23. D. Jacob *et al.*, *Geochim. Cosmochim. Acta* **58**, 5191 (1994).
24. K. S. Viljoen, unpublished data.
25. C. J. Hatton, thesis, University of Cape Town, Cape Town, South Africa (1978).
26. I. D. MacGregor and W. I. Manton, *J. Geophys. Res.* **91**, 14063 (1986).
27. N. V. Sobolev, *Peep-Seated Inclusions in Kimberlites and the Problem of the Composition of the Upper Mantle* (at the American Geophysical Union, Washington, DC, 1977).
28. G. P. Pearson *et al.*, *Geochim. Cosmochim. Acta* **59**, 959 (1995).
29. D. Jacob *et al.*, *N. Jahrb. Miner. Abh.* **172**, 357 (1998).
30. D. Jacob and E. Jagoutz, in *Kimberlites, Related Rocks and Mantle Xenoliths*, CPRM Special Publication 1/95, H. O. A. Meyer and O. H. Leonardos, Eds. (Companhia de Pesquisa de Recursos Minerais, Brasilia, Brazil, 1995), vol. 1, pp. 304–317.
31. K. S. Viljoen *et al.*, *Chem. Geol.* **131**, 235 (1996).
32. D. Mattey and C. McPherson, *Chem. Geol.* **105**, 305 (1993).
33. P. Cartigny, thesis, Université Paris 7 (1997).
34. G. A. Snyder *et al.*, *J. Petrol.* **38**, 85 (1997).
35. C. B. Smith *et al.*, in *Kimberlites and Related Rocks*, J. Ross *et al.*, Eds., *Geol. Soc. Am. Spec. Publ.* **14**, 853 (1989).
36. S.-S. Sun and W. F. McDonough, in *Magmatism in the Ocean Basins*, A. D. Saunders and M. J. Norry, Eds., *Geol. Soc. London Spec. Publ.* **42**, 313 (1989).
37. H. P. Longerich *et al.*, *J. Anal. At. Spectrom.* **11**, 899 (1996). Trace element data are available at www.sciencemag.org/feature/data.1051804.shl.
38. This study was supported by Deutsche Forschungsgemeinschaft grants Ja 781/2-1 and Ja 781/2-2 to D.J. We thank D. Mattey for providing access to his efficient stable isotope lab and P. Cartigny for measuring the carbon isotopes. Framesite samples were provided by DeBeers. We thank three anonymous reviewers for constructive criticism.

1 May 2000; accepted 20 June 2000

Degradation of Outer Membrane Protein A in *Escherichia coli* Killing by Neutrophil Elastase

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In determining the mechanism of neutrophil elastase (NE)–mediated killing of *Escherichia coli*, we found that NE degraded outer membrane protein A (OmpA), localized on the surface of Gram-negative bacteria. NE killed wild-type, but not OmpA-deficient, *E. coli*. Also, whereas NE-deficient mice had impaired survival in response to *E. coli* sepsis, as compared to wild-type mice, the presence or absence of NE had no influence on survival in response to sepsis that had been induced with OmpA-deficient *E. coli*. These findings define a mechanism of nonoxidative bacterial killing by NE and point to OmpA as a bacterial target in host defense.

After bacterial infection, neutrophils engulf and kill bacteria by oxidative and nonoxidative pathways. Nonoxidative mechanisms are less well defined, but they predominantly relate to the ability of peptides to alter the bacterial membrane permeability (*1*). NE has long been regarded as an antibacterial protein, but its mechanism of bacterial killing remains unclear (*2*). NE is a potent serine proteinase whose catalytic activity relies on the His-Asp-Ser triad (*3*). Recently, we have demonstrated that NE is required for host defense against Gram-negative, but not Gram-positive, bacteria (*4*). These findings prompted us to delineate the mechanism by which NE kills *E. coli*, a common and virulent pathogen.

Using immuno–electron microscopy (immuno-EM), we confirmed that NE was localized within the phagolysosomes following ingestion of *E. coli* by neutrophils (Fig. 1A) (*5, 6*). To determine whether the antibacterial activity of NE was related to its catalytic activity, we cultured *E. coli* [10^6 colony-forming units (CFUs)] with or without human NE (2 μM), and bacterial viability was monitored over time by plating serial dilutions. The addition of NE markedly decreased *E. coli* growth (Fig. 1B). This decrease in bacterial growth was dependent on the time of

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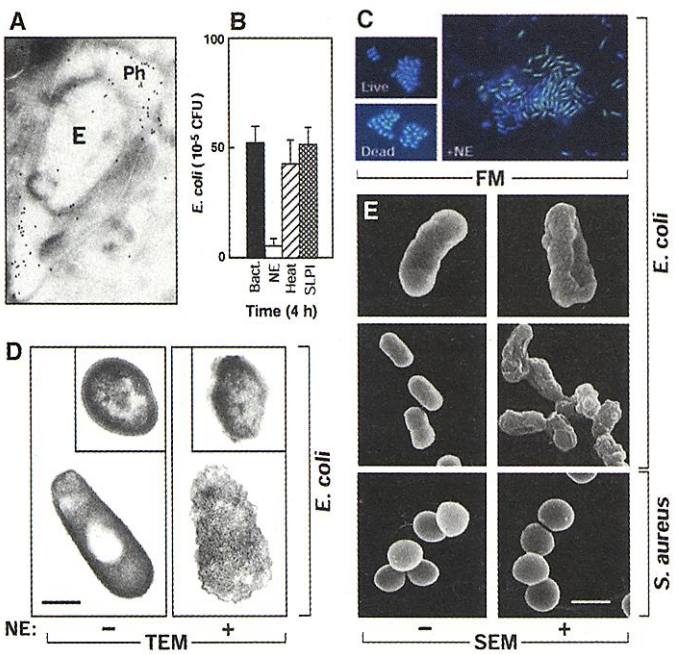

Fig. 1. NE localization and effect on bacteria. **(A)** Immuno-EM localization of NE in neutrophils. Human neutrophils were incubated with freshly grown bacteria at a 1:100 ratio, and the reactions were processed for immuno-EM with antibody to NE. A phagolysosome containing an *E. coli* bacterium is shown. There is an increased number of gold particles inside the phagolysosome. The estimated size of (A) is 3.5 μm. Ph, phagolysosome; E, *E. coli*. **(B)** NE kills *E. coli* through its catalytic activity. *E. coli* were incubated with or without NE. Similar experiments were repeated where NE was inactivated and viable bacterial counts were determined. SLPI (at 0.05 μM) inhibited NE activity (>95%) but exhibited unsubstantial antibacterial activity against *E. coli*. Results represent the mean of four experiments; error bars indicate the standard error of the mean. **(C)** Fluorescence microscopy (FM) of bacteria. *E. coli* were incubated without (live) or with NE (+NE) as described above, and the reactions were stained with a mixture of DAPI and SYTOX. DNA of live (intact cell membranes) and dead (disrupted membranes) bacteria fluoresce blue with DAPI and bright green with SYTOX (magnification, ×2000). **(D and E)** Electron micrographs of bacteria incubated with (+) or without (−) NE. *E. coli* and *S. aureus* were cultured with or without NE, and the reactions were processed for SEM and TEM. Single cells and aggregates of bacteria are shown. **(E)** In the presence of NE, the coccus morphology (*S. aureus*) remained intact, and the bacillus morphology (*E. coli*) was distorted (determined by SEM) (scale bar, 1.5 μm). **(D)** In the absence of NE, the outer and inner membranes are intact, but the addition of NE resulted in a distorted structure (determined by TEM) (scale bar, 0.5 μm). Insets represent cross sections of bacteria.

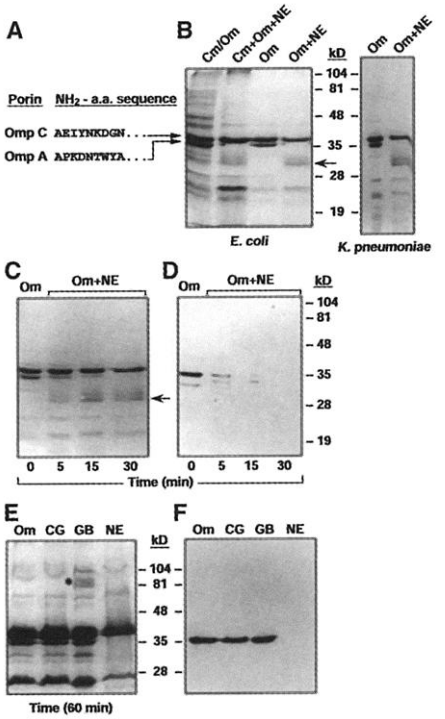

Fig. 2. NE, but not GB and CG, degrades OmpA. *E. coli* were grown and subjected to subfractionation. (**A**) Although complex, the Om fraction contains two major proteins that were identified by NH_2-terminal amino acid (a.a.) sequencing (*24*). (**B**) Membrane fractions (Cm/Om and Om) were incubated with NE for 1 hour. (**C** and **E**) The Om fraction was incubated with NE, GB, and CG for varying times. These reactions were performed in duplicate. Reactions (**C**) and (**E**) were resolved with SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and were stained with Coomassie blue. Duplicate reactions (**D**) and (**F**) were processed for Western blotting by using antibody to OmpA. Arrow indicates NE; the asterisk shows GB.

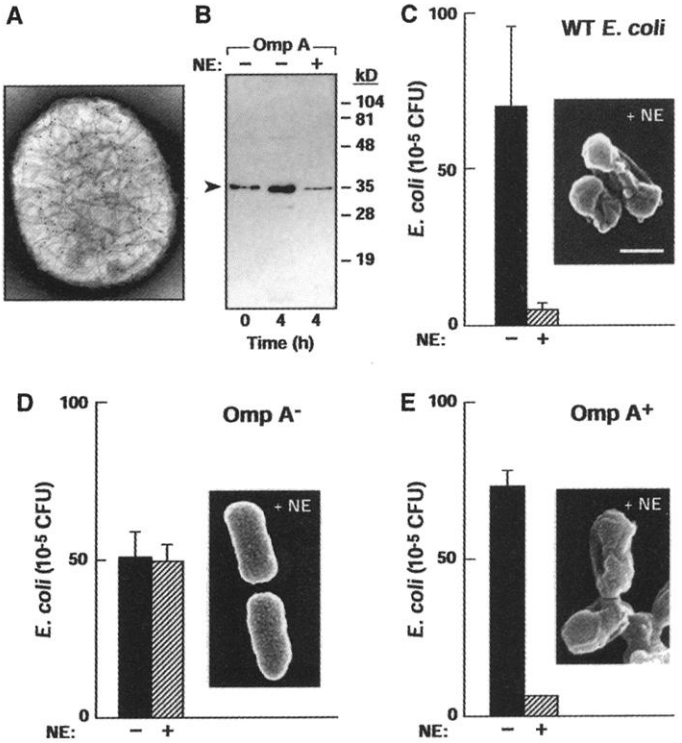

Fig. 3. Characterization of *E. coli* and isogenic mutant strains after incubation with NE. (**A**) WT *E. coli* were processed for immunogold negative labeling using antibody to OmpA. Shown is an *E. coli* bacterium (2.5 μm) with OmpA evenly distributed on the Om. (**B**) *E. coli* (10^6 CFUs) were cultured with or without NE (2 μM) for 4 hours, boiled, and resolved directly with SDS-PAGE and processed for Western blotting using antibody to OmpA. There is a decreased level of OmpA in the presence of NE (arrowhead). (**C**) WT, (**D**) $OmpA^-$, and (**E**) $OmpA^+$ *E. coli* were cultured with or without NE. Viability and morphology of bacteria were followed after 4 hours of incubation. Bacterial growth data represent the mean of four different experiments; error bars indicate the standard error of the mean [scale bar, 1.5 μm [applicable to SEM images in (C) through (E)]].

incubation (*4*) and NE concentration but was independent of the growth phase and salt concentration. Upon heat inactivation or active site inhibition of NE with secretory leukocyte protease inhibitor (SLPI) (*7*), the bacteria cultured with NE grew similarly to the bacteria cultured without it. Thus, NE catalytic activity was required for bactericidal activity. This was further confirmed by using fluorescent staining to distinguish live versus dead bacteria (*8*). In the presence of NE, DNA of dead bacteria with damaged membranes fluoresce bright green, whereas DNA of live bacteria with intact membranes stains blue (Fig. 1C). Neither cathepsin G (CG) (*9*) nor gelatinase B (GB) (*10*) was able to kill this strain, suggesting selective antibacterial activity of neutrophil granule proteinases.

Unlike Gram-positive bacteria, Gram-negative bacteria typically have an outer membrane (Om). We hypothesized that Om proteins were susceptible to cleavage by NE, resulting in cell death. To address this, we cultured *E. coli* with or without NE, and the reactions were processed for both scanning and transmission electron microscopy (SEM and TEM) (*5, 11*). Upon addition of NE, the rodlike morphology of *E. coli* was perturbed, with the appearance of "nodules" and collapsed architecture (Fig. 1E). Loss of membrane integrity was further confirmed with TEM. In the presence of NE, *E. coli* lost its discernable inner and outer membranes, resulting in a disrupted and frayed appearance (Fig. 1D). In contrast, the coccus morphology of *Staphylococcus aureus* was intact after incubation with NE, consistent with the inability of NE to kill this Gram-positive bacterium (Fig. 1E).

To determine the bacterial target(s) of NE, we grew *E. coli* and subjected it to fractionation (*12, 13*). As previously shown (*14*) and because of its potent activity, NE degraded several proteins in both cytoplasmic (Cm) and outer membranes. The Om fraction, although complex, comprised two major bands identified as Om protein C and A (Omp C and OmpA) (Fig. 2A). Strikingly, OmpA, but not OmpC, was completely and rapidly degraded by NE (Fig. 2, A, C, and D). Similar findings were observed with *Klebsiella pneumoniae* (Fig. 2B). CG and GB (*15*) did not cleave OmpA (Fig. 2, E and F).

OmpA is highly conserved in a wide range of Gram-negative bacteria, and multiple potential functions have been ascribed to it, including maintenance of virulence, structural integrity, and porin activity (*16*). To determine the importance of OmpA cleavage for NE-mediated killing of *E. coli*, we first verified that OmpA was evenly distributed on the cell surface by immuno-EM (*17, 18*) (Fig. 3A). Incubation of intact bacteria with NE was accompanied by considerable degradation of OmpA, as demonstrated by immunoblotting (*19*) (Fig. 3B), coinciding with bacterial death (Fig. 3C). Next, to assess the contribution of OmpA to NE-mediated killing, we incubated NE with wild-type (WT) *E. coli*, isogenic mutants where OmpA expression was disrupted ($OmpA^-$), and $OmpA^-$ *E. coli* that were rescued by complementation ($OmpA^+$) (*8, 11, 20*). Exposure of the WT strain to NE for 4 hours resulted in altered morphology and a decrease in growth (Fig. 3C). However, in the absence of OmpA, NE had no effect on viability or the morphology of $OmpA^-$ (Fig. 3D). Reconstitution of OmpA expression restored WT *E. coli* growth characteristics and NE-mediated killing for $OmpA^+$ (Fig. 3E).

Next, we compared the ability of WT ($NE^{+/+}$) and NE-deficient ($NE^{-/-}$) neutrophils to kill WT *E. coli* and its isogenic $OmpA^-$ in vitro (*4, 21*). Both neutrophils recognized and interacted with both *E. coli* strains equally (time 0 in Fig. 4, A and C). But, $NE^{-/-}$ exhibited less bactericidal activity than $NE^{+/+}$ after incubation with WT *E. coli* (Fig. 4A) (*4*). In Fig. 4C, both $NE^{+/+}$ and $NE^{-/-}$ killed $OmpA^-$. However, there was no difference in the killing when we compared $NE^{-/-}$ to $NE^{+/+}$, suggesting that

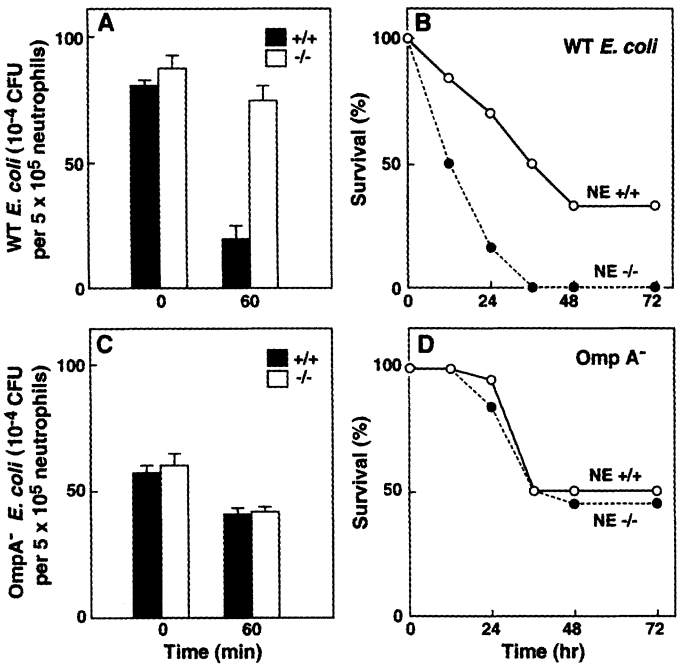

Fig. 4. (**A** and **C**) Capacity of $NE^{+/+}$ and $NE^{-/-}$ neutrophils to kill WT and $OmpA^-$ *E. coli*. Bacteria (10^7 CFUs) were incubated in the presence of 10^5 freshly isolated $NE^{+/+}$ and $NE^{-/-}$ neutrophils. After 15 min of exposure (time 0), nonadherent bacteria were removed. At that time (time 0) and 60 min later, neutrophils were solubilized with 0.1% Triton, and the number of viable bacteria (intracellular and cell-associated bacteria) was determined. Data shown are expressed as the mean of four independent experiments; error bars indicate the standard error of the mean. (**B**) Survival curves for $NE^{+/+}$ and $NE^{-/-}$ mice in response to being ip challenged with WT *E. coli* ($P = 0.0018$, Wilcoxon test). (**D**) Survival curves for $NE^{+/+}$ and $NE^{-/-}$ mice in response to being ip challenged with $OmpA^-$ *E. coli* ($P = 0.6852$, Wilcoxon test).

bacterial death was independent of NE and that the presence of OmpA was required for NE bactericidal killing.

To confirm the relevance of this mechanism in vivo, we infected $NE^{-/-}$ mice and $NE^{+/+}$ littermates intraperitoneally (ip) with either WT *E. coli* or $OmpA^-$, and their survival was monitored over time (*22*). Infection with 4×10^4 CFUs of WT *E. coli* resulted in the death of all $NE^{-/-}$ mice, whereas 35% of $NE^{+/+}$ mice survived (Fig. 4B) ($P = 0.04$, Fisher's exact test) (*4*). $OmpA^-$ were less virulent than WT *E. coli*, but a dose that caused 50% of $NE^{+/+}$ mice to die [median lethal dose (LD_{50})] was achieved with 3.5×10^5 CFUs. The same LD_{50} was observed with $NE^{-/-}$ mice (Fig. 4D) ($P = 0.5$, Fisher's exact test) (*23*). Thus, the absence of OmpA negates the role of NE in host defense against *E. coli*.

These data confirm the role of NE in host defense against *E. coli* and demonstrate that NE-mediated killing requires the presence of OmpA. NE degradation of OmpA results in cell death either by a loss of bacterial integrity or by localized weakening of the cell wall followed by osmotic lysis. Alternatively, OmpA cleavage could allow NE access to internal protein(s), resulting in further proteolysis and bacterial death. These findings highlight the importance of NE as a host defense molecule and demonstrate bacterial killing through the proteolytic attack of a specific Om protein. Thus, the function of NE needs to be reconsidered if intracellular NE inhibition is pursued to treat destructive diseases, including cystic fibrosis. Also, OmpA may represent a target in the design of therapeutic strategies against Gram-negative bacteria.

References and Notes

1. W. M. Shafer, F. Hubalek, M. Huang, J. Pohl, *Infect. Immun.* **64**, 4842 (1996); T. Ganz, *Proc. Assoc. Am. Physicians* **111**, 390 (1999).
2. J. Blondin, A. Janoff, J. C. Powers, in *Neutral Proteases of Human Polymorphonuclear Leukocytes*, J. Havemann and A. Janoff, Eds. (Urban and Schwartzenberg, Baltimore, MD, 1978), pp. 39–55.
3. M. Zimmer *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8215 (1992).
4. A. Belaaouaj *et al.*, *Nature Med.* **4**, 615 (1998).
5. J. W. Slot and H. J. Geuze, *Methods Microbiol.* **20**, 211 (1988).
6. Healthy human neutrophils were isolated, and differential counting and trypan blue exclusion were performed to determine that the cell suspension contained >95% viable cells. *E. coli* were freshly grown in brain heart infusion (BHI) media at 37°C and washed twice with phosphate-buffered saline (PBS). Bacteria (10^6 CFUs) and 10^5 neutrophils were incubated in suspension for 1 hour in a humidified incubator (at 37°C with 5% CO_2). The cells were spun gently and processed for immuno-EM (*5*).
7. R. J. Zitnik *et al.*, *Biochem. Biophys. Res. Commun.* **232**, 687 (1997).
8. Bactericidal activity was quantified as previously reported (*4*). In parallel, NE was heat inactivated for 10 min at 100°C or inhibited with SLPI as previously described (*7*), and the bactericidal assay was performed. Serial dilutions were immediately spread on agar plates, and the number of CFUs was determined after overnight incubation at 37°C. For each assay, elastase activity was confirmed by the spectrophotometric method (*4*). The bacterial viability was further assessed with two-color visualization of dead and live cells. After a short incubation with NE-treated *E. coli*, 4′,6-diamidino-2-phenylindole (DAPI) (blue fluorescent live-cell stain) and SYTOX (green fluorescent dead-cell stain) nucleic acid stains discriminate between bacteria with intact membranes and those with damaged membranes, respectively (Molecular Probes, Eugene, OR). As a control for death, bacteria were killed with heat (100°C for 10 min) or 70% isopropyl.
9. D. M. MacIvor *et al.*, *Blood* **94**, 4282 (1999).
10. T. Betsuyaku, J. M. Shipley, Z. Liu, R. Senior, *Am. J. Respir. Cell Mol. Biol.* **20**, 1303 (1999).
11. After incubation with or without NE, *E. coli* were processed for TEM as previously described (*5*). For SEM, the reactions were seeded onto coverslips, which had been treated with 1% aqueous polyethylenimine (P-3134, Sigma, St. Louis, MO), and gently slid into a fixative solution of 2.5% glutaraldehyde. After ethanol dehydration, the cover slips were dried, mounted on aluminum SEM stubs, rimmed with colloidal silver paint, coated with a 30-nm gold layer, and viewed with a Hitachi S-450 scanning electron microscope. Micrographs were recorded on Polaroid 55 P/N film.
12. H. Nikaido and E. Y. Rosenberg, *J. Bacteriol.* **153**, 241 (1983).
13. *E. coli* were grown in 100 ml of BHI media and subjected to subfractionation by using sarkosyl (*12*). Five micrograms of Om proteins was incubated for varying times with 2 μM NE, GB (*14*), and CG in a 20-μl reaction containing PBS. The reactions were performed in duplicate and resolved by gel electrophoresis. One gel was stained with Coomassie blue, and the other was processed for enhanced chemoluminescence (ECL) Western blotting (NEN, Boston, MA) using antibody to OmpA (dilution 1:20,000). For amino acid sequencing, the two major proteins were sequenced by automated Edman degradation with an Applied Biosystem 473 sequenator.
14. R. F. Rest and E. Pretzer, *Infect. Immun.* **34**, 62 (1981).
15. S. D. Shapiro, D. K. Kobayashi, T. J. Ley, *J. Biol. Chem.* **268**, 23824 (1993).
16. M. Klose *et al.*, *J. Biol. Chem.* **263**, 13291 (1988); J. N. Weiser and E. C. Gotschlich, *Infect. Immun.* **59**, 2252 (1991).
17. J. E. Beesley, S. E. Day, M. P. Betts, C. M. Thorley, *J. Gen. Microbiol.* **130**, 1481 (1984).
18. Freshly grown *E. coli* were allowed to attach on a copper grid (mesh, 400 lines/inch), and then processed for immuno-gold negative labeling (*17*).
19. Bacteria were incubated with or without NE as described above. Next, the reactions were resolved, and the gel was processed for ECL Western blotting (NEN, Boston, MA) using antibody to OmpA.
20. N. V. Prasadarao *et al.*, *Infect. Immun.* **64**, 146 (1996).
21. Bactericidal activity of neutrophils was determined as described earlier (*4*).
22. OmpA and WT *E. coli* were passaged twice in 129/Sv mice before use. For each organism, at least 20 WT mice were injected ip with varying amounts of bacteria (CFUs), and their survival was followed over time. From these studies, the LD_{50} from bacteria was determined by interpolation for each bacterial strain. Next, 10 $NE^{-/-}$ mice and 10 of their $NE^{+/+}$ littermates were infected ip, and their survival was monitored over time.
23. Survival probabilities of mice as a function of time were estimated by using the Kaplan-Meier method. Survival curves were compared by the Wilcoxon test. Survival rates at the end of the experiment were compared by using Fisher's exact test.
24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; G, Gly; I, Ile; K, Lys; N, Asn; P, Pro; T, Thr; W, Trp; and Y, Tyr.

25. We thank J. Gaither-Ganim, J. Campbell, and M. Veith for their excellent technical support, R. M. Senior and M. Caparon for critical review of the manuscript, and A. A. Todorov for statistical analysis. All of the animal experiments were in accordance with Washington University Animal Studies Committee guidelines. This work was supported by a Barnes-Jewish Hospital foundation grant (to A.B.) and by the NIH (to S.D.S. and K.S.K.).

17 May 2000; accepted 5 July 2000