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Adherence Assay of Uropathogenic *Escherichia coli* In Vivo and In Vitro

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Purpose: This study aims to investigate the specific pathogenic properties or virulent determinant characteristics of uropathogenic *Escherichia coli* (UPEC) as bacterial adherence in tissue culture cells in vitro and the pathogenicity in animal model in vivo.

Materials and Methods: Thirty strains of *E. coli* were isolated from urine of patients with acute pyelonephritis. Four cell lines—HeLa cells, HEp-2 cells, A-498 cells, and J-82 cells—were used for bacterial adherence assay. Histologic examination and scanning electron microscopy examination of pyelonephritis or cystitis, which was caused by *E. coli*, in mice was performed.

Results: Sixteen (53.3%) strains of *E. coli* appeared to adhere to at least one or more kinds of four cell lines, and seven strains were able to adhere to all four cell lines. All of the tested *E. coli* strains were adhered to the mouse bladder and kidneys. The number of bacteria colonized in the kidney was greater than that of bladders in the following 5 strains of *E. coli*: TME104, TME107, TME113, TME306, and TME119. There was no difference in the number of bacteria colonized in the bladder and kidneys in the aspects of adherence patterns to tissue culture cells.

Conclusions: Although there was no best choice cell lines in the adherence assay to identify the adherence patterns, combined assays of in vitro cell culture and in vivo model of mouse urinary tract infection appeared to be efficient methods to investigate the role of bacterial adherence in the pathogenesis of UPEC.

Keywords: Urinary tract infections; Virulence factors; *Escherichia coli*

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INTRODUCTION

Urinary tract infections (UTI) are usually caused by gram-negative micro-organisms. *Escherichia coli*, a member of *Enterobacteriacea*, is the most frequent agent of UTI, regardless of patients age.

Some potential virulence factors of *E. coli* that have been suggested so far are particular structures or components of bacteria, including fimbriae, hemolysin, aerobactin, and specific O antigen, as well as functional properties, including serum resistance and adherence to the host cells [1]. Since the ability to adhere to host epithelial cells in the urinary tract represents the most important determinant of pathogenicity [1], there have been numerous reports about adherence in UTI. The adhesins of E coli exhibiting mannose resistant hemagglutination were divided into two types of adhesins: P and X. P, F, and O-negative-A-positive belong to the P-related adhesins. Group of Dr, afimbrial adhesin (AFA)-I, AFA-III, S, and nonfimbrial adhesin was X related adhesins. Among the adhesins reported thus far, P-pili have

thought to be a major virulence factor in uropathogenic *E. coli* (UPEC) [2].

The adherence of bacterial strains to tissue culture cells has been used to detect virulent strains that implicated bacterial pathogens. The adherence assay was used to differentiate the specific pathogenic properties or characteristics as virulent determinant first for the purpose of screening the enteropathogenic *E. coli* (EPEC) strains [3], and Cravioto et al. [4] reported the application of adherence assay by using HEp-2 cells to characterize EPEC. This study was designed primarily in order to investigate the correlation between bacterial adherence in tissue culture cells in vitro and the pathogenicity in an animal model in vivo. Secondly, this study aimed to provide data for selecting an adequate cell line of tissue culture cell model to determine the uropathogenicity of *E. coli* for accompanying with the trial to figure out potential virulence factors that might be associated with tissue tropism of bacteria.

MATERIALS AND METHODS

1. Bacterial Strains

Thirty strains of *E. coli* were isolated from urine of patients with acute pyelonephritis (APN). These strains were identified via the API 20E (bioMérieux, Paris, France) method. All isolated strains were inoculated into the trypticase soy broth (TSB) and grown overnight, in which, 20% glycerol was added; it was then kept at -70°C until further use.

2. Cell Culture Adherence Assay

Four cell lines, HeLa (cervix), HEp-2 (larynx), A-498 (kidney), and J-82 (bladder), were used for bacterial adherence assay. The adherence assay was performed by the method of Nataro et al. [5]. Each cell line was grown in Eagle's minimum essential medium (EMEM; Gibco, Carlsbad, CA, USA) with conventional antibiotics and 10% fetal bovine serum. Cell monolayers were obtained after growth in 1 ml of growth media in each well of 24 well plates. The cells were fixed with absolute methanol for 20 minutes and stained with the Giemsa solution for an additional 20 minutes. After rinsing with sterile distilled water, cell culture plates were examined for bacterial adherence via inverted microscope. The adherence index was expressed as the mean value of the *E. coli* cell numbers, which adhered to each of 10 HeLa or other tissue culture cells. These adherent strains were classified into the following three types [6]. The term, localized adherence (LA), described *E. coli* strains bound to the localized areas of the tissue culture cells, that resulted in the formation of particulate microcolonies. Diffuse adherence (DA) was defined as a group of *E. coli* strains bound to the dispersed surface of the tissue culture cells. The aggregative adherence (AA) pattern was defined as a characteristic aggregation

Table 1. Adherence of *Escherichia coli* strains to the host cells

Strain no.	HeLa	HEp-2	A-498	J-82
TLE102	DA (54.5±14.8)	DA (82.0±7.4)	AA (68.2±5.3)	AA (45.3±9.6)
TLE103	NA	NA	LA (17.0±4.4)	LA (27.8±6.5)
TLE104	NA	NA	NA	LA (34.3±7.1)
TLE105	NA	NA	NA	LA (17.0±0.8)
TME102	LA (11.2±5.8)	LA (16.2±2.3)	NA	NA
TME103	NA	NA	DA (18.2±6.1)	DA (19.5±4.2)
TME104	DA (178.3±16.5)	DA (208.8±17.4)	DA (37.5±12.4)	DA (147.8±22.4)
TME107	LA (9.7±3.9)	LA (7.8±1.3)	NA	NA
TME108	DA (70.2±20.0)	DA (53.5±10.6)	AA (70.0±19.7)	DA (40.5±10.8)
TME113	LA (31.2±9.8)	LA (35.2±8.6)	DA (15.2±4.1)	DA (16.0±2.8)
TME114	AA (39.5±5.9)	AA (32.0±5.8)	AA (34.8±7.2)	AA (30.5±8.0)
TME116	DA (19.0±5.0)	DA (31.0±4.6)	DA (24.0±5.5)	DA (17.0±5.5)
TME303	LA (29.3±4.8)	LA (45.5±5.0)	LA (35.8±14.8)	LA (24.2±5.7)
TME304	NA	NA	LA (27.8±5.4)	LA (32.7±15.2)
TME306	AA (14.0±2.4)	AA (28.0±5.3)	NA	NA
TME307	AA (22.5±7.4)	AA (43.0±3.4)	ND	ND

The number of bacteria adhered to each host cells are presented as mean±standard error of the mean.
DA: diffuse adherence, AA: aggregative adherence, NA: non-adherence, LA: localized adherence, ND: almost all cultures cells were detached and lysed, and adherence pattern and degree of *E. coli* strains could not be determined.

of bacteria frequently showing an appearance of "stacked-brick."

3. Adherence to Mouse Uroepithelial Cells In Vivo

The adherence assay to mouse uroepithelial cells was examined using the modified method of Hagberg et al. [7] and Linder et al. [8]. Six-to-eight week-old female BALB/c mice, weighing about 25 g, were used. Bacteria cells for experimental infection were cultured in TSB at 37°C, harvested, and suspended in phosphate buffered saline (PBS, pH 7.4). The inoculum size for infection was adjusted to 1.3×10^8 colony forming unit (CFU)/ml with Spectronic 20 spectrophotometer (Bausch & Lomb; Rochester, New York, USA) after standardization of bacterial cell concentration by counting the number of viable colonies on the agar plate.

Each bacterial suspension was inoculated directly into the urinary bladder of mice via surgery. After ethyl ether anesthesia, abdominal wall of the mouse opened, and 0.1 ml of bacterial suspension (1.3×10^8 CFU) were inoculated directly into the urinary bladder with a 26 gauge needle. Animals were sacrificed 24 hours after infection. The two kidneys and bladder were removed and homogenized with Polytron handled homogenizer. Tenfold serial dilution of homogenates in PBS was plated on eosin methylene blue (EMB) agar. The number of bacteria per 0.1 ml of tissue homogenate was meticulously counted after selecting EMB agar plates, which was shown to be between 20 and 100 colonies.

4. Light Microscopy of Lesion In Vivo

Histologic examination of pyelonephritis or cystitis caused by *E. coli* in mice was performed. Paraffin embedded specimens were dissected with 3 μm of thickness, and stained with hematoxylin and eosin.

5. Scanning Electron Microscopy

For an *in vitro* study of adherence, J-82 cells was cultured on a slide glass and added to 50 μl bacteria culture. After repeated washing with PBS three times, the cells were fixed with osmium tetroxide. For an in vivo study, bacteria were inoculated into the mouse urinary bladder. After sacrificing

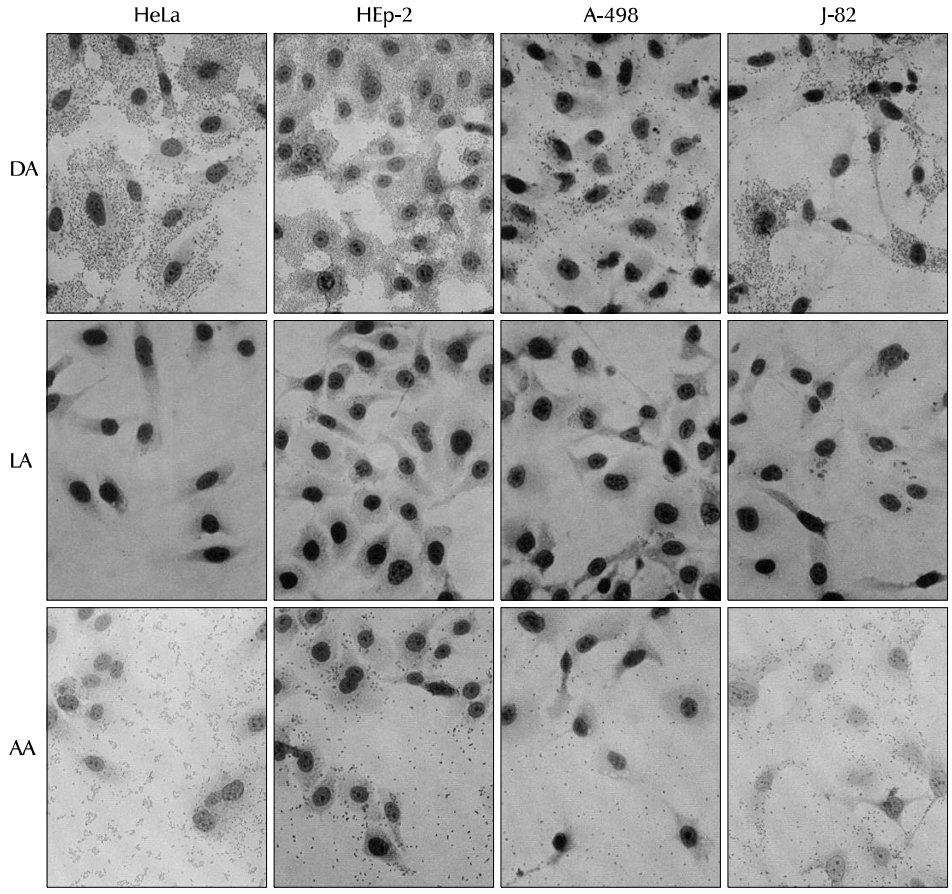

Fig. 1. Light micrographs showing 3 types of adherence pattern in HeLa, HEp-2, A-498, and J-82 cells. DA denotes a diffuse adherence to the TME104 strain on the dispersed surface of the cultured cells. LA represents a localized adherence of the TME303 strain to the localized areas of cultured cells and formed to particulate microcolonies. AA denotes an aggregative adherence of TME114 strain to the cultured cells. Note the characteristic aggregates of bacteria that showed "stacked-brick" appearance. They bound aggregately to both of cultured cells and bottom of cell culture dish (×500).

the animals 24 hours after infection, the bladder was removed, and then fixed with osmium tetroxide. The fixed samples for scanning electron microscopy (SEM) were sequentially dehydrated with ethanol, critical point dried, and coated with platinum. The samples were analyzed by Hitachi S-800 (Hitachi, Tokyo, Japan) scanning electron microscope.

RESULTS

1. Adherence to Tissue Culture Cells

A total of 30 *E. coli* strains isolated from APN patients were subjected for the assessment of virulence in the aspects of adherences to HeLa cells, HEp-2 cells, J-82 cells, and A-498 cells.

The adherence patterns and numbers of bacteria adhered to HEP-2 cells and HeLa cells are summarized in Table 1. Sixteen (53.3%) strains of *E. coli* appeared to adhere to at least one or more kinds of the cell lines, and seven strains were able to adhere to all four cell lines. Eleven (36.7%) strains adhered to both HEp-2 and HeLa cells, but the number of bacteria adhered to each of the HEp-2 and HeLa cells (adherence capacity) were more or less different. Eight of 11 *E. coli* strains, except three strains (TME107, TME108, and TME114), showed greater likelihood of adhering to HEp-2 cell than to HeLa cell with respect to the number of bacteria adhered per each cell of HEp-2 or HeLa. The number of strains that showed DA, LA, and AA patterns were 4, 4, and 3 strains, respectively. The average number of bacteria adhered to HEp-2 cells, except 2 strains (TME102 and TME107). The DA pattern of HEp-2 cells of the strain TME104 was most striking (Table 1, Fig. 1).

Table 2. In vivo adherence of *Escherichia coli* strains to mouse bladder and kidneys

Strain no.	Adherence pattern	No. of bacteria (/0.1 ml of tissue homogenate)[a]	
		Bladder	Kidneys
TME104	DA	5.5×10^7	1.7×10^8
TME108	DA	9.4×10^4	3.5×10^4
TME116	DA	2.1×10^5	6.7×10^4
TME107	LA	3.5×10^4	9.5×10^4
TME113	LA	3.2×10^4	1.3×10^5
TME303	LA	7.1×10^6	2.2×10^3
TME114	AA	7.4×10^8	5.2×10^8
TME306	AA	3.1×10^5	3.3×10^5
TME307	AA	1.2×10^4	6.0×10^2
TME115	NA	9.2×10^5	7.2×10^5
TME117	NA	5.1×10^5	6.3×10^3
TME119	NA	1.2×10^4	6.1×10^6

DA: diffuse adherence, LA: localized adherence, AA: aggregative adherence, NA: non-adherence.
[a]The numbers of bacteria adhered to bladder and kidneys were the mean numbers of double experiments.

Table 3. Histological changes of mouse urinary tract after intravesical inoculation with *Escherichia coli* strains

Strain no.	Adherence pattern	No. of mice with		
		Cystitis & pyelitis	Only cystitis	Only pyelitis
TME116	DA	3/5	0/5	2/5
TME303	LA	2/3	1/3	0/3
TME114	AA	0/4	3/4	0/4
TME115	NA	0/3	1/3	1/3

DA: diffuse adherence, LA: localized adherence, AA: aggregative adherence, NA: non-adherence.

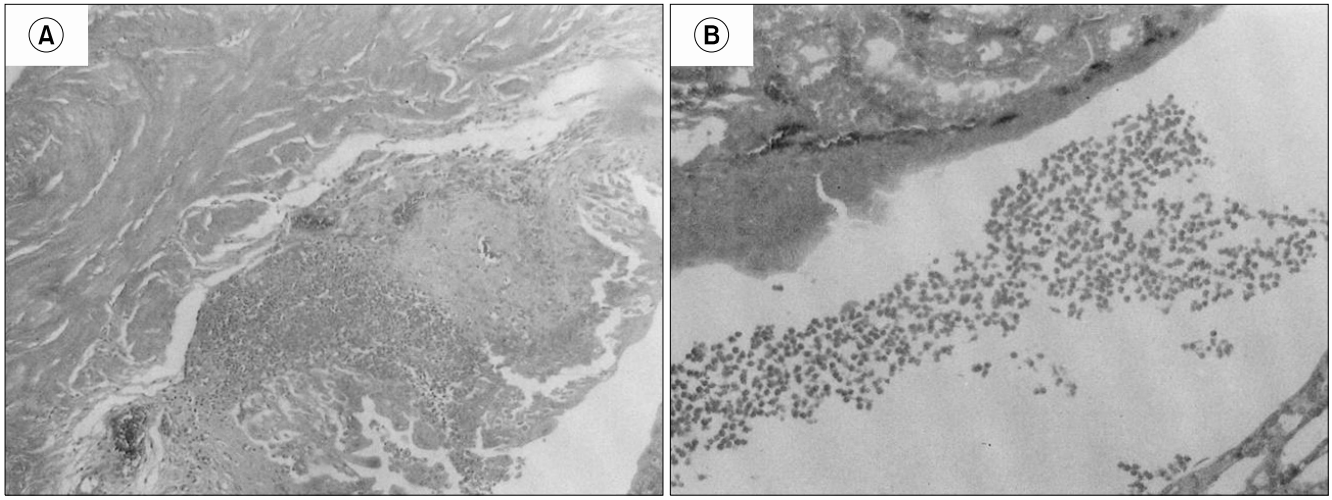

Fig. 2. Inflammation of the mouse bladder (A) and kidney (B) caused by bacterial infections. H&E, ×100.

2. Adherence In Vivo Assay of *E. coli* Strains to Mouse Uroepithelium

Twelve strains of *E. coli* that exhibited different adherence patterns compared with HEp-2 cells were tested for the adherence of mouse urinary bladder and kidney (Table 2). Animals were inoculated with 1.3×10^8 CFU for each *E. coli* strain grown on the agar plates, which was counted. All strains of *E. coli* tested adhered to the mouse bladder and kidneys. The number of bacteria colonized in the kidney was greater than that in the bladders for 5 strains of *E. coli*, TME104, TME107, TME113, TME306, and TME119. TME114 strain that exhibited the AA pattern on HEp-2 cells showed outstanding adherence to kidneys and bladder. There was no difference in the number of bacteria colonized in the bladder and kidneys regarding the adherence patterns of tissue culture cells.

3. Inflammatory Response of Experimentally Infected Mouse

Four strains of *E. coli* (TME116, TME303, TME114, and TME115) underwent the virulence assay in vivo through the observation of intravesical inoculations of *E. coli* strains (Table 3, Fig. 2). After injection with the TME116 strain, all five animals showed inflammation and pathologic change in the renal pelvis, and overt cystitis was developed in three animals. The histological finding of renal pelvis wall and in the muscle layers of the bladder was infiltrated with polymorphonuclear leukocytes and mononuclear cells. A

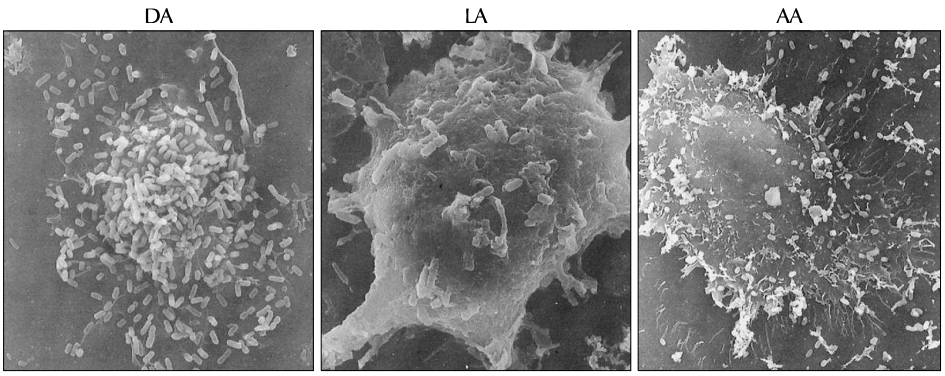

Fig. 3. Scanning electron micrographs showing diffuse adherence (DA) of TME104, localized adherence (LA) of TME303, and aggregative adherence (AA) of TME114 *Escherichia coli* strains adhered to J-82 cells. Magnification, ×1,500.

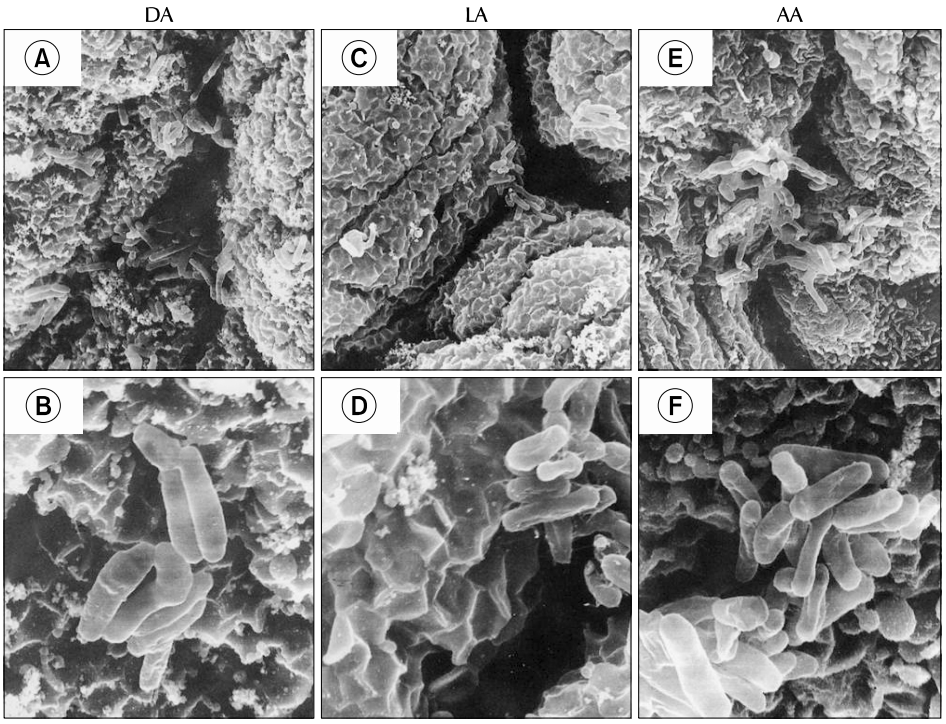

Fig. 4. Scanning electron micrographs showing TME116 (A, B), TME303 (C, D), and TME114 (E, F) *Escherichia coli* strains adhering to mouse urinary bladder. Magnification, A, C, E: ×2,200; B, D, F: ×15,000. DA: diffuse adherence, LA: localized adherence, AA: aggregative adherence, NA: non-adherence.

gross finding of the bladder of one animal revealed a micro-abscess in the submucosal layer of the bladder. Through SEM, microbe adherence to the surface of J-82 cells was shown in an in vivo study (Fig. 3); and in an in vivo study of adherence in an animal (Fig. 4), bacteria were adhered to the uroepithelial cell surface of the mouse bladder.

DISCUSSION

On the basis of both the prevalence theory and the special pathogenicity theory [9], UPEC strains seemed to be quantitatively dominant over normal floral *E. coli* and appeared to be resident on the fecal flora before ascending to the urinary tract; conversely, the theory of special pathogenicity suggested that the strain causing UTI should have special virulence determinants distinguishable from non-pathogenic *E. coli* [10]. Pathologic change can start with an interaction between the direct effects of the bacteria and counteraction of the host's immune response [11]. The severity and rapid progress of the disease can be decided, in part, by several factors, such as the site of infection, host immune responsiveness to pathogenic bacteria, and other physiological condition of UTI [2].

Because intact uroepithelial cells are relatively refractory to the bacterial adherence, *E. coli* strains bind to the scars of uroepithelial surfaces [12]. The surface hydrophobicity of bacteria confers loose attachment to the epithelial surface on themselves; however, many kinds of adhesins of bacteria and reciprocal receptors of the host cells are known to mediate strong adherence of *E. coli* strains to the epithelial surfaces [13,14].

Adherence of *E. coli* strains to uroepithelial cells is the essential step in UTI. Bacterial adherence in the urinary tract has been studied via both in vivo and in vitro approaches. Experimental animals have been selected in the aspects of similar condition with specificity of adherence for in vivo studies. Various animals, including monkeys, mice, rats, or rabbits, have been used in UTI in vivo studies. Rat uroepithelial cells express the Gal(1-3)Gal form of globoside instead of the Gal(1-4)Gal form of globoside, and thus, cannot make critical receptors for P-pili [15,16]. Since P-pili is one of the most important virulence factors among pyelonephritogenic *E. coli* strains, a rat model is an unacceptable animal for the evaluation of P-pili related pathogenic mechanism.

The two types of cell lines, HeLa and HEp-2, are widely used to investigate bacterial adherence and invasiveness of other gram negative pathogenic organisms [17]. Bacterial adherence to the cultured cells was also used to differentiate virulent *E. coli* strains. Scaletsky et al. [6] reported that EPEC strains adhered to HeLa cells by two different patterns. Since the first report by Scaletsky, a new category of adherence pattern was described in the cell culture adherence test [18]. At present, HeLa cells and HEp-2 cells were frequently used to detect diarrheagenic *E. coli* strains; however, there have been no best cell lines for studying *E. coli* strains isolated from the urinary tract.

We examined the availability of A-498 cells and J-82 cells originated from urinary tract carcinoma as well as HeLa cells and HEp-2 cells for in vitro adherence assay of UPEC strains. It was found the more numbers of UPEC were adhered to J-82 cells than A-498 cells, HeLa cells, and HEp-2 cells. Of the 30 *E. coli* strains, apart from the type of adherence pattern, twelve strains adhered to J-82 cells, eleven to HeLa and HEp-2 cells, and ten to A-498. Some *E. coli* strains that did not adhere to HEp-2 cells adhered to J-82 cells, and other strains that adhered to HEp-2 cells did not adhere to J-82 cells. This suggested that the discordance of bacterial adherence to different cell lines seemed to be related to the difference of each receptor for bacterial adhesion expressed on the cell surface.

The adherence pattern of HeLa cells and HEp-2 cells did not show a good correlation with J-82 cells and A-498 cells; however, both of J-82 cells and A-498 cells showed the same adherence pattern. Two strains (TLE102 and TME108) that exhibited the DA pattern on HEp-2 cells showed the AA pattern on J-82 cells. One strain (TME113) that exhibited the LA pattern on HeLa cells caused the DA pattern on J-82 cells; nonetheless, TME113 strain was positive in dot hybridization with EPEC adherence factor (EAF) probe gene coding LA. Since EAF probe for detecting the LA pattern has been originally designed on the basis of adherence assay using HeLa cells or HEp-2 cells, it was unable to rule out the possibility of DA pattern on J-82 cells. One strain (TME307), when tested with the cultured cell adherence assay, caused the J-82 cells and A-498 cells to detach from the cell culture plate and adherence assay itself was impossible because making cell monolayer was unsuccessful, and all cells were lysed. These phenomena

were repeated many times, implicating that the presence of any toxins or other factors attributed to cell detachment.

Whether alive or dead, *E. coli* strains that had an adherence capacity were injected into the urinary tract of mice, and previously reported data showed that mucosal inflammation was triggered [8]. This reaction could be estimated by measuring the number of leukocytes excreted into the urine or by bacterial counts after homogenization of kidney and urinary bladder of mice. Hagberg et al. [7] explained the in vivo role of adhesin for persistence of bacteria in the kidney and bladder of mice. They found that mannose resistant adhesin enhanced bacterial adherence to both the kidney and bladder, but mannose sensitive adhesin enhanced bacterial adherence to only the bladder. They concluded that the adhesins determined the localization and retention of bacteria in the urinary tract of mice, and aside from the known adhesins, the presence of unknown adhesive properties could be associated with the adherence and localization of bacteria.

Twelve strains of *E. coli* that exhibited different adherence patterns to HEp-2 cells were subjected to the in vivo assay of bacterial adherence using the mouse model. Regardless of the kinds of adherence pattern, all *E. coli* strains tested were detected from the bladder and kidneys of mice; however, the number of bacterial count differed by the adherence pattern of *E. coli* in this study. There was no significant difference in the number of bacteria detected in the bladder and kidneys in accordance with the adherence patterns on tissue culture cells. This result suggested that the adherent capacity of *E. coli* to HEp-2 cells was unlikely to be associated with mice uroepithelial adherence. Although all *E. coli* strains tested adhered to uroepithelial cells of mice, every mouse did not show inflammatory changes of the urinary tract.

Linder et al. [8] reported that the inflammatory response was regulated via the genes that control the reactivity to lipopolysaccharide (LPS). They used two kinds of mice, C3H/HeN mice that responded to LPS and C3H/HeJ mice that do not respond to LPS. In the C3H/HeN mice, a significant influx of leukocytes into the urine occurred within 24 hours after bacterial injection, whereas, a significant increase of leukocytes in the urine did not occur in the C3H/HeJ LPS non-response mice. The specific adherence was required for the optimal delivery of LPS into the uroepithelial cells. With all the above findings in mind, they concluded that inflammation could be induced by *E. coli* in the urinary tract as a consequence of endotoxin, and bacterial adherence was the prerequisite for delivery of LPS.

TME303 strain induced an infection in both the bladder and kidney, and inflammatory changes were confirmed in both organs. The data obtained in this study suggested that the presence of AA adhesin or unknown additional adhesins might be involved in urinary tract inflammation. A greater number of bacteria were found in the kidney and bladder after injection of TME114 strains. However, strain TME114 (AA pattern on HEp-2 cells) did not cause pathologic changes of the kidney, whereas strain TME116 (DA pattern on HEp-2 cells) affected both the bladder and kidney. The above findings of different localization of bacteria (tissue tropism) were associated with bacterial special virulence factors, including two kinds of adhesins, adherence to cultured cells.

CONCLUSIONS

Of the 30 *E. coli* strains, irrespective of the type of adherence pattern, twelve strains adhered to J-82 cells, eleven to HeLA and HEp-2 cells, and ten to A-498 cells, respectively. The adherence patterns of *E. coli* on HEp-2 cells and HeLa cells showed an excellent agreement. When tested with *E. coli* strains that exhibited the DA pattern, more number of bacteria adhered to HEp-2 cells than to other cells tested. The number of microcolonies in the LA pattern on J-82 and A-498 cells was found to be greater than that on HeLa and HEp-2 cells. The typical AA pattern appeared to be more prominent on HeLa cells than on other cells.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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