Innate Resistance of Mice to Experimental Infection with
Naegleria fowleri

R. M. HAGGERTY AND D. T. JOHN*

Department of Microbiology, Virginia Commonwealth University, Richmond, Virginia 23298

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The mouse system provides an excellent model for studying host resistance to Naegleria fowleri, the agent of primary amoebic meningoencephalitis. Innate resistance to infection with N. fowleri was examined with respect to infecting dose and the age, sex, and strain of mice. Intravenous inoculation with 10⁷ amoebae per mouse produced 100% mortality in 9 days, whereas inoculation with fewer amoebae reduced the cumulative mortality. Male and female DUB/ICR mice of varying ages were inoculated intravenously with 2.5 × 10⁵ N. fowleri per g of body weight. The youngest mice died first, with 100% mortality for both males and females, and mortality decreased with increasing age. Female mice were significantly more resistant to infection than males. Five strains of mice weighing approximately 20 g were inoculated intravenously with weight-adjusted doses; mortality ranged from 10% in C57BL/6 mice to 95% in A/HeCr mice.

Primary amoebic meningoencephalitis is a recently identified, fatal human disease caused by the amoeboid flagellate Naegleria fowleri. The disease may be experimentally produced in laboratory animals by using various routes of inoculation (6, 13). Factors of innate resistance to N. fowleri infection are undefined. Relatively few human infections have occurred even though large numbers of individuals have been exposed to similar environmental conditions (4). In reviews of primary amoebic meningoencephalitis (6, 7), factors of host age and sex have been given considerable attention; however, definitive statements have not been made regarding these factors and relative susceptibility to infection. In many published accounts of experimental primary amoebic meningoencephalitis, investigators often failed to give the age, sex, or strain of mice used. The importance of identifying and controlling for these factors cannot be overemphasized when the virulence of an organism is being tested.

In this study we have demonstrated that age, sex, and strain of mice are important variables that markedly affect innate resistance of mice to infection with N. fowleri.

MATERIALS AND METHODS

N. fowleri (LEE strain), used throughout this study, was isolated from human brain by E. C. Nelson (Department of Microbiology, Virginia Commonwealth University). Amoebae were cultured axenically in cotton-stoppered, 2.5-liter, siliconized Fernbach flasks by using 1 liter of Nelson medium (1) inoculated with 10⁴ amoebae per ml. Cultures were adjusted to pH 5.5 and incubated at 37°C in a gyratory shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 100 rpm.

Amoebae were harvested by centrifugation (2,000 × g, 10 min, 20°C) at 72 h. The cells were washed twice with Page amoeba saline (1) and suspended in 0.15 M NaCl for counting and mouse inoculation. Amoebae were judged viable by using trypan blue exclusion.

For counts, 0.2 ml of cell suspension was added to 9.8 ml of electrolyte solution consisting of 0.5% (vol/vol) Formalin and 0.4% (wt/vol) NaCl in distilled water and Vortex shaken to disperse cell aggregates. Cells were then counted in a Coulter Counter (model ZB1, Coulter Electronics, Inc., Hialeah, Fla.) by using settings described elsewhere (18).

Mouse strains used were male DUB/ICR mice weighing 13 to 18 g and male and female DUB/ICR mice weighing approximately 10, 20, 30, and 40 g (Flow Research Animals, Inc., Dublin, Va.); 20-g male A/HeCr mice (Charles River Breeding Laboratories, Wilmington, Mass.); and 20-g male DBA/2Cr, BALB/c, and C57BL/6 mice (Simonsen Laboratories, Inc., Gilroy, Calif.). All mice were allowed to adjust to their new environment for 3 days before experimentation. The mice were given free access to water and feed (Purina Lab Chow, Ralston Purina Corp., St. Louis, Mo.).

Mice were inoculated intravenously (i.v.) with a 0.2-ml cell suspension containing 1.5 × 10⁴ amoebae per g of body weight, or 2.5 × 10⁵ amoebae per g of body weight, or with a total inoculum of 10⁶, 2.5 × 10⁶, 5 × 10⁶, or 10⁷ amoebae per mouse of live N. fowleri (LEE strain). There were 20 mice in each experimental or control group unless otherwise specified. Mice were held 21 days after inoculation, and the cumulative percent dead was recorded on a daily basis. Infection was verified by culturing amoebae in Nelson medium from brain tissue of dead or dying mice. Statistical analysis of cumulative mortality for male and female
DUB/ICR mice of varying ages was performed by using the chi-square test.

RESULTS

After i.v. inoculation, the course of *N. fowleri* infection and percent mortality were dependent upon the infecting dose of amoebae as shown in Fig. 1. Inoculation with $10^5$ amoebae per mouse resulted in 100% mortality by day 9. Reducing the inoculum by one-half, $5 \times 10^4$ amoebae per mouse, caused 75% mortality in 11 days. Further reductions in inocula produced 60% mortality for $2.5 \times 10^4$ amoebae per mouse and 20% mortality for $10^4$ amoebae per mouse. As the size of inoculum was increased, the survival time for mice decreased.

The lethal dose required to kill 50% of the test animals was calculated using the Karber (Spearman) method (2). By using this method, the calculated 50% lethal dose (LD$_{50}$) for *N. fowleri* (LEE strain) was $2.4 \times 10^4$ amoebae per 0.2 ml inoculated i.v. for 13- to 18-g male DUB/ICR mice.

Male DUB/ICR mice of varying ages inoculated with $2.5 \times 10^5$ *N. fowleri* per g of body weight exhibited a wide range of mortality. The youngest mice (10 g) died first, with 100% mortality in 8 days as shown in Fig. 2. With increasing mouse age there was a corresponding decrease in mortality. The oldest males responded with 55% mortality at 15 days. In this and in the other experiments, amoebae were cultured from brain tissue of all dead and dying mice.

By using the same route and dose of amoebae, female mice aged 3, 4, 6, and 24 weeks and weighing an average of 10, 20, 27, and 36 g, respectively, were inoculated with *N. fowleri*. Figure 3 shows again that the youngest mice (10 g) died first, with 100% mortality by day 9. Mortality was less for the older mice. From these results it appears that age plays a significant role in host resistance in both male and female mice. When the sexes are compared, however, the females exhibited greater resistance with age than the males. Table 1 gives a summary of cumulative dead for male and female mice of various ages together with a chi-square analysis. The observed differences in mortality were statistically significant ($P \sim 0.05$ or less) for all groups but the youngest, in which mortality was 100% for both males and females. By using the Mantel and Haenszel (15) summary chi-square analysis for the four age groups, the overall chi-square value with one degree of freedom is 18.86 and $P < 0.001$.

Five mouse strains were examined to deter-
TABLE 1. Comparison, by the chi-square test, of the cumulative mortality for various ages of male and female DUB/ICR mice inoculated i.v. with 2.5 x 10^6 N. fowleri (LEE) per g of body weight

<table>
<thead>
<tr>
<th>Sex of Mice a</th>
<th>No. of mice dead</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 Week (10 g)b</td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
</tr>
<tr>
<td>Female</td>
<td>20</td>
</tr>
<tr>
<td>Chi-square value c</td>
<td>0</td>
</tr>
<tr>
<td>Level of significance</td>
<td>0</td>
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</tbody>
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a There were 20 mice in each group.
b Age and weight at inoculation.
c One degree of freedom.

mine whether there were strain differences in susceptibility to N. fowleri infection. A total of 40 mice per strain, with each mouse weighing approximately 20 g, were inoculated with 1.5 x 10^6 N. fowleri per g of body weight. Figure 4 shows differences in susceptibility for the five strains of mice tested. Among the inbred mice, mortality was 95% for A/HeCr, 55% for DBA/2Cr, 45% for BALB/c, and 10% for C57BL/6 mice. Mortality was 85% for DUB/ICR mice, the only outbred strain tested. Deaths occurred earlier and reached maximum cumulative death sooner for A/HeCr mice than for the other four strains. The other strains exhibited prolonged survival with lower mortality.

DISCUSSION

Factors of host resistance to N. fowleri infection are unclear both in human and in experimentally induced primary amoebic meningoencephalitis. This study was undertaken to examine certain variables of innate resistance by using mice as models.

The course of disease and cumulative mortality after i.v. inoculation with N. fowleri amoebae were shown to be dose dependent. Earlier studies (5, 10) have used intranasal (i.n.) inoculation because the nasal mucosa is the suspected route of entry in human infection. In this study we have chosen the i.v. route of inoculation to administer a more accurate and consistent dose. After i.n. inoculation, mice, even when under ether anesthesia, tend to sneeze out a portion of the inoculum, and although it is possible to calculate the dose given, it is difficult to determine the number of amoebae retained by the host. Mice that die after i.v. inoculation exhibit characteristic meningoencephalitis (D. T. John and A. J. Martinez, J. Protozool. 22:39A, 1975), and at the time of death amoebae have been cultured only from brain and lungs.

The cumulative percent mortality increased with each increase in dose and, correspondingly, the survival time for mice decreased as the number of amoebae increased. These findings are in agreement with those of Adams et al. (1) in which older mice were inoculated intraperitoneally and i.v. with N. fowleri and those of Cerva (8) in which guinea pigs were inoculated i.n. with increasing doses of the Vitek strain of N. fowleri. However, in both of the above studies, the amoebae were cultured under unagitated conditions rather than agitated as in this series of experiments.

Culbertson et al. (10) determined that i.n. inoculation with 10^2 to 10^3 N. fowleri (HB-1 strain) killed all mice in 8 to 10 days. Singh and Das (16) obtained similar results using the same strain. From our results, the calculated LD50 for mice inoculated i.v. was 2.4 x 10^6 N. fowleri (LEE strain) per 0.2 ml for 13- to 16-g male DUB/ICR mice. Carter (5) found the LD50 for N. fowleri isolated from a fatal human infection in Australia to be 278 amoebae per female Swiss mouse when inoculated i.n. Using the Vitek strain of N. fowleri, Cerva (8) calculated an LD50 of 100 amoebae per white mouse after i.n. inoculation. The substantially higher LD50 for i.v.-inoculated mice probably can be attributed to clearance of the amoebae by phagocytic cells of the reticuloendothelial system, or it may reflect loss of virulence of amoebae maintained in axenic culture (21).

The age of test mice affects the outcome of N. fowleri infection. Mice of different ages were inoculated with a dose of amoebae based upon weight to account for increased size with age. The youngest mice died first as might be expected, and then with increasing mouse age.

![Figure 4. Mortality of various strains of 20-g male mice inoculated i.v. with 1.5 x 10^6 N. fowleri (LEE) per g of body weight. There were 40 mice per group. Symbols: (△) A/HeCr; (□) DUB/ICR; (●) DBA/2Cr; (△) BALB/c; and (■) C57BL/6.](http://iai.asm.org/)

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there was a corresponding decrease in mortality. Early and 100% mortality for the youngest mice (approximately 3 weeks old and weighing 10 g) may be attributed to several factors including the following: (i) the infecting dose was based upon the calculated LD₃₀ for 13- to 18-g (15-g average) mice and may have been too great for a smaller mouse; and (ii) it is probable that the immune system was not fully developed at this age. The most resistant male mice were the 24-week-old (40-g average) retired breeders that received 10⁷ amoebae i.v. It would be instructive to test the susceptibility of mice between 1 and 2 years of age to determine whether the immune response declined with senescence as it does in humans.

Studies conducted with 27 Old World monkeys infected intracereally with N. fowleri showed that age played a definite role in host susceptibility (20). When inoculated with amoebae of reduced virulence, five of six monkeys that died of meningoencephalitis were 5 years old or less, whereas only one of six survivors was less than 5 years old. Inoculation with amoebae of increased virulence produced death in all but one old monkey.

Inoculation of various-aged female mice on a weight basis provided results similar to those obtained with male mice. With increased age of mice, there was a noticeable decrease in mortality. The survival time for the youngest female mice was comparable to that of male mice of the same age. Comparison of the cumulative mortality of male and female mice, however, revealed greater resistance in females with increasing age. Chi-square analysis showed statistical significance between all groups but the youngest.

Many studies dealing with experimental N. fowleri infections have not given the age or sex of the mice used. Clearly, both are important when considering host susceptibility to infection. Willaert (19) in reviewing the world literature on primary amoebic meningoencephalitis, reported the sex distribution of cases to be 50 male and 29 female; of the infections in which N. fowleri was identified, 67% occurred in males and 33% in females. These percentages include the four New Zealand fatalities (14) initially attributed to infection with amoebae of myxomycetales (slime molds) and later identified as N. fowleri (11). The apparent selection for males has been attributed to the vigorous swimming, diving, and adventuresomeness of males and, thus, the probability of greater exposure to N. fowleri rather than to the possibility of greater susceptibility. If the ratio of 2:1 of male to female is an accurate reflection of the sex distribution of primary amoebic meningoencephalitis in humans, then perhaps males indeed are more susceptible to infection than are females. Our experimental results from infected mice would support this hypothesis.

The state of sexual maturity of the host appears to influence the course of infection. We have suggested that immune competence may be involved in susceptibility to N. fowleri infection, i.e., as the mice (both male and female) become older and, thus, immunologically competent, they also become increasingly resistant to infection. Human infections have almost always occurred in previously healthy children or adolescents who, although immunologically competent, may not have had sufficient exposure to Naegleria antigens to acquire resistance to N. fowleri infection. John et al. (13) have demonstrated that exposure of mice to N. fowleri and to nonpathogenic N. gruberi afforded protection against lethal challenge with virulent N. fowleri.

Susceptibility may also be affected by the hormones of the host. Sexual maturity in mice normally occurs after 4 weeks of age—precisely when maturity occurs is highly variable and depends upon the interaction of a series of hormonal and neural events (12). Using weight-adjusted inocula, we obtained 100% mortality for 3-week-old mice and observed reduced mortality for mice 4 weeks of age or older. Perhaps specific hormones (or hormone levels) of females contributed to the significantly greater resistance of female mice over males. Again, most of the human infections have occurred in pubescent or pubescent individuals.

The various strains of mice used for virulence studies have included Swiss white (3, 5), specific pathogen free (9), and ICR (14, 17). In the present study, one random-bred and four inbred strains of mice were examined for susceptibility to N. fowleri after i.v. inoculation. All mice were approximately the same weight and were given weight-adjusted doses. Although weights were similar, there may have been age variation among the strains that could affect host resistance as shown earlier; for example, a 20-g ICR mouse is a younger animal than the same-weight BALB/c. Nonetheless, susceptibility varied greatly among the five strains tested. It is instructive to note that the most sensitive mouse strain (A/HeCr) was complement (C'5) deficient, whereas the most resistant strain (C57BL/6) was complement (C'5) competent. Obviously, it is premature to suggest that complement is involved in host resistance. However, Carter (5) demonstrated that an amoebacidal factor was present in fresh human serum and that heat treatment of the serum for 30 min at 56°C inactivated the factor. Clearly, this area warrants further investigation.
In summary, mortality for mice after i.v. inoculation with N. fowleri was dose dependent and decreased with increasing host age, female mice were significantly more resistant to infection than males, and a wide range of susceptibility occurred among various strains of mice. These results show that factors of innate resistance influence the susceptibility of mice to infection and also probably alter the outcome of human exposure to N. fowleri.

ACKNOWLEDGMENT

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LITERATURE CITED

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