Resistance of C3H/HeJ Mice to the Effects of Haemophilus pleuropneumoniae

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Comparisons were made in the mortality associated with an inhaled dose of viable Haemophilus pleuropneumoniae type 5, strain J45, between adult C3H/HeN and C3H/HeJ mice. Mice of both strains were also challenged with Escherichia coli strains O111:B4 and J5. The 50% lethal dose (LD₅₀) of H. pleuropneumoniae in C3H/HeN mice was calculated to be 10⁶.5 CFU. At a mean dose of 10⁶.7 CFU a 46% mortality rate occurred in C3H/HeN mice, whereas only 10% of the C3H/HeJ mice died (P < 0.01). Deaths occurred significantly earlier in C3H/HeN mice (P < 0.01). No deaths occurred later than 12 h postinfection in either group. Pulmonary lesions in the mice that died were similar to those in pigs that die during the acute phase of H. pleuropneumoniae infection. In surviving mice of both strains, a mild resolving interstitial and bronchopneumonia was present which was not typical of subacute H. pleuropneumoniae infections in swine. Quantitative bacterial isolations from the lungs, liver, and spleen indicate that H. pleuropneumoniae did not multiply in the lungs, was rapidly cleared, and did not become systemic. No deaths occurred in the mice inoculated with E. coli J5 or O111:B4 at mean doses of 10⁶.3, 10⁷.2, and 10⁸.5 CFU, and 10⁶.4, 10⁷.5, and 10⁸.2 CFU, respectively. The difference in the mortality rate between the C3H/HeN and C3H/HeJ mice suggests that endotoxin may be involved in acute deaths in pigs infected with H. pleuropneumoniae. As indicated by the E. coli challenge, however, other factors are also likely to be involved. Because of the differences in the pathology and microbiology following H. pleuropneumoniae pulmonary infections in mice and pigs, mice do not appear to be an accurate model of the overall disease in swine.

Haemophilus (Actinobacillus) pleuropneumoniae is the cause of a severe and often fatal infectious pneumonia with pleuritis in pigs (20, 22, 23). Prominent features of the pulmonary lesions caused by H. pleuropneumoniae in pigs are venous thrombosis and necrosis. Results of previous studies suggest that toxic factors associated with the bacteria are important in the pathogenesis of the disease and are likely to be the cause, either directly or indirectly, of the necrosis and acute deaths (1, 4). The identity of the toxin or toxins and their role in the disease have yet to be defined. Direct cytotoxic effects, endotoxins, and exotoxins have been suggested (1, 4, 8, 17).

C3H/HeJ mice are relatively refractory to the toxic and mitogenic effects of endotoxin due to a defect in the lps allele on the fourth chromosome and other undefined genetic influences (15, 27, 28). On the other hand, the syngeneic and fully histocompatible C3H/HeN mice are fully responsive to the effects of endotoxin (15). This difference (approximately 1,000:1) has proven to be a powerful tool in the study of the role of endotoxin in the pathogenesis and immunopathology of gram-negative infections.

C3H/HeJ mice are hypersusceptible to Salmonella typhimurium infections, while C3H/HeN mice are innately resistant (19). This difference has been interpreted as an indication that endotoxin is not a major factor in salmonellosis (2). By using the same approach, the potential involvement of endotoxin in the pathogenesis of H. pleuropneumoniae was studied, and at the same time a proposed mouse model of H. pleuropneumoniae infections in swine was evaluated (24, 25).

MATERIALS AND METHODS

Bacterial strains and growth conditions. An encapsulated strain of H. pleuropneumoniae serotype 5 (strain J45) that had been originally isolated from a naturally occurring case of porcine pleuropneumonia was obtained from E. L. Biberstein, University of California, Davis. Bacteria were grown on chocolate agar plates at 37°C in 10% CO₂ for 18 h. The cells were harvested with sterile saline, washed twice with cold saline, and stored on ice. Bacterial numbers were spectrophotometrically adjusted to the desired concentration, as determined in previous experiments, with cold sterile saline. Escherichia coli O111:B4 was purchased from the American Type Culture Collection, Rockville, Md., and E. coli J5 was provided by E. Ziegler, University of California, San Diego. They were prepared in the same manner as H. pleuropneumoniae, except blood agar was used.

Mice. Male and female adult C3H/HeJ and C3H/HeN mice were purchased from Bantin and Kingman Inc., Fremont, Calif. Mice were separated by sex and housed in groups of 8 or 10 and acclimatized for several weeks. They weighed 22 to 28 g at the start of the experiment. Prior to the start of the experiment, a few mice were randomly selected and killed, their lungs were cultured for mycoplasma and bacteria and examined histologically. Serum collected from these mice was evaluated for antibodies to H. pleuropneumoniae J45 and E. coli O111:B4 and J5 by a microtubate agglutination assay (12). Positive control sera were produced in mice by repeated subcutaneous injection of viable organisms.

LD₅₀ determination. Mice were anesthetized with halothane to light narcosis. Replicate groups of 10 C3H/HeN mice were inoculated intranasally with various concentrations of bacteria diluted in 0.05 ml of sterile saline, as described by Rushton (21). The concentrations of the cultures were periodically determined between inoculations by

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techniques described by Miles and Misra (11), and the mean concentration of organisms per 0.05 ml of inoculum was calculated. Clinical signs and mortality rates were recorded at 30-min intervals for the first 18 h and then twice daily for 5 days. The 50% lethal doses (LD_{50}) were calculated based on cumulative mortality at 5 days postinoculation.

**Experimental infection.** The calculated LD_{50} of *H. pleuropneumoniae* for C3H/HeN mice was simultaneously given intranasally to both C3H/HeN and C3H/HeJ mice, as described previously (21). In a separate experiment, groups of 16 mice from both strains were inoculated intranasally with various concentrations of *E. coli* O111:B4 and J5. Ten control mice were inoculated with sterile saline.

Clinical signs and deaths were continuously recorded for the first 18 h postinoculation and then twice daily for 5 days. Pathologic or microbiologic examination was alternately performed on all animals that died. Six mice from both strains that were challenged with *H. pleuropneumoniae* were randomly selected at 24 and 72 h postinoculation and examined. Two mice from each strain that were given the *E. coli* or saline were examined at 4, 24, and 72 h postinoculation. Five days after the inoculation all remaining mice were sacrificed and divided into equal groups for pathologic and microbiologic examinations. For pathologic examination, the lungs were insufflated with 10% Carlson-modified Formalin before they were removed from the thorax.

**Bacterial recovery from tissues.** The lungs, liver, and spleen were removed from each mouse and kept on ice. The weight of the lungs and the combined weight of the liver and spleen were determined. The lungs or the liver and spleen were placed into chilled sterile Ten Broeck tissue homogenizers, cold sterile saline was added to equal 10 ml, and the tissues were thoroughly homogenized. Undiluted homogenate or appropriate dilutions were plated onto chocolate agar and incubated at 37°C. Cultures were performed in triplicate. The plates with optimal numbers of organisms were counted 24 h later, and the total number of organisms present in the lung or in the combined spleen and liver was calculated.

**Statistics.** The LD_{50} was calculated, as outlined by Reed and Muench (18). Mortality rates and time to death data were analyzed by challenge experiment analysis techniques (9), using analysis of variance, the Student *t* test, and Mann-Whitney techniques to verify population uniqueness. Differences in bacterial recovery rates were evaluated by the chi-square test.

**RESULTS**

**Susceptibility of mice.** Antibody in serum against *H. pleuropneumoniae* or *E. coli* O111:B4 was not detected by the bacterial agglutination assay in control mice. A few mice of both strains had low bacterial agglutination titers (never greater than 1:8) against *E. coli* J5. The LD_{50} for an inhaled challenge of *H. pleuropneumoniae* 145 in C3H/HeN mice was calculated to be 10^{6.5} CFU. At a mean dose of 10^{6.7} CFU the mortality rate for C3H/HeN mice was 46.7% (28 of 60), whereas only 10% of the endotoxin-hyporesponsive C3H/HeJ mice died (6 of 60) (*P* < 0.01). In addition, the mean time to death was shorter in the C3H/HeN mice than in the C3H/HeJ mice (2.43 versus 6.75 h, respectively) (*P* < 0.01) (Fig. 1). No deaths in either strain of mice occurred later than 12 h postinoculation. No deaths (0 of 10) occurred with either *E. coli* J5 or O111:B4 at mean doses of 10^{6.3}, 10^{7.2}, 10^{8.3} CFU, and 10^{6.4}, 10^{7.2}, and 10^{8.2} CFU, respectively.

**Clinical disease.** Most of the mice recovered rapidly from the anesthesia. A small number of mice from both strains (5.0% C3H/HeJ and 8.3% C3H/HeN) died because of anesthetic overdose and were replaced. All mice that recovered from anesthesia were clinically normal by 15 min postinoculation. The mice from both strains that died during the first few hours after inoculation initially showed roughened hair coats, weakness, and reluctance to move. In a short time they rapidly developed severe respiratory distress with gasping, and they became comatose and died. The time from the initial sign of illness to death in C3H/HeN mice averaged 25 min but was as short as 5 min (Table 1). This progression appeared to be slower in the C3H/HeJ mice, but because of the small number of animals that died the observation was not statistically significant.

The mice that died between 7 and 12 h postinoculation were slower to develop severe respiratory distress, and the progression to death was delayed (mean of 48 min between first clinical sign and death). Approximately one-half of the animals that survived the inoculation also showed roughened hair coats and a tendency to huddle together, but only a few showed any signs of respiratory disease which was, for the
most part, upper respiratory in nature. There was no apparent difference between the mouse strains in the severity of the clinical signs in the mice that survived. The mice inoculated with one of the E. coli strains did not show clinical disease for approximately 24 h. From 24 to 72 h postinoculation approximately 25% of the mice from both strains at any one time had roughened hair coats, were reluctant to move, and huddling together. No evidence of respiratory disease was apparent and no difference between bacterial or mouse strain was noted. All mice were clinically normal by 5 days postinoculation. The control mice were normal throughout the experiment.

Pathology. All control mice and those failing to recover from anesthesia were free of histologic pulmonary lesions. The pulmonary lesions from both strains of mice that died as a result of the H. pleuropneumoniae inoculation were similar, regardless of the time of death. Gross examination showed that the lungs were uniformly consolidated, wet, and heavy and they failed to collapse. Histological examination showed that (Fig. 2) alveoli were flooded with sero-hemorrhagic exudate, marked vascular congestion was present, and lymphatics were dilated by fibrin and edema fluid. Inflammatory cell infiltration was minimal.

The animals surviving the H. pleuropneumoniae and E. coli challenges that were examined on days 1, 3, and 5 postinoculation had normal lungs on gross examination. Histologically, a mild to focally moderate interstitial and bronchopneumonia, with neutrophils, macrophages, and small amounts of fibrin, was present. The inflammation was centered on conducting airways, and only rarely were alveoli affected and then only in close association with affected airways. Neither vascular thrombosis nor parenchymal necrosis was evident. The lesions were most severe on day 1, with considerable improvement and mild focal inflammation by day 3 and with near complete resolution by day 5 postinoculation (Fig. 3).

Microbiology. The concentrations of viable H. pleuropneumoniae or E. coli with which the mice were inoculated was determined by averaging the concentration before, during, and at the end of the inoculation period. The total time involved was approximately 1.75 h. No organisms were cultured from the mice that were examined prior to the start of the experiment or from the controls.

Isolations were not made from the livers or spleens of any of the mice, irrespective of the organism or time of exami-
necrosis. The mean number of organisms present in the lungs of mice from the various treatment groups by time is presented in Table 2. Pulmonary clearance of *H. pleuropneumoniae* and both *E. coli* strains occurred rapidly and was complete by postinoculation day 5 in all but one group; C3H/HeJ give *E. coli* O111:B4 at $10^{8.5}$ CFU. There was no detectable difference in the rate of pulmonary clearance between the mouse strains, regardless of the organism. The isolations that did occur were on day 3 from one infected mouse and on day 5 from two infected mice.

**DISCUSSION**

In these studies we used the inherent difference in endotoxin responsiveness between C3H/HeJ and C3H/HeN mice to evaluate the involvement of endotoxin in the pathogenesis of *H. pleuropneumoniae* infections and at the same time to test the validity of a mouse model for the disease in swine.

The results demonstrate that the endotoxin-hyporesponsive C3H/HeJ mice are significantly more resistant to the acute effects of inhaled *H. pleuropneumoniae*, as measured by mortality and mean time to death, than the normally endotoxin-responsive C3H/HeN mice. This observation implicitly suggests a role of endotoxin in the early stages of *H. pleuropneumoniae* infections; but as indicated by the difference between the effects of *H. pleuropneumoniae* and *E. coli*, other factors are likely to be involved. Moreover, mice do not appear to be an accurate model of *H. pleuropneumoniae* infections in pigs in that inhaled *H. pleuropneumoniae* acts more as a toxin than a true infection, and both the pathologic and microbiologic findings differ from those seen in the later stage of *H. pleuropneumoniae* infections.
infections in pigs. However, the clinical disease and pulmonary lesions during the first few hours after *H. pleuropneumoniae* exposure in mice are similar to acute lethal infections in swine.

C3H/HeJ mice are hypersusceptible to *S. typhimurium* septicemia and to *E. coli* urinary tract infections (2, 3, 15, 19). The reason for their unusually high susceptibility to gram-negative infections is uncertain but has been attributed to defective autosomal codominant *lps* genes and is associated with their hyporesponsiveness to the biologic and immunologic effects of endotoxin (27–29). In contrast, C3H/HeJ mice were more resistant to the lethal effects of an inhaled dose of *H. pleuropneumoniae* than were C3H/HeN mice. However, infections evidenced by significant bacterial multiplication and dissemination did not take place.

The effect of an inhaled dose of *H. pleuropneumoniae* in C3H/HeJ mice is strongly dose dependent and results in one of three events: rapid acute death within a few hours, mild clinical disease, or no observable effect. A similar *H. pleuropneumoniae* dose-related effect has been experimentally demonstrated in swine (26) and in other strains of mice (24, 25). However, in mice, as opposed to in swine, *H. pleuropneumoniae* did not multiply significantly, did not become systemic, and was rapidly cleared from the lungs. Interestingly, at the same or higher dose the two strains of *E. coli* were cleared as fast as *H. pleuropneumoniae* without notable toxicity.

The acute toxicity of *H. pleuropneumoniae* in mice, as well as the pathology and dose-related nature of the effect, suggests that endotoxin may be involved in acute *H. pleuropneumoniae* infections. If these effects were related only to the concentration of lipid A, it would be expected that *E. coli* at the same or higher dose would also be toxic. The fact that a similar toxic reaction commonly occurs when swine are immunized with heat-killed (and other) *H. pleuropneumoniae* vaccines (S. Henry, Abilene, Kans., personal communication, 1985) further supports the inherent toxic nature of *H. pleuropneumoniae*. The lesions similar to those which occur in pigs that die during the acute stages of pleuropneumonia can be reproduced in pigs by the intratracheal administration of culture supernatants (S. Rosendal, W. R. Mitchell, M. Weber, M. R. Wilson, and M. R. Zamen, Proc. Int. Pig Vet. Soc. Congr., p. 221, 1980) or the purified lipopolysaccharide (LPS) of *H. pleuropneumoniae* 345 (2a). Inhalation of *H. pleuropneumoniae* LPS by mice also induces lesions similar to those seen in the mice that die after being inoculated intranasally with *H. pleuropneumoniae* (data not shown). In addition, ultrastructural examination of the lesions induced by *H. pleuropneumoniae* in pigs showed endothelial changes similar to those of the Shwartzman reaction preceding vascular thrombosis (16). The content and composition of the LPS among gram-negative bacteria vary widely (6, 10), as do their toxigenic and thrombogenic properties (13). *Haemophilus* sp. and related organisms are known to have vesicular structures, or "blebs," on their outer cell walls which are morphologically similar to LPS vesicles (7). These structures are implicated in tissue damage and inflammation associated with gram-negative bacterial infections.

In the mice, all deaths occurred during the first few hours after inoculation with *H. pleuropneumoniae*. In the acute form of *H. pleuropneumoniae* infections in swine, death also commonly occurs within the first 24 h, often with a very rapid clinical course (4, 5). In these pigs, as in the mice that died, the lungs are characterized by widespread alveolar flooding, vascular congestion, dilated fibrin-filled lymphatics, focal necrosis, and minimal inflammatory cell infiltration. The principle pathologic difference between the acute lesions in swine and mice is the greater amount of fibrin present in the lungs of swine.

Swine that survive the acute stages of an *H. pleuropneumoniae* infection commonly develop focal areas of necrosis in the lung associated with venous thrombosis without any recognizable airway association. These pulmonary infarcts commonly contain *H. pleuropneumoniae*, thus resulting in the occurrence of chronic carriers (14). The lesions in the mice surviving the *H. pleuropneumoniae* inoculation and those given *E. coli* were distinctively airway oriented and lacked areas of necrosis, and the presence of *H. pleuropneumoniae* or *E. coli* could not be demonstrated.

Sebunya and Saunders (24, 25) proposed a mouse model of *H. pleuropneumoniae* infection in which they suggested its usefulness in studying the pathogenesis of the disease in swine as well as in evaluating potential vaccines. The results of the present experiment are similar, with the differences in LD50, mortality rates, and time to death easily explained by the use of different bacterial and mouse strains. Both studies show that in the strains of mice examined, *H. pleuropneumoniae* in general fails to multiply significantly; deaths mainly occur shortly after inoculation; very large doses of *H. pleuropneumoniae* are required for infection; deaths are closely associated with the inoculating dosage; and except for the mice that died acutely, the lesions are not similar to those in *H. pleuropneumoniae*-infected pigs. In a previous study (25), when *H. pleuropneumoniae* was inoculated intranasally into mice at low dosages it multiplied for a short time (24 h) and then was rapidly cleared. We found that at reasonable challenge doses (equal to the infective dose for pigs) mice can rapidly clear *H. pleuropneumoniae* 345 from the lungs and show few clinical signs with minimal pathologic changes in the lungs.

The findings in this report suggest that the acute toxic effects of *H. pleuropneumoniae* infection in mice, and possibly in swine, are due to endotoxin and that *H. pleuropneumoniae* is inherently more toxic than *E. coli*, for an as yet to be determined reason. Utilization of a mouse model of *H. pleuropneumoniae* infections may be helpful in elucidating the pathogenesis of rapid and apparently toxic deaths that occasionally occur in the early stages of *H. pleuropneumoniae* infections in pigs. The murine model may also be helpful in the development of less toxic *H. pleuropneumoniae* vaccines. It is apparent, however, that because of an innate resistance to *H. pleuropneumoniae* infections in the strains of mice that have been studied, the mouse appears not to be an appropriate model for the overall study of the pathogenesis and immunology of *H. pleuropneumoniae* infections in swine.

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LITERATURE CITED


H. PLEUROPNEUMONIAE IN MICE


