

# Monoclonal Antibodies Provide Protection Against Ocular *Pseudomonas aeruginosa* Infection

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**A panel of well characterized monoclonal antibodies (MAbs) directed against outer membrane proteins H2, or F (porin) of *Pseudomonas aeruginosa* were examined to determine whether they exhibited any protective effect against subsequent ocular challenge with the bacteria topically applied to the scarified corneal surface. Mice were observed macroscopically following bacterial challenge and the degree of ocular disease graded on a scale of 0 to 4 (0, normal, fully protected cornea; 4, corneal perforation or phthisis, not protected). Mice treated intravenously with either MAb MA1-6 (anti-H2) or MA2-10 (anti-F), or a combination of these two MAbs and MAb MA4-4 (anti-F), two hours before corneal challenge with the viable bacteria, exhibited significantly less corneal disease than mice either not treated with the MAbs, treated with MA4-4 alone or treated with MAb MA1-3 (anti-I). The latter MAb is directed against an outer membrane epitope that is not surface exposed. Light and transmission electron microscopic histopathology also was employed and provided confirmatory evidence to support the macroscopic analyses. Invest Ophthalmol Vis Sci 29:1277-1284, 1988**

Ocular disease produced by the opportunistic bacterial pathogen *P. aeruginosa* often leads to a fulminating and highly destructive infection resulting in rapid liquefaction of the cornea and blindness.<sup>1</sup> Antibiotic treatment is not always successful due to the resistance of many clinical strains.<sup>2,3</sup> Thus, development of agents with therapeutic efficacy against various outer membrane antigens and exoproducts of the organism have begun to receive considerable attention, in an attempt to provide protection from infection. Active or passive immunization with flagella,<sup>4</sup> slime capsule,<sup>5</sup> lipopolysaccharide (LPS)<sup>6-8</sup> and high molecular weight polysaccharide,<sup>7,9</sup> outer membrane proteins,<sup>10-12</sup> as well as exoenzymes such as exotoxin A<sup>13,14</sup> and bacterial proteases<sup>14,15</sup> have been used in a variety of animal models with various degrees of success. Experimental studies suggest that purified outer membrane components may prove efficacious as immunogens, since they, unlike LPS, are nontoxic, contain conserved surface-located epi-

topes<sup>16-18</sup> and provide protection against a broad spectrum of *P. aeruginosa* strains, regardless of serotype.<sup>11</sup> Recent work by Kreger et al<sup>15</sup> demonstrated protection in rabbits actively immunized with *P. aeruginosa* LPS endotoxin and to a lesser extent with elastase. Passive protection with anti-elastase polyclonal antisera was also somewhat effective in protecting both rabbits and mice from severe corneal damage. In an extension of these studies, the present report has shown that protection against severe corneal damage can be achieved in mice by passive immunization with monoclonal antibodies (MAbs) directed against outer membrane proteins H2 and F (porin) of *P. aeruginosa*.

## Materials and Methods

### Bacterial Cell Cultures

Stock cultures of *P. aeruginosa* American Type Culture Collection (ATCC, Rockville, MD) 19660 stored at 25°C on tryptose agar slants (Difco Laboratories, Detroit, MI) were used for inoculation of 60 ml of broth medium containing 5% peptone (Difco) and 0.25% trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD). The culture was mucoid, hemolytic, proteolytic and produced lecithinase and exotoxin A. Cultures were grown on a rotary shaker at 37°C for 18 h, centrifuged at 27,000 g for 20 min at 4°C, washed with saline (Travenol Laboratories, Inc., Deerfield, IL) and resuspended to a concentration of  $2.0 \times 10^{10}$  colony forming units (CFU) per ml, using a standard curve relating viable counts to optical density at 440 nm.<sup>19</sup>

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## Mice

Female adult C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) 6–8 weeks of age were used in these experiments. This particular mouse strain was used since it has been shown to be highly susceptible to ocular *P. aeruginosa* experimental infection.<sup>20</sup> In a typical experiment, animals fail to restore corneal clarity within 4–6 weeks after corneal scarification and topical bacterial challenge and a high percentage of infected eyes become phthisical. All experiments with these animals were carried out according to the ARVO Resolution on the Use of Animals in Research.

## Infection of Mice

Mice were anesthetized with ether and placed beneath a stereoscopic microscope at  $\times 40$ . The ocular surface of the left eye of each animal was wounded, penetrating no deeper than the superficial corneal stroma just beneath the epithelial basal lamina. This was achieved by making three 1 mm incisions to the corneal surface of the eye using a sterile 26 gauge needle. The depth of the wound was randomly ensured with routine scanning and transmission electron microscopy. A bacterial cell suspension (5  $\mu$ l), containing a final concentration of  $5.0 \times 10^7$  CFU of *Pseudomonas aeruginosa* ATCC 19660 was topically delivered onto the surface of each incised cornea using a calibrated micropipette (Oxford Laboratories, Foster City, CA) with a sterile, disposable tip.

## Monoclonal Antibody Preparation

Hybridoma cells inoculated intraperitoneally into BALB/cByJ mice produced ascitic fluid from which MAbs were purified by ammonium sulphate precipitation. In brief, the ascitic fluid was diluted 1:1 in saline and 40% (vol/vol) saturated ammonium sulphate added slowly with stirring at 4°C for 4 hr. The precipitate was removed by centrifugation at 1200 g and redissolved at the original volume in phosphate buffered saline (PBS). The antibodies were then reprecipitated by the addition of 45% (vol/vol) saturated ammonium sulphate as before. The precipitate was then dissolved in a minimal amount of PBS and dialysed against 5:1 PBS overnight. Any precipitate was removed by low-speed centrifugation. Sodium dodecyl sulphate polyacrylamide gel electrophoresis indicated that at least 90% of the remaining protein in the supernatant was antibody. Western immunoblots<sup>21</sup> were performed to confirm the purity of the monoclonal antibodies. The following outer membrane protein specific monoclonal antibodies were used: protein F-specific monoclonal antibodies

MA2-10 and MA4-4,<sup>18,21</sup> the lipoprotein H2-specific monoclonal antibody MA1-6<sup>16,17</sup> and the putative lipoprotein I-specific monoclonal antibody MA1-3.<sup>16,22</sup> Monoclonal antibodies MA2-10 and MA1-3 were of the IgG1 subclass, MA4-4 was an IgG2a, whereas MA1-6 was an IgG antibody of uncharacterized subclass. The MAb MA1-3 was used as a negative control since its specificity is directed at a nonsurface exposed outer membrane epitope.<sup>12</sup> It also provided a control for antibody protein subjected to the same purification procedures as the other MAbs. Further, passive immunization with this MAb has been shown not to provide protection against *P. aeruginosa* infection in other systems.<sup>12</sup>

## Passive Immunization

Each mouse received 0.1 ml of MAb or PBS by intravenous injection into a dorsolateral tail vein. The total amount injected per mouse (as determined by spectrophotometric protein assay, Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA) was approximately 500  $\mu$ g for MAbs MA1-3, MA1-6 and MA4-4 and was 50  $\mu$ g for MAb MA2-10. No higher concentration could be achieved for the latter antibody. A combination of MAbs (MA1-6, MA4-4 and MA2-10) also was administered similarly at a final concentration of 500  $\mu$ g per animal. The administration of each MAb or a combination of MAbs was performed approximately 2 hr prior to experimental ocular infection.

## Macroscopic Evaluation

The ocular response to *P. aeruginosa* infection was macroscopically evaluated "blindly" by two of the investigators (LDH and MMM) at 24 hr after bacterial challenge, and then every other day through the first week following infection. Animals were then examined at weekly intervals over a 4 week time period. The ocular response of the infected animals passively immunized with each of the MAbs, a combination of MAbs, or given PBS, as described above, was graded on a scale of 0 to 4 (Table 1) and these data are presented in Table 2. Since the PBS and MAb MA1-3 data were similar, only the MA1-3 data is presented in Table 2. Figure 1 comparatively illustrates the severity of the grossly observable ocular lesions at 30 days after infection.

## Light and Electron Microscopy

Mice were ether anesthetized and sacrificed by cervical dislocation. The eyes were enucleated, washed briefly with sodium phosphate buffer (0.1 M, pH 7.4) and fixed intact for 3 hr at 4°C in a 1:1:1 solution of 2% osmium tetroxide, 2.5% glutaraldehyde and 0.2

M sodium phosphate buffer, pH 7.4. After fixation, specimens were rinsed in sodium phosphate buffer (0.1 M, pH 7.4) and dehydrated through a graded series of ethanols to 100% and then to propylene oxide. Specimens were infiltrated with a mixture of Epon-araldite resin and propylene oxide (1:1 mixture for 24 hr, followed by 3:1 for 24 hr) and infiltrated with fresh resin for 1 week before embedding in fresh resin containing the polymerizing agent DMP-30. Thick sections (1.5  $\mu$ m) were cut and stained with a modified Richardson stain<sup>23</sup> and were examined and photographed with a Zeiss automatic photomicroscope equipped with standard bright field optics. Thin sections (90 nm) were cut, stained with aqueous uranyl acetate and lead citrate and observed and photographed using a JEM-100CX transmission electron microscope, operating at 60 kV.

#### Statistical Analysis

Comparison of the incidence of a grade 0 or 1 response versus all other ocular response grades was performed by the Chi square test with no Yates continuity correction for the data expressed in Table 2.

### Results

#### Passive Immunization of Mice Against *Pseudomonas Keratitis*

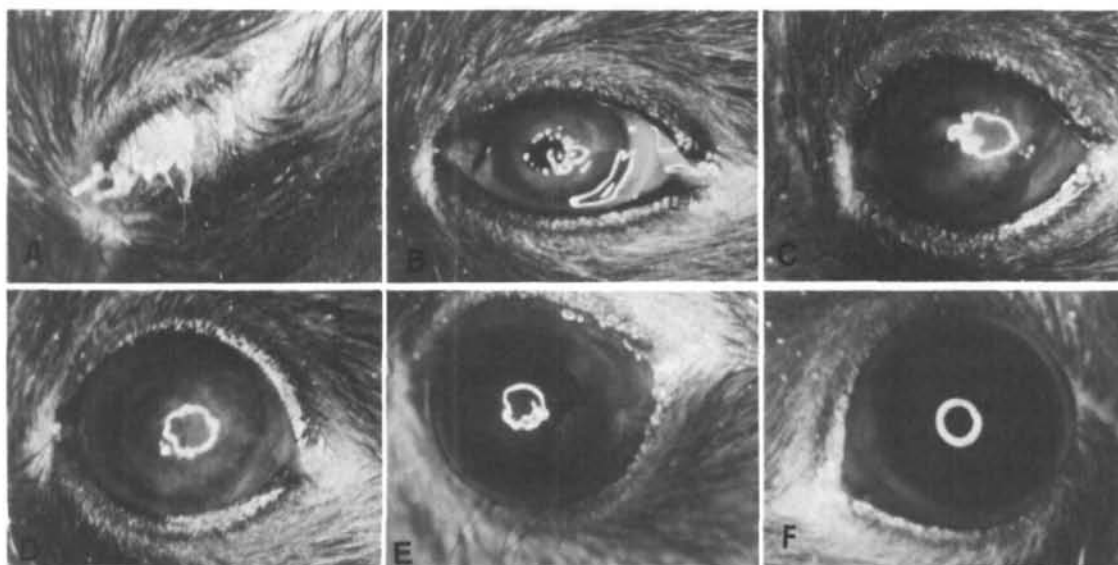
Mice passively immunized with the MAbs MA1-6 (anti-H2) or MA2-10 (anti-F) or a combination of

**Table 1.** Grading of grossly observable ocular disease in infected mice

0	= Clear or slight opacity, partially covering pupil
1	= Slight opacity fully covering anterior segment
2	= Dense opacity, partially or fully covering pupil
3	= Dense opacity, covering entire anterior segment
4	= Corneal perforation or phthisis

these two MAbs and MA4-4 (anti-F) prior to corneal challenge with the viable bacteria exhibited significantly less corneal damage than mice given PBS or immunized passively with MA4-4 alone or the non-specific MAb MA1-3 (Fig. 1 and Table 2). Two of the monoclonals (MA2-10 and MA1-6) or a combination of these and MA4-4 protected a significant number of mice from phthisis, but not all of the mice were fully protected against corneal opacification (Fig. 1).

The results of three separate, but similarly designed experiments were combined to allow sufficient numbers of animals for statistical analysis. These data are presented in Table 2. The administration of MAb MA1-3 provided little or no protection from corneal disease. As shown in Table 2, the corneas of 22 of 30 mice (73%) receiving MAb MA1-3 prior to bacterial challenge underwent perforation (grade 4) within 1 month post-challenge. The remaining eight mice (27%) of this experimental group showed corneal disease of various degrees (grades 1 to 3). These data were not statistically different from results obtained



**Fig. 1.** Ocular disease observed in nonimmunized or passively immunized mice challenged with *P. aeruginosa* ATCC 19660. Results shown are 30 days after challenge with the viable bacteria suspended in PBS. (A) Corneal perforation and phthisis (grade 4) typically observed in nonimmunized mice. (B) Phthisis (grade 4) typically observed in mice not protected by antibody treatment (MAb MA1-3). (C) and (D) Slight opacity over anterior segment (grade 1) typically observed in mice partially protected by antibody treatment (C, MAb MA1-6; D, MAb MA2-10). (E) Normal cornea (grade 0) typically observed in mice fully protected by antibody treatment (MAb MA2-10 + MAb MA1-6 + MAb MA4-4). (F) Normal mouse cornea not infected with *P. aeruginosa*. All  $\times 260$ .

**Table 2.** Passive immunization of mice against corneal disease produced by *P. aeruginosa*

Mouse strain	Bacterial inoculum	Monoclonal antibody specificity	Ocular response	Time postinfection in days	
				1	30
C57BL/6 <sup>30</sup> *	5.0 × 10 <sup>7</sup>	MA1-3 (anti-I) (500 µg/mouse)	0	—	—
			1	1/30	4/30
			2	16/30	2/30
			3	13/30	2/30
			4	—	22/30
C57BL/6 <sup>24</sup> *	5.0 × 10 <sup>7</sup>	MA2-10 (anti-F) (50 µg/mouse)	0	2/24	4/24
			1	17/24	13/24 (≤0.0005)†
			2	5/24	—
			3	—	—
			4	—	7/24
C57BL/6 <sup>20</sup> *	5.0 × 10 <sup>7</sup>	MA1-6 (anti-H2) (500 µg/mouse)	0	—	8/20
			1	15/20	10/20 (≤0.0005)
			2	4/20	—
			3	1/20	—
			4	—	2/20
C57BL/6 <sup>18</sup> *	5.0 × 10 <sup>7</sup>	MA4-4 (anti-F) (500 µg/mouse)	0	—	3/18
			1	12/18	2/18 (≤0.0005)
			2	4/18	9/18
			3	2/18	—
			4	—	4/18
C57BL/6 <sup>18</sup> *	5.0 × 10 <sup>7</sup>	MA2-10 + MA1-6 + MA4-4 (anti-F and H2) (500 µg/mouse)	0	—	6/18
			1	10/18	9/18 (≤0.005)
			2	5/18	—
			3	3/18	—
			4	—	3/18

\* The number of animals shown in parentheses reflects data pooled from three similar, separate experiments.

† *P* value for grades 0 and 1 combined is in parentheses. The 95% significance level is *P* = 0.05.

using PBS as an alternate negative control (data not shown).

Groups of mice that received MAbs specific for either outer membrane porin protein F (MA2-10, MA4-4) or lipoprotein H2 (MA1-6) demonstrated various degrees of protection against bacterial induced ocular disease. As shown in Table 2, 17 of 24 mice (70%) that received MAb MA2-10 were evaluated as grade 0 or 1 at 30 days post-challenge. Of those mice receiving MAb MA1-6, 18 of 20 animals (90%) were evaluated as grade 0 or 1 for the same time period. The group receiving a combination of MAbs MA2-10, MA1-6 and MA4-4 demonstrated similar results (83% as grade 0 or 1), while the group receiving MAb MA4-4 alone showed the fewest number of animals (5 of 18) in groups 0 and 1 (27%) and these data were not significant when compared with MA1-3 values. Protection was significant for two of the MAbs (MA1-6 and MA2-10) either separately or for a combination of these and MA4-4 (*P* ≤ 0.0005; the 95% significance level being *P* = 0.05) when compared with values for MAbs MA1-3 or MA4-4 alone.

The ability of the MAbs to passively protect against severe corneal damage produced by experimentally induced *P. aeruginosa* keratitis was confirmed by both light (Figs. 2–4) and transmission electron microscopy (Fig. 5A–F). A comparison of Figure 2

(MAb MA1-3, or unprotected) and Figure 3 (partially protected) and 4 (protected) illustrates the immunotherapeutic capabilities of the MAbs observable at the light microscopic level. Unprotected eyes (Fig. 2) exhibited anterior segment histopathology including corneal stromal cellular infiltrate, corneal vascularization and anterior synechia. Cataractous changes in the ocular lens were also notable. Partially protected eyes (Fig. 3) showed less severe pathological changes, including blood vessel ghosts in the corneal stroma. Protected eyes (Fig. 4) exhibited normal morphology at the light microscopic level. Similar comparisons can be made at the transmission EM level. Corneas of unprotected eyes (Fig. 5A) exhibited goblet cells, widened epithelial intercellular junctions, and (Fig. 5B) disorganized stroma with inflammatory cell infiltrates. The histopathology noted in partially protected eyes (Fig. 5C) included widened epithelial intercellular junctions, tortuous epithelial basal lamina and (Fig. 5D) minimal stromal disorganization. Corneas of protected eyes exhibited normal morphology of both epithelium and stroma (Fig. 5E and F, respectively).

## Discussion

The present study describes the use of several well characterized monoclonal antibodies<sup>12,16–18,22</sup> di-



Fig. 2. Light microscopic histopathology of nonimmunized or passively immunized and not protected mouse cornea challenged with *P. aeruginosa* at 30 days after infection. Cornea not protected (grade 4) by the MAbs or after PBS injection exhibited stromal cellular infiltrate, blood vessels and anterior synechia. Cataractous changes in ocular lens (arrows) are also evident. Original magnification  $\times 95$ .

rected against outer membrane proteins H2 and F (porin) to protect against experimental keratitis caused by *P. aeruginosa* in a highly susceptible mouse strain.<sup>20</sup> Previously, it has been demonstrated that rabbits vaccinated with heat-killed *P. aeruginosa*,<sup>24</sup> with slime extracts of *P. aeruginosa*<sup>5</sup> and with gamma globulin obtained from rabbits vaccinated with the killed bacteria<sup>25</sup> were protected against severe corneal disease by the homologous bacterial strains. In addition, mice have been actively immunized and protected against pseudomonas keratitis by oral or intraperitoneal administration of phenol-killed suspensions of the homologous challenge strain.<sup>26</sup> Recently, Kreger et al<sup>15</sup> demonstrated that active immunization with *P. aeruginosa* LPS protected rabbits from keratitis. However, bacterial outer membrane proteins may potentially be more useful than LPS in immunization against keratitis, since they appear to lack the inherent toxicity of LPS,<sup>12</sup> are considered important in pathogenesis<sup>10-12,27</sup> and are antigenically related or identical in all 17 serotype-specific strains of *P. aeruginosa*.<sup>16-18,21</sup> Thus, the outer membrane antigens may act as "common" antigens for use in vaccine development, thereby obviating the need for specific serotypic antigens.

In the present study, passive immunization with MAbs to outer membrane proteins H2 and F provided ocular protection. The results are consistent with the demonstration of production of antibodies to outer membrane proteins F and H2 after subcutaneous and lung infections of mice<sup>27</sup> and rats<sup>28</sup> respectively with *P. aeruginosa*. In addition, antibodies to outer membrane proteins F and H2 have been demonstrated in the sera of cystic fibrosis (CF) patients<sup>28-30</sup> and immunofluorescent staining of autopsy-derived lung sections of two CF patients revealed that proteins F and H2 are exposed in these patients.<sup>31</sup>

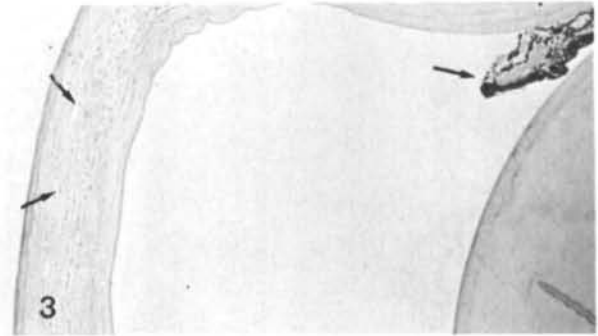


Fig. 3. Light microscopic histopathology of a passively immunized (MAB MA2-10), partially protected mouse cornea (grade 1) at 30 days after infection. Numerous blood vessel ghosts (arrows) are seen within the stroma and Descemet's membrane is somewhat tortuous. The ocular lens appears normal and the iris is mildly hyperemic (arrow). Original magnification  $\times 95$ .

Of the three proteins used as antigen to produce the MAbs in the presently described ocular studies, it is noteworthy that one of the proteins was F. This protein is the major constitutive porin protein of *P. aeruginosa*. Previously, it has been demonstrated that porin preparations from *P. aeruginosa*,<sup>10,11</sup> *Neisseria meningitidis*<sup>32</sup> and *Salmonella typhimurium*<sup>33</sup> act as protective antigens in non-ocular animal models of infection. MAb MA4-4 directed against *Pseudomonas* outer membrane protein F was protective in mouse peritoneal and burn infection models, although neither MA4-4 nor other protein F-specific MAbs promoted complement-mediated killing of the bacteria.<sup>12</sup> These antibodies were, however, capable of acting as effective opsonins for phagocytosis by human polymorphonuclear leukocytes,<sup>12</sup> mouse peritoneal macrophages<sup>22</sup> and human monocyte-derived macrophages.<sup>22</sup> Although in our studies MA4-4 failed to provide significant protection (at  $P = 0.05$  level), it did protect more mice than the nonspecific MA1-3. Another anti-F directed MAB, MA2-10, did

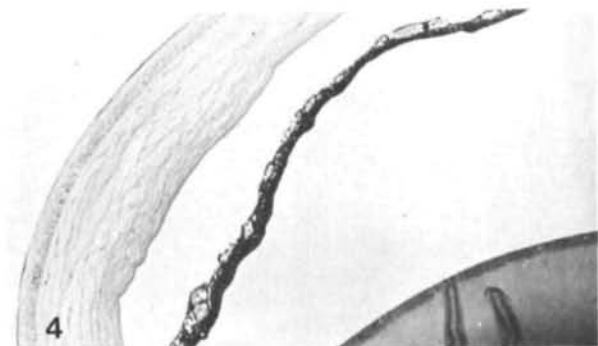
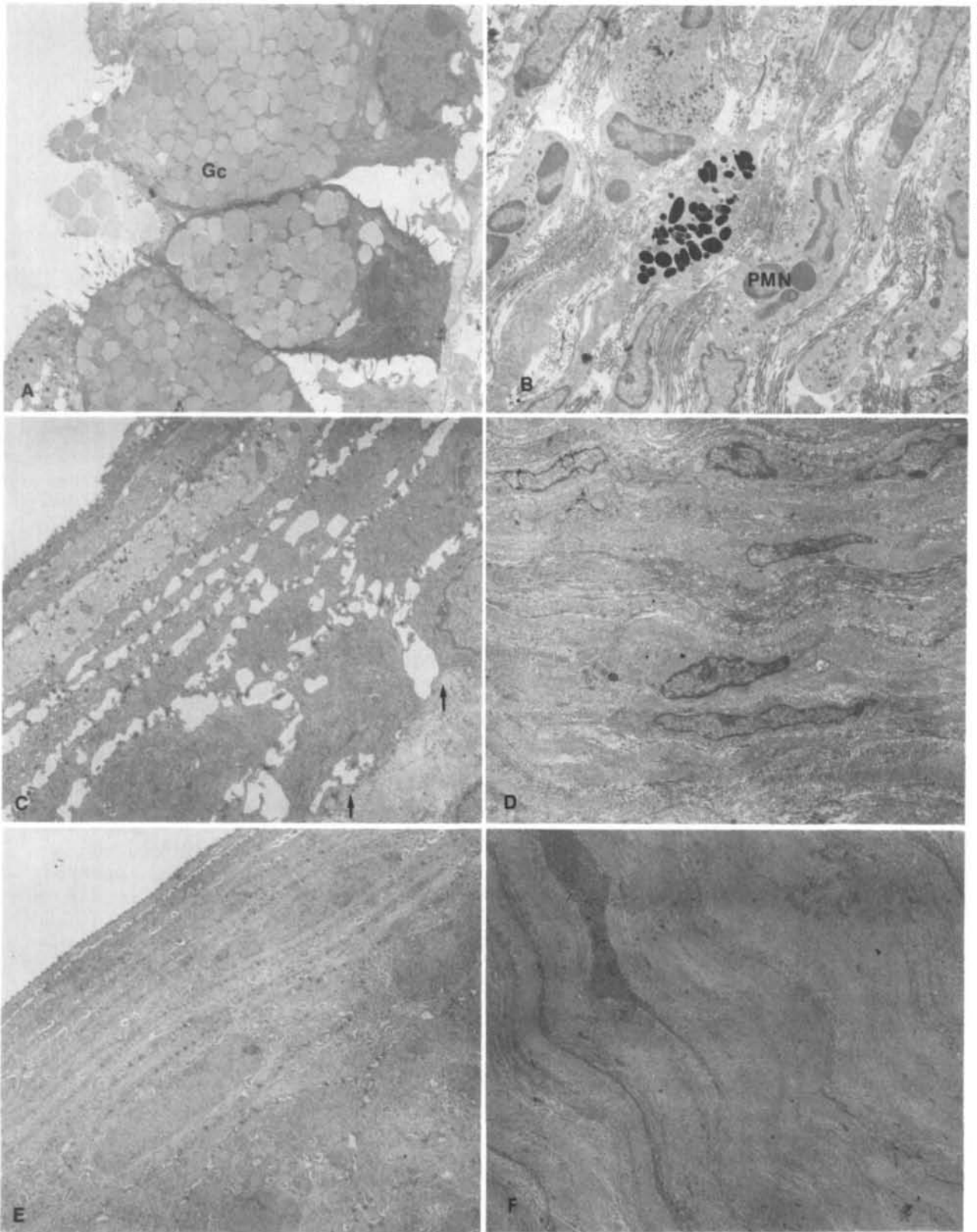


Fig. 4. Light microscopic histopathology of a passively immunized and protected mouse cornea (grade 0) (MAB MA2-10 + MA1-6 + MA4-4) at 30 days after infection. Corneal morphology appears normal as does the iris and ocular lens. Original magnification  $\times 95$ .





**Fig. 5.** Transmission electron microscopy (TEM) of a nonimmunized or passively immunized and not protected mouse cornea challenged with *P. aeruginosa* at 30 days after infection (A, B). Cornea not protected (grade 4) shows several goblet cells (Gc) and widened epithelial intercellular junctions (A). The superficial stroma (B) of this type of cornea (grade 4) was typically disorganized, containing numerous neutrophils (PMN) as well as a pigmented cell, presumably from the iris. TEM of a passively immunized (MAb MA1-6) partially protected cornea (grade 1) at 30 days after infection (C). Widened epithelial intercellular junctions and a tortuous basal lamina (arrows) are seen. Stromal disorganization is minimal in this grade 1 cornea (D). TEM of a passively immunized, fully protected cornea (grade 0) at 30 days after infection. Both the epithelium (E) and stroma (F) are of normal morphology. All original magnifications  $\times 3800$ .

provide significant ocular protection even at comparatively lower antibody concentration than MA4-4. These data suggest that MA4-4 may be directed at an antigenic epitope which is not significant for virulence in the eye infection model. Interestingly, in other studies, MA2-10 was significantly more effective at promoting phagocytosis than MA4-4, while MA1-3 was totally ineffective.<sup>22</sup> These functional defects were not related to antibody isotype, since MA2-10, like MA1-3, is of the IgG1 subclass. The results presented herein support these data with regard to the relative effectiveness of the anti-porin antibody MA2-10 in protecting against ocular infection, although the other anti-porin antibody, MA4-4, provided no significant protection when compared to the nonspecific MAb, MA1-3 (at the  $P = 0.05$  confidence interval). These collectively suggest that a possible mechanism of ocular protection may involve enhanced opsonic killing of the bacteria. Alternatively, preimmunization with these MAbs, directed at bacterial outer membrane proteins, may functionally interfere with the ability of the bacteria to adhere to the scarified corneal surface. Unfortunately, at this time we have no direct data to support either of these hypotheses.

Nonetheless, the data presented in a well established mouse model of ocular *P. aeruginosa* infection do support the hypothesis that one or more of the outer membrane proteins of *P. aeruginosa*, or monoclonal antibodies against these proteins, may have potential therapeutic efficacy against ocular bacterial infection with this pathogen. Their potential value in non-ocular infections is less clear. In this regard, Pennington et al<sup>34</sup> failed to protect guinea pigs by passive immunization with a MAb to outer membrane protein F (porin), whereas, MAb to LPS provided significant protection in experimental *P. aeruginosa* pneumonia. A significant difference between this study and our own is that in the former, animals were immunized 2 hr after bacterial challenge, whereas in the studies presented in this paper, animals were immunized 2 hr prior to ocular bacterial challenge. In addition, it was not demonstrated that their monoclonal antibody was directed against a surface-exposed epitope. The importance of these differences with regard to future patient prophylaxis and treatment prompts further study of these MAbs in both animal models.

**Key words:** monoclonal antibodies, cornea, *P. aeruginosa*, protection

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