* demonstrating that, contrary to accepted wisdom, eosinophils may actually not be major components of host defense against helminthic parasites in vivo; numerous recent studies have confirmed or refuted this point *(4–10)*, which remains perhaps the most controversial subject in eosinophil research today. Other positive roles suggested for eosinophils currently under consideration in the literature include wound healing *(11,12)* and antigen presentation *(13–16)*. Although there may not be one, all-encompassing answer to the question of a positive role for eosinophils, in our laboratory we have begun to look at some of the intriguing associations that link eosinophils to diseases caused by respiratory viral pathogens, most notably respiratory syncytial virus (RSV). As in other cases, eosinophils are currently perceived as the villains of RSV disease, and have been shown to contribute to the bronchospasm, wheezing, and tissue damage characteristic of this condition. Based on our recent findings, we believe that the eosinophilic inflammation characteristic of this disease may actually represent a double-edged sword, as eosinophils, via the actions of their unique secretory ribonucleases can, and perhaps do, provide some level of innate host defense against this viral pathogen.

Eosinophils and Ribonucleases

The cytoplasmic granules of the human eosinophil were recognized as containing ribonuclease activity by Archer and Hirsch as early as 1963, although the full significance of this observation was not appreciated at that point *(17)*. The granules were later shown to contain four major secretory effector proteins,

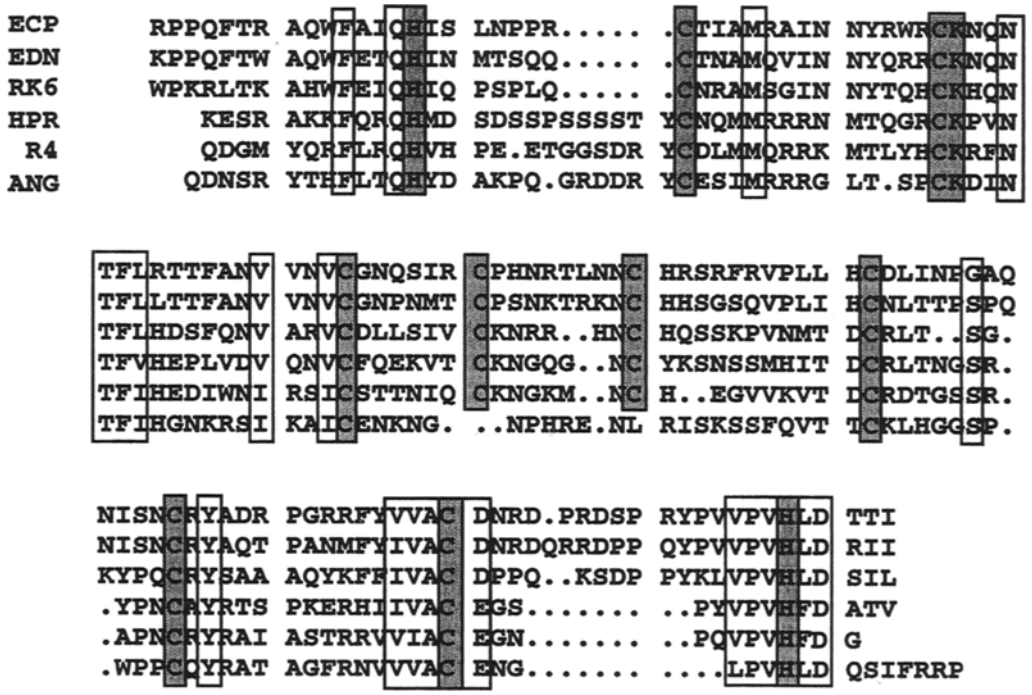

Fig. 2. Alignment of the amino acid sequences of the six known human members of the Ribonuclease A gene superfamily. The shaded boxes enclose the eight cysteines and catalytic lysine and histidines that are characteristic of members of this gene superfamily; the open boxes enclose additional conserved residues. Reprinted with permission from: Rosenberg HF, Dyer KD: Molecular cloning and characterization of a novel human ribonuclease (RNase k6): increasing diversity in the enlarging ribonuclease gene family. Nucleic Acids Res. 1996;24:3507-3513, Oxford University Press.

including two originally described by Olsson and colleagues *(18,19)* and Durack and colleagues *(20,21)* and given the names eosinophil cationic protein (ECP) based on its high apparent net charge, and eosinophil-derived neurotoxin (EDN), based on its observed toxicity to rabbit Purkinje cells, respectively. Gleich and colleagues *(22)* were the first to purify ECP and EDN and to remark upon the similarity of their amino terminal sequences to that of bovine Ribonuclease A. Molecular cloning of both EDN and ECP confirmed these proteins as members of a larger group, namely the emerging Ribonuclease (RNase) A gene superfamily *(23–26)*; Slifman and colleagues *(27)* and Gullberg and colleagues *(28)* demonstrated that EDN and ECP were both generalized ribonucleases with activity against standard polymeric RNA substrates. At current count, the human genome contains six unique sequences encoding RNase A superfamily ribonucleases (Fig. 2), including the eosinophil ribonucleases EDN (RNase 2) and ECP (RNase 3), pancreatic ribonuclease (RNase 1), RNase 4, angiogenin (RNase 5), and RNase 6, all of which map to the long (q) arm of chromosome 14 *(29)*. Among features common to all members of this family, EDN and ECP have amino terminal signal sequences, eight spaced cysteines that form four disulfide bonds in the correctly folded, catalytically active form of the protein, and two histidines and one lysine (the latter within a conserved motif) that comprise the active site.

Ribonuclease Activity and Cytotoxicity

Given that ECP and EDN are ribonucleases, the obvious question emerges: Does ribonuclease activity play a role in any of the characterized functions of these eosinophil granule proteins? Two groups working independently came to the conclusion that ribonuclease activity was essential to the neurotoxicity of EDN *(30,31)*, but given the unlikelihood of this representing a physiologic phenomenon, the conclusions from this with respect to eosinophil function remain unclear. ECP had been characterized as a generalized cytotoxin, with activity against parasites, bacteria, and mammalian cells *(32,33)* via what was initially characterized a membrane-disruptive lytic mechanism *(34)*. We began by asking a simple question: Does ribonuclease activity contribute to the observed cytotoxicity of ECP?

Much to our surprise, the answer was no. To perform this work, we prepared recombinant ECP, both wild-type and with mutations in two of the three aforementioned catalytic residues. As expected, the wild-type was ribonucleolytically active, while the mutant form was not. Yet both forms were equally effective at reducing the number of colony forming units of a characterized target strain of *Staphylococcus aureus (35)*. Similar results were obtained by Molina and colleagues *(36)* in their study of the anti-helminth activity of native ECP. The results of this work—that ribonuclease activity was not essential for cytotoxicity—was not only surprising but counterintuitive from an evolutionary perspective. If ribonuclease activity *per se* is not required for function, what are the constraints that have permitted this activity to be retained intact?

Evolution of Primate Eosinophil Ribonucleases

At about this time, several of our colleagues urged us to consider studying this in vivo, via targeted gene disruption of these eosinophil ribonucleases in mice. However, we found ourselves unable to identify genes orthologous to EDN or ECP in mouse genomic DNA, even under the least stringent of DNA–DNA hybridization conditions. On further investigation, we were surprised to discover that sequences homologous to EDN and ECP could be detected only in primate genomes [Fig. 3 *(37)*]. We proceeded to isolate these (intronless) coding sequences from several nonhuman primates, and found that EDN and ECP arose as a gene pair rather recently, some time after the divergence of the Old World from the New World monkeys, a date estimated at ~50 million years ago. We also noted that, since duplication, the genes encoding EDN and ECP have been diverging at a very rapid pace, with nearly 30% amino acid sequence divergence noted between human and New World monkey orthologs of the EDN gene. To put this in some perspective, most human–mouse coding pairs differ by no more than 10–15% *(38)*, with the divergence between these two mammalian species estimated to have occurred ~80 million years ago. Upon a thorough analysis of the GenBank database, we found that EDN and ECP were incorporating nonsilent (nonsynonymous) mutations at rates exceeding those of all other functional coding sequences studied among primates. Most significant to us was the fact that, despite the rapid rate at which these sequences were incorporating mutations, each retained the eight cysteines, the two histidines, and the catalytic lysine, all features that are crucial to maintaining ribonuclease activity among members of this gene superfamily. Zhang and colleagues *(39)* have provided a more thorough evolutionary analysis of this lineage, and have concluded that the evolution of ECP represents one of the rare examples of positive (Darwinian) selection contributing to diversity at the molecular level. We have evaluated the catalytic activity of the various primate orthologs *(40,41)* and found that the recombinant version of the New World

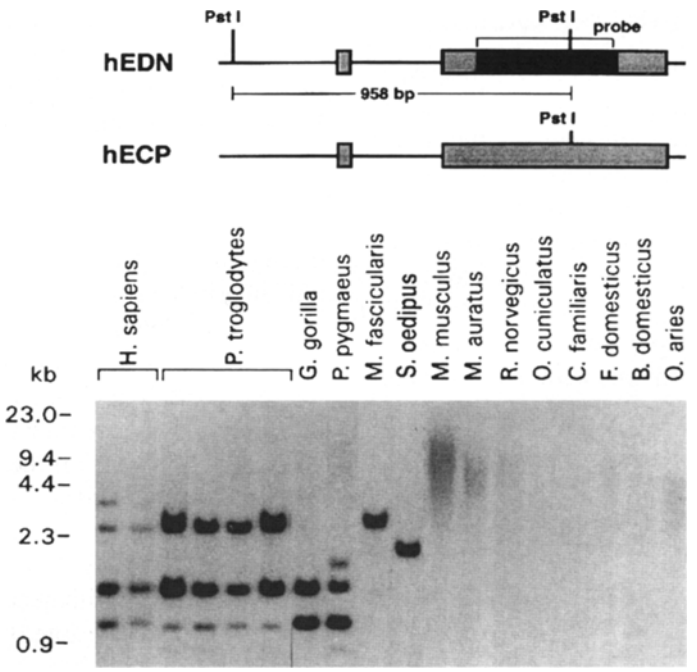

Fig. 3. Above, restriction maps of hEDN and hECP. The (intronless) coding sequence of EDN (shown in black) was used to probe the genomic blot shown in below. Below, Pst I- digested mammalian genomic DNAs probed with the coding sequence of EDN. The genus/species identification is noted above each lane. Lanes 1–10 are from the order Primata; lanes 11–13, from the order Rodentia; lane 14, the order Lagomorpha; lanes 15–16, the order Carnivora; lanes 17–18, order Ariodactyla. Reprinted with permission from: Rosenberg HF, Dyer KD, Tiffany HL, Gonzalez M. Rapid evolution of a unique family of primate ribonuclease genes. Nature Genet 1995;10:219–223.

monkey single-sequence EDN/ECPs have significantly less ribonuclease activity than their higher primate counterparts. We have traced this differential activity in part to a tripeptide sequence present at the carboxy-terminus, a region not previously recognized as part of the catalytic site as it is currently defined *(41)*. Finally, while establishing the unusual evolutionary history of primate EDNs and ECPs, we have isolated and characterized a previously unidentified ribonuclease, RNase k6, a single copy gene in primate genomes that is the next most closely related ribonuclease to EDN and ECP *(42,43)*.

Taken together, these evolutionary studies lead us to the conclusion that ribonuclease activity must indeed be crucial to one or more important physiologic functions of EDN and ECP, and by extension, to the physiologic function of the eosinophilic leukocyte.

Evolution of Rodent Ribonucleases

Our initial hypothesis gained strength in light of several findings on the evolution of the EDN/ECP lineage in rodents. This work was initiated by Larson and colleagues *(44)* who identified mouse eosinophil ribonucleases (mEARs) 1 and 2 by amino acid sequencing of proteins isolated directly from mouse eosinophils. We have since identified six additional related ribonucleases in mice, forming what

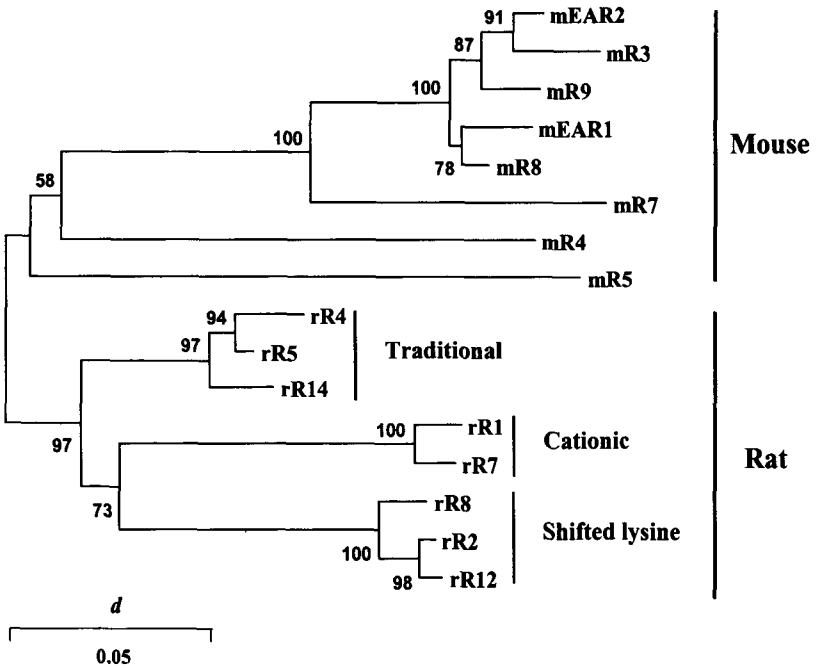

Fig. 4. Evolutionary dendrogram depicting the relationships among the mouse mR and rat rR cluster ribonucleases. The tree was constructed using the neighbor joining method with Kimura's distances *(65)* and is rooted on the interior branch between the two clusters using the sequences of human EDN and human ECP as outgroups. Values at the interior nodes are bootstrap percentages derived from 500 replications *(66)*. Reprinted with permission from: Singhania NA, Dyer KD, Zhang J, Deming MS, Bonville CA, Domachowske JB, Rosenberg HF. Rapid evolution of the Ribonuclease A superfamily: adaptive expansion of independent gene clusters in rats and mice. J Mol Evol, 1999;49:721–729, Springer-Verlag, Inc.

we call the mouse ribonuclease (mR) cluster. All mR cluster ribonucleases have the requisite cysteines and catalytic residues, and range from 60% to 94% amino acid sequence similarity to one another, demonstrating an unusual expansion of the EDN/ECP lineage within a single species. This has only been compounded by what has occurred in rats. We have recently identified an analogous ribonuclease cluster in rats, and have demonstrated that distinct ribonuclease clusters have evolved independently in inbred species of rats and mice (*46*; Fig. 4). The results shown here imply that within the last 10–15 million years, the estimated divergence time of rats and mice, each species has undergone at least eight independent gene duplication events. Clearly, both primate and rodent ribonucleases of this lineage are undergoing similar but independent styles of rapid evolution, suggesting the possibility that they may be responding to similar but independent evolutionary constraints.

Putting It All Together

EDN and ECP, both ribonucleases, are among the major effector proteins of the human eoisnophilic leukocytes, cells whose role in host defense remains a subject of controversy. The evolutionary studies suggest that the function of these proteins must somehow take into account the conserved ribonuclease activity. Furthermore, the astounding amount of evolutionary energy that has been expended on these specific ribonuclease lineages suggests not only that they are important, but that they are in the process of being tailored for a specific,

ribonuclease-related goal. This takes us to our most recent work, in which we have begun to explore the associations linking eosinophils and diseases caused by respiratory viral pathogens, most notably that caused by the single-stranded RNA virus, respiratory syncytial virus.

Eosinophils, Eosinophil Ribonucleases, and Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is a member of the Pneumovirus subfamily of the family Paramyxoviridae. Its genome is a molecule of single-stranded RNA consisting of ~15,200 nucleotides encoding 11 viral proteins *(47)*. RSV has been characterized as one of the most important respiratory pathogens worldwide, resulting in a seasonal bronchiolitis affecting primarily infants and toddlers, as well as the institutionalized elderly *(48,49)*. Some examples of the associations linking eosinophils and RSV—during severe RSV infection, eosinophils have been shown to be recruited to and degranulate into the lung parenchyma *(50,51)*, and RSV-infected epithelial cells express several prominent eosinophil chemoattractants *(52–56)*. RSV-infected epithelial cells also support the increased adherence of eosinophils *(57)*, and pulmonary eosinophilia has been observed in the Balb/c mouse model of RSV infection *(58,59)*. As in other disease states, eosinophils are universally perceived as the villains of RSV disease, and the role of eosinophils in promoting tissue damage and bronchospasm has been the focus of this area of research. Looking at this from a new perspective, we have begun to consider the possibility that eosinophils, via their unique collection of ribonucleases, may also have a beneficial role to play here, and that the eosinophilic inflammation associated with RSV disease may actually represent more of a "double-edged sword."

Our in vitro experiments have yielded intriguing results. When introduced into RSV viral suspensions, we found that purified eosinophils mediate a dose-dependent reduction in viral infectivity (Fig. 5; *50*). Furthermore, we have determined that this antiviral effect is mediated by the eosinophil secretory ribonucleases; addition of placental ribonuclease inhibitor completely eliminates the antiviral effect. Taken one step further, we have found that recombinant human EDN, the more powerful of the two eosinophil ribonucleases, acting alone, also mediates reduction in viral infectivity in vitro. Again, ribonuclease activity seems to be essential—EDN's antiviral activity is not shared with the ribonucleolytically inactivated point mutant of EDN, EDNdK^{38}. At the same time, this antiviral activity is not shared by RNase A, a highly potent ribonuclease that is the prototype of this gene family. It is also not shared with bovine seminal ribonuclease, nor with the amphibian ribonuclease onconase, both ribonucleases with antiviral activity characterized in different contexts *(61,62)*. These results imply that EDN interacts with its target (as yet unknown) with some degree of specificity above and beyond its role as a generalized ribonuclease, as discussed in the section on mechanism of action.

EDN and RSV: Implications for Therapy

As EDN clearly functions as an antiviral agent in a cell-culture based, in vitro assay system, it is intriguing to consider the possibility that it might function as an antiviral agent in a clinical setting. Working in its favor, EDN is a thermostable protein, with no toxicity to respiratory epithelial cells observed in culture *(63)* and a very short half-life in the bloodstream *(64)*. We envision the use of EDN and/or its derivatives as an aerosol agent to be administered as prophylaxis against (or treatment of) RSV and similar respiratory viral pathogens; it is conceivable that EDN might function synergistically with antiviral agents currently in use. Once we have a clearer picture of its mechanism of action,

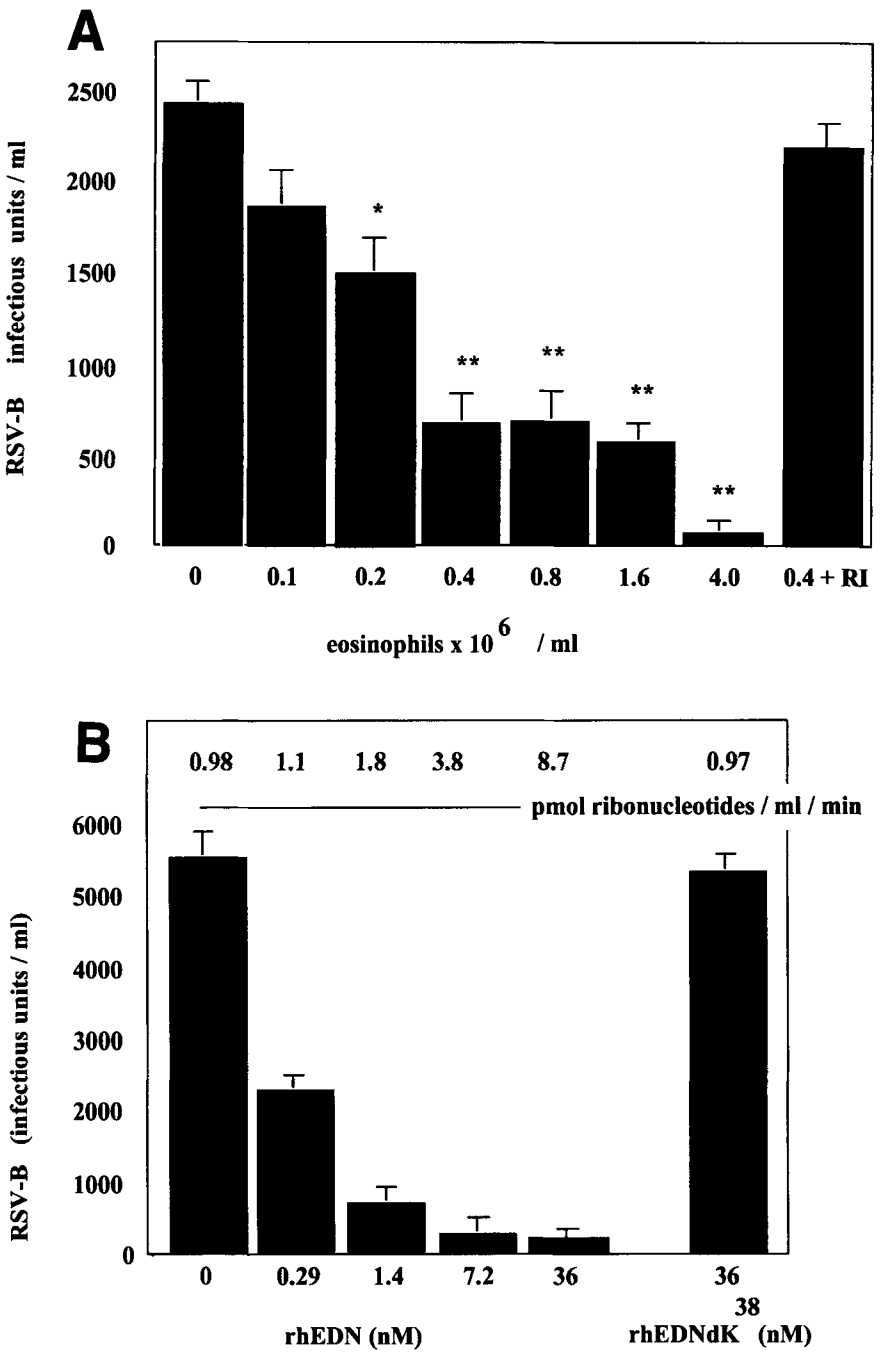

Fig. 5. (A) Dose-dependent reduction in infectivity of RSV-B in response to exposure to human eosinophilic leukocytes alone (0 to 4×10^6 /mL) or in the presence of placental ribonuclease inhibitor (200 U/mL), +/– standard deviation. (B) Dose-dependent reduction in infectivity of RSV-B in response to recombinant human EDN (rhEDN; 0–50 n*M*) and to ribonucleolytically inactivated point mutant of EDN (rhEDNdK[38]; 36 nM) +/– standard deviation. Reprinted with permission from: Domachowske JB, Dyer KD, Bonville CA, Rosenberg HF. Recombinant human eosinophil-derived neurotoxin / RNase 2 functions as an effective antiviral agent against respiratory syncytial virus. J Infect Dis 1998;177:1458–1464.

modifications designed to enhance its antiviral activity and/or improve tolerance can be envisioned (US Patent Serial No. 60/052,986).

Ribonucleases and RSV: Mechanism of Action

Certainly one of the most pertinent questions that emerges is that of mechanism of action. How does EDN mediate this antirival effect? Is EDN interacting with the virions alone, with a target on the cell surface, or both? Is EDN acting directly during infection, or is it creating perturbations that have some effect later down the line? How does the catalytic activity, apparently crucial to the antiviral effect, play a role in this mechanism of action? Although it is tempting to assume that there is a direct interaction between the ribonuclease and the viral RNA genome, the existence of such an interaction is not yet clear. Whereas these questions remain to be answered, we do have evidence demonstrating that the interaction between EDN and its target (be it cellular and/or viral) is both specific and saturable (*62*; Fig. 6). Specifically, when the antiviral activity of rhEDN is measured in the presence of increasing concentrations of the ribonucleolytically inactivated rhEDNdK[38], we observe a dose-dependent inhibition of the antiviral effect. In contrast, increasing concentrations of rhRNase k6, a ribonuclease with no antiviral activity in this assay, has no effect whatsoever on the drop in infectivity mediated by rhEDN. The implications of this result reach out in many directions. First, it would be difficult to consider a specific, saturable interaction between EDN and its target as anything but physiologically relevant. This single result stands in greatest support for our hypothesis regarding the double-edged sword of eosinophilic inflammation, and has suggested the existence of a specific target molecule to be identified. Second, even if the pharmacologic potential of EDN proves to be less than anticipated, the elements of specificity might be harnessed and linked to some other form of antiviral therapy. And finally, the suggestion of a specific interaction between a EDN and its as yet unidentified target provides support for our more general hypothesis regarding ribonuclease and viral pathogens, below.

Ribonucleases and Host Defense: a Hypothesis

We believe that the unusual evolutionary constraints acting on the EDN/ECP lineages of the RNase A gene superfamily have promoted the acquisition of specialized antiviral activity. We believe that, when we begin to look carefully, we will find that these rapidly evolving ribonucleases have diverged to interact specifically with independent targets relating to host defense against viral pathogens. The degree to which this hypothesis represents foresight or fantasy will emerge as our work continues.

Eosinophils and Host Defense Against Respiratory Viral Pathogens

The role of eosinophils and their ribonucleases in host defense again respiratory viral pathogens will require experimentation with relevant animal models. In this particular situation, the comparison problems are quite complex. The first issue is the fact that human eosinophils and murine eosinophils are quite different from one another, most pronouncedly so with respect to their component ribonucleases. The two mR cluster ribonucleases known to be associated with eosinophils—mEAR-1 and mEAR-2—have only about 50% amino acid sequence identity to their human counterparts. Further-

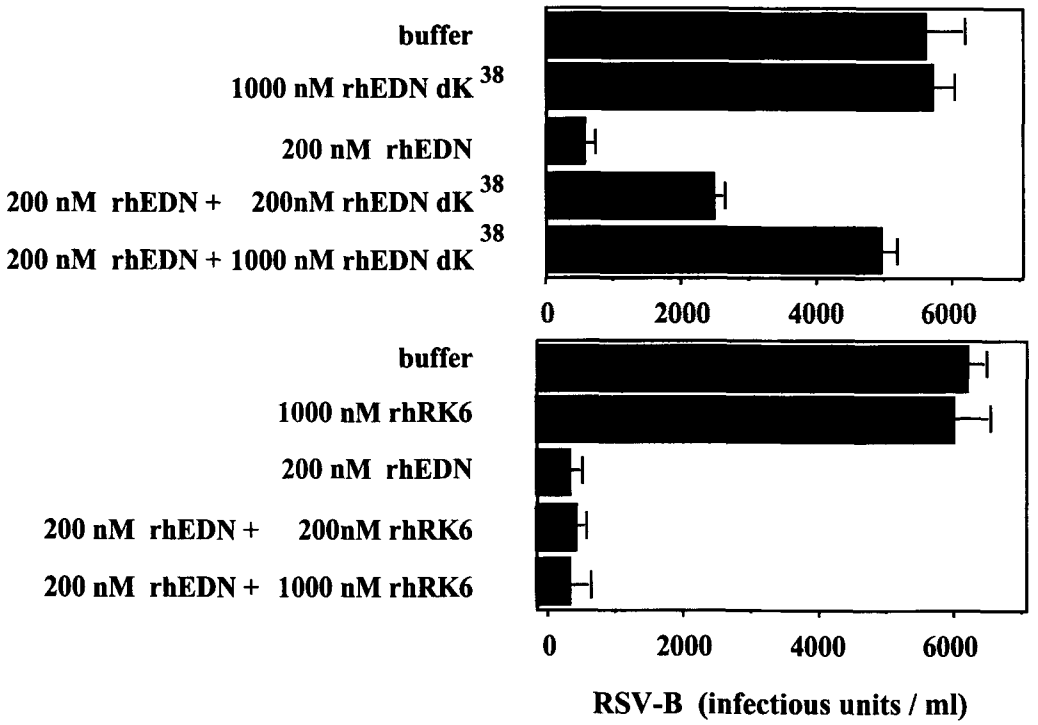

Fig. 6. Antiviral activity of rhEDN measured in the presence of increasing concentrations of the ribonucleolytically inactivated point mutant, rhEDNdK[38], or the related human ribonuclease, recombinant human RNase k6 (rhRK6) +/– standard deviation. The results of this experiment suggest that the interaction between rhEDN and its target is both specific and saturable. Reprinted with permission from: Domachowske JB, Bonville CA, Dyer KD, Rosenberg HF. Evolution of antiviral activity in the ribonuclease A gene superfamily: evidence for a specific interaction between eosinophil-derived neurotoxin (EDN / RNase 2) and respiratory syncytial virus. Nucleic Acids Res 1998;26:5327–5332, Oxford University Press.

more, RSV is not a mouse pathogen, and has extremely limited infectivity in this species. In order to address this question, we will need to examine the activity of murine ribonucleases against murine paramyxoviral pathogens in vitro, as well as to evaluate murine paramyxoviral pathogens for their ability to promote pulmonary eosinophilia. Armed with this knowledge, we can then approach cell ablation and gene ablation experiments in a more coherent and substantive way.

Conclusions

Our recent work suggests that eosinophils and their secretory ribonucleases may have a significant role to play in host defense against respiratory viral pathogens. In our work, we continue to address both the physiologic as well as the pharmacologic implications of these findings.

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