INFECTION AND IMMUNITY, Aug. 1982, p. 749-754
0019-9567/82/080749-06$02.00/0

Vol. 37, No. 2

Serological Response to *Escherichia coli* Pili in Pyelonephritis

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Received 16 February 1982/Accepted 16 April 1982

The serological response to *Escherichia coli* pili was studied in 4 adult male patients with pyelonephritis and in 11 uninfected controls. Antibody to the specific pilus antigen of the infecting strain of each patient was measured with indirect immune electron microscopy. Antibody to a cross-reacting antigen of the type 1 pili of heterologous *E. coli* 346 was quantitated with an enzyme-linked immunosorbent assay. Few antipilus antibodies were found in the serum of control patients with either technique. All pyelonephritic patients developed an increase in specific antipilus antibodies belonging to the immunoglobulin classes G, A, and M. Antibodies to the cross-reacting type 1 pilus antigen were found in the serum of three of the four patients and were predominantly immunoglobulin G. Few or no antipilus antibodies were present in the urine of infected or control patients. These results suggest that a serological response to the pili of infecting organisms develops after pyelonephritis.

Pili are filamentous, nonflagellar appendages found on many gram-negative bacteria. There is increasing evidence that pili are important for bacterial virulence in the urinary tract. Pili mediate adherence to the surface of many cells, including uroepithelial cells (3, 5, 10), and the adhesiveness of different isolates of *Escherichia coli* can be correlated with their uropathogenicity (11). In experimental ascending pyelonephritis, heavily piliated strains are more virulent than lightly piliated organisms (6), and immunity to pili has been shown to confer protection (7).

These considerations have encouraged speculation that pili may prove to be useful vaccine antigens to prevent urinary tract infection (UTI). Little is known, however, about naturally acquired immunity to pili. In this study, we investigated the antibody response to the pili of *E. coli* in patients with pyelonephritis by using two immune assays. Immune electron microscopy (IEM) permitted the detection of antibodies to the specific pilus antigen of each infecting strain, and enzyme-linked immunosorbent assay (ELISA) detected antibodies to type 1 pili.

MATERIALS AND METHODS

The serum and urine of four consecutive patients admitted to the Veterans Administration Medical Center of Sepulveda, Calif. with *E. coli* pyelonephritis were studied. The diagnosis of pyelonephritis was supported by the presence of appropriate clinical findings and by a positive antibody-coated bacterium test (12). Each patient denied any previous UTI, and none had any clinical or radiological evidence of structural abnormality. The initial serum was obtained within 24 h of the bacteremic episode of each patient, but it was not possible to date precisely the onset of the infection. Additional samples were obtained serially for 4 to 14 weeks. Urine samples were concentrated fivefold by ultrafiltration. Eleven patients who were admitted to the orthopedic ward and who denied any present or previous history of UTI were included as a control group. A single serum and urine specimen was obtained from each of the uninfected individuals. The mean ages of the patients with pyelonephritis and of the uninfected individuals were 60.6 and 52.8 years, respectively. All were male.

The ELISA test was performed by the following modification of the method of Buchanan (2). Polystyrene tubes were coated by incubation overnight at 37°C with 1 μg of purified *E. coli* 346 pili per ml, suspended in 0.005 M Tris buffer (pH 8). Observation in our laboratory indicated that the type 1 pili of this strain cross-react with many other strains (data not shown). After the tubes were washed three times with a mixture containing 0.01 M phosphate buffer (pH 7.0), 0.85% normal saline, and 0.05% Tween 20 (PBST), a sample of patient serum diluted 1:10 or of concentrated urine was added and incubated at 37°C for 30 min. The tubes were washed again three times in PBST, and 1:250 dilution of peroxidase-conjugated goat antibodies to human immunoglobulin G (IgG), IgM, or IgA (Miles Laboratories, Inc., Elkhart, Ind.) was added. The tubes were then incubated as before. After a final threefold rinse with PBST, the peroxidase substrate *o*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) plus H_2O_2 was added, and the tubes were incubated at room temperature in the dark. The reaction was stopped after 30 min by adding 8 N sulfuric acid, and the intensity of the color that developed was quantitated spectrophotometrically. All determinations were done in duplicate. Each serum sample was assayed on two separate occasions.

Pili were purified by a modification of the method of Brinton (1). In brief, bacteria were collected from static broth cultures of tryptic soy broth, and the pili were removed by mechanical shearing and purified by

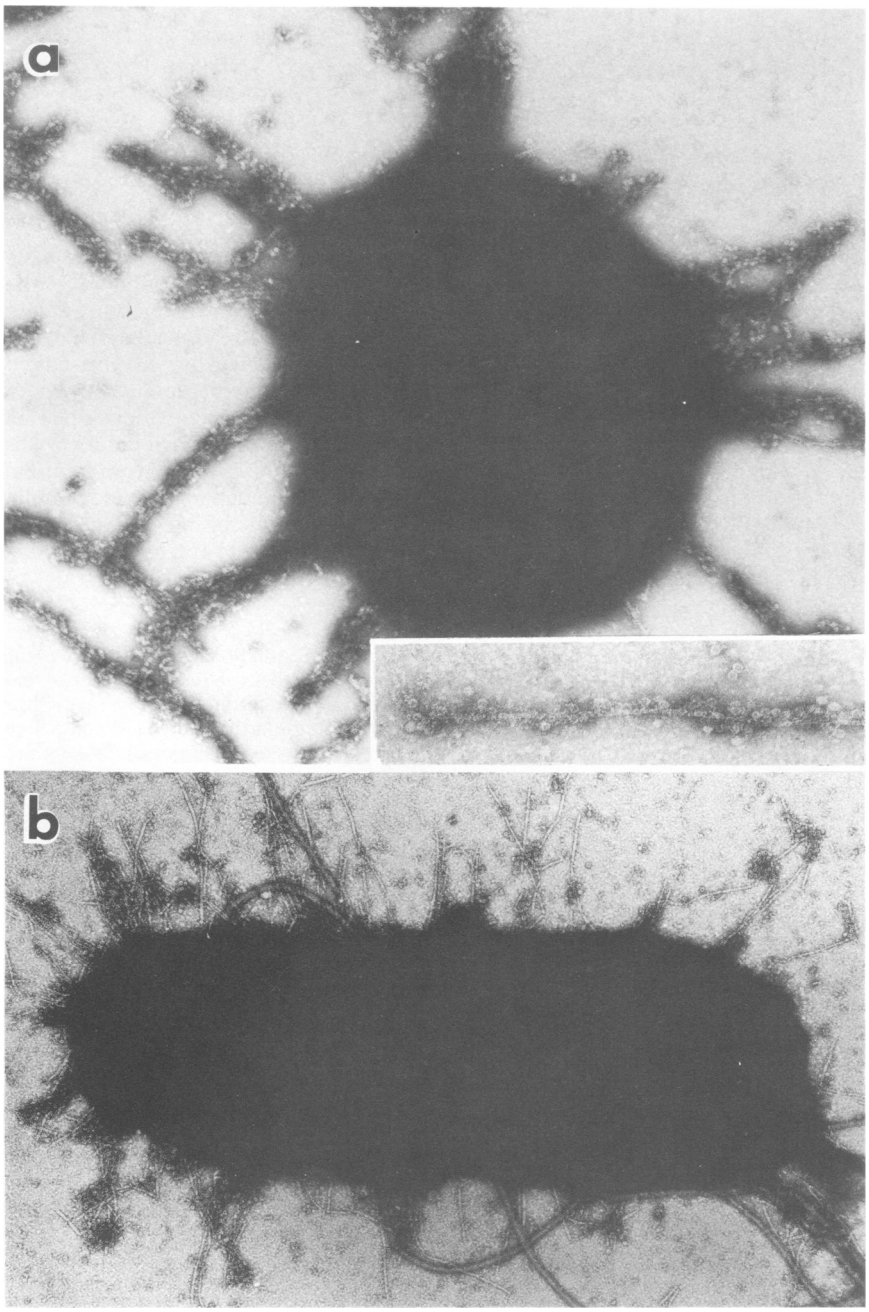

FIG. 1. Representative examples of antipilus antibodies whose titers were determined by IEM. Bacteria were exposed sequentially to serum and ferritin-conjugated anti-immunoglobulin and were then negatively stained. (a), ++++ reaction. All pili are heavily coated all along their length. ×50,000. (Inset), Doughnut-shaped ferritin molecules aligned along pilus. ×112,500. (b) ++ reaction with definite labeling of some but not all pili. ×50,000.

repeated cycles of precipitation with magnesium chloride and differential centrifugation. The purity of the pilus antigen was evaluated by electron microscopy and gel electrophoresis.

Because the ELISA technique is sensitive enough to give a positive reaction to minor contaminants of the pilus antigen, we extracted both lipopolysaccharide (LPS) and outer membrane protein (OMP) and measured antipilus antibodies in a high-titer serum before and after absorption with these components. LPS and

TABLE 1. Initial and maximum antipilus antibody activity in sera of patients with pyelonephritis

Patient	ELISA[a] (optical densisty units)			IEM[b] (reciprocal dilution)		
	IgG	IgM	IgA	IgG	IgM	IgA
1						
Initial	0.10	0.00	0.02	32	16	0
Maximum	0.47	0.00	0.02	512	256	256
2						
Initial	0.58	0.32	0.12	ND[c]	ND	ND
Maximum	0.76	0.42	0.18	128	32	32
3						
Initial	0.01	0.00	0.00	ND	ND	ND
Maximum	0.10	0.00	0.00	2	Undiluted	Undiluted
4						
Initial	0.03	0.01	0.00	4	0	0
Maximum	0.04	0.02	0.01	64	16	16
Controls (mean ± SD)	0.03 ± 0.01	ND	ND	0	ND	ND

[a] ELISA used heterologous pili from *E. coli* 346 as the antigen.
[b] IEM used the homologous strain as the antigen.
[c] ND, Not done.

OMP were obtained by the phenol extraction method of Westphal as modified by Sutherland (19). The *E. coli* isolated from patient 1 was grown to log phase in tryptic soy broth, and 100 ml of the bacterial suspension was mixed with phenol (45%, wt/vol) for 1 h at 60°C. The mixture was centrifuged at 10,000 rpm for 30 min at 0°C. A diphasic mixture was obtained, with the upper aqueous layer containing the LPS and OMP and the lower phenolic layer containing the remaining proteinaceous material. The aqueous phase was aspirated, dialyzed for 48 h against water, and concentrated 10-fold with polyethylene glycol. A 1-ml portion of the extract was added to glass tubes and incubated at 37°C for 24 h to allow the LPS and OMP to adhere to the tube walls. The contents of the tubes were poured out, and 1 ml of the serum of patient 1 was added and incubated at 25°C for 10 min. The absorption was repeated five times. The serum was clarified by centrifugation, and the antipilus activity of the absorbed serum was compared with unabsorbed serum by ELISA as described above.

IEM was performed as follows. Copper grids coated with carbon and Parlodion were placed on drops of bacteria grown for 24 to 48 h in tryptic soy broth. Bacteria adhered spontaneously to the grid surface. The grids were next washed in a drop of distilled water. Each grid was then placed sequentially on drops of diluted serum or urine from the patient, distilled water, and a 1:250 dilution of ferritin-conjugated goat anti-human immunoglobulin. Ferritin conjugates of antibodies to IgG, IgM, and IgA (Miles Laboratories) were used to identify antibodies of these classes. The amount of ferritin labeling of the pili was evaluated by electron microscopy and graded on a relative scale. The titer was defined as the reciprocal of the highest dilution in which labeling of the pili could still be discerned (Fig. 1).

The hemagglutination pattern of each organism was evaluated as follows. A drop of bacterial suspension (about 10^{10} organisms) and a drop of guinea pig or human erythrocytes (3% [vol/vol] in normal saline) were mixed on a porcelain tile and rocked gently for 10 min. Mannose-sensitive (MS) hemagglutination was defined as agglutination of guinea pig erythrocytes at 22°C that could be inhibited by addition of 2.5% D-mannose. Mannose-resistant (MR) hemagglutination was defined as agglutination of human erythrocytes at 4°C which could not be inhibited by 2.5% D-mannose.

RESULTS

The specificity of the two immunoassays used in this study was supported by the observation that few or no antibodies to type 1 pili of strain 346 were found in the serum of control patients by either ELISA or IEM (Table 1). Furthermore, absorption of a serum sample with LPS and OMP did not reduce its ELISA titer, indicating that antibodies detected by this technique were not directed to these potential contaminants (Table 1). The comparability of the two techniques was supported by the observation that antibodies to the type 1 antigen of strain 346 were detected in the serum of patient 1 by both IEM and ELISA (Fig. 2 and Table 1).

Although there was a considerable range of variation in the maximal titers, each of the four patients studied had an increase in antipilus antibodies demonstrated by one or both immunoassays (Table 1). Antipilus antibodies were detected in three of the four patients by ELISA and in all patients by IEM. IgG antibodies were found with both techniques, but IgM and IgA antibodies were more consistently demonstrated with IEM. Few or no antipilus antibodies were

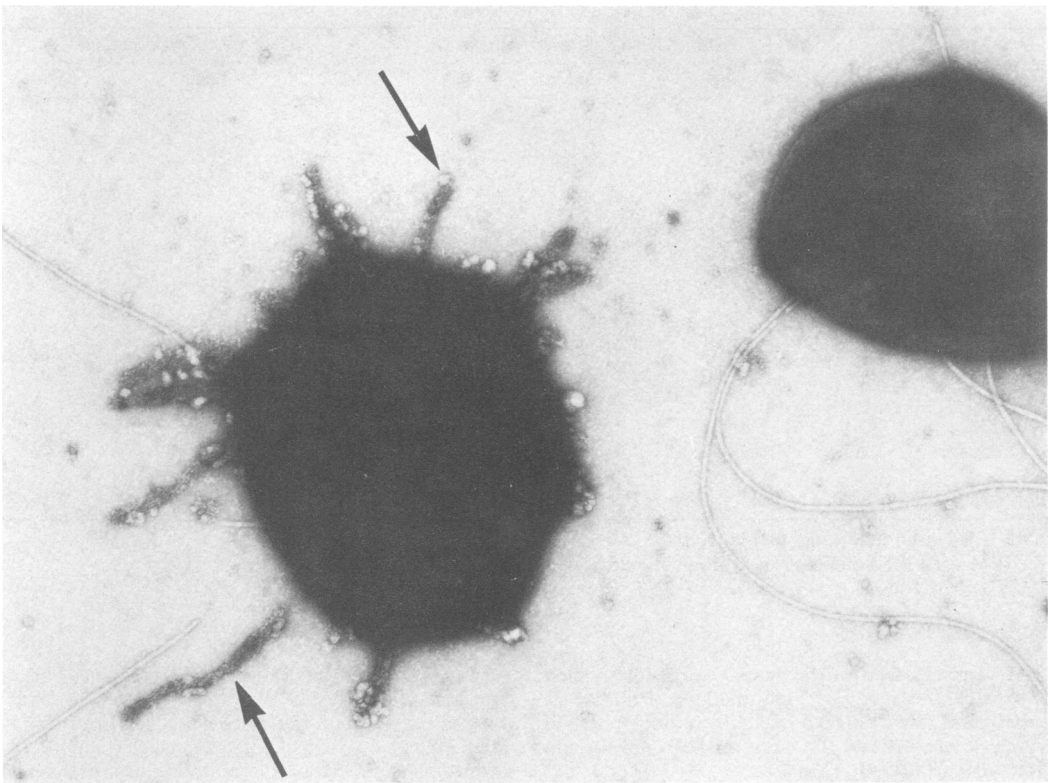

FIG. 2. Negative-stain electron micrograph of strain 346 that was incubated with a serum sample from patient 1 diluted 1:32 and ferritin conjugate as described in the legend to Fig. 1. Pili are completely ensheathed with antibody and ferritin complexes (arrows). Note that the labeling is specific for the pili and that an adjacent nonpiliated organism is not labeled at all. ×50,000.

found in the urine of any patient or control (data not shown).

The serological response of patient 1 to the type 1 pili of strain 346 as detected by ELISA is shown in Fig. 3. Some IgG antibodies were present in the initial sample, obtained 2 days after admission. IgG activity reached its maximum at 4 weeks and returned to the initial level by 14 weeks. No IgA or IgM antibody activity was noted. In contrast, with IEM, a 16-fold or greater increase in the titer of strain-specific antibodies was observed in all three immunoglobulin classes; this gradually declined over the next 12 weeks (Fig. 4).

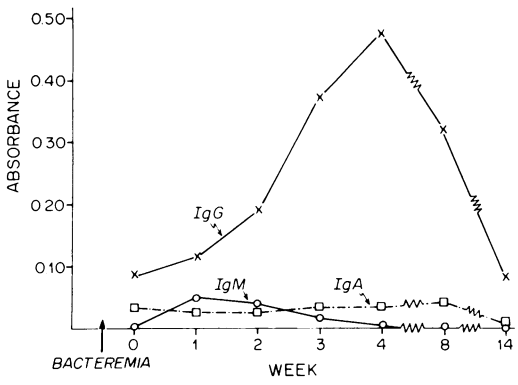

FIG. 3. Change in the titer of antibody to heterologous type 1 pili in the serum of patient 1 (age, 62 years) determined by ELISA. Only IgG antibodies are present.

Figure 5 shows the serological response of patient 4. During the first 6 weeks of infection, few IgG antibodies to type 1 pili of strain 346 were measured by ELISA. In contrast, a 16-fold increase in IgG antibodies to the specific pili of the strain infecting the patient was detected by IEM. Subsequent studies have shown that the pili of this strain do not react with serum that contain antibodies to the pili of strain 346, and so the pili of these two strains are antigenically distinct.

The hemagglutination pattern of each patient's strain and of 346 is shown in Table 2. All strains produced mannose-sensitive agglutination of guinea pig erythrocytes. Two of the infecting strains gave mannose-resistant hemag-

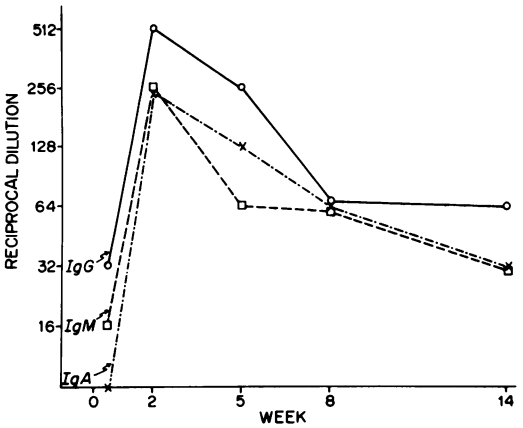

FIG. 4. Change in the titer of strain-specific antipilus antibodies in the serum of patient 1 determined by IEM. In contrast to antibodies directed against heterologous type 1 pili (Fig. 2), strain-specific antibodies belonged to classes IgG, IgM, and IgA.

glutination of human erythrocytes as well.

DISCUSSION

From these results it appears that significant amounts of antipilus antibodies are not present in the serum of adult males who deny a prior history of UTI. After acute pyelonephritis, however, each of our patients manifested a rise in serum antipilus antibodies. Antibodies against heterologous type 1 pili were predominantly IgG, whereas strain-specific antibodies belonged to classes IgG, IgM, and IgA. The reason for this difference is not known, but it may reflect previous exposure to gut organisms with type 1 pili, as antibodies synthesized in a recall response are predominantly IgG. Alternately, it is possible that the IgM peak was missed, as we could not date precisely the onset of infection.

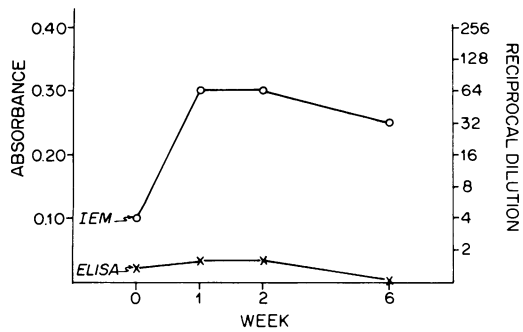

FIG. 5. Change in the amount of IgG antipilus antibodies in patient 4 (age, 64 years). Although there was little response to heterologous type 1 pili found by ELISA (left-hand ordinate), there was a brisk antibody response to the specific pilus antigens of this strain detected by IEM (right-hand ordinate).

TABLE 2. Hemagglutination pattern of *E. coli* strains used in this study

Strain	Hemagglutination	
	MS[a]	MR[b]
Patient 1	+	+
Patient 2	+	−
Patient 3	+	−
Patient 4	+	+
346	+	−

[a] Agglutination of guinea pig erythrocytes inhibited by 2.5% D-mannose.

[b] Agglutination of human erythrocytes not inhibited by 2.5% D-mannose.

Although the role of antipilus antibodies for host defense in the urinary tract is not known for certain, they may serve to protect against subsequent reinfection by inhibiting attachment of piliated bacteria to the uroepithelium. Antipilus antibodies have been shown to block adherence of *E. coli* to mammalian cells in vitro (6) and to confer protection against experimental *E. coli* pyelonephritis in rats (7). To block adherence in vivo, however, antipilus antibodies should be present on the uroepithelial surface. Therefore, it was surprising to find few antipilus antibodies in the urine of our patients, especially as antipilus antibodies are known to be synthesized in the kidney during the course of experimental pyelonephritis (8). It is possible, however, that antipilus antibodies were present in the genitourinary secretions but were diluted by the urine to concentrations below our ability to detect them.

The pili of *E. coli* are known to be antigenically and functionally diverse. Leffler and Svanborg-Edén have reported that strains of *E. coli* isolated from female patients with uncomplicated pyelonephritis bear pili which mediate MR hemagglutination (4). Although our sample is small, it would appear that MR pili are not present on all strains of *E. coli* that cause pyelonephritis in men (Table 2). The fact that our patients manifested a serological response to MS (type 1) pili indicates that MS pili are expressed in vivo and suggests further that MS pili may contribute to the uropathogenicity of these strains. It is interesting that one patient, patient 4, did not develop antibodies to the heterologous type 1 pili despite the fact that his infecting strain had type 1 pili. This observation implies that type 1 pili are antigenically diverse. Future studies which use the ELISA technique to detect antibodies to type 1 pili should use either a pool of pilus antigens from different strains or pili from a single strain with a more broadly cross-reacting antigenic determinant. The antigenic diversity of pili should also be

considered in determining the composition of any future pilus-based vaccine for UTI.

ACKNOWLEDGMENTS

This study was supported by the Medical Research Service of the Veterans Administration.

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