Novel Model To Study Virulence Determinants of Escherichia coli K1

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It is shown here for the first time that locusts can be used as a model to study Escherichia coli K1 pathogenesis. E. coli K-12 strain HB101 has very low pathogenicity to locusts and does not invade the locust brain, whereas the injection of $2 \times 10^8$ E. coli K1 strain RS218 (O18:K1:H7) kills almost 100% of locusts within 72 h and invades the brain within 24 h of injection. Both mortality and invasion of the brain in locusts after injection of E. coli K1 require at least two of the known virulence determinants shown for mammals. Thus, deletion mutants that lack outer membrane protein A or cytotoxic necrotizing factor 1 have reduced abilities to kill locusts and to invade the locust brain compared to the parent E. coli K1. Interestingly, deletion mutants lacking FimH or the NeuDB gene cluster are still able to cause high mortality. It was argued that the likely existence of additional virulence determinants can be investigated in vivo by using this insect system.

Escherichia coli sepsis and meningitis are among the leading causes of neonatal bacterial infections, affecting from 0.5 to 5 per 10,000 live births in developed countries, with far greater numbers in developing countries, and with case fatality rates of up to 30 to 40%. It is estimated that E. coli K1 meningitis alone results in 50,000 deaths per year worldwide despite advances in antimicrobial chemotherapy (3, 4, 8, 36). One reason for this is an inadequate understanding of the pathogenesis and pathophysiology of this disease. Primary requirements in bacterial meningitis include a high degree of bacteremia by circulating E. coli, followed by invasion of the central nervous system (CNS). However, the precise mechanisms associated with bacterial survival in the bloodstream and invasion of the CNS are not clear. Research over the last few decades has identified, at different regions on the E. coli chromosome, a few bacterial virulence determinants (such as outer membrane protein A [OmpA], FimH, cytotoxic necrotizing factor 1 [CNF1], Ibe proteins, TraJ, and As1A) that are associated with virulence determinants (such as outer membrane protein A [OmpA], FimH, cytotoxic necrotizing factor 1 [CNF1], Ibe proteins, TraJ, and As1A) that are associated with virulence determinants shown for mammals. Thus, deletion mutants that lack outer membrane protein A or cytotoxic necrotizing factor 1 have reduced abilities to kill locusts and to invade the locust brain compared to the parent E. coli K1. Interestingly, deletion mutants lacking FimH or the NeuDB gene cluster are still able to cause high mortality. It was argued that the likely existence of additional virulence determinants can be investigated in vivo by using this insect system.

Current studies have relied upon vertebrate models to study E. coli K1 meningitis. Although physiologically relevant, mammalian models are expensive, are not routinely available in many laboratories, require labor-intensive management, and have ethical implications. The remarkable parallels between the innate immune responses of mammals and insects (6, 30) and the fact that E. coli K1 meningitis is limited mostly to neonates suggest that insects could make useful models in which to study the pathogenesis of E. coli K1. The physiology of Locusta migratoria, the African migratory locust, has been studied extensively in our laboratories (9–11), and this insect was chosen as a model to study E. coli K1 pathogenesis in vivo. The overall aim of the study was to determine how well E. coli infection can be modeled in the locust by studying the role in the insect of some of the virulence factors known currently to be required for E. coli K1 pathogenesis in mammals.

MATERIALS AND METHODS

Locusts. African migratory locusts (Locusta migratoria) were fed on bran, wheat seedlings, and fresh grass. Adult locusts live an average of 80 to 100 days, and the adult males used in the experiments described here were between 15 and 30 days old with a fully developed innate immune system (9, 10, 25).

Bacterial strains and culture conditions. E. coli K1 strain RS218 (O18:K1:H7) is a cerebrospinal fluid isolate from a neonate with meningitis (1, 31). Several isogenic gene deletion mutants of K1 were used, including ΔfimH, constructed by deleting the entire fimH gene and replacing it with the chloramphenicol resistance cassette (17, 33). The fimH gene codes for a lectin-like adhesin, located at the tip of the shaft of type 1 fimbriae, i.e., filamentous surface organelles produced by E. coli. Other mutants included the ΔompA (28) and Δcnf1 (see references 15 and 16) mutants. The OmpA protein is a major component of the outer membrane exposed on the surface of K1 encapsulated E. coli and is important in E. coli K1 binding to and invasion of human brain microvascular endothelial cells. Cytotoxic necrotizing factor 1 (CNF1) is a bacterial toxin known to activate Rho GTPases and induce host cell cytoskeleton rearrangements. Of note, the omp4 gene was replaced with a chloramphenicol resistance cassette, while the cnf1 gene was replaced with a kanamycin resistance cassette as described previously (15, 16). In addition, an isogenic K1 capsule deletion mutant generated from K1 was used (14, 20). This strain lacks the neuDB gene cluster that is necessary for the production of cytoplasmic precursors to the exopolysaccharide capsule, and this cluster was replaced with a chloramphenicol resistance cassette as detailed previously (14, 20). E. coli K12 strain HB101, a noninvasive isolate, was used as a negative control. It is important to indicate that the mutants described here were constructed and their isogenicity was confirmed by using genetic complementation in vitro with human brain microvascular endothelial cells and in vivo with the rat model as described in the aforementioned studies, except in the case of the fimH and neuDB deletion mutants, which were tested only using assays in vitro (14, 17, 20, 33). In addition, the growth kinetics together with the protein profiles of mutants were similar to those of the wild-type strain as determined in previous studies (indicated above). E. coli strains were routinely grown at 37°C in Luria-Bertani (LB) broth. Where appropriate to the strain being cultured, the medium was supplemented with ampicillin (100 μg/ml), kanamycin (40 μg/ml), chloramphenicol (25 μg/ml), or rifampin (50 μg/ml).

Assays to determine bacteremia and mortality. Locusts were randomly allocated to different groups: except where indicated otherwise, groups of 10 injected locusts were set up in replicates of 6 (in total, 60 locusts were used per strain). Each locust was injected with 20 μl of a suspension of E. coli containing $2 \times 10^{10}$ CFU in LB broth. Injections were performed by using an automatic pipette in which the plastic tip was modified by the insertion of a short length of stainless...
plated on nutrient agar plates. The brains were then lysed by the addition of sodium dodecyl sulfate before being gentamicin) and lysed by the addition of sodium dodecyl sulfate (0.5%, final concentration) together with vigorous vortexing. Bacterial counts were determined by plating the samples onto nutrient agar plates.

To determine whether locusts developed bacteremia, hemolymph was collected at various intervals after injection of 2 × 10^8 bacteria: the cuticle and air sacs, and surgical instruments were surface sterilized with methanol moistened in 70% ethanol. After the cuticle surface was allowed to air dry, a small puncture was made in the membrane by using a sterile needle, and hemolymph was collected into glass capillaries calibrated to contain 5 μl. The hemolymph was expelled into 100 μl of LB broth, and bacterial counts were determined by plating the samples onto nutrient agar plates.

**CNS invasion assays in vivo.** To determine *E. coli* association with the CNS, locust brains were isolated. Briefly, locusts were injected with *E. coli* as described earlier. At 24 h after injection, the locusts were killed; the left side of head was removed by a sagittal cut through the base of the left antenna; and the brain dissected by using fine forceps after severing the circum-esophageal connectives and the (remaining) optic tract to the right compound eye. Care was taken to ensure that both cerebral ganglia were present and that they were free of fat body tissue and air sacs, and surgical instruments were surface sterilized with methylated spirit between dissections. Each brain was washed by placing it in a microcentrifuge tube containing 1 ml of phosphate-buffered saline (PBS). Tubes were centrifuged by a brief pulse at 750 × g, and the supernatants were discarded. This washing process was repeated three times. In initial experiments, the brains were then lysed by the addition of sodium dodecyl sulfate (0.5%, final concentration) together with vigorous vortexing. Bacterial counts were determined by plating the lysates on nutrient agar plates. In later experiments, after the initial wash in PBS, brains were incubated with gentamicin (100 μg/ml, final concentration) at 37°C for 60 min to kill extracellular bacteria. The brains were then washed a further three times (to remove gentamicin) and lysed by the addition of sodium dodecyl sulfate before being plated on nutrient agar plates.

**RESULTS**

*E. coli* K1 produced more than 90% locust mortality within 72 h. Approximately 20% ± 7% of the locusts injected with K1 died within 24 h of injection, and this figure increased to 85% ± 7% within 48 h and to more than 95% ± 3% within 72 h. On the other hand, injections of K-12 resulted in only ca. 5% ± 2% mortality after 72 h, but this was variable, with some replicates showing zero mortality. Indeed, 95% of the K-12-injected locusts were still alive up to 7 days after injection.

The levels of mortality induced by injection of the capsule and fimH deletion mutant were not significantly different from that induced by the wild type, but mortality was significantly lower in locusts injected with the cnf1 or ompA deletion mutants (Fig. 1).

**Injections of E. coli K1, but not K-12, produced bacteremia in locusts.** As shown in Fig. 2, both K1 and K-12 were reduced dramatically in numbers (bacterial CFU per μl of hemolymph) within the first 24 h after injection. Subsequently, the numbers of K-12 reduced to zero by 36 h, but K1-injected locusts showed a persistent level of bacteremia at 24 and 36 h, and the numbers of bacteria had increased by 48 h (Fig. 2). Bacterial counts were not obtained for later times because >80% of the K1-injected locusts died within 48 h.

*E. coli* K1 but not K-12 invaded the locust brain. Locusts were injected with K1 or K-12 as described above, and the brains were dissected from each locust 24 h later. Brain lysates from K1-injected locusts were positive for *E. coli* with counts of ca. 25,000 CFU per brain. In contrast, brain lysates from K-12-infected locusts showed less than 5 CFU per brain (data not shown).

To confirm that *E. coli* K1 had penetrated into the locust brain, brains were removed from the K1- and K-12-injected locusts, washed in PBS, and then incubated with gentamicin to kill extracellular bacteria. For K1, the average numbers of colonies recovered from each gentamicin-treated brain were reduced by a factor of 2.5 (to leave ~10,000 CFU per brain) compared to the gentamicin-negative group. In contrast, lysates of gentamicin-treated brains from K-12-infected locusts were negative for bacterial cultures.

OmpA and CNF1 were critical determinants in *E. coli K1*-mediated locust infection. The results of experiments in which
deletion mutants were injected into locusts demonstrated clearly that ompA or cnf1 deletion almost completely abolished K1-mediated locust death, but neither fimH nor neuDB deletion reduced locust mortality significantly (Fig. 1).

When the abilities of the deletion mutants to invade the locust brain were determined, the results correlated with the earlier findings on mortality. E. coli K1 strains that induced high mortality showed a high presence in brain lysates, whereas the ompA deletion mutant was not found in brain lysates, and the cnf1 deletion mutant was present in low numbers (Fig. 3). In contrast, penetration of the brain by the fimH deletion mutant was not significant ($P = 0.1$), whereas that of the neuDB deletion mutant was less ($P < 0.05$). Overall, these findings suggest that there may be a link between E. coli K1 invasion of the locust brain and mortality (Fig. 3).

**DISCUSSION**

Important similarities between the pathogenetic determinants in mammals and a locust are demonstrated in the present study. For example, a high level of bacteremia is seen as a prerequisite for bacterial invasion of the CNS in the rat of E. coli K1 meningitis (21). In the present study, large numbers of bacteria were injected into locusts, and it is clear that the locust’s immune system is capable of removing large numbers of them from the hemolymph within the first 24 h of infection (11). The mechanisms by which this is achieved have been well studied (12, 29) and involve direct phagocytosis by hemocytes, entrapment by groups of hemocytes forming nodules, and the production of antimicrobial compounds (for a review, see reference 7). By such mechanisms, it appears that E. coli K-12

**FIG. 2.** E. coli K1 produced bacteremia in locust. Injections of E. coli K1, but not K-12, produced bacteremia in locusts. Within the first 24 h after injection, both K1 and K-12 were reduced dramatically in numbers (bacterial CFU per μl of hemolymph), and the numbers of K-12 were reduced to zero by 36 h, but K1-injected locusts showed an increase in bacterial counts by 48 h. Values are mean ± the SE.

**FIG. 3.** E. coli K1 but not K-12 invaded locust brain, and OmpA and CNF1 were critical determinants in E. coli K1 invasion of locust brain. Locust brains were removed 24 h after injection of bacteria into the hemocoel, treated with gentamicin, washed, and lysed, and bacterial counts determined as described in Materials and Methods. Penetration of the brain by the fimH deletion mutant was not significantly ($P = 0.1$) lower than that of K1, whereas that of the neuDB deletion mutant was less ($P < 0.05$). Thus, E. coli K1 strains that induced high mortality (see Fig. 1) showed a high presence in brain lysates, whereas the ompA deletion mutant was not found in brain lysates, and the cnf1 deletion mutant was present in significantly ($P < 0.01$) reduced numbers. Values are mean ± the SE.
disappears completely from the hemolymph within 36 h. This is not true, however, for *E. coli* K1: beyond 24 h after injection, the numbers of these cells in the hemolymph increase. It may be that within the first 24 h, many bacteria are trapped in nodules and therefore are not circulating freely in the hemolymph, but while the hemocytes kill K-12 in nodules, they fail to do so with K1, which would explain why bacteremia develops between 24 and 48 h.

In vertebrates, the penetration of circulating bacteria into the brain is limited by the presence of the blood-brain barrier. The vertebrate blood-brain barrier acts at two levels: as a barrier between blood and brain extracellular fluid located at the cerebral capillary endothelium and as a barrier between blood and cerebrospinal fluid located at the choroid plexus epithelium and the arachnoid membrane (18). The barrier at each site is formed by a single layer of cells that are joined by tight junctions (comprising three major integral membrane proteins—claudin, occludin, and junction adhesion molecule—and the cytoplasmic proteins zonula-1 [ZO-1], ZO-2, and ZO-3) and adherens junctions (composed of membrane protein and cadherin that binds to catenin and is linked to the actin cytoskeleton, forming adhesive contacts between the cells). Zonula proteins are accessory proteins that provide structural support and bind to all three integral membrane proteins of the tight junctions, further linking membrane proteins to the actin cytoskeleton (13, 19, 34), making it highly selective. Insects have an open circulatory system, with the hemolymph bathing all tissues, but the brain and peripheral nervous system are protected by a blood-brain or blood-nerve barrier. The physical nature of this insect barrier depends also on the existence of tight junctions between glial and/or perineurial cells (2) and, at least in *Drosophila*, the molecular components of these tight junctions are similar to those in vertebrates (5, 35). Although the development of the insect blood-brain barrier and its ion selectivity have been well studied (27, 32), there appears to be no published information on its resistance to penetration by microorganisms. However, it is to be expected that in insects as in mammals (26), bacteria would cross the blood-brain barrier via a transcellular route. At the molecular level, it is likely that there will be similarities between the mechanisms by which *E. coli* K1 invades the CNS of mammals (24) and of locusts, and this speculation is supported by the results of the present study.

Thus, it is of great interest that brain lysates of K1-injected locusts, but not K12-injected locusts, yield significantly large bacterial cultures. It may be that this reflects the inability of K-12 to survive in the hemolymph and/or in the brain, rather than an inability to penetrate the blood-brain barrier. However, the balance of evidence presented here concerning the ability of mutants of K1 that lack known pathogenicity factors to invade the locust brain would favor the latter possibility. It is significant that injection of *E. coli* K1 causes locust death, with almost complete mortality within 72 h, and also gives the highest recovery of bacterial cultures from lysates of locust brain. It is tempting to speculate that K1-mediated locust death is, in part, associated with the bacterium’s ability to invade the locust CNS. However, other factors may also contribute to locust mortality. For example, K-12-injected locusts can show up to 8% mortality, even though K-12 are never recovered from locust brains.

While the requirement of bacterial invasion of the CNS in the development of meningitis is well documented, the precise mode of bacterial traversal of the blood-brain barrier remains incompletely understood. Nevertheless, using the rat model, several *E. coli* K1 determinants have been identified that are required for *E. coli* K1 interactions with human brain microvascular endothelial cells in vitro and/or invasion of the CNS in vivo using a rat model of *E. coli* K1 meningitis. These determinants include FimH, OmpA, CNF-1, and NeuDB: FimH is a 29-kDa protein that is expressed at the tip of bacterial fimbiae (17, 33), OmpA is a 35-kDa protein expressed in the outer membrane of *E. coli* (16), CNF1 is a 110-kDa AB type bacterial toxin (15), and the neuDB gene cluster necessary for the production of cytoplasmic precursors to the exopolysaccharide capsule (14, 20).

In the locust in vivo model studied here, deletions of *fimH* or *neuDB* cause only slight reductions in the ability of *E. coli* K1 to kill locusts, even though *neuDB* is less able to invade the locust brain. These particular pathogenicity factors have been demonstrated to be important in vitro using human brain microvascular endothelial cells (15, 16, 20), but there appears to be no published evidence that they are required in vivo in mammals. Thus, it remains to be determined whether the locust in vivo model is at variance with in vivo mammalian models in this respect. An alternative explanation is that *fimH* and *neuDB* may play important role(s) in infection, other than the phenotypes tested in the present study. It is significant, however, that the *ompA* deletion mutant does not cause high mortality in locusts and is not recovered from brain lysates of *ompA* mutant-injected locusts. At present, it is not clear whether the presence of OmpA is a requirement for bacterial survival in the bloodstream, bacterial penetration of the blood-brain barrier, bacterial survival in locust brain, or some combination of these factors. Studies are in progress to address this question. Deletion of CNF1 results in an intermediate condition, in which both the ability of the *cnf1* deletion mutant to kill locusts and to invade the brain is reduced significantly compared to the parent strain.

A whole-organism approach to the study of disease is recognized as essential to gaining a full understanding of the interrelationships between infectious agents and their hosts. Although vertebrate model systems are seen as immediately more relevant, it is proposed here that the use of an invertebrate model at an early stage can offer several advantages in terms of speed, cost, technical convenience, and ethical acceptance. The locust model studies here could be a valuable tool to discriminate molecules participating from both sides of the host-bacterium interaction. In this way the locust model could generate potentially useful leads that can be tested subsequently in mammalian systems, thus reducing the numbers of mammals required overall. In conclusion, we have shown here for the first time that locusts can be used as a model for studying *E. coli* K1 pathogenesis in vivo. Because insects rely for their protection against infection on an entirely innate immune system, the use of an insect model is particularly relevant in the study of human newborn *E. coli* K1 meningitis, the control of which has significant dependency on the innate immune system. The studies described here concerning the requirement of several known virulence determinants support strongly the proposition that *E. coli* infection can be modeled...
in locusts, and it is anticipated that the likely existence of additional virulence determinants can be investigated in vivo by using this insect system.

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