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Role of Type 1 and S Fimbriae in the Pathogenesis of *Escherichia coli* O18:K1 Bacteremia and Meningitis in the Infant Rat

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The role of fimbriae in the pathogenesis of *Escherichia coli* infection was studied in the infant rat model. Rat pups were challenged intraperitoneally at the age of 5 days with *E. coli* K1 (strain IH3080, O18:K1:H7) and three different subpopulations (type 1, type S, or nonfimbriated) of it. All bacterial subpopulations were able to produce peritonitis, bacteremia, and meningitis. However, the type 1 fraction was the least virulent and the type S fraction was the most virulent, as judged by the bacterial counts in body fluids and by the mortality rates of the pups. Fimbrial phase variation to mainly the type-S-fimbriated forms was observed in all body fluids. An initially type-S-fimbriated inoculum remained predominantly type S fimbriated in the peritoneal fluid and blood. In the cerebrospinal fluid, however, about 50% of the bacteria were type S fimbriated and 50% were nonfimbriated 1 h after challenge with the type-S-fimbriated subpopulation; at later times the share of type-S-fimbriated bacteria also increased in the cerebrospinal fluid.

Many gram-negative bacteria, including *Escherichia coli*, possess filamentous surface appendages known as pili or fimbriae (6). The fimbriae may have different functions, depending on the route, stage, or type of infection. Fimbriae mediate adhesion of the bacteria to epithelial cell surfaces, the logical first step in the pathogenesis of infection (1). Enterobacterial strains often are able to synthesize many types of fimbriae (23), but one cell usually expresses only one type at a time (25). It has been suggested (3, 8, 11) that the variability of fimbriation itself plays a role in pathogenesis. It could alternately enable the bacteria to adhere to tissue surfaces or to avoid phagocytes or antibodies (3, 8, 11). The genetic mechanisms responsible for fimbrial variation are complex and have not yet been fully elucidated. The variation has mainly been studied in vitro (20, 26, 28). In a recent study (22), however, it has been shown that fimbrial phase variation also occurs in vivo.

In the present study we examined the role of fimbriae and fimbrial phase variation in an infant rat meningitis model (17, 30). Five-day-old infant rats were challenged intraperitoneally (i.p.) with different doses of *E. coli* K1 (strain IH3080, O18:K1:H7). Strain IH3080 possesses type S and type 1 fimbriae. In the challenge experiments it was used either in an unfractionated form or was fractionated into subpopulations of type-S- or type-1-fimbriated or nonfimbriated bacteria. Type 1 fimbriae bind to α-D-mannosides and are found on most *E. coli* strains (6), whereas type S fimbriae bind to sialic acid-containing glycoconjugates and are typically present on many *E. coli* strains that cause infant meningitis (10, 12). Such sialyl galactoside structures occur widely in human (14) and rat (9) tissues, including the brain, and could thus act as receptors to the type S fimbriae.

MATERIALS AND METHODS

Animals. Pregnant female, outbred, pathogen-free albino Wistar rats were obtained from the Department of Genetics, University of Helsinki (Helsinki, Finland). The animals were housed under standard conditions (25°C; relative humidity, 40%), with a 6 a.m. to 9 p.m. light schedule. Rat chow (Purina) and water were available ad libitum. The rat pups remained with their mothers after parturition and were used at the age of 5 days (±1 day), when they weighed 10 to 15 g.

Bacteria. The *E. coli* K1 strain IH3080 ($O18_{ac}$:K1:H7) was a clinical isolate from the cerebrospinal fluid (CSF) of a human neonate with meningitis (22); it belongs to the major O18:K1:H7 meningitis clone that is capable of expressing type 1 and type S fimbriae (13). It was stored in skim milk at −70°C in small portions until use.

The standard inoculum for challenge (referred to as the unfractionated inoculum) was prepared by first plating the thawed bacterial suspension onto L agar (agar containing 10 g of tryptone per liter [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract per liter [Difco], and 5 g of NaCl per liter [pH 7.0]), followed by overnight growth at 37°C. Two colonies were transferred to 20 ml of L broth (as described above but without agar) and grown overnight with shaking at 37°C, diluted 10-fold with L broth, and grown for 2 h at 37°C with shaking. Appropriate dilutions for injection were made in 0.9% saline, and the number of viable bacteria in the inoculum was confirmed as CFU by plating serial dilutions onto L agar.

Type-1-, type-S-, and nonfimbriated subpopulations of IH3080. The inoculum for the culture to be fractionated was first enriched for type-S-fimbriated cells by aseptic adsorption to human erythrocytes (blood type O) at 0°C in an ice bath, followed by elution at 37°C (19). The bacteria were then grown for 24 h at 37°C without shaking in tissue culture flasks (80 cm^2; Nunc, Copenhagen, Denmark) containing 70 ml of L broth. The culture was then fractionated into the specific subpopulations by serial adsorptions and elutions with yeast cells and human type O erythrocytes, as described previously (19). The fractionation was repeated until less than 1% cross-contamination was observed by immunofluorescence (IF) (18) of approximately 10^3 cells with fimbria-specific antibodies (see below). The bacteria were kept in an ice bath during the fractionation and then overnight at 4°C before use.

The inoculum for the injection was prepared by diluting

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the fractionated cells 10-fold in broth and culturing them for 2 h at 37°C without shaking. The numbers of bacteria were then estimated by counting them in a Burker chamber, and the appropriate dilutions for injection were made in 0.9% saline. Before each experiment the presence of the strain K1 capsule was confirmed by the latex agglutination test (16), and 10 μl containing approximately 10^3 cells was placed as triplicate drops onto a glass slide for IF.

i.p. infection model. In each experiment, more than one litter (the number of pups in each litter varied between 7 and 15) was used, and the litters were divided into groups of six pups (all six pups were from the same litter). The desired number of bacteria was injected i.p. in a final volume of 0.1 ml. After the injection the pups were returned to their mothers. At the indicated times, body fluids were collected from some of the pups, while others were observed for 4 days for survival. The dose of bacteria that killed half of the animals was determined (24).

Sampling of body fluids. CSF was collected by a modification of a previously described method (17, 30). The unanesthetized rat was held in an immobile position with its head flexed forward. The suboccipital region was identified by palpation; and the cisterna magna was entered by puncturing the skin with a sterile, disposable, 30-gauge needle. CSF flowed into the hub of the needle and could be easily seen with appropriately positioned lighting. Approximately 15 to 20 μl of CSF was allowed to accumulate before the needle was withdrawn. The CSF was then drawn into a micropipette, from which 10 μl was placed onto a glass slide for IF studies, and the rest was plated in serial dilutions onto L agar plates to count the CFU. For blood cultures, either the tail or the lateral marginal vein of the hind leg was cleansed with 70% alcohol, and blood was collected in a 10-μl calibrated microcapillary pipette and handled as described above for the CSF samples. In some cases the erythrocytes in the samples used for the IF experiments were lysed by adding cold water.

Peritoneal cavity washings were obtained after i.p. injection of 0.5 ml of 0.9% saline into the peritoneal cavity. The abdomen of the pup was lightly massaged, and about 100 μl of the fluid was drawn out and processed as described above for CSF.

IF. Fluorochrome-conjugated rabbit immunoglobulin G specific for type 1 or type S fimbriae of *E. coli* were the same as those described earlier (21). Fluorescein isothiocyanate (FITC; Sigma Chemical Co., St. Louis, Mo.) was used for the anti-type-1 and tetramethylrhodamine B isothiocyanate (TRITC) was used for anti-type-S-fimbrial antibodies. The immunoglobulin fraction isolated by precipitation with $(NH)_2SO_4$ (15) from rabbit anti-O18 antiserum (J. Vuopio-Varkila, M. Karvonen, and H. Saxen, J. Med. Microbiol., in press) was conjugated with FITC and TRITC (18).

The IF samples on glass slides, which were dried at room temperature and stored at 20°C, were double stained in the following combinations: (i) anti-type-1 FITC and anti-type-S TRITC, (ii) anti-type-1 FITC and anti-O18 TRITC, and (iii) anti-type-S TRITC and anti-O18 FITC. The stained samples were examined in a standard microscope (Zeiss) equipped with an epiluminator IV FL and filter systems for FITC and TRITC (18). The total number of bacteria in a microscope field was counted as the number of cells stained with the anti-O18 conjugate, and the percentage of fimbriated cells was calculated from the fraction of cells stained with the anti-fimbria conjugates. The total number of cells counted varied from 50 to 200 at different times.

Statistical analysis. Student's *t* test for two means was used to study the statistical significance between different groups. The variances shown in the figures represent the standard errors of the mean.

RESULTS

***E. coli* K1 infection in the infant rat after i.p. challenge.** The development of bacteremia and meningitis was studied 24 h after i.p. injection of 10^1 and 10^2 of unfractionated strain IH3080 bacteria. A challenge dose of 10 bacteria was able to cause bacteremia in all animals and meningitis in 80% of the animals, whereas with a challenge dose of 10^2, all animals had both bacteremia and meningitis 24 h after challenge. The number of bacteria found in the blood and CSF was high, and no significant difference between these two inocula was seen in the bacterial counts of body fluids. With these inocula there was no mortality in 24 h, but after 3 to 4 days, about 50% of the pups had died.

The number of bacteria detected in the peritoneal fluid, blood, and CSF as a function of time after administration of a larger inoculum (3×10^5 bacteria per rat) is shown in Fig. 1. Bacteremia occurred in 100% of the pups at all time points between 2 and 12 h; and meningitis occurred in about 50% of the pups at 3 h, 80% at 6 h, and 100% at 9 h. With this inoculum size, all pups were dead at 24 h. At all time points the bacterial counts in the peritoneal fluid were 1 or 2 log units higher than those in the blood or CSF. The number of bacteria in all body fluids increased rapidly, and the maximum bacterial densities were obtained by 9 to 10 h (Fig. 1).

The kinetics of infection was also studied with a very large inoculum (10^8 bacteria per animal). The bacteria spread very rapidly from the peritoneal cavity to the blood and the CSF. The parameters of infection were quite similar to those obtained with a lower inoculum and a longer incubation time.

Behavior of type-1-, type-S-, or nonfimbriated subpopulations of *E. coli* K1 in the infant rat. The fractionation procedure yielded subpopulations of the bacteria with only type 1 or type S fimbriae or with neither type 1 nor type S fimbriae (the nonfimbriated fraction). Before injection into the rat, the fractions were grown for 2 h as described above for the unfractionated inoculum, but without shaking. Immunostaining of the inocula showed that they were 85 to 100% pure with respect to the fimbriation phase that was selected.

The behavior of the type-S- and type-1-fimbriated populations in the infant rat 12 h after i.p. injection of relatively

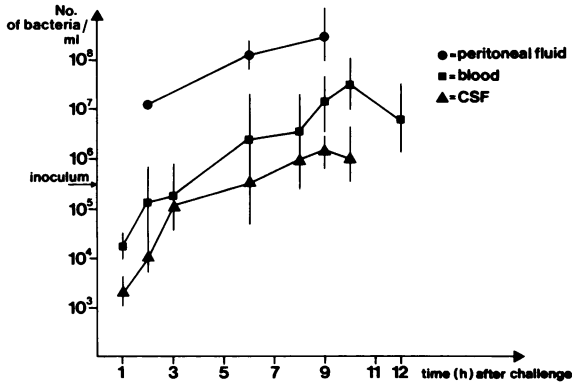

FIG. 1. Kinetics of bacteremia, meningitis, and peritonitis after i.p. challenge of 5-day-old rats with 3×10^5 unfractionated *E. coli* IH3080 (O18:K1).

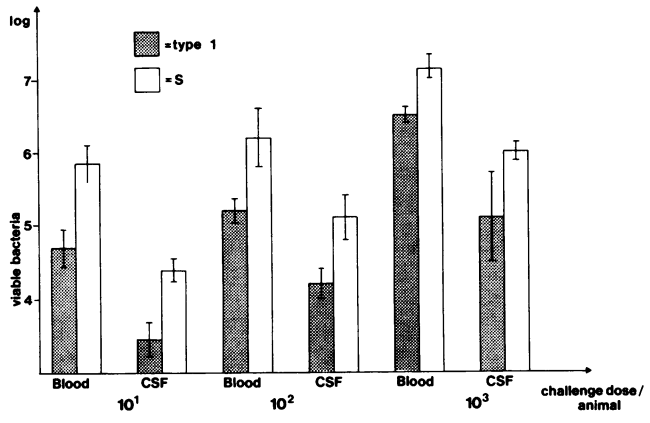

FIG. 2. Bacterial counts as viable bacteria per milliliter in blood and CSF 12 h after i.p. challenge of 5-day-old rats with different doses of type-1- and type-S-fimbriated fractions of *E. coli* IH3080 (O18:K1).

small inocula (10^1, 10^2, and 10^3 bacteria per rat) is shown in Fig. 2. The rate of bacteremia and meningitis was 100% with both subpopulations, and the bacterial counts were comparable to those in infections caused by unfractionated bacteria (Fig. 1). However, the number of bacteria in the blood and CSF was significantly ($P < 0.01$) lower after challenge with the type-1 fimbriae than that after challenge with type S fimbriae. Mortality after 24 h was 70% in rats infected with type-S-fimbriated bacteria, but only 10% in those infected with type-1-fimbriated bacteria.

In the next experiment we used a higher inoculum dose (10^5 per rat) and a shorter assay time (6 h). Again (Fig. 3), the type-1-fimbriated bacteria were significantly ($P < 0.01$) less virulent, as judged by the number of bacteria in the blood and CSF. They also produced markedly less meningitis (43%) than did the type S fimbria fraction (100%). The type-S-fimbriated bacteria were most virulent, as judged by bacterial counts in the blood and CSF, as well as by the rates of meningitis and mortality, although no significant differences in bacterial counts between the type-S- and the nonfimbriated inocula existed.

Fimbrial variation in vivo. The fimbrial phase of the organisms, as determined by direct immunostaining of the inocula or samples from the blood and CSF after challenge with 10^5 bacteria of the three subpopulations, is shown in Fig. 4. The type-S-fimbriated inoculum contained about 85% type-S-fimbriated cells and 15% nonfimbriated cells. At 6 h after the challenge, 100% of the cells found in CSF and blood were type S fimbriated. Ten percent of the bacteria in the blood had also type 1 fimbriae. The nonfimbriated fraction inoculum consisted of 100% nonfimbriated cells. Still at 6 h, 96% of the bacteria in the blood were nonfimbriated. The bacterial numbers in the CSF obtained at this time were too low ($<10^5$ bacteria per ml) for reliable counting by immunostaining.

The inoculum of the type 1 fraction had 90% type-1-fimbriated, 9% nonfimbriated, and 1% type-S-fimbriated cells. After 6 h there were no type 1 cells in the blood samples, which instead contained type-S-fimbriated and nonfimbriated cells in almost equal proportions. Again, the number of bacteria in CSF were too low for immunostaining.

The data suggest that at least the bacteria with type 1 fimbriae undergo phase variation in the infant rat. To test this hypothesis, a new experiment was performed to obtain samples from the early phases of infection. Therefore, a very high inoculum (10^8 bacteria per rat) and short incubation times (0.5, 1, 2, and 3 h) were used, and the kinetics as well as the fimbrial phase of the bacteria were followed (Fig. 5 and 6). In this experiment there was no significant difference between type S and nonfimbriated fractions in the bacterial counts of peritoneal cavity and blood; they increased in the peritoneal cavity in the first 3 h, and a considerable number of bacteria were also present in the blood (Fig. 5). The number of bacteria in CSF after challenge with the type S- and nonfimbriated fractions differed very significantly, however ($P < 0.001$), from each other. The CSF counts after the challenge with nonfimbriated bacteria declined sharply after 1 h, but remained at a high level for the 3 h of observation after challenge with the type-S-positive fraction.

The type S fimbriated inoculum remained predominantly type S fimbriated in the peritoneal fluid and also in the blood throughout the experiment. By contrast, in CSF only 56% of the cells were type S fimbriated and 40% were nonfimbriated 1 h after infection. At later time points the numbers of type-S-fimbriated cells increased and those of nonfimbriated

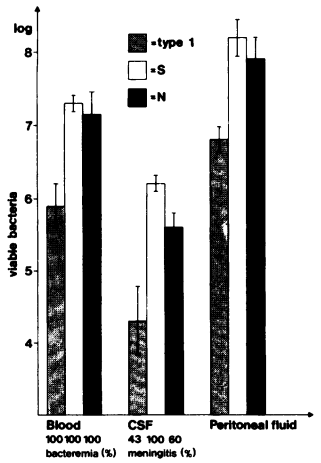

FIG. 3. Bacterial counts as viable bacteria per milliliter recovered from the blood, CSF, and peritoneal fluid 6 h after i.p. challenge of 5-day-old rats with 10^5 bacteria of type-1-, type-S-, and nonfimbriated (N) fractions of *E. coli* IH3080 (O18:K1).

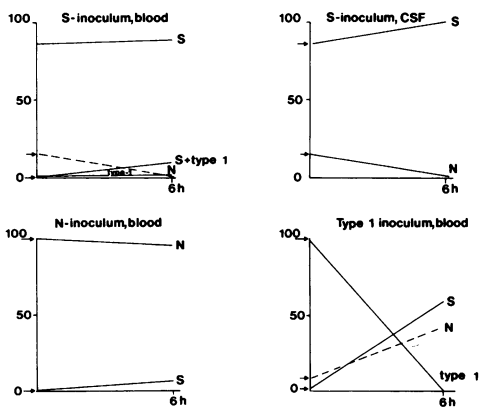

FIG. 4. The shares of type-1-, type-S-, and nonfimbriated (N) bacteria in blood and CSF 6 h after i.p. challenge of 5-day-old rats with 10^5 type-1-, type-S-, or nonfimbriated fractions of *E. coli* IH3080 (O18:K1).

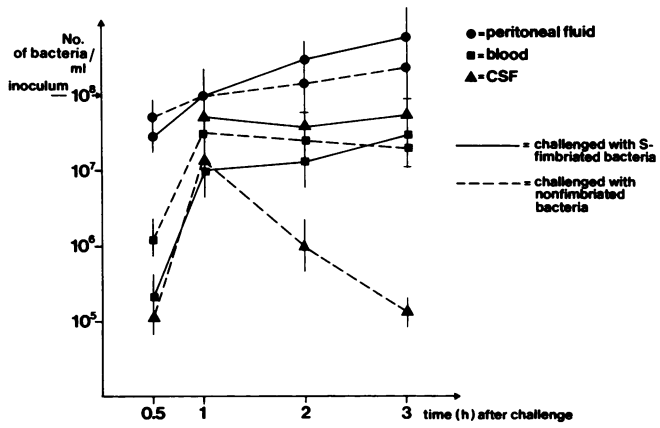

FIG. 5. Kinetics of bacteremia, meningitis, and peritonitis after i.p. challenge of 5-day-old rats with 10^8 bacteria of type-S- and nonfimbriated (N) fractions of *E. coli* IH3080 (O18:K1).

cells decreased (Fig. 6). After challenge with the nonfimbriated fractions, all body fluids showed a similar trend, with the proportion of nonfimbriated cells decreasing and that of type-S-fimbriated cells increasing.

DISCUSSION

E. coli IH3080 (O18:K1:H7) is a representative of the *E. coli* clone most commonly found as the cause of meningitis in human infants (13). This clone possesses several proven or presumed virulence factors, including the K1 capsule (2, 4, 27) and type S fimbriae (12, 13). In this study we focused on the possible role of the fimbriae in the well-established model of infant rat meningitis (17, 30).

Under our experimental conditions IH3080 was highly virulent, so that 10 bacteria administered i.p. were able to cause rapid and fatal bacteremia and meningitis in 5-day-old rat pups. The bacteria multiplied in the peritoneal cavity and apparently spread from there to the blood and CSF, since the concentrations of bacteria were highest in the peritoneal cavity at all time points. A high-grade bacteremia (>10^5 CFU/ml) seemed to be a prerequisite for the development of meningitis, as has also been shown in *Haemophilus influenzae* type b infection in the infant rat model (17). With challenge doses higher than 10^5 bacteria, bacteremia and meningitis developed in a few hours and the infection led to death in less than 24 h. The rates of bacteremia and meningitis were very similar to those found by Bortolussi et al. (5).

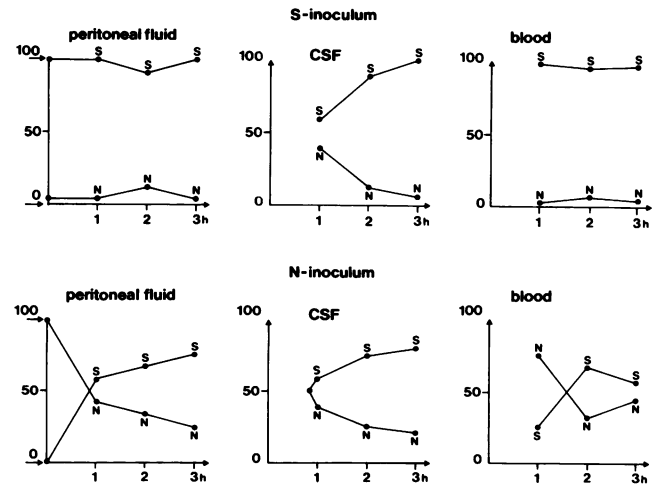

FIG. 6. Shares of type-S- and nonfimbriated (N) bacteria in blood, CSF, and peritoneal fluid 1, 2, and 3 h after i.p. challenge of 5-day-old rats with 10^8 type-S- or nonfimbriated fractions of *E. coli* IH3080 (O18:K1).

When type-1, type-S-, and nonfimbriated subpopulations of strain IH3080 were used for challenge, differences in the virulence between these three subpopulations were observed. In all experiments the type-1-fimbriated bacteria were less virulent than the two other subpopulations. The number of the type 1 bacteria in the peritoneal cavity was lower than that attained after injection of type-S- or nonfimbriated bacteria. This difference is probably due to destruction of the type 1 bacteria in the peritoneal cavity by phagocytosis. Type 1 bacteria bind to phagocytic cells (29), whereas type-S- and nonfimbriated fractions do not (22). Consistent with the lower number of bacteria in the peritoneal cavity, both the mortality and the meningitis rates were the lowest for the type-1-fimbriated fraction. Both the type-S- and nonfimbriated subpopulations had high levels of virulence. However, the rates of meningitis and mortality were somewhat lower with the nonfimbriated than with the type-S-fimbriated challenge bacteria, especially at high inoculum doses.

Fimbrial phase variation was found to occur in the body fluids of the rat, in a manner similar to that seen previously in mice (22), with type-S-fimbriated forms favored in vivo in both adult mice and newborn rats. Six hours after the inoculation of 10^5 type 1 bacteria, the entire population recovered from the rats had shifted to type-S- (approximately 55%) and nonfimbriated (approximately 45%) forms. Inocula of type-S-fimbriated bacteria (10^5 or 10^8 bacteria per rat) remained type S fimbriated in both the peritoneal fluid and blood for the following 6 h. The behavior of the fractionated nonfimbriated bacteria varied. In one experiment (Fig. 4) they remained nonfimbriated for 6 h, whereas in another experiment (Fig. 6) there was considerable shift toward type-S-fimbriated forms. Type S fimbriae are known to bind in vitro to human epithelial and endothelial tissues (10). Importantly for the present study, they bind to the vascular endothelium and to the epithelial lining of the choroid plexus and ventricles of the neonatal rat brain, whereas the binding to corresponding tissues of an adult rat, which is not susceptible to *E. coli* meningitis, is much less (J. Parkkinen, T. K. K. Korhonen, A. Pere., J. Häikiö, and S. Soila, J. Clin. Invest., in press). Moreover, sialic acid-containing glycoproteins occur in the endothelia of a number of rat organs (9). These findings suggest that type S fimbriae might aid the development of meningitis by binding to capillaries in the brain and, thus, binding the bacteria in close association with the vascular endothelium, which they must penetrate to reach the CSF. This is consistent with the present finding that the type-S-fimbriated subpopulation gave higher and more persistent bacterial counts in CSF than did the other subpopulations (Fig. 2, 3, and 5) or the unfractionated inoculum.

On the basis of the results presented above, we would have expected to see only type-S-fimbriated bacteria in the CSF. This was, however, not the case. In CSF samples taken a short time (1 h) after i.p. inoculation, approximately half of the bacteria were nonfimbriated and half were type S fimbriated. At later times (2, 3, or 6 h), the proportion of type-S-fimbriated bacteria increased to approximately 100%.

This pattern was seen with inocula of both type-S- and nonfimbriated subpopulations. A possible mechanism to account for these observations could be that only nonfimbriated bacteria can actually pass through the vascular endothelium to the CSF; once there, they undergo a phase shift to type S fimbriation as they do in the other body fluids. If the bacteria that bind to the vascular endothelium are type S fimbriated, as suggested above, this would mean that of the type-S-fimbriated bacteria, only those that shifted to the nonfimbriated phase would pass through the vascular endothelium. It can be speculated that this complicated scheme might be possible because of the high rate of fimbrial phase variation (20, 26).

The role of S fimbriae in experimental infections has been studied with another *E. coli* strain (O6:K15) which, however, is much less virulent and does not cause meningitis (7a). The data from that study suggested that encapsulated *E. coli* strains possessing the type S fimbrial antigen are virulent in a murine respiratory infection assay and in a subcutaneously induced septicemia; however, the role of type S fimbriae could not be separated from the contribution of other virulence factors, serum resistance, and hemolysin production.

In all experiments the type-1-fimbriated bacteria were much less virulent than the type-S- or nonfimbriated bacteria, and among the latter, the type-S-fimbriated fraction was somewhat more virulent than the nonfimbriated fraction. These conclusions should, however, be taken with some caution, because the fractionation procedure (19) required to produce the differently fimbriated subpopulations could have caused differences in other components, too. The presence of the K1 capsule was controlled in all inocula, but the fractions could have differed in its relative amount or in other components. There was little variation from experiment to experiment in the behavior of the type-1- or type-S-fimbriated fractions, whereas the behavior of the nonfimbriated fraction was less consistent.

Results of this study provide evidence for a role of both type S fimbriae and fimbrial phase variation in the pathogenesis of septicemia and meningitis in the rat. The tendency of the inoculated bacteria, irrespective of their fimbriation, to shift to type-S-fimbriated forms makes it difficult to obtain conclusive evidence of this. We tried to overcome this problem by using very large inocula that produced meningitis in 1 h, but even then considerable shift to type S fimbriation took place. The use of stable type-1-, type-S-, and nonfimbriated forms would be of definite advantage, but so far we have been unable to obtain such mutants of the virulent *E. coli* clone. The isogenic series of stable mutants would be even more necessary for testing the role of the fimbriae by peroral inoculation, when the bacteria would need to first colonize the gut and then penetrate the gut epithelium to start the systemic infection (7).

We have suggested, on the basis of the results of this study and previous data of type-S-fimbriae-mediated tissue binding (10), a possible mechanism by which type S fimbriae could mediate a close association of the bacteria to the vascular epithelium in the brain. Here, the continuously occurring fimbrial phase variation would produce some nonfimbriated bacteria, and these would be the forms that penetrate the endothelium. The bacteria that reached the CSF would again shift to type-S-fimbriated forms and adhere to the endothelium of the cisternae. The latter hypothesis is formulated to explain the fimbriation patterns observed in the CSF during this study and needs to be tested experimentally. The stable fimbriation variants would be of great help to such tests, too.

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