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Heterologous Protection against Invasive *Escherichia coli* K1 Disease in Newborn Rats by Maternal Immunization with Purified Mannose-Sensitive Pili

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Heterologous protection against *Escherichia coli* K1 bacteremia with antibody to purified mannose-sensitive (MS) pili was demonstrated in a neonatal rat model. The serological relatedness of purified MS pili from 17 *E. coli* K1 clinical isolates was examined by an enzyme-linked immunosorbent assay. Five pilus serogroups were identified, with the pili in each group showing 50% or greater cross-reactivity with the typing serum of the group. The MS pili from 12 of 17 (70%) strains belonged to just two serogroups. Pregnant Sprague-Dawley rats (dams) were immunized with purified pili, and their newborns (pups) were challenged with heterologous *E. coli*. Bacteremia was significantly reduced when the pili used for immunization were from the same serogroup as the pili expressed by the challenge bacteria. Thus, immunization with C94 pili and challenge with E03 (71% cross-reactivity) or E04 (50% cross-reactivity) resulted in bacteremia rates of 12 of 17 (17%) versus 51 of 79 (65%) in controls and 0 of 75 (0%) versus 28 of 70 (40%) in controls, respectively ($P < 0.001$ for each comparison). With lower cross-reactivity, less protection was observed ($P < 0.05$ for 22 to 37% pilus serological relatedness). No protection was seen in pups suckled by dams immunized with MS pili having only 5% serological relatedness to the pili on the challenge strain.

The major cause of gram-negative bacterial sepsis and meningitis in newborn infants is *Escherichia coli* K1 (10, 16, 17). Although the unique pathogenicity of this organism is due in part to the K1 polysaccharide capsule (21), other factors may also be responsible for *E. coli* K1 virulence, including O antigen serotype, hemolysin production, presence of the ColV plasmid, and expression of different classes of pili (3, 7, 15, 18, 24, 25). Of these bacterial components, the polysaccharide capsule and pili are of particular interest as potential vaccine agents. Antibodies to the K1 polysaccharide capsule provide some protection against experimental *E. coli* K1 infections (4, 16), but the polysaccharide is a very poor immunogen, making it an undesirable vaccine candidate. Pili, also known as fimbriae, are generally good immunogens, and our other studies suggest that antibodies against purified pili prevent invasive *E. coli* K1 disease (N. G. Guerina et al., submitted for publication).

The role of pili and capsule in experimental *E. coli* K1 disease in a well-defined neonatal rat model has been studied (9, 11, 19). The findings suggested that mannose-sensitive (MS) pili, also referred to as type 1 pili, are important in the pathogenesis of *E. coli* K1 infections. These pili bind specific epithelial cell receptors and mediate hemagglutination in a manner which is inhibited by mannose or mannose derivatives (6, 14). Newborn rats (pups) challenged with *E. coli* K1 were heavily colonized with bacteria in the MS piliated phase, whereas bacteria recovered from the bloodstream were nonpiliated (11). Immunization of pregnant rats (dams) with purified homologous MS pili prevented invasive disease in their suckling pups (Guerina et al., submitted). Colonization (oral cavity and small intestine) was not prevented, but the colonizing bacteria recovered from pups suckled by immunized dams were nonpiliated, suggesting that protection was partly due to antibody-mediated selection for nonpiliated bacteria at mucosal sites.

These studies indicate that MS pili may participate in mucosal events leading to bacteremia and that invasive *E. coli* K1 disease may be prevented by passive immunization of newborns with MS pilus-specific antibody. However, the development of an MS pilus vaccine would depend in part on the serological diversity of *E. coli* K1 MS pili. A large number of serologically distinct pilus types would make a pilus vaccine impractical. Alternatively, if most clinical isolates express pili with shared antigenicity sufficient to provide protection against heterologous infections, the development of an MS pilus vaccine against invasive *E. coli* K1 disease may be possible. In this study the serological diversity of MS pili from clinical *E. coli* K1 isolates was examined, and pairs of strains with MS pili showing various degrees of serological relatedness were selected for heterologous challenge studies in the neonatal rat model.

MATERIALS AND METHODS

Bacterial strains. Seventeen *E. coli* K1 strains were used in this study. Strains C94 and B1A were isolated from the cerebrospinal fluid of human neonates with meningitis and were supplied by M. Glode (Division of Infectious Diseases, Children's Hospital, Denver, Colo.) and J. Robbins (National Institutes of Health, Bethesda, Md.), respectively. Strain LH was isolated from urine and was also provided by M. Glode. All of the other strains were obtained from the Bacteriology Laboratory, The Children's Hospital, Boston, Mass. Strains E01, E02, E03, E04, and E19 were isolated from the cerebrospinal fluid of human neonates with meningitis; strains E20, E33, E34, E35, E37, E39, E40, and E41 were isolated from the blood of human neonates with sepsis; and strain E31 was isolated from the oropharynx of a human adult. All strains were stored in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) with 1% di-

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methyl sulfoxide at −70°C. The presence of the K1 polysaccharide capsule was confirmed by observing the presence of a halo of capsule-antibody precipitate around colonies grown on Davis minimal agar (Difco Laboratories, Detroit, Mich.) supplemented with 0.2% Casamino Acids and containing horse group B meningococcal antiserum as previously described (9). Streptomycin-resistant mutants of all *E. coli* K1 strains used in neonatal rat bacteremia studies were prepared by spreading dense bacterial suspensions on Trypticase soy agar (BBL Microbiology Systems) containing 1,000 μg of streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml.

Bacteria used for pilus purification were selectively grown for the MS piliated phase as previously described (11). Bacteria were grown on Davis minimal agar supplemented with 0.1% glucose (MG agar), and colonies corresponding to bacteria in the MS piliated phase were identified with a 10× binocular dissecting microscope and subcultured onto fresh MG agar. In this way cultures containing >95% bacteria in the MS piliated phase were obtained, as verified by MS hemagglutination of guinea pig erythrocytes and electron microscopy (EM) (11). We previously found that bacteremia rates in newborn rats were independent of pilus expression, even when the challenge inocula contained as great as 100% bacteria in a single phase, as determined by EM (11). Similarly, we found no difference in MS pilus antibody-mediated protection against bacteremia in newborn rats challenged with either piliated or nonpiliated *E. coli* K1 (Guerina et al., submitted). Thus, multiple challenge studies incorporating single-phase bacteria were not required, and challenge inocula which contained an equal mixture of piliated and nonpiliated bacteria were prepared.

Purification of MS pili. MS pili were purified by repeated cycles of solubilization and precipitation coupled with differential centrifugation as previously described (11). All pilus preparations used for immunization were judged pure by homogeneity and the absence of particular impurities, as determined by dark-field microscopy and EM, and by the presence of a single major Coomassie blue-staining protein band corresponding to a molecular mass of 17,000 daltons, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8, 11). Each pilus preparation also showed typical minor pilus protein bands (<1% total protein) on grossly overloaded gels (2, 12, 13).

EM. Bacteria were examined by EM by the agar filtration-negative staining technique as previously described (11). This procedure was also used to examine pilus preparations. The technique sandwiches all macromolecules or particulate materials in a suspension between two Parlodion membranes. Thus, a reliable assessment of bacterial piliation can be made without concern for the selective adherence of piliated bacteria as compared with nonpiliated bacteria to EM grids. The procedure also allows accurate evaluation of the homogeneity of pilus preparations. Specimens were stained with 0.5% uranyl acetate and examined in a JEOL 100S transmission EM at 60 to 80 kV.

Hemagglutination assay. Hemagglutination of guinea pig erythrocytes was carried out to test for the expression of MS pili by selectively grown *E. coli* K1. The guinea pig cells were freshly washed and suspended in 0.01 M phosphate-buffered saline (pH 7.2) to 3% of the total volume, and a 0.2-ml sample was thoroughly mixed on a glass slide with an equal volume of bacteria at a concentration of 10^8 CFU/ml. MS hemagglutination was confirmed by the presence of erythrocyte clumping which was inhibited by 0.1 M mannose.

Pilus antiserum production and serological assays. Antisera for serotyping were produced in young virgin female New Zealand White rabbits (Pine Acre Rabbitry, Norton, Mass.) by three subcutaneous injections of 500 μg of purified MS pili administered at 1-week intervals. Prior to each injection the pili were suspended in phosphate-buffered saline containing 0.01 M $MgCl_2$ and mixed 1:1 with Freund incomplete adjuvant (BBL Microbiology Systems). Animals were sacrificed 1 week after the final inoculations by intravenous injection of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, Ill.), and sera were obtained by intracardiac puncture. The titers of the antisera were determined with purified MS pili by an enzyme-linked immunosorbent assay (ELISA). In this assay purified MS pili (0.1 mg/ml in 50 mM $NaCO_3$ [pH 9.5]) were added to polyvinyl chloride microdilution plates (Dynatech Laboratories, Inc., Alexandria, Va.) and incubated overnight at 4°C. The microdilution wells were blocked with 1% gelatin in 50 mM $NaCO_3$ and incubated at 4°C overnight. Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Miles Laboratories, Inc., Elkhart, Ind.) was added for 3 h at 37°C, followed by the substrate *o*-phenylenediamine. The reaction with the substrate was stopped after 20 min with 3.5 M HCl, and the optical density was determined at 490 nm on an enzyme immunoassay reader (Bio-Tek Instruments).

Immunization of pregnant rats (dams) and bacteremia studies in neonatal rats. Synchronized-time pregnant pathogen-free Sprague-Dawley rats (Taconic Farms, Germantown, N.Y.) were sequentially immunized on days 3, 10, and 17 (normal gestation, 21 days) with 300 μg of purified MS pili per kg administered intramuscularly. Prior to immunization, pili were suspended in phosphate-buffered saline containing 0.01 M $MgCl_2$ and mixed 1:1 with Freund incomplete adjuvant. Control dams were sequentially injected with buffer-adjuvant alone. The dams were allowed to give birth naturally, and each litter of newborn rats (pups) within an experimental group (immunized versus control) was equally distributed among the dams within the same group. Pups were fed streptomycin-resistant *E. coli* K1 atraumatically, and bacteremia was determined at 5 days postchallenge by intracardiac puncture as previously described (11). Prior to blood sampling, animals were sacrificed by intraperitoneal injection of 0.2 ml of pentobarbital sodium. All cultures were plated on MG agar containing 100 μg of streptomycin sulfate per ml and incubated overnight at 37°C. Differences in bacteremia rates between immunized and control animals were compared by Student's paired *t* test.

RESULTS

Serological relatedness of *E. coli* K1 MS pili. The serological relatedness of *E. coli* K1 MS pili was established by determining the titers of rabbit antipilus antisera against purified MS pili from randomly selected *E. coli* K1 clinical isolates. Our initial studies showed very little serological relatedness between C94 and B1A MS pili, so antisera against these pili were used to initiate serotyping. When MS pili from a new strain were found to have very low reactivity against these antisera they were used to produce new typing sera. In this way five antisera were obtained (Table 1).

Rabbits sequentially immunized with purified MS pili produced high homologous antibody titers ranging from 161,000 ± 62,000 to 369,000 ± 220,000 in the ELISA. Heterologous titers varied from 4,000 ± 3,000 to 456,000 ± 167,000 (Table 1), but overall there was considerable homol-

TABLE 1. Homologous and heterologous titers of five pilus antisera against purified *E. coli* K1 MS pili

Pili	Titer (10^3)[a] with the following pilus antiserum:				
	C94	B1A	E02	E35	E39
C94	203 ± 84 (100)	23 ± 14 (6)	40 ± 36 (10)	8 ± 6 (5)	10 ± 4 (3)
E01	203 ± 84 (100)	16 ± 12 (4)	25 ± 10 (8)	6 ± 3 (4)	32 ± 24 (11)
E03	102 ± 42 (50)	18 ± 10 (5)	51 ± 21 (16)	10 ± 4 (6)	20 ± 8 (7)
E04	144 ± 80 (71)	16 ± 12 (6)	51 ± 21 (16)	6 ± 6 (4)	8 ± 6 (3)
E19	203 ± 84 (100)	81 ± 33 (22)	40 ± 36 (12)	25 ± 23 (16)	8 ± 6 (3)
E20	144 ± 80 (71)	54 ± 43 (15)	40 ± 36 (12)	25 ± 23 (16)	10 ± 4 (3)
E34	102 ± 42 (50)	128 ± 96 (35)	14 ± 11[b] (4)	20 ± 8 (12)	20 ± 8 (7)
B1A	10 ± 4 (5)	369 ± 220 (100)	64 ± 48 (20)	16 ± 12 (10)	20 ± 8 (7)
LH	76 ± 19[b] (37)	323 ± 133 (100)	81 ± 33 (25)	10 ± 4 (6)	8 ± 6 (3)
E31	5 ± 2 (2)	406 ± 167 (110)	81 ± 33 (25)	8 ± 6 (5)	23 ± 12[b] (8)
E33	6 ± 2 (3)	406 ± 167 (110)	323 ± 133 (100)	5 ± 2 (3)	5 ± 2 (2)
E37	9 ± 2 (4)	456 ± 92 (124)	40 ± 36 (12)	10 ± 4 (6)	16 ± 12 (6)
E02	20 ± 8 (10)	64 ± 48 (17)	323 ± 133 (100)	13 ± 5 (8)	32 ± 24 (12)
E40	4 ± 3 (2)	91 ± 58 (25)	161 ± 66 (50)	25 ± 10 (16)	20 ± 8 (7)
E35	8 ± 6 (4)	32 ± 24 (9)	32 ± 24 (10)	161 ± 62 (100)	32 ± 24 (11)
E41	13 ± 5 (6)	40 ± 17 (11)	36 ± 28 (11)	114 ± 63 (71)	32 ± 24 (11)
E39	4 ± 2 (2)	40 ± 17 (12)	40 ± 36 (12)	13 ± 5 (8)	287 ± 159 (100)

[a] Log normal average ± standard deviation calculated from three replicate ELISA titers for each pilus-antiserum pair, except as otherwise stated. Numbers in parentheses are average titers normalized to homologous titers, which were designated 100.
[b] Calculated from duplicate titers only.

ogy, with 12 of 17 (70%) strains showing 50% or more serological relatedness with *E. coli* K1 C94 or B1A.

Antisera were not raised against the MS pili from all 17 *E. coli* K1 strains, so it was not possible to determine reciprocal titers for the 5 typing pili against antisera to the MS pili from the other 12 strains. However, antisera were raised against MS pili from *E. coli* K1 E04 and E31, and the titers of each of these were determined against E04, E31, and the five typing pili. Furthermore, reciprocal titers for the five typing pili against their corresponding antisera were determined (Table 1) and are summarized along with the E04 and E31 titers in Table 2. All of the reciprocal titers were similar when normalized to homologous titers of 100. For example, C94 MS pili against B1A antiserum produced a titer of 23 ± 14 (6% of the homologous B1A titer), and B1A MS pili against C94 antiserum produced a titer of 10 ± 4 (5% of the homologous C94 titer). Symmetry in the reciprocal titers was seen not only with strains showing low cross-reactivity but also with strains showing higher heterologous cross-reactivity (Table 2).

Although five pilus typing groups were identified among the 17 *E. coli* K1 isolates studied, strain E33 showed very high cross-reactivity with both B1A and E02 pilus antisera. This result suggests that some pili may contain multiple major antigenic determinants. It is also possible that the actual number of unique pilus serogroups may be fewer than five for the 17 *E. coli* K1 strains examined.

Heterologous challenge studies. Based on the serotyping results, combinations of strains showing various degrees of serological relatedness were selected for heterologous protection studies in the neonatal rat model. Dams were immunized with purified MS pili from one strain of a pair, and their suckling pups were challenged with the other strain. Control dams were immunized with buffer only. In all studies control pups had high bacteremia rates which varied with the strain and inoculum size (Table 3). Immunization with C94 MS pili resulted in highly significant protection ($P < 0.001$) against bacteremia in animals challenged with strains E03 and E04 (Table 3). The MS pili from strains E03 and E04 showed 50 and 70% cross-reactivity, respectively, with antiserum against C94 MS pili (Table 1). Protection against invasive disease was lower when the serological relatedness of MS pili was <50% (Table 3). Nevertheless, protection against bacteremia was significant for pairs of strains having MS pili with 22, 35, and 37% cross-reactivity ($P < 0.05$ for each). No protection was seen in pups suckled by dams immunized with C94 MS pili and challenged by *E. coli* K1 B1A; the MS pili from these strains showed only 5% cross-reactivity (Table 3).

TABLE 2. Homologous and heterologous titers for seven pilus antisera against the corresponding MS pili

Pili	Titer (10^3)[a] with the following pilus antiserum:						
	E04	E31	C94	B1A	E02	E35	E39
E04	512 ± 161 (100)	45 ± 23 (12)	144 ± 80 (71)	16 ± 12 (5)	51 ± 21 (16)	6 ± 6 (6)	8 ± 6 (7)
E31	91 ± 46 (18)	362 ± 185 (100)	5 ± 2 (2)	406 ± 167 (110)	81 ± 33 (25)	8 ± 6 (5)	23 ± 12 (8)
C94	362 ± 185 (71)	23 ± 12 (6)	203 ± 84 (100)	23 ± 14 (6)	40 ± 36 (10)	8 ± 6 (5)	10 ± 4 (3)
B1A	23 ± 12 (4)	362 ± 185 (100)	10 ± 4 (5)	369 ± 220 (100)	64 ± 48 (20)	16 ± 12 (10)	20 ± 8 (7)
E02	91 ± 46 (18)	91 ± 46 (25)	20 ± 8 (10)	64 ± 48 (17)	323 ± 133 (100)	13 ± 5 (8)	32 ± 24 (12)
E35	32 ± 10 (6)	23 ± 12 (6)	8 ± 6 (4)	32 ± 24 (9)	32 ± 24 (10)	161 ± 62 (100)	32 ± 24 (11)
E39	11 ± 6 (2)	23 ± 12 (6)	4 ± 2 (2)	40 ± 17 (12)	40 ± 36 (12)	13 ± 5 (8)	287 ± 159 (100)

[a] Titers for C94, B1A, E02, E35, and E39 antisera are log normal average values ± standard deviations from Table 1. Titers for E04 and E31 antisera are log normal values ± standard deviations for duplicate determinations by the ELISA. Numbers in parentheses are the log normal titers normalized to a homologous titer of 100.

TABLE 3. Heterologous protection against *E. coli* K1 bacteremia in newborn rats suckled by mothers immunized with MS pili

Challenge strain	Challenge inoculum (CFU/ml)	Immunizing pili[a]	% Serological relatedness[b]	Bacteremia rate (%) in:	
				Control rats[c]	Immunized rats
E04	5×10^7	C94	71	51/79 (65)	12/72 (17)
E03	1×10^7	C94	50	28/70 (40)	0/75 (0)
B1A	5×10^6	E34	35	32/74 (43)	21/97 (22)
C94	8×10^7	LH	37	56/110 (64)	25/73 (34)
E03	6×10^7	E31	37	70/110 (64)	64/108 (43)
B1A	5×10^6	E19	22	29/80 (36)	21/93 (23)
B1A	5×10^6	C94	5	35/81 (43)	35/79 (44)

[a] Pregnant rats (dams) were immunized with purified MS pili on days 3, 10, and 17 of gestation (normal gestation, 21 days).
[b] Determined from normalized titers in Tables 2 and 3.
[c] Control dams injected with buffer vehicle.

DISCUSSION

If MS pili function in the pathogenesis of gram-negative bacterial infections, they must act in concert with other virulence factors, since they are prevalent among enteric organisms, including nonpathogenic strains. Previous studies have suggested that MS pili contribute to the pathogenicity of bacterial diseases (23), including *E. coli* urinary tract infections (20; J. D. S. Fusco, P. C. Fusco, J. A. Kramarik, M. J. Carter, and C. C. Brinton, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, B216, p. 65). We have found that MS pili may be important in experimental *E. coli* K1 infections, since there is a rapid selection for the MS piliated phase of *E. coli* K1 at mucosal sites of colonization and since maternal immunization with purified MS pili protects suckling rats against homologous invasive *E. coli* K1 disease (11; Guerina et al., submitted).

Heterologous protection against invasive disease is an absolute requirement for the development of a pilus vaccine against *E. coli* K1. Our current findings demonstrate heterologous protection in neonatal rats by use of purified intact MS pili. Furthermore, our serological survey of 17 clinical isolates indicates that there are a limited number of major pilus serogroups. This result is consistent with those of previous studies suggesting a small number of major MS pilus serogroups among *E. coli* strains isolated from diverse sources (5; C. Brinton and P. Fusco, U.S. patent 4,725,435, February 1988).

Heterologous protection correlated with the degree of shared major antigenicity between the MS pili used for immunization and those expressed on the challenge strain. Although significant protection was observed with as low as 22% cross-reactivity, the greatest protection was seen with 50% or greater serological relatedness. In another study highly significant protection against bacteremia caused by *E. coli* K1 C94 was also seen in pups suckled by dams immunized with MS pili from *Klebsiella pneumoniae* Fader (bacteremia rates, 33 of 81 [40%] controls versus 1 of 80 [1%] immunized) (Guerina et al., submitted). The MS pili from strain Fader showed 50% serological relatedness to C94 MS pili. These results suggest that an optimal multivalent pilus vaccine would consist of the minimum number of MS pilus types showing at least 50% cross-reactivity with all naturally occurring *E. coli* K1 strains. Although extensive serological testing would be required to identify all of the serogroups necessary for an effective pilus vaccine, it is encouraging that 12 of 17 strains in this study belonged to just two serogroups. Alternative vaccine candidates would be synthetic peptides corresponding to immunogenic pilus protein epitopes showing common antigenicity (22) or the tip adhesin protein for MS pili, which appears to be antigenically conserved (1, 12).

Although our results suggest a role for MS pili in *E. coli* K1 infections, they do not demonstrate that MS pili are required for invasive disease. Pilus-mediated bacterial-mucosal interactions may be important for bloodstream invasion, but it is also possible that invasion depends on other bacterial factors which may be under coordinate expression with MS pili. Our other finding that antipilus antibody selects for nonpiliated-phase *E. coli* K1 at mucosal sites is consistent with both hypotheses. Thus, antipilus antibody may prevent invasive disease by selecting for a noninvasive phase of *E. coli* K1 or by blocking pilus function. It is unlikely that antipilus antibody directly interferes with the growth of piliated-phase bacteria, since we have not demonstrated in vitro phase selection or growth inhibition by incubating piliated *E. coli* K1 in the presence of antipilus antibody (unpublished results). Studies incorporating pilus-deficient or phase-locked mutants are likely to further define the role of pili and the mechanism of action of antipilus antibody in *E. coli* K1 infections.

A recent study by Saukkonen et al. (18) suggested that type S pili may be important in *E. coli* K1 infections and that MS pili were unlikely to be virulence factors. In that study selection for type S piliated bacteria and against MS piliated bacteria was found in the bloodstreams of experimentally infected infant rats. However, that study incorporated intraperitoneal inoculations of challenge bacteria, and thus the mucosal events critical to the pathogenesis of *E. coli* K1 infections were not examined. Our study indicates an important role for MS pili or the MS piliated phase in bacterium-host interactions which occur before *E. coli* K1 enters the bloodstream.

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