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Experimental *Escherichia coli* Ascending Pyelonephritis in Rats: Active Peroral Immunization with Live *Escherichia coli*.

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Peroral immunization with a live strain of *Escherichia coli* O6K13H1 against experimental ascending pyelonephritis caused by the same strain was studied in rats, and the effect of immunization on antibody titers against the O and K antigens and lipid A was determined. Peroral immunization with live bacteria protected significantly against pyelonephritis. Sera collected 1 week after infection from the immunized group were increased in immunoglobulin G (IgG) anti-O6 and IgM anti-K13 in comparison with the nonimmunized group. The peroral immunization did not correspondingly affect the response to lipid A. In urine, there was an IgG antibody response to the O6 antigen. In bronchopulmonary secretion, IgM, IgG, and IgA antibodies to O6 were detected. Perorally immunized animals had significantly higher levels of IgG and IgA anti-O6 compared with the nonimmunized group 1 week after infection. Passive transfer of anti-lipid A did not increase resistance against pyelonephritis.

In spite of a follow-up and treatment program aimed at preventing renal scarring, the risk of developing renal parenchymal reduction for girls followed from their first urinary tract infection (UTI) was about 5% (39). Febrile recurrences increased the risk of attracting renal damage (U. Jodal, Ph.D. thesis, University of Göteborg, Göteborg, Sweden, 1974).

Parenteral as well as intravesical immunization with killed *Escherichia coli* protects against experimentally induced pyelonephritis in rats (16), suggesting that vaccination may prevent pyelonephritis in humans also. The protection may be mediated not only by serum antibodies but also via local immunity in the form of a secretory antibody response, as observed in the urine of patients with acute pyelonephritis (17, 33, 36). Orally administered vaccines may induce a secretory antibody response not only in the intestine but also in the secretions from other mucous membranes remote from the intestine, such as lacrimal, salivary, and mammary secretions (7, 30; B. Kaijser, submitted for publication). This route of vaccination has not been tried against experimental pyelonephritis.

E. coli O and K antigens may be suitable components of a vaccine against UTI, as antibodies to these antigens protect against experimental pyelonephritis (16). Lipid A, a component common to gram-negative bacteria (5), may be another component of such a vaccine.

This investigation analyzed the antibody response to *E. coli* O and K antigens and lipid A as measured in serum, urine, and bronchopulmonary secretions after peroral immunization with live bacteria. The protective effects of the peroral immunization and of passively transferred anti-lipid A antibodies against experimental pyelonephritis were tested in rats.

MATERIALS AND METHODS

Bacterial strains. *E. coli* O6K13H1 (World Health Organization designation Su 4344/41) was used for infection and peroral immunization. The bacteria were cultured overnight in nutrient broth. The concentration was determined spectrophotometrically and was adjusted to the desired level after the cultures were washed with phosphate-buffered saline.

Experimental ascending pyelonephritis. Female Sprague-Dawley rats (200 to 250 g) were used. They were infected by injecting the bacteria into the urinary bladder as described earlier (18), and the grade of renal lesions was determined (18) by a modified procedure based on that of McLaren (29).

Bacteria were isolated from the kidneys by pressing a cut surface of the middle part of the kidney onto a Drigalski agar plate.

Immunization procedure and protection against pyelonephritis. (i) Peroral immunization with live bacteria. Two weeks before infection, animals were perorally immunized by tube feeding with live *E. coli* O6 K13H1 diluted in tap water or phosphate-buffered saline. The bacteria were administered on 2 consecutive days, with about 5×10^9 bacteria in a volume of 0.4 to 0.5 ml. Control animals received tap water or phosphate-buffered saline. Immediately before the feeding the animals were slightly anesthetized with ether. The immunized and control animals were kept in separate cages to prevent transfer of the immunizing

strain. The experiment was repeated three times (10 to 20 rats in each group of immunized or control animals per experiment). The immunization procedure did not result in bacteremia, as tested by culturing blood samples taken from 10 rats by heart puncture 5 min after the intubation.

Urine was collected 4 to 7 days after injection of bacteria into the urinary bladder, and serum and bronchoalveolar fluids were taken when the animals were sacrificed. In one experiment fecal specimens were taken 3 days after immunization to determine whether *E. coli* O6K13 bacteria were present.

To probe possible antibody secretion from the urinary bladder, the bladders were removed from eight rats in one immunized group and eight rats in one control group. The bladders were washed in Eagle medium and then incubated with 1 ml of RPMI 1640 (20 mM HEPES [*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid]–1% penicillin-streptomycin; Flow Laboratories) for 24 h at 37°C. The buffer solution was then removed and centrifuged. The supernatants were kept frozen until used.

(ii) Control experiment. The experiment was performed to test whether the infecting organism was transferred from five rats infected in the urinary tract to five noninfected animals kept in the same cage. An additional five rats were kept in a separate cage. Serum and bronchopulmonary lavage were collected 2 weeks after the infection.

(iii) Protection against experimental pyelonephritis by passively transferred anti-lipid A antibodies. Rats were injected intraperitoneally with 0.5 ml of rabbit anti-lipid A serum 3 h before intravesical injection of bacteria. Control rats received normal rabbit serum. This passive transfer experiment was also performed with heat-inactivated sera (56°C, 15 min). Serum samples were collected just before the injection of bacteria from each of five rats in the immunized group and five animals in the control group to check antibody activity to lipid A. The animals were sacrificed 1 or 3 weeks after the infection.

Collecting samples. Metabolic cages were used for collection of urine, and sodium azide was added to the collecting cups to inhibit bacterial growth. Bronchoalveolar fluid was obtained by injecting 5 ml of phosphate-buffered saline into the trachea with a blunt syringe. The solution was withdrawn, and the procedure was repeated 10 times. All samples were stored at −20°C until used.

Serotyping of bacteria. The *E. coli* O and K antigen determinations of the bacterial strains isolated from the kidneys and feces of the rats were performed as described earlier (15, 21).

Antiserum to lipid A. Rabbit antisera were produced by immunizing rabbits with human erythrocytes coated with lipid A (25).

Antibody determination. The enzyme-linked immunosorbent assay (4) was used, as described earlier (23). Anti-rat immunoglobulin G (IgG), IgM, and IgA and anti-rabbit IgG were purchased from Nordic Laboratories. Crude O6K13 polysaccharide and lipid A were used as antigens (23).

Serum, urine and bronchoalveolar lavage fluid were diluted in 10-fold steps. The starting dilutions for anti-O and -K were 1:100 for sera from infected animals, 1:10 for sera collected from rats in the control experiment, 1:1 for urine, and 1:5 for bronchial lavage. The urinary bladder incubation samples were diluted 1:4, and the sera analyzed for anti-lipid A antibodies were diluted 1:10.

The enzyme-linked immunosorbent assay titer was defined as the $\log_{10}$ of the sample dilution that gave an extinction value at 405 nm of 0.125 above the background. Each run was adjusted relative to a reference serum, which was always included. A difference of 0.45 $\log_{10}$ unit (2 standard deviations) or more in the titer between two samples was considered significant (23).

Anti-*E. coli* O6 antibodies were not found in sera (diluted 1:100) or in urine from nonimmunized and noninfected animals. Low anti-O6 titers in bronchoalveolar lavage fluid and lipid A titers in sera diluted 1:10 were infrequently found.

Quantitation of immunoglobulins and rat serum albumin. Quantitation of immunoglobulins and rat serum albumin in serum, bronchoalveolar lavage, and urinary bladder incubation samples was performed by using the Mancini technique with the same antisera as for the enzyme-linked immunosorbent assay (22). Only the relative amounts of the immunoglobulins were measured. Anti-rat serum albumin produced in rabbits was purchased from Nordic Laboratories.

Statistics. The Wilcoxon test for two samples, the chi-square test, and the Spearman coefficient of rank correlation were used.

RESULTS

Protection against experimental pyelonephritis by peroral immunization with live *E. coli* O6K13H1. Significant protection against pyelonephritis ($P < 0.01$) as well as decrease of bacterial growth from the kidneys ($P < 0.05$) were induced by peroral immunization (Table 1). None of the 17 rats had *E. coli* O6K13 bacteria in their stool before immunization, but 10 of 17 (59%) had such bacteria 3 days later.

(i) Antibody response to O6 antigen. The serum antibody response 1 week after UTI is shown in Fig. 1. A significant difference was observed for IgG antibodies ($P < 0.01$) comparing the perorally immunized group with the control group.

Urinary antibodies of the IgM class were only

TABLE 1. Protection against ascending pyelonephritis in rats orally immunized with live *E. coli* O6K13H1[a]

Oral immunization	Rats with given condition/total no. infected (%)			
	Pyelonephritis	*P*[b]	Bacterial growth in the kidneys	*P*
+	5/36 (14)	<0.01	19/36 (53)	<0.05
−	15/33 (45)		26/33 (79)	

[a] Accumulated data from three experiments.
[b] Statistical evaluation performed with chi-square test.

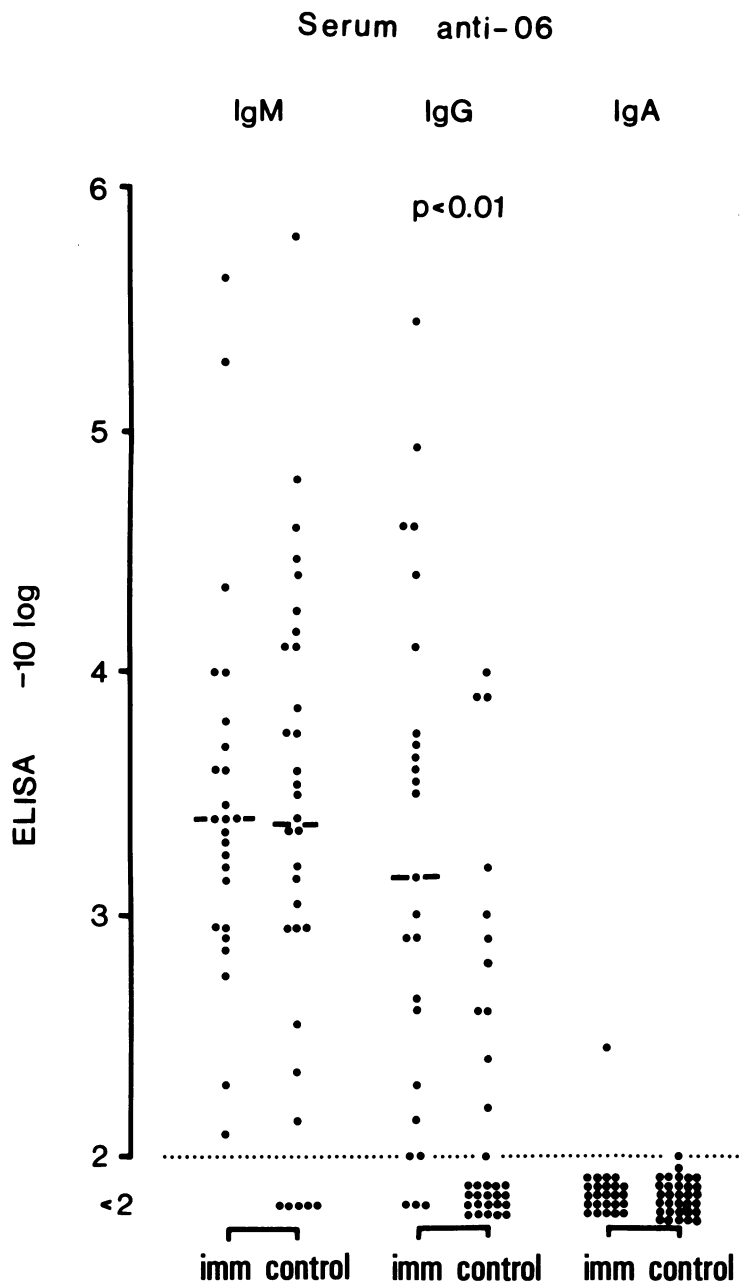

FIG. 1. Serum antibody response to O6 antigen in rats preimmunized orally with live *E. coli* O6K13H1 2 weeks before experimental induction of UTI with the same bacteria (imm). The other group (control) was not preimmunized. The serum samples were collected 1 week after the injection of bacteria into the urinary bladder. Median values for the different groups are indicated. ELISA, Enzyme-linked immunosorbent assay.

observed in 3 of 10 nonimmunized rats. IgG urine antibodies were seen in 5 of 10 immunized and in 2 of 16 nonimmunized rats. No IgA antibodies were recorded. There was no correlation between serum and urinary IgG anti-O6 antibody levels.

The supernatants from the bladder incubations showed IgG anti-O6 antibodies in six of eight perorally immunized rats which had neither pyelonephritis nor bacteria present in their kidneys and in three of eight nonimmunized rats (Fig. 2). Two of these three rats with IgG

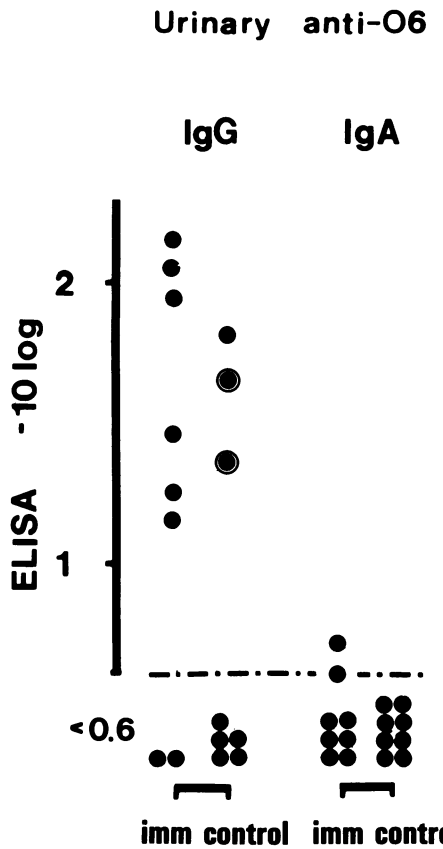

FIG. 2. IgG and IgA anti-O6 antibody titers in the supernatants from rat urinary bladder incubations. The urinary bladders were removed from rats 1 week after the injection of live *E. coli* O6K13H1 into the urinary bladder. One group (imm) was immunized orally 2 weeks before infection Symbol: ◉, pyelonephritis.

antibodies in the nonimmunized group were the only animals analyzed which had macroscopically visible pyelonephritis. The third had bacteria in the kidneys. The serum IgG correlated with the urinary IgG antibody levels ($P < 0.01$, seven paired samples). The amount of IgG relative to rat albumin in the supernatants was roughly twice as large as that in the sera. IgA anti-O6 antibodies were found in two of the immunized rats (Fig. 2).

Pulmonary antibodies were seen in both the perorally immunized and the nonimmunized groups (Fig. 3). Significantly higher levels were observed for IgG and IgA anti-O6 antibodies ($P < 0.02$, $P < 0.01$) among the perorally immunized rats. The pulmonary IgG antibodies correlated with the serum IgG antibodies ($P < 0.01$). Pulmonary and serum IgM anti-O6 antibodies did not correlate. In spite of the pulmonary IgA anti-O6 antibodies recorded, there were no detectable serum IgA anti-O6 antibodies. The concentration of total IgA was about 1.5 times higher in serum than in bronchoalveolar fluid. The concentration of albumin and total IgG in the bronchoalveolar fluid was approximately 1% of that in serum.

(ii) Antibody response to K13 antigen. The IgM antibody response to K13 in serum differed significantly between the perorally immunized and the control groups (Fig. 4). One of the rats in the control group also had IgG antibodies. Bronchopulmonary IgA antibodies were exhibited by three of seven rats in the perorally immunized group compared with none of the seven controls. IgM anti-K13 antibodies were recorded in bronchial lavage fluid in two rats, one from each group. No bronchopulmonary antibodies of the IgG class were detected.

(iii) Antibody response to lipid A. Serum IgM antibodies to lipid A were seen in immunized (11 of 21) as well as in nonimmunized (15 of 24) animals 1 week after the infection, with no difference between the two groups. IgG antibodies were recorded in only two rats, one from each group. IgA antibodies to lipid A were not found in the serum or bronchopulmonary lavage.

Control experiments. Low levels of serum anti-O6 antibodies of the IgG and IgM classes were found in three of five rats kept in the same cage as the infected ones and in two of five unexposed control rats. One rat from each group showed only serum IgM antibodies. With the exception of one rat of those kept in the same cage as the infected rats, the rats of both groups had antibody levels detectable only at a serum dilution of <1:100, whereas the infected rats had higher antibody levels. Bronchopulmonary antibodies were not found in the two control groups, and only one of the infected rats showed these antibodies.

Protection against experimental pyelonephritis by anti-lipid A antibodies. No significant protective capacity of anti-lipid A serum was found with 15 of 49 rats which developed pyelonephritis compared with 21 of 48 rats given only normal rabbit serum.

DISCUSSION

In this study peroral immunization increased the resistance against pyelonephritis and lowered the number of cases with bacteria present in the kidneys. In the orally immunized animals, an enhanced antibody response to O6 and K13 was seen which may have increased by the challenge infection. Since the oral immunization gave rise to increased levels of systemic antibody compared with controls, a transiently bacteremic state could not be excluded. It does not seem likely, however, since no bacteria were

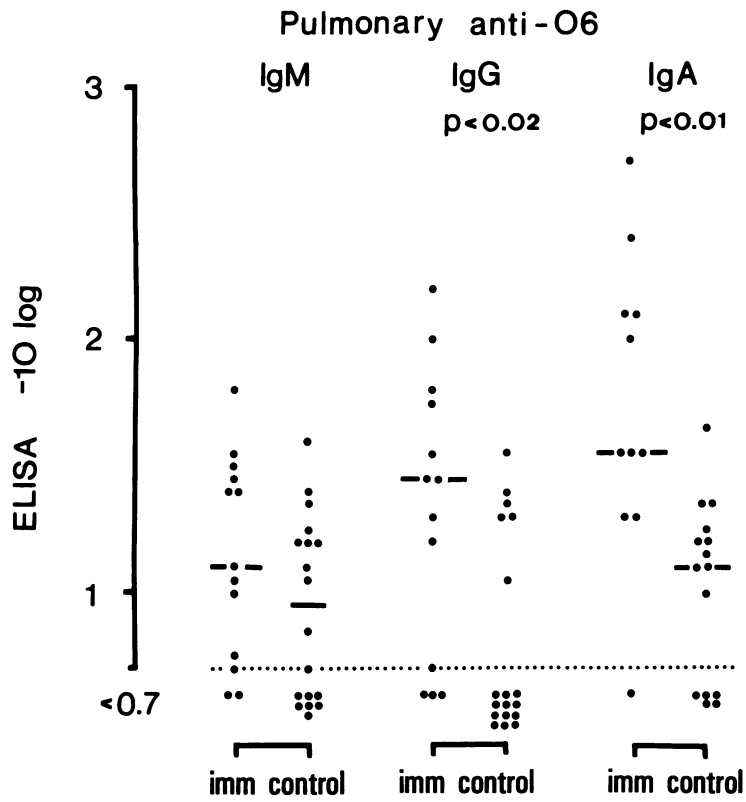

FIG. 3. Pulmonary anti-O6 antibody titers in *E. coli* O6K13H1-infected rats with (imm) or without (control) oral preimmunization. The bronchoalveolar washings were collected 1 week after the urinary bladder injection of live *E. coli* O6K13H1. Median values are indicated for the different groups. ELISA, Enzyme-linked immunosorbent assay.

present in the blood immediately after immunization. In addition, Glode et al. could induce bacteremia by intragastric intubation only in rats aged 15 days or younger and only with *E. coli* K1 strains at a volume of 0.5 ml (6). Furthermore, live *E. coli* O4K12 given perorally to healthy humans also gave rise to systemic as well as secretory antibodies and no adverse reactions were observed (Kaijser, submitted for publication). There are several reports of serum antibodies appearing after oral immunization in humans as well as in experimental animals (3, 11, 12).

The lack of urinary IgA antibodies or significant differences in the IgG levels between the perorally immunized and the nonimmunized groups could be due to the diluted urines used for antibody analysis. The urinary bladder specimens were also diluted before antibody analysis, possibly contributing to the low frequency of IgA antibodies.

The urinary bladder incubation specimens also contained IgG anti-O6 antibodies which correlated to serum antibody titers. The ratio of IgG in these specimens to serum IgG was higher than for that of rat albumin, possibly indicating the appearance of locally produced IgG antibodies. Some studies have shown IgG antibodies to be the predominate antibody locally produced during UTI, with less IgA being found (8, 19, 34). Our present experiments do not permit any conclusions as to the contribution to the protection by serum and urine antibodies or other host defense factors.

The hypothesis of a common mucosal defense system, as proposed by McDermott and Bienenstock (28), is attractive for explaining the protective effect of peroral immunization against the experimentally induced UTI and the appearance of the pulmonary IgA antibody response. In this hypothesis the gut-associated lymphoid tissue and also the bronchus-associated lymphoid tissue play central roles in the development of lymphoid cells homing to various mucosal sites.

The pulmonary IgG antibody titers correlated to the serum titers. This was not the case for the pulmonary IgA antibodies, which might be locally produced since no serum IgA antibodies could

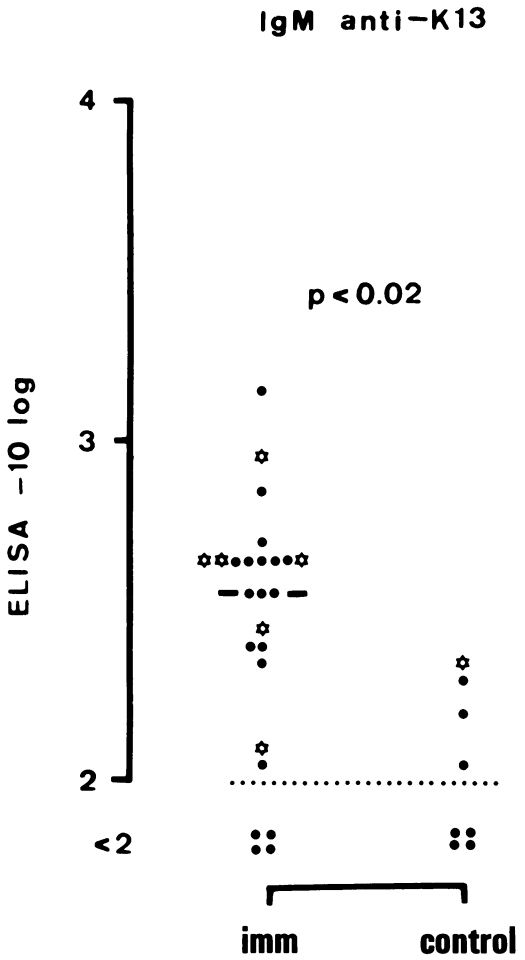

FIG. 4. Serum IgM anti-K13 antibody levels in rats orally preimmunized (imm) with live *E. coli* O6K13H1 and infected 2 weeks later with the same organism. The control group was not preimmunized. Median values are indicated. Symbol: ☆ no significant increase compared with serum titer before immunization and infection. ELISA, Enzyme-linked immunosorbent assay.

be recorded (9). The measurement of total concentrations of IgA and IgG in serum and bronchoalveolar fluid also implicates local production of IgA. It is possible, however, that IgA antibodies could have been recorded at a lower serum dilution and also that the IgA antibodies might have been cleared from the circulation by binding to secretory components on epithelial cells being transported into the secretions of the bronchi and other mucosal sites. Secretory component-producing epithelial cells have been found in many organs, including the bronchi and the renal tubuli (20, 35). Furthermore, the serum IgA concentration in rats is very low, and the IgA is present mostly in monomeric form (1, 13).

Vaccination via the oral route against infections starting on mucous membranes might be useful since both mucosal and systemic immune responses may be achieved. Adverse effects by parenteral administration of a vaccine might also be avoided. Peroral immunization favors the synthesis of secretory antibodies (secretory IgA), and such antibodies may contribute to the protection (10).

As the *E. coli* acidic polysaccharide capsular antigens are regarded as a virulence factor (14), it is of importance to evoke an antibody response against such antigens. Only IgM antibodies were detected in serum, in agreement with the findings of only IgM anti-K13 in the serum of rats with long-term pyelonephritis caused by *E. coli* O6K13 (23). The finding of pulmonary IgA anti-K13 antibodies in three of seven orally immunized rats is notable.

Passive transfer of mainly IgG anti-lipid A antibodies did not protect animals against pyelonephritis, in agreement with previous protection experiments in other animal models (2, 26, 27, 31). Our results suggest that serum anti-lipid A is not preventing bacteria from invading the urinary tract and support the suggestion that lipid A is not exposed on the bacterial surface (24).

Nevertheless, antibodies to lipid A have been found in humans with UTI, and particularly high titers have been observed in patients with acute upper UTI and renal scarring (22a, 32, 38). On the basis of experimental data, Westenfelder et al. have suggested lipid A to be involved in the pathogenesis of renal damage (37).

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