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Neutrophil Serine Proteinases Cleave Bacterial Flagellin, Abrogating Its Host Response-Inducing Activity

Yolanda S. López-Boado,† Marcia Espinola,‡ Scott Bahr,‡ and Abderrazzaq Belaaouaj2§

After bacterial infection, neutrophils dominate the cellular infiltrate. Their main function is assumed to be killing invading pathogens and resolving the inflammation they cause. Activated neutrophils are also known to release a variety of molecules, including the neutrophil serine proteinases, extracellularly. The release of these proteinases during inflammation creates a proteolytic environment where degradation of different molecules modulates the inflammatory response. Flagellin, the structural component of flagella on many bacterial species, is a virulence factor with a strong proinflammatory activity on epithelial cells and other cell types. In this study we show that both human and mouse neutrophil serine proteinases cleave flagellin from Pseudomonas aeruginosa and other bacterial species. More important, cleavage of P. aeruginosa flagellin by the neutrophil serine proteinases neutrophil elastase and cathepsin G resulted in loss of the biological activity of this virulence factor, as evidenced by the lack of innate host defense gene expression in human epithelial cells. The finding that flagellin is susceptible to cleavage by neutrophil serine proteinases suggests a novel role for these enzymes in the inflammatory response to infection. Not only can these enzymes kill bacteria, but they also degrade their virulence factors to halt the inflammatory response they trigger. The Journal of Immunology, 2004, 172: 509–515.

Neutrophils represent the earliest phagocytic cells that dominate the sites of bacterial infection. Their main function is assumed to be the killing of invading pathogens. Various antimicrobial molecules have been identified in neutrophil granules and grouped into oxygen-dependent and -independent systems (1). This latter system comprises the readily active serine proteinases (NSPs), neutrophil elastase (NE), cathepsin G (CG), and protease 3 (PR3), among other polypeptides known to alter the bacterial structural integrity. NSPs are structurally related and share the conserved charge-relay triad, His57-Asp102-Ser195, where Ser is the active residue (chymotrypsin numbering) (2). Previously, we have shown that NE is required for host defense against Gram-negative bacteria (3, 4) and demonstrated that NE kills Gram-negative Escherichia coli by degrading its major outer membrane protein A (5). Together, these findings demonstrate that NSPs serve a physiological role in the intracellular killing of microbes.

Activated neutrophils are also known to release a variety of molecules, including NSPs, during their egress from the vasculature, when their defensive functions (e.g., phagocytosis) are impaired or the bacterial dose is overwhelming. The release of these proteinases during inflammation creates a proteolytic environment where degradation of different molecules modulates the inflammatory response. For example, NE is capable of cleaving a panoply of substrates in vitro. These include extracellular matrix proteins, coagulation factors, and Igs (6). NE degrades proinflammatory mediators such as TNF-α and IL-1β (7) and hence down-regulates inflammation. NE cleaves ICAM-1, indicating that the enzyme might regulate neutrophil adhesion and migration (8, 9). NE induces the secretion of GM-CSF and IL-8 (10), which could amplify the inflammation. This substrate repertoire, which continues to expand, has already led to the incrimination of NE in the pathogenesis of various tissue-destructive diseases, including pneumonia-mediated acute lung injury and cystic fibrosis (CF) (6).

At sites of infection, bacteria and/or their components modulate the inflammatory response as well. In recent years, flagellin, the structural component of flagella in many bacterial species (11), has been demonstrated to have a strong proinflammatory activity on epithelial cells and other cell types (12–14). With respect to epithelial cells, flagellin induces IL-8 secretion, inducible NO synthetase expression, and NF-κB activation in several models of cells (15–19). In addition, the secretion of flagellin is necessary to induce the activation of proinflammatory signaling pathways and neutrophil trans-epithelial migration (20). We and others have recently demonstrated that flagellin up-regulates the expression of the innate host defense genes, matrilysin and human β-defensin-2 (hBD-2), in epithelial cells (21, 22). This bacterial protein can also activate other cell types, including monocytes, to produce proinflammatory cytokines (23, 24). Finally, flagellin can trigger adaptive immune responses both by stimulating chemokine secretion by epithelial cells and subsequent migration and maturation of dendritic cells (25) and by modulating T cell activation in vivo (26). Although the presence and role of flagellin in mounting an inflammatory response are documented, mechanisms that control its inflammation-inducing activities are still unknown.

The goals of these studies were, then, 2-fold. We wanted to determine whether NSPs cleave flagellin and alter its ability to induce host responses. We have found that NE and CG degrade flagellin, rendering it inactive. Indeed, our data demonstrate that

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flagellin, when degraded to completion, failed to induce the expression of the innate host defense genes, matrilysin and hBD-2. Taken together, these results point to flagellin as a novel substrate of NE and CG and highlight a new mechanism by which NSPs modulate the inflammatory response in the setting of bacterial infection.

Materials and Methods

Reagents

Purified NSPs, NE, CG, and PR3 and their specific peptide substrates were obtained from Elastin Products (Owensville, MO). Purified matrix metalloproteinases (MMPs), MMP-1, MMP-7, and MMP-8 were obtained from Chennicon (Temecula, CA). Activation of MMP proenzymes was achieved by exposure to 1 mM 4-aminophenylmercuric acetate at 37°C for 1 h or by a combination of 4-aminophenylmercuric acetate and trypsin as recommended by the manufacturer. The purity and activity of each enzyme were confirmed by a spectrophotometric method using specific substrates and/or SDS-PAGE analysis. Regarding NSP preparations, the proteases were also incubated with the broad serine proteinase inhibitor PMSF (100 μM) or the cysteine protease inhibitor E64 (10 μM) for 15 min at 37°C before addition of their peptide substrates to verify their specific activities. The anti-flagellin polyclonal Ab was provided by A. Prince (Columbia University, New York, NY). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cells, mice, and bacteria

The human lung carcinoma cell line Calu-3 was obtained from American Type Culture Collection (Manassas, VA) and was maintained in RPMI 1640 medium supplemented with 10% FBS without antibiotics or in the presence of 50 μg/ml gentamicin. To obtain neutrophils, wild-type mice and mice deficient in NE, CG, or both were injected i.p. with 15% glycogen (1 ml/mouse) to elicit the cells to the peritoneum. Mice deficient in NE or CG were generated by gene-targeting technique and interbred to obtain mice doubly deficient in NE and CG (3, 4). All mice are in the C57/Sv129 background. Four hours after i.p. injection, mice were sacrificed by CO2 narcosis according to the animal studies committee of Washington University School of Medicine (St. Louis, MO). Next, the peritoneum was lavaged with 5 ml of HBSS (1× HBSS). The lavage fluid was spun and resuspended in a hypotonic lysis solution to remove red cell contamination. Neutrophils represented >95% of the cell population, and >98% were viable as judged by differential counting and trypan blue dye exclusion. Aliquots of cells (2 × 10⁶ cells) were prepared in a total volume of 0.2 ml of HBSS and were kept on ice until use. Pseudomonas aeruginosa and Salmonella typhimurium strains were also obtained from American Type Culture Collection (Manassas, VA) and routinely grown at 37°C in 3% tryptic-soy broth (TSB).

Purification of flagellin

P. aeruginosa and S. typhimurium flagellin were purified as described previously (21) with some modifications. A 400-ml sample of an overnight culture was centrifuged at 10,000 × g for 15 min. Ammonium sulfate was added to the bacterial supernatant to reach 40% saturation over a period of 2 h at room temperature. After centrifugation at 20,000 × g for 40 min, the pellets were resuspended in 6 ml of 50 mM Tris-HCl, pH 9.5, containing 0.5 mM DTT and dialyzed extensively against the same buffer. Flagellin was collected by centrifugation at 27,000 × g for 40 min and was resuspended in 2 ml of 50 mM Tris-HCl, pH 7.5, containing 0.5 mM DTT. To obtain a preparation of completely detached monomeric flagellin, the pH of the protein solution was adjusted to 3.5 by the addition of 0.25 M HCl for 5 min at room temperature and was immediately restored to a neutral pH by the addition of 0.25 M NaOH. Finally, flagellin was analyzed by SDS-PAGE and native gel electrophoresis on 12% acrylamide gels.

Degradation of flagellin by proteolytic enzymes

All enzymes tested were used at equimolar concentrations. Flagellin (5 μM) was incubated with each enzyme in a 20-μl reaction volume at 37°C for designated times. In separate experiments, NE and CG were incubated with PMSF or E64 as described above before addition of flagellin. When using NSPs, the reactions were conducted in PBS, pH 7.4. The reactions with MMPs were, however, performed in a buffer containing 20 mM Tris-HCl, pH 7.5, supplemented with 10 mM CaCl₂, 150 mM NaCl, and 1 μM ZnCl₂. Unless indicated, the reactions were performed in duplicate. After designated times of incubation, they were subjected to protein electrophoresis under reducing conditions. The gels were stained with Coomassie Brilliant Blue and photographed. Duplicate gels were processed for ECL Western blotting (NE, Boston, MA) using specific Abs.

In other experiments, degradation of flagellin by NE was assessed directly in intact bacteria. Briefly, P. aeruginosa bacteria were freshly grown in TSB medium at 37°C and washed twice with and resuspended in FBS, and the OD of the culture was determined at 600 nm (1 OD = 10⁷ bacterial/ml). Mid-log grown bacteria (10⁸ bacteria) were incubated in the presence of the absence of NE (2.5 μM) in a total volume of 100 μl of PBS containing 1% (v/v) TSB for varying times (0, 2, and 4 h) (5). Next, the reactions were processed for ECL Western blotting (NE) using flagellin-specific Abs as described below.

Degradation of flagellin by neutrophils

Aliquots of peritoneum-isolated neutrophils (2 × 10⁶) were primed with LPS for 15 min and stimulated with N-MLP for an additional 15 min to allow activation and release of lysosomal enzymes (27). Next, flagellin was incubated alone and with nonactivated or activated cell aliquots at 37°C. After overnight incubation, levels of flagellin degradation were determined by Western blotting using an Ab specific to flagellin. To ensure the release of active proteinases, including NE, from neutrophils, NE activity (Aabsorbance/min at 410 nm) was determined by the spectrophotometric method using NE-specific chromogenic substrate Meo-Suc-Al-Al-Pro-Val-pNA (3), as recommended by the manufacturer (Elastin Products). As controls, this peptide substrate was degraded by purified NE, but not CG or PR3.

Immunoblotting

In general, samples were separated on 12% SDS-polyacrylamide gels and transferred by semidry electrophoretic transfer at 15 V for 20 min to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Little Chalfont, U.K.) in a total volume of 0.2 ml of TBS. The membranes were incubated with either a 1/10,000 dilution of anti-Pseudomonas flagellin polyclonal antisemur (12) or a 1/1,000 dilution of an Ab against E. coli flagellin (IBEN International, Gaithersburg, MD), which cross-reacts with S. typhimurium flagellin. In the OD of the culture was determined at 600 nm (1 OD = 10³ bacterial/ml). Next, samples were incubated with either an appropriate dilution of peroxide-linked secondary IgG (Amersham Pharmacia Biotech) in blocking buffer for 1 h, washed twice with TBS containing 0.1% Tween 20 for 10 min. Membranes were subsequently incubated in a blocking buffer for 1 h, washed twice, and developed with the ECL system according to the manufacturer’s instructions.

N-terminal sequence analysis of flagellin degradation products

Flagellin (10 μM) was incubated with increasing concentrations of NE or CG at 37°C for 30 min. The samples were then subjected to SDS-PAGE, and the OD of the culture was determined at 600 nm (1 OD = 10³ bacterial/ml). Mid-log grown bacteria (10⁸ bacteria) were incubated in the presence of 10% FBS without antibiotics or in the presence of 50 μg/ml gentamicin, and incubated at 37°C for 90 min. Epithelial monolayers were then washed extensively with PBS, and the OD of the culture was determined at 600 nm. Total RNA was isolated from the culture using Qiagen RNeasy kit (Hilden, Germany). RT-PCR was performed using specific primers: for hBD-2: forward, 5′-GAG TCA TGC TGA ACG CTA CAC CAG-3′; reverse, 5′-GAG TTC TCA GTG TAC ATG GTC CTT CCT GCA GCT GGC CCT CC-3′; for hBD-3: forward, 5′-GAG TCC CCT CCA AGC CAT CAG-3′; and the reverse primer, 5′-GAA ATG GTC GTG TAC ATG GTC TGG CTT TGT CTT GCA GAA TTT TG-3′.
Reactions were conducted for 25 cycles at an annealing temperature of 57°C and analyzed on 3% agarose gels.

**Mouse pneumonia model**

Mice (n = 6) were intranasally (i.n.) challenged with *P. aeruginosa* as previously described (28). Briefly, mice were anesthetized by i.p. injection of ketamine hydrochloride (75 mg/kg) and medetomidine hydrochloride (1 mg/kg), followed by i.n. administration of *P. aeruginosa* bacteria (4 × 10⁶ CFU/mouse). At this dose, mice exhibit signs of infection, including ruffled fur and lethargy, but the majority recover by 48 h postinfection (Y. S. Lopez-Boado, M. Espinola, S. Bahr, and A. Belaaouaj, manuscript in preparation). As a control, mice (n = 6) were instilled i.n. with *P. aeruginosa* LPS (10 μg/mouse) in 50 μl of PBS to incite a neutrophilic response (29). At designated time points (24 h postchallenge), mice were sacrificed, and their lungs were gently perfused with saline via the right ventricle. The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge catheter (BD Biosciences, Mountain View, CA). The lungs were lavaged in situ (bronchoalveolar lavage (BAL)) with a total of 2 ml of HBSS (pH 7.4). The BAL fluids were centrifuged for 10 min at 4°C, and the supernatants were aliquoted and stored at −80°C until use.

**Elastin zymography**

The elastolytic activity in cell-free BAL fluids was assessed by elastin zymography as previously described (3). BAL supernatants (20 μl) were migrated under nonreducing conditions at 4°C on SDS-PAGE gels (12%), containing 1 mg/ml elastin. Purified NE (0.1 μg) was used as a control. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 30 min and incubated at 37°C for 48 h in 50 mM Tris-HCl (pH 8.2) containing 5 mM CaCl₂. The gels were then stained with Coomassie Brilliant Blue and destained in 5% acetic acid and 10% methanol. Active NE appears as a transparent lysis band at ~29 kDa.

**Results**

**Neutrophil serine proteinases cleave *P. aeruginosa* flagellin**

The ability of NSPs to cleave the virulence factor flagellin was examined using purified flagellin from *P. aeruginosa*. As shown in Fig. 1A, flagellin was effectively degraded by NE and CG, suggesting the high susceptibility of this protein to proteolysis by these enzymes. However, degradation of flagellin by proteinase 3 was very limited and was not further characterized. Preincubation of NE and CG with the serine proteinase inhibitor PMSF, but not with the cysteine protease-specific inhibitor E64, prevented flagellin degradation (Fig. 1, B and C). This serine proteinase-specific inhibition was further confirmed using the secretory leukocyte proteinase inhibitor (data not shown). In contrast, using experimental conditions comparable to those of NSPs, none of the MMPs tested cleaved flagellin (Fig. 1A). These included neutrophil collagenase (MMP-8), interstitial collagenase (MMP-1), and matrixilysin (MMP-7). Of note, we have previously shown that flagellin can up-regulate the expression of MMP-7, an MMP involved in host defense mechanisms (21, 30).

**NSPs degrade flagellin present in intact bacteria**

To determine whether NSPs degrade flagellin in intact *P. aeruginosa* bacteria, we incubated whole bacteria with NE and analyzed the reaction by Western blotting using a flagellin-specific Ab. Our data demonstrate that in the absence of NE, flagellin remained intact and that its expression increased slightly over time (0 vs 2 h) as judged by immunoblotting (Fig. 2A). However, incubation of

![FIGURE 1](http://www.jimmunol.org/)  
**FIGURE 1.** Neutrophil serine proteinases cleave *P. aeruginosa* flagellin. A, *P. aeruginosa* flagellin (5 μM) was incubated with NE, CG, and PR3 (2.5 μM) for 1 h at 37°C, separated on 12% SDS-PAGE gels under reducing conditions, and stained with Coomassie Blue. Purified flagellin was also incubated with purified and activated MMPs for 1 h at 37°C, resolved on SDS-PAGE, and analyzed by Western blotting using a flagellin-specific Ab. Note that NSPs, but not MMPs, degraded flagellin. B and C, NE and CG were incubated alone or with the inhibitor PMSF or E64. Next, flagellin was added to the reactions, and the incubation was conducted for 1 h at 37°C. The reactions were then resolved on 12% SDS-PAGE and analyzed by Western blotting with anti-flagellin polyclonal antiserum. Note that PMSF, but not E64, blocked flagellin degradation by NE and CG. Molecular mass standards are shown on the right. The arrowhead points to the locations of NE and CG proteins.
bacteria with NE was accompanied by complete flagellin degradation as early as 2 h. These results indicate that NSPs, including NE and CG, are capable of cleaving not only soluble flagellin that is secreted or leaked by the bacteria, but also when the protein is assembled at the cell surface.

Relative importance of neutrophil serine proteinases in flagellin degradation

To further characterize flagellin degradation by NSPs, we incubated flagellin with wild-type (WT) mouse neutrophils directly. In parallel experiments the cells were treated with LPS followed by fMLP to maximize the release of proteases and then added to flagellin. As shown in Fig. 2B, flagellin was entirely degraded in the presence of unstimulated or stimulated neutrophils. These data suggest that flagellin was degraded by released proteases, including NE and CG. To address the relative importance of NSPs to degrade flagellin, we performed similar ex vivo experiments, this time using neutrophils that were isolated from WT and NE-, CG-, or NE- and CG-deficient mice and activated with LPS/fMLP (Fig. 2C). Although flagellin was completely degraded in the presence of WT and NE-deficient neutrophils, incubation of this protein with neutrophils derived from CG- or NE/CG-deficient mice resulted in incomplete degradation of the protein, suggesting that NSPs, in particular CG, play an important role in flagellin cleavage by neutrophils. Of note, overnight incubation of flagellin alone did not cause a spontaneous degradation of the protein (Fig. 2, B and C, lane a), and flagellin Ab did not immunoreact with neutrophil proteins (Fig. 2, B and C, lane b).

Relative contributions of NSPs to degrade bacterial flagellin

To compare the relative contributions of NSPs to cleave flagellin, P. aeruginosa flagellin was incubated with NE or CG, and degradation was monitored as a function of enzyme concentration and time. Fig. 3A shows that flagellin was degraded by both NE and CG. However, at similar concentrations the rates of catalysis were different for NE and CG. CG catalyzed rapid and complete cleavage of flagellin (100% of flagellin was completely degraded by 0.25 μM CG within 20 min). In contrast, degradation of flagellin by NE occurred at a somewhat slower rate (0.25 μM of NE generated multiple flagellin fragments within 60 min). Next, to determine whether NSPs could cleave flagellin from other bacterial species, we purified flagellin from S. typhimurium cultures and exposed it to NE and CG. Similarly to P. aeruginosa, S. typhimurium flagellin was degraded by NE in an enzyme concentration- and time-dependent manner (Fig. 3B). Like NE, CG degraded S. typhimurium flagellin, but rapidly (data not shown). These data suggest that although NSPs effectively cleave flagellin from different pathogens, their degradation rates are different.

Cleavage sites of flagellin

To attempt to identify the cleavage sites of NSPs in flagellin, we incubated both P. aeruginosa- and S. typhimurium-purified flagellin with low concentrations of NE and CG for 30 min (Fig. 4, A and B). Major cleavage products were identified by Coomassie Blue staining and were processed for N-terminal sequencing. Analysis of our results showed that NE catalyzed an initial cleavage in S. typhimurium flagellin at Ile-Ala, 50 residues from the N terminus of the protein, and within the D0 N-terminal domain (Fig. 4C) (31). Of note, two bands of NE-cleaved S. typhimurium flagellin migrating at ~40 and 45 kDa had the same N terminus. Notably, we identified a cleavage site in P. aeruginosa flagellin 59 residues from the N terminus of the protein, which by simulation of the proposed structure of Salmonella flagellin could be located within the D0 N-terminal domain (31) (Fig. 4D). Interestingly, CG cleaved S. typhimurium flagellin at Phe-Thr and P. aeruginosa flagellin at Asn-Ala, 54 and 63 residues from the N terminus, respectively. This suggests that CG, like NE, targets flagellin within the D0 N-terminal domain.

NSPs cleave flagellin, altering its biologic activity

We have determined by size exclusion chromatography that our purification procedure of flagellin usually results in a mix of monomers and aggregates of higher m.w. (data not shown). As reported previously (32), this could be due to the autopolymerization of the protein that occurs at high concentrations of flagellin or in the presence of salt (e.g., ammonium sulfate) during the purification process. When these protein preparations were subjected to a transitory acidification or heat treatment, flagellin became essentially monomeric, as shown by native PAGE (data not shown). Of importance, these treatments did not affect flagellin activity, as illustrated by the similar induction of expression of both matrilysin and hBD-2 (data not shown). These data also indicate that monomeric and/or polymerized flagellin have similar biological activity as bacteria-associated flagellin.

To determine whether the biologic activity of flagellin is altered after its degradation by NSPs, we incubated the protein with different concentrations of NE and CG and examined the ability of the degradation products to induce the expression of innate host defense genes in human lung epithelial cells. In Fig. 3, we showed that treatment of flagellin with increasing concentrations of NE and CG led to progressive degradation of the protein. This cleavage of flagellin resulted in a parallel loss in its ability to induce the expression of host defense genes in epithelial cell lines, Calu-3 and A549 (Fig. 5 and data not shown). Indeed, degradation of flagellin by NE or CG resulted in the loss of induction of matrilysin and hBD-2 gene expression (Fig. 5, A, B, and D). Furthermore, when the catalytic activity of NE or CG was blocked with PMSF and the secretory leukocyte protease inhibitor, flagellin was not degraded and induced the expression of both matrilysin and hBD-2 (Fig. 5, A and B, and data not shown). The cysteine protease inhibitor E64,

**FIGURE 3.** A. NE and CG catalyze flagellin degradation at different rates. Purified flagellin (5 μM) from P. aeruginosa was incubated with different concentrations of NE or CG for various times, and the reactions were resolved on 12% SDS-PAGE and processed for Western blotting using P. aeruginosa flagellin-specific Ab. The arrow points to flagellin. Molecular mass standards are shown on the right in the upper panel. B, NE and CG also degrade S. typhimurium flagellin in an enzyme dose- and time-dependent manner. As described in A, purified S. typhimurium flagellin (5 μM) was incubated with NE or CG, and the reactions were processed for Western blotting using S. typhimurium flagellin-specific Ab. Shown are the data for NE only. Molecular mass standards are shown on the right.
FIGURE 4. Cleavage sites of NE in *P. aeruginosa* and *S. typhimurium* flagellin. Purified flagellin from *P. aeruginosa* (A) and *S. typhimurium* (B) was incubated with low concentrations of NE or CG (data not shown for CG) for 30 min. Samples were resolved in 12% SDS-PAGE and transferred to ProBlott membranes, and distinct bands were excised and processed for sequencing by automated Edman degradation. C, N-terminus sequences of NE-cleaved *P. aeruginosa* or *S. typhimurium* flagellin fragments. Arrowheads point to sequenced fragments. D, Schematic structure of flagellin (adapted from Ref. 41) and localization of the initial cleavage site in the D₀ N-terminal domain. The arrow on the left indicates the location of the NE cleavage site; the arrow on the right points to characteristic domains of flagellin protein.

however, did not protect flagellin from cleavage, and therefore its ability to induce the expression of host defense genes was lost (Fig. 5, A and B). This effect of flagellin on host gene expression was dependent on NSP concentration. For example, the capacity to induce the expression of matrilysin and hBD-2 genes was retained in partially degraded flagellin (e.g., NE at 0.25 μM) and was lost in totally degraded flagellin (e.g., NE at 2.5 μM; Fig. 5, C and D). Similar results were observed for CG (data not shown). Of note, the level of hBD-1 expression, which does not change in response to bacterial infection (33) or flagellin, was not altered in the presence of NE-degraded flagellin (Fig. 5E) or CG-degraded flagellin (data not shown).

In vivo colocalization of flagellin and NSPs

To determine the in vivo significance of our findings, we examined whether flagellin and free active NE colocalize in infected milieu. We challenged mice i.n. with *P. aeruginosa* bacteria and checked for the presence of flagellin and free active NE in the cell-free BAL fluids of infected mice 24 h postchallenge (Fig. 6A). Immunoblotting data showed the presence of flagellin in cell-free BAL fluids of *P. aeruginosa*-infected mice. Different flagellin bands were detected, including intact (band migrating similar to control), degraded (lower bands), and high m.w. complex (upper bands) bands. These latter could result from autopolymerization of flagellin or flagellin that interacted with bacterial or other proteins in the BAL. As expected no signal for flagellin was detected in the samples from LPS-instilled mice, demonstrating the high specificity of flagellin Ab. With respect to NE, there was a single lysis band in cell-free BAL of *P. aeruginosa*-infected mice that migrated similarly to control NE (Fig. 6B). These findings demonstrate that flagellin (free or bacteria-associated) and NSPs, including NE and CG, colocalize in the lung in the setting of infection and support the hypothesis that NSPs could encounter and degrade flagellin in vivo.

Discussion

Using different biochemical approaches, we demonstrated that NSPs cleave free or *P. aeruginosa*-associated flagellin, a cleavage that was prevented when the catalytic activity of the enzymes was specifically inhibited. We also found that NE and CG degrade *S. typhimurium* flagellin, a very potent cytokine inducer (24), suggesting that NSPs could target flagellin from other microbial species. Characterization of degraded flagellin of both *P. aeruginosa* and *S. typhimurium* found that flagellin cleavage was catalyzed by NE and CG at different rates and occurred in peptide bonds that are preferred by the enzymes (34). Also, the identification of two NE-degraded fragments in *S. typhimurium* flagellin with the same N-terminus, but different molecular masses, suggests that additional C-terminal processing of flagellin by NSPs may occur. Interestingly, both NE and CG generated a cleavage site within the D₀ domain for the presence of NE-degraded flagellin (Fig. 5E) or CG-degraded flagellin (data not shown).

FIGURE 5. Flagellin loses its innate host defense gene expression-inducing activity after exposure to NSPs. A and B, Flagellin was incubated with NE or CG for 1 h at 37°C. In parallel experiments, NE and CG were incubated with either PMSF or E64 before addition of flagellin. Next, Calu-3 lung epithelial cells were induced with untreated or treated flagellin. After 90 min of incubation, cells were washed and incubated in fresh medium for 6 h. At this time point, the expression of matrilysin and GAPDH was analyzed by Northern blotting with specific probes. Ctrl, untreated cells. C, Calu-3 lung epithelial cells were induced with flagellin that was pretreated with different concentrations of NE at 37°C for 1 h. The expression of matrilysin was analyzed as described in A and B. D and E, The expression of hBD-1 and hBD-2 genes in Calu-3 cells was analyzed by RT-PCR. Amplified products were resolved on 3% agarose gels and stained with ethidium bromide. The sizes of the amplified products for hBD-2 and hBD-1 are 241 and 258 bp, respectively. D, Data corresponding to flagellin that was not treated (0) or was treated with 0.25 and 2.5 μM. E, hBD-1 expression was unaltered by intact or degraded flagellin (NE at 0.025, 0.25, and 2.5 μM). M, 100-bp DNA ladder.
The N-terminal domain of flagellin. Together, these results suggest that NSPs have a common cleavage mechanism of flagellin, but degrade the protein at different rates within discrete and specific areas. More physiologically relevant, flagellin was completely degraded when incubated with neutrophils, indicating that NSPs could contribute to neutrophil-mediated flagellin cleavage.

Flagellin is a member of the pathogen-associated molecular patterns group, a collection of bacterial products that activate various cell types. We have shown that P. aeruginosa flagellin is the factor that induces the expression of matrilysin and hBD-2 in lung epithelial cells (Ref. 21 and this work). This finding was double confirmed using the isogenic mutant of P. aeruginosa deficient in filC, the gene that encodes flagellin (35), which failed to induce the expression of the matrilysin gene (21). In addition, we demonstrated that preparations of flagellin containing either monomeric and/or complex forms (i.e., flagella) induce a similar response in lung epithelial cells (data not shown). These findings suggest that flagellin, independently of its quaternary structure, is able to signal through its receptor, Toll-like receptor 5 (TLR5) in targeted cells (36). The loss of flagellin activity after exposure to NSPs could be attributed to the degradation of a motif that interacts directly with its receptor, Toll-like receptor 5 (TLR5) in targeted cells or alteration of the ligand domain conformation (37). Interestingly, under our experimental conditions, the presence of NSPs did not alter TLR5 expression in epithelial cells (data not shown), further suggesting that the loss of TLR5-mediated cell response is due to flagellin degradation. The basic structure of flagellin is composed of two C- and N-terminal constant domains and a central hypervariable domain, which varies in length among bacterial species (38). Although the role of flagellin as an immune response inducer is well established, the nature of the peptide that confers the protein its activity is still debatable. Recently, the active region in flagellin has been attributed to the central hypervariable domain by McDermott et al. (39) and to the constant N- and C-terminal domains by Eaves-Pyles et al. (40) and Donnelly et al. (41). Studies are underway to identify NSP-generated flagellin fragment(s), which retain their ability to induce the expression of innate host response genes.

In the setting of bacterial infections, NSPs could degrade flagellin inside the phagocytic vacuoles. This possibility is supported by a recent work from Zychlinsky and his group (42). They demonstrated that NE targets virulence factors of Shigellae, engulfed in the phagolysosomes (42). Alternatively, degradation of flagellin could take place extracellularly. In fact, there is accumulating evidence for the presence of flagellin and NSPs, particularly NE, in the infected milieu. For example, a high Ab titer against P. aeruginosa flagellin and elevated levels of active NE (up to 20 μM in CF sputum) have been detected in CF patients (43, 44). Separate studies have also documented the secretion of flagellin by bacteria and NE release from neutrophils in the course of infections (45–47). In accordance, we detected active NE and different forms of flagellin (intact, complex, and degraded) in mouse lungs in our pneumonia model with P. aeruginosa. Together, these observations suggest that active NSPs are likely to encounter and cleave flagellin. It should be emphasized that we used NSPs at concentrations below their physiological values, which have been reported to exceed the millimolar range (48, 49). Furthermore, the inability of CG or NE/CG-deficient neutrophils to completely degrade flagellin suggests the relative importance of NSPs in the degradation of flagellin.

Several studies demonstrated that flagellin signals through TLR-5 to induce the gene expression of host defense and proinflammatory molecules in various cell types (13, 16, 36). The purpose of the flagellin-TLR5 interaction is to reinforce the host antimicrobial systems. If uncontrolled, this interaction could also lead to excessive inflammatory response and injury of the host. In view of our findings, degradation of flagellin by NSPs could be regarded as beneficial to the host. In other words, neutrophils are called in when the first line of host defense is breached (e.g., epithelial lining). This neutrophil recruitment represents one of the means by which the host attempts to curb not only bacterial infection, but also the associated inflammation. However, under overwhelming conditions (e.g., uncontrolled infection), neutrophils and their secreted products could damage the surrounding tissues as well. In sum, our present work points to the virulence factor, flagellin, as a novel substrate targeted by NSPs. It also highlights the potential role of NSPs in modulating the host inflammatory response to bacterial infection. Taken altogether, these findings add new insights to the role of NSPs during the complex host-pathogen interactions. Future studies using mice deficient in NSPs will allow us to determine the relative importance of these proteinases in the resolution of flagellin-induced inflammation.

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References


