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Inverse Relationship between Severity of Experimental Pyelonephritis and Nitric Oxide Production in C3H/HeJ Mice

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The contribution of nitric oxide to host resistance to experimental pyelonephritis is not well understood. We examined whether the inhibition of nitric oxide synthesis alters the sensitivity of lipopolysaccharide (LPS) responder (C3H/HeN) and nonresponder (C3H/HeJ) mice to experimental *Escherichia coli* pyelonephritis. C3H/HeJ and C3H/HeN mice were implanted subcutaneously with minipumps containing an inhibitor of nitric oxide, N^G-nitro-L-arginine methyl ester (L-NAME), or a corresponding vehicle. Ascending urinary tract infection by bladder catheterization with two strains of *E. coli*, an O75 strain bearing Dr fimbriae and an O75 strain bearing P fimbriae, was developed in tested animals. Twenty-four hours following bladder infection, the kidneys of C3H/HeN and C3H/HeJ mice were colonized at a similar rate. However, 5 weeks postinoculation, C3H/HeN mice cleared infection while C3H/HeJ mice showed persistent colonization. Twenty-four hours following infection, C3H/HeN mice treated with L-NAME showed no significant increase of renal tissue infection compared to the saline-treated control group. However, L-NAME-treated C3H/HeJ mice showed an approximately 100-fold increase in *E. coli* infection rate compared to the saline-treated controls in the Dr^+ group but showed no change compared to those in the P^+ group. Dissemination of Dr^+ *E. coli* but not P^+ *E. coli* to the liver and uterus was significantly enhanced with L-NAME treatment in C3H/HeJ mice only. Nitric oxide had no direct killing effect on *E. coli* in vitro. Nitrite production by various organs was found to be significantly lower in C3H/HeJ mice than in C3H/HeN mice. Alteration of nitric oxide and LPS responsiveness was significantly associated with the increased sensitivity of C3H/HeJ mice to experimental Dr^+ but not to P^+ *E. coli* pyelonephritis. These findings are consistent with the hypothesis that nitric oxide synthase activity in concert with LPS responsiveness may participate in the antibacterial defense mechanisms of the C3H mouse urinary tract. This phenomenon is strain dependent and possibly related to the invasive properties of *E. coli*.

Nitric oxide (NO), which has many functions, is being recognized as a critically important mediator during infectious processes (21). Interestingly, suggested effects on infection may be either positively or inversely related to NO production. For example, NO may protect a host from infection or contribute to increased microbial virulence (21). Synthesis of NO is required for the severe respiratory tract effects of *Bordetella pertussis* tracheal cytotoxin on epithelial cells (11). It has been suggested that NO may contribute to the maintenance of microbial latency (21). On the other hand, NO may mediate microbiostasis, especially in infections with the intracellular pathogens *Salmonella typhimurium*, *Mycobacterium*, *Plasmodium*, and *Leishmania* (4, 21, 42). In patients with some of these infections, NO production appears to be increased in macrophages and correlate with better clinical outcome (27, 32).

Urinary tract infection (UTI), with its most severe clinical form, pyelonephritis, is one of the diseases that most frequently affect females. Pregnancy appears to provide the highest risk for developing ascending UTI and associated complications. The most prevalent microbial pathogen responsible for UTIs is *Escherichia coli* (24, 28).

The host mechanisms responsible for increased risk for developing pyelonephritis are only partially understood. The roles of both urinary tract tissue receptors and *E. coli* colonization factors, P fimbriae, in the pathogenesis of receptor-adhesin-mediated ascending acute pyelonephritis have been well documented (1, 10, 20, 24, 33, 36, 37, 39). However, in chronic, recurrent, and gestational renal infections the contributions of the tissue receptors and virulence factors remain underinvestigated, and only recently *E. coli* with Dr fimbriae was shown to play an important role in these clinical forms of UTI (2, 6–8, 17, 30, 35).

One of the key steps observed in the development of pyelonephritis is parenchymal invasion by microbes. This process implies that the pathogen, *E. coli*, must possess the capacity to invade parenchymal cells. The Dr fimbriae are a type of adhesin associated with recurrent *E. coli* UTI, cystitis, and chronic diarrhea in children and pyelonephritis in pregnant patients (6–8, 30). Our recent studies showed that *E. coli* strains that express Dr fimbriae, which function as a colonization factor, invade renal interstitium and establish chronic interstitial nephritis in C3H/HeJ mice (8, 9). Dr fimbria-bearing *E. coli* strains were also shown to possess the capacity to invade epithelial cells originating from the lower genital tract (9). Furthermore, mutation of the Dr operon prevented C3H/HeJ lipopolysaccharide (LPS) nonresponder mice from developing interstitial colonization and abolished the invasion of *E. coli* into HeLa cells in vitro (8).

C3H/HeJ LPS nonresponder mice are unable to resist and clear parenchymal renal invasion with Dr^+ *E. coli* (8); however, the host defense mechanisms involved in this process are not clear. It was postulated that NO could contribute to bacteriostasis, especially against invasive intracellular pathogens (21). The contribution of NO to the *E. coli* virulence in as-

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cending pyelonephritis is not known and was only recently investigated in experimental uterine infections (31). We hypothesize that modulation of NO expression could be one of the key host mechanisms in preventing *E. coli* renal parenchymal invasion. If so, then inhibition of NO or inherently low production of NO could increase the rate of renal infection. In the present investigation, we assessed the relationship between severity of experimental pyelonephritis, measured by quantitative renal cultures, and NO production status in C3H/HeJ LPS nonresponder mutant mice and C3H/HeN mice that respond to LPS. The results presented in this report are consistent with the proposed hypothesis and show that inherently low or experimentally reduced production of NO in C3H/HeJ mice inversely correlates with severity of experimental pyelonephritis and that this phenomenon is *E. coli* strain dependent.

MATERIALS AND METHODS

Strains. *E. coli* IH11128 serotype O75:K5:H−, which expresses Dr fimbriae, and *E. coli* 2525 serotype O75, which expresses P fimbriae, isolated from female patients with pyelonephritis, were selected for the experiments (29, 35). *E. coli* IH11128 was invasive while *E. coli* 2525 was noninvasive to the HeLa monolayer (9). The strains were stored in 20% glycerol L-broth medium at −70°C and were subcultured on L-agar plates overnight before the experiments were conducted.

Experimental UTI model. An experimental ascending pyelonephritis model with minor modifications was used to test C3H/HeJ (LPS-nonresponder) and C3H/HeN (LPS-responder) mice, as previously described (10). Briefly, 2 days before experimental infection, each animal received one dose of streptomycin (7.0 mg/g of body weight) to eliminate possible infections that may have occurred naturally in the urogenital tract. Twenty-four hours before infection, animals in one group were implanted with Alzet osmotic minipumps (1 μl/h; Alza Corporation, Palo Alto, Calif.) that contained the NO inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 50 mg/day/100 g of body weight), and mice in the control group were implanted with minipumps containing corresponding amounts of saline. L-Arginine is the substrate necessary for the production of NO (12), and L-NAME has been shown to inhibit the synthesis of NO in both in vitro and in vivo systems. L-NAME at 50 mg/day/100 g of body weight has been shown to inhibit NO synthesis (46). Suspensions of 50 μl of Dr^+ or P^+ *E. coli* with a total of 5×10^8 bacterial cells were inoculated into the bladder of each mouse by catheterization; the catheter was immediately withdrawn, and no further manipulations were performed.

Quantitative cultures. The organs isolated from each mouse were the kidney, spleen, liver, and uterus. Tissues were homogenized in phosphate-buffered saline (pH 7.2) and the number of CFU per gram of homogenate were calculated for each organ and collectively for each mouse. Tenfold dilutions of homogenates were plated on MacConkey agar and L agar to assess both the morphology of the colony and the identity of isolated colonies by the testing of O serotype and expressed fimbrial factors (28, 29). The serotype of each colony was identified with anti-O75 immunoglobulin G in an agglutination assay. The fimbrial types were tested by hemagglutination with human O-group erythrocytes and by the blockage of hemagglutination with inhibitors such as chloramphenicol for Dr fimbriae or Gal-Gal receptor for P fimbriae.

Evaluation of nitrate production in mouse organs. Tissues of the kidney, uterus, and spleen were cut into small pieces (2-mm strips), rinsed thoroughly, placed in minimum essential medium (GIBCO, Grand Island, N.Y.) containing 1% penicillin and 1% streptomycin, and incubated in a CO_2 incubator with a humidified chamber at 37°C for an initial 1-h equilibration period. Then the medium was replaced with fresh medium. After 24 h of incubation, the medium was collected for a nitrite assay. Medium nitrite concentrations were measured in triplicate by the microplate assay method with the Griess reagent, as described earlier (3, 5). Briefly, the Griess reagent (0.5 sulfanilamide and 0.05% naphthalene diamine dihydrochloride in 2.5% orthophosphoric acid, 100 μl) was added to 100-μl aliquots of medium, and optical densities were measured at 550 nm in a microplate reader (Molecular Devices Co., Sunnyvale, Calif.) after 10 min of incubation at room temperature. Nitrite values were determined with sodium nitrite as a standard value. Background nitrite values of media without tissues were subtracted from those with tissues, and the values were expressed as nanomoles per gram of wet weight of uterine, kidney, and spleen tissue. The inter-assay and intra-assay coefficients of variation for the nitrite assay were <10%, and the sensitivity of nitrite determination was 1 μmol/liter.

Statistical analysis. The data were analyzed with analysis of variance followed by the Bonferroni test. The Mann-Whitney U test was used where the data were nonparametrically distributed.

RESULTS

Because studies on the role of LPS responsiveness in experimental *E. coli* UTI reported contradictory results, we first evaluated the course of experimental pyelonephritis in LPS responder (C3H/HeN) and nonresponder (C3H/HeJ) mice. C3H/HeJ and C3H/HeN mouse groups were sacrificed 24 h and 5 weeks following infection with Dr^+ *E. coli* IH11128. Figure 1 shows that within 24 h after inoculation, both LPS responders and nonresponders developed a similar rate of kidney colonization (3×10^3 CFU/g in C3H/HeJ and 5×10^2 CFU/g in C3H/HeN). Although the colonization rate was higher in C3H/HeJ mice the observed differences between the two groups of mice did not reach statistical significance (analysis of variance, $P > 0.05$). At 5 weeks following inoculation, the C3H/HeJ mice maintained a significant amount of kidney colonization (2×10^4 CFU/g), while the C3H/HeN mice completely cleared the infection.

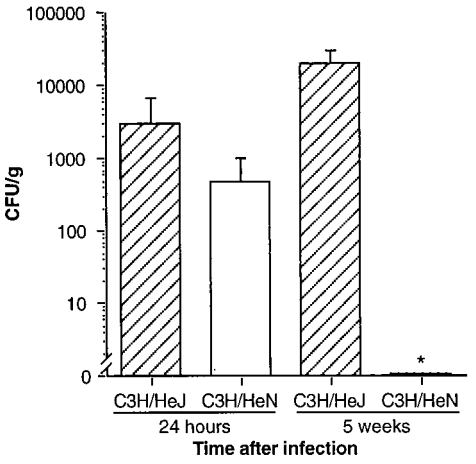

FIG. 1. Quantitative bacterial counts in kidneys of C3H/HeJ and C3H/HeN mice challenged with Dr^+ *E. coli* IH11128 at 24 h and 5 weeks postinoculation. *, clearance of infection in C3H/HeN mice at 5 weeks; all cultures at 5 weeks were found to be negative. Error bars are indicated for each tested group.

Because significant differences were observed between LPS responder (C3H/HeN) and nonresponder (C3H/HeJ) mice with respect to kidney infection, we examined if these differences were due to the differential NO generation by the tissues in these animals. Therefore, we evaluated NO production in the tested organs of these mouse strains. Significantly lower levels of production of nitrites, metabolic products of NO, were observed in the kidneys, spleens, and uteri of LPS nonresponder (C3H/HeJ) mice than in organs of LPS responder (C3H/HeN) mice (Fig. 2). This suggests that decreased NO activity in C3H/HeJ mice could be an important host factor contributing to the decreased resistance of LPS nonresponders to experimental pyelonephritis.

We next evaluated whether an inhibitor of NO synthesis, L-NAME, would alter the sensitivity of C3H/HeJ and C3H/HeN mice to renal infection within 24 h postinoculation. Data from three separate experiments, presented in Fig. 3, show that in all groups tested, the majority of mice were found to have Dr^+ *E. coli* or P^+ *E. coli* in the kidney. The frequency of infection was similar in the L-NAME-treated animals and the control groups.

In the homogenates of kidneys from C3H/HeN mice receiving saline, the number of *E. coli* IH11128 (Dr^+) CFU varied from 10^2 to 10^3/g, with a median of 3.1×10^2 CFU/g. The mean number of CFU in the L-NAME-treated C3H/HeN mice (1.1×10^3/g) was higher (Mann-Whitney U test, $P < 0.03$) than in the saline-treated control mice. More interestingly, homogenates of kidneys from C3H/HeJ mice (LPS nonresponders)

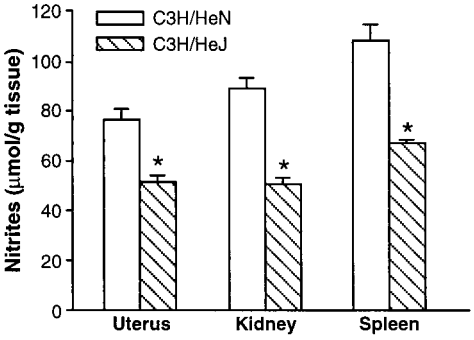

FIG. 2. Nitrites production by uterus, kidney, and spleen in C3H/HeJ and C3H/HeN mice. Analysis of data within each tissue was done by the Student *t* test, and C3H/HeJ mice showed statistically lower production of nitrites in all tissues (*, $P < 0.05$).

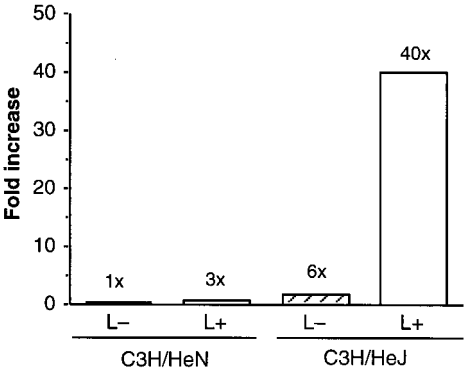

FIG. 4. Increase in renal colonization rates with respect to LPS responsiveness and L-NAME treatment. L−, control mice not treated with L-NAME. L+, mice treated with L-NAME.

receiving L-NAME showed approximately sixfold-higher colonization rates (1.1×10^4 CFU/g) ($P < 0.007$) than those from saline-treated (1.8×10^{-3} CFU/g) animals (Fig. 3A).

Since sensitivity to infection differed both between the tested mouse strains and with the L-NAME treatment, we compared whether these effects were equally or more pronounced in LPS^d mutant mice. Figure 4 shows the fold increase in the renal colonization rate with respect to LPS responsiveness and L-NAME treatment. L-NAME treatment increased the infection about threefold in C3H/HeN mice. Mutation of the LPS gene of C3H/HeJ mice was associated with a sixfold-increased infection rate compared to the rate in LPS responder C3H/HeN mice. Surprisingly, LPS mutant mice (LPS nonresponders) treated with L-NAME showed a 40-fold increase in the rate of infection of the kidneys. These effects exceed the expected additive effects of LPS mutation (sixfold) and L-NAME treatment (threefold), which would be only 18-fold. These results suggest that mice with a primary deficiency of antibacterial defense (LPS^d mutation) become even more sus-

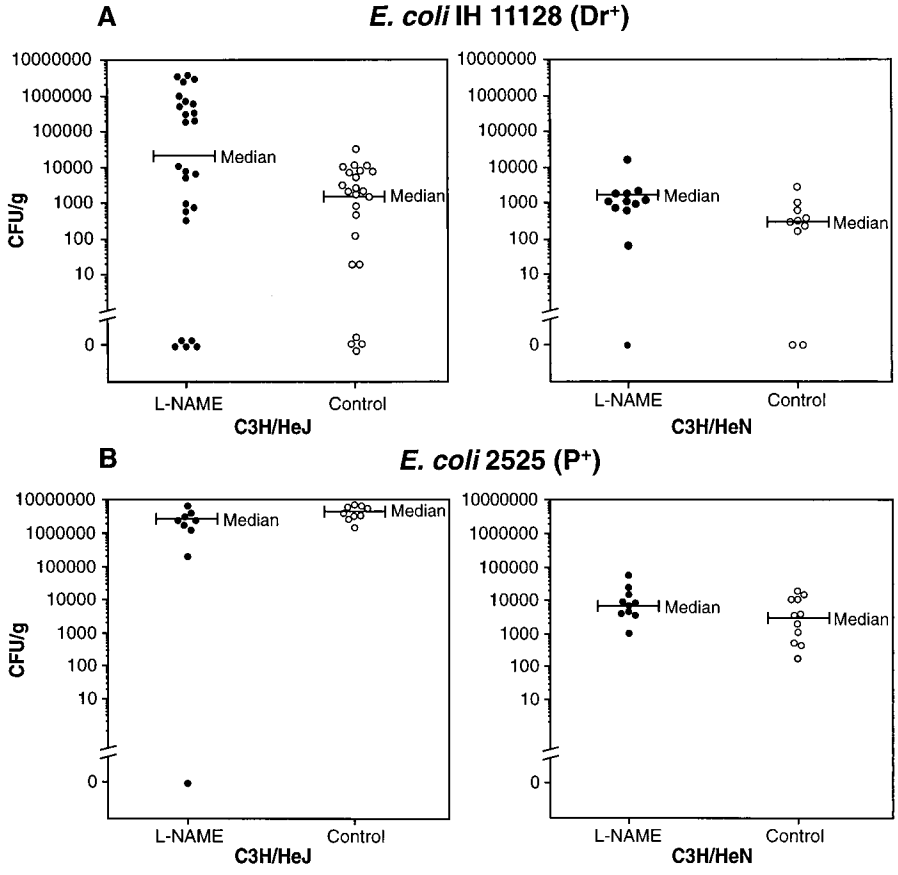

FIG. 3. Effect of L-NAME on quantitative bacterial counts in kidneys of C3H/HeJ and C3H/HeN mice at 24 h postinoculation.

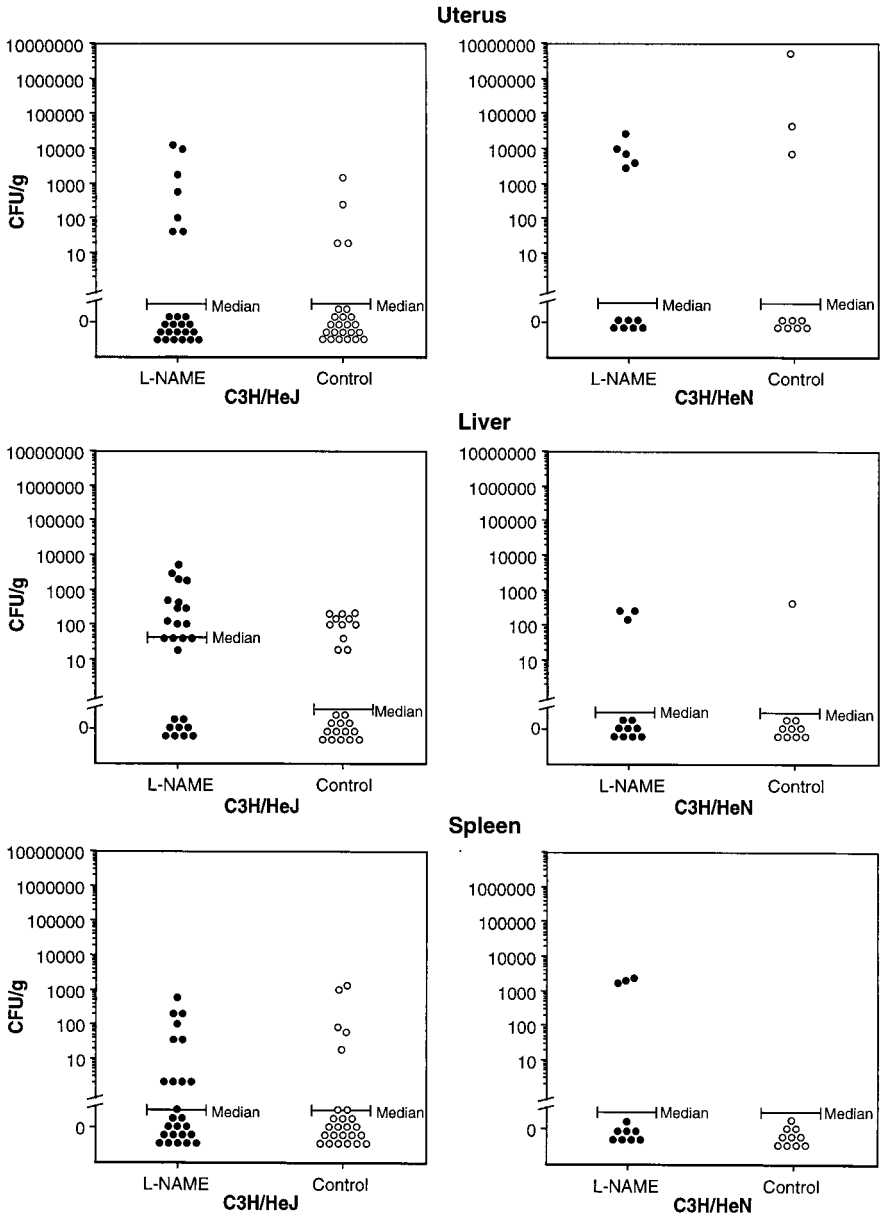

FIG. 5. Effect of L-NAME on quantitative bacterial counts in uterus, liver, and spleen of C3H/HeJ and C3H/HeN mice challenged with Dr^+ *E. coli* IH11128 at 24 h postinoculation.

ceptible to the pyelonephritogenic strain *E. coli* IH11128 (Dr^+) if NO is blocked.

The reported changes in the UTI rate in C3H/HeJ versus that in C3H/HeN mice are contradictory (5, 10, 14). We consider the possibility that the observed differences may be associated with the use of different *E. coli* strains. To evaluate whether LPS-NO defense is equally effective against different *E. coli* strains, we performed experiments with pyelonephritogenic *E. coli* of the same serotype (O75) but expressing P instead of Dr fimbriae (Fig. 3B). These two strains differed in invasiveness to HeLa cells, with the Dr^+ strain being invasive and the P^+ strain being noninvasive. Similar to mice infected with Dr^+ *E. coli*, CH3/HeJ mice infected with P^+ *E. coli* had a significantly higher rate of kidney colonization than C3H/HeN mice. However, contrary to the situation with Dr^+ *E. coli* IH11128, L-NAME treatment did not increase the counts of renal CFU of P^+ *E. coli* 2525 in either C3H/HeJ or C3H/HeN mice ($P > 0.05$).

Because of the significant increase in the rate of renal infection with the inhibition of NO in C3H/HeJ mice inoculated with Dr^+ *E. coli*, we investigated if dissemination of infection to other organs, such as the liver, uterus, and spleen, occurs in both C3H/HeJ and C3H/HeN animals, with and without L-NAME treatment. In the C3H/HeJ mice infused with L-NAME, dissemination of infection to the liver occurred in 64% of the animals (Fig. 5). The median number of CFU of positive liver cultures was 40 CFU/g. On the other hand, the infection of the liver was substantially less extensive (Mann-Whitney U test, $P = 0.08$) in the control saline-treated group (1 CFU/g) of C3H/HeJ mice. This indicated the enhanced

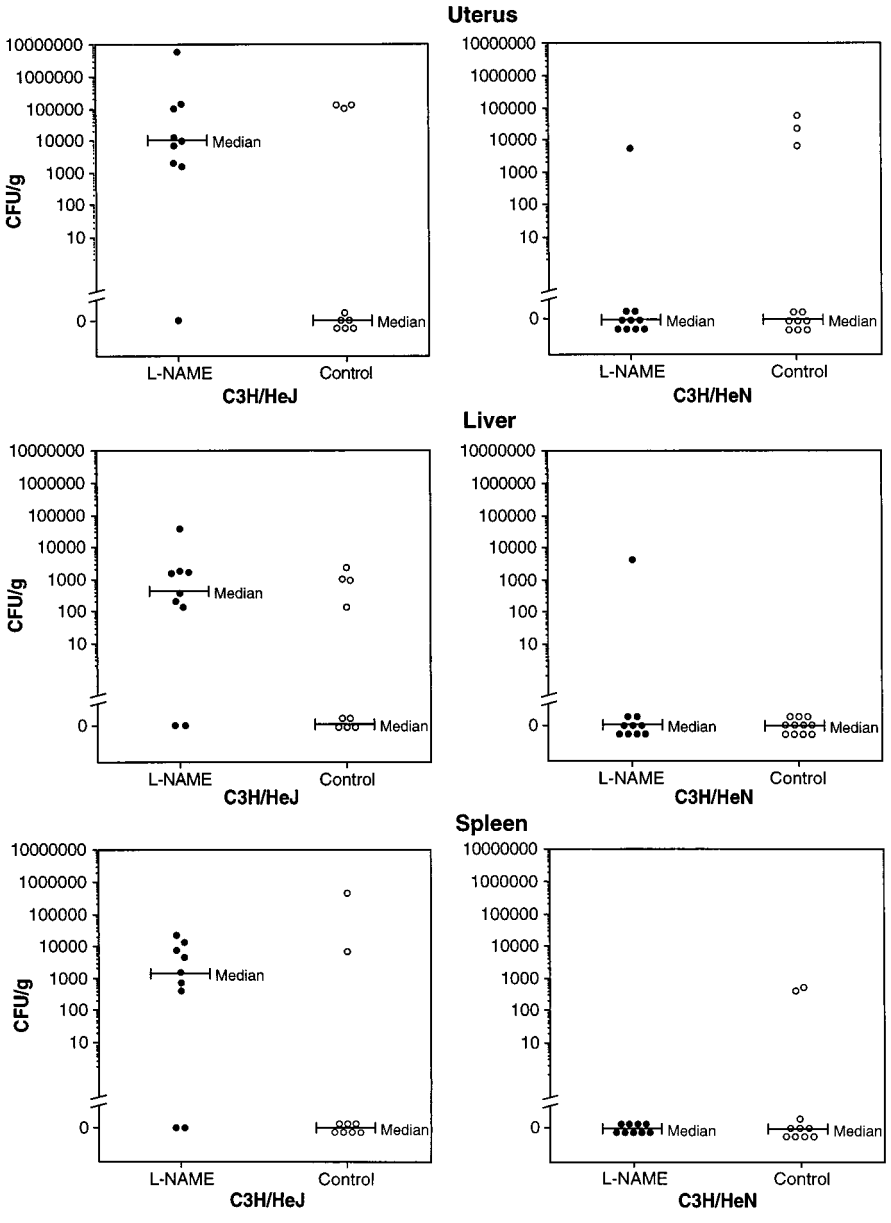

FIG. 6. Effect of L-NAME on quantitative bacterial counts in uterus, liver, and spleen of C3H/HeJ and C3H/HeN mice challenged with P^+ *E. coli* 2525 at 24 h postinoculation.

spread of Dr^+ *E. coli* infection from the urinary tract to the liver in the L-NAME-treated C3H/HeJ group. Livers from only a few C3H/HeN mice were infected at a very low rate, and L-NAME did not significantly alter the liver infection rate.

Uteri from 28% of the C3H/HeJ mice treated with L-NAME were found to be positive for Dr^+ *E. coli* culture (Fig. 5), while in C3H/HeJ mice treated with saline, only 17% ($P \leq 0.149$) of uteri were positive for Dr^+ *E. coli*. In C3H/HeN mice, the rate of uterine infection was similar to that of C3H/HeJ animals and L-NAME did not increase the infection rate or number of CFU per gram of uterus.

There were no significant differences with respect to CFU counts for Dr^+ *E. coli* in the spleens of C3H/HeJ mice in the L-NAME- and saline-treated groups. Similar results were also obtained for C3H/HeN mice.

Similar evaluation of organ dissemination was performed for P^+ *E. coli* 2525 (Fig. 6).

Cultures of the livers, spleens, and uteri of C3H/HeN mice were negative in the majority of tested animals, and L-NAME had no significant effect on dissemination ($P > 0.05$).

In C3H/HeJ mice, although more positive cultures or higher CFU counts for P^+ *E. coli* were observed in the spleen, liver, and uterus and with L-NAME treatment, the observed increases were not statistically significant. *P* values for L-NAME treatment in this group were 0.1.

To investigate whether a NO synthase (NOS) inhibitor would have a direct effect on *E. coli* growth, we analyzed, in vitro, the kinetics of the Dr^+ *E. coli* IH11128 growth in the presence of three different concentrations (10^{-3}, 10^{-4}, and 10^{-5} M) of L-NAME; L-arginine, the substrate of NO gener-

ation; and D-arginine, a control isomer of L-arginine that is not a substrate for NO synthesis. Growth was assessed at 0, 1, 2, 3, and 6 h, and growth curves were similar in all four groups, suggesting a lack of direct effect of the NO substrate and inhibitor on the growth of Dr^+ *E. coli* IH11128 in vitro.

Ten individual colonies were isolated from one kidney of each animal and tested for the serological identity with the inoculated strain of *E. coli* O75 IH11128, and with *E. coli* O75 2525. A slide agglutination assay with anti-O75 immunoglobulin G confirmed that bacterial colonies from the kidneys belonged to the O75 serotype of *E. coli* in the liver, uterus, and spleen and were also serologically identical to the inoculated O75 isolate. Similarly, fimbrial types expressed were tested with human O-group erythrocytes in the presence or absence of chloramphenicol or Gal-Gal as an inhibitor. Hemagglutination assay confirmed that the isolated colonies of bacteria expressed Dr or P fimbriae as expected. These tests confirmed that the isolates from different organs were identical to those which were introduced by bladder catheterization (no organ contamination with natural flow), indicating the etiology of disseminated infection.

DISCUSSION

The role of NO in host resistance to renal infection was evaluated in LPS responder (C3H/HeN) and nonresponder (C3H/HeJ) mice with experimental pyelonephritis induced by two *E. coli* strains of the O75 serotype that differed in fimbrial adhesin and invasiveness. Significant increases in colonization of various organs, including the kidneys, were observed in mice in which either NO generation was lower or NO synthesis was inhibited by L-NAME treatment. Interestingly, C3H/HeJ mice with a primary deficiency of the antibacterial defense became more susceptible to pyelonephritis with NO inhibition when infected with Dr^+ *E. coli* IH11128 but not when infected with P^+ *E. coli* 2525. Furthermore, a lack of a direct effect of NO on *E. coli* growth in vitro further indicates that the host defense-related mechanisms are involved in colonization, probably by modulation of NO and LPS responsiveness. Therefore, we suggest that NO together with LPS responsiveness plays a major role in host defense against experimental Dr^+ *E. coli* IH11128 infection of the kidneys and that this phenomenon is strain dependent.

C3H/HeJ mice lacking the LPS gene (Lps^d/Lps^d) do not develop rapid polymorphonuclear leukocyte response and are more sensitive to UTI (10). In contrast, a recent study showed that LPS responders and nonresponders did not differ in their capacity to clear UTI (14). Also, experimental septicemia in C3H/HeJ and C3H/HeN mice shows similar sensitivity with respect to the lethal outcome in both groups (5). Therefore, it is not clear whether differences between the above-mentioned results were associated with the use of different *E. coli* isolates or with other factors in addition to LPS responsiveness that contribute to the differences between C3H/HeJ and C3H/HeN mice (15, 34).

It is of interest that C3H/HeN LPS responder mice cleared experimental pyelonephritis within a few weeks, while in C3H/HeJ LPS nonresponder mice, pyelonephritis developed into persistent infection (8). The results presented here are in agreement with reported observations that LPS nonresponder mice are more susceptible to UTI (10). The potential explanation for the lack of differences observed in recent reports (5, 14) is that investigators used different *E. coli* strains. For example, P-fimbriated *E. coli* was used in a study by Hopkins et al. (14), while Dr- and P-fimbriated *E. coli* strains were utilized in our experiments. Dr^+ *E. coli* strains are associated with 30 to 50% of cystitis in nonpregnant females and 30 to 40% of pyelonephritis in third-trimester pregnant patients (28, 30, 35). It is important to note that Dr^+ *E. coli* possess the capacity to invade HeLa epithelial cells, while P-fimbriated *E. coli* are less likely to have such a capacity (9, 18). Interestingly, our experiments with two different *E. coli* strains revealed that NO-dependent virulence for the kidney was true for Dr^+ but not for P^+ *E. coli*. It is conceivable that P-fimbriated *E. coli* binds to the cellular receptor of mouse renal tubules without internalization, while Dr^+ *E. coli* invades the epithelium. Thus, modulation of the NO system would have a more profound effect on invasive Dr^+ *E. coli* than on extracellular P^+ *E. coli*. In humans, invasion of renal parenchyma is an important component of the pathogenesis of pyelonephritis. In this context, the L-NAME-enhanced infection rate may suggest contribution of local tissue NOS activity to the invasion of the renal tissue by the specific *E. coli* strain.

Surprisingly, inhibition of NO produced a greater increase in renal infection in LPS-nonresponder C3H/HeJ mice than in LPS-responder C3H/HeN mice. This observed increase in the rate of infection was higher than the predicted additive effect of NO inhibition and Lps^d mutation. These observations are consistent with the hypothesis that adequate NO production and LPS responsiveness work synergistically to provide a mechanism of renal resistance to pyelonephritis associated with *E. coli* strains causing recurrent, chronic, or third-trimester infections.

In several organs, including the kidney, the liver, and the uterus, the NOS activity was significantly higher in C3H/HeN mice than in C3H/HeJ mice. The underlying mechanism for the lower NOS activity in C3H/HeJ mice is not clear, but it is consistent with the hypothesis that mutation of the LPS gene(s) alters NO generation. It is therefore conceivable that NO produced in the organs of C3H/HeN mice may provide defense against the *E. coli* infection (16, 21, 22, 25, 26, 38). The effect of L-NAME on the bacteremic spread of Dr^+ *E. coli* to organs in C3H/HeJ mice may be explained by the significantly lower concentration of NO in the kidneys and other organs of the mutant animals. A physiologic condition during pregnancy is associated with decreased NO production at parturition (41, 43–46). Therefore, decrease in NO generation prior to labor hypothetically represents a risk for increased bacterial infections (13, 23, 40).

In summary, the underlying mechanism of host sensitivity to UTI and spreading of infection is not completely understood but may involve (i) the potential differences in NOS activity in the tissues of mice responding and not responding to LPS, (ii) the synergistic effect of NO and LPS responses to infection, and (iii) the characteristics of a strain, including, for example, the invasive properties of *E. coli* (9, 19). The underlying molecular mechanisms of NO-LPS-mediated resistance of the kidney to Dr^+ and P^+ *E. coli* infection are currently being studied.

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