

## Age-Related Susceptibility to *Pseudomonas aeruginosa* Ocular Infections in Mice

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The susceptibility of newborn and infant mice to eye infection by *Pseudomonas aeruginosa* was studied in 5-, 10-, 15- to 16-, and 21-day-old mice. In the first of three age-related susceptibility experiments, inoculation of *P. aeruginosa* under the unopened eyelids of infant (5- and 10-day-old) mice in the absence of prior corneal wounding resulted in acute infection and rapid death of many of the animals. However, endophthalmitis was observed in about 30% of bacteremic animals that survived to age 14 to 15 days. In the second experiment, 15- to 16-day-old mice whose eyes were open received *P. aeruginosa* topically onto either wounded or unwounded corneas. At least 50% of the mice that received both corneal wounding and the bacteria exhibited keratitis, endophthalmitis, and subsequent phthisis bulbi. None of the infected mice died of bacteremia. In addition, mice infected in the absence of corneal wounding did not exhibit any eye damage. In the third experiment, the wounded-cornea responses of 21-day-old mice to *P. aeruginosa* were more variable. Thirty seven percent of the mice exhibited an intermediate response of decreased eye size and cataracts which was not observed in 15- to 16-day-old mice, 32% recovered spontaneously, and 29% exhibited complete shrinkage of the infected eyes. The variability of the latter responses may reflect a transitional maturation period of natural immunity to the organism in some of the animals, since all 4- to 6-week-old adult mice respond routinely to ocular wounding and similar infections with the organism by undergoing a spontaneous resolvable keratitis (3 to 4 weeks).

Previously, it has been demonstrated that *Pseudomonas aeruginosa* cannot infect normal eyes of experimental animals unless corneal abrasions precede the bacterial insult (6). Such experiments with *P. aeruginosa* in our laboratory have shown that corneas of adult Swiss-Webster mice (4 to 6 weeks old) respond to intracorneal wounding and topical application of the bacteria with detectable opacity within 18 to 24 h. The keratitis remains localized, does not spread to the uninfected eyes, and resolves spontaneously within several weeks (6). Direct topical application of the organisms onto unwounded corneas of adult mice fails to provoke any of the above responses. Other laboratories (4, 20) have reported similar results for mice.

Although the responses of adult Swiss-Webster mice to *Pseudomonas* corneal infection are well characterized, the immunocompetency of eyes in young animals to this infection is unknown. Therefore, the purpose of this investigation was to characterize the response of infant and young (5-, 10-, 15- to 16-, and 21-day-old) Swiss-Webster mice to experimental *Pseudomonas* ocular infection (with and without ocular

trauma) in an attempt to compare these responses with those previously reported (6) for adult mice.

### MATERIALS AND METHODS

**Bacterial cell cultures.** Stock cultures of *P. aeruginosa* ATCC 19660 stored at 25°C on tryptose agar (Difco, Detroit, Mich.) slants were used for inoculation of 50 ml of broth medium containing 5% peptone (Difco) and 0.25% Trypticase soy broth (Baltimore Biological Laboratories, Cockeysville, Md.). The culture was hemolytic and proteolytic and produced lecithinase and exotoxin A. The culture was grown on a rotary shaker at 37°C for 18 h, centrifuged at 27,000 × g for 20 min (4°C), and suspended in 0.9% sterile nonpyrogenic saline (Travenol Laboratories Inc., Deerfield, Ill.) to the desired concentration of 5.0 × 10<sup>10</sup> colony-forming units (CFU)/ml using a predetermined curve that related viable counts to optical density at 440 nm. To establish the number of viable cells, serial dilutions were plated onto tryptose agar plates and read for growth after 24 h.

**Infection of mice.** Before initiation of the experiments, 15 midterm pregnant female Swiss-Webster mice (22 to 25 g) were obtained from Spartan Research Animals, Inc., Haslett, Mich. A total of 199 infant mice were obtained from the resulting litters. On either day

5 (six litters, 75 mice) or day 10 (six litters, 87 mice) after birth, but before the eyelids were opened, the mice were injected with 2.5  $\mu$ l of various bacterial dilutions (final concentrations,  $2.0 \times 10^5$  to  $2.5 \times 10^5$  CFU) beneath the unopened lids, using a 50- $\mu$ l syringe equipped with a sterile 30-gauge needle. Care was taken not to scratch the corneas or adjacent ocular tissues by gently lifting the eyelids away from the eyes by use of a small forceps before inoculation into the resultant air spaces. Three control litters (41 mice) received sterile saline in a similar volume and manner. The eyelids of mice with unopened eyes were independently observed, using a stereoscopic microscope at  $\times 40$ , by two of the investigators before infection and at daily intervals until the eyes opened. As a control for the inoculation procedure, examination (stereomicroscope at  $\times 40$ ) of an additional litter of eight mice indicated no microscopic or histologically visible damage to the corneal surfaces of surgically opened eyelids 30 min after inoculation. No corneal opacity or other abnormalities (such as needle tracks) were observed that might indicate trauma to the eye surfaces due to the inoculation procedure. The 50% lethal dose determinations of CFU for 5- and 10-day-old mice were calculated by the Reed and Muench method, using a 48-h end point (13).

In the second experiment, litters from female Swiss-Webster mice were obtained as described above. At 15 to 16 days after birth (10 litters, 92 mice), the animals were routinely anesthetized with ether and placed beneath a stereoscopic microscope, and the corneas were either wounded (4, 6) or left unwounded. In the former mice (46 animals), bacterial cell suspensions of various dilutions (final concentrations,  $10^4$  to  $10^8$  CFU) were delivered topically, using a micropipette with a sterile disposable tip, onto the wounded corneas. The wounding was accomplished by making three 1-mm corneal incisions, using a sterile 26-gauge needle, taking care not to penetrate the anterior chambers (6). In the latter mice (46 animals), bacterial suspensions (final concentrations,  $10^4$  to  $10^8$  CFU) were delivered similarly onto unwounded corneas. Control animals (two litters, 25 mice) received sterile saline topically onto either wounded or unwounded corneas as described. Corneas from all mice were examined visually with a  $\times 40$  stereoscopic microscope.

In the third experiment, 21-day-old, weaned mice received  $10^6$  to  $10^8$  CFU topically onto wounded (37 mice) and unwounded (34 mice) corneas (as described for experiment two). Saline controls (15 mice) were treated as described above.

Each of the three individual experiments was repeated three times with similar numbers of litters.

**Blood cultures.** Animals were anesthetized with ether and exsanguinated from the axillary vessels. Blood was cultured in broth (5% peptone, 0.25% Trypticase soy) and subcultured at 24 h on tryptose agar plates at 37°C. The identification of *P. aeruginosa* was confirmed by cultural characteristics and inoculation of Oxi-Ferm tubes.

## RESULTS

**Experiment 1.** In the first experiment, 5- and 10-day-old mice were infected under the eyelids

with varying numbers of *P. aeruginosa*. Care was taken to avoid trauma to the ocular surfaces.

Table 1 shows the response of 5-day-old mice infected with  $2.0 \times 10^5$  to  $2.5 \times 10^5$  CFU. Six litters (75 mice) were infected under the unopened lids. Those receiving  $2.5 \times 10^5$  CFU unexpectedly died of severe bacteremia and were cannibalized by the mother within less than 24 h after infection. The percentages of surviving mice at 24 to 48 h after infection with  $2.5 \times 10^4$  or  $2.5 \times 10^3$  CFU were 13.3 and 76.4%, respectively. Lower numbers of bacteria failed to produce bacteremia or death in any of the mice. Consequently, the 48-h 50% lethal dose was found to be  $6.6 \times 10^3$  CFU. Observations of eyes of surviving animals (at 14 to 15 days, when eyelids opened) showed no visible abnormalities in either controls or lower-dose-infected ( $2.0 \times 10^2$  to  $2.5 \times 10^2$  CFU) mice. On the other hand, 30% (4/13) of the surviving mice infected with at least  $2.5 \times 10^3$  CFU exhibited corneal opacity, with eventual endophthalmitis and eye shrinkage (phthisis bulbi). Pure cultures of *P. aeruginosa* were obtained from the blood of surviving 5-day-old mice at 24 h after infection.

Table 1 also shows the response of 10-day-old (six litters of 87 mice) mice infected with  $2 \times 10^5$  to  $2.5 \times 10^5$  CFU under the unopened eyelids. Those receiving  $2.5 \times 10^3$  to  $2.5 \times 10^5$  CFU of bacteria died of severe pseudomonas bacteremia within 24 to 48 h after infection. The percentage of surviving mice 48 h after infection at  $2.5 \times 10^5$  CFU was 17%, at  $2.5 \times 10^4$  it was 19%, and at  $2.5 \times 10^3$  it was 85%. Lower numbers of the bacteria ( $2.0 \times 10^2$  to  $2.5 \times 10^2$  CFU), on the other hand, failed to produce bacteremia or death of any of the mice. Calculation of the data as listed in Table 1 yielded a 48-h 50% lethal dose of  $1.1 \times 10^4$  CFU. Visual observation of eyes in surviving mice at the time the lids opened showed no abnormalities in control or lower-dose animals. Most of the surviving mice (2/3 at  $2.5 \times 10^5$ ; 2/3 at  $2.5 \times 10^4$  CFU) infected with greater numbers of the organism, however, showed corneal opacity, endophthalmitis, and, eventually, shrunken

TABLE 1. Survival period of 5- and 10-day-old mice infected under the eyelid

No. of bacteria (CFU) used in inoculum	No. surviving/no. infected for periods (day[s]) indicated					
	5-Day-old mice			10-Day-old mice		
	1	2	3-21	1	2	3-21
$2.5 \times 10^5$	0/17	0/17	0/17	3/17	3/17	3/17
$2.5 \times 10^4$	2/15	2/15	0/15	4/16	3/16	3/16
$2.5 \times 10^3$	13/17	13/17	13/17	13/13	11/13	11/13
$2.5 \times 10^2$	12/12	12/12	12/12	15/15	15/15	15/15
$2.5 \times 10^1$	14/14	14/14	14/14	16/16	16/16	16/16
$2.5 \times 10^0$	13/13	13/13	13/13	10/10	10/10	10/10

eyes (phthisis bulbi). At 24 h after infection, blood cultures obtained from surviving 10-day-old animals receiving greater numbers of the bacteria yielded pure cultures of *P. aeruginosa*.

**Experiment 2.** In the second experiment, 15- to 16-day-old animals whose eyes had opened were infected by topical application of the bacteria, at various dilutions, onto wounded and unwounded corneas. It is apparent from the results (Table 2) that the response to the organisms was dose dependent and that the ocular response was most severe in animals receiving greater numbers ( $10^7$  to  $10^8$  CFU) of bacteria. None of the infected mice died of pseudomonas bacteremia. In addition, only those mice receiving corneal wounding before infection exhibited keratitis and eye shrinkage. No cataract formation or microphthalmia was detectable in these animals, indicating a more severe response to infection than noted in 21-day animals as described below.

**Experiment 3.** In experiment three, 21-day-old mice were infected by topical application of pseudomonas ( $10^6$  to  $10^8$  CFU) onto wounded and unwounded corneas. The host response (Table 3) to the organisms on day 21 after birth was extremely variable in mice with experimentally wounded corneas. Twenty-nine percent of the mice suffered complete loss of infected eyes, 37% immediately developed microphthalmia and cataracts, and 32% recovered spontaneously. Corneas of the latter experimental group, as well as control mice with wounded corneas which had received sterile saline topically, were clear upon routine stereoscopic microscope examination. As expected, no keratitis was observed in animals with unwounded corneas which received topical application of similar numbers of the bacteria. No bacteremia or death as observed in 5- and 10-day-old mice occurred in any of the 21-day-

TABLE 2. Infection in wounded and unwounded eyes of 15- to 16-day-old mice

No. of bacteria (CFU) used in inoculum	Infection in:			
	Wounded corneas <sup>a</sup>			Unwounded corneas (clear; 1-21 days)
	Microphthalmia and cataract (21 days)	Phthisis bulbi (10-12 days)	Clear (21 days)	
$10^8$	0/10	10/10	0/10	10/10
$10^7$	0/10	5/10	5/10	10/10
$10^6$	0/8	1/8	7/8	8/8
$10^5$	0/10	1/10	9/10	10/10
$10^4$	0/8	0/8	8/8	8/8

<sup>a</sup> Number of mice exhibiting microphthalmia and cataract, phthisis bulbi, or clear corneas/total infected at each dose.

TABLE 3. Eye infection in wounded and unwounded corneas of 21-day-old mice

No. of bacteria (CFU) used in inoculum	Infection in:			
	Wounded corneas <sup>a</sup>			Unwounded corneas (clear; 1-21 days)
	Microphthalmia and cataract (21 days)	Phthisis bulbi (10-21 days)	Clear (21 days)	
$10^8$	5/13	4/13	4/13	13/13
$10^7$	5/12	3/12	4/12	11/11
$10^6$	4/12	4/12	4/12	10/10

<sup>a</sup> Expressed as those exhibiting microphthalmia and cataract, phthisis bulbi, or clear corneas/total infected at each dilution.

old animals. Phthisis bulbi production in 15- to 16- and 21-day-old mice was examined for independence of response at  $10^6$  to  $10^8$  CFU by the chi-square test (Tables 2 and 3). Only the values for  $10^8$  CFU were statistically significant, with a  $P < 0.005$ . At  $10^6$  and  $10^7$  CFU, the differences were not significant even at  $P < 0.05$ .

## DISCUSSION

In recent years (18, 19) it has been shown that attainment of immunological competency in some species may occur well before birth, whereas in others, such as laboratory rodents, this condition is achieved only after birth (16). It is significant that the immunological apparatus does not seem to mature as a single event that endows the young animals with an active defense against all types of antigens. Thus, an antigenic stimulus such as an infectious agent, which may be relatively innocuous to adults, could prove devastating to infant or immature animals (1, 14).

Although mice are considered to lack systemic immunocompetency only on the first postnatal day (16), the present study has shown that the ability to resist *P. aeruginosa* infection in young animals is not effectively developed in eyes until as late as 21 days after birth.

The susceptibility of 5- and 10-day-old mice to injection of *P. aeruginosa* under the unopened eyelids, in the absence of corneal trauma, was unexpected and resulted in severe bacteremia and death within 24 to 48 h in a high percentage of the animals. With increased numbers of the organism, surviving mice (both 5 and 10 days old) exhibited corneal infection with subsequent endophthalmitis and phthisis bulbi (14 to 15 days). The true incidence of ocular disease may have been even higher in either or both groups, since some of the animals that died before eye opening could have had endophthalmitis.

The mechanism by which pseudomonas induces these ocular infections in the absence of corneal trauma is not clear. However, the infection does not appear to spread by metastatic seeding of the eyes by the bacteria, since the keratitis always is unilateral, being confined only to the inoculated eyes. On the other hand, bilateral infections have been reported in experiments with infant rats, using *Haemophilus influenzae* administered by intraperitoneal or intranasal routes (12). Thus, as alternate considerations, the surface of the eyes of young mice may be more permeable to the organism, or perhaps local levels of lysozyme or other tissue factors are insufficient (or absent) to protect against bacterial invasion. These and other considerations such as neutrophil function or vascular permeability should be examined in future studies. Also, it is interesting that responses (bacteremia and death) similar to those observed in 5- and 10-day-old mice have recently been reported for experimentally immunosuppressed (cyclophosphamide-treated) adult Swiss-Webster mice (7). However, infant animals (5 and 10 days old), as compared with immunosuppressed adults, appear highly susceptible to very low doses of pseudomonas. Similar numbers of the organism are incapable of producing bacteremia, death, or eye infection in normal adult mice, even when corneal trauma precedes infection.

Mice infected by topical application of the organisms onto wounded corneas only, on day 15 to 16 after birth, were incapable of clearing the bacteria. Eyes were shrunken (phthisis bulbi) 10 to 12 days after infection, particularly with a greater number of the bacteria ( $10^7$  to  $10^8$  CFU). The response of 21-day-old mice, on the other hand, was variable, with most animals (38%) exhibiting microphthalmia and cataracts, 30% undergoing phthisis bulbi, and 32% resolving the infection, presenting clear corneas within 3 to 4 weeks after infection. This broad range of responses, as well as the absence of microphthalmia and cataracts in 15- to 16-day-old animals, suggests a period of transition from total susceptibility to the organism, as observed in younger mice, to total resistance (with recovery of a clear cornea) to pseudomonas, as reported for adult animals (4, 6, 20). Adult (30- to 42-postnatal-day Swiss-Webster) mice recover spontaneously from corneal wounding and topical administration of the organism and never exhibit systemic infection or death. Many experimental studies of bacterial-induced endophthalmitis have utilized intraocular trauma and application of bacteria (4-7, 10, 11, 17, 20). In contrast, other experiments have demonstrated that intravascular (15) and intraperitoneal or

intranasal (11) routes of bacterial administration can cause ocular infection. Indeed, exposure to almost any bacterium able to cause severe bacteremia can result in ocular infection (2, 3, 8, 9). To our knowledge, however, this is the first report of ocular infection induced by injecting bacteria under the unopened eyelid in the absence of trauma to the eye surface.

In conclusion, our study has shown that the ocular response of infant Swiss-Webster mice to pseudomonas infection is strikingly different from the adult response, resulting in bacteremia, death, and ocular infection in some survivors even in the absence of corneal trauma preceding bacterial infection. Further studies to clarify the sequential histopathology of the ocular infection are underway in our laboratory.

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