Development of Experimental Model of Chronic Pyelonephritis with *Escherichia coli* O75:K5:H-bearing Dr Fimbriae

Mutation in the dra Region Prevented Tubulointerstitial Nephritis

Pawel Goluszko,* Steve L. Moseley,§ Luan D. Truong,‖ Anil Kaul,* John R. Williford,§ Rangaraj Selvarangan,*‡ Stella Nowicki,*‡ and Bogdan Nowicki*‡

**Department of Obstetrics and Gynecology, and ‡Department of Microbiology and Immunology, The University of Texas Medical Branch, Galveston, Texas 77555-1062; §Department of Microbiology, University of Washington, School of Medicine, Seattle, Washington 98195; and ‖Department of Pathology, Renal Pathology Laboratory, Baylor College of Medicine, Houston, Texas 77030*

Abstract

***Escherichia coli* that express Dr fimbriae and related adhesins recognize the common receptor decay accelerating factor. *E. coli* strains that express adhesins of the Dr family were postulated to be associated with cystitis (30–50%), pregnancy-associated pyelonephritis (30%), and chronic diarrhea (50%). In this study, we investigated the hypothesis that *E. coli* renal interstitial binding mediated by the Dr adhesin may be important for the development of chronic pyelonephritis. An insertional *dra* mutant, *E. coli* DR14, of the clinical *E. coli* isolate IH11128 bearing Dr fimbriae, was constructed and used to characterize persistence of infection and interstitial tropism in an experimental model of ascending pyelonephritis. Quantitative cultures of kidney homogenates indicated that Dr hemagglutinin positive (Dr+) *E. coli* IH11128 established a 1-yr colonization of renal tissue. In the Dr hemagglutinin negative (Dr−) group, 50% of animals cleared infection within 20 wk and 100% between 32 to 52 wk. Dr+ *E. coli* colonized the renal interstitium. Significant histological changes corresponding to tubulointerstitial nephritis including interstitial inflammation, fibrosis, and tubular atrophy were found in the kidney tissue of the Dr+ but not the Dr− group. A substantial amount of fimbrial antigen was detected in the parenchymal regions affected by interstitial inflammation and fibrosis. The obtained results are consistent with the hypothesis that mutation within the dra region, affecting *E. coli* binding to tubular basement membranes, prevented renal interstitial tropism and the development of the changes characteristically seen in tubulointerstitial nephritis. (*J. Clin. Invest.* 1997. 99: 1662–1672.) Key words: decay accelerating factor • attachment • collagen type IV • *Escherichia coli* • Dr fimbriae**

Introduction

Escherichia coli is the most frequent causal agent of pyelonephritis (1). Studies on *E. coli* adhesins and their correspondent uroepithelial receptors provided an adhesin–ligand-based mechanism of ascending urinary tract infection (2). The presence of receptor binding sites in the colon may be crucial for endogenous *E. coli* to establish colonization of the gastrointestinal tract. Endogenous *E. coli* may ascend to the lower and the upper urinary tract and to the kidney via uroepithelial binding sites (3).

Pyelonephritogenic *E. coli* may express various fimbriae-containing adhesins that have been found to recognize different renal receptors and structures (3–13). P fimbriae associated with acute pyelonephritis provide an attachment mechanism to α D-Gal-(1,4)-β-D-Gal-containing receptors residing on the luminal apical surface of the renal tubules (7, 10, 12, 13). In contrast to P fimbriae, Dr fimbriae mediating the recognition of the receptors decay accelerating factor (DAF)[1] and collagen type IV, located in the tubular basement membranes and Bowman's capsule, may account for the renal interstitial tropism of *E. coli* (3, 13–15). In 1987, we proposed that renal interstitial tropism of Dr hemagglutinin positive (Dr+) *E. coli* may be important for establishing chronic pyelonephritis (16).

Chronic pyelonephritis has been defined histologically as a destructive inflammatory process involving both the pyelocaliceal system and renal parenchyma (17). The renal parenchymal lesions include tubular atrophy, interstitial inflammation, and interstitial fibrosis. Parenchymal lesions may be relentlessly progressive and may result in end-stage kidney. Although the etiology and pathogenesis of acute pyelonephritis are well known, factors responsible for the progression of the acute phase into chronic pyelonephritis are poorly understood. Current knowledge emphasizes the importance of humoral and cellular immune responses to the antigenically altered renal tissue but downplays the role of bacterial virulence and tissue invasion by bacteria (18).

To investigate a possible role of bacterial virulence factors in the persistence of bacteria in renal tissue and in the pathogenesis of chronic pyelonephritis, development of an appropriate animal model is necessary. We propose that an animal model of experimental chronic pyelonephritis that mimics human counterpart should fulfill two requirements: (*a*) colonization receptors should be expressed in the renal interstitium and/or adjacent tubular basement membranes and resemble

Address correspondence to Bogdan Nowicki, M.D., Ph.D., Department of Obstetrics and Gynecology, The University of Texas Medical Branch at Galveston, Galveston, TX 77555-1062. Phone: 409-772-7599; FAX: 409-747-0475; E-mail: bnowicki@marlin.utmb.edu

Received for publication 4 April 1996 and accepted in revised form 22 January 1997.

J. Clin. Invest.

0021-9738/97/04/1662/11 $2.00
Volume 99, Number 7, April 1997, 1662–1672

1. *Abbreviations used in this paper:* Dr+, Dr hemagglutinin positive; Dr−, Dr hemagglutinin negative; DAF, decay accelerating factor; IF, immunofluorescence.

human kidneys; and (*b*) the *E. coli* strain should express an adhesin that can attach to the receptors located within the interstitial tissue and/or basement membranes resulting in the interstitial inflammation. This study provides evidence for the importance of bacterial virulence in establishing persistent colonization and chronic pyelonephritis. We report here that by using an insertional mutagenesis technique, we found that *E. coli* with Dr fimbriae persisted in the kidney tissue and was associated with significant tubulointerstitial nephritis, whereas the mutant *E. coli* DR14 without Dr fimbriae was gradually cleared from kidney tissue which displayed significantly less pathology.

Methods

Experimental model of mouse urinary tract infection (UTI). To study whether Dr hemagglutinin and its receptor facilitate bacterial colonization in kidney tissue and whether this event plays a role in the development of chronic pyelonephritis, we used an ascending urinary tract infection model developed by L. Hagberg et al. (19). Female mice 8–10 wk old (strain C3H/HeJ, The Jackson Laboratory, Bar Harbor, ME) were infected by urethral catheterization and instillation of a single dose of bacterial suspension into the urinary bladder as described before (19). The urinary bladder was voided by gentle compression of the abdomen. A drop of urine was obtained from the urethral orifice with an automatic pipetmen and spread on MacConkey and blood agar plates to test sterility. After the animals were anesthetized with isoflurane in a sealed glass jar for $\sim$ 30 s, the inoculum of Dr+ and Dr hemagglutinin negative (Dr−) *E. coli* prepared and characterized as described below was instilled into the urinary tract through a soft polyethylene catheter of outer diameter 0.30 mm (Norton Performance Plastics, Akron, OH) adapted to a 0.4 × 20-mm needle on a tuberculin syringe. A 0.05-ml sample of the appropriate microbial inoculum was injected. The catheter was then immediately withdrawn without any further manipulations. The animals were subsequently allowed free access to food and drink.

Quantitative tissue cultures and detection of bacterial antigens. Groups of mice were sacrificed at 2–4, 6–8, 12–16, 24–28, and 32–52 wk, and renal tissues were subjected to bacteriologic and morphologic methods to detect live bacterial cells and/or bacterial antigens, and to quantify the changes of chronic colonization. After killing the mice by cervical dislocation, their kidneys were aseptically removed and equally divided. One half was snap frozen for staining, and the other half was homogenized in 0.5 ml PBS in a Teflon tissue grinder (Frank A. Thomas Co., Philadelphia, PA). Viable counts of 0.05 ml of serial dilutions of mouse tissues were tested on L-agar and MacConkey plates. The number of bacteria was expressed as the number of CFUs per gram of tissue. The establishment and persistence of bacterial infection in the mouse urinary tract (UT) was monitored by both quantitative tissue cultures and by immunofluorescence (IF) staining of frozen kidney tissue sections for the presence of Dr+ and Dr− *E. coli* cells. IF staining was performed with monospecific rabbit anti-Dr hemagglutinin IgG and/or anti-O75LPS IgG, and secondary anti–rabbit IgG labeled with FITC. Controls for nonspecific staining were performed with preimmune rabbit IgG and/or replacing each step of staining by corresponding buffers.

Hemagglutination and agglutination assays. A 3% (vol/vol) suspension of human O erythrocytes with or without chloramphenicol at 10-mM concentration was used to detect expression of Dr fimbriae (20). A slide agglutination assay with anti-O75 IgG was used to detect *E. coli* LPS according to the World Health Organization (WHO)-approved protocol.

Histological evaluation. Half of each kidney was fixed in 10% buffered formalin, paraffin-embedded, sectioned at 4 μm, stained with hematoxylin-eosin, and examined by light microscopy. Renal parenchymal changes characteristic for pyelonephritis including tubular atrophy, interstitial fibrosis, and interstitial inflammation were sought. The renal pelvis was also evaluated for inflammation and fibrosis. To facilitate comparison of the renal and pelvic changes in Dr+ and Dr− groups, the severity of each of the lesions mentioned above was graded semiquantitatively using the following score: 0 = no change; 1 = < 5% of renal or pelvic tissue involved; 2 = between 5 and 15%; and 3 ≥ 15%. The degree of glomerulosclerosis was also semiquantitated. Because the number of animals in either group was almost the same (60 vs. 61), the total score for each lesion and/or time point was calculated. Statistical analysis was performed by the Fisher exact test, the Kruskal-Wallis test, and by the one-sided Chi-square test.

Bacterial strains and plasmids. E. coli IH11128 was isolated from a human with pyelonephritis and has been described previously (13). *E. coli* strain SM10λ*pir* (21) encodes the mobilization functions for RP4-related plasmids, and the π protein required for replication of plasmid R6K-related replicons. The suicide vector pGP704 is an R6K replicon and contains *oriT* of plasmid RP4 (22). pSSS1 contains the daa operon which encodes expression of the F1845 fimbrial adhesin, a member of the Dr family of *E. coli* adhesins (23). *E. coli* strain K12 derivative CGSC6630 (24) was used as a negative control in Northern hybridizations. pCC51XB contains the 3′ portion of the dra operon including *draE* (25). The *dra* genes have been recently renamed to correspond to related *daa* and *afa* genes; the structural gene formerly designated *draA* is now *draE* (25).

DNA probes and hybridization. Preparation of genomic DNA and plasmid DNA, restriction endonuclease digestion, and transformation were performed using standard methods. DNA restriction fragments isolated from pBJN406 dra coding region and pSSS1 daa coding region were used as gene probes for the presence of homologous sequences in tested mutants and parent strain.

Northern hybridizations. Total RNA was prepared as follows: A 1.5-ml volume of exponential phase bacterial cells grown in LB broth was added to a 3-ml volume of lysis solution consisting of a 1:1 mixture of phenol (saturated with 20 mM sodium acetate, pH 4.8) with 7.5 M guanidinium thiocyanate, 1 mM EDTA, and 0.5% SDS. The resulting suspension was thoroughly mixed and frozen in a dry ice/ethanol bath. The suspension was then thawed and extracted once with chloroform. RNA was precipitated with isopropanol, and the precipitate was washed with 70% ethanol. The RNA pellet was resuspended in water and extracted with a 1:1 mixture of phenol and chloroform. RNA was then ethanol precipitated, washed in 70% ethanol, and resuspended in 25 μl water. An equal volume of formamide was added, and the solution was frozen for storage. Electrophoresis of RNA and transfer to nylon membranes were performed as previously described (25). The *draE* hybridization probe was prepared as follows: An XbaI-BamHI fragment from pCC51XB containing *draE* was inserted into pBluescript II KS (+) (Stratagene Inc., LaJolla, CA.). The resulting construct was linearized with XbaI, and a ^{32}P-labeled antisense RNA probe was generated using α-^{32}[P]UTP (New England Nuclear, Boston, MA), T3 RNA polymerase, and reagents provided in kit form (Maxiscript transcription kit; Ambion Inc., Austin, TX). Hybridization conditions were as previously described (26). After hybridization, the nylon membranes were washed in 300 mM NaCl, 30 mM sodium citrate at 75°C for 4 h, then incubated at room temperature in the same buffer with 25 μg/ml Rnase A and 10 U/ml Rnase T1. The membranes were then exposed to a storage phosphor screen and analyzed with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). For photographic representation, a membrane was exposed to x-ray film.

Construction of the Dr− mutant of clinical isolate IH11128. General strategies for the use of pGP704 for mutagenesis have been described in detail previously (22). A 1.5-kb KpnI-SstI fragment was isolated from pSSS1, which encodes the F1845 fimbrial adhesin. This fragment encodes the 3′ end of *daaC* and shares extensive homology with the 3′ end of *draC* (data not shown). The fragment was inserted into the suicide vector pGP704 and the resulting construct was designated pJMA14. pJMA14 was introduced by transformation into *E.*

coli SM10λ*pir*, and then to *E. coli* IH11128 by conjugation, selecting for ampicillin resistance encoded by pGP704. The donor was counterselected by plating on minimal media. Because pJMA14 cannot replicate independently in the absence of the product of *pir*, ampicillin resistant transconjugants are likely to have resulted from insertion of pJMA14 into the homologous region of the dra operon. One transconjugant was designated DR14, and Southern blot analysis confirmed insertion of pJMA14 into *draC.* Strain DR14 has lost adherence functions of the Dr hemagglutinin but retain O75 LPS and similar growth curves.

Results

Construction of a Dr− mutant. An insertion mutant was constructed in the dra operon of *E. coli* IH11128 using the suicide plasmid pGP704. A derivative of pGP704, designated pJMA14, was constructed which contains a portion of *daaC* from the operon encoding F1845, a fimbrial adhesin related to the Dr hemagglutinin (see Methods for details). Conjugation of pJMA14 into IH11128 yielded a mutant which no longer expressed Dr hemagglutinin-mediated adherence. The mutant was designated DR14. When the 1.5-kb KpnI-SstI fragment from the daaC region of the daa operon, which was used in the construction of the pJMA14, was hybridized with SphI-restricted total DNA from IH11128, a DNA fragment of ∼ 4.8 kb was detected (Fig. 1), consistent with the published sequence of the *afa-3* operon which is highly homologous with *dra.* When the same probe was hybridized with DNA from DR14, the 4.8-kb fragment was absent, and a major hybridizing fragment of 15–20 kb was detected, and a minor fragment of ∼ 10 kb was also observed. A single insertion of pJMA14 into the dra operon would have resulted in an increase in size of 5.2 kb, thus the expected fragment hybridizing with the probe would be 9.9 kb. Our observations are consistent with two or three tandem insertions of pJMA14 yielding the 15–20 kb hybridizing fragment, with resolution occurring at a low frequency producing the minor fragment of ∼ 10 kb representing a single insertion of pJMA14 in *dra.*

The mutant strain DR14 does not produce the Dr hemagglutinin due to an insertional inactivation of *draC.* The product of this gene is essential for assembly of functional adhesins of the Dr family (23, 26). An insertional mutation of this nature could have polar effects on unknown genes downstream of the dra operon, although we considered this unlikely because transcriptional studies of the closely related daa operon indicate that transcription does not proceed beyond the operon (27). However, to assess the potential for polar effects of the transposon insertion of DR14, we examined by Northern blot analysis the expression of *draE*, the structural subunit of the Dr hemagglutinin. The insertion in DR14 is located between the dra promoter and *draE*, thus a polar effect of the insertion may block downstream transcription of *draE* mRNA. The results are shown in Fig. 2. An RNA species of ∼ 1 kb was detected by the draE antisense probe in both IH11128 and the mutant strain DR14. This transcript is of the size previously shown to result from endonucleolytic processing of the *dra* and *daa* transcripts (26). Our results indicate that the transposon insertion in DR14 does not block downstream transcription, although there is a decrease in levels of *draE* mRNA. Densitometric analysis of two independent Northern hybridizations using different RNA preparations indicate that the mutation resulted in expression of *draE* at 36 and 37%, respectively, of wild-type levels.

Growth rates of both Dr+ *E. coli* IH11128 and Dr− *E. coli* DR14 were tested at 1, 2, 3, 4, 6, and 8 h. Both strains showed almost identical growth curves. Slide agglutination of IH11128 and DR14 with anti-O75 antibody was positive for both strains indicating that tested isolates expressed the same LPS.

Experimental pyelonephritis and quantitative tissue cultures of infected animals. We investigated whether Dr fimbriae bearing *E. coli* IH11128 (O75:K5:H-) and DR14, the insertional Dr− mutant of clinical *E. coli* IH11128 that lost binding to Dr receptor, differ in their persistence in the mouse urinary tract. Because mouse kidney resembled human kidney in the location of tubular basement membrane and interstitial Dr receptors binding sites (see below), mouse strain C3H/HeJ (LPS

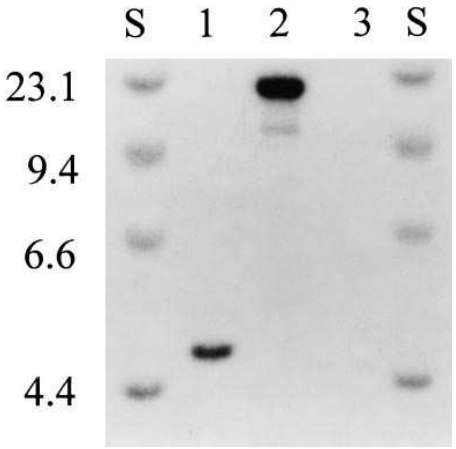

Figure 1. Southern blot analysis of the insertional mutation in *dra.* Total DNA from *E. coli* strains IH11128 (lane *1*), DR14 (lane *2*), and CGSC6630 (negative control; lane *3*) was digested with SphI, and a Southern blot of the digest was probed with a ^{32}P-labeled 1.5-kB KpnI–SstI fragment of pSSS1 encoding region of *daaC.* Size standards (lanes *S*) are ^{32}P-labeled HindIII fragments of bacteriophage λ DNA, and the sizes are indicated in kilobases.

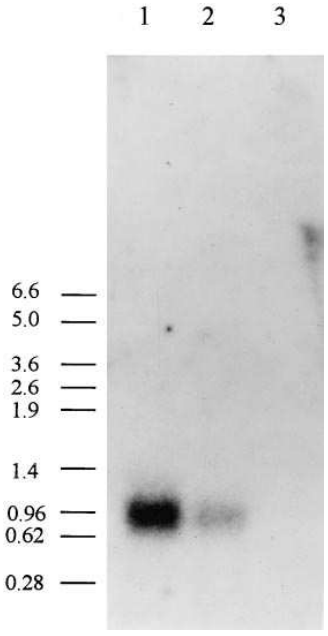

Figure 2. Northern analysis of *draE* expression in 1H11128 and its mutant derivative DR14. Total RNA was separated by formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane. The blotted RNA was hybridized to a single stranded RNA probe corresponding to the antisense sequence of *draE.* Size standards were visualized ultraviolet illumination of the ethidium bromide-stained gel. The mobility of the size standards, expressed in kilobases, are shown on the left. (Lane *1*) IH11128; (lane *2*) DR14; (lane *3*) *E. coli* CGSC6630 (negative control laboratory strain).

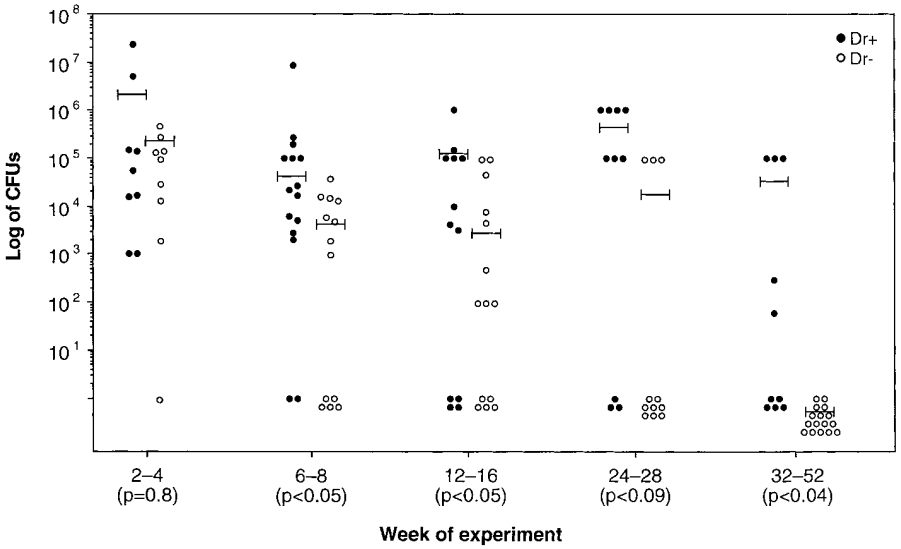

Figure 3. Quantitative bacterial counts in kidneys of mice challenged with Dr+ *E. coli* IH11128 and Dr− *E. coli* Dr-14 with respect to duration of infection. (*Closed circles*) Mice challenged with Dr+ *E. coli.* (*Open circles*) Mice challenged with Dr− *E. coli* Dr-14. A total number of animals in Dr+ group was 60 and in the Dr− group, 61. Bars indicate mean value for the tested group. Statistical analysis was performed by the Kruskal-Wallis test. The *P* values for each time point were evaluated for positive cultures only. *P* values of < 0.05 were considered statistically significant. Overall *P* value indicated significant difference ($P \leq 0.04$) between Dr+ and Dr− group.

nonresponder) was selected for ascending colonization experiments. It was formerly suggested that LPS contributes to scarring of the kidney (28). Use of C3H/HeJ mice with decreased response to LPS and potential effect of LPS on renal pathology allowed evaluation of the role of non-LPS factors such as Dr fimbriae in pathogenesis of experimental infection.

For the pilot study, we used different inocula ranging from 5×10^4 to 5×10^8 CFU/ml (4). With lower concentrations used, the number of infected animals decreased to 40–60%. An inoculum of 5×10^7 CFU/ml used throughout these experiments was the one that resulted in infection on the majority of animals. Higher concentrations of *E. coli* were not further used for infection due to the potential risk of generating systemic dissemination. Two groups of female mice were infected with the parent clinical Dr+ *E. coli* IH11128 and Dr− *E. coli* DR14 by bladder catheterization (single dose of $5x10^7$ CFU/ml in 50 μl) with no further manipulations. Infected animals were randomly removed from each Dr+ and Dr− group during weeks 2–4, 6–8, 12–16, 24–28, and 32–52 and were killed. Two independent experiments, both lasting 52 wk, were performed. Because both long-term experiments showed similar outcomes, the pertinent figures present combined results.

The animals killed in weeks 2–4 included nine mice in the Dr+ and nine mice in the Dr− group. Tissue cultures taken during weeks 2–4 showed efficient colonization of kidneys in both Dr+ and Dr− groups ($P = 0.8$; Fig. 3). Chronic inflammatory changes were found in one animal in the Dr+ group, and three animals in the Dr− group (see Fig. 7). The mice

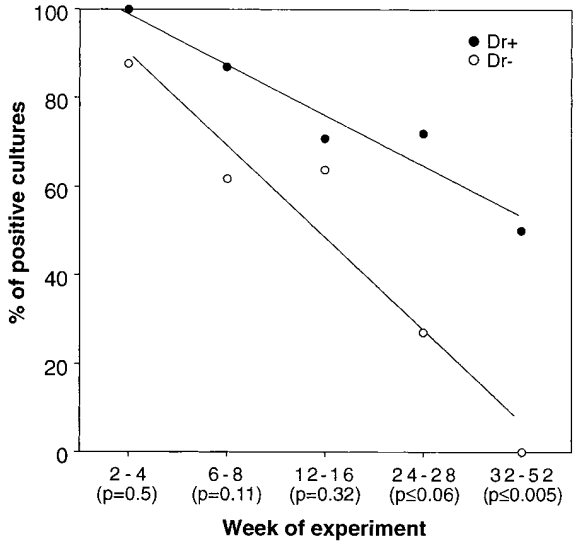

Figure 4. Frequency of positive cultures of renal tissue homogenates with respect to duration of infection. (*Open circles*) Mice in the Dr+group; (*closed circles*) Mice in the Dr−group. Overall *P* value was $P \leq 0.04$ (evaluated by Fisher exact test).

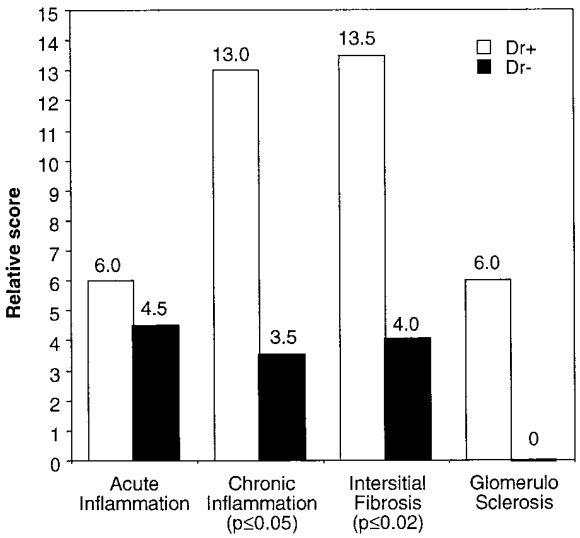

Figure 5. Relative score of renal parenchymal lesions in the mouse infected with Dr fimbriae + and Dr fimbriae − mutant of *E. coli* IH11128. (*Open bars*) Mice group infected with Dr+ *E. coli*; (*solid bars*) Mice group infected with Dr− *E. coli.*

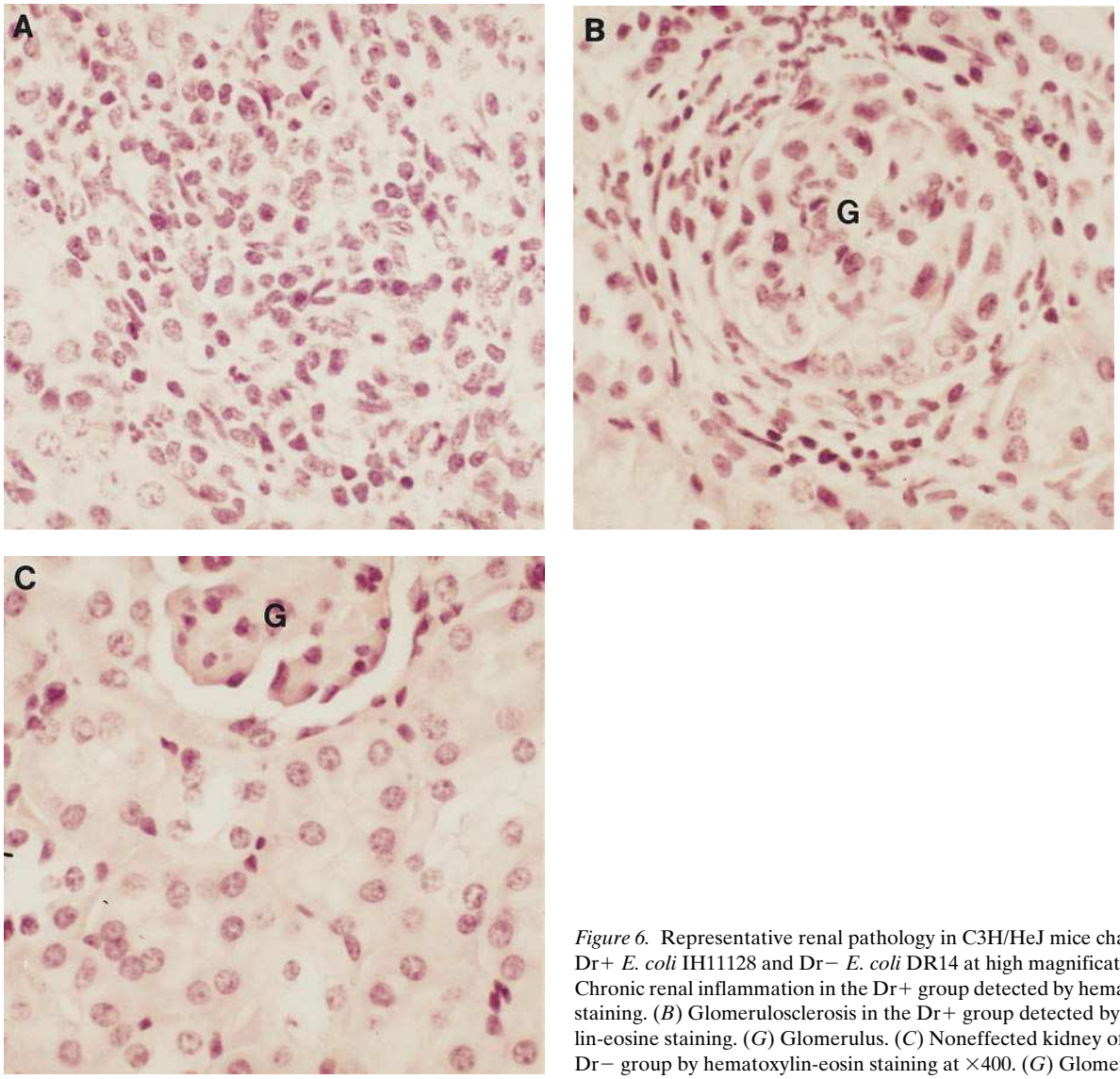

Figure 6. Representative renal pathology in C3H/HeJ mice challenged with Dr+ *E. coli* IH11128 and Dr− *E. coli* DR14 at high magnification. (*A*) Chronic renal inflammation in the Dr+ group detected by hematoxylin-eosine staining. (*B*) Glomerulosclerosis in the Dr+ group detected by the hematoxylin-eosine staining. (*G*) Glomerulus. (*C*) Noneffected kidney of the Dr− group by hematoxylin-eosin staining at ×400. (*G*) Glomerulus.

killed during the 2–4 wk period were the only ones group in which Dr− infected animals had higher incidence of chronic inflammation than the Dr+ group.

The animals killed in weeks 6–8 of the urinary tract infection included 15 mice in the Dr+ and 13 in the Dr− group. Decreasing trends in CFU counts were observed in the Dr− group. Kidneys in the Dr− group showed about one log lower mean CFU count than in the Dr+ group ($P \leq 0.05$). The number of animals that developed features of chronic inflammatory infiltration and fibrosis was 6 of 15 (40%) in the Dr+ group and 4 of 13 (30%) in the Dr− group. The relative histological score of chronic inflammation was significantly higher in the Dr+ than the Dr− group ($P < 0.05$; Fig. 4). In the Dr+ group, three animals developed features of glomerulosclerosis (Figs. 5 and 6). Glomerulosclerosis was not found in animals in the Dr− group. Two animals in the Dr+ group and none in the Dr− group died at the 6–8 wk period. Hematoxiline and eosine staining of kidneys in the Dr+ group showed presence

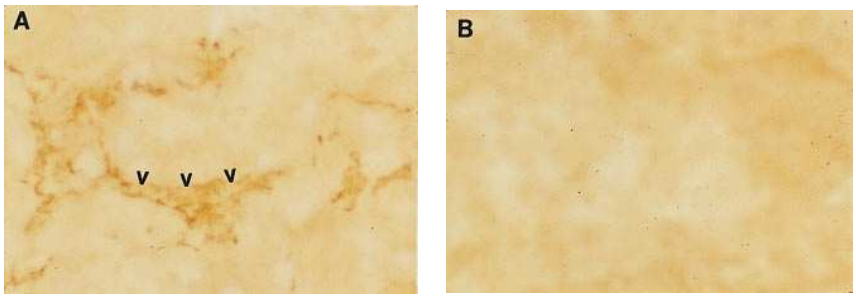

Figure 8. Binding of Dr fimbriae to the mouse kidney. (*A*) (*V arrow*) Basement membrane binding of purified recombinant Dr fimbriae. (*B*) Control negative staining in which Dr fimbriae were replaced with corresponding buffer.

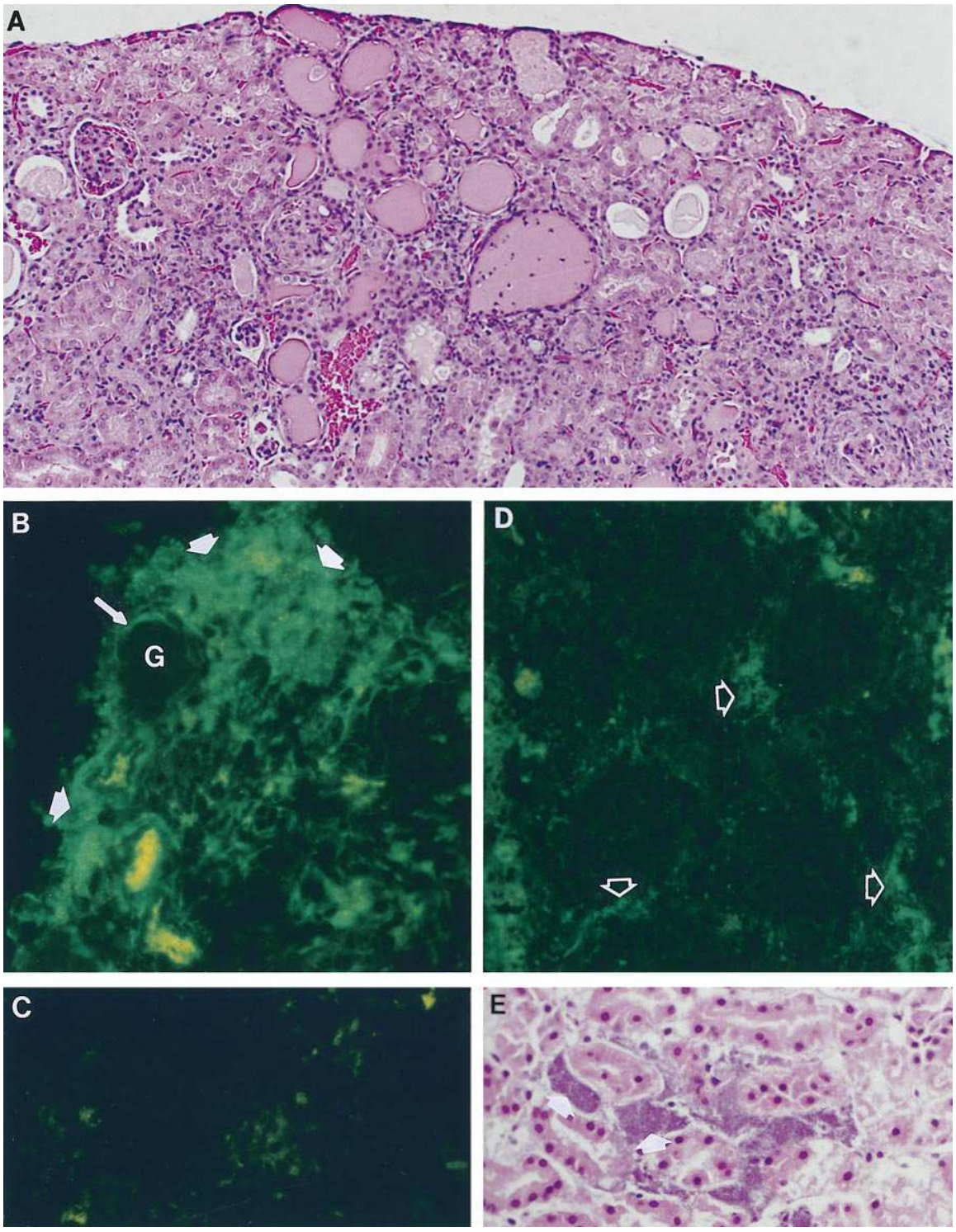

Figure 7. Representative renal pathology in C3H/HeJ mice challenged with Dr+ *E. coli* IH11128. (*A*) Shows features of chronic tubulointerstitial nephritis and glomerulosclerosis. (*B*) Shows IF staining of kidney section affected by chronic interstitial fibrosis with anti-Dr adhesin IgG. (*Thick arrows*) Region of strong fluorescence consistent with accumulation of substantial amounts of fimbrial antigen. (*G*) Area corresponding to glomerulus. (*Thin arrow*) Stained structure resembling Bowman's capsule. (*C*) Control IF staining with anti-Dr adhesin IgG of kidney section from Dr− group. (*D*) Shows interstitial colonization by Dr+ *E. coli* detected by IF staining. (*E*) Interstitial colonization by Dr+ *E. coli* detected by hematoxylin-eosin staining.

of Dr+ *E. coli* in the renal interstitium (Fig. 7). The kidney of the animal of the Dr+ group that was found dead also showed significant interstitial colonization. The cross-sections of the kidney arteries of the dead animal contained significant number of bacterial cells, which may suggest a possible dissemination of infection to the circulation. In the Dr− group, interstitial colonization was not detected.

The animals killed during weeks 12–16 included 12 mice of

the Dr+ and 14 mice of the Dr− group. During weeks 12–16, a two log reduction in CFU/g of renal tissue in the Dr− group as opposed to the efficient persistence of infection in the renal tissue in Dr+ group was observed ($P \leq 0.05$). Four mice of Dr+ group (4/12) (33%) and one of Dr− (1/14) (7%) developed histological changes of chronic inflammation and fibrosis. One mouse died in the Dr+ group.

The group of animals killed between weeks 24 and 52 (1 yr) included 17 mice in the Dr+ and 26 mice in the Dr− group. Analysis of animals killed between 32 and 52 wk showed that 50% of mice in the Dr+ group but none in the Dr− group remained infected ($P < 0.005$) (Figs. 3 and 4). The rate of persistence in the Dr+ group in several animals was in the range of 10^5 CFU/g. Histological changes of interstitial chronic inflammation and fibrosis was found in six mice of the Dr+ group (35%) and one mouse of the Dr− group (4%). In the 24–52 week group, five mice died in the Dr+ group and none in the Dr− group. Irrespective of colony counts, the frequency of positive cultures in the Dr− group gradually decreased during 52 wk of observation (Fig. 4). The kinetics of bacterial persistence in the kidney suggested that insertional mutation within the dra region that resulted in the Dr− phenotype was associated with gradual elimination of the mutant strain by the mouse urinary tract.

The total number of mice that died in the Dr+ group during the 52-wk observation was 8 of 60 (13%). In the Dr− group, no mice died during the 52-wk experiment. Kidney tissue in dead animals was tested for histology and cultured. The histological distribution of bacteria was the same (interstitial) as in remaining Dr+ infected animals. Infection rates of kidneys varied between 9.1×10^3 to 1.6×10^5 CFU/g. Histology revealed progressive autolysis of the kidney tissue in some specimens. This was most likely due to the postmortem process.

E. coli colonies recovered from homogenized kidneys were tested for expression of Dr fimbriae. 10–20 colonies from each kidney were examined by hemagglutination in the presence and absence of chloramphenicol and by agglutination with anti-075 LPS antibody. With few exceptions, the type of fimbriae and serotype of isolates obtained from kidneys did not change during infection and were the same as those introduced by catheterization. However, in mouse No. PG-DK70, in the Dr− group killed at week 16, Dr+ revertants at 10^5 CFU/g were isolated. Because Dr+ revertants were found in the mouse infected with Dr− mutant, this animal did not fulfill criteria of the Dr− group. Histologic changes in the kidney of this animal were described in corresponding sections of this manuscript, but for statistical analysis, the animal was excluded from experimental Dr− group.

An experiment of 6-wk duration was performed to evaluate colonization capacity of *E. coli* DR14, a Dr− mutant of IH11128, in which adherence of the strain was restored by complementing dra mutation with plasmid pBJN406 (16). Experiments shown in Fig. 3 indicated that a significant difference in colonization capacity between Dr+ and Dr− *E. coli* was observed during 6–8 wk of infection. Three groups of C3H/HeJ mice, six animals each, were infected with Dr+ *E. coli* IH11128, Dr− *E. coli* DR14, and Dr+ *E. coli* DR14 (pBJN406), respectively. Animals used for the complementation experiment were killed during the sixth week of infection. All mice infected with Dr+ *E. coli* IH11128 showed colonization of the kidney at the rate of 10^3 to 10^4 CFU/g with a mean value of 4.0×10^4/CFU/g. Mice infected with Dr+ *E. coli* DR14 (pBJN406) were found to be colonized at the similar rate of 10^3 to 10^4 CFU/g with a mean value of 2.4×10^4 CFU/g. None of the mice infected with Dr− *E. coli* DR14 was found to have a positive culture at the sixth week past infection. This experiment indicates that complementation of insertional mutation of *E. coli* DR14 restored its virulence to the level of the clinical isolate IH11128.

Detection of Dr binding sites and bacterial antigens. Immunohistochemical analysis of Dr receptor binding sites for recombinant Dr fimbriae cloned from the *E. coli* IH11128 (16) indicated that mouse kidney expressed Dr binding sites in the tubular basement membranes and Bowman's capsule (Fig. 8). The presence of Dr binding sites in the mouse tubular basement membranes and Bowman's capsule suggested similarity to human kidney.

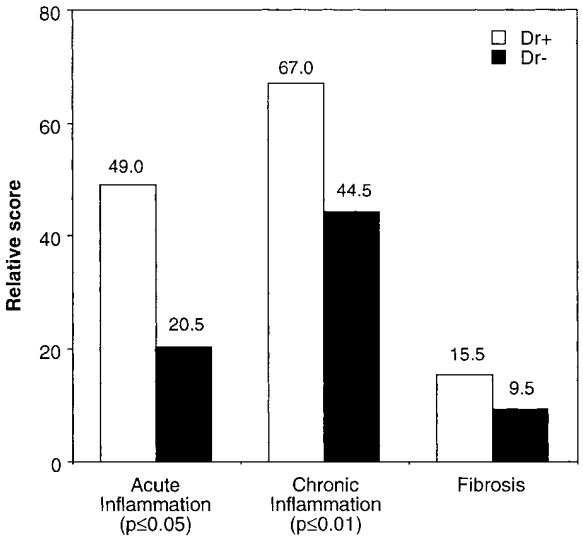

Figure 9. Relative score of renal pelvic lesions in mice infected with Dr fimbriae+ and Dr fimbriae– mutant of *E. coli* IH11128. (*Open bars*) Mice group infected with Dr+ *E. coli*; (*solid bars*) Mice group infected with Dr− *E. coli.*

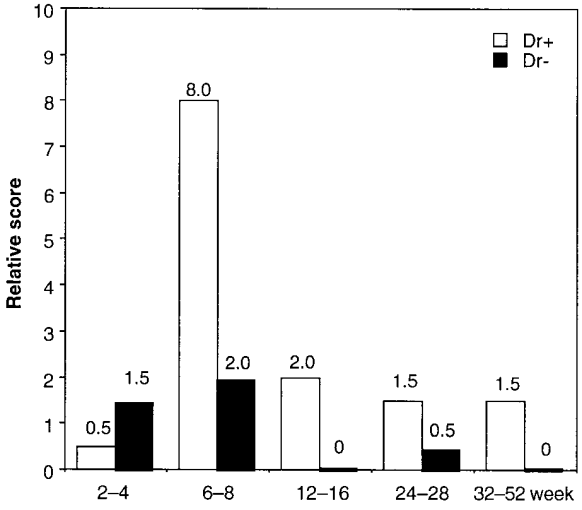

Figure 10. Relative score of interstitial fibrosis in the mouse renal tissue with respect to duration of infection.

Hematoxylin and eosin staining of kidney sections revealed bacterial colonization of interstitium in the Dr+ group but not in the Dr− group. Fig. 8 *E* shows extensive interstitial colonization (*arrow*), further confirmed by IF staining with anti-Dr fimbriae IgG (Fig. 7 *D*). Immunostaining with polyclonal monospecific anti-Dr IgG for the presence of Dr fimbrial antigen showed strong positive fluorescence within the renal tissue affected by chronic inflammation and fibrosis (Fig. 7 *B*). No interstitial staining in kidneys in the control Dr− group was found (Fig. 7 *C*).

Histologic changes. Chronic pyelonephritis was noted in 17 of 60 (28%) mice from the Dr+ group and 8 of 61 mice (13%) in the Dr− group ($P \leq 0.03$). These changes included interstitial inflammation, which focally involved both cortex and medulla. However, in almost all samples, the inflammation was more severe in the medulla than in the cortex. In the medulla, the inflammation was seen not only in the interstitium but also focally extended into the tubular lumen and the pyelocaliceal wall. There was also tubular atrophy associated with the interstitial fibrosis of variable severity. The inflammatory infiltrates were composed of both neutrophils and mononuclear inflammatory cells including both lymphocytes and a smaller number of macrophages. The chronic interstitial inflammation seemed to be more pronounced in the cortex, whereas the acute inflammation seemed to be more pronounced in the medulla (Fig. 6).

Mice in the Dr+ and Dr− groups were assessed for renal histological lesions by analyzing relative histological scores as described in Methods. Because the number of animals included for analysis in the Dr+ ($n = 60$) and Dr− groups ($n = 61$) was almost identical, the quantitative scores represent cumulated values per group, lesion, or time points. The semiquantitative scores for the renal parenchymal lesions and for the renal pelvic lesions of the Dr+ and Dr− groups are shown in Figs. 5, 9, and 10. Overall, the relative score of chronic inflammation and fibrosis in the renal parenchyma in the Dr+ group was significantly higher than in the Dr− group, $P \leq 0.05$ and $P \leq 0.02$, respectively. Similar differences in chronic inflammation were found for renal pelvis ($P \leq 0.01$; Fig. 9). Fig. 10 shows the scores of tubular atrophy and interstitial fibrosis, with respect to the duration of infection. Except for the early stage of infection (weeks 2–4) interstitial fibrosis in the Dr+ group was higher than in the Dr− group. The most pronounced changes were observed during weeks 6–8. In addition to the interstitial and pelvic changes described above, glomerulosclerosis or mild glomerular hypercellularity was observed in some mice in the Dr+ group but not in the Dr− group. Interestingly, in mouse PG-DK70 of the Dr− group, in which Dr+ revertants were recovered, histologic examination of the kidney revealed chronic interstitial inflammation.

Discussion

We report here that chronic pyelonephritis was developed in C3H/HeJ mice by intravesical inoculation of an *E. coli* strain that expresses Dr fimbriae, an adhesin that recognizes tubular basement membrane and Bowman's capsule receptors. We also note that *E. coli* IH11128 bearing the Dr adhesin persistently colonized renal interstitium, whereas the mutant strain DR14 without the Dr adhesin was gradually eliminated from the mouse kidney. The persistence of *E. coli* was associated with histologic evidence of chronic pyelonephritis, whereas few persistent renal changes were noted for the Dr− mutant *E. coli* DR14.

The colonization rate of renal tissue by *E. coli* was estimated in mice up to 52 wk. It is an intriguing phenomenon that in the Dr+ group, renal colonization lasted at least 1 yr. The kinetics of renal infection suggest that the early colonization process was similarly efficient for both Dr+ and Dr− *E. coli.* Therefore, at least in the mouse urinary tract (UT), the presence of the Dr adhesin seems not to be crucial for establishing the early colonization/infection process. Interestingly, *E. coli* DR14, the Dr− mutant was eliminated by the mouse urinary tract significantly sooner than the Dr+ parent strain. The frequency of animals with positive kidney cultures in the Dr+ group decreased from 100% in week 2 to 50% during weeks 32–52. In the Dr− group, this process occurred much earlier, reaching a 50% decrease at around week 20 and resulting in clearance of infection in 100% of animals during weeks 32–52. This process occurred gradually and was documented both by decrease of the mean CFU counts per gram of renal tissue as well as by the number of animals that clear infection in the Dr+ versus Dr− group. Clearance of bacterial infection in the Dr− group suggests that mutation of the *dra* coding region that resulted in a nonadherent clinical strain was associated with reduced virulence of the strain. Our results are consistent with the hypothesis that the function of the *dra* operon is important for the long term colonization of the mouse kidney. The mutant strain used for these studies (DR14) was constructed by insertional inactivation of the dra operon. Although insertional mutations are subject to polar effects, we have demonstrated that transcription downstream of the insertion was not blocked. We therefore consider it unlikely that the inability of DR14 to produce pathology in the chronic pyelonephritis model was due to polar effects of the insertion on unknown genes transcriptionally coupled to *dra.* There was, however, a modest reduction in levels of transcription downstream of the insertion, so we could not rigorously rule out this possibility.

To further address this possibility, an experiment evaluating renal colonization capacity of the mutant DR14 in which adherence was restored with plasmid pBJN406 encoding the Dr adhesin was performed. Dr+ DR14 (pBJN406) regained its virulence to the level of clinical isolate IH11128. This experiment seemed to fulfill the molecular Koch's postulates and suggests that Dr+ phenotype played an important role in establishing chronic colonization of the mouse renal tissue.

E. coli type 1 fimbriae may bind to leukocytes and trigger oxidative burst-and-release lytic enzymes (29, 30, 31). Renal scarring has been suggested to occur due to type 1 fimbriae-mediated activation of leukocytes (31). Gupta et al. attempted to develop an ascending model of chronic pyelonephritis with type 1 fimbriae-positive and type 1-negative mutant *E. coli* strains (32). Renal colonization in a study by Gupta et al. lasted 12 wk for both type 1 positive and type 1 negative *E. coli.* Unexpectedly, the severity of the histological score was higher in the group infected with the type 1-negative mutant than with the type 1-positive strain (32) suggesting a limited role for type 1 fimbriae in chronic colonization/infection in mice.

Recently, construction of P fimbriae mutants was used to test the role of P fimbriae of *E. coli* in the pathogenesis of ascending pyelonephritis (33). Interestingly, no differences in CFU counts between the parent and mutant strains were observed between P fimbriae-positive and negative *E. coli.* In this

study Mobley et al. showed that, similar to culture results, renal pathology scores were similar for P positive and P fimbriae-negative mutant. It remains to be investigated whether long-term colonization with P fimbriae-positive *E. coli* would be similar to the P fimbriae mutant.

One of the important differences between Dr and P fimbriae *in vitro* is that the Dr adhesin recognizes receptors in tubular basement membranes adjacent to interstitium histologically unavailable for colonization. P adhesins may bind to the receptors directly available for colonization at the apical luminal sites of selected types of renal tubules (13). We have proposed to call this phenomenon a differential tissue tropism (2). We hypothesize that differential tissue tropism could be a factor in determining development of the clinical form of infection. P+ *E. coli* binding to tubular epithelium may induce aggressive inflammatory responses leading to acute pyelonephritis. Dr+ *E. coli*, if capable of reaching interstitial tissue receptors, may evoke a less aggressive renal response leading to chronic colonization and interstitial inflammation. Hematoxylin-eosin and IF staining revealed that Dr+ *E. coli* colonized interstitium in vivo. This intriguing observation is in agreement with interstitial binding studies and suggests that in vivo Dr+ *E. coli* was able to reach and colonize tissue substructures which contain high-density Dr binding sites. It is likely that interstitial colonization due to function of the *dra* region allowed Dr+ *E. coli* to efficiently avoid host attack and resulted in persistence in the kidney. Contrary to type 1 fimbriated *E. coli*, binding to human leukocytes mediated by Dr fimbriae has been recently found not to result in killing of Dr+ strains (34). Resistance to killing by leukocytes may be an important factor, contributing to long-term survival in the mouse kidney.

The lack of DAF receptor in the lumen of the renal tubule does not eliminate an option of interstitial infection by Dr+ *E. coli.* The hypothetical mechanism of reaching interstitium by Dr fimbriae-positive *E. coli* may include colonization of the renal pelvis, followed by invasion of the uroepithelium and spreading of infection into interstitial substructures. The observed decrease of colonization by the mutant DR14 may result from altering two associated properties of Dr fimbriae-adherence and invasion. Invasion of IH11128 is mediated by interaction between Dr fimbriae and DAF and is prevented by mutations that affect expression of Dr fimbriae (Goluszko, P., and B.J. Nowicki, manuscript in preparation). Dr+ *E. coli* IH11128 is hypothetically capable of invading pyelocaliceal epithelium and reaching interstitium while Dr− *E. coli* DR14 is less adherent and invasive and is gradually eliminated.

The involvement of the pyelocaliceal system was more severe than that of renal parenchyma, indicating that infection progressed (ascended) from the renal pelvis to the renal parenchyma. Estimation of histological lesions in the parenchyma indicated the development of significantly more frequent and severe chronic tubulointerstitial damages in the Dr+ than in Dr− group. Immunostaining of frozen sections of the kidney area affected by chronic tubulointerstitial damages revealed that substantial amounts of Dr fimbrial debris (fimbrial antigen; Fig. 8) were present in these areas, but not in areas without tubulointerstitial damages. Presence of bacterial debris in histologic lesions may suggest their potential pathogenic role in chronic pyelonephritis. For example, interstitial colonization and/or presence of Dr fimbrial antigens may evoke chronic inflammatory responses in the interstitium. Interestingly, development of chronic pyelonephritis in a mouse strain C3H/HeJ, a LPS nonresponder, may indicate that observed histological lesions did not occur due to the host response to LPS (28). Chronic interstitial colonization of bacteria as a cause of chronic pyelonephritis could be of therapeutic importance and suggests potential for antibiotic treatment even in cases where advanced renal lesions already occurred.

It is noted that glomerulosclerosis was present in kidneys of some mice in the Dr+ group, but was absent in the Dr− group. This interesting observation in the current model has some human relevance. Although the glomerulus is known to be well preserved in the early phase of chronic pyelonephritis, development of glomerulosclerosis has been well documented in advanced chronic pyelonephritis related to intrarenal reflux of urine, with or without infection, in pigs and humans (35). The pathogenesis of the glomerulosclerosis in the current model is not clear, but may be related to a progressive tubulointerstitial damage, which subsequently resulted in glomerular sclerosis through interrupting of glomerular blood supply. Alternatively, significant tubulointerstitial damage may lead to an overloading of the remaining intact nephrons as a pathway to glomerulosclerosis (36). In this aspect, it is pathogenetically relevant to note in our model that the kidneys with the largest number of glomeruli with sclerosis also displayed the highest degree of chronic tubulointerstitial damage. Whether localization of Dr fimbrial antigen in glomerulus plays a role in causing glomerulosclerosis remains to be seen. It is interesting to note that Korhonen and colleagues reported a presence of Dr+ fimbrial antigens in the rat glomerulus several months after intravenous injection of this purified antigen. On the other hand, a process called intrarenal reflux has been known to be capable of transporting molecules from renal pelvis to the proximal parts of the nephron including glomerulus (18, 37, 38).

A family of *E. coli* Dr adhesins with common receptor specificity (binding to decay accelerating factor) was postulated to be associated with cystitis (30–50% of isolates) (26, 39), gestational pyelonephritis (30% isolates; 40), and protracted diarrhea in Mexican children (50% cases associated with diffused adherence; 41). Although association of Dr adhesins with chronic infections remains to be further investigated, association of Dr-related strains with chronic diarrhea suggests that the Dr family may possess virulence features that predispose to chronic infection. The Dr adhesin recognizes two receptors, DAF and collagen type IV, both expressed in the renal tissue (42, 43). Recognition of matrix proteins such as collagen type IV and DAF, a complement regulatory protein protecting host tissue from autolytic affect of the complement cascade, may represent important virulence features contributing to chronic processes. We have recently described a higher incidence of Dr+ *E. coli* in pregnant women in the III trimester pyelonephritis (44). In the preantibiotic era, gestational pyelonephritis has been associated with a risk of developing hypertension and end-stage renal disease (ESRD; 45, 46). Tubulointerstitial nephritis is among the main causes of end-stage renal disease (45, 47, 48). Whether a Dr fimbriae-bearing *E. coli*–mediated infection process may progress towards the end-stage renal disease remains to be investigated.

Our experiments offer a complementary explanation to the views associating chronic pyelonephritis with anatomical and/or functional changes in the urinary tract. We provide experimental evidence that specific microorganisms and genes encoding virulence factor(s) could be important in the natural history of chronic pyelonephritis. Mutation within the *dra* re-

gion coding for Dr fimbriae prevented development of tubulointerstitial nephritis. The chronic pyelonephritis model shall allow studies on novel approaches to block interstitial colonization and therefore may contribute to future attempts of preventing serious, life-threatening consequences of some renal diseases.

Acknowledgments

We thank Dr. Elbert B. Whorton, Jr., for statistical analysis, Linda Morrow Poindexter, and Robert G. McConnell, and his staff of Publication, Grant, and Media Support for the Department of Obstetrics and Gynecology, The University of Texas Medical Branch (Galveston, TX), for their assistance in the preparation of this manuscript.

This project was funded in part by grants from the National Institutes of Health (NIH) DK-42029 and the John Sealy Memorial Endowment Fund to B. Nowicki, and NIH DK-49862 to S. Moseley.

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