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Serum Antibody Response to *Pseudomonas aeruginosa* Antigens during Corneal Infection

MICHAEL J. PRESTON,[1] JULIANNA M. BERK,[2] LINDA D. HAZLETT,[2] AND RICHARD S. BERK[1]*

Departments of Immunology/Microbiology[1] and Anatomy/Cell Biology,[2] Wayne State University School of Medicine, Detroit, Michigan 48201

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Previous studies in our laboratory have indicated that naturally resistant, inbred DBA/2J mice mount a greater serum antibody response to *Pseudomonas aeruginosa* 19660 than susceptible C57BL/6J mice. However, the specificity of the antibody produced was not known. The present study examines the specificity and kinetics of the humoral response of these mouse strains to potential virulence factors produced by the organism during both a primary and a secondary corneal infection administered 4 weeks after the primary infection. Serum antibody levels specific for lipopolysaccharide (LPS), exotoxin A, phospholipase C (PLC), alkaline protease, elastase, and flagella were measured by enzyme-linked immunosorbent assay. Little or no antibody to either alkaline protease or elastase was detected during either primary or secondary infection. Immunoglobulin G (IgG) antibodies specific to exotoxin A, PLC, and flagella were detected 2 weeks after primary infection, and a rapid response to these antigens was measured 1 week after secondary infection. During primary infection, detectable LPS-specific antibody was only IgM, while IgG appeared only after secondary infection. The kinetics of the humoral response in susceptible C57BL/6J mice were similar to those in resistant DBA/2J mice, although the magnitude of the response varied according to the antigen tested. These results indicate that LPS, exotoxin A, PLC, and flagella are present or produced in amounts that are immunogenic during corneal infection by *P. aeruginosa* 19660 in the mouse strains tested.

Pseudomonas aeruginosa is an opportunistic pathogen which causes severe corneal infections in humans and experimental animals (6, 8, 14, 37, 38). The infection is usually preceded by corneal trauma. Thereafter, the infection rapidly spreads and may result in permanent ocular damage. Previous experimental studies from our laboratory have indicated that DBA/2J mice can spontaneously restore corneal clarity within a few weeks after infection and are therefore classified as naturally resistant (4, 18). On the other hand, C57BL/6J mice initially exhibit the same severity of ocular infection at 24 to 72 h postinfection but are unable to restore corneal clarity within a period of 4 weeks and are classified as susceptible (4, 18). Consequently, corneal infection in these mice usually leads to corneal perforation, phthisis bulbi (shrinkage), or both. However, when the mice were reinfected in the contralateral control eye 2 months after the primary corneal infection, most of the mice restored corneal clarity within 3 to 6 days after the secondary infection (7). Recent studies suggest that genetic factors of the host regulate the corneal response of the two mouse strains and that one to two resistance and susceptibility genes may be involved (2, 5). Extending these studies further, we have recently described a correlation between the dissimilar corneal response of the two mouse strains with the ability to mount a specific, rapid, and protective humoral response to *P. aeruginosa* prior to the development of permanent corneal damage (7). Thus, the susceptible C57BL/6J mice were initially unresponsive or hyporesponsive to infection, while the resistant DBA/2J mice mounted a rapid and heightened antibody response specific to *P. aeruginosa*, thereby preventing permanent corneal damage. The mechanism by which corneal damage occurs in vivo has not been definitely established. However, studies with purified exoenzymes, cellular factors, and infection with various mutants have implicated endotoxin, proteases, and exotoxin A as potential sources of gross ocular damage as seen in pseudomonas keratitis (17, 21, 25, 29, 30, 33). Host factors have also been implicated in causing ocular damage during infection (19, 27, 28, 35, 37). Therefore, the purpose of the present study was to determine which cellular and exocellular products of *P. aeruginosa* stimulate a specific antibody response during both a primary and secondary corneal infection of resistant DBA/2J mice. Also, we determined whether susceptible C57BL/6J mice are hyporesponsive to specific bacterial antigens other than the whole organism during corneal infection by *P. aeruginosa*.

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. *P. aeruginosa* ATCC 19660 possesses b-type flagella (32) and shares lipopolysaccharide (LPS) O-type antigens with immunotype 7 LPS (33a). Cultures were grown on a rotary shaker at 37°C for 18 h, centrifuged at 7,000 × *g* for 20 min (4°C), washed three times with saline, and suspended in 0.9% sterile, nonpyrogenic saline (Travenol Laboratories, Deerfield, Ill.) to a concentration of 2×10^{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 6 weeks of age. Prior to infection, they were lightly anesthetized with ether and

* Corresponding author.

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. A bacterial suspension (5 μl) containing 10^8 CFU was topically delivered onto the surface of the incised corneas with a micropipette (Oxford Labs) with a sterile, disposable tip. The mice were then examined 24 h later to determine that they were all infected. At 4 weeks after the primary corneal infection, the uninfected contralateral control eye was used to establish a secondary infection.

Antigens. Purified exotoxin A was purchased from the Swiss Serum and Vaccine Institute, Berne, Switzerland. Purified *P. aeruginosa* elastase and alkaline protease were obtained from K. Morihara, Yokohama, Japan, and Nagase Biochemicals, Fukuchiyama-shi, Kyoto, Japan. Purified flagellum type b was obtained from T. C. Montie, Knoxville, Tenn. Phospholipase C was purified from strain 19660 as previously described (3). Each protein antigen gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Purified LPS of immunotype 7 was obtained from G. B. Pier, Boston, Mass.

Sera. A total of 135 mice of each strain were divided into 27 groups of 5 mice each. Three groups of five mice were bled via the tail vein each week. Blood was collected each week for 4 weeks following both the primary and secondary infections. Three groups were also bled for normal sera. Sera were pooled from blood collected from the five animals within each group. This protocol ensured that no animal was bled more than once, to avoid the possibility that prior bleeding may alter subsequent antibody levels. Sera from each weekly bleeding were kept frozen at −70°C until the end of the experiment when antibody titers were determined.

Enzyme-linked immunosorbent assay (ELISA). Immunlon II polystyrene microplates (Dynatech Laboratories Inc., Alexandria, Va.) were coated overnight at 22°C with, per well, 100 μl of exotoxin A (2 μg/ml), elastase (2 μg/ml), alkaline protease (10 μg/ml), phospholipase C (10 μg/ml), or LPS (10 μg/ml) in 0.02 M phosphate-buffered saline with 0.02% (wt/vol) sodium azide, pH 7.2, as previously described (16, 36) with minor modifications in the antigen concentrations. Nunc immunoplates (Nunc, Thousand Oaks, Calif.) were coated with, per well, 100 μl of flagella (1 μg/ml) in 0.05 M carbonate coating buffer with 0.02% sodium azide, pH 9.6, as previously described (32). The pooled mouse serum was diluted for testing in Tris-buffered saline–0.05% Tween 20, pH 7.4 (TBS-Tween). The plates were then washed three times with TBS-Tween prior to the addition of serum. The diluted serum was then added in triplicate to the inner wells and incubated at 22°C for 90 min. After incubation, the plates were washed three times with TBS-Tween, and a 1:1,000 dilution of either alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (IgG) (γ-chain-specific) or IgM (μ-chain-specific) (Sigma Chemical, St. Louis, Mo.) conjugate was added. The plates were then incubated again for 90 min at 22°C and washed, and 100 μl of the enzyme substrate was added. The enzyme substrate consisted of 10% diethanolamine solution, pH 9.7, with 0.01% $MgCl_2$ and 0.02% sodium azide with 1 mg of *p*-nitrophenolphosphate per ml. The enzyme substrate reaction was allowed to proceed at room temperature until the positive control reference sera for each antigen reached a predetermined absorbancy. The positive control sera for phospholipase C, exotoxin A, and LPS were taken from mice given both a primary and secondary corneal infection and were screened for high titers to these antigens. Antielastase and anti-alkaline protease antisera were obtained from rabbits immunized with these antigens. These positive control sera were themselves standardized to a positive control serum pool from C.D2-Idh/Pep-3 animals with high IgG and IgM titers specific to whole *P. aeruginosa* coated on microtiter plates to ensure reproducibility between assays and to allow comparison of the titers reported in this study with those of past and future studies. The enzyme substrate reaction generally took approximately 15 min to reach the stopping point. Enzyme activity was inhibited by the addition of 50 μl of 3 N NaOH to each well. Substrate conversion was measured spectrophotometrically at 405 nm with a plate reader (model EL307; Biotek, Burlington, Vt.). ELISA units were determined by multiplying the A_{405} by the dilution of the test sera and subtracting the nonspecific background values for normal mouse sera.

Statistical analysis. The statistical significances of the differences between antibody titers of each mouse strain were determined over the 4 weeks following the primary and secondary infections by analysis of variance.

RESULTS

Exotoxin A. IgG antibodies to exotoxin A first appeared in serum of DBA/2J mice 3 weeks after primary infection. These titers returned to background levels by 4 weeks, as shown in Fig. 1. In contrast, high titers were measured in C57BL/6J mice by 2 weeks, with the levels peaking at 3 weeks and declining by 4 weeks. Upon secondary infection, DBA/2J mice mounted a very rapid secondary IgG response by 1 week that peaked by 3 weeks and decreased rapidly by 4 weeks. C57BL/6J mice mounted a less rapid IgG secondary response at 1 week. This response peaked at 2 weeks and declined by weeks 3 and 4. The difference in titers over the 4-week primary infection was significant ($P = 0.0114$), whereas the difference over the 4-week secondary infection was not. Neither mouse strain mounted a detectable IgM response to exotoxin A.

Phospholipase C. IgG antibodies specific for phospholipase C first appeared by 2 weeks following the primary infection and increased steadily over the 4 weeks tested in both mouse strains (Fig. 2). C57BL/6J mice mounted a more rapid secondary IgG response than DBA/2 mice 1 week after the secondary infection. The anti-phospholipase C IgG response peaked at 2 weeks in both mouse strains. Little or no IgM was detected upon either primary or secondary infection. Differences in the IgG responses between the two mouse strains upon both primary and secondary infection were not significant.

Elastase and alkaline protease. Very few or no IgG or IgM antibodies specific for alkaline protease or elastase were detected in either mouse strain at any time point tested during either primary or secondary infection (≤14 ELISA units) (data not shown). Controls consisting of rabbit antielastase and anti-alkaline protease sera gave high titers when measured on the same plates as the test sera, indicating that the antigen was coated on the wells and not totally degrading the specific immunoglobulins (data not shown).

LPS. IgM was the only immunoglobulin class detected in both mouse strains upon primary infection (Fig. 3). IgM could be detected in C57BL/6J mice only for the first 2 weeks after primary infection, while in DBA/2J mice the titers peaked at 2 weeks and remained elevated during the last 2 weeks tested. During secondary infection, DBA/2J mice again produced measurable IgM titers for the first 2 weeks

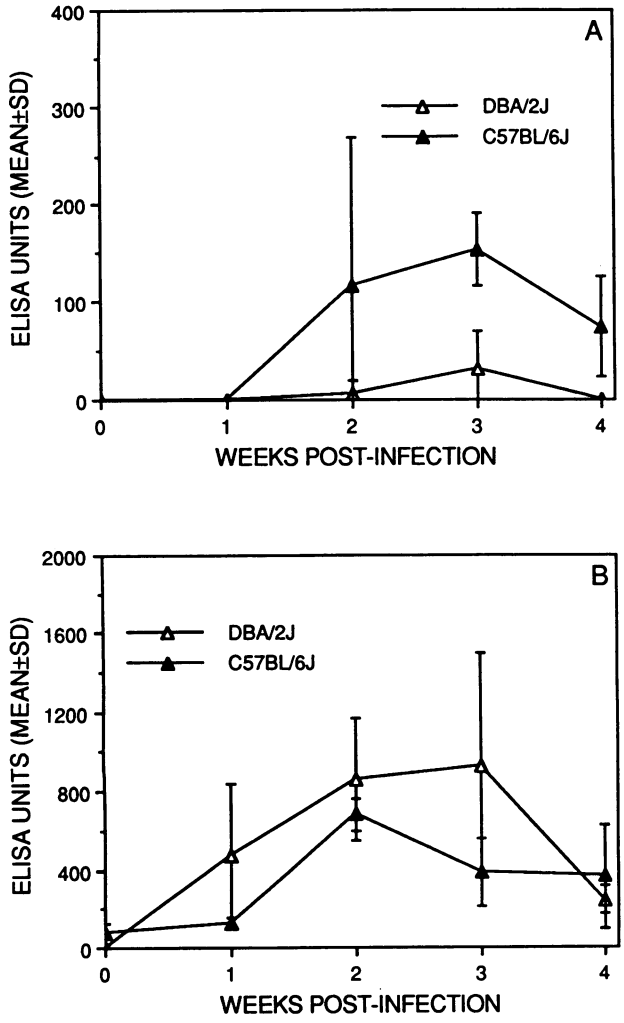

FIG. 1. Serum IgG antibody response to exotoxin A during primary (A) and secondary (B) corneal infection. Secondary infection was administered in the contralateral eye 4 weeks after the primary infection. Data were determined from the sera of three groups of five animals each at each time point. The sera within each group were pooled.

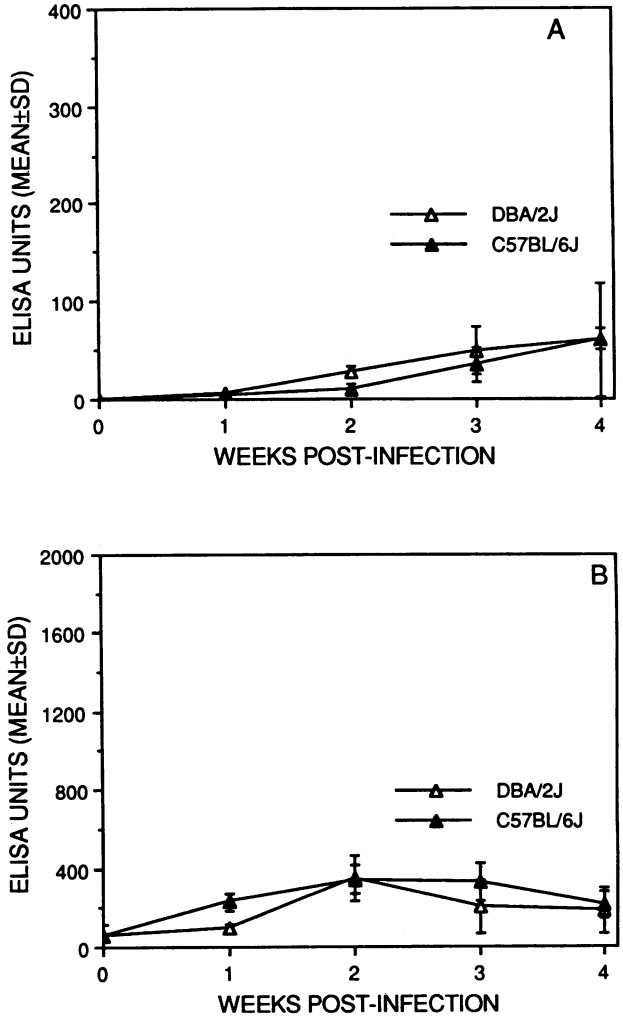

FIG. 2. Serum IgG antibody response to phospholipase C during primary (A) and secondary (B) corneal infection. Data were determined as described in the legend to Fig. 1.

after infection, but the titers remained negligible in C57BL/6J mice. These differences during both primary (P = 0.0001) and secondary (P = 0.0002) infection were significant. IgG antibodies specific for LPS were not detected in either mouse strain upon primary infection (Fig. 4). However, both mouse strains produced detectable, but moderate, IgG antibodies during secondary infection, although differences between the mouse strains were not significant.

Flagella. During primary infection, C57BL/6J mice produced very high antiflagellum IgG antibodies starting at 2 weeks, with titers rising throughout the remaining 2 weeks, while DBA/2J mice produced significantly lower (P = 0.0001) titers that appeared by weeks 3 and 4 (Fig. 5). This pattern continued during secondary infection, with the C57BL/6J mice producing a very high and rapid secondary IgG response; although DBA/2J mice mounted a fairly strong secondary response, the difference was highly significant (P = 0.0001). No IgM response was detected in either mouse strain during either primary or secondary infection.

DISCUSSION

Previous studies by Berk et al. indicated that resistant DBA/2J mice can mount a rapid and elevated antibody response specific to *P. aeruginosa*, while susceptible C57BL/6J mice were initially hyporesponsive (7). However, C57BL/6J mice were able to mount a substantial humoral response within 30 days after secondary infection. Consequently, the basis of resistance or susceptibility was attributed to the ability to mount a protective humoral response prior to the development of permanent corneal damage. Garzelli et al. have reported that heat-killed *P. aeruginosa* inhibited antibody responses in C57BL/6J mice (13). It was suggested that whole heat-killed *P. aeruginosa* acted as a mitogen on B cells that, when strongly stimulated to proliferate, temporarily lost their capacity to mount a normal antibody response. It is not known whether this phenomenon plays a role in C57BL/6J mice infected intraocularly. However, since *P. aeruginosa* produces a number of cell-associated antigens and exocellular products, it is important to determine the specificity of the immune response to these substances during corneal infection. This type of information

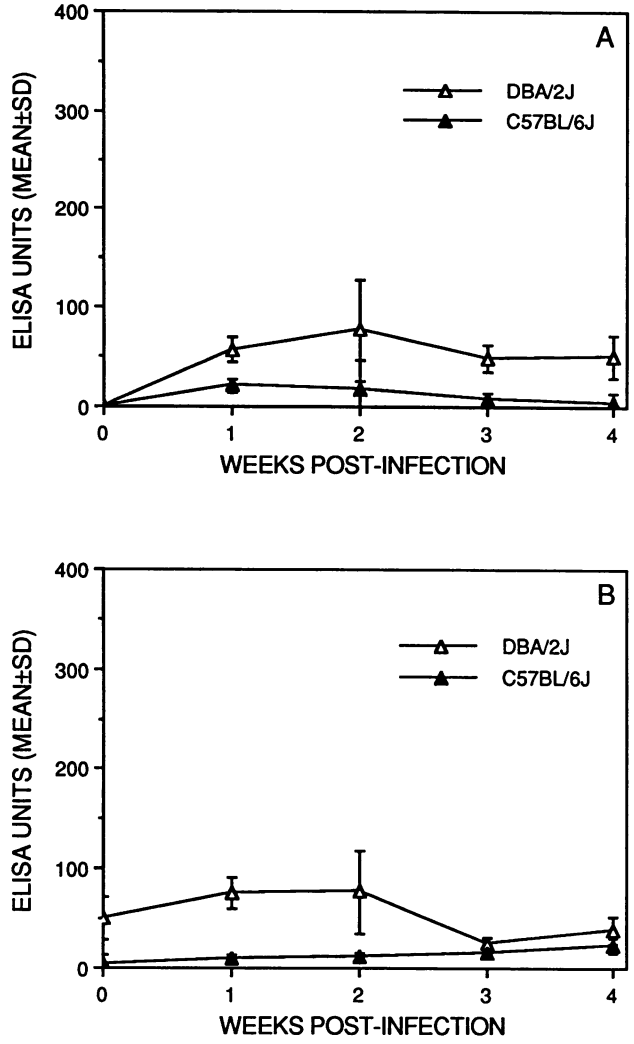

FIG. 3. Serum IgM antibody response to LPS during primary (A) and secondary (B) corneal infection. Data were determined as described in the legend to Fig. 1.

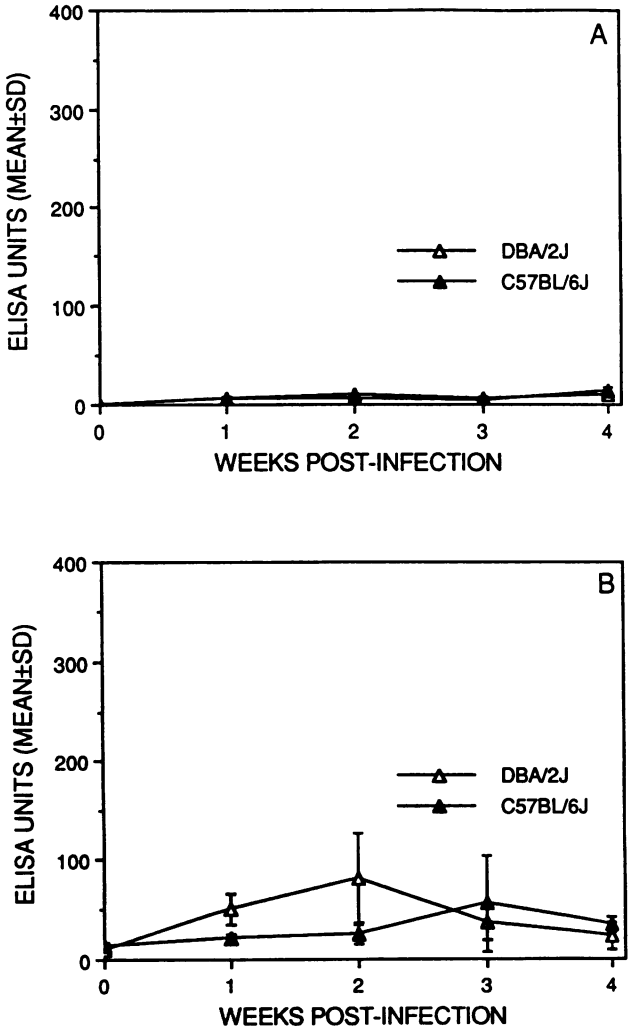

FIG. 4. Serum IgG antibody response to LPS during primary (A) and secondary (B) corneal infection. Data were determined as described in the legend to Fig. 1.

could then aid in identifying potential virulence factors and in the development of a protective immunization regimen and could also shed further light on the factors that might affect the resistance-susceptibility phenotypic response.

A number of unexpected findings occurred when we determined the antibody specificity to a number of cellular and exocellular antigens in both mouse strains. For example, there was little or no antibody response to either elastase or alkaline protease during either primary or secondary infection. These results are surprising, since the potential roles of the proteases in corneal infection have been extensively studied and alluded to by numerous investigators (21, 25, 26, 29, 30, 33, 35). For example, previous studies by Kreger et al. demonstrated that elastase was immunogenic in rabbits and could stimulate the production of neutralizing antibody which reduced the severity of the infection compared with that in control rabbits (30). A similar ocular response was obtained in naive mice receiving passive transfer of antielastase antibody prior to corneal infection (30). Several investigators found that intrastromal injection or topical application of purified elastase or alkaline protease to the excised cornea elicited ulcerations similar to those seen during *P. aeruginosa* corneal infection (25, 29, 35). Both proteases have been shown to degrade the proteoglycan matrix rather than the collagen fibers (26). Other studies with an elastase-deficient mutant, PA103, that produces low levels of alkaline protease revealed no differences in the course of infection and the resulting corneal damage from that of the wild-type strain (33, 35). However, Howe and Iglewski recently reported that an alkaline protease-deficient mutant, PA103-AP1, derived from an elastase-negative mutant, PA103, was avirulent in the corneal infection model, suggesting that alkaline protease is required for the establishment of infection (21). The data of Granstrom et al. (16) support our findings. They found that patients with *Pseudomonas* infections (septicemia, pneumonia, pancreatitis, and osteitis), in contrast to chronically infected cystic fibrosis or burn wound patients, showed no serological response to alkaline protease and elastase. Our current antibody studies suggest that *P. aeruginosa* 19660 does not produce either protease in amounts necessary to stimulate an antibody response during primary and/or secondary infection in either

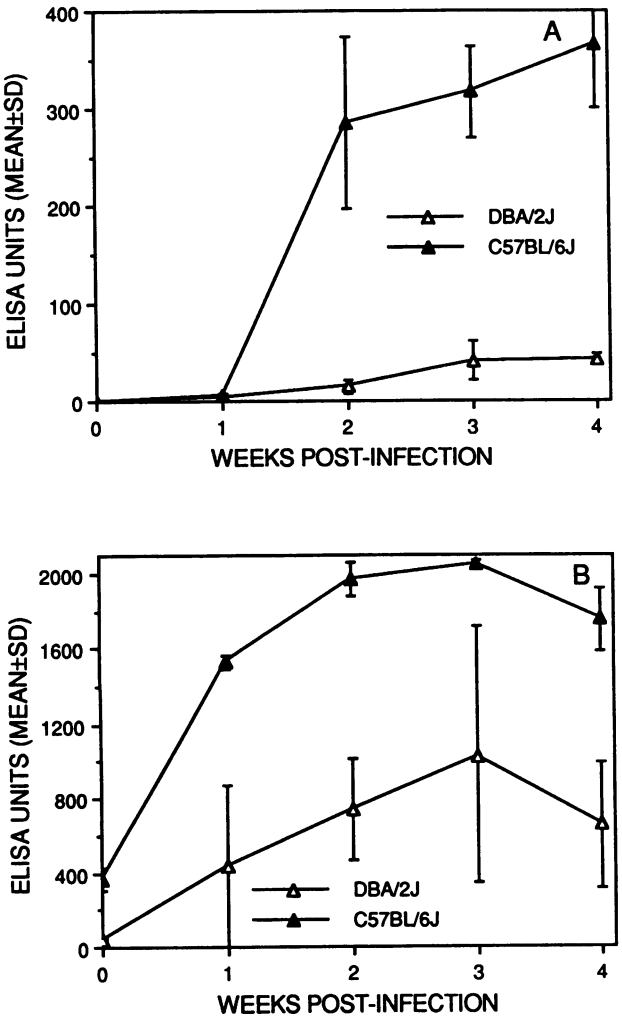

FIG. 5. Serum IgG antibody response to flagella during primary (A) and secondary (B) corneal infection. Data were determined as described in the legend to Fig. 1.

mouse strain. While this would seem to contradict the genetic studies of Howe and Iglewski, the results obtained may be due to differences in the bacterial strains used in the studies, and while alkaline protease may be required for initiation of infection, it does not seem to be a major virulence factor during the infection, at least with *P. aeruginosa* 19660. It is possible that one or both of the bacterial proteases are produced in amounts necessary to initiate the infection but are quickly degraded by host or other bacterial factors as fast as they are produced so that no stimulation of the humoral response can occur. A less likely explanation is that the proteases are nonimmunogenic in the mouse strains tested. While this study demonstrates the absence of a serum antibody response to the proteases by ELISA, it does not preclude the possibility of local IgA production in the tears that neutralizes the proteases. Studies are currently under way to examine this possibility.

Steuhl et al. have suggested an alternate explanation for the cause of tissue damage during corneal infection (35). They contend that host-derived inflammatory processes may be an important cause of corneal destruction during *P. aeruginosa* infection. Tissue-damaging factors such as lysosomal enzymes and oxidative substances may originate from keratocytes, corneal epithelial cells, and polymorphonuclear leukocytes. Their release may result in damage to the proteoglycan matrix and collagen fibers. The data of Steuhl et al. suggest that the collagenase and proteoglycanase activity of ocular cells is more important than direct damage by *P. aeruginosa* exoenzymes, especially within 24 h post-infection. Similar confirmatory results have been described by others as well (19, 37).

LPS has been demonstrated to be a protective antigen in a rabbit model of *P. aeruginosa* corneal infection as well as other nonocular models (10, 12, 23, 30, 34). Since we have previously demonstrated that resistant DBA/2J mice exhibit a rapid and heightened response to the whole organism, we predicted that there should be a similar response to LPS as well, since it is a major component of the outer membrane (7). Additionally, there should be little or no response to LPS in the susceptible C57BL/6J mice. The results described herein for IgM support this proposition and mimic the results obtained with antibody directed toward the whole organism. However, the failure to detect an IgG response to LPS during the primary infection in DBA/2J mice was surprising, since they mount a fairly strong IgG response to the whole organism within 1 week following primary infection. Enzyme-substrate incubation times of up to 1 h in our ELISA system still failed to yield detectable anti-LPS IgG antibodies during primary infection. C57BL/6J mice produced minimal anti-LPS IgG antibodies upon secondary infection, while DBA/2J mice produced heightened levels during the first 2 weeks only after secondary infection. Therefore, it remains to be determined which major outer membrane antigens are being recognized by DBA/2J mice during the primary infection.

Another potential virulence factor postulated to play a role in corneal infections by *P. aeruginosa* is exotoxin A. Exotoxin A inhibits cellular protein synthesis by catalyzing the transfer of the ADP ribose portion of NAD to mammalian elongation factor 2 (22). It has been shown that the toxin inhibits the host's ability to clear the bacteria as well as destroys corneal tissue (17, 33). However, previous experimental data suggest that it is not involved in the establishment of corneal infection (33). Also, Tox^- strains produced much less corneal damage than toxigenic parental strains (33). From our antibody studies, it is quite apparent that *P. aeruginosa* actively produces exotoxin A during the infectious process.

Another exoenzyme that stimulated an antibody response was phospholipase C. Up to now there have been no studies describing a possible role for this enzyme in ocular infection, although the glycolipid hemolysin from *P. aeruginosa* has been demonstrated to play a role in the severity of infection (24). However, studies in cystic fibrosis patients infected with *P. aeruginosa* indicated that all patients so far tested appear to produce antibodies to phospholipase C (15). The presence of these antibodies has been considered as a diagnostic indicator of chronic *P. aeruginosa* infection (15). Previous studies from our laboratory indicate that phospholipase C is lethal for mice and is responsible for dermonecrosis, footpad swelling, and vascular permeability (3). In addition, it produced platelet aggregation and was shown to be a potent inflammatory agent (9, 31). The present studies suggest a potential role for phospholipase C during corneal infection.

The antigen that stimulated the strongest IgG response in the present study was flagella. Naturally susceptible

C57BL/6J mice produced very high antiflagellum titers upon both primary and secondary corneal infection. However, the role for flagella in ocular infections is unclear at this time. Numerous studies in the burn wound sepsis model suggest that flagellum-mediated motility is important to the virulence of *P. aeruginosa* and that antiflagellum antibodies could protect animals from lethal challenge (11, 20). Anderson and Montie also demonstrated that antiflagellar antiserum was able to enhance the uptake of radiolabeled *P. aeruginosa* by mouse polymorphonuclear leukocytes compared with normal serum (1). In unpublished observations in our laboratory, immunization with 2 μg of purified flagella intramuscularly generated high (approximately 500 ELISA units) antiflagellum IgG titers 2 weeks postimmunization in C57BL/6J mice. When these 2-week postimmunization sera were then tested against the whole organism, low (approximately 25 ELISA units) IgG titers were measured. Our data suggest that the high antiflagellum antibody titers observed in these studies play a relatively minor role in opsonophagocytic clearing of the organism by polymorphonuclear leukocytes and macrophages compared with the role of the total IgG directed against whole *P. aeruginosa* 19660.

In summary, these studies have demonstrated that both mouse strains can mount an immune response to exotoxin A, phospholipase C, LPS, and flagella during primary corneal infection. Antibodies directed specifically towards the exocellular bacterial products may neutralize the toxicity of the exoenzymes but may not enhance bacterial clearance or provide adequate protection during the infectious process. In addition, the relatively late appearance of these antibodies (2 weeks) after primary infection may not allow them to prevent damage to the tissue by these toxins early in infection. The data described herein shed light on the substrate-specific humoral response of both mouse strains during primary and secondary *P. aeruginosa* corneal infection. In addition, C57BL/6J mice are not hyporesponsive to all antigens during *P. aeruginosa* corneal infection and, in fact, respond very well to some antigens.

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