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Enhancement of In Vitro Growth of Pathogenic Bacteria by Norepinephrine: Importance of Inoculum Density and Role of Transferrin

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Norepinephrine is a stress hormone that enhances bacterial growth. We examined the effects of a small inoculum on the norepinephrine-induced growth of species previously reported to be unaffected by norepinephrine. The results indicated that a reduced inoculum density is essential for observing norepinephrine-induced effects. Additional studies using serum-free media suggested that transferrin plays a role in norepinephrine-induced growth.

Determining the direct effect of catecholamines on the in vitro growth response of bacteria is one interdisciplinary approach that has been utilized to increase our understanding of the role that stress hormones play in the establishment and progression of infection in a host (21). Although a great deal of evidence suggests that stress-induced neurohormones play a critical role in the outcome of infections (1, 3, 4, 5, 7, 21, 31), the mechanisms by which these hormones act in the host remain unclear. Studies with human and animal models have indicated that increased levels of stress hormones, including norepinephrine (NE), as well as other catecholamines, alter the immune response and physiology of the host (2). High circulatory levels of these hormones are detected in individuals exposed to a variety of physically and/or mentally stressful situations, including trauma, space flight, and sepsis (13, 29, 31, 32). The increases not only may alter the immune function but also may contribute to host morbidity and increased risk of infection (1, 7, 34). In the current study we reexamined the in vitro growth responses of a variety of bacterial species that were previously tested and reported not to be enhanced by the addition of NE (6, 8).

The conditions employed in this study include a minimally nutritive low-iron medium previously shown to maintain bacteriostasis (21), a low initial inoculum density of bacteria (10 CFU/ml) in order to capture the lag phase of the bacterial growth curve typically observed in a bacteriostatic medium (21, 26), and a concentration of NE (0.0001 M) (14, 15, 26) which corresponds to target tissue levels and not mere plasma spillover (16, 18, 20). These rigorous conditions better represent in vivo milieus and also allow more suitable evaluation of a growth enhancement effect without the camouflage of rapid bacterial growth encountered when rich medium and large inocula are employed.

Using a lower initial inoculum density (approximately 10 CFU/ml), each species tested exhibited NE-induced enhancement of in vitro growth compared to nontreated controls. Cultures of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Shigella sonnei*, and *Staphylococcus aureus* grown in the presence of NE had shortened lag times and exhibited significant increases in bacterial counts (CFU/ml) at 18 and 24 h (Fig. 1) compared to the control. Moreover, for all of the gram-negative pathogens there were other times when there were significant increases in growth (Fig. 1A to D). *S. aureus* growth was only moderately affected by NE treatment, and there were significant differences in growth (slightly more than 1 log) only at 18 and 24 h (Fig. 1E). Interestingly, the nontreated *S. sonnei* cultures did not achieve amplitude (stationary phase) within 92 h (Fig. 1D), suggesting that the presence of NE may be extremely important in establishing an in vivo infection with low initial densities of this overt pathogen.

To investigate the possible mechanisms of NE-induced bacterial growth, we focused on two important elements involved in bacterial growth, iron and transferrin. Most aerobic bacteria require iron for metabolism and growth (17, 35, 36) and produce endogenous siderophores to assist in assimilating this essential element from the host (19, 37). Due to its oxidative toxicity in the mammalian host, iron is solubilized and sequestered by a variety of iron-binding proteins, including transferrin (30). This iron sequestration provides major protection to the host, keeping bacterial growth at bay (10, 33). To test whether transferrin plays a role in the NE-induced enhancement of bacterial growth, serum was replaced with either 1.5 mg/ml bovine apotransferrin or 1.5 mg/ml bovine holotransferrin in SAPI medium. This concentration was chosen since adult sera contain 1 to 2 mg of transferrin per ml (9). As expected, the growth of *K. pneumoniae* in the iron-deficient apotransferrin-containing medium (negative control) was severely restricted in both nontreated and NE-treated cultures (Fig. 2). Growth of *K. pneumoniae* cultured in iron-saturated holotransferrin-containing medium was recoverable with >500-fold enhancement of growth in the presence of NE (Fig. 2), similar to the results for NE-treated SAPI serum levels of growth at this time. Bacteria grown in the presence of holotransferrin supplemented with free iron (21 μM $FeCl_3$ in 50 mM sodium bicarbonate–50 mM sodium citrate [pH 8.6]) (9)

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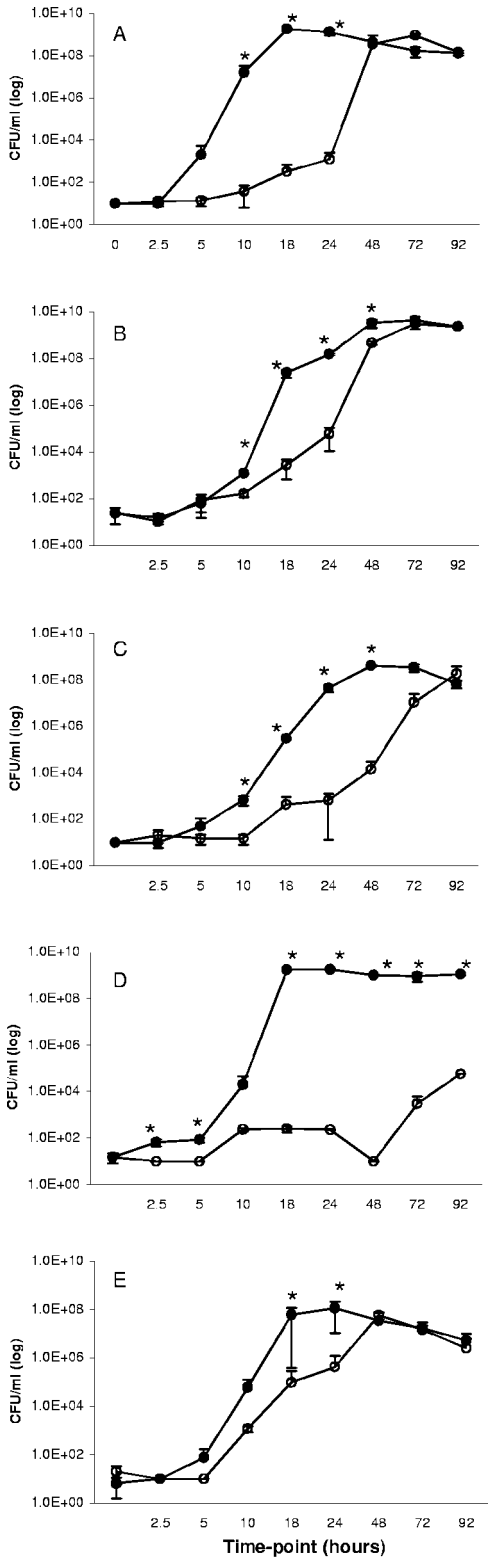

FIG. 1. Growth curves for *K. pneumoniae* (A), *P. aeruginosa* (B), *Enterobacter* species (C), *S. sonnei* (D), and *S. aureus* (E) cultured in SAPI medium containing 30% adult bovine sera in the absence of NE (control) (○) or in the presence of 0.0001 M norepinephrine (+)-bitartrate salt (●). The symbols indicate the means, and the error bars indicate the standard deviations. An asterisk indicates that there is a significant difference between the growth of the control culture and the growth of the NE-treated culture, as determined using the Student *t* test ($P \leq 0.05$). The concentrations of nondiluted cultures that produced no colonies on Trypticase soy agar plates were recorded as 10 CFU/ml (limit of detection).

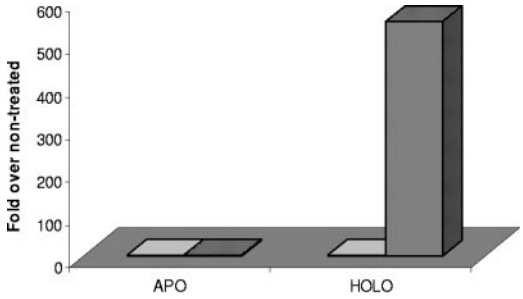

FIG. 2. Fold induction of growth of *K. pneumoniae* cultured in serum-free, apotransferrin-containing (APO) or holotransferrin-containing (HOLO) SAPI medium in the absence of NE (control) (light gray bars) or in the presence of 0.0001 M NE (dark gray bars) harvested at 18 h postinoculation. The differences in growth in holotransferrin-containing medium are significant for control and NE-treated cultures, as determined using the Student *t* test ($P \leq 0.05$). (The iron content specification for apotransferrin [essentially free] is 50 ppm, and the lot concentration was 31 ppm; the iron content specification for holotransferrin [saturated] is 0.1% or greater, and the lot concentration was 0.14% [Sigma, St. Louis, MO].)

in the absence of NE served as a positive control for growth under serum-free conditions (data not shown).

It is clear from this and other studies that bacterial growth is enhanced in the presence of NE (9, 22, 26, 28). However, the underlying mechanisms remain poorly understood. Studies of *Escherichia coli* have suggested that NE acts by inducing de novo synthesis of various bacterial virulence factors (23, 24, 27). Additional studies have looked for evidence of cognate bacterial receptors that may utilize NE as a growth-activating ligand (22), but no such mammalian-type adrenergic receptors have been identified yet (25). Finally, it is possible that NE may also exert its effect by modifying existing constituents, such as ferrisiderophores. Further investigation is required to determine if NE acts similarly to induce or stimulate unidentified endogenous bacterial activity for each of the species tested in this study. From the results shown in Fig. 2, it is apparent that NE fosters a mechanism for iron availability to bacteria, including members of the genus *Klebsiella*. Interestingly, each member of the structural group of siderophores called the catecholates contains a hydroxylated benzene ring(s) or catechol moiety(ies) (17). NE also has a catechol moiety in its molecular structure (9). The prospect that NE itself may act as an exogenous siderophore or may be involved in liberating iron from this complex is intriguing. NE has previously been shown to complex with the iron-sequestering molecules belonging to the transferrin family (12). Freestone et al. found that in *E. coli*, NE promotes iron shuffling between transferrin molecules, resulting in iron availability for the siderophore enterobactin (11). Burton et al. reported, but did not show, that the growth of *E. coli* strain JPN10 was enhanced using iron-loaded and dialyzed transferrin in the presence of NE (9).

In conclusion, the results of the present study indicate that culture conditions, including the inoculum densities of bacteria, are important factors that should be considered when the effects of neurohormones on bacterial growth are evaluated. In previous in vitro studies that employed experimental inoculum densities of greater than 10^2 CFU/ml, it was concluded that the growth of some species of bacteria, including *Enterobacter* sp., *S. aureus*, and *Shigella*, was not enhanced in the presence of NE (6, 8). Because these species are capable of causing severe diseases in humans, it is very important to more accurately assess if growth can be affected by stress hormones, such as NE. We established that these species were in fact NE responsive when a lower initial inoculum density (approximately 10 CFU/ml) was used. NE-induced bacterial growth enhancement appears to be evolutionarily important across species and therefore requires further attention. This work also provided evidence that supports the critical role of transferrin in the NE-induced enhancement of bacterial growth. We extended the observation that transferrin is involved in NE-induced growth phenomena to include bacteria belonging to the genus *Klebsiella*. Together, the results have profound implications for our understanding of how microorganisms can potentially utilize stress hormones to make a host susceptible to an increased risk of infection and disease.

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