Prevention of Renal Scarring from Pyelonephritis in Nonhuman Primates by Vaccination with a Synthetic Escherichia coli Serotype O8 Oligosaccharide-Protein Conjugate

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Received 9 February 1993/Returned for modification 19 March 1993/Accepted 13 September 1993

Rhesus monkeys were vaccinated with a synthetic Escherichia coli serotype O8 oligosaccharide-protein conjugate. Using our experimental pyelonephritis monkey model, we tested whether such immunization was protective against the renal damage from inflammation following experimental infection with a P-fimbriated O-antigenically homologous E. coli strain. The vaccination did not significantly alter the duration of bacteruria or interfere with the infection. However, the vaccine was efficient in renal protection, as vaccinated animals showed significantly less intratubular infiltration of neutrophils (P < 0.02) and the degree of renal scarring was also significantly less in these animals (P > 0.05) than in the control animals. Total kidney involvement in the vaccinated animals was 16.9%, compared with 32.5% in the control animals (P = 0.07).

Acute pyelonephritis is a cause of renal damage. Ten percent of the end-stage renal disease cases children and adolescents are due to chronic pyelonephritis. In most patients without urinary tract obstruction, pyelonephritis is an ascending infection caused by certain clones of P-fimbriated Escherichia coli (29).

To study the pathogenesis of acute ascending E. coli-induced pyelonephritis, we have established an experimental monkey model which closely resembles the disease in humans, as nonhuman primates possess the same P fimbriae receptors as humans (17). P fimbriae enable the infecting organism to adhere to the uroepithelium by specific recognition of glycolipids containing the α-D-Galp-(1-4)-β-D-Galp carbohydrate moiety. This appears to be an essential virulence factor for pyelonephritogenic strains, particularly in the initiation of the infection and its ascent to the kidney in the absence of vesicoureteral reflux (22). Another bacterial component that is important in the pathogenesis of E. coli-induced pyelonephritis is the bacterial endotoxin (lipopolysaccharide [LPS]), which is the major stimulus for tissue inflammation and damage (3).

Since both P fimbriae and LPS are important virulence factors, they may also be important molecules in the design of a subcellular vaccine against pyelonephritis. Several investigators have studied immune responses to LPS and suggested that anti-LPS immunity is essential for protection against pyelonephritis. For example, Braude and Siemieniasky showed that immunization of rats with O-antigenically homologous LPS reduced the severity of later experimentally induced E. coli pyelonephritis (4). It could not be determined in these earlier experiments how much of the protection was due to actual anti-LPS immunity rather than induced endotoxin tolerance, which McCabe has shown to occur in experimental pyelonephritis (12). The lipid A moiety is the portion of the LPS molecule that is responsible for its toxic and inflammatory activities. We have previously shown that in the monkey model, immunization with purified P fimbriae yields protection against experimental pyelonephritis (8, 16, 19). The lipid A moiety is the portion of the LPS molecule that is responsible for its inflammatory activity. We have, however, found that it is possible to specifically down-regulate the fever response induced by endotoxin in rabbits by prior immunization with a lipid A-free synthetic oligosaccharide-carrier protein conjugate, provided that it was O-antigenically homologous to the endotoxin used for the challenge (24). These findings prompted us to test whether vaccination with an E. coli O-antigen-specific carrier protein conjugate might abrogate some of the local adverse inflammatory responses in our acute pyelonephritis monkey model and whether this vaccination might diminish renal scarring.

MATERIALS AND METHODS

Bacteria. A nonhemolytic O8 E. coli strain with P fimbriae but not type 1 fimbriae was used. It was obtained from a patient with acute pyelonephritis.

Preparation of LPS, O polysaccharide, and oligosaccharides. O polysaccharide from E. coli O8 LPS was prepared by weak alkaline treatment of the isolated LPS (0.15 M aqueous NaOH, 100°C, 3 h), after which the pH was adjusted to 5.0 and released previously ester-bound fatty acids were removed by extraction with petroleum ether. After subsequent centrifugation, the clear supernatant was extensively dialyzed against distilled water and the partially delipidated LPS was then subjected to acidic hydrolysis (0.1 M aqueous HCl, 95°C, 2 h). After renewed extraction and high-speed centrifugations as described above, the supernatant was extensively dialyzed against distilled water and finally lyophilized.

Heptasaccharide of E. coli serotype O8 specificity was

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obtained from the O polysaccharide by bacteriophage O-8-associated endomannosidase-mediated cleavage as previously described (7). The purity and identity of the heptasaccharide preparation were checked by sugar and methylation analysis followed by gas-liquid chromatography-mass spectrometry of the partially methylated alditol acetate derivatives (10, 11). The identity of the heptasaccharide was also ascertained by high-resolution nuclear magnetic resonance. Spectra in solutions in deuterium oxide were recorded at 27°C (13C) or 70°C (1H) with a JEOL GX 270 instrument. Chemical shifts were expressed in parts per million relative to internal 1,4-dioxane 67.4 downfield from external tetramethylsilane for 13C and internal acetone 2.21 downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate for 1H. Both the 1H and the 13C spectra were in accordance with those earlier reported.

Preparation of oligosaccharide-BSA conjugate. Synthesis of the heptasaccharide 2-(4-isothiocyanatophenyl)-ethylamine derivative was performed as described earlier (24–28). The heptasaccharide isothiocyanatophenyl-ethylamine derivative was then reacted with the free ε-aminosyl groups of bovine serum albumin (BSA) for 48 h at room temperature, and the conjugate was extensively dialyzed against distilled water and lyophylized. After concentration, the conjugates were purified by gel chromatography on a Bio-Gel P150 column (2.5 by 90 cm) eluted with phosphate-buffered saline. The degree of substitution for the heptasaccharide-BSA conjugate was 20 mol/mol as estimated from its apparent mobility upon sodium dodecyl sulfate polyacrylamide gel electrophoresis and its protein and carbohydrate contents.

Immunization of rabbits. To test the specificity of the conjugate two rabbits were inoculated with the E. coli serotype O8 oligosaccharide-BSA (O8 Os-BSA) conjugate. The conjugate, which had been suspended in Freund's complete adjuvant (1/1), was injected directly into the popliteal lymph nodes of New Zealand White rabbits (2 to 3 kg each) in order to establish that the conjugate-elicited antibodies recognized the specific target O8 LPS antigen. Both the primary dose and the booster doses on days 14 and 21 were 20 μg per rabbit. The rabbits were bled before priming and on day 28.

Vaccination and experimental infection of monkeys. Adult female rhesus monkeys (Macaca mulatta) were used. The protocol for immunization with the O8 Os-BSA conjugate was similar to that used in previous studies with purified P fimbriae (8, 9, 16). The first injection was given in the inguinal lymph nodes and was followed by monthly subcutaneous injections of 100 μg per animal per injection. Injections were continued until the serum gave an antibody titer of 3,000 or more. Then the monkeys were infected. Eight rhesus monkeys were used for this study, four controls and four immunized monkeys.

With a cystoscopically introduced ureteral catheter, one kidney was inoculated with 0.6 ml of a solution containing 106 E. coli, sodium diatrizoate, and Hippuran 1 131. Inoculation was at the point of pyelotubular backflow as monitored by fluoroscopy.

In addition, blood samples taken at 1, 10, and 60 min were cultured and counted in a gamma counter to ensure that pyelovenous inoculation did not occur. Urine samples for culture were obtained by suprapubic bladder puncture. All challenge experiments were done by using ketamine tranquilization with the P-fimbriated E. coli strain SS385 (serotype O8), which was isolated from a patient suffering from acute pyelonephritis.

Immunological studies. Sera from the animals were tested in enzyme-linked immunosorbent assays (ELISA) for anti-O8 LPS immunoglobulin G antibodies, with the corresponding purified LPS as the coating antigen. Total serum hemolytic complement was determined by using rabbit anti-sheep erythrocyte antiserum. Sheep cells were sensitized by incubation with the rabbit antiserum, and the amount of hemolytic complement in the monkey sera was then assessed by the ability of the sera to lyse the sensitized sheep erythrocytes as measured by the optical density of the supernatants at 541 nm (20).

Radiologic studies. Radiologic studies prior to and after infection which included voiding cystograms showed that no animal had vesicoureteral reflux. The contrast material was introduced by suprapubic bladder puncture, and urine for culture was obtained in the same way.

Quantitative renal scintillation studies were done by using Hippuran I 131 (injected intravenously) for determination of effective renal plasma flow (ERPF) and excretion time of radionuclide, using a nuclear Searle scintillation camera (23).

Histology. Animals were sacrificed 4 weeks after infection by an overdose of intravenous barbiturate. Kidneys, ureters, and bladder were obtained aseptically. Kidneys were weighed, samples were taken for histology, and the remainder was used for culture. The standard section was through the midportion to contain the cortex, medulla, and papilla. (Samples from ureters and bladders were also taken.) For histology, the tissue was fixed in 10% formalin and stained with hematoxylin and eosin. Histologic sections were graded according to established histologic parameters (6) on hematoxylin- and eosin-stained slides. The late response with fibrosis and scarring and a mononuclear cell infiltrate, especially in the subcapsular, pelvic, and periglomerular regions, were considered to indicate subacute to chronic pyelonephritis and are the typical findings following an untreated infection in our experimental model. Scores ranged from 1 to 4, with 4 being the most severe. The sections were rated for the following parameters: tubular and/or interstitial neutrophils, tubular and/or interstitial mononuclear cells, fibrosis, scarring, pelvic infiltrate, glomerular change, subcapsular invasion, tubular dilatation, and tubular atrophy. In addition, the percentage of kidney involved was estimated on the basis of the amount of kidney on the hematoxylin- and eosin-stained slide of the standard section. This rating was done in a blinded fashion by the pathologist (19). The renal cortex and the renal medulla were rated separately for statistical analysis, for pathology sometimes occurs in one area and not the other.

Statistics. Statistics were determined by the student t test for independent samples and two-way analysis of variance.

RESULTS

The E. coli serotype O8 Os-BSA conjugate was quite efficient in eliciting high-level immunoglobulin G antibody responses in rabbits when administered with Freund's complete adjuvant. After two booster injections (days 14 and 21), the two rabbits exhibited anti-E. coli O8 LPS titers of 25,000 and 32,000 at 4 weeks. The elicited antibodies bound specifically to the same O8 oligosaccharide conjugated to edestin in ELISA. The specificity of the rabbit antibodies was further ensured by ELISA inhibition experiments with the isolated and purified E. coli serogroup O8 O-polysaccharide side chain as the inhibitor. In the nonhuman primate experiments the vaccine was administered without any adjuvant. When the rhesus monkeys vaccinated with the O8 Os-BSA
conjugate vaccine showed an anti-O8 immunoglobulin G titer of at least 3,000, they were experimentally infected with the P-fimbriated E. coli strain SS385 (serotype O8) through a cystoscopically introduced ureteral catheter. Fluoroscopy performed during the infection showed that renal inoculation occurred in all animals by pyelotubular backflow. That pyelovenous inoculation did not occur during infection was evidenced by the finding that all blood samples taken at 1, 10, and 60 min after injection were negative upon culture and also by the fact that no Hippuran I 131 could be detected in the same blood samples.

As expected, the O8 Os-BSA conjugate vaccine did not decrease the duration of bacteriuria (7 days in vaccinated animals and 8.8 days in controls). The ERPF in both groups of animals was measured before infection and at days 2, 7, 14, and 21 by quantitative renal scintillation with Hippuran I 131. In the group of vaccinated animals the ERPF had returned to baseline values by day 21, while the controls still showed lower ERPF values (Fig. 1). The groups were not different statistically when analyzed by analysis of variance (P = 0.23).

The pathology of the animals that were vaccinated with the O8 Os-BSA conjugate vaccine included a significant decrease in renal scarring at sacrifice compared with that in control animals (P < 0.0065) (Table 1). The degree of total kidney involvement was 16.9% in the vaccinated animals, compared with 32.5% in the control animals (P = 0.07). This might explain the fact that renal weights were essentially the same while the amount of neutrophils was significantly less in the immunized group: neutrophils were found only in the involved areas, and this inflammatory response is assumed to be the reason for the weight. Both groups of animals showed loss of infected renal weight following the infection, and there was no statistical difference between kidney weights in the animals immunized with the O8 synthetic conjugate vaccine and those in control animals.

The subsequent experimental infection caused a further anti-O8 antibody response in the vaccinated animals (Fig. 2). In the vaccinated group the mean titer at 1 week after infection rose from an initial 5,800 to 10,400, while the corresponding figures for the control animals were 100 and 1,000, respectively.

**DISCUSSION**

In this study a synthetic E. coli serotype O8 oligosaccharide-protein conjugate vaccine protected against renal dam-

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**TABLE 1. Vaccination with synthetic vaccines**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control infection (n = 4)</th>
<th>Vaccinated monkeys (n = 4)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular</td>
<td>0.9</td>
<td>0.4</td>
<td>0.02</td>
</tr>
<tr>
<td>Interstitial</td>
<td>0.2</td>
<td>0.1</td>
<td>0.28</td>
</tr>
<tr>
<td>Mononuclear cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular</td>
<td>0.5</td>
<td>0.1</td>
<td>0.06</td>
</tr>
<tr>
<td>Interstitial</td>
<td>2.2</td>
<td>1.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Scarring</td>
<td>1.8</td>
<td>0.8</td>
<td>0.006</td>
</tr>
<tr>
<td>Pelvic infiltrate</td>
<td>1.5</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Glomerular change</td>
<td>1.8</td>
<td>1.0</td>
<td>0.08</td>
</tr>
<tr>
<td>Subcapsular invasion</td>
<td>2.5</td>
<td>1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Tubular atrophy</td>
<td>2.1</td>
<td>1.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Kidney involvement</td>
<td>32.5</td>
<td>16.9</td>
<td>0.07</td>
</tr>
</tbody>
</table>

* Scores were assigned as described in the text.

* Calculated by Student's t test.
age induced by a P-fimbriated O-antigenically homologous \textit{E. coli} strain in an ascending model of acute pyelonephritis in monkeys. The synthetic conjugate vaccine significantly decreased the degree of renal scarring and also decreased the intratubular infiltration of neutrophils.

All earlier studies of the protective effect of LPS immunity in pyelonephritis as well as other studies of the biological effects of LPS have employed products containing lipid A, either in its native state or after various chemical modifications (1, 5, 12, 13). Therefore, it has previously been impossible to determine whether immunity directed solely against the O polysaccharide can mediate protection against experimental pyelonephritis. The present studies demonstrate unequivocally that the O-polysaccharide antigen alone, when made immunogenic by covalent linkage to a carrier protein, is sufficient for the induction of protection against renal scarring.

With the same model we have previously investigated the series of systemic and local inflammatory events that ultimately lead to renal scarring and dysfunction (15, 21). From these as well as other studies, it became evident that the bacterial endotoxin was an important trigger of the inflammatory response both locally in the kidney and in the more systemic reactions (2, 18). It is known that the portion of the LPS molecule that endows it with its strong ability to induce fever and inflammation is exclusively associated with its lipid A moiety. Therefore, it was interesting that the synthetic O8 Os-BSA conjugate vaccine significantly reduced the renal damage. We believe this is due to interference of the anti-O8 antibodies with the presentation of endotoxin to responsive host cells. They neutralized the endotoxin effect, or induced "endotoxin tolerance."

In contrast to our previous studies with P-fimbria vaccines, the \textit{E. coli} O8 Os-BSA conjugate vaccine did not decrease the duration of bacteriuria. This was expected, since the conjugate vaccine was not designed to interfere with adherence, which is the most important factor for persistent bacteriuria (8, 9, 16, 19).

\textit{E. coli} clones which cause acute pyelonephritis in humans and monkeys are of a limited number of different O serotypes. Thus, from these and previous studies (29), we suggest that a subcellular vaccine against pyelonephritis should contain a combination of P fimbriae (to prevent colonization and ascent to the kidney) and a repertoire of the common O serotypes to prevent endotoxin-induced inflammation.

ACKNOWLEDGMENTS

This work was supported by USPHS grants RR 00164 and DK 14681.

REFERENCES


