

Serum Immunoglobulin Response and Protection from Homologous Challenge by *Proteus mirabilis* in a Mouse Model of Ascending Urinary Tract Infection

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We tested the hypothesis that experimental *Proteus mirabilis* urinary tract infection in mice would protect against homologous bladder rechallenge. Despite production of serum immunoglobulin G (IgG) and IgM (median titers of 1:320 and 1:80, respectively), vaccinated (infected and antibiotic-cured) mice did not show a decrease in mortality upon rechallenge; the survivors experienced only modest protection from infection (mean log₁₀ number of CFU of *P. mirabilis* NaI^r HI4320 per milliliter or gram in vaccinated mice versus sham-vaccinated mice: urine, 100-fold less [3.5 versus 5.5; *P* = 0.13]; bladder, 100-fold less [3.1 versus 5.1; *P* = 0.066]; kidneys, 40-fold less [2.7 versus 4.3; *P* = 0.016]). Western blots using protein from the wild-type strain and isogenic mutants demonstrated antibody responses to MR/P and PMF fimbriae and flagella. There was no correlation between serum IgG or IgM levels and protection from mortality or infection. There was a trend toward elevated serum IgA titers and protection from subsequent challenge (*P* ≥ 0.09), although only a few mice developed significant serum IgA levels. We conclude that prior infection with *P. mirabilis* does not protect significantly against homologous challenge.

While *Proteus mirabilis* causes less than 10% of uncomplicated urinary tract infections (UTIs), it is much more frequently isolated from patients with complicated UTIs, i.e., those with functional or anatomic abnormalities of or with chronic foreign bodies in the urinary tract (17, 21, 29). For example, in those with long-term catheters in place, nearly half of urine specimens contain *P. mirabilis* at concentrations of ≥10⁵ CFU/ml (24). This bacterium causes not only cystitis and acute pyelonephritis (5–7, 23) but also urinary stones, a result of expression of a highly active urease. Stone formation, a hallmark of infection with this organism, adds another dimension to the already complicated urinary tract (8, 18, 19).

Prevention of *P. mirabilis* UTIs is clearly a worthy goal, and thus, the concept of a vaccine has been pursued (15, 20). A vaccine against this organism may be feasible for several reasons. First, the species is quite homogeneous with respect to expression of surface antigens (14). Second, *P. mirabilis* is present in the fecal flora of <5% of individuals (25) and, thus, preventing its colonization of the host should not result in disruption of normal bowel flora. Finally, patient populations that would benefit from such a vaccine are well defined and include those with known anatomically or functionally abnormal urinary tracts, possibly women with recurrent UTIs, and those early in the course of long-term catheterization. As a first step toward the development of a vaccine, we assessed antibody response to whole bacteria and specific antigens and immunity to homologous reinfection in mice that had been inoculated transurethraly with a virulent *P. mirabilis* strain and subsequently cured by antibiotic treatment.

Experimental infection (vaccination). Live *P. mirabilis* HI4320, a strain recovered from the urine of a patient with catheter-associated bacteriuria and a mouse uropathogen (11), was used to assess immunity following transurethral challenge (vaccination). A nalidixic acid-resistant mutant of *P. mirabilis* HI4320 (*P. mirabilis* NaI^r HI4320; nalidixic acid MIC of 512 µg/ml) was used to challenge mice 5 weeks later (challenge). For mouse vaccination and challenge, *P. mirabilis* was grown overnight on Trypticase soy agar (TSA) (BBL, Cockeysville, Md.). Bacteria were harvested into phosphate-buffered 0.9% sodium chloride, pH 7.2 (PBS; BBL), and adjusted to approximately 2 × 10⁸ CFU/ml for *P. mirabilis* HI4320 and approximately 2 × 10⁷ CFU/ml for *P. mirabilis* NaI^r HI4320, using McFarland turbidity standards confirmed by spread plate enumeration (Spiral Systems, Bethesda, Md.). On day 1, mice were divided into vaccination (60 mice) and sham vaccination (30 mice) groups (Fig. 1). Vaccination group mice were challenged by the transurethral route using a previously described procedure (10). Sham-vaccinated mice were similarly infused with 50 µl of PBS. The catheter was removed immediately after transurethral infusion, and mice were returned to their cages and cared for by the normal routine. As described previously (10), in each experiment, one mouse was used to assess whether the inoculum refluxed into the kidney during the challenge procedure. Vaccinated and sham-vaccinated mice were observed daily for 4 weeks. During the observation period, sick and moribund mice were sacrificed by exposure to an overdose of CO₂. On days 28 to 31, ampicillin (500 mg/ml) was added to the mouse drinking water daily to eradicate residual *P. mirabilis* from the urinary tract. On day 32, tap water use was restored and mice were held for an additional 3 days to allow washout of the ampicillin. On day 35, urine samples were collected from all of the mice and cultured.

Homologous challenge. Thirty mice in each of the vaccinated and sham-vaccinated groups were challenged transurethraly

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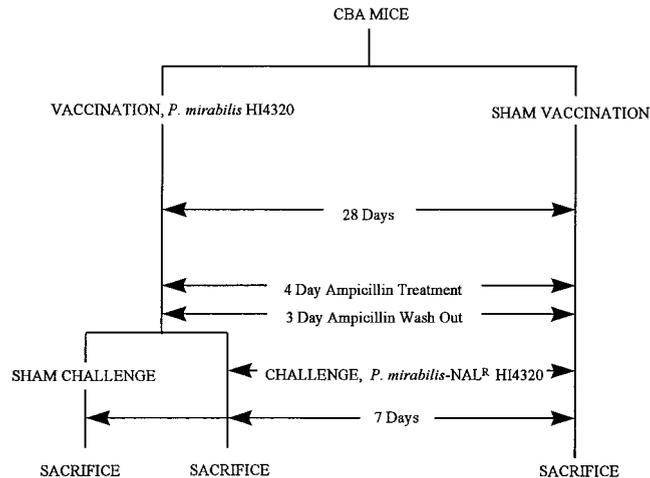


FIG. 1. Flow chart of transurethral vaccination and challenge of mice with *P. mirabilis*.

with 10^6 CFU of *P. mirabilis* Nal^r HI4320 as described above. An additional 10 vaccinated mice were challenged only with 50 μ l of PBS (sham challenge). Mice were examined daily and sacrificed 7 days after challenge (day 42) by using an overdose of CO₂. At sacrifice, the abdomen was opened aseptically by a midline incision and urine was aspirated from the bladder with a tuberculin syringe for quantitative bacteriologic culture. Then, after tying of the proximal end of each ureter, the bladder was washed by injecting and aspirating sterile saline. The bladder and kidneys were removed aseptically: the bladder and one half of each kidney were separately homogenized in PBS using a sterile glass grinder (Kontes, Inc., Vineland, N.J.). Urine and the homogenized tissues were quantitatively cultured on TSA and TSA containing nalidixic acid at 50 μ g/ml by the spread plate technique. The mean number of CFU per milliliter of urine or gram of bladder or kidney was determined after 24 h of incubation at 37°C. Nalidixic acid-containing TSA was used to distinguish the nalidixic acid-resistant challenge strain from any residual nalidixic acid-susceptible strains of the original infection (i.e., the vaccination).

Statistical methods. Mean numbers of CFU per milliliter or gram from cultures of urine or tissue homogenates and mean histologic scores were compared by Student's *t* test. Differences in the numbers of mice with the bladder or kidneys colonized by the challenge organism were compared by chi-square analysis. The correlation between immunoglobulin response and quantitative infection in each mouse was measured by Spearman's rank order correlation coefficient.

Mortality and assessment of colonization. Inoculation (vaccination) with approximately 10^7 CFU of live *P. mirabilis* HI4320 delivered via the urethra into the bladder resulted in the death of 16 (27%) of 60 mice. Of 30 sham-vaccinated (control) mice, none died over the next 35 days. Following 4 days of ampicillin administration, urine cultures of 4 of 44 surviving vaccinated mice were still positive for *P. mirabilis* HI4320, these 4 were not further evaluated. No bacteria were detected in day 35 urine cultures of sham-vaccinated mice. Of the 40 surviving animals whose *P. mirabilis* infection was eradicated by ampicillin, 10 were randomly assigned to a sham challenge group and received only PBS transurethrally. The rest of the vaccinated animals ($n = 30$) and the 30 sham-vaccinated animals were challenged with *P. mirabilis* Nal^r HI4320. Eight (27%) of the vaccination group and nine (30%) of the sham-vaccinated group died within 7 days after the challenge.

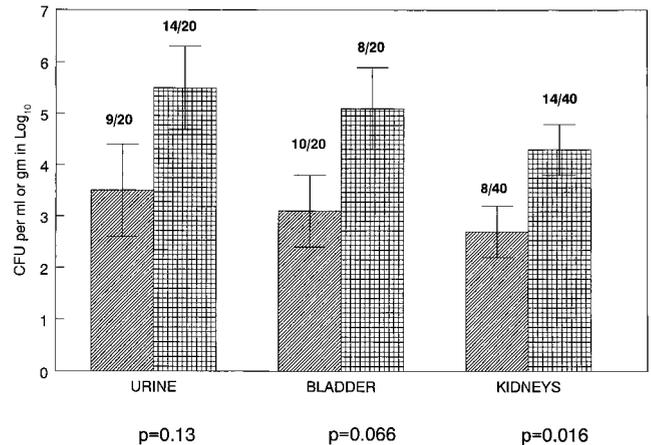


FIG. 2. Colonization of the urine, bladder, and kidneys 7 days after transurethral challenge with *P. mirabilis* Nal^r HI4320 in vaccinated (▨) or sham-vaccinated (▩) mice. The *P* values are for comparisons of mean numbers of CFU per milliliter or gram of urine or bladder or kidney homogenates from vaccinated and sham-vaccinated mice. The fractions above the bars are the numbers of mice colonized at a level of $\geq 10^3$ CFU/ml or g of urine, bladder, or kidney over the total number of specimens examined.

Of the surviving animals on day 42, 20 were selected from each group for assessment of infection by quantitative culture. There was a trend suggesting a modest decrease in *P. mirabilis* UTI among the vaccinated animals (Fig. 2). Mean log₁₀ numbers of CFU of *P. mirabilis* Nal^r HI4320 per milliliter or gram were lower in vaccinated mice than in sham-vaccinated mice: urine, 100-fold less (3.5 [log₁₀] versus 5.5; *P* = 0.13); bladder, 100-fold less (3.1 versus 5.1; *P* = 0.066); kidneys, 40-fold less (2.7 versus 4.3; *P* = 0.016). Examining the prevalence of infection at 7 days, we found that insignificantly fewer vaccinated mice were colonized with *P. mirabilis* Nal^r HI4320 at a level of $\geq 10^3$ CFU/ml or g than were sham-vaccinated mice (urine, 8 of 20 versus 14 of 20 mice [*P* = 0.053]; bladder, 10 of 20 versus 14 of 20 mice [*P* = 0.19]) and mice with at least one infected kidney (10 of 20 versus 14 of 20 mice [*P* = 0.19]).

Histopathology. On day 42, half of each kidney was processed for light microscopy and evaluated by a previously described procedure (10). Acute pyelitis and pyelonephritis were observed in 13 of 20 sham-vaccinated mice, with a mean histologic score of 1.48 ± 1.38 (standard error of the mean [SEM]). No mononuclear infiltrates or fibrosis was observed in the kidneys of those mice. Moderate-to-severe dilatation of the renal pelvis was also observed in those mice. In sham-challenged mice, no acute renal inflammation was observed. Mononuclear inflammation, predominantly lymphocytes (>90% of the cells) confined to the subepithelial connective tissue of the pelvis, was observed in 10 of 10 of those mice, with a mean histologic score of 1.8 ± 1.03 (SEM). There was mild (1+) fibrosis and mild (1+)-to-moderate (2+) dilatation of the pelvis. The renal parenchyma did not show fibrosis or scarring.

In contrast, both acute and chronic changes were seen in the kidneys of mice in the vaccinated group. We observed a mixed pattern of acute pyelitis-pyelonephritis and chronic inflammation in 13 of 20 mice, solely acute pyelitis-pyelonephritis in 2 of 20 mice, and mild-to-moderate chronic inflammation confined to the pelvis and characterized by mononuclear inflammation in 5 of 20 mice. In the mice exhibiting only chronic inflammation, plasma cells accounted for almost half of the inflammatory cells, compared to the predominately lymphocyte response seen in the sham-challenged group. Pelvic dilatation was proportional to the acute inflammation. Mice with chronic

inflammation had normal pelvises or minimal dilatation. The mean histology scores for the vaccinated group were 2.03 ± 1.31 (SEM) for chronic changes and 1.65 ± 1.59 (SEM) for acute changes.

Antibody response to *P. mirabilis*. On days 0 and 35, mice were bled retro-orbitally and serum was evaluated by enzyme-linked immunosorbent assay (ELISA) for antibodies to whole *P. mirabilis* cells. Flat-bottom microtiter wells were coated with antigen prepared as a formalin-inactivated broth culture containing approximately 10^8 CFU/ml of 0.06 M carbonate buffer, pH 9.6. After the wells had been washed three times with PBS containing 0.05% Tween 20 (PBS-T), the nonbinding sites in the wells were blocked by using 3% bovine serum albumin (BSA) in PBS. After the wells had been washed three times with PBS-T, serial twofold dilutions of serum in PBS-T containing 1% BSA were added to the wells and they were incubated at 37°C for 1 h. After the wells had been washed five times with PBS-T, alkaline phosphate-conjugated goat anti-mouse immunoglobulin (Ig) in PBS-T containing 1% BSA was added to the wells and they were incubated for 1 h at 37°C. After the wells had been washed five times with PBS-T, *p*-nitrophenylphosphate in 10% diethanolamine buffer, pH 9.8, was added to the wells and they were incubated for 30 min at 37°C. After the reaction had been stopped by addition of 3 N NaOH, the A_{405} was read (9).

Although all mice tested before vaccination had no detectable titers of antibodies against *P. mirabilis*, by day 35 following vaccination, there was a substantial increase in the serum antibodies among the survivors of the vaccination with live *P. mirabilis* HI4320. Figure 3 shows serum IgG, IgM, and IgA levels for each vaccinated mouse in sera collected on day 35 prior to homologous rechallenge. IgG levels increased in 19 of 20 mice, with a minimum increase of 1:40; 12 of 20 mice had an IgG titer of $\geq 1:360$. Serum IgM levels increased in 16 of 20 mice; 5 of 20 had an IgM titer of $\geq 1:360$. However, serum IgA levels increased in only 3 of 20 vaccinated mice; none had an IgA titer of $\geq 1:360$. The median Ig titers of those 20 vaccinated mice were as follows: IgG, 1:320; IgM, 1:80; IgA, <1:40. The median titers on day 35 of vaccinated mice that died between days 35 and 42 were as follows: IgG, 1:320 (range, 1:80 to 1:2,560); IgM, 1:80 (range, <1:40 to 1:320); IgA, <1:40 (seven mice, <1:40; one mouse, 1:40).

We assessed mortality and infection caused by the *P. mirabilis* rechallenge according to prechallenge titers of Igs to *P. mirabilis* (data not shown). Protection from neither infection nor mortality was associated with elevated titers of either IgG or IgM.

Antibody response to specific antigens. Isogenic mutants of *P. mirabilis* HI4320 were used for detection of serum antibodies directed against specific proteins, including MR/P fimbriae (2), PMF fimbriae (13), urease (11), and flagella (16), by Western blot analysis. For preparation of bacterial proteins, *P. mirabilis* strains were passaged three times in nutrient broth statically for 48 h at 37°C, conditions that favor the expression of MR/P fimbriae (1). The same cultures were used for preparation of PMF fimbriae. For preparation of flagella, bacteria were grown overnight on TSA plates. For preparation of urease, cultures were induced overnight with 100 mM urea. Whole-cell preparations from the wild-type strain and isogenic mutants were solubilized in sodium dodecyl sulfate (SDS)-gel sample buffer, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) as described by Towbin et al. (28). Immunoblots were developed with serum from vaccinated mice with elevated Ig titers. For preparation of MR/P fimbrial antigens, the whole-cell preparation was pretreated

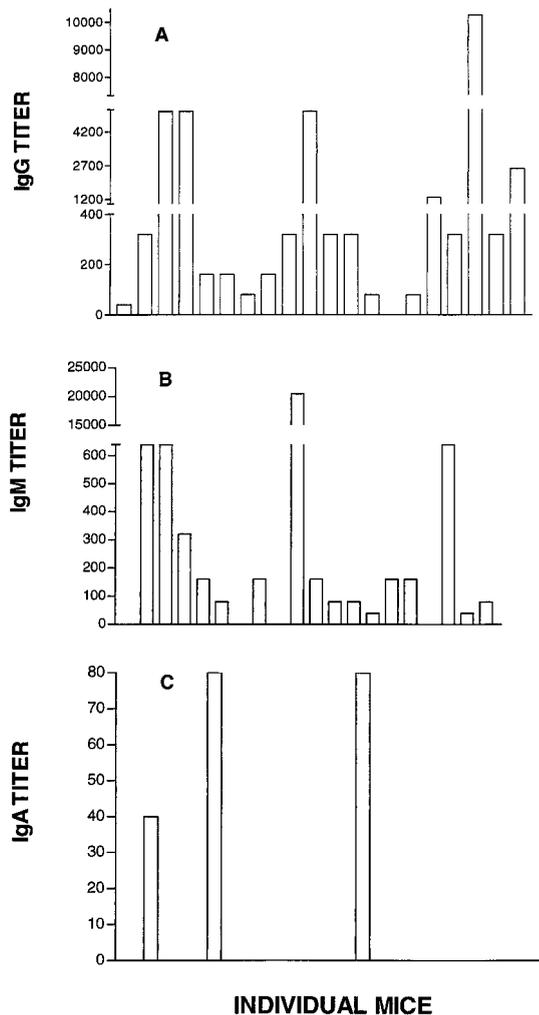


FIG. 3. Serum anti-*P. mirabilis* responses in individual mice 35 days after transurethral vaccination with live *P. mirabilis* HI4320.

with 10% trichloroacetic acid, necessary to denature MR/P fimbriae (1), prior to solubilization in SDS-gel sample buffer.

To identify an antibody response to specific *P. mirabilis* antigens, sera from mice with elevated Ig titers were used for Western blot analysis of protein preparations (Fig. 4). Protein from both the wild-type strain and isogenic mutants deficient in each of four virulence-associated proteins were used to assess a specific Ig response. Strong Ig responses were identified for PMF fimbriae (Fig. 4) and MR/P fimbriae (data not shown) and flagella (Fig. 4), in addition to numerous other, unidentified, surface antigens. No serum Ig response to urease was detected; this was not unexpected, as urease is a cytoplasmic protein (11).

A significant serum Ig response to *P. mirabilis* does not correlate with protection from homologous challenge. The live vaccination resulted in the development of measurable levels of IgG and IgM in the sera of 15 of 20 animals; only 4 animals developed detectable serum IgA levels. Others have reported increases in antibody responses following *P. mirabilis* vaccination. Pazin and Braude (21) reported the development of immobilizing serum antibody to H antigen following intravenous vaccination of rats with formalin-killed *P. mirabilis*. The immobilizing antibody prevented the spread of *P. mirabilis* infection of one kidney through the urinary tract to the uninfected kid-

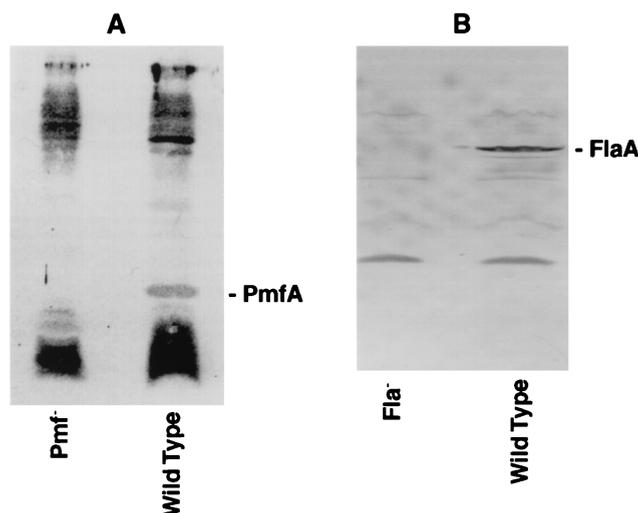


FIG. 4. Western blots using serum from a mouse 35 days after vaccination by transurethral challenge with *P. mirabilis* HI4320 and isogenic mutants. Shear preparations from *P. mirabilis* HI4320 (wild type) and its PMF-negative (A) or flagellum-negative (B) isogenic mutants were used to prepare Western blots. Sera from vaccinated mice were used as primary antiserum for detection of antigen.

ney. Domingue et al. (4) reported substantial increases in hemagglutinin titers (Ig classes not defined) following intravenous vaccination of rabbits with enterobacterial common antigen which resulted in protection from both retrograde and hematogenous pyelonephritis. In our mice, although most of the animals developed measurable serum IgG and/or IgM titers following live vaccination, quantitative titers did not correlate with protection from infection or mortality. Indeed, for those animals with serum IgG levels of $>1,000$, five of six sacrificed on day 42 were infected; two of eight mice that died between days 35 and 42 had IgG titers of $>1:1,000$. On the whole, IgM titers were lower than IgG titers; however, the one animal with a very high IgM titer (1:20,480) became infected and five of eight mice that died had titers of $\geq 1:80$. None of three mice with elevated IgA titers sacrificed on day 42 were infected, but one mouse that died on day 41 had a titer of 1:40.

Experience with many other infectious diseases indicates that natural infection is frequently sufficient to protect against subsequent infection with homologous or similar strains. Why is that not the case in this model of *P. mirabilis* UTI? There are several possible reasons. One is that the use of the whole organism as a live vaccination elicits a variety of Igs, many of which may not be protective. The facts that numbers of women experience recurrent UTIs (26, 27) and that *P. mirabilis* may persist for months in a catheterized urinary tract (30) suggest that there are human analogues to our experimental findings. Might this be different if a pertinent antigen ordinarily present at a low concentration during an infection were presented at a high concentration as a vaccine? There are some indications that this is the case from experimental work with fimbrial tip adhesins of both P fimbriae and type 1 fimbriae in *Escherichia coli* (12, 22). One may need, in addition to the right antigen, the right antibody response to it. Results from the present study are similar to observations by Bluestone and colleagues (3), who evaluated mice 1 and 4 weeks after experimental *E. coli* UTI. At 1 week after infection, serum Ig levels in infected mice were similar to those in uninfected mice. At 4 weeks after challenge, a significant elevation in *E. coli*-specific IgG, but not IgM or IgA, was detected in mice with pyelonephritis; no

significant increase in IgG was detected in mice without pyelonephritis.

Data in the present study suggest that there is a correlation between elevated serum anti-*P. mirabilis* IgA levels and protection from urinary tract colonization upon homologous rechallenge. Four mice demonstrated elevated serum anti-*P. mirabilis* IgA levels following transurethral vaccination, and no organisms were recovered from the urinary tracts of those mice 7 days after homologous rechallenge but one mouse died (data not shown). Although these data are suggestive of a correlation between elevated serum IgA levels and protection from urinary tract colonization, additional studies in which IgA responsiveness is optimized are required.

Use of isogenic mutants to identify Ig responses to specific antigens. As demonstrated in this study, the use of isogenic mutants allows clear identification of the host immune response to specific antigens of the infecting organism through the use of cell lysates containing or not containing a target antigen. We are able to detect the time after challenge when specific bacterial surface antigens are recognized by the host immune response. These results provide information that will likely lead to a more effective immunization strategy by identifying bacterial antigens the host recognizes as important as evidenced by a postinfection antibody response. Vaccination with a purified targeted protein identified by this technique and perhaps the use of a more effective route of antigen presentation for antibody production may lead to enhanced protection from UTI. *Proteus* fimbriae, which might induce antibodies that block bacterial attachment to the uroepithelium, or flagella, which might induce antibodies that immobilize the organisms, may represent vaccine candidates.

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