Serum Antibody and Ocular Responses to Murine Corneal Infection Caused by *Pseudomonas aeruginosa*

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The serum antibody response and differential corneal response to primary and secondary infections by *Pseudomonas aeruginosa* were investigated in DBA/2J (resistant) and C57BL/6J (susceptible) mice, since they respond differently to intracorneal challenge. Using an enzyme-linked immunosorbent assay, we found that naturally resistant DBA/2J mice mounted a significant immunoglobulin M (IgM) and IgG response to *P. aeruginosa* within 7 days postinfection of one eye; this was subsequently followed by a drop in the IgM response. Of 31 mice, 30 were able to restore corneal clarity within 3 to 4 weeks. However, when C57BL/6J mice were infected intracorneally, their levels of serum antibody developed more slowly than did those of the DBA/2J mice, and they were unable to restore corneal clarity within 8 to 12 weeks. None of the mice from either test strain mounted a detectable serum IgA response to *P. aeruginosa* over a 90-day holding period. However, infection of the contralateral, normal cornea of mice of both test strains resulted in a heightened IgG response to *P. aeruginosa* within 30 days after the secondary infection. Many (50%) of the susceptible C57BL/6J mice recovered or exhibited less severe corneal damage within the 30-day holding period. If the C57BL/6J mice were reinfected 60 days after the primary infection instead of after 30 days, most (89%) of the mice had restored corneal clarity within 3 to 6 days. Passive transfer of immune serum from either recovered DBA/2J or C57BL/6J mice to naive C57BL/6J mice resulted in the restoration of corneal clarity in many of the recipients following infection.

Pseudomonas aeruginosa is an opportunistic pathogen which causes severe corneal infection in humans. The infection rapidly spreads and often results in blindness. Many of the clinical features can be reproduced in rabbits, guinea pigs, and mice (8, 14, 25, 26). Previous studies from our laboratory indicated that some inbred mouse strains, such as DBA/1J and DBA/2J, can spontaneously restore corneal clarity within a few weeks after infection and are therefore classified as naturally resistant (3–6, 8). On the other hand, mice of the BALB/cJ, C3H/HeJ, A/J, and C57BL/6J strains are classified as susceptible, since corneal infection may lead to perforation, phthisis bulbi, or both (3–6, 8). The factor(s) that regulates the corneal response to infection has not been completely identified. However, it has been demonstrated that infant DBA/2J mice are initially susceptible and progressively develop resistance to corneal infection and to exotoxin A as they enter young adulthood (7, 13). Other studies indicated that resistance is dependent on polymorphonuclear leukocytes (12, 25) and that temporary depletion of C3 complement component levels in DBA/2J mice result in a transitory loss of resistance until C3 levels return to normal. C5 complement seems to play a less important role in the natural resistance to intracorneal infection by DBA/2J mice (9).

Recent studies suggest that genetic factors of the host regulate the corneal response of DBA/2J mice to infection and that one to two resistance genes may be involved. In addition, the susceptible C57BL/6J and BALB/cJ mice also carry one or more complementary resistance genes, since all of their F₁ progeny are resistant (4). However, C57BL/6J mice also carry dominant susceptibility genes which mask their resistance gene(s) (3). Studies with congenic BALB/c

mice carrying genes from DBA/2J mice and isoenzyme studies indicate that the corneal response may be dependent on the *Igh-1* locus on chromosome 12 and possibly a gene, at or near the *Ric* locus, on chromosome 5 (5, 6) which is thought to be associated with macrophage function (a macrophage gene). The gene product of the *Ric* locus and the mechanism of action remain unknown (11). Since *P. aeruginosa* is an extracellular pathogen, one would expect that the dominant immune response to intracorneal infection would be a humoral one. We propose, therefore, that the corneal response of resistant mouse strains is based on their ability to mount a rapid and protective humoral response to infection prior to permanent corneal damage, while susceptible mouse strains are initially unresponsive or hyporesponsive to infection. Therefore, the purpose of this study was to compare the serum humoral response of DBA/2J and C57BL/6J mice over a 30-day holding period. In addition, we wanted to determine whether mice of a susceptible strain can develop protective immunity as the result of a primary or secondary corneal infection as well as by the passive transfer of serum from animals that have recovered from corneal infections.

MATERIALS AND METHODS

Bacterial cell cultures. Stock cultures of *P. aeruginosa* ATCC 19660 were stored at 25°C on tryptose agar slants (Difco Laboratories, Detroit, Mich.) and were used for the inoculation of 50 ml of broth medium containing 5% peptone (Difco) and 0.25% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). The culture was hemolytic and proteolytic and produced lecithinase and exotoxin A. Cultures were grown on a rotary shaker at 37°C for 18 h, centrifuged at 7,000 × g for 20 min (4°C), washed with saline, and suspended in 0.9% sterile nonpyrogenic saline (Travenol

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Laboratories, Deerfield, Ill.) to a concentration of 2.0×10^{10} CFU/ml by using a standard curve relating viable counts to the optical density at 440 nm.

Infection of animals. Two inbred strains of mice, DBA/2J and C57BL/6J (Jackson Laboratory, Bar Harbor, Maine) (weight, 18 to 22 g), were infected at 5 to 6 weeks of age. Prior to infection, they were lightly anesthetized with ether and placed beneath a stereoscopic microscope. The corneal surface of only the left eye was incised (three 1-mm-long incisions) with a sterile 26-gauge needle, taking care not to penetrate the anterior chamber or to damage the sclera. Accidental infection of the anterior chamber of the resistant DBA/2J mice resulted in an inability to restore corneal clarity. A bacterial cell suspension (5 μ l) containing a final concentration of 10^8 CFU was topically delivered onto the surface of incised corneas by using a micropipette (Oxford Laboratories, Foster City, Calif.) with a sterile disposable tip. Control animals received a similar wound and 5 μ l of sterile saline. All experimental data represent the results of three to four independently performed experiments. The infected eyes were examined 24 h after bacterial challenge and then at 3-day intervals over a 3- to 4-week time period and compared with the uninfected, contralateral control eye as well as the eyes of control mice treated with saline. At 30 or 60 days after the primary corneal infection, the contralateral eye was reinfected and the corneal response was again evaluated every 3 days for a 30-day holding period. The ocular response of infected animals was graded macroscopically on a scale of 0 to 4 on the following basis: grade 0 (resistant); the cornea was clear and macroscopically similar to the contralateral control eye; grade 1 (resistant), the cornea had slight or faint opacity partially covering the pupil; grades 2 and 3 (susceptible), dense corneal opacity covering the pupil and entire anterior segment, respectively; grade 4 (susceptible), corneal perforation, phthisis bulbi (shrinkage following inflammation), or both. Animals were handled in accordance with the resolution on the use of animals in research established by the Association for Research in Vision and Ophthalmology.

Determination of antibody directed against *P. aeruginosa*. Twenty mice of each strain were bled from the retroorbital plexus prior to corneal infection and thereafter were bled weekly for 4 weeks from the contralateral, uninfected control eye. After infection of the contralateral eye, mice were bled from the tail vein on a weekly basis for an additional 4 weeks. Sera from each weekly bleeding of each set of 20 mice were pooled and frozen until the end of the experiment. Specific antibody directed against *P. aeruginosa* in each pool of mouse serum was then determined by enzyme-linked immunosorbent assay. A polystyrene microtitration plate (Nunc, Thousand Oaks, Calif.) was coated with heat-killed organisms and incubated overnight at 37°C. For coating, a suspension of 10^8 washed, heat-killed organisms per ml was diluted 1:20 in 0.05 M carbonate coating buffer (pH 9.6) with 0.2% (wt/vol) sodium azide. The pooled mouse serum was diluted for testing in phosphate-buffered saline–0.05% Tween 20. The plates were then washed 3 times with phosphate-buffered saline–0.05% Tween 20 prior to the addition of serum. The diluted serum was added in triplicate to the inner wells and incubated at 37°C for 90 min. After incubation, the plates were washed 3 times with phosphate-buffered saline–0.05% Tween 20, and a 1:1,000 dilution of either goat anti-mouse immunoglobulin G (IgG; gamma chain specific) alkaline phosphatase (Cappel Laboratories, Malvern, Pa.), goat anti-mouse IgM (mu chain specific) alkaline phosphatase (Sigma Chemical, St. Louis, Mo.), or

goat anti-mouse IgA (alpha chain specific) alkaline phosphatase (Sigma) conjugate was added. The plates were incubated again for 90 min at 37°C and washed, and the enzyme substrate was added. The enzyme substrate consisted of 10% diethanolamine solution (pH 9.7) with 0.01% (wt/vol) MgCl_2 and 0.02% (wt/vol) NaN_3 with 1 mg of *p*-nitrophenolphosphate per ml (27). The enzyme substrate reaction was allowed to proceed at room temperature until the optical density reached at least 0.9 in the positive control well. Enzyme activity was inhibited by the addition of 50 μ l of 3 M NaOH to each well. Substrate conversion was measured spectrophotometrically at 405 nm by using a plate reader (model EL307; Biotek, Burlington, Vt.) against the reference blank (substrate-NaOH in the outer wells).

On each plate the same negative and positive reference controls were used (day 0 serum from DBA/2J mice and week 8 [4 weeks after reinfection] serum from DBA/2J mice) to monitor the plates. When the titer was determined, wells were considered positive when their optical density was at least double that of the negative control well. Additional sera from five different pools of uninfected mice were also tested, and the titers of the serum obtained from each strain on day 0 fell within the optical density range of those of the other uninfected serum pools. Titers of sera from mice that were hyperimmunized with an acetylcholine receptor preparation were also tested in the assay and fell within the optical density range of those of the other uninfected serum pools. Titers of sera were determined by starting with a 1:100 dilution. If the serum was negative at that dilution, it was retested starting at a dilution of 1:10.

Passive transfer of sera. Serum from recovered DBA/2J or C57BL/6J mice was collected and pooled and complement inactivated (56°C, 30 min), and 0.2 ml was administered via the dorsolateral tail vein to naive C57BL/6J mice 1 h prior to corneal infection with 5×10^8 CFU. Before some passive transfers of immune serum to naive C57BL/6J recipients, immune serum was adsorbed twice with 10^{10} *P. aeruginosa* organisms.

Statistical analysis. Comparison of the incidence of a grade 0 or 1 response (corneal recovery) versus all other ocular responses (grades 2 to 4) in C57BL/6J mice was performed by the chi-square test (contingency analysis) with no Yates continuity correction for the data expressed in Tables 3 and 4. Yates continuity correction was used for the data expressed in Table 5. *P* values were not calculated for infected DBA/2J mice, since they were expected to recover from intracorneal challenge (3–6, 8).

RESULTS

Initial experiments were designed to compare the humoral response specific to *P. aeruginosa* during corneal infection of resistant (DBA/2J) and susceptible (C57BL/6J) mice. Weekly serum samples from 20 mice of each strain were assayed by enzyme-linked immunosorbent assay for IgM, IgG, and IgA. The results are given in Table 1 and indicate that the DBA/2J mice were able to mount both an IgM (titer, 1:800) and IgG (titer, 1:400) response within the first 7 days postinfection. Thereafter, the IgM response dropped, while the IgG response remained constant. At the end of 4 weeks postinfection, the recovered mice were reinfected in the contralateral eye and were held for an additional 4-week holding period. At the end of that time, titers of 1:400 and 1:6,400 were obtained for IgM and IgG, respectively. No IgA titers in serum were detected over a 90-day holding period during the primary and secondary corneal infections.

TABLE 1. Specific antibody response to *P. aeruginosa* after primary and secondary corneal infections in resistant (DBA/2J) and susceptible (C57BL/6J) mice

Time	Titers in the following mice ^a :					
	DBA/2J			C57BL/6J		
	IgM	IgG	IgA	IgM	IgG	IgA
Day 0	— ^b	—	—	—	—	—
Week 1	1:800	1:400	—	1:80	1:20	—
Week 2	1:200	1:400	—	1:10	1:40	—
Week 4	1:200	1:400	—	1:10	1:200	—
Week 1 reinfection	1:200	1:400	—	1:80	1:320	—
Week 4 reinfection	1:400	1:6,400	—	1:200	1:6,400	—

^a Antibody titers were determined by enzyme-linked immunosorbent assay on pooled sera from 20 mice of each strain, which were repeatedly sampled at the indicated times.

^b —, No measurable titer at a serum dilution of 1:10.

Concomitantly with the antibody studies, we monitored the ocular response of DBA/2J mice on a weekly basis and found that 30 of 31 mice were able to restore corneal clarity within 3 to 4 weeks (Table 2). Restoration of corneal clarity became macroscopically observable within 7 to 10 days postinfection. When the recovered mice receiving a primary infection were reinfected in the contralateral control eye 60 days later, corneal recovery again occurred as expected; however, the rate of healing was accelerated (Table 2). Initially, 30 of 31 mice exhibited corneal response grades of 2 or 3 within the first 24 h after infection. However, 9 of 31 and 16 of 31 of the mice had restored corneal clarity as exhibited by respective corneal response grades of 0 and 1 at the end of 3 days. At the end of 6 days, 29 mice had restored corneal clarity and 1 of the remaining 2 mice recovered within 14 days.

For comparative purposes, the studies described above were repeated with susceptible C57BL/6J mice. Antibody studies indicated that the humoral response in these mice was of a lower order of magnitude than that obtained with the resistant DBA/2J mice and suggested that the C57BL/6J mice were hyporesponsive. The IgM and IgG titers at the end of week 1 were 1:80 and 1:20, respectively, while titers at the end of the 4-week holding period were 1:10 and 1:200, respectively. However, 4 weeks following reinfection of the contralateral eye, the IgM (1:200) and IgG (1:6,400) titers were similar to titers from the DBA/2J mice that received the same treatment (Table 1).

TABLE 2. Rate of recovery of DBA/2J mice intracorneally infected with *P. aeruginosa*

Time after infection	No. of mice with the following ocular response grades ^a :				
	0	1	2	3	4
Primary infection					
Week 1	0	0	12	18	1
Week 2	0	17	11	2	1
Week 3	18	12	0	0	1
Week 4	24	6	0	0	1
Secondary infection ^b					
Day 3	9	16	5	0	1
Day 6	22	7	1	0	1

^a A total of 31 mice were initially infected.

^b Mice were reinfected 30 days after the primary infection.

TABLE 3. Corneal response of C57BL/6J mice to a primary infection and a secondary infection (30 days later)

Time after infection	No. of mice with the following ocular response grades ^a :				
	0	1	2	3	4
Primary infection					
Week 1	0	0	0	5	18
Week 4	0	0	4	2	17
Secondary infection (30 days later)					
Week 1	0	0	0	15	8
Week 4	5	1	7	2	8

^a A total of 23 mice were initially infected. The *P* value for grades 0 and 1 combined was 0.05. The 95% significance level was *P* = 0.05.

Examination of the eyes of C57BL/6J mice that received the primary corneal infection indicated that 23 of 23 mice were unable to have corneal clarity restored after 4 weeks, and 17 of these exhibited corneal response grades of 4, thereby signifying corneal perforation, phthisis bulbi, or both (Table 3). In addition, the severity of the infection during the first few days after primary infection was macroscopically indistinguishable from that of DBA/2J mice. However, when the mice were administered a secondary corneal infection by using the contralateral control eye, 6 of 23 mice had restored corneal clarity of only the contralateral eye within the next 30-day holding period (Table 3). An additional seven mice showed some slight improvement over the response noted with a primary infection. However, in a subsequent experiment, when 28 mice were reinfected in the contralateral eye 60 days rather than 30 days after the primary infection, the response (Table 4) was similar to that observed with the DBA/2J mice that were reinfected 60 days after they were given the primary infection (Table 2). Thus, 8 of 28 mice restored complete corneal clarity (corneal grade 0) within 3 days, while 17 of 28 exhibited a faint corneal opacity (corneal grade 1). By the end of 6 days, 18 and 7 of 28 mice exhibited grades of 0 and 1, respectively. Only three mice were unable to restore corneal clarity within this time period, but these mice recovered their corneal clarity within the next 10 days. Interestingly, none of the C57BL/6J mice that received a primary infection were able to restore corneal clarity in the primary infected eye even after they received a secondary corneal infection. The damage in these corneas was permanent and was unlike the slow and complete recovery previously observed in BALB.B and BALB.K mice at 6 weeks after the primary infection, but not at 4 weeks postinfection (4).

In order to establish that protective antibody was responsible for the recovery of DBA/2J mice after a primary corneal infection and of C57BL/6J mice that received a

TABLE 4. Recovery in C57BL/6J mice that were given a secondary corneal infection 60 days after they were given the primary infection

Time (day) after secondary infection	No. of mice with the following ocular response grades ^a :				
	0	1	2	3	4
3	8	17	2	1	0
6	18	7	2	1	0

^a A total of 28 mice were initially infected. The *P* value for grades 0 and 1 combined was <0.0001. The 95% significance level was *P* = 0.05.

TABLE 5. Effect of passive transfer of sera to naive C57BL/6J mice^a

Serum from the following mice:	No. of mice with the following ocular response/total no. tested:				
	0	1	2	3	4
Normal C57BL/6J					12/12
Immune C57BL/6J	6/11			1/11	4/11
Immune DBA/2J	1/10	4/10	1/10		4/10
Immune DBA/2J or C57BL/6J adsorbed					12/12

^a In each case, 0.2 ml of complement-inactivated serum (56°C, 30 min) from recovered mice was administered via the dorsolateral tail vein 1 h prior to intracorneal infection with 5×10^8 CFU of *P. aeruginosa*. The serum was adsorbed twice with 10^{10} *P. aeruginosa* organisms prior to transfer to naive recipients. The results were recorded 2 weeks postinfection. The *P* value for grades 0 and 1 combined was 0.01. The 95% significance level was *P* = 0.05.

secondary infection, 0.2 ml of immune serum (titer, 1:6,400) obtained from either donor strain after the restoration of corneal clarity was transferred intravenously to naive C57BL/6J mice 1 h prior to corneal challenge. In order to test the efficacy of the immune serum, a higher than usual inoculum (5×10^8 CFU) was used for infection, and final evaluation of the results was made at the end of 2 weeks rather than the usual 4 weeks. Of 11 of the C57BL/6J mice receiving immune C57BL/6J serum, 6 either recovered completely or showed a moderation in the severity of the ocular damage (Table 5). Similar results were obtained with passively transferred immune serum from the DBA/2J mice to naive C57BL/6J recipients, while 12 of 12 control animals that received 0.2 ml of normal serum or antibody-adsorbed immune serum exhibited corneal perforation (corneal grade 4).

DISCUSSION

Although *P. aeruginosa* is an important cause of eye infections, little is known about ocular immunity to this pathogen. The normal cornea is unique in that it contains no blood vessels or lymphatic drainage prior to infection, but it develops blood vessels after infection with *P. aeruginosa* (24). However, the immune system most likely plays a role in normal corneal defenses, since IgG, IgM, IgA, and complement components are normally present prior to infection (1, 2, 17, 19). In addition, inflammatory cells actively invade the cornea when it is infected with *P. aeruginosa* (13, 25). Previous studies from our laboratories indicated that the ability of DBA/2J mice to restore corneal clarity is dependent on C3 levels (9). Components of both the classical and alternate complement pathways are present in infected corneas (1, 9, 17, 19) and potentiate phagocytosis of *P. aeruginosa* (1, 28). Other studies indicated that active immunization with lipopolysaccharide enhances the clearance of *P. aeruginosa* from infected corneal tissues, whereas anti-elastase antibody reduces tissue damage because of the elastase, without the elimination of the bacteria (14). Findings by other investigators also lend credence to the importance of humoral immunity in both corneal and noncorneal infections caused by *P. aeruginosa* (10, 14, 16, 21, 22, 28, 29; M. M. Moon, L. D. Hazlett, R. E. W. Hancock, R. S. Berk, and R. Barrett, Invest. Ophthalmol. Vis. Sci., in press). However, recent nonocular studies indicate that cellular immunity may also play a role in infections, depending on the nature and dosage of the immunogen (15, 23). Thus, exposure of mice to low numbers of live *P. aeruginosa* or

polysaccharide generates protective cell-mediated immunity in the absence of an antibody response (15, 23).

Previous genetic studies indicated that DBA/2J mice were able to restore corneal clarity within 3 to 4 weeks postinfection, while most other inbred strains that we examined were classed as susceptible (3–6, 8). Since *P. aeruginosa* is an extracellular agent, we expected that immunity would be due to protective antibody. Based on this premise, we had two objectives in the present study. Our first objective was to determine whether there was any correlation between the ability or inability to restore corneal clarity with the ability to mount a humoral response. Our working hypothesis was based on the fact that DBA/2J mice should mount a rapid and significant humoral response to intracorneal infection, which would permit the animals to recover from infection with concomitant corneal repair. Presumably, the presence of protective antibody acting as opsonins promote efficient opsonophagocytosis and killing of *P. aeruginosa* (28) and controls the outcome of infection. Our findings supported this concept since significant IgM and IgG titers in serum were detected by 7 days postinfection. Conversely, we expected that the C57BL/6J mice would be unable to restore corneal clarity if the mice were humorally hyporesponsive or nonresponsive compared with the DBA/2J mice; our antibody data support this hypothesis. Thus, corneal damage due to the infectious process in C57BL/6J mice appears to exceed the ability of the animals to mount a protective humoral response during the primary infection, thereby leading to permanent ocular damage. The interval between the time of infection and week 4 postinfection appears to be critical in the relationship between host and parasite, because the mice either clear the infection and restore corneal clarity or suffer permanent ocular damage.

It is interesting that neither mouse strain mounted a measurable serum IgA response over a 60-day holding period. These results are consistent with the observations of Mondino et al. (18), who studied the serum antibody response to *Staphylococcus aureus* corneal infection as well as to ocular immunization with ribitol teichoic acid. In their experimental model, they also found no serum IgA, but did find it in rabbit tears. We were unsuccessful in aspirating tear film from our mice for determining immunoglobulin class and titers. These types of studies are hampered by the small size of the test animal plus the drying of the corneal surface because of the infection. In future studies we will use immunocytochemical techniques to demonstrate corneal antibodies.

The second goal of this study was to determine whether a primary corneal infection in one eye would lead to the development of protective immunity in the contralateral eye of susceptible C57BL/6J mice that were reinfected either 30 or 60 days after the primary infection. The results indicated that many of the C57BL/6J mice are able to restore corneal clarity or moderate the severity of corneal damage when they are reinfected 30 days after the primary infection. However, these results also showed that many of the animals do not develop a mature protective immunity until 60 days after the primary infection, when the collective IgG titer in serum was 1:6,400. After secondary infection at that time, all of the mice not only recovered but many exhibited an accelerated recovery within 3 to 6 days. The same response was noted with the DBA/2J mice, which were reinfected after 60 days. Thus, our contention is that the ability to develop protective immunity in susceptible C57BL/6J mice is causally related to the ability to mount a protective

humoral response, as determined by immunoglobulin titers in serum.

To give further support to our protective humoral hypothesis, we passively transferred the immune sera from recovered C57BL/6J or DBA/2J mice to naive C57BL/6J recipient mice and demonstrated that the mice were able to restore corneal clarity or show less severe corneal damage compared with control animals, despite a larger than usual inoculum and a shorter holding period. Similar findings were observed in our laboratories when monoclonal antibodies to outer membrane proteins were passively transferred to naive C57BL/6J mice (Moon et al., in press).

The results presented here are consistent with our genetic findings, in that they suggest that genes coding for heavy-chain immunoglobulin (*Igh-1*) and possibly macrophage function (5, 6) may play a regulatory role in antibody formation. The innate susceptibility of C57BL/6J mice is most likely a consequence of a genetically conferred defect in delayed and/or diminished antibody response, as observed in a nonocular system (20). Presumably, the presence of one or more dominant susceptibility gene(s) in C57BL/6J mice acts to suppress antigen processing or presentation so that protective humoral immunity is delayed or hyporesponsive after initial corneal challenge. This humoral hyporesponsiveness to *P. aeruginosa* polysaccharide has been attributed to T suppressor cells (15, 23). Future studies will investigate the potential role of these regulatory cells in our ocular model.

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