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Contribution of Cloned Virulence Factors from Uropathogenic *Escherichia coli* Strains to Nephropathogenicity in an Experimental Rat Pyelonephritis Model†

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***Escherichia coli* 536 (O6:K15:H31), which was isolated from a case of urinary tract infection, determines high nephropathogenicity in a rat pyelonephritis system as measured by renal bacterial counts 7 days after infection. The loss of S fimbrial adhesin formation (Sfa$^-$) (mannose-resistant hemagglutination [Mrh$^-$] and fimbria production [Fim$^-$]), serum resistance (Sre$^-$), and hemolysin production (Hly$^-$) in the mutant 536-21 led to a dramatic reduction of bacterial counts from almost 10^5 to only 40 cells per g of kidney. The reintroduction of the cloned S fimbrial adhesin determinant (*sfa*) increases the virulence of the avirulent mutant strain by a factor of 20; almost the same effect was observed after restoration of serum resistance by integration of an *sfa*$^+$ recombinant cosmid into the chromosome. Additional reintroduction of the Hly$^+$ phenotype by transformation of two *hly* determinants increased the virulence of the strains. Hemolysin production determined increased renal elimination of leukocytes and erythrocytes. Thus all three determinants investigated, S fimbriae, serum resistance, and hemolysin, contribute to the multifactorial phenomenon of *E. coli* nephropathogenicity.**

Escherichia coli is the causative organism in more than 80% (30) of urinary tract infections (UTI). UTI *E. coli* isolates generally exhibit several characteristics which contribute to urinary tract virulence, including the ability to adhere to urinary tract epithelial cells (31, 36) and to become serum resistant (14, 17, 32, 37) or cytotoxic (3, 9).

A specific binding of uropathogenic *E. coli* strains is mediated by adhesins which also agglutinate erythrocytes of various species. Whereas such adhesion factors which do not agglutinate erythrocytes in the presence of 2% mannose (mannose-sensitive hemagglutination, Msh phenotype) are common in *E. coli* cells of different sources, the mannose-resistant hemagglutination (Mrh phenotype) factors are specific for pathogenic *E. coli* strains (5, 17, 39). Such adhesin determinants code for different adhesin-specific proteins, and one of these represents the fimbria subunits (pilin) which form long filamentous cell appendices. One has to distinguish these fimbriae (Fim phenotype) from the actual adhesion proteins responsible for the specific binding to eucaryotic receptor structures (12, 38). Among uropathogenic *E. coli* different groups of Mrh adhesins have been identified. Adhesins which bind a Gal-Gal receptor molecule identical to the human P blood group antigen are called P-fimbria antigens (18, 28). Mrh factors which recognize a sialic acid-containing receptor have been termed S-fimbria antigens (22, 29). The contribution of cloned determinants coding for Msh and P adhesins to colonization of *E. coli* strains in the mouse bladder and kidney has already been demonstrated (13).

The ability of UTI *E. coli* strains to survive and multiply in human serum depends on the presence of specific O and K antigens (41), and perhaps other factors, including components of the outer membrane, also contribute to serum resistance (37; Hughes et al., submitted for publication).

The *E. coli* hemolysin, an extracellular protein which lyses erythrocytes of different species, can often be detected in UTI *E. coli* isolates (9, 17, 27). The contribution of this protein to virulence of uropathogenic *E. coli* strains has been demonstrated by cloning the chromosomal determinant for hemolysin synthesis and determining its influence in a rat peritonitis model (42) and by toxicity tests in mice (9, 10).

Here we provide evidence that the cloned hemolysin (*hly*) as well as the products of the determinants for S-specific hemagglutination (*mrh*) and S fimbriae (*fim*) also contribute to nephropathogenicity in a rat pyelonephritis model. Because in this model bacteria are injected into the bladder via the urethra and because they survive and multiply in the rat kidney leading to the histopathological picture of a pyelonephritis and resulting in pyuria, this in vivo system closely reflects upper urinary tract infections and not mere colonization. In addition we show that a serum resistance factor that has not yet been characterized also influences the level of virulence of this uropathogenic *E. coli* strain.

MATERIALS AND METHODS

***E. coli* wild-type strains, transformants, and recombinant DNAs.** The *E. coli* wild-type strain tested, 536, was isolated from a patient in Würzburg suffering from a UTI (11; J. Hacker, T. Jarchau, S. Knapp, R. Marre, G. Schmidt, T. Schmoll, and W. Goebel, *in* D. Lark, S. Normark, H. Wolf-Watz, and E.-E. Uhlin, ed., *Molecular Biology of Microbial Pathogenicity*, in press). It was identified by standard methods and characterized as described previously (11). All mutants and transformants tested derive from the uropathogenic *E. coli* strain 536, which belongs to the serotype O6:K15:H31, and are listed in Fig. 1. Strain 536 exhibits a mannose-resistant, neuraminidase-sensitive, S-specific hemagglutination (Mrh$^+$) and production of protein

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† Dedicated to Professor H. Brandis on the occasion of his 70th birthday.

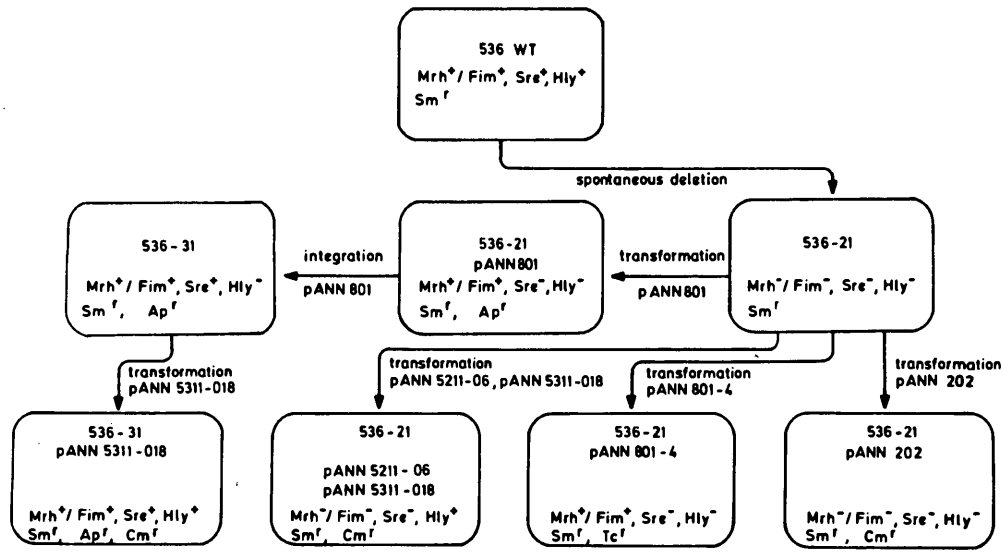

FIG. 1. Construction of *E. coli* strains which derive from the urinary pathogen 536 (O6:K15:H31). The strains were manipulated in the following characteristics: mannose-resistant hemagglutination of S type (Mrh^+), S-fimbria formation (Fim^+), serum resistance (Sre^+), and hemolysin production (Hly^+). Other abbreviations used: Sm^r, streptomycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance.

fimbriae (Fim^+). The strain is also serum resistant (Sre^+) and produces hemolysin (Hly^+).

The spontaneous mutant 536-21, described elsewhere (11), has lost the ability to produce fimbriae (Fim^-) and to hemagglutinate (Mrh^-) and is sensitive to human serum (Sre^-) and hemolysin negative (Hly^-). The Hly^- phenotype of mutant 536-21 is due to a specific deletion which involves both of the *hly* determinants (11, 20). Lack of the S-fimbrial adhesin (Mrh^-/Fim^-) is the result of a block in transcription of the S-fimbria determinant (G. Hacker et al., in press). Strain 536-21 was transformed with hly^+ and S-fimbrial adhesin (sfa^+) recombinant plasmids by a modified $CaCl_2$ procedure for wild-type strains (24). All hly^+ plasmids (pANN5211-O6, pANN5311-O18) contain the *hlyC* gene from the plasmid pHly152 and the *hlyA*, *hlyB* (former $hlyB_a$), and *hlyD* (former $hlyB_b$) (6) genes from the chromosomes of the O6 strain 536 and the O18:K5:H^- strain 764 (1, 7, 9). The transformants exhibit the phenotype Mrh^-/Fim^- Sre^- Hly^+. As a control the recombinant plasmid pANN202 was used which consists of the vector (pACYC184) and the *hlyC* part of pHly152.

In addition we introduced plasmid pANN801-4 into strain 536-21. This recombinant DNA codes for the S-fimbrial adhesin of strain 536. pANN801-4 consists of the vector pBR322 and 8.0-kilobase *Pst*I fragments coding the Mrh^+/Fim^+ phenotype (12).

We also transformed the sfa^+ cosmid pANN801 into the 536-21 mutant. pANN801 only codes for the S-fimbrial adhesin, and no other virulence determinants could be detected on this recombinant plasmid. From these transformants we isolated a strain (536-31) which carried cosmid pANN801 integrated into the chromosome and exhibited the following properties: Mrh^+/Fim^+ Sre^+ Hly^-. The phenotypic markers of the manipulated strains are given in Fig. 1. The in vitro segregation rate of the introduced recombinant DNAs was 6 to 9% in 20 generations after the strains were grown without antibiotic pressure in Luria broth. The growth rates of the strains in rat urine were identical.

Isolation of plasmid DNA. Plasmid DNA from transformants carrying recombinant DNAs was screened by the cleared lysate procedure (2). Preparative DNA isolation was achieved as described previously (8).

Animals. Female Wistar rats (strain Han-WIST; initial weight, 200 g) were used in the experiments. The animals were kept in an air-conditioned animal house. Water was provided ad libitum.

Pyelonephritis model. The nonobstructed pyelonephritis model (26, 32) was used as the in vivo assay. The rats were anesthetized with penobarbital (Nembutal; Abbott Laboratories), at a dose of 25 mg/kg of body weight administered intraperitoneally. The *E. coli* strains were grown overnight on blood agar and suspended in phosphate buffer (0.1 M, pH 7.0) to give a final concentration of 5×10^7 CFU/ml. Of this suspension 1.5 ml was injected via the urethra into the bladder of the rats. In previous experiments it was shown that this leads to a vesicoureteric reflux, followed by local injuries in the forniceal region and subsequent inflammation (14, 26, 32). A reduction of the injected volume results in low infection rates; an increase of the bacterial concentration is associated with higher mortality rates (34).

The rats were sacrificed 7 days later, and the kidneys were removed. Viable counts of the *E. coli* were made from kidney homogenates on McConkey agar. The number of CFU per gram of kidney of each group was calculated and served as the measure of nephropathogenicity; 20 to 70 rats were used per bacterial strain.

On certain days after infection renal elimination of leukocytes and erythrocytes was determined. After 10 ml of 0.9% saline had been given to the rats intraperitoneally the rats were placed singly into metabolic cages for 2 h. Furosemide (10 mg/kg) was added to the saline to increase diuresis. The urine was collected, the number of cells was counted in a Fuchs-Rosenthal chamber, and cell elimination per minute was calculated. Each group of rats consisted of 10 animals.

Renal histology. Kidneys of rats killed 7 days after infection were prepared for light microscopic examination by

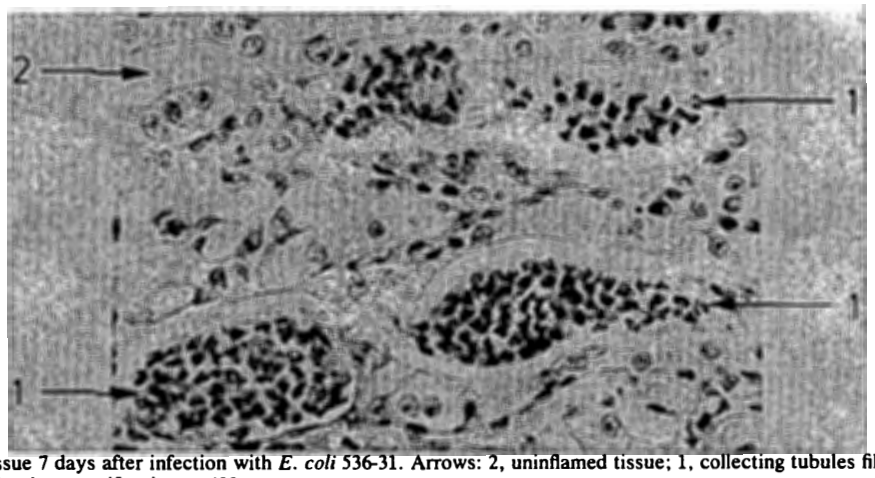

FIG. 2. Renal tissue 7 days after infection with *E. coli* 536-31. Arrows: 2, uninflamed tissue; 1, collecting tubules filled with leukocytes. Periodic acid-Schiff stain; magnification, ×400.

fixation in 5% formaldehyde. Paraffin sections 5 μm thick were stained with the periodic acid-Schiff stain.

Screening of transformants and strains reisolated from rats. Transformed strains and those isolated from the kidneys of rats were checked for the presence of plasmid DNA and markers on the recombinant DNA. Antibiotic resistance markers were identified on suitable plates. Hemolysin production was detected on plates and confirmed in a liquid assay (40). Serum resistance of the strains was measured in 90% human serum as described previously (17). The hemagglutination ability was tested with human and bovine blood and by agglutination with specific antisera. Fimbria production was detected by the use of antisera and by electron microscopy (1, 12). The strain 536 derivatives were further tested for streptomycin resistance and production of K15 and O6 antigens as described previously (10). Since there is an in vivo segregation rate of 10 to 15% over a period of 7 days, only those colonies were counted which still retained the plasmid-encoded markers.

Statistics. The mean number of CFU per gram of kidney of each group of rats was calculated. The renal bacterial counts, which were not normally distributed, were statistically evaluated by the Kruskal-Wallis test, followed by the Nemenyi test, if statistically significant differences ($P < 0.05$) were found (4).

RESULTS

Nephropathogenicity of the *E. coli* wild-type strain 536 and the mutant 536-21. We assessed the nephropathogenicity of the uropathogenic strain 536 in the rat pyelonephritis model. Intravesical injection of bacteria resulted in an acute pyelonephritis persisting for at least 7 days with bacterial cell counts of 10^5/g of kidney (see Fig. 3). Like pyelonephritis in humans, the renal infection led to urinary shedding of bacteria, leukocytes, and erythrocytes.

The inflammation was primarily localized in the parenchyma adjacent to the fornix region. Almost invariably coagulated blood projecting into the calyx could be found at the fornix. Many collecting tubules were filled with leukocytes, and some polymorphs could be seen in the interstitial tissue (Fig. 2). This corresponded to the histological picture of an acute pyelonephritis.

As described elsewhere (11, 20) the strain 536-21 used here derives from strain 536 and results from a deletion in the region containing the *hly* determinants. In addition the strain has lost the ability to produce the S-fimbrial antigen and to survive in 90% human serum (Hughes et al., submitted for publication). In contrast to the parent wild-type strain, there was a 2,000-fold reduction in renal counts in the rat pyelonephritis model with strain 536-21. Because strain 536-21 has lost the presumed virulence factors S fimbriae, serum resistance, and hemolysin, the reintroduction of cloned genes and the test of these transformants in the rat model seems to be an extremely suitable system to quantitate the contribution of these genes to nephropathogenicity.

Nephropathogenicity of *E. coli* 536-21 carrying the cloned S-adhesin determinant. The cloned S-adhesin determinant (Sfa) of the strain 536 consists of the mannose-resistant hemagglutination (Mrh) factor and protein fimbriae (Fim) with subunits of 16.5 kilodaltons in size (12; Hacker et al., in press). The recombinant plasmid pANN801-4 carrying the entire *sfa* determinant was transformed into the Mrh^-/Fim^- Sre^- Hly^- mutant strain 536-21. The entire *sfa* determinant coding for fimbriae (Fim^+) and hemagglutination (Mrh^+) strongly contributes to the nephropathogenicity of the strain 536-21 (Fig. 3). The number of bacteria isolated from the kidneys of infected rats was more than 20 times higher for the Mrh^+/Fim^+ strains than for the Mrh^-/Fim^- mutant.

All *E. coli* bacteria isolated from the kidneys of the infected rats showed the chromosomally encoded markers (streptomycin resistance, O6 and K15 antigens). More than 90% of the bacteria still carried the recombinant DNA, as indicated by the retention of tetracycline resistance, hemagglutination, and fimbria production and also by its direct identification by gel electrophoresis of cleared cell lysates. Only those colonies which retained the S-adhesin phenotype were counted in the test.

Nephropathogenicity of serum-resistant *E. coli* 536-31. The cosmid pANN801 which codes for S-adhesin formation (12) was introduced into the Mrh^-/Fim^- Sre^- Hly^- mutant 536-21, and a variant (536-31) was isolated which carried the cosmid DNA integrated into the chromosome. As recently demonstrated (19; Hacker et al., in press), strain 536-21 still retains the structural genes for the Mrh^+/Fim^+ phenotype, and it is therefore likely that the integration of cosmid pANN801 occurred via homologous recombination. The resulting strain 536-31 not only regained mannose-resistant hemagglutination and fimbriae of the S type but was also able

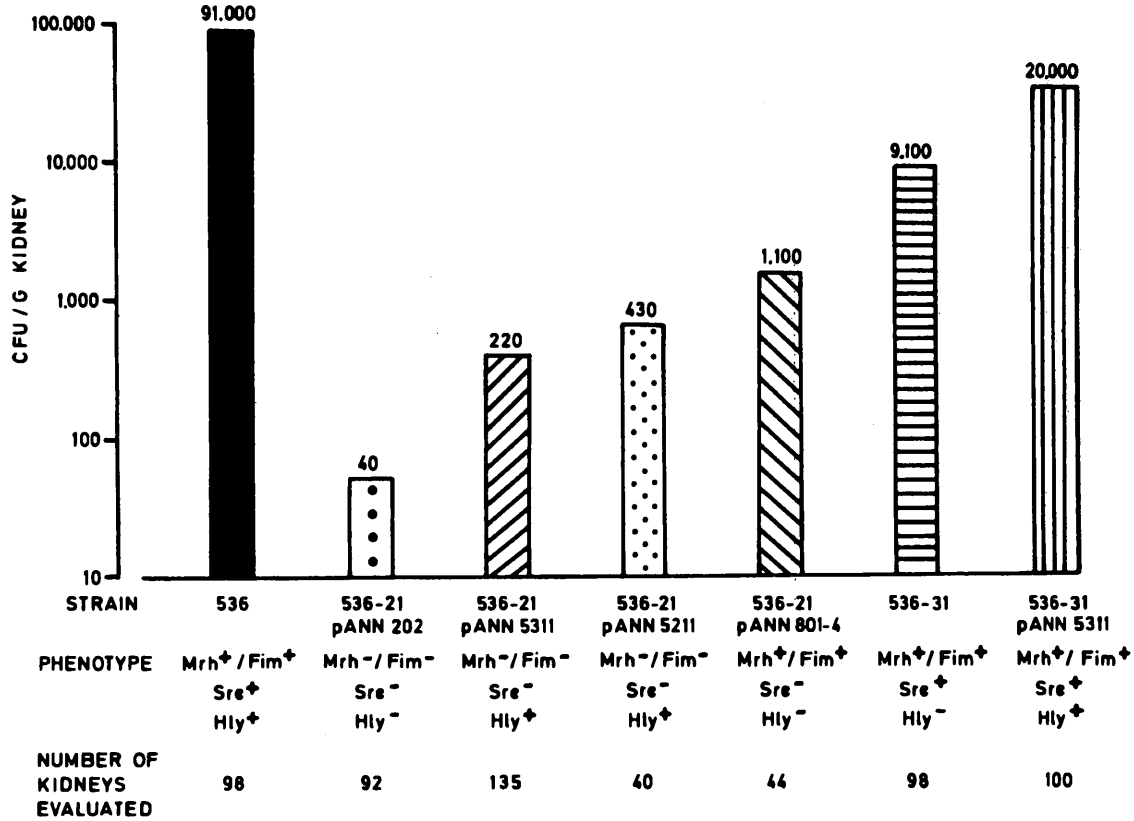

FIG. 3. Nephropathogenicity in a rat pyelonephritis model of wild-type strain 536 (O6:K15:H31), mutant 536-21 and its derivatives showing the influence of S-specific mannose-resistant hemagglutination (Mrh^+) and fimbria production (Fim^+), serum resistance (Sre^+), and hemolysin (Hly^+) on the CFU per gram of kidney. Statistically significant differences: 536 and 536-21, 536-21(pANN5311-O18), 536-21(pANN5211-O6), 536-21(pANN801-4), and 536-31; 536-21 and 536-21(pANN5211-O6), 536-21(pANN801-4), 536-31, and 536-31(pANN5311-O18).

to survive in 90% human serum. We, tested the nephropathogenicity of strain 536-31 bacteria.

The bacterial counts in the rat kidneys of the Mrh^+/Fim^+ Sre^+ Hly^- strain 536-31 were 200 times higher than those of the Mrh^-/Fim^- Sre^- Hly^- strain 536-21 (Fig. 3). Because the number of bacteria per kidney reached a level 10 times higher than those of the Mrh^+/Fim^+ Sre^- Hly^- transformant (see above), the results indicate a contribution of the yet uncharacterized serum resistance determinant to the nephropathogenicity of the strain 536.

Nephropathogenicity of *E. coli* strains carrying cloned hemolysin determinants. We tested the contribution to nephropathogenicity of the cloned hemolysin (*hly*) determinants derived from the chromosomes of the O6 strain 536 and the O18 strain 764 in the rat pyelonephritis model.

All strains harboring the cloned *hly* determinants were more virulent than their nonhemolytic counterparts in the rat model. The renal bacterial counts of the 536-21 strains carrying one of the two chromosomal *hly* determinants (pANN5311-O18 or pANN5211-O6) were 5 and 10 times higher, respectively, than those of the Hly^- mutant. Transformation of the O18 hemolysin determinant into the serum-resistant variant 536-31 also increased the renal counts by a factor of 2.3. As described above for the S-adhesin transformants, the Hly^+ strains exhibited the chromosomally encoded markers, and only those colonies which still retained the hemolytic phenotype were counted in the animal test.

From the hemolytic clone 536-21(pANN5311-O18) and the nonhemolytic variant 536-21 (Sre^- Mrh^- Fim^-) the renal elimination of leukocytes and erythrocytes was determined after transurethral infection (Fig. 4). In parallel to the renal bacterial concentration the hemolytic strain provoked higher urinary shedding of leukocytes and erythrocytes, indicating an effect of hemolysin on the inflammatory reaction of a UTI.

DISCUSSION

In this paper we provide evidence that rat nephropathogenicity of an uropathogenic *E. coli* strain depends on at least three factors present on the cell surface (S adhesin, serum resistance factor) or secreted into the medium (hemolysin).

The contribution of cloned P and Msh pili and hemolysin determinants to the virulence of extraintestinal *E. coli* strains was observed earlier over a short time period (24 h) in several animal models (9, 10, 13, 35, 42, 43). The rat pyelonephritis model used here fulfills the histological and bacteriological criteria of an acute pyelonephritis. Since the bacteria were introduced via the urethra, the model is better suited for detecting the influence of adherence to uroepithe-

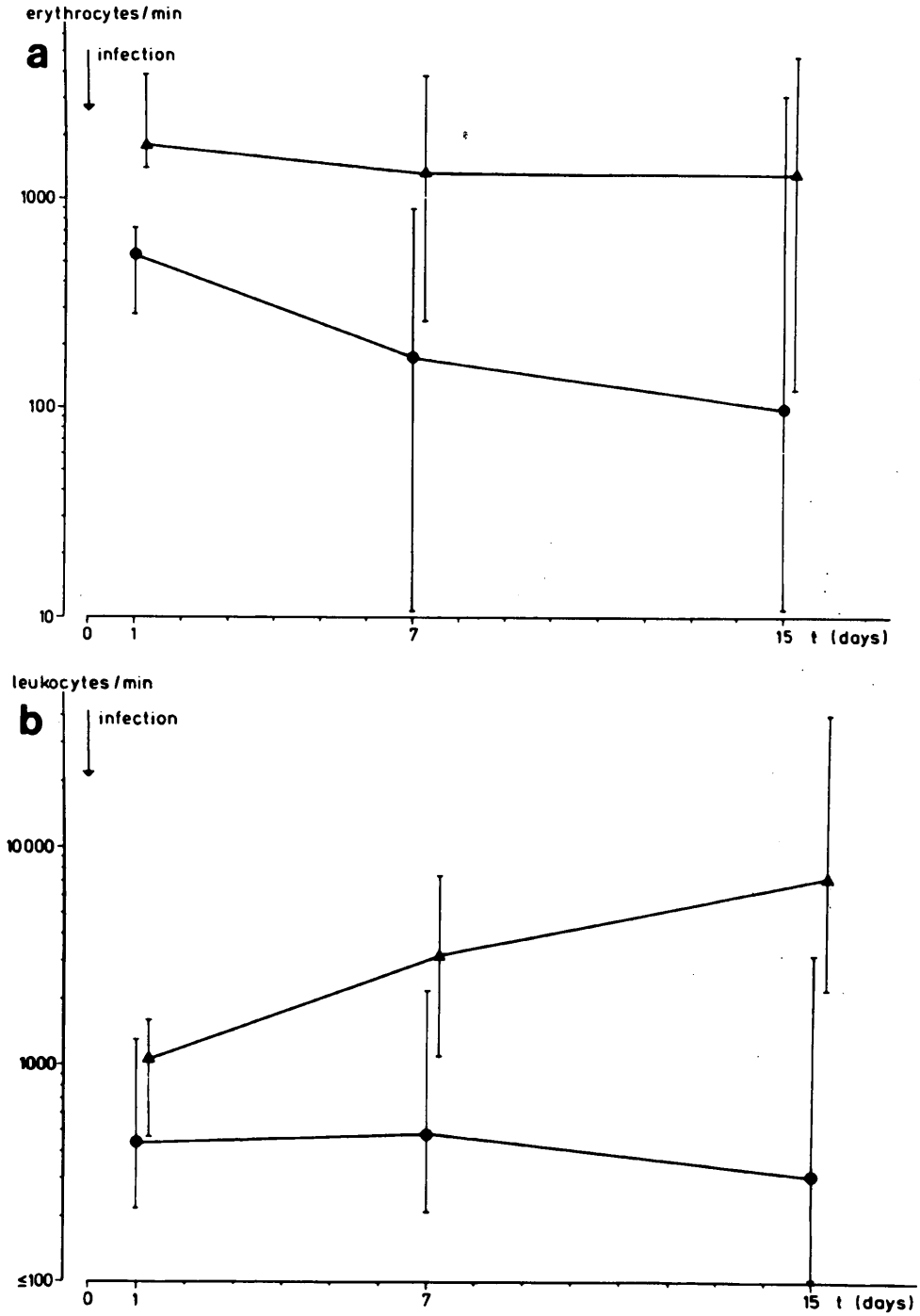

FIG. 4. Elimination of erythrocytes (a) and leukocytes (b) from urine of rats infected with strain 536-21 (●) or 536-21(pANN5311-O18) (*hly*$^+$) (▲). Median values and 90% confidence limits are given. Each group consisted of 10 animals.

lial cells on nephropathogenicity than UTI models using the hematogenous route. Because of the vesicoureteral reflux, *E. coli* strains without the virulence factors studied here are able to produce an infection. This corresponds to experiences with patients with vesicoureteral reflux (25). Furthermore the long renal persistance of the bacteria makes it possible to monitor the interaction of the host defense mechanisms and the bacterial virulence factors. The experimentally induced inflammation can persist for more than 3 months, exhibiting the morphological characteristics of a chronic pyelonephritis (33). Furthermore, inflammation can be evaluated continuously over several days in single ani-

mals by determining the urinary shedding of leukocytes and erythrocytes.

The differences in virulence between the wild-type isolate 536, which still retained S-adhesin formation, serum resistance, and hemolysin production, and the mutant 536-21, which has lost all of these characters, was highly significant as demonstrated by the reduction of bacterial counts per gram of kidneys 7 days after infection from nearly 100,000 to only 40 (Fig. 3). After reintroduction of the three determinants into the avirulent mutant strain by means of recombinant DNA techniques, the combined action of the factors led to a significant increase in renal bacterial counts, which exceeded the additive effects of single virulence properties. Compared with other *E. coli* animal models (9, 10, 13, 35, 42, 43) the differences in nephropathogenicity seen here were very strong, and this was true without any manipulation of the rats like ligation of the ureters, application of drugs, starvation, and others. However, the virulence of clone 536-31(pANN5311-O18), which exhibits the same phenotype as the wild-type isolate 536, does not reach the plateau of this parental strain. Besides the fact that in 536-21 more than three factors could be involved in the switch of the phenotype, these virulence differences could come from the different hemolysins produced by the two strains. Whereas strain 536 codes for two O6-specific hemolysins, its manipulated counterpart only excretes the O18-specific hemolysin.

As demonstrated by Svanborg-Edén and co-workers (14) the P fimbriae of *E. coli*, which recognize a Gal-Gal globoside as receptor on the target cells (18, 28), contribute to the persistence of the *E. coli* strains in a mouse model. Here we show that S fimbriae that belong to another group of adhesin factors found on *E. coli* strains from different extraintestinal sources (23) contribute to colonization by bacteria of the kidney of rats. This effect seems to indicate that S-specific sialyl acid-containing receptors are present on the surface of rat kidney cells. One cannot exclude, however, the possibility that S fimbriae may play another role in rat UTI as recently suggested for mouse septicemia (B. Nowicki, J. Vuopio-Varkila, P. Viljanen, T. K. Korhonen, and P. H. Mäkelä, Microb. Pathogenesis, in press). For P fimbriae it has been shown that Gal-Gal rceptor structures are not present on rat tissue cells (31). Therefore it is not surprising that cloned P(F8) fimbriae also appear to have no influence on the colonization of bacteria in the rat urinary tract (Hacker et al., in press).

Strain 536-31, which carried the cosmid pANN801 integrated into the chromosome and thereby regained serum resistance, displayed a significantly higher nephropathogenicity than its serum-sensitive counterpart. This indicates a considerable contribution of serum resistance to virulence of *E. coli* as already assumed on the basis of experimental pyelonephritis with *E. coli* strains of different serotypes and different serum susceptibility (14).

Several lines of evidence also indicate that the hemolysin determinants play a role in this model of infection. Differences in the renal counts observed after the introduction of bacteria harboring the different *hly* determinants could only result from differences in the structural gene (*hlyA*) or the genes *hlyB* and *hlyD* involved in secretion of hemolysin but not from different promoters which are constant in these *hly* determinants (1, 7, 16). Rats being infected with hemolytic *E. coli* have elevated renal bacterial counts. In addition signs of inflammation such as leukocyturia and erythrocyturia are more pronounced in these animals than in those infected with the Hly^- counterpart. The contribution to virulence might be partially outweighed by the fact that the hemolysin also acts as a bacterial suicide protein, since it promotes the release of leukotriene, thus stimulating the antiinfectious host response (21).

The fact that all three virulence factors, S fimbriae, serum resistance factor, and hemolysin production, contribute to rat nephropathogenicity supports the view that extraintestinal *E. coli* virulence is a multifunctional phenomenon. Each of the factors seems to play a significant role in the process of infection: S fimbriae mediate attachment of bacteria to eucaryotic cells, the serum resistance factor promotes protection against the serum complement system, and hemolysin acts as a cytolysin (3) which specifically interacts with blood cells.

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