Extraintestinal Pathogenic *Escherichia coli* Survives within Neutrophils

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Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains are common causes of a variety of clinical syndromes, including urinary tract infections, abdominal infections, nosocomial pneumonia, neonatal meningitis, and sepsis. ExPEC strains are extracellular bacterial pathogens; therefore, the innate immune response (e.g., professional phagocytes) plays a crucial role in the host defense against them. Studies using the model ExPEC strain CP9 demonstrated that it is relatively resistant to neutrophil-mediated bactericidal activity. Although this could be due to resistance to phagocytosis, the ability of CP9 to survive the intracellular killing mechanisms of neutrophils is another possibility. Using a variation of the intracellular invasion assay, we studied the survival of CP9 within peripheral blood-derived human neutrophils. Our results indicated that CP9 did survive within human neutrophils, but we were unable to demonstrate that intracellular replication occurred. This finding was not unique to CP9, since when a conservative assessment of survival was used, four of six additional ExPEC strains, but not an *E. coli* laboratory strain, were also capable of survival within neutrophils. Initial studies in which we began to decipher the mechanisms by which CP9 is able to successfully survive intracellular neutrophil-mediated bactericidal activity demonstrated that CP9 was at least partially susceptible to the neutrophil oxidative burst. Therefore, absolute resistance to the oxidative burst is not a mechanism by which ExPEC survives within neutrophils. In addition, electron microscopy studies showed that CP9 appeared to be present in phagosomes within neutrophils. Therefore, avoidance of phagosomal uptake or subsequent escape from the phagosome does not appear to be a mechanism that contributes to CP9’s survival. These findings suggest that survival of ExPEC within neutrophils may be an important virulence mechanism.

Extraintestinal pathogenic *Escherichia coli* (ExPEC) strains are a clinically significant group of pathogens that cause a variety of clinical syndromes. ExPEC strains are a major cause of urinary tract infections, abdominal infections, nosocomial pneumonia, neonatal meningitis, and sepsis (16). The direct and indirect costs of ExPEC infection amount to billions of dollars each year (30).

ExPEC strains are considered to be classic extracellular bacterial pathogens, and the host innate immune system plays a critical role in defense against infections due to this group of bacteria. Professional phagocytes (e.g., macrophages, monocytes, and neutrophils) are a critical component of the innate immune system. Shortly after an organism enters the host, a combination of host and bacterial factors stimulates neutrophil chemotaxis toward the site of infection. Traditionally, ExPEC’s primary defense against neutrophil-mediated bactericidal activity is thought to be the presence of surface polysaccharides (e.g., capsule and O-specific antigen) that enhance resistance to phagocytosis (4, 6, 13). The host counters these bacterial virulence factors through opsonization, mediated by complement components and pathogen-specific antibodies, which optimize phagocytosis. After engulfment of extracellular pathogens, such as ExPEC, by neutrophils (polymorphonuclear leukocytes [PMNs]), the pathogens are incorporated into phagosomes and subsequently exposed to reactive oxygen species and a variety of antimicrobial peptides and enzymes, presumably resulting in bacterial death.

However, the interaction between extracellular bacterial pathogens and neutrophils has proven to be more complex. Although in many cases, neutrophils successfully eradicate the bacteria from the site of infection, it is becoming increasingly clear that pathogens have evolved additional strategies to modulate this crucial host-pathogen interaction. *Streptococcus pyogenes*, for example, utilizes multiple mechanisms for evading destruction by human neutrophils, including inhibiting PMN recruitment, blocking opsonization by host proteins, and exploiting neutrophil apoptosis (37). Some recent studies have also shown that organisms such as *Staphylococcus aureus* and group A streptococci may survive in neutrophils and that this persistence in neutrophils may enhance their overall survival (1, 11).

Data generated in our laboratory have shown that ExPEC is also capable of minimizing neutrophil-mediated bactericidal activity. We have demonstrated that the O antigen and capsule modulate human neutrophil chemotaxis (27). More recently, we have established that the *E. coli* toxin α-hemolysin (Hly), depending on the bacterial titer, induces either neutrophil apoptosis or necrosis/lysis both in vitro and in a rat pneumonia model (26). In these ways, ExPEC is able to both diminish encounters with neutrophils and prematurely induce neutrophil death, thereby maximizing its chances for survival. Studies reported here, using ExPEC model strain CP9, demonstrated that this strain is relatively resistant to neutrophil-mediated bactericidal activity. Although this could be due to resistance to phagocytosis, it is also possible that CP9 is able to survive the intracellular killing mechanisms of neutrophils. Further-
more, while exploring the potential effects of ExPEC on neutrophil apoptosis and necrosis/lysis, we found in neutrophil morphological studies that ExPEC strain CP9 appeared to be able to survive within neutrophils. Therefore, we hypothesized that an additional mechanism by which ExPEC subverts PMN function involves the ability to survive within PMNs after phagocytosis.

In the study described here we tested the hypothesis that a wild-type extraintestinal pathogenic strain of *E. coli* (CP9) and other ExPEC strains can survive within neutrophils after phagocytosis. Using a variation of the intracellular invasion assay, we demonstrated that most, but not all, ExPEC isolates were able to survive within neutrophils. Here we also describe initial studies in which we began to decipher the mechanisms by which CP9 is able to survive exposure to intracellular neutrophil-mediated bactericidal activity.

**MATERIALS AND METHODS**

**Bacterial strains and media.** The model pathogen CP9 (O4:K54) is an *E. coli* blood isolate cultured from a patient with sepsis and has been described previously (19, 28). CP9 possesses many of the characteristics of typical ExPEC strains (29), an intraperitoneal infection model (31), and a pneumonia model (24, 25). The survival of six additional ExPEC strains within neutrophils was also evaluated. One strain was an isolate with an unknown serotype that was obtained from a patient with bacteremia in Buffalo, NY (strain 104), and two bacteremic isolates. One strain was an isolate with an unknown serotype that was obtained from a patient with bacteremia in Buffalo, NY (strain 104), and two bacteremic isolates. Other ExPEC strains were grown in iron-chelated LB broth (5 ml) (39), IA2 [O4:K12] (15), and CFT073 [O6:K2] (40), as well as K-12 laboratory strain HB101. Bacterial strains were grown in iron-chelated LB broth (5 g/liter yeast extract, 10 g/liter tryptone, 10 g/liter NaCl, 31.24 mg/liter 22-dipalmitoyl-sn-glycerol-3-phosphatidylcholine). Pilot experiments determined that incubation of neutrophils with 105 neutrophils were mixed with approximately 105 to 3x107 CFU of CP9, 1% autologous serum, and 1% rabbit anti-CP9 serum in polystyrene tubes for 60 min and mixed with a Nutator at 37°C. Subsequently, gentamicin (final concentration, 200 μg/ml) was added, and the tubes were incubated for an additional 60 min. Next, the neutrophils were lysed, and the number of CFU was determined. Surprisingly, in repeated assays, approximately 1x103 to 3x107 CFU were consistently found. Together, these experiments suggested that under our assay conditions, either treatment with gentamicin did not kill all extracellular bacteria or cytosol D did not completely inhibit phagocytosis. To try to determine which of these possibilities was correct, experiments were first performed to determine if increasing the gentamicin concentration or incubation time eradicated residual extracellular bacteria that survived treatment with 200 μg/ml of gentamicin for 60 min. Data obtained in these experiments confirmed that treatment with gentamicin did not eradicate all extracellular bacteria and that treatment with 200 μg/ml of gentamicin for 30 min was as effective as treatment with higher concentrations for longer incubation times (data not shown). Therefore, in all subsequent experiments 30 min of incubation with 200 μg/ml of gentamicin was utilized. Next, we assessed whether cytosol D completely inhibited phagocytosis, as incomplete inhibition would allow some bacteria to be internalized and therefore escape killing by gentamicin. In pilot experiments we determined that incubation of 105 CFU of CP9 with 105 neutrophils pretreated with cytosol D for up to 60 min was as effective as a 10-min incubation with decreasing background titers (data not shown). Therefore, incubation with 20 μg/ml cytosol D for 10 min was used in all subsequent experiments. (ii) A total of 1x107 CFU of CP9 were incubated for 60 min with neutrophils that were pretreated with cytosol D or were not treated. Next, a cytoside was prepared and examined by light microscopy. Intracellular CP9 cells were readily observed in many of the untreated neutrophils but were never observed in neutrophils pretreated with cytosol D. However, the sensitivity of this method is not optimal, and low titers of intracellular bacteria may not be identified. (iii) Finally, using a flow cytometry-based assay that measured uptake of fluorescein isothiocyanate-labeled zymosan particles (23), we found that phagocytosis in neutrophils treated with 20 μg/ml cytosol D was mostly but not completely inhibited (Fig. 1). Using our flow cytometric data, we could not precisely determine the degree to which phagocytosis was inhibited by cytosol D, but even if phagocytosis was inhibited by 99% (which we estimated), a measurable number of intracellular bacteria may still have been present. Therefore, we could not be sure whether under the conditions of our assay the bacteria enumerated when neutrophils were pretreated with cytosol D were intracellular or were extracellular and not killed by gentamicin. The most conservative course was to calculate survival by subtracting this number of bacteria from the number of CFU enumerated when neutrophils were pretreated with cytosol D and that it was better to underestimate survival than to overestimate survival. Therefore, we opted to calculate intracellular survival by determining the difference between the bacterial titer obtained for untreated neutrophils and the bacterial titer obtained for cytosol D-pretreated neutrophils.

**Standard assay used to measure survival of *E. coli* within neutrophils.** As a result of these findings, assays to measure the survival of *E. coli* within neutrophils were performed as follows. A 1 ml mixture consisting of (i) 5x107 purified human neutrophils (resuspended in 50 μl of 1× PBS–20% autologous human serum heated at 56°C for 30 min to inactivate complement) that were either incubated with 20 μg/ml cytosol D for 10 min at 37°C prior to the addition of bacteria and subsequently throughout the assay or not treated, (ii) approximately 5x106 CFU of the strain being assessed (resuspended in 100 μl of 1× PBS). (iii) 10 μg of autologous serum (final concentration, 1%), (iv) 10 μl of rabbit anti-CP9 serum (final concentration, 1%), and (v) 1× PBS (380 μl) was incubated for 60 min at 37°C on a Nutator. For assays in which we assessed the survival of HB101, the autologous serum and rabbit anti-CP9 serum were omitted since HB101 is serum sensitive. Next, gentamicin (final concentration, 200 μg/ml) was added; in addition, 20 μg/ml cytosol D was added to the untreated neutrophils in order to inhibit further phagocytosis. Then the preparations were incubated for an additional 30 min at 37°C. Pilot experiments established that addition of cytosol D at this time did not add any significant effect on survival. Samples were then placed on ice, and neutrophils were washed twice with 5 ml of 1× PBS to remove the gentamicin, which could kill intracellular bacteria when they were subsequently released by lysis of neutrophils. Initially, neutrophils were lysed with saponin (final concentration, 0.5 mg/ml) to release intracellular bacteria. How-
ever, a comparison of the bacterial titers obtained for neutrophils that were lysed with saponin and the bacterial titers obtained for neutrophils that were not treated with saponin showed that the titers were similar; therefore, saponin-mediated lysis was not performed. Presumably, neutrophils lyse when they are treated with saponin according to the manufacturer’s instructions. In brief, 1 \times 10^7 CFU of live CP9 (Phagoburst [Orpegen Pharma], distributed by BioCarta, San Diego, CA) according to the manufacturer’s instructions to confirm the effectiveness of DPI using the Phagoburst assay (Phagoburst [Orpegen Pharma], San Diego, CA). 

Transmission electron microscopy (TEM) of the neutrophil interaction with E. coli. (A) Quenched neutrophils not pretreated with cytochalasin D and not exposed to zymosan particles. (B) Quenched neutrophils pretreated with cytochalasin D and not exposed to zymosan particles. (C) Quenched neutrophils pretreated with cytochalasin D and not exposed to zymosan particles. (D) Quenched neutrophils pretreated with cytochalasin D and exposed to zymosan particles. cyto. D, cytochalasin D.

Intracellular survival assay for inhibition of macrophagoctosis. We examined the effects of the macrophagoctosis inhibitors methylamine (10 to 100 mM), amiloride (3 mM), dimethyl amiloride (500 \mu M), and rottlerin (2 \mu M) in our intracellular survival assays to determine their effects on the intracellular survival of CP9. Unfortunately, methylamine was found to be toxic to our bacteria and therefore could not be used in our assay. In a recent paper the researchers noted that cytochalasin D, amiloride, and dimethyl amiloride lacked specificity for blocking phagocytosis and/or macrophagoctosis in other phagocytic cells (33), but the protein kinase C inhibitor rottlerin was found to be an effective specific inhibitor of macrophagoctosis; thus, we used this inhibitor in our assay as well. The standard survival assay for measuring E. coli survival within neutrophils was carried out with either 8.1 \mu M phorbol 12-myristate 13-acetate (positive control) or 5 \times 10^7 CFU of live CP9 (wild type) for 10 min at 37°C. Untreated neutrophils or neutrophils exposed to DMSO (DPI solvent) were used as negative controls. The generation of reactive oxygen species was measured by flow cytometry by gating on 10,000 neutrophil events and determining the proportion of these cells for conversion of the fluorogenic substrate dihydrodihydrodihydramine 123 to rhodamine 123. In the presence of DPI, nearly complete inhibition of the generation of rhodamine 123 was observed (Fig. 2), confirming the effectiveness of DPI as an inhibitor of the oxidative burst.

Measurement of ExPEC binding to neutrophils. Differences in bacterial binding to untreated neutrophils may affect phagocytosis, which in turn may affect the survival. Therefore, we examined whether the strains whose neutrophils was assessed in this study bound differentially to neutrophils. A total of 5 \times 10^7 CFU of E. coli were mixed with 8.0 \times 10^8 to 1.2 \times 10^5 CFU of CP9, CFT073, IA2, K1/Y, 743, 470, and 104 at 4°C for 30 min. At this temperature bacteria can bind to but are not phagocyted by neutrophils. Subsequently, differential centrifugation (110 \times g for 11 min) was performed to separate neutrophil-bound and nonbound bacteria, and the bacteria bound to neutrophils were enumerated. The percentage of bacteria bound was calculated by determining the ratio of the number of neutrophil-bound bacteria to the initial bacterial input titer. The percentages of bacteria that bound to neutrophils were similar for all strains, as follows: CP9, 3.3%; CFT073, 2.7%; IA2, 4.2%; K1/Y, 3.5%; 743, 3.0%; 470, 2.7%; and 104, 4.2%. Therefore, this factor did not appear to be a confounding variable in our survival assay.

Serum sensitivity assay. To determine the effect of complement-mediated bactericidal activity on CFT073 and IA2, serum sensitivity assays were performed as described previously (32), except that 1% active serum and 1% active anti-C9 rabbit polyclonal antibody were utilized. Bacterial titers were determined at 0, 30, and 60 min.

Assessment of neutrophil phagocytosis. Laser scanning confocal microscopy was employed to confirm the phagocytosis of various ExPEC strains. ExPEC strains were stained with 400 \mu M carboxyfluorescein diacetate (succinimidyl ester) (Invitrogen) (2,2,5,7,8) and then washed three times in 1× PBS. Neutrophils were isolated from human blood, and the intracellular survival assay was performed as described above using bacteria stained with carboxyfluorescein diacetate (succinimidyl ester). After the final wash, the neutrophil pellet was resuspended in 20 \mu l of 1× PBS and allowed to adhere to poly-L-lysine (Sigma)-pretreated coverslips for 20 min at room temperature. Nonadherent neutrophils were removed by washing the preparations with 1× PBS. In order to identify the outer membrane of neutrophils, cells were incubated for 5 min in 1× PBS containing 2,4,6-triamin-1,3,5-triazine at 37°C. Cells were washed twice in 1× PBS and were then fixed using 3.5% paraformaldehyde (Sigma) for 5 min at 37°C. Fixed cells were followed by 3 min of permeabilization in 0.3% Triton-X-1% PBS. After washing, cell nuclei were stained with 1 \mu M TO-PRO-3 (Invitrogen) for 15 min at room temperature. The cells were then washed and mounted on glass slides using Vectashield (Vector) mounting medium. Neutrophils were examined at the Confocal Microscopy and 3-D Imaging Facility (School of Medicine and Biomedical Sciences, University at Buffalo) using a Zeiss LSM 510 Meta confocal microscope. Z stacks were analyzed in order to distinguish intracellular bacteria from extracellular bacteria. One hundred neutrophils were counted per slide, and the percentage of phagocytosis was calculated. For assessment of phagocytosis by light microscopy we used the same suspensions. One hundred neutrophils were counted per slide for the presence of intracellular bacteria (within the limits of the methodology).
RESULTS

ExPEC model pathogen CP9 is relatively resistant to neutrophil-mediated bactericidal activity. Neutrophil-mediated bactericidal activity against various titers of CP9 was assessed in the presence of complement and CP9-specific antisera as opsonins. While some killing was observed from 60 to 90 min, the magnitude was only approximately 0.25 log (Fig. 3). These data demonstrate that CP9 is relatively resistant to neutrophil-mediated bactericidal activity.

ExPEC model pathogen CP9 survives within human neutrophils. Strains of ExPEC are thought to be classical extracellular bacterial pathogens, whose primary defense against neutrophil-mediated bactericidal activity is avoidance of phagocytosis; once phagocytosed, ExPEC strains are believed to be killed. However, if strains of ExPEC were able to avoid intracellular killing and survive within neutrophils, this would give them a significant advantage and increase their pathogenic capability. In neutrophil morphological studies we noted that ExPEC strain CP9 appeared...
to be able to survive within neutrophils (26). Therefore, we hypothesized that an additional mechanism by which ExPEC may subvert PMN function involves the ability to survive within PMNs after phagocytosis. To test this hypothesis, we assessed the survival of ExPEC wild-type strain CP9 within human neutrophils. To assess the survival of CP9 within neutrophils, we used a modification of the cell invasion assay (14). The premise of this assay is that bacteria that are able to survive within host cells can be identified by adding gentamicin, which kills all extracellular bacteria but not intracellular bacteria since gentamicin is unable to enter cells. To control for extracellular bacteria that may not have been killed by gentamicin, intracellular survival was calculated by determining the difference between the bacterial titer obtained for untreated neutrophils and the bacterial titer obtained for neutrophils pretreated with cytochalasin D, an inhibitor of phagocytosis. However, this provided a conservative estimate of survival since some (or all) of the bacteria counted when neutrophils were pretreated with cytochalasin D may actually have been intracellular since we were not able to completely inhibit phagocytosis (see Materials and Methods). The survival of CP9 within neutrophils is shown in Fig. 4. The number of CP9 CFU obtained for untreated neutrophils reflected both the number of intracellular CP9 cells and the number of extracellular CP9 cells (if any) that survived treatment with gentamicin (Fig. 4A), and the number of CP9 CFU obtained for neutrophils pretreated with cytochalasin D included the extracellular CP9 cells that were not killed by gentamicin and/or the intracellular bacteria that may have been present if cytochalasin D did not mediate complete inhibition of neutrophil phagocytosis (Fig. 4A). The calculated difference between these titers was a conservative estimate of intracellular survival (Fig. 4B). The level of intracellular survival was dependent on the input titer of CP9, increasing as the input titer increased (Fig. 4B). When 1.5 × 10^7 CFU of CP9 were added to 5 × 10^5 neutrophils, we calculated that 1.6 × 10^5 ± 6.7 × 10^4 CFU of CP9 survived. However, since the efficiency of phagocytosis of CP9 is unknown, it is not clear what proportion of CP9 cells survived within neutrophils once they were phagocytosed. At the lowest input titer at which we were able to detect intracellular survival of CP9 (3.5 × 10^5 CFU), 1.8 × 10^2 ± 2.5 × 10^2 CFU of CP9 were calculated to survive. Whether survival occurs at lower input titers is not clear, since we were not able to decrease the background titer of gentamicin-resistant CP9 determined when neutrophils were pretreated with cytochalasin D to less than 1 × 10^3 CFU. These results clearly demonstrate that the model ExPEC strain CP9 is able to survive within human neutrophils.

**ExPEC strain CP9 survives but does not replicate within neutrophils.** In previous studies (26) microscopic examination of neutrophils that had interacted with CP9 revealed multiple intracellular bacteria. The observation that CP9 was able to survive within neutrophils raised the question of whether CP9
could also replicate within neutrophils. To answer this question, we performed the gentamicin protection assay as described above but assessed survival over time. A total of 5 x 10^6 CFU of CP9 were incubated with 5 x 10^5 purified human neutrophils that either were pretreated or were not pretreated with cytochalasin D. After 1 h of incubation with CP9, neutrophils were treated with gentamicin to kill the extracellular bacteria and with cytochalasin D (only neutrophils that were not pretreated) to prevent further phagocytosis. Neutrophils were then harvested to assess the intracellular survival of CP9 at 30, 60, 90, 120, and 150 min after the addition of gentamicin (Fig. 5A). For the times examined, the survival of CP9 over time was constant. These data can be explained by either (i) a lack of intracellular replication of CP9, (ii) an equilibrium between intracellular bacterial replication and death, and/or (iii) intracellular bacterial replication and lysis of neutrophils over time, since it was shown in previous studies (26) that CP9 was capable of lysing neutrophils. Neutrophil lysis would expose intracellular CP9 to gentamicin, which in turn would decrease the titer of CP9. To assess possible neutrophil lysis under the conditions used in the survival assay, neutrophils were counted in parallel with the enumeration of CP9. As Fig. 5B shows, the numbers of neutrophils were constant during this experiment. Therefore, CP9 did not undergo intracellular replication, or intracellular replication and death were in equilibrium. These results also demonstrated that CP9 was able to survive within neutrophils for up to 210 min.

Survival of ExPEC within neutrophils was observed when additional ExPEC strains were used. Although previous studies suggested that various strains of ExPEC have common virulence factors that enable them to cause extraintestinal infections (18), it was important to examine whether CP9 was unique or whether other ExPEC strains could survive in neutrophils. Therefore, six additional ExPEC strains and E. coli laboratory strain HB101 were assessed to determine whether they could survive within neutrophils. Not surprisingly, HB101 was not able to survive within human neutrophils. In contrast, four of the six additional ExPEC strains were capable of survival within neutrophils (Fig. 6). These studies demonstrated that even when our conservative method was used, the majority of ExPEC strains assessed to date (5 of 7 strains, or 71%) are able to survive within human neutrophils and that this phenotype is not limited to CP9. We were not able to demonstrate that CFT073 and IA2, which are well-studied ExPEC isolates, survived. One potential explanation for this is that these strains actually survive within neutrophils but we were unable to establish this fact with our methodology and because of the sensitivity of our assay. Alternative explanations were also considered. Therefore, studies were performed to determine if serum sensitivity, resistance to phagocytosis, rapid neutrophil-mediated killing, or toxic effects of neutrophils on the strains could explain our findings. However, CFT073 and IA2 were resistant to the concentration of serum used in the survival...
assay, were not killed rapidly by neutrophils, did not lyse neutrophils, and were not resistant to phagocytosis (data not shown). Therefore, the reason(s) for the lack of survival of CFT073 and IA2 within human neutrophils is unclear.

**ExPEC wild-type strain CP9 is partially susceptible to the oxidative burst of neutrophils.** One mechanism by which CP9 may be able to survive within neutrophils involves complete or partial resistance to the oxidative burst. To examine this possibility, we measured the intracellular survival of CP9 in neutrophils in which NADPH oxidase, the enzyme responsible for production of reactive oxygen species, was and was not inhibited by DPI. The results of these studies demonstrated that inhibition of NADPH oxidase resulted in an approximate 0.5-log increase in the intracellular survival of CP9 (the mean survival titer increased from $6.77 \times 10^4 \pm 2.2 \times 10^4$ CFU/ml to $4.56 \times 10^5 \pm 5.15 \times 10^4$ CFU/ml) (Fig. 7). These findings demonstrate that CP9 is at least partially susceptible to the neutrophil oxidative burst. Therefore, absolute resistance to the oxidative burst is not the mechanism by which CP9 survives within neutrophils.

**ExPEC appears to be incorporated into phagosomes within neutrophils.** To assess whether avoidance of uptake into a phagosome and escape from the phagosome were mechanisms that contributed to CP9’s survival within human neutrophils, we performed TEM with neutrophils that had phagocytosed CP9 under the conditions used in our standard survival assay. The findings of these studies demonstrated that CP9 always appeared to be within a phagosome. Some of the phagosomes appeared to be spacious (Fig. 8A), while others appeared to be tight phagosomes (Fig. 8B). Therefore, avoidance of neutrophil uptake via the phagosome and subsequent escape from the phagosome do not appear to be mechanisms by which CP9 survives within neutrophils.

**ExPEC is not taken up via macropinocytosis.** To assess whether CP9 was taken up by macropinocytosis and was subsequently confined in a protective vacuole within the neutrophil (a potential survival mechanism), we inhibited neutrophil macropinocytosis using three known inhibitors, amiloride, dimethyl amiloride, and rottlerin. No change in intracellular survival was observed with dimethyl amiloride or rottlerin, but a slight increase in survival was observed with amiloride (Fig. 9). These data do not support the hypothesis that uptake via macropinocytosis is a mechanism that contributes to the survival of ExPEC within neutrophils.

**DISCUSSION**

ExPEC strains are a significant cause of human morbidity and mortality and are capable of causing infections in normal hosts. It has been established for a long time that in order to accomplish this, ExPEC strains are relatively resistant to the host’s innate defense system. ExPEC strains have been considered to be extracellular pathogens, whose main defense against the bactericidal activity of neutrophils is avoidance of phagocytosis. The findings described in this report alter this paradigm. Our results indicated that the model ExPEC strain CP9...
was capable of survival within human neutrophils, but we were unable to demonstrate that intracellular replication of CP9 occurs. This finding was not unique to CP9, since, using our conservative method to establish survival, four of six additional ExPEC strains, but not an E. coli laboratory strain, were also capable of survival within neutrophils. Initial studies that began to decipher the mechanisms by which CP9 is able to successfully survive exposure to intracellular neutrophil-mediated bactericidal activity demonstrated that CP9 was at least partially susceptible to the neutrophil oxidative burst. Therefore, absolute resistance to the oxidative burst is not a mechanism by which ExPEC survives within neutrophils. In addition, TEM studies showed that CP9 appeared to be present in a phagosome within a neutrophil. Therefore, avoidance of neutrophil uptake via the phagosome and subsequent escape from the phagosome do not appear to be mechanisms which contribute to CP9’s survival. Finally, our studies showed that ExPEC does not appear to be taken up by neutrophils via macroinocytosis, making this mechanism an unlikely means by which ExPEC survives within neutrophils. Together, our findings suggest that survival of ExPEC within neutrophils may be an important virulence mechanism, which in combination with other virulence factors contributes to the pathogenesis of infections due to ExPEC.

Intracellular survival within neutrophils has previously been described for two gram-positive extracellular pathogenic bacteria, S. aureus (11) and S. pyogenes (22). S. aureus has been shown to resist intracellular neutrophil killing through modification of its cell wall components. The negative charge of the bacterial membrane is neutralized, which prevents binding of the positively charged antimicrobial defense peptides within the phagosome. S. aureus may also survive through the creation of “spacious” phagosomes in the neutrophils, in which it can survive by avoiding granule fusion (10, 11). S. pyogenes (group A streptococci) has been shown to up-regulate oxidative stress response genes in order to avoid intracellular killing within PMN phagosomes, and it also up-regulates cell envelope components, which may suggest that a group A streptococcus may also repair itself within the PMN (37, 38).

ExPEC has been shown in previous studies to modulate PMN function. Clinical strain 1177 was shown to trigger oxygen-dependent apoptosis in human neutrophils, and the ExPEC toxin CNF1 was shown to modulate phagocytosis and the release of reactive oxygen species (2, 7). Another gram-negative bacterium, Neisseria gonorrhoeae, was shown to survive and replicate within neutrophils, which may partially account for its persistence within the genital tract (34). ExPEC may employ a similar mechanism, which could contribute to its pathogenicity in extraintestinal sites of infection.

In support of our findings, Fexby et al. recently described the survival of E. coli within neutrophils in a paper published after our paper was submitted (9). This study suggested that aggregation of the bacteria might be responsible for the bacterial resistance to PMN-mediated intracellular killing. However, we have not observed bacterial aggregation with our strains, and we do not believe that this is a factor in the survival of our ExPEC strains within neutrophils. Therefore, we believe that intracellular survival of ExPEC may be attributed to another mechanism that has yet to be elucidated. Studies are currently under way in our laboratory to determine the bacterial and host factors involved in ExPEC survival.

The in vitro studies described here established that the ExPEC model pathogen CP9 was able to survive within neutrophils at titers ranging from $3.5 \times 10^5$ to $1.5 \times 10^7$ CFU/ml. While in our in vitro studies we were not able to establish that ExPEC was able to survive within neutrophils at titers below $3.5 \times 10^5$ CFU/ml, it is also possible that at lower titers of bacteria the sensitivity of our assay does not detect lower levels of intracellular survival, particularly if the bacteria enumerated when neutrophils are pretreated with cytochalasin D are not truly extracellular bacteria that are resistant to gentamicin treatment but are actually intracellular bacteria that were internalized because cytochalasin D did not completely inhibit neutrophil phagocytosis. However, even if survival of ExPEC does not occur at lower titers, this observation is still physiologically relevant for certain infections. Titters of $3.5 \times 10^5$ CFU/ml occur within the urinary tract and abscesses and probably at other sites infected with extraintestinal E. coli (8, 20). Since urinary tract infections caused by ExPEC are a major cause of morbidity in humans, we viewed these concentrations of bacteria as physiologically important in the study of neutrophil-ExPEC interactions.

We do not know why we were unable to demonstrate survival of the well-studied ExPEC strains CFT073 and IA2 within neutrophils. It is possible that these strains actually are able to survive within neutrophils but we were unable to demonstrate this fact using our conservative methodology for establishing survival, which may not be optimally sensitive. We did establish that differences in serum sensitivity, phagocytosis, toxic effects of the strains on neutrophils, or rapid killing of the strains by neutrophils between CFT073, IA2, and CP9 did not appear to be responsible. It is also possible that since CFT073 and IA2 are well-studied strains,
they lost some genetic material important for survival within neutrophils during laboratory passage before our laboratory received them. Regardless, the fact that not all of the ExPEC strains studied exhibited survival within neutrophils does not mean that this is not an important virulence mechanism. Clearly, ExPEC strains that have this phenotype should have a survival advantage; however, it is likely that this is just one of multiple phenotypes or virulence factors that contribute to the overall pathogenesis of ExPEC strains.

In order to determine whether CP9 evaded phagosomal uptake or escaped from the phagosome as a mechanism for survival within neutrophils, we performed TEM. From electron micrographs we determined that following phagocytosis, CP9 was always observed to be within an apparent phagosome to the overall pathogenesis of ExPEC strains. One of multiple phenotypes or virulence factors that contribute to the overall pathogenesis of ExPEC strains is resident neutrophils during laboratory passage before our laboratory received them, and it is likely that this is just one of multiple phenotypes or virulence factors that contribute to the overall pathogenesis of ExPEC strains. One of multiple phenotypes or virulence factors that contribute to the overall pathogenesis of ExPEC strains is resident neutrophils during laboratory passage before our laboratory received them, and it is likely that this is just one of multiple phenotypes or virulence factors that contribute to the overall pathogenesis of ExPEC strains.

In summary, data presented here demonstrate that ExPEC strains can survive within human neutrophils. Studies in progress were designed to identify bacterial virulence factors that enable this to occur and to identify the neutrophil killing mechanism(s) that are subverted by ExPEC strains.

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