INFECTION AND IMMUNITY, Mar. 1984, p. 1068–1071
0019-9567/84/031068-04$02.00/0

Vol. 43, No. 3

Vero Cell Invasiveness of *Proteus mirabilis*

PAUL G. H. PEERBOOMS,* A. MARIAN J. J. VERWEIJ, AND DAVID M. MACLAREN

Research Group for Commensal Infections, Departments of Medical Microbiology and Oral Microbiology, Vrije Universiteit, Amsterdam, The Netherlands

Received 5 July 1983/Accepted 8 December 1983

Vero cell invasiveness was studied for a group of *Proteus mirabilis* strains isolated from the urinary tract and feces and for a limited group of urinary isolates of *Escherichia coli*. Experimental conditions affecting this invasiveness were studied. All of the *P. mirabilis* strains tested were capable of cell invasion, whereas none of the *E. coli* strains was. Correlation between the hemolytic activity of the *P. mirabilis* strains and their invasive ability suggested that the bacterial hemolysin may be involved in the invasion process. Other experimental evidence supporting this hypothesis is discussed. The differences in the invasive capacities of *P. mirabilis* and of *E. coli* may be important for the apparent differences in the pathogenesis of urinary tract infection by both species.

Proteus mirabilis and *Escherichia coli* are important pathogens of the urinary tract (6). Several properties of both species, especially of *E. coli*, have been studied in relation to virulence for the urinary tract. The importance of the *P. mirabilis* urease as a virulence factor, both in human and experimental urinary tract infections, is well documented (10, 11, 13). The importance of other possible virulence factors of *P. mirabilis* such as adhesive ability (20), the possession of flagellae (15), and sensitivity to the bactericidal effect of human serum (9) is much less clear.

As there is an increasing interest in cell invasiveness as an important step in the pathogenesis of infections by different bacteria (7, 12, 14, 19), we decided to study invasiveness by *P. mirabilis* as a part of the study of virulence factors of *P. mirabilis* in urinary tract infections. In 1960, Braude and Siemienski (3) described the invasion of a *P. mirabilis* strain in mammalian cells both in vivo and in vitro. A urinary strain of *E. coli* did not show this ability to invade cells. Except for a confirmation by Silverblatt (20), little attention has been paid to this phenomenon. Other authors have also reported differences in the pathogenesis of urinary tract infections by both species in experimental models. Infections caused by *P. mirabilis* tend to be more persistent than infections caused by *E. coli* (5). Renal infections are more easily established with *P. mirabilis* than with *E. coli* in mice (16, 22).

The purpose of the present study was to investigate the experimental conditions affecting cell invasiveness by *P. mirabilis*. We also investigated the possible role of the *P. mirabilis* hemolysin in the invasion process since Kihlström and Edebo (8) suggested that surface-bound catalysts on bacteria might enhance invasion. Furthermore, we tested a group of urinary isolates of *E. coli* to confirm the observation of Braude and Siemienski (3) on the lack of invasive ability of a urinary strain of *E. coli*.

MATERIALS AND METHODS

Bacterial strains. The *P. mirabilis* strains used in this study were isolated from the urine of patients with urinary tract infections or from the feces of persons without urinary tract infections. Strains T1 and M12 are a wild-type urinary isolate and its urease-negative mutant, respectively, as described by MacLaren (10). All *E. coli* strains were urinary isolates. Only strains susceptible to kanamycin were used in the experiments (MIC $\leqq$ 10 μg/ml).

Cultivation of bacteria. All strains were kept in glycerol-broth mixture (3/7) at −70°C. Before use, a strain was inoculated on cystine-lactose electrolyte-deficient (CLED) agar plates (Oxoid Ltd.). From these plates, the bacteria were inoculated in nutrient broth and grown overnight in a shaking water bath (100 rpm) at 37°C. This culture was then diluted in the incubation medium for the invasion experiments.

Vero cell culture. The Vero cells (African green monkey kidney cells; Flow Laboratories) were passaged weekly by trypsinization and grown as monolayers at 37°C. The culture medium was the Glasgow modification of Eagle medium (Flow Laboratories) supplemented with glutamine (2 mM), fetal calf serum (10%), and penicillin-streptomycin. For invasion experiments, the cells were cultured in multiwell trays (Falcon 3008; BD Labware). Confluent monolayers were used in the experiments.

Invasion test. The wells containing the confluent monolayers were washed twice with Hanks balanced salt solutions (HBSS). One milliliter of the incubation medium with bacteria was added to each well with Vero cells. The incubation medium was composed of HBSS (80%, vol/vol), HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate) buffer (200 mM [10%, vol/vol]), and minimal medium (10%, vol/vol). The pH of the incubation medium was set at 7.5. A 1:200 dilution of the overnight bacterial culture was used in all experiments unless otherwise stated. This resulted in ca. 5×10^7 CFU/ml for the *P. mirabilis* strains and ca. 2.5×10^7 CFU/ml for the *E. coli* strains, with a variation of 15%. After incubation for 2 h at 37°C, the bacterial growth during this period was determined by serial dilution of the supernatant fluid and plating of the cells onto CLED agar plates. After removal of the incubation medium, the cells were washed with HBSS, and tissue culture medium with kanamycin (250 μg/ml) was added to the wells to kill the extracellular bacteria (14). The trays were reincubated for 1.5 h at 37°C. The wells were then washed twice with HBSS and checked for integrity of the monolayer, and the cells were lysed with a slightly modified blood-lysing solution (23) which was nontoxic for the bacteria (NaH_2PO_4, 0.01 M; Tween 20, 1% [vol/vol]; trypsin, 0.025% [wt/vol] [pH 8.0]). A 30-min period at 37°C was sufficient to lyse the Vero cells. Bacterial counts were made by plating a suitable dilution of the lysed cell solution onto CLED agar plates. At least three wells were

* Corresponding author.

TABLE 1. Effect of pH of the incubation medium on cell invasion by *P. mirabilis* AM24

pH[a]	Invasion (10^3 CFU per well)[b]	Growth (%)[c]
6.5–6.7	1.43 (0.43)	250
7.0–7.2	2.40 (0.30)	220
7.5–7.6	5.32 (0.31)	243
8.0–8.0	4.70 (0.11)	226

[a] pH of the incubation medium before and after incubation.
[b] Mean (standard error of the mean) of four wells.
[c] Expressed as a percentage of the inoculum at the start of the experiment (2.5×10^7 CFU/ml).

used for each test. All experiments were performed at least twice. Variations in the composition of the incubation medium or in the incubation times used in some experiments are discussed below.

Hemolytic activity. The values for the hemolytic activities of the *P. mirabilis* strains used were measured as described previously (17). Briefly, hemolytic titers were recorded as the highest dilution of a late-log-phase culture which produced complete hemolysis of a standardized suspension of horse erythrocytes.

Urease inhibition. Acetohydroxamic acid (Sigma Chemical Co.) was used as an inhibitor of urease at a concentration of 0.5 mg/ml.

RESULTS

We studied first the effect of various experimental conditions on cell invasion by *P. mirabilis*.

Influence of pH of the incubation medium. The pH of the incubation medium was varied from 6.5 to 8.0. The effect on cell invasiveness was tested with *P. mirabilis* AM24 and AM65. The results for strain AM24 are shown in Table 1. Whereas variation of the pH had no significant effect on the bacterial growth during the incubation period, there was an optimal pH 7.5 to 8 for cell invasion. Similar results were obtained with strain AM65 (data not shown). Higher pH values induced cell damage. A pH of 7.5 was used in all other experiments.

Incubation time and bacterial numbers. The number of intracellular bacteria was also dependent on the number of bacteria in the incubation medium and on the length of the incubation period. The results of a typical experiment with *P. mirabilis* AM65 are shown in Fig. 1. However, both the number of bacteria and the length of the incubation period had to be limited by the occurrence of cytotoxic effects depending on the hemolytic activity of the strains used. This effect has been described previously (17).

Control experiments with a prolonged incubation time with the kanamycin medium did not show significant intracellular growth (<50%) of bacteria in the first 5 h after invasion; therefore, the increase in numbers of intracellular bacteria must be considered as the result of increased invasion.

Effect of urea on cell invasion. Braude and Siemienski (3) described a stimulating effect of urea on cell invasion by *P. mirabilis* at an optimal concentration of ca. 0.2%. We had to carry out experiments with urea in the incubation medium in a CO_2 incubator as otherwise the pH rose to cytotoxic levels (>8.5) despite the presence of the HEPES buffer. The effect of urea on strain AM24 with or without the urease inhibitor acetohydroxamic acid and on strain T1 and its urease-negative mutant M12 was studied (Fig. 2). As shown in Fig. 2, we could not find any evidence for a stimulating effect of urea on cell invasion. No effect was found with other urea concentrations (0.05 to 0.5% [data not shown]).

Reproducibility of the invasion experiments. The variation in the number of intracellular bacteria found in different wells in one experiment was about 10%. The variation between different experiments was much greater. However, the relative effect of different incubation circumstances was constant in different experiments. Comparing the invasive capacities of different strains was possible by expressing this ability as a percentage of the invasive ability of a standard strain. The variation between different experiments was most probably because of variations in the number and condition of the Vero cells.

Role of *P. mirabilis* hemolysin in cell invasion. Preliminary screening of a few *P. mirabilis* strains suggested that their hemolysin might be involved in the invasion process. To confirm this hypothesis, we compared 32 strains for their invasive ability (Fig. 3). Strain AM24 was used as a reference strain. By using the Spearmann rank correlation test, we found a significant correlation between the hemolytic

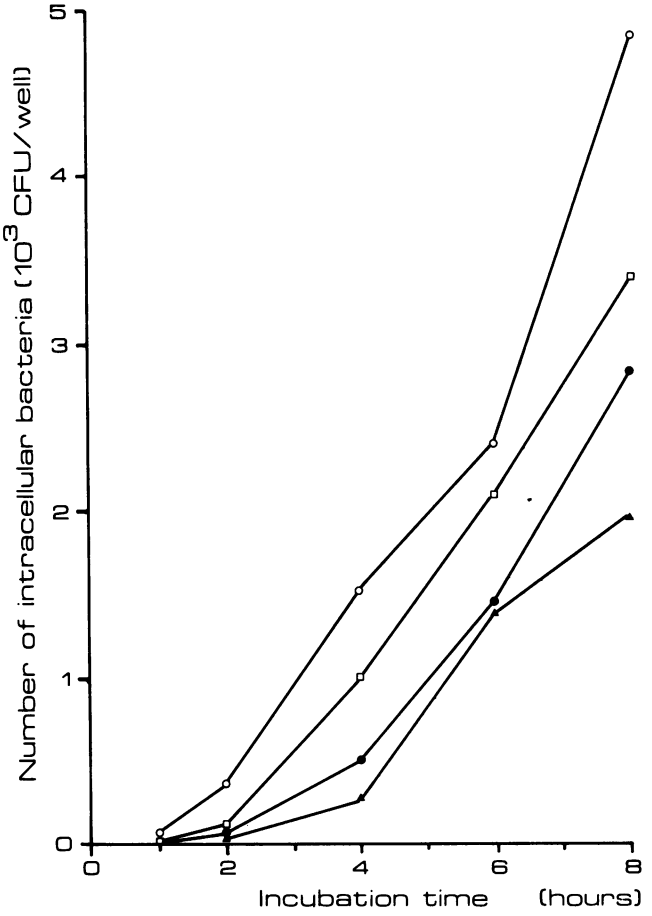

FIG. 1. Numbers of intracellular bacteria per well for different numbers of bacteria in the incubation medium: 5×10^7 CFU/ml (○), 2.5×10^7 CFU/ml (□), 1.3×10^7 CFU/ml (●), and 6.3×10^6 CFU/ml (▲). At $t = 8$ h, only the difference in numbers of intracellular bacteria in the wells with the highest and the lowest numbers of bacteria in the incubation medium was significant ($P < 0.05$; Student t-test).

activity of a strain and its invasive ability ($P < 0.01$). Strains with a hemolytic titer over 256 were not used because of the occurrence of cytotoxic effects under the conditions used in these experiments.

Further evidence for an effect of the hemolysin was obtained from the results with a mutant strain of AM24. This mutant strain, obtained by treatment with ethyl methane sulfonate (Sigma Chemical Co.), had an increased hemolytic activity (5- to 10-fold) compared with the parent strain. Apart from this increased hemolytic activity, we could not detect any differences between the two strains. Biochemical characteristics (determined with the API 20E), growth rate in nutrient broth or in the incubation medium, MIC values for 12 antibiotics, and agglutinating antibody titers against O and H antigens in mouse immune sera raised against both strains were identical for both strains. Cell invasion by the mutant was consistently two to four times higher than invasion by the parent strain in three independent experiments.

Also in agreement with the hypothesis were the observations that invasion by overnight-grown bacteria started slowly and coincided with the development of hemolytic activity and that an inhibitory effect was seen with chloramphenicol which, as we previously showed, inhibits efficiently the production of the hemolysin. Chloramphenicol in the incubation medium (50 μg/ml) reduced cell invasion to 20% of the level without chloramphenicol (data not shown). In a typical experiment, we measured invasion with strain AM24 as 1.3×10^4 CFU per well, which was reduced to 2.6×10^3 CFU per well in the presence of chloramphenicol.

Cell invasion by urinary isolates of *E. coli*. We tested 13 urinary *E. coli* strains. Four strains were α-hemolytic, and total cell destruction resulted with these strains in the standard invasion procedure. By lowering the number of bacteria, the cytotoxic effects could be avoided, but no invasion was seen. The nine nonhemolytic strains did not cause cytotoxic effects in the standard invasion procedure but, again, no invasion of the Vero cells occurred.

DISCUSSION

Incubation of bacteria with tissue culture cells, followed by the use of an aminoglycoside antibiotic to kill extracellular but not intracellular (21) bacteria and recovery of the intracellular bacteria by lysing the cells, is now used by many authors to study cell invasion by bacteria (8, 14, 19). Several observations support the suitability of this method for *P. mirabilis*. The fact that the number of bacteria recovered after an invasion experiment remained nearly constant during incubation in a kanamycin medium for at least 5 h can only be explained by an intracellular localization of these bacteria since, in control experiments under similar conditions, this concentration of kanamycin caused a dramatic decrease in viable count (>1,000-fold in 60 min). Furthermore, the cells with intracellular bacteria should be viable as the survival of bacteria in cells in the presence of kanamycin in the extracellular medium is based on the impermeability of living cells to aminoglycoside antibiotics (21). This impermeability, as shown by trypan blue exclusion, is lost in damaged cells. A major advantage over microscopic methods to establish the number of intracellular bacteria is that extracellular adhering bacteria do not interfere in the assay.

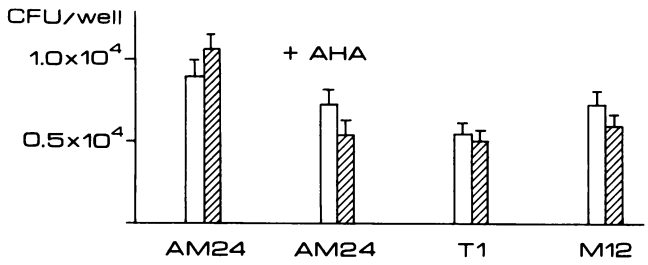

FIG. 2. Effect of the presence (▨) or absence (□) of urea (0.25%, wt/vol) in the incubation medium. Acetohydroxamic acid was added to a concentration of 0.5% (wt/vol). Strain M12 is a urease-negative mutant of strain T1. Vertical bars represent the standard error of the mean.

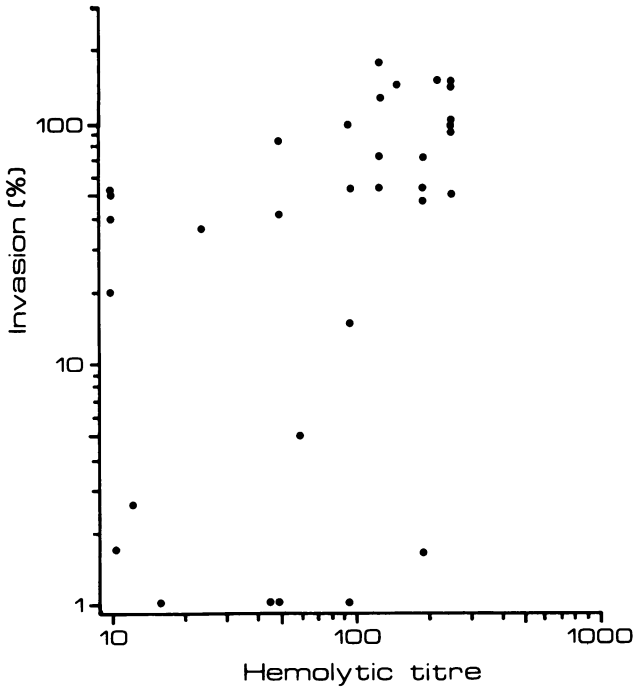

FIG. 3. Cell invasion by *P. mirabilis* strains, expressed as percentage of the invasion by strain AM24, in relation to the hemolytic titer of these strains.

All *P. mirabilis* strains tested by this method were able to invade Vero cells. The exact nature of this process remains, however, unclear, just as it is for other invasive bacteria such as *Salmonella* (7), *Shigella* (14), and *Yersinia* (19) species. Kihlström and Edebo (8) suggest that cell invasion is a phagocytosis-like process since dead bacteria are also ingested by cultured cells. Our observation that chloramphenicol-inhibited bacteria invaded Vero cells is comformable to this hypothesis. Nevertheless, it is clear that bacterial properties are important in the invasion process. For *Shigella* spp., it has been shown that a plasmid is involved (18). Furthermore, Bhogale et al. (2) presented evidence that heat-labile antigens of *Shigella flexneri* are important in HeLa cell invasion. The hypothesis of Kihlström and Edebo (8) on the involvement of a bacterial surface-bound catalyst has already been mentioned above. For *P. mirabilis*, we brought forward evidence in this study which supports the role of the cell-associated hemolysin (17) as such a catalyst. The correlation between the hemolytic activity and the invasive ability for a large group of strains, the increased invasion by a mutant strain with an increased hemolytic

activity, the inhibitory effect of chloramphenicol, and the kinetics of the invasion process which parallel the development of the hemolytic activity are all in agreement with this hypothesis.

We were not able to confirm the observation of Braude and Siemienski (3) that urea stimulates cell invasion by *Proteus* spp. A likely explanation is that we performed our experiments at a constant pH, whereas in experiments of Braude and Siemienski, a significant rise in the pH occurred in the presence of urea. It is tempting to speculate that the increased pH of the urine during *P. mirabilis* urinary tract infections, because of the breakdown of urea, might stimulate cell invasion in vivo.

As to the invasive ability of urinary *E. coli*, strains we could confirm the observation by Braude and Siemienski that these strains do not invade cells. In a recent observation, Sansonetti (18) showed that only *E. coli* strains harboring a plasmid, very similar to the plasmid in invasive *S. flexneri* strains, are invasive. These strains are isolated from patients with a dysenteric syndrome. Probably cell invasion is not an important aspect in the pathogenesis of urinary tract infections by *E. coli*. Interestingly, Coles et al. (4) showed that T-lymphocytes have no protective effect in urinary tract infections caused by *E. coli*, whereas Araki (1) showed just such a protective effect in pyelonephritis in rats caused by *P. mirabilis*. T-lymphocytes are known to be important in the host defense mechanisms against intracellular bacteria. Possibly, the differences in invasive abilities between *E. coli* and *P. mirabilis* are responsible for these contradictory findings of Coles et al. and Araki. More experiments, especially in vivo, are necessary to elucidate the importance of the invasive ability of *Proteus* strains in the pathogenesis and treatment of urinary tract infections. These studies are now in progress.

LITERATURE CITED

1. **Araki, T.** 1977. Cell-mediated immunity in pyelonephritis. Jpn. J. Urol. **68**:771–779.
2. **Bhogale, S. R., K. D. Sharma, and R. S. Kamat.** 1983. Role of heat-labile antigens of *Shigella flexneri* in HeLa cell invasion. J. Med. Microbiol. **16**:37–43.
3. **Braude, A. I., and J. Siemienski.** 1960. Role of bacterial urease in experimental pyelonephritis. J. Bacteriol. **80**:171–179.
4. **Coles, G. A., S. Chick, M. Hopkins, R. Ling, and N. J. Radford.** 1974. The role of the T cell in experimental pyelonephritis. Clin. Exp. Immunol. **16**:629–636.
5. **Gorrill, R. H., and S. J. DeNavasquez.** 1964. Experimental pyelonephritis in the mouse produced by *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus mirabilis*. J. Pathol. Bacteriol. **87**:79–87.
6. **Gould, J. C.** 1968. The comparative bacteriology of acute and chronic urinary tract infections, p. 43–48. *In* F. O'Grady and W. Brumfit (ed.), Urinary tract infection. Oxford University Press, London.
7. **Kihlström, E.** 1977. Infections of HeLa cells with *Salmonella typhimurium* 395 MS and MR10 bacteria. Infect. Immun. **17**:290–295.
8. **Kihlström, E., and L. Edebo.** 1976. Association of viable and inactivated *Salmonella typhimurium* 395 MS and MR10 with HeLa cells. Infect. Immun. **14**:851–857.
9. **Larsson, P., and Olling.** 1977. O-antigen distribution and sensitivity to the bactericidal effect of normal human serum of *Proteus* strains from clinical specimens. Med. Microbiol. Immunol. **163**:77–82.
10. **MacLaren, D. M.** 1969. The significance of urease in *Proteus* pyelonephritis: a bacteriological study. J. Pathol. **96**:45–56.
11. **MacLaren, D. M.** 1969. The significance of urease in *Proteus* pyelonephritis: a histological and biochemical study. J. Pathol. **97**:43–49.
12. **Mehlham, I. J., E. L. Eide, A. C. Sanders, M. Fishbein, and C. C. Aulislo.** 1977. Methodology for recognition of invasive potential of *Escherichia coli*. J. Assoc. Anal. Chem. **60**:546–562.
13. **Musher, D. M., D. P. Griffith, D. Yawn, and R. D. Rossen.** 1975. Role of urease in pyelonephritis resulting from urinary tract infection with *Proteus*. J. Infect. Dis. **131**:177–181.
14. **Okamura, N., T. Nagai, R. Nakaya, S. Kondo, M. Murakami, and K. Hisatsune.** 1983. HeLa cell invasiveness and O antigen of *Shigella flexneri* as separate and prerequisite attributes of virulence to evoke keratoconjunctivitis in guinea pigs. Infect. Immun. **39**:505–513.
15. **Pazin, G. J., and A. I. Braude.** 1974. Immobilizing antibodies in urine. II. Prevention of ascending spread of *Proteus mirabilis*. Invest. Urol. **12**:129–133.
16. **Peerbooms, P. G. H., A. M. J. J. Verweij, and D. M. MacLaren.** 1982. Urinary virulence of *Proteus mirabilis* in two experimental mouse models. Infect. Immun. **36**:1246–1248.
17. **Peerbooms, P. G. H., A. M. J. J. Verweij, and D. M. MacLaren.** 1983. Investigation of the haemolytic activity of *Proteus mirabilis* strains. Antonie van Leeuwenhoek J. Microbiol. **49**:1–11.
18. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1982. Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect. Immun. **35**:852–860.
19. **Schiemann, D. A., and J. A. Devenish.** 1982. Relationship of HeLa cell infectivity to biochemical, serological and virulence characteristics of *Yersinia enterocolitica*. Infect. Immun. **35**:497–506.
20. **Silverblatt, F. J.** 1974. Host-parasite interaction in the rat renal pelvis. J. Exp. Med. **140**:1696–1711.
21. **Tronet, A., and P. Tulkens.** 1981. Intracellular penetration and distribution of antibiotics, p. 337–351. *In* L. Ninet (ed.), The future of antibiotherapy and antibiotic research. Academic Press, Inc., London.
22. **Van den Bosch, J. F., J. de Graaff, and D. M. MacLaren.** 1979. Virulence of *Escherichia coli* in experimental hematogenous pyelonephritis in mice. Infect. Immun. **25**:68–74.
23. **Zierdt, C. H.** 1982. Blood-lysing solution nontoxic to pathogenic bacteria. J. Clin. Microbiol. **15**:172–174.