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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

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ABSTRACT

We have demonstrated that the human eosinophil-derived neurotoxin (EDN, RNase 2), a rapidly evolving secretory protein derived from eosinophilic leukocytes, mediates the ribonucleolytic destruction of extracellular virions of the single-stranded RNA virus respiratory syncytial virus (RSV). While RNase activity is crucial to antiviral activity, it is clearly not sufficient, as our results suggest that EDN has unique structural features apart from RNase activity that are necessary to promote antiviral activity. We demonstrate here that the interaction between EDN and extracellular virions of RSV is both saturatable and specific. Increasing concentrations of the antivirally inactivated, ribonucleolytically inactivated point mutant form of recombinant human EDN, rhEDNdK^{38}, inhibits rhEDN's antiviral activity, while increasing concentrations of the related RNase, recombinant human RNase k6, have no effect whatsoever. Interestingly, acquisition of antiviral activity parallels the evolutionary development of the primate EDN lineage, having emerged some time after the divergence of the Old World from the New World monkeys. Using this information, we created ribonucleolytically active chimeras of human and New World monkey orthologs of EDN and, by evaluating their antiviral activity, we have identified an N-terminal segment of human EDN that contains one or more of the sequence elements that mediate its specific interaction with RSV.

INTRODUCTION

The eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) are two RNase toxins secreted from the large cytoplasmic granules of human eosinophilic leukocytes. While EDN was named for its ability to promote the Gordon phenomenon (1,2), a non-physiological syndrome of cerebellar dysfunction associated with loss of Purkinje cells, its physiological function was until recently completely uncertain. Gleich and colleagues (3) were the first to note N-terminal sequence similarities shared by EDN, ECP and bovine pancreatic RNase (RNase A). Molecular cloning (4–7) confirmed both EDN and ECP as members of the enlarging RNase A gene superfamily, a group that also includes angiogenin and RNases 4 and 6 (8,9).

The evolutionary history of the genes of the EDN/ECP lineage suggest an inextricable link between these eosinophil proteins and RNase activity. Although the genes encoding primate EDNs and ECPs have incorporated non-silent mutations at rates exceeding those of all other coding sequences studied in primates, they maintain all structural and catalytic residues necessary for RNase activity (10). In addition, we have shown that the enhanced RNase activity was acquired some time after the divergence of the Old World and New World monkeys and has been retained in higher primate species (11,12).

Working on the hypothesis that RNase activity must be crucial in some way to EDN (and thus eosinophil) physiology, we have begun to explore the associations linking eosinophils, the eosinophil RNases and respiratory disease caused by the single-stranded RNA virus respiratory syncytial virus (RSV; family Paramyxoviridae). Among these associations, eosinophils are recruited to and degranulate into the lung parenchyma in response to RSV infection (13–15) and eosinophils have been shown to interact with and to be activated directly by RSV virions (16,17); several groups have reported that respiratory epithelial cells produce and secrete eosinophil chemoattractants in response to RSV infection (18–21). While most researchers currently regard eosinophils as the villains of RSV disease, we have begun to consider the possibility that eosinophilic inflammation may have beneficial as well as detrimental features and may represent more of a 'double-edged sword'. In support of this hypothesis, we have recently shown that eosinophils promote the destruction of extracellular virions of RSV *in vitro*, an antiviral effect that is reversed in the presence of soluble RNase inhibitor. Recombinant human EDN, acting alone, also promoted a significant reduction in viral infectivity, an effect that was not shared by the ribonucleolytically inactive form of the protein, rhEDNdK^{38} (22). Interestingly, while RNase activity is clearly crucial to antiviral activity, RNase activity alone is not sufficient; no antiviral activity was observed with the same and

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higher concentrations of several potent RNase A family RNases (22,23), suggesting that the antiviral activity depends in part on unique structural features characteristic of EDN alone.

In this work, we demonstrate the existence of such a structural feature, as we show that the interaction between EDN and extracellular virions of RSV is both specific and saturatable. We also show that acquisition of antiviral activity parallels the evolutionary development of the primate EDN gene lineage and, by constructing human/owl monkey EDN chimeras, we have identified an N-terminal segment of human EDN that contains one or more of these specific sequence elements.

MATERIALS AND METHODS

Preparation of recombinant RNases and RNase chimeras via bacterial expression

For the experiments described in Tables 1 and 5, recombinant proteins were prepared from cDNAs encoding EDNs of human (*Homo sapiens*; GenBank accession no. M24157), orangutan (*Pongo pygmaeus*; U24104), macaque (*Macaca fascicularis*; U24096), tamarin (*Saguinus oedipus*; U24099) and owl monkey (*Aotus trivirgatus*; U88827) in the pFCTS bacterial expression vector as described (11). With this system, recombinant RNases are directed to the bacterial periplasm and can be isolated in enzymatically active form. Culture, induction and isolation of recombinant protein via M2 agarose chromatography were as described previously (11). We have recently shown that limited induction with a reduced concentration of IPTG (1 µM) followed by bacterial freeze–thaw and sonication has permitted quantitative harvest of ribonucleolytically active recombinant protein, avoiding the losses necessitated by gentle periplasmic stripping (24). Human/owl monkey EDN chimeras were prepared by overlap PCR mutagenesis as described (12) and prepared as recombinant proteins via the pFCTS expression vector.

Preparation of recombinant RNases via baculovirus expression

For the experiments described in Tables 3 and 4, recombinant human RNase k6 (9; GenBank accession no. U64998), human EDN and human EDNdK^{38} (ribonucleolytically inactived EDN via conversion of the catalytic residue K38 to R; 11) were prepared from supernatants of *Spodoptera frugiperda* (Sf9) cells infected with recombinant baculoviral constructs. The recombinant baculoviral vectors included the full-length coding sequences of each of these three RNases inserted in-frame with the C-terminal FLAG octapeptide (analogous to the pFCTS expression constructs) into the *Bam*HI and *Xba*I sites of the pVL1393 transfer vector (Invitrogen, San Diego, CA). Three micrograms of recombinant vector and 0.5 µg linear wild-type baculovirus AcNPV (Pharmingen, San Diego, CA) were used to co-transfect Sf9 insect cells by the lipofectin method (Gibco BRL, Gaithersburg, MD). Polyhedrin-deficient recombinant viruses were selected and cloned by standard plaque assay and the protein product was confirmed by immunoblotting with the M2 monoclonal anti-FLAG antibody. Large scale production of each RNase was achieved by infecting 100 ml suspension cultures of Sf9 cells (2×10^6/ml) with recombinant virions. Supernatants containing each RNase were harvested at 96 h post-infection, at >60% cell lysis, and frozen at –80°C prior to use. Defrosted supernatants (100–200 ml) were dialyzed overnight at 4°C against 20 vol 50 mM Tris, pH 8.0, 1 mM NaCl. Recombinant RNases were initially concentrated by heparin–Sepharose FPLC chromatography and eluted via a salt gradient (1–1000 mM NaCl). Fractions containing recombinant protein were concentrated (Centricon 10; Amicon, Beverly, MA) and subjected to size fractionation (Superdex G-75; Pharmacia Biotech, Brussels, Belgium) in a 50 mM Tris, 150 mM NaCl mobile phase, resulting in purified protein by gel electrophoretic analysis. Protein concentration was determined by BCA assay (Pierce, Rockford, IL) against bovine serum albumin standards.

Determination of viral infectivity

The antiviral activity of each recombinant RNase versus extracellular virions of RSV group B was determined as previously described (22). Briefly, recombinant protein at the final concentrations indicated (or buffer control) was added directly to suspensions containing virions ($2–5 \times 10^3$ infectious units/ml) in culture medium (Iscove's Modified Dulbecco's Medium with 10% heat-inactivated fetal calf serum and 2 mM glutamine) and incubated with gentle rotation at room temperature. After a 2 h incubation, 200 µl of the suspension was used to infect target cells (human respiratory epithelial HEp-2) present in confluent monolayers ($3–4 \times 10^5$ cells/monolayer) on coverslips within a one dram shell vial (Viromed, Minneapolis, MN). After spin amplification (700 *g* at 22°C) and 16 h incubation (37°C, 5% CO_2), the primary infected cells were identified by immunofluorescent staining (mouse anti-RSV blend, FITC-labeled; Chemicon International, Temencula, CA). Data is presented as infectious units ± SD. We have defined the infectious unit as the active component of the viral suspension that results in the detectable infection of a single cell within the HEp-2 monolayer. We have shown (i) a linear relationship between infectious units detected by this method and viral dilution ($r^2 = 0.99$; 22) and (ii) that infectious units correspond to plaque-forming units generated by the more traditional plaque-forming assay over the range of concentrations utilized in these experiments (25). No toxicity to the HEp-2 monolayer was observed at any of the RNase concentrations indicated. In the specific blocking assay (Table 3), rhEDN was added after either rhEDNdK^{38} or rhRNase k6 in the combinations as indicated.

RNase assay

The RNase assay has been described previously in detail (11,12,26). Briefly, the concentration of perchloric acid-soluble ribonucleotides generated from acid-insoluble yeast tRNA (Sigma, St Louis, MO) in a 40 mM sodium phosphate, pH 7.5, buffer by a given quantity of RNase was measured spectrophotometrically at 260 nm, with the conversion to pmol tRNA as described (11). In the experiments described here, 10 µl of viral suspension, to which 100 nM recombinant RNase had been added, was included in a 0.8 ml reaction volume with 10 µl of 4 mg/ml yeast tRNA. Presented here (Tables 1, 3 and 5) are initial rates calculated from four sequential time points, each assayed in triplicate.

Evolutionary analysis

Analysis of the molecular evolution of primate RNases (Figure 1 and Table 2) was based on several previous studies (10,11,27). Values for synonymous and non-synonymous nucleotide substitution were calculated by the method described by Nei and Gojobori (28). Values for non-synonymous (K_a) and synonymous (K_s) substitution are calculated by the equations $K_a = N_d/N$ and $K_s = S_d/S$,

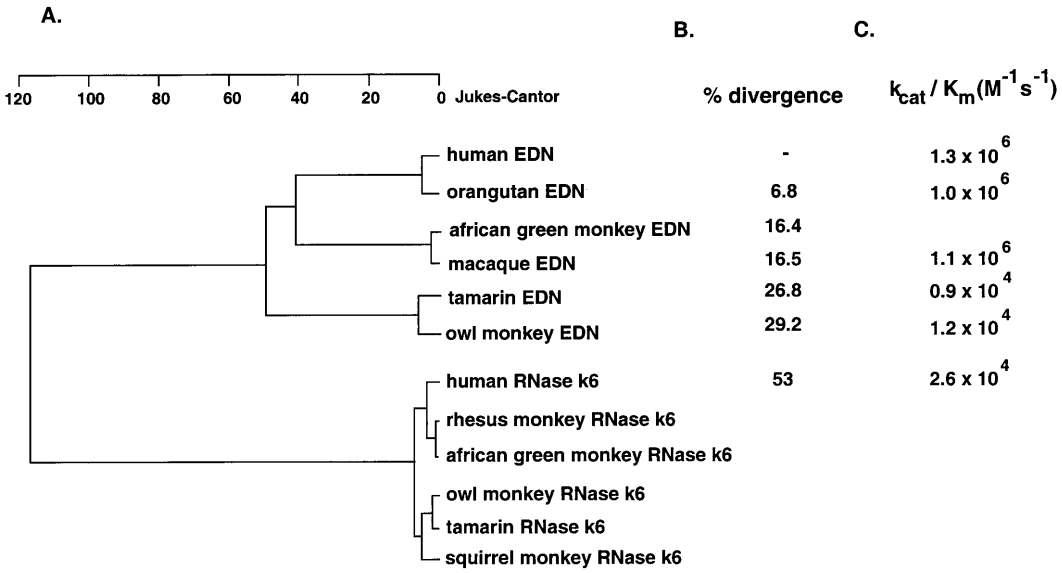

Figure 1. (**A**) Dendrogram depicting the relationships among human and non-human primate EDNs and RNase k6s. GenBank accession nos of primate sequences are listed in Materials and Methods and in Deming *et al.* (36), save for African green monkey EDN, GenBank accession no. AF078127. Distances calculated by a version of the Jukes–Cantor method as per the DISTANCES algorithm of the Wisconsin Genetics Computer Group (WGCG) program on-line at the National Institutes of Health. Signal sequences were not included in the calculations. The 'ancestral' sequence noted at the node demarcating the divergence of the Old World from the New World monkeys was inferred by maximum parsimony included 263 informative sites. (**B**) Amino acid sequence divergences (1 – % identity) as per the GAP algorithm of WGCG (signal sequences not included). (**C**) Catalytic constants determined via double reciprocal plots of RNase activities of each cDNA-encoded protein expressed in recombinant form (data obtained from refs 9,11,12).

respectively, where *N* and *S* are the probabilities that any single nucleotide change in a given coding sequence will be either non-silent (*N*) or silent (*S*) multiplied by the total number of nucleotides. N_d and S_d are determined via direct comparison of the sequences with one another, with each nucleotide sequence change scored appropriately. The data were also analyzed using the method described by Li (29,30), which yielded analogous results.

RESULTS

Evolution of primate RNase genes

We have previously shown that the genes encoding the two eosinophil RNases, EDN and ECP, are incorporating non-silent mutations at rates exceeding all other functional coding sequences studied among primates (10); Zhang and colleagues (27) have provided evidence for Darwinian selection at the molecular level for the ECP genes of this lineage. The dendrogram in Figure 1A and the divergence calculations in Figure 1B depict the relationships among the primate EDN orthologs included in the present study. In Figure 1C, the catalytic coefficients (k_{cat}/K_m) derived from double reciprocal plots for each protein in recombinant form (8,11,12) are listed. As reported previously (11,12), acquisition of enhanced RNase activity parallels the split between the Old World (macaque and african green monkey) and the New World (tamarin and owl monkey) monkeys; the enhanced RNase activity is maintained throughout the higher primate lineages.

Evolution of antiviral activity

A comparison of the antiviral activities of the various primate orthologs of human EDN is shown in Table 1. Human, orangutan and macaque EDNs are equally effective against extracellular virions of RSV in suspension, with a 12- to 14-fold reduction in infectious units/ml observed in response to 100 nM recombinant protein in each case. As anticipated from their catalytic coefficients (Fig. 1C), the RNase activities measured in the viral suspensions to which 100 nM recombinant EDN had been added were indistinguishable from one another. In contrast, neither of the two New World monkey RNases, tamarin EDN and owl monkey EDN, displayed any antiviral activity in this assay. The amino acid sequences of these EDNs differ from human EDN by 27 and 29%, respectively, and they are not as ribonucleolytically active as the EDNs from higher primates.

In Table 2, we have calculated the rates of non-synonymous (K_a) and synonymous substitution (K_s), respectively, for four Old World monkey/New World monkey EDNs. This type of analysis proceeds under two simplifying assumptions: (i) that synonymous, or 'silent', substitution proceeds without specific constraints; (ii) that non-synonymous, or 'non-silent', substitution can be observed only when response to evolutionary constraints can be reconciled with the need to maintain structural integrity (positive selection). A K_a/K_s ratio >1.0 or a rate of non-synonymous substitution exceeding the rate of synonymous substitution has been accepted as an indication of positive selection, although there are very few examples of this phenomenon (27,32–35). While not exceeding unity, the K_a/K_s ratios determined for the four EDN pairs were relatively high, ranging from 0.68 to 0.82. However, similar ratios were calculated for a series of Old World/New World monkey pairs encoding RNase k6, an RNase A family RNase that has displayed a more conservative rate of sequence divergence during primate evolution (36; Discussion).

Table 1. Antiviral activity of primate orthologs of EDN

Ortholog	Concentration (nM)	Infectious units/ml (±SD)	RNase activity (pmol/min)
Human EDN	0	3220 ± 208	
	25	2432 ± 93	
	50	500 ± 58[a]	
	100	258 ± 30[a]	35
Orangutan EDN	0	3768 ± 88	
	25	2823 ± 235	
	50	603 ± 93[a]	
	100	260 ± 91[a]	30
Macaque EDN	0	3900 ± 219	
	25	3268 ± 214	
	50	583 ± 26[a]	
	100	302 ± 60[a]	28
Tamarin EDN	0	3008 ± 78	
	25	3015 ± 184	
	50	3128 ± 68	
	100	2951 ± 63	1.8
Owl monkey EDN	0	3122 ± 87	
	25	3093 ± 86	
	50	3078 ± 138	
	100	3122 ± 106	2.7

Sequences orthologous to human EDN were isolated from species indicated as described (10); genus/species designations include *Pongo pygmaeus* (orangutan), *Macaca fascicularis* (macaque), *Saguinus oedipus* (tamarin) and *Aotus trivirgatus* (owl monkey). Antiviral activity was measured via the quantitative shell vial assay (22,25). RNase activity was measured as generation of acid-soluble ribonucleotides from acid-insoluble tRNA substrate as described in Materials and Methods. Baseline RNase activity measured in the absence of added EDN is 0.6 pmol/min. Results presented are the average of triplicate samples ± SD from a representative experiment.

[a]The value differs significantly from baseline (0 nM) at the $P < 0.01$ level.

Antiviral activity of other RNase A family RNases

While we have found that RNase activity is essential to this antiviral effect (22), we have also shown previously that other RNase A family RNases have no antiviral activity against extracellular virions of RSV in this assay (22,23), including RNase A, human RNase k6 and onconase. We extend this list (Table 3) to include bovine seminal RNase (BSR), a dimeric RNase shown to have antiviral activity against intracellular forms of HIV-1 (37). These results support our hypothesis regarding regions of primary sequence unique to EDN (and shared at least somewhat with ECP) that are necessary to promote this antiviral effect.

Specific interaction between EDN and extracellular virions of RSV

We evaluated the antiviral activity of rhEDN in the presence of increasing concentrations of the ribonucleolytically inactivated point mutant rhEDNdK[38]; we have shown previously that this form of inactivated rhEDN has no antiviral activity in this assay (22). In Table 4 we show that rhEDN's antiviral activity is blocked by increasing concentrations of rhEDNdK[38]. Increasing concentrations of rhRNase k6, a RNase with no demonstrable antiviral activity (23; Table 3), did not block the antiviral activity of rhEDN.

Antiviral activity of human/owl monkey EDN chimeras

As an initial step towards identifying the region or regions of EDN that are crucial for the specific, saturatable interaction with RSV, we assembled two chimeras utilizing complementary regions of human and owl monkey EDNs, as shown in Figure 2. An analysis of the antiviral effects of each chimera is shown in Table 5. Chimera OR5 has low level ribonucleolytic activity and, not surprisingly, has no measurable antiviral activity. Interestingly, chimera EG5 is a RNase that is nearly as potent as human EDN but that has little to no antiviral activity, suggesting the presence of a crucial region or regions somewhere within the first 50 residues of human EDN.

Table 2. Evolutionary analysis of New World and Old World monkey EDNs

Coding sequence pairs New World/Old World	K_a (substitutions/site)	K_s (substitutions/site)	K_a/K_s
EDN (RNase 2)			
Tamarin/macaque	0.20 ± 0.05	0.30 ± 0.09	0.68
Tamarin/african green monkey	0.20 ± 0.05	0.27 ± 0.08	0.74
Owl monkey/macaque	0.22 ± 0.05	0.29 ± 0.09	0.74
Owl monkey/african green monkey	0.21 ± 0.09	0.26 ± 0.08	0.82
RNase k6			
Tamarin/rhesus monkey k6	0.045 ± 0.021	0.096 ± 0.033	0.47
Tamarin/african green monkey k6	0.056 ± 0.024	0.123 ± 0.037	0.45
Owl monkey/rhesus monkey k6	0.046 ± 0.019	0.062 ± 0.025	0.74
Owl monkey/african green monkey k6	0.048 ± 0.021	0.089 ± 0.030	0.54
Squirrel monkey/rhesus monkey k6	0.077 ± 0.022	0.095 ± 0.054	0.82
Squirrel monkey/african green monkey k6	0.080 ± 0.024	0.122 ± 0.056	0.65

Ratios of synonymous substitution per synonymous site (K_s) and non-synonymous substitutions per non-synonymous site (K_a) were calculated using the MEGA (Molecular Evolutionary Genetics Analysis) program (46) and the DIVERGE algorithm (29,30) of the Wisconsin Genetics Computer Group program on-line at the National Institutes of Health. Analysis of the coding sequences did not include N-terminal signal sequences.

Table 3. Antiviral activity of other RNase A superfamily RNases

RNase	Concentration (nM)	Infectious units/ml (±SD)	RNase activity (pmol/min)
RNase A	0	2480 ± 66	
	25	2423 ± 230	
	50	2502 ± 65	
	100	2482 ± 140	114
BSR[a]	0	3900 ± 219	
	25	4035 ± 147	
	50	4015 ± 58	
	100	3995 ± 139	80
RNase k6	0	2480 ± 66	
	25	2313 ± 206	
	50	2288 ± 62	
	100	2485 ± 111	20

Antiviral activity was measured via the quantitative shell vial assay (22,25) and RNase activity by generation of acid-soluble ribonucleotides from acid-insoluble tRNA substrate as described in Materials and Methods. Baseline RNase activity measured in the absence of added RNase was 0.6 pmol/min. Results presented are the average of triplicate samples ± SD from a representative experiment.
[a]BSR, bovine seminal RNase.

Table 4. Antiviral activity of rhEDN in the presence of rhEDNdK^{38} or rhRNase k6

	rhEDN (nM)	Infectious units/ml (±SD)
rhEDNdK^{38} (nM)		
0	0	5625 ± 220
1000	0	5698 ± 324
0	200	573 ± 126[a]
200	200	3273 ± 167
1000	200	2482 ± 315
rhRNase k6 (nM)		
0	0	6380 ± 145
1000	0	6205 ± 318
0	200	522 ± 46[a]
200	200	626 ± 37[a]
1000	200	502 ± 114[a]

Antiviral activity against extracellular virions of RSV-B measured by quantitative shell vial assay as described (22,25). Results presented are the average of triplicate samples ± SD from a representative experiment.
[a]The value differs significantly from baseline (0 nM) at the $P < 0.01$ level.

DISCUSSION

In this work we show that the emergence of antiviral activity parallels the acquisition of enhanced RNase activity among EDN genes, as both features emerge some time after the divergence of the Old World from the New World monkey species. Interestingly, Zhang and colleagues (27) evaluated the evolutionary history of the primate genes encoding ECP and concluded that ECP's antimicrobial function was acquired as a result of positive (Darwinian) selection at the molecular level. The mathematical analysis shown here (Table 2) does not provide definitive support for positive selection with respect to the antiviral function of EDN, however, the conclusions from this type of analysis are based on the assumption that mutations that do not alter the

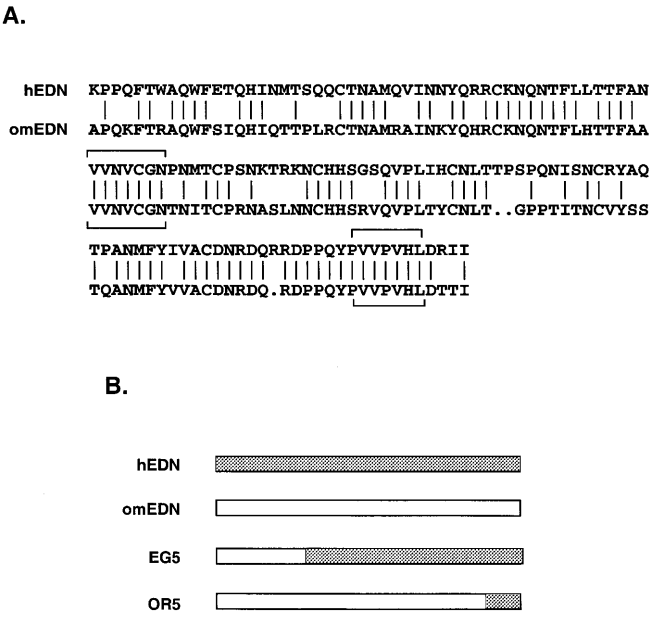

Figure 2. (**A**) Alignment of coding sequences of human and owl monkey EDN genes, with brackets indicating divisions between segments used to generate individual chimeras. (**B**) Segment maps of chimeras EG5 and OR5.

protein sequence ('silent' mutations) occur freely and at random. While this may be the case for some genes, it is likely to be an over-simplification; several groups have shown that sequences with elevated rates of non-silent mutations also tend to have elevated rates of silent mutations (38–40) and others have suggested a correlation between rates of synonymous ('silent') substitution and nucleotide and/or amino acid composition (41–43). A clearer understanding of the evolutionary constraints promoting both 'silent' and 'non-silent' mutations both in general and for individual protein coding sequences may ultimately result in a reassessment of the conclusions drawn here.

Table 5. Antiviral activity of human/owl monkey EDN chimeras

Chimera	Concentration (nM)	Infectious units/ml (±SD)	RNase activity (pmol/min)
OR5	0	5112 ± 415	
	100	5615 ± 690	5
EG5	0	5112 ± 415	
	25	5717 ± 755	
	50	5247 ± 420	
	100	2641 ± 539[a]	20

Antiviral activity was measured via the quantitative shell vial assay (22,25). RNase activity was measured as generation of acid-soluble ribonucleotides from acid-insoluble tRNA substrate as described in Materials and Methods. Baseline RNase activity measured in the absence of added EDN is 0.6 pmol/min. Results presented are the average of triplicate samples ± SD from a representative experiment.
[a]The value differs significantly from baseline (0 nM) at the $P < 0.05$ level.

The most important findings presented here are those that address the mechanism of EDN's antiviral activity. In our previous work, we concluded that RNase activity was crucial to the antiviral effect, but that it was not sufficient to explain EDN's

apparent unique activity among the RNase A RNases. We demonstrate here that EDN's antiviral activity is blocked specifically by increasing concentrations of a ribonucleolytically inactivated point mutant of EDN, rhEDNdK^{38}, while identical concentrations of ribonucleolytically active, but antivirally inactive, human RNase k6 had no effect whatsoever. The implications of this result reach out in many directions. The presence of specific, saturatable binding suggests physiological relevance and stands in greatest support of our hypotheses regarding the physiological function of EDN and the 'double-edged sword' of eosinophilic inflammation. In terms of mechanism, identification and characterization of the elements of specificity, both within EDN and within the as yet to be identified viral surface target(s), may provide some insight into the way in which EDN successfully penetrates the viral capsid and gains access to the viral RNA genome. As most antiviral agents in current use function by inhibiting viral replication intracellularly, information on how and where one might attack virions extracellularly might ultimately be harnessed to the creation of a novel class of antiviral agents. We have begun this search by delimiting an N-terminal region of human EDN that appears to be crucial to its antiviral effect.

The specificity of this interaction is intriguing from a more global perspective as well. In our previous studies, we have documented the astonishing amount of evolutionary energy that has served both to expand and to diversify the EDN/ECP lineage of the RNase A gene superfamily. Not only are EDN and ECP the most rapidly evolving coding sequences known among primates (10), the orthologous mouse and rat RNase genes have also undergone multiple independent gene duplication events, forming two unusual, species-specific multigene clusters (44,45; Singhania and Rosenberg, unpublished results). When considered together with our findings on the specific antiviral activity of primate EDNs, a novel hypothesis emerges. We believe that the unusual evolutionary constraints acting on these lineages of the RNase A gene superfamily have promoted the aquisition of *specialized* antiviral activity. By specialized antiviral activity, we predict that we will find that each individual RNase of the EDN/ECP lineage has diverged and acquired features that permit specific interaction with one individual (or closely related group of) single-stranded RNA viral pathogen(s). To date, we have identified two paramyxoviruses, RSV and parainfluenza virus (22), as potential targets of EDN. Studies in progress in our laboratories will serve to evaluate, elucidate and extend this hypothesis.

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