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M Söderhäll, ... , J Winberg, R Möllby

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### Research Article

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# Induction of Protective Immunity After *Escherichia coli* Bladder Infection in Primates

## Dependence of the Globoside-specific P-fimbrial Tip Adhesin and Its Cognate Receptor

Mats Söderhäll,\* Staffan Normark,<sup>‡§</sup> Kiyohito Ishikawa,<sup>‡</sup> K.-A. Karlsson,<sup>||</sup> Susann Teneberg,<sup>||</sup> Jan Winberg,<sup>¶</sup> and Roland Möllby<sup>‡</sup>

\*Department of Nephrology, Karolinska Hospital, Karolinska Institute, S-171 76 Stockholm; <sup>‡</sup>Microbiology and Tumorbiology Center, Karolinska Institute, S-171 75 Stockholm; <sup>§</sup>Swedish Institute for Infectious Disease Control, S-105 21 Stockholm; <sup>||</sup>Institute of Medical Biochemistry and Microbiology, Department of Medical Biochemistry, Göteborg University, S-413 90 Göteborg; and <sup>¶</sup>Department of Woman and Child Health, Karolinska Hospital, Karolinska Institute, S-171 76 Stockholm, Sweden

### Abstract

Clinical observations suggest that immune mechanisms affect etiology and course of recurrent cystitis. A primate infection model was used to show that primary bladder infection with a uropathogenic P-fimbriated strain (binding to globoside present in the bladder wall) protects against rechallenge with homologous as well as heterologous *Escherichia coli* strains for up to 5–6 mo. In contrast, mutant derivatives producing P-fimbriae either lacking the tip adhesin protein or carrying an adhesin for which no bladder receptor was present, were unable to induce protection, even though they generated bladder infections of similar duration as the wild type. Therefore, the protective effect mediated by the adhesin seemed to depend upon the presence of its cognate receptor. Since the wild strain also mediated protection against mutants that lacked the adhesin, our data suggest that the globoside-binding PapG adhesin acts as an adjuvant during infection to enhance a specific response against other bacterial antigens. In fact, the globoside-binding strain DS17, but not the mutant DS17-1, unable to bind to membrane-bound globoside, elicited a secretory IgA response to LPS in urine. These in vivo findings suggest that bacterial adhesin–ligand interactions may have signaling functions of importance for the immune response. (*J. Clin. Invest.* 1997. 100:364–372.) **Key words:** bacterial adhesion • mucosal immunity • globoside • glycosphingolipids • urinary tract infections

### Introduction

Symptomatic urinary tract infection (UTI)<sup>1</sup> is a major infectious disease that causes considerable morbidity, suffering, and costs. In a recent prospective study, the incidence of symptomatic UTI per person-year of follow-up among young women

varied between 0.5 and 0.7 (1). The estimated cost for the care of each episode is 40–80 US dollars (2) (many times higher for childhood pyelonephritis), and in addition there are costs for the loss of working days. A better insight into the pathogenesis of these infections is needed as a basis for effective prevention (2). The role of immunity in the defense against recurrent UTI is unsettled (3–5). When a small child falls ill with an acute pyelonephritis, the sickness may be followed by another pyelonephritis, then by a couple of attacks of acute symptomatic cystitis, and later by periods of asymptomatic bacteriuria. This course of events is compatible with a change of host defense. Furthermore, about half of those girls that fall ill with a first symptomatic infection, will have no further infections during several years of followup (6). In patients with a number of recurrent *Escherichia coli* infections, the causative *E. coli* successively either become phenotypically altered (7), or become replaced by more unusual bacteria. These findings are consistent with a modification of the host defense upon repeated *E. coli* infections.

The *E. coli* strain causing the first urinary tract infection usually expresses a series of virulence attributes, such as additional adhesins and siderophores normally not found in commensal *E. coli*. The strongest correlation to uropathogenicity is the expression of so-called P-fimbriae–recognizing glycolipids in the urinary tract containing a Gal $\alpha$ (1–4)Gal moiety (globoseries of glycolipids). Thus, in anatomically healthy children suffering from their first pyelonephritis or cystitis, the *E. coli* isolates expressed P-fimbriae in 90 (8, 9) and 50% (10) of the respective cases. In individuals with recurrent bladder infections, the frequency of P-fimbriated *E. coli* was lower (8).

P-fimbriae are heteropolymeric structures composed of a rigid stalk making up the bulk of the surface organelle, and a thin flexible tip (fibrillum) containing several proteins, one of which is the receptor-binding PapG adhesin (11–13). In clinical materials, one finds P-fimbriated *E. coli* with two distinctly different tip-associated adhesins. The PapG adhesin of class II dominates in acute pyelonephritis, but is also common in first-time cystitis. This adhesin preferentially binds to globoside (GalNAc $\beta$ 3Gal $\alpha$ [1–4]Gal $\beta$ 4Glc $\beta$ cer) that is abundant in the upper urinary tract of humans. P-fimbriae with a PapG adhesin of class III are common in human cystitis, but are rare in pyelonephritic isolates (14). The class III adhesin preferentially binds to the Forssman antigen and to globoA, both of which carry extensions on the terminal GalNAc $\beta$  residue of globoside. It has been observed that UTI caused by P-fimbriated *E. coli* with a class III adhesin frequently occur in blood group A<sub>1</sub>P<sub>1</sub> secretor positive individuals that carry globoA on their uroepithelial cells (15).

In a previous study it was shown that the presence of the

Address correspondence to Jan Winberg, Department of Pediatrics, Karolinska Hospital, S-171 76 Stockholm, Sweden. Phone: 46-8-517-729-85; FAX: 46-8-517-740-34.

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1. Abbreviations used in this paper: PBS-T, PBS containing 0.05% Tween 20; UTI, urinary tract infection.

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class II PapG adhesin is necessary for pyelonephritis to occur when cynomolgus monkeys are challenged intraureterally with the P-fimbriated, pyelonephritogenic *E. coli* strain DS17 (16). We have also found that this strain, as well as its two isogenic mutants (DS17-8 producing P-fimbriae devoid of its native PapG class II adhesin, and DS17-1 with P-fimbriae carrying a class III PapG adhesin binding to the Forssman antigen), was able to cause cystitis, with a mean duration of 9–10 d (16, 17). When the wild strain and the mutant without the tip adhesin were inoculated simultaneously into the bladder, however, the wild strain outcompeted the mutant within a few days, suggesting that an interaction between bacteria and globoside provides *E. coli* with a competitive edge in primary bladder infection (17).

In this study we show, using cynomolgus monkeys, that a primary bladder infection with P-fimbriated *E. coli* DS17 induces protective immunity to a subsequent challenge infection. To determine whether the globoside-binding PapG adhesin plays a role in this protective response, we made use of mutant DS17-8 lacking the adhesin, as well as the class-switch mutant DS17-1, carrying a class III PapG unable to interact with globoside when present in biological membranes (18).

## Methods

The experimental procedures, the bacterial strains, and the characteristics of the monkeys (healthy, adult, female *Macaca fascicularis*) have been described in detail previously (16, 17). A brief summary follows.

**Bacterial strains.** The wild-type *E. coli* strain DS17 was originally isolated during an epidemic outbreak of pyelonephritis on a neonatal ward (19). It is of serotype O6:K5:H<sup>-</sup>, expresses P-fimbriae with a class II G adhesin and type 1 fimbriae, but lacks S-fimbriae and Afa-1 adhesin. The strain produces hemolysin and the siderophore aerobactin, is resistant to ampicillin and trimethoprim sulphphonamide, but is sensitive to ciprofloxacin. The mutant derivatives DS17-8 and DS17-1 (see below) were of the same serotype and resistance pattern as the parent strain DS17.

The construction of these mutants was described earlier in detail (16, 17). Strain DS17 carries a *pap* gene cluster encoding P-fimbriae with a class II PapG-adhesin. Through introducing a 1-bp deletion early in the *papG* gene, an isogenic knock-out mutant, DS17-8, was created (16). This *papG* mutant cannot mediate any Gal $\alpha$ (1–4)Gal-specific attachment, and is negative for hemagglutination with human erythrocytes. The other mutant, DS17-1, was created through a substitution of the *papG* class II allele, with a gene encoding for PapG class III, which mediates binding to Forssman antigen. This class switch mutant efficiently agglutinates sheep erythrocytes, but is negative for hemagglutination of human erythrocytes (17). All bacterial strains were grown on CF-agar plates overnight, freshly harvested before the experiments, and suspended in PBS.

*E. coli* strain JR1, provided by Dr. James Robert (Tulane University, New Orleans, LA) is of serotype O4, K:NT, H5, expresses P-fimbriae with a class II adhesin and type 1 fimbriae, and produces hemolysin (20).

Recombinant *E. coli* strain HB101/pPil291-15, carrying a plasmid-born *pap* gene cluster with a class II *papG* allele, was a kind gift from Dr. Irma Van Die (University of Limburg, The Netherlands) and Dr. Benita Westerlund (University of Helsinki, Finland).

**Bladder colonization.** 19 out of 24 monkeys were outbred, and the other five were inbred. They were housed separately with free access to water and food until 8 h before the experiments, when water was withheld. All experiments were done under ketamine and metazolam anesthesia.

Bladder infection was induced through inoculation of a bacterial

suspension (1 ml, 10<sup>7</sup> CFU/ml) via a urethral catheter. The inoculates were prepared directly from CFA-agar plates, and were consistently devoid of type 1 fimbriae. Upon repeated serial subcultivation in urine and in broth, it was possible to obtain expression of type 1 fimbriae without any demonstrable systematic differences between DS17 and its two mutants. Suprapubic bladder aspiration was used to obtain urine cultures usually at days 2, 7, 9, 14, 16, and 21, until two negative urine cultures were obtained. 20–30 min before bladder aspiration, the monkeys were hydrated with 30 ml lukewarm 0.9% sodium chloride solution administered subcutaneously to secure an optimal diuresis. The *E. coli* strains were identified as described under vaginal colonization. Leukocyte excretion was measured (Ecur<sup>4</sup>-Test sticks; Boehringer Mannheim, Mannheim, Germany). The methodology has been described in detail (16). Persisting infections were eliminated by either local instillation, or by intramuscular injection of ciprofloxacin (ciprofloxacin test substance; Bayer, Leverkusen, Germany) diluted in PBS. The mean intervals between initial infection and the first re-exposure were 39.2 d (DS17→DS17), 25.1 d (DS17→DS17-8), 23.8 d (DS17-8→DS17), and 22.2 d (DS17-1→DS17).

**Vaginal colonization.** All 16 monkeys were outbred. The procedure has been described in detail elsewhere (17, 21, 22). In summary, a catheter was introduced into the vagina, and 3 ml of an *E. coli* suspension (10<sup>9</sup> CFU/ml) with either DS17, DS17-8, or DS17-1 was flushed over the mucous membranes.

Sampling of vaginal specimens for bacterial examination was performed by rotating a sterile cotton-tipped swab against the vaginal wall. The sample was suspended in 1 ml of PBS, and was vortexed for 20 s, giving a suspension referred to as vaginal fluid. A 0.1-ml sample was then spread on a Cled agar plate (Oxoid Ltd., Hampshire, United Kingdom) and incubated for 18 h at 37°C. Colonies with *E. coli* appearance were semiquantitated and identified as DS17, or its mutants by biochemical tests, antibiotic sensitivity ampicillin (resistant), trimethoprim-sulphonamide (resistant), ciprofloxacin (sensitive), and biochemical fingerprinting (23). DS17 and its mutants were distinguished by P-specific particle agglutination test (PPA-test) (24) identifying P-fimbriae (DS17 only) and sheep red cell agglutination identifying class III *papG* proteins binding to the Forssman antigen (DS17-1 only). DS17-8 did not agglutinate either. A successful vaginal colonization was defined as persistence of > 10<sup>4</sup> CFU/ml vaginal fluid for six subsequent days or more.

**Treatment of monkeys after a colonization experiment.** Often the vaginal *E. coli* bacteria disappeared spontaneously. When they did not, the vagina was flushed with ciprofloxacin in a concentration of 40 mg/liter. We attempted to restore the normal vaginal flora by direct transfer of 3 ml vaginal fluid from a healthy monkey not exposed to antibiotics or a DS-strain. A new experiment started not earlier than 4 d after the end of this procedure.

**Purification of LPS from wild-type *E. coli* strain DS17.** LPS from *E. coli* strain DS17 cultured overnight on CF-agar was extracted with phenol/water (9:1 vol/vol) (25), precipitated by 6 vol of diethylether/acetone (1:5 vol/vol) and 1 volume of phenol phase. The extract was dialyzed against running tap water for 3 d, centrifugated at 9,000 g for 20 min, dialyzed against dH<sub>2</sub>O, and finally freeze-dried.

**ELISA.** Serum levels of IgA and IgG and urine levels of IgA and secretory IgA against LPS were determined by indirect ELISA. The four different sets of ELISAs used the same initial coating procedure: flat-bottom polystyrene microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with LPS (100  $\mu$ l, 10  $\mu$ g/ml in PBS, pH 7.4) overnight at 22°C. For analysis of serum antibodies, the remaining binding sites were blocked with 1.5% (wt/vol) freeze-dried milk powder (Semper AB, Stockholm, Sweden) for 1 h at 22°C and thereafter washed three times with PBS containing 0.05% Tween 20 (PBS-T).

**Analysis of IgA in serum.** Serum samples diluted 1:400 in PBS-T were added to the plates, incubated for 1 h at 37°C, and then washed three times with PBS-T. 0.1 ml of the conjugate (goat anti-monkey IgA Fc-specific; Nordic Immunologic Laboratories, Tilburg, The Netherlands) labeled with horseradish peroxidase, diluted 1:5,000 in PBS-T, was applied to each well, incubated for 2 h at 37°C, and the

plates were washed three times with PBS-T. After addition of the substrate 2',2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium (0.22 mg/ml) in citric acid (0.05 M, pH 4.0), the plates were incubated and read at 405 nm using a microtiter plate spectrophotometer (integrated EIA Management System; Labsystems Tyresö, Sweden).

**Analysis of IgG in serum.** The conjugate (goat anti-human IgG  $\gamma$ -specific; Sigma Chemical Co., St. Louis, MO), labeled with alkaline phosphatase, was diluted 1:2,000 in PBS-T, applied to each well, incubated for 1 h in 37°C, and the plates were washed three times with PBS-T. The substrate *para*-nitro-phenylphosphate (1 mg/ml; Sigma) solubilized in diethanolamine (1 M, pH 9.8) was added, and the plates were incubated and read at 405 nm.

**Analysis of IgA and secretory IgA in urine.** Undiluted urine samples were added to the plates, incubated for 1 h at 37°C, and then washed three times with PBS-T. 0.1 ml of one of the two conjugates (goat anti-monkey IgA Fc-specific or goat anti-monkey secretory component-specific (both Nordic Immunology) labeled with horseradish peroxidase, diluted 1:1,500 in PBS-T, was applied to each well, incubated for 2 h in 37°C, and the plates were washed three times with PBS-T. After addition of the substrate ABTS (0.22 mg/ml) in citric acid (0.05 M, pH 4.0), the plates were incubated and read at 405 nm.

**In vitro adhesion studies.** Adherence to formalin-fixed tissue from monkey kidney, bladder dome, and vagina was performed by using fluorescein isothiocyanate-labeled DS17, DS17-8, and DS17-1 as described (16).

For epithelial cell binding, cells were obtained by scraping a cotton-tipped swab against the vaginal wall. The swab was stirred in 1.0 ml of PBS, vortexed, and spun down at 200 *g* at 4°C for 10 min. The sediment was washed in 5 ml PBS and centrifuged twice. The washed cells were resuspended in PBS and adjusted to a final concentration of 10<sup>3</sup>–10<sup>4</sup> cells/ml, using an optical scale. To optimize expression of P-fimbriae, bacteria were cultured overnight on CF-agar (26), harvested, suspended, washed in PBS, and adjusted to a concentration of 10<sup>8</sup>–10<sup>9</sup> CFU/ml. Adhesion of *E. coli* strains were examined as described earlier (27, 28). In short, the epithelial cells were mixed with the bacterial suspension, filtered onto a large pore (8.0  $\mu$ m) polycarbonate membrane filter (Nucleopore Corp., Pleasanton, CA), washed with 30 ml PBS, and stained with Diff-Quick (Svenska Labex AB, Helsingborg, Sweden). The filter was placed on a microscope slide, and adherent bacteria were counted visually in the microscope on 40 cells.

**Preparation of glycosphingolipids.** One female cynomolgus monkey was killed by an intracardial injection of pentobarbital, and the kidney, ureters, bladder, and vaginal wall tissue were immediately taken out and frozen at -70°C. Isolation of glycosphingolipids was done as described (29) with minor modifications. In brief, the tissues were lyophilized, and thereafter extracted with chloroform and methanol. The extracts were partitioned according to Folch-Pi (30), and the upper phase thus obtained was dialyzed, and thereafter separated into acid and nonacid glycolipid fractions on a DEAE cellulose column. The nonacid fraction was added to the lower phase obtained from the Folch partitioning. This fraction was subjected to mild alkaline hydrolysis, and was further separated by chromatography on silicic acid and DEAE cellulose column. In summary, three final fractions were obtained: acid glycosphingolipids from the upper phase (acid-U), acid glycosphingolipids from the lower phase (acid-L), and total nonacid glycosphingolipids.

**Labeling of bacteria.** For the binding assays to immobilized, glycosphingolipids, *E. coli* DS17 and its mutant DS17-8, were cultured on CF-agar with the addition of 10  $\mu$ l [<sup>35</sup>S]methionine (400  $\mu$ Ci; Amersham International, Little Chalfont, United Kingdom) at 37°C for 24 h, whereas the recombinant *E. coli* strain HB101/pPil 291-15 was grown in Luria broth supplemented with ampicillin (100  $\mu$ g/ml) and [<sup>35</sup>S]methionine (400  $\mu$ Ci/10 ml) at 37°C for 12 h. The bacteria were harvested by scraping and centrifugation, respectively. After two washes with PBS, pH 7.3, the cells were resuspended to 10<sup>9</sup> CFU/ml in PBS. The specific activities of the suspensions were ~ 100 CFU/cpm.

**Chromatogram binding assay.** Binding of bacteria to glycosphingolipids on thin-layer chromatograms was done as described (31). In brief, glycosphingolipids were separated on alumina-backed silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) using chloroform/methanol/water 60:35:8 (by vol) as the solvent system. The dried chromatograms were treated with 0.5% polyisobutylmethacrylate (Röhm, GmbH; Darmstadt, Germany) in diethylether (wt/vol), and thereafter soaked in PBS containing 2% bovine serum albumin and 0.1% NaN<sub>3</sub> (solution A) for 2 h. Thereafter, the chromatograms were incubated with suspensions of <sup>35</sup>S-labeled bacteria for 2 h at room temperature, followed by washings with PBS. Autoradiography was done for 12–72 h using XAR-5 x-ray films (Eastman Kodak Co., Rochester, NY). The same method was used for binding of monoclonal antibodies directed against the Forssman epitope (clone M1/22.25.8.HL; Sera-Lab, Crawley Down, United Kingdom). After coating with solution A, the chromatograms were incubated for 2 h with suspensions of the anti-Forssman antibody (diluted 10 times in solution A). After rinsings there followed another 2-h incubation with <sup>125</sup>I-iodinated (labeled by the Iodogen method [32], rabbit anti-mouse antibodies (DAKOPATTS, Copenhagen, Denmark) diluted to 2  $\times$  10<sup>5</sup> cpm/ml in solution A. Finally, the chromatograms were rinsed, dried, and autoradiographed for 12–24 h.

## Results

### Primary bladder infections

Both the wild-type strain DS17 and its two mutant derivatives DS17-8 and DS17-1 induced bladder infections at primary inoculation in all monkeys except one (Table I). 16 infections were spontaneously cured; six were eliminated by ciprofloxacin locally installed into the bladder, while two infections were eliminated by parenteral administration of ciprofloxacin. The median duration of the infection was not significantly different for the three strains (14.0, 9.0, and 9.5 d for DS17, DS17-1, and DS17-8, respectively). Mean durations were 17.2, 12.5, and 11.0 d, respectively. Two infections with DS17 were of unusually long duration; 35 and 44 d. If these infections are excluded, the mean duration for DS17 infections was 12.7 d.

For comparison of bacterial numbers in infections caused by the three strains, we selected the first three cultures on days 2, 7, and 9 after inoculation. Median numbers were 4.1  $\times$  10<sup>5</sup>, 2.9  $\times$  10<sup>4</sup>, and 4.5  $\times$  10<sup>4</sup> CFU/ml for DS17, DS17-1, and DS17-8, respectively. Corresponding mean values were 1.1  $\times$  10<sup>6</sup>,

Table I. Bladder Infections After a Primary Intravesical Inoculation with DS17 and Its Mutants DS17-8 and DS17-1

	Inoculated strain		
	DS17	DS17-8	DS17-1
No. of monkeys ( <i>n</i> )			
Total study group	12	6	6
Infected	12	6	5*
With positive leukocyte esterase test <sup>‡</sup>	11	6	3
Spontaneous cure	7	3	6
Median duration of bacteriuria (d)	14	10	9

\*The sixth monkey voided immediately after the first inoculation, and the day 2 urine culture was negative. A reinoculation with the same strain induced an abortive infection (3  $\times$  10<sup>3</sup> CFU/ml on day 2, followed by negative cultures). <sup>‡</sup>The leukocyte esterase test, when showing +1 or more, was graded as positive. In urine specimens of 20 monkeys obtained before the first inoculation, the leukocyte esterase test was negative in 19, and was graded +1 in a single monkey.

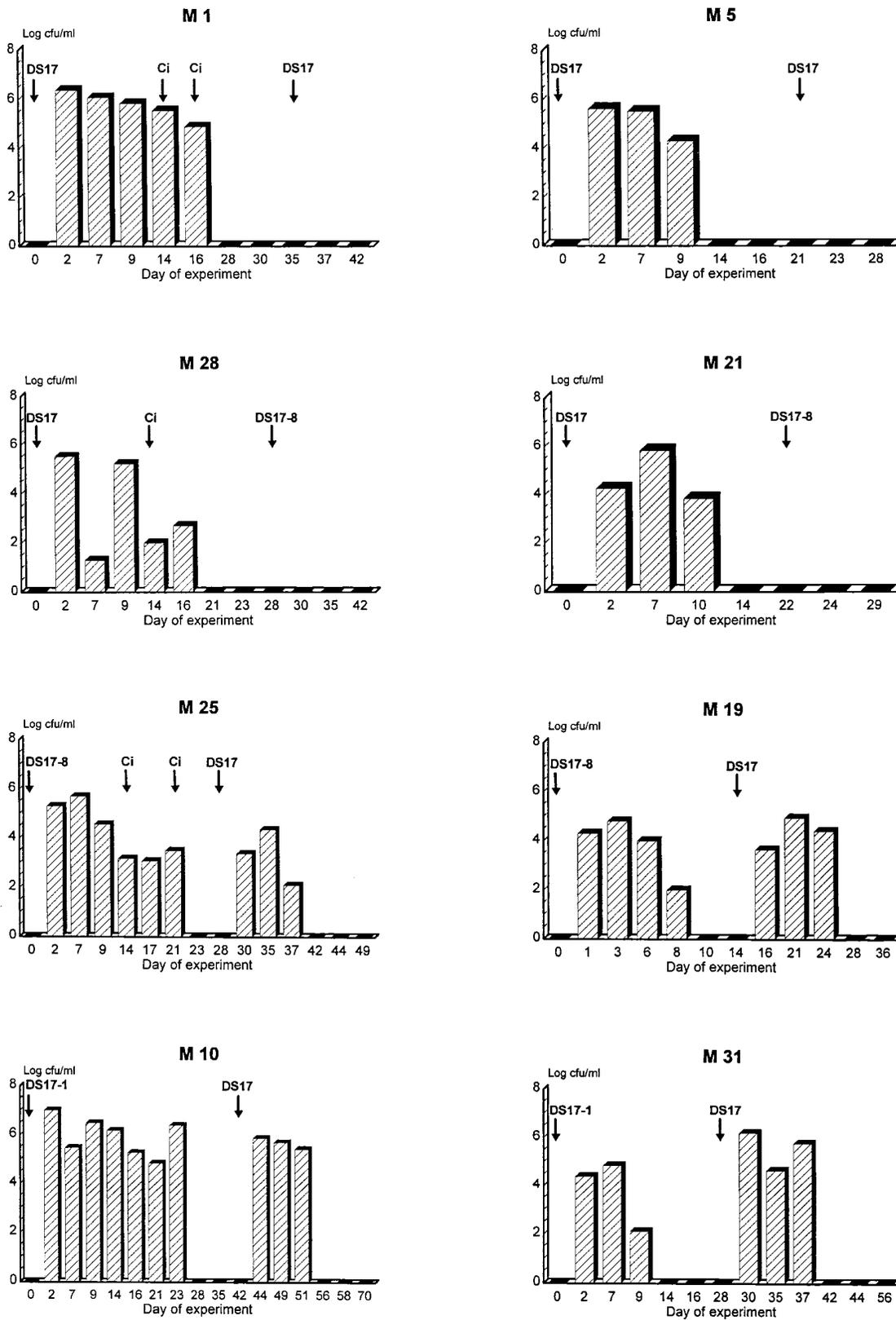


Figure 1. Inoculation of various combinations of strains into the bladder of eight monkeys (*M* indicates the monkey individual). For each of the four sets of experiments, two panels are shown, one where the initial infection is of longer duration (16–23 d) and one of shorter duration (8–10 d). Primary infection with the wild strain DS17 protected against challenge with DS17 and DS17-8. Primary infections with the isogenic mutants DS17-8 or DS17-1 did not protect against challenge with DS17. Inoculation of 1 ml of  $10^7$  CFU/ml of each strain. Summaries of the results are shown in Tables I and II. Ci, intravesical inoculation of ciprofloxacin.

Table II. Protection Against Bladder Infection at a Subsequent Intravesical Challenge

Initial inoculation	Subsequent inoculation	No. of monkeys		
		Total	Protection	No protection
DS17	DS17	5	4	1*
DS17	DS17-8	7	7‡	0
DS17-8	DS17	5	0	5
DS17-1	DS17	5	0	5
Mutant + DS17§	DS17	12	12 <sup>  </sup>	0

Mean intervals between initial and challenge infections are given in the text. \*No protection at second challenge, but complete protection at third challenge. ‡Complete protection in five monkeys and partial protection in two ( $2.5 \times 10^2$  CFU/ml at day 2 respective negative at day 2, and  $2.2 \times 10^3$  CFU/ml on day 7). Negative leukocyte esterase tests in both monkeys. §The initial inoculations consisted of a first exposure to either DS17-8 or DS17-1, followed by exposure to DS17. The subsequent inoculation consisted of DS17. The procedure is illustrated in Fig. 2 a. <sup>||</sup>Partial protection in three monkeys:  $2.0 \times 10^3$ ,  $2.3 \times 10^3$ , and  $1.5 \times 10^5$  CFU/ml at day 2, and then negative cultures. Negative leukocyte esterase tests in two out of three experiments.

$7.8 \times 10^5$ , and  $1.6 \times 10^5$  CFU/ml. Neither of these differences was significantly different by the quantitative Student's *t* test, but DS17 caused infection with significantly higher number of bacteria as calculated by the Mann-Whitney ranking test ( $P = 0.04$  against DS17-1,  $P = 0.02$  against DS17-8).

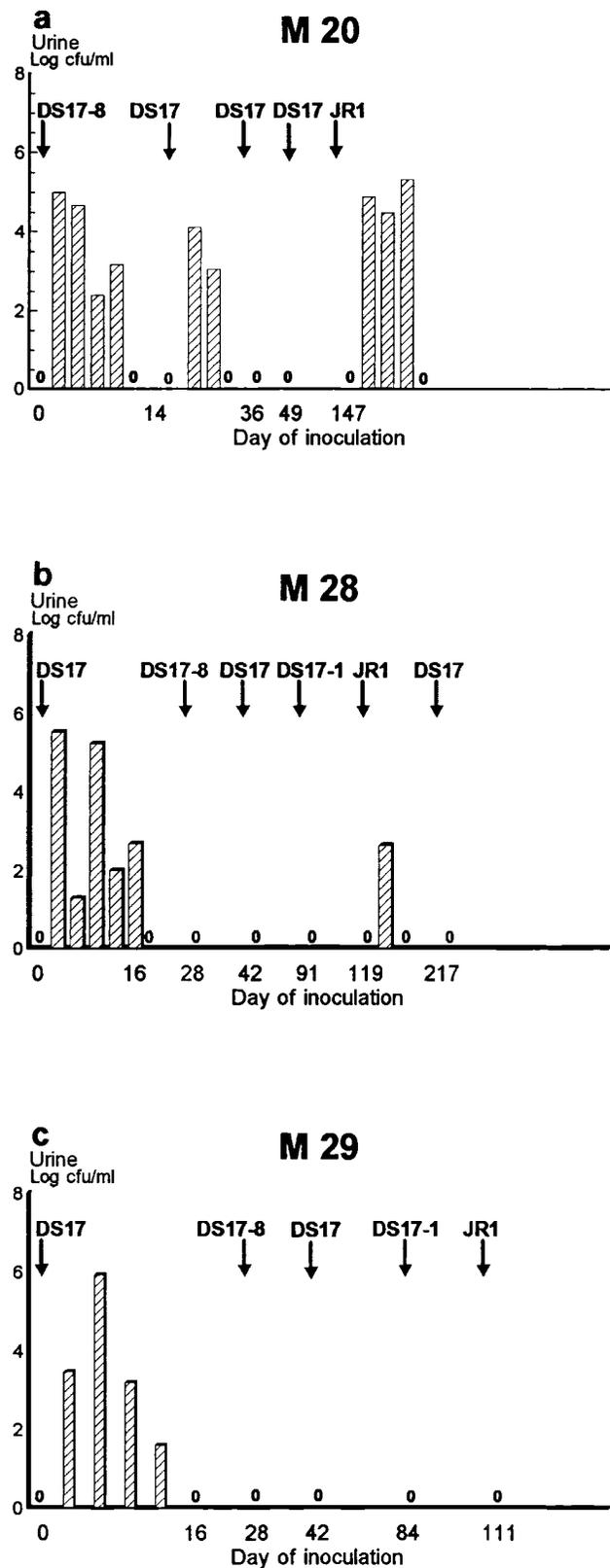
Significant differences in degree of inflammation as measured by the leukocyte esterase test were not found between the three strains, nor did polymorph white blood cell count on day 2 differ between infections caused by the three strains.

*Wild-type DS17, but not its papG mutants, protects against subsequent bladder inoculations*

After the primary infection and two subsequent negative urine cultures, the monkeys were challenged with a second intravesical inoculation of either the wild-type strain or one of its mutants. Monkeys primarily inoculated with DS17 were completely (CFU  $< 10^2$ /ml in all urine cultures after challenge) or partially (CFU  $\geq 10^2$ /ml in one, but not two urine cultures after challenge) protected against reinfection with both DS17 and the mutant DS17-8. In contrast, neither of the two mutants gave rise to protection against challenge with the parent wild-type strain (Fig. 1, Table II). In 12 monkeys that had been inoculated primarily with a mutant followed by inoculation of DS17, a second intravesical inoculation of DS17 was given (Fig. 2 a). In 9 out of 12 such experiments, the second challenge with DS17 did not establish a bladder infection. The three remaining monkeys were partially protected.

In this series of repeated inoculations, we could demonstrate that previous bladder exposure to DS17 also generated protection against the heterologous P-fimbriated strain JR1 in 12 (11 complete, 1 partial, i.e.,  $4.5 \times 10^2$  CFU/ml at day 2, then negative cultures) out of 19 experiments. Examples are given in Fig. 2. The mean interval between the last positive DS17 in-

Figure 2. (a) In 12 experiments (Table I) the initial inoculation consisted of a mutant strain followed by the wild strain DS17. The subsequent inoculation consisted of DS17. In this experiment, the monkey was protected at the second challenge with DS17 on day 36. In this monkey there was no protection against heterologous challenge with



the wild strain JR1 at day 147. (b) This monkey was infected initially with DS17. After heterologous challenge with JR1 at day 119, there was a partial protection ( $4.5 \times 10^2$  CFU/ml at day 121), then negative cultures. (c) Analogous with b, this monkey was infected initially with DS17. Heterologous challenge with JR1 at day 111 showed a total protection.

fection and challenge was 86.3 d (range 42–156 d) for the protected monkeys, and 91.6 d (range 42–135 d) for those who did not experience protection.

After intravesical inoculation, strains DS17, DS17-8, and DS17-1 can be later found in the gut. Interestingly, even after protection has been generated in the bladder, the organism may be recovered from feces (data not shown).

#### Serum and urine immune response after primary bladder inoculation with DS17 and DS17-1

A serum or urine response was regarded as positive when the peak absorbance value was at least twice the initial one. Peak values < 0.200 were always considered as a negative response.

**Serum immune response.** A positive serum IgG response to DS17 was seen in 9 out of 11 monkeys infected with DS17, and in 6 out of 6 with DS17-1. Corresponding figures for serum IgA were 6 out of 11, and 3 out of 6.

**Urine immune response.** A local immune response as measured by secretory IgA against LPS in urine was seen in 8 out of 11 monkeys infected with DS17, and in 0 out of 5 monkeys with DS17-1. Corresponding figures were seen for total IgA against LPS. When the five monkeys, primarily inoculated with DS17-1, were challenged with DS17, they all showed a significant secretory IgA response.

Thus, there was a systemic immune response to DS17 as well as to DS17-1, whereas a local response was seen only after DS17 infections. The data suggest that specific ligand–receptor interaction is important for the local IgA immune response.

#### Local protection is not induced after repeated vaginal and rectal colonizations

It was shown previously that strain DS17 is an excellent colonizer of the monkey vagina (21, 33). Repeated vaginal colonizations with the wild type as well as with the two mutants were now performed in four additional monkeys (Fig. 3). No local protection was observed. Moreover, the strains used for vaginal colonization invariably spread to the intestinal tract, and it was possible to recover them from feces during the experi-

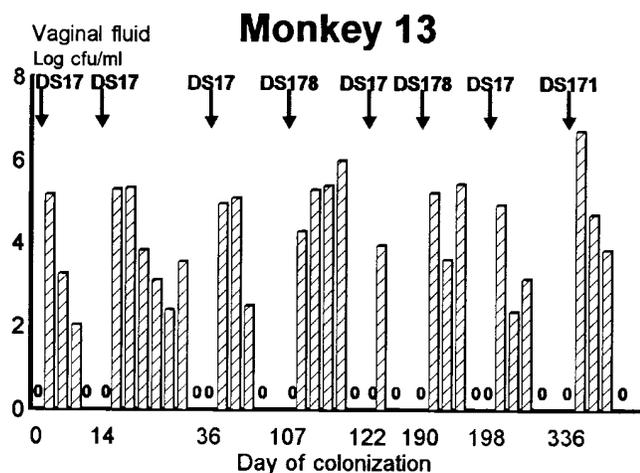


Figure 3. Vaginal cultures in one monkey after local inoculations with the wild strain DS17 and its mutant derivatives. Cultures were taken five days a week. Only results of two of the weekly cultures are shown in the figure. DS17 did not protect against further colonizations with itself, DS17-8, or DS17-1. Altogether, such studies were performed in four monkeys.

ment. Thus, none of the strains were able to generate protection in the gut after vaginal inoculation.

#### In vitro binding to monkey urogenital tissues and cells of E. coli strain DS17 and its mutant derivatives DS17-8 and DS17-1

We have shown previously that DS17, but not its *papG* adhesin–negative mutant DS17-8, efficiently binds to kidney sections (16). Now we found that the class switch mutant DS17-1, recognizing the Forssman antigen rather than globoside, was likewise unable to bind to kidney sections (data not shown).

None of the three strains bound to sections of formalin-fixed bladder tissue (data not shown), suggesting that the level of Gal $\alpha$ (1–4)Gal-containing receptor active glycoconjugates could be absent, lower, or unavailable for binding in the bladder wall compared to the kidney.

In contrast, all three strains bound avidly to vaginal tissue sections (data not shown), and to washed and unwashed vagi-

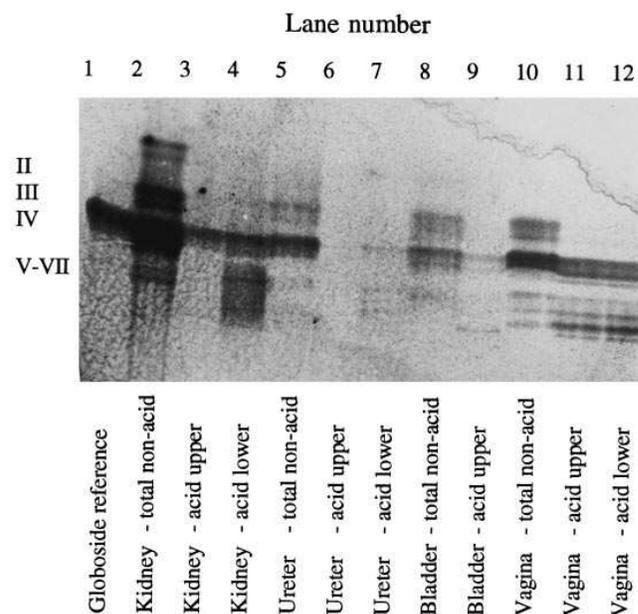


Figure 4. Binding of P-fimbriated *E. coli* to glycosphingolipids from monkey urogenital tract on thin-layer chromatogram. Lane 1 contained reference globoside from human erythrocytes, 2  $\mu$ g; lanes 2–4 were glycosphingolipids isolated from cynomolgus monkey kidneys; lanes 5–7 were glycosphingolipids from monkey ureters; lanes 8 and 9 were glycosphingolipids from monkey bladder; and lanes 10–12 were glycosphingolipids from monkey vaginal wall tissue. The roman numbers to the left indicate the number of carbohydrate residues in the bands. Dark bands on the autoradiogram indicate bacterial binding to receptor active compounds. The autoradiogram shown was obtained using  $^{35}$ S-labeled recombinant *E. coli* HB101/pPil 291-15. An identical binding pattern was obtained using  $^{35}$ S-labeled DS17, albeit with a higher background. Based on several years of experience with this technique, binding of a P-fimbriated strain in this assay is solely to Gal $\alpha$ (1–4)Gal-containing isoreceptors (34). Even though no single glycolipid was isolated and chemically characterized in the present study, the location on the thin-layer plate compared to mobilities for known structures was considered sufficient to ascribe a structure to some of the binding components shown in Fig. 2 of a previous paper (17). A number of additional receptor active components appeared that migrated differently than the known standard compounds. These components, however, were present in too low amounts to allow for a structural analysis.

nal epithelial cells (median values for all strains were  $\sim 50$  bacteria/cell [washed cells, data not shown]). This binding was not inhibited by 2% methyl- $\alpha$ -D-mannopyranoside (MMP; Sigma), supporting the existence of another adhesin in strain DS17 and its two derivatives, different from both PapG and FimH on type 1 fimbriae.

*Receptor-active glycoconjugates for G-adhesins in tissues from the monkey urogenital tract*

The binding data given above could be interpreted to mean that only the kidney, but not the bladder, contains receptor-active glycoconjugates. Our intravesical inoculation experiments, however, clearly implicated a role for the globoside-binding PapG adhesin in the local protective response evoked by strain DS17. We therefore tested for receptor-active glycosphingolipids based on Gal $\alpha$ (1-4)Gal sequences in relevant target tissues in the monkey. Several glycosphingolipids were shown to act as receptors for DS17.

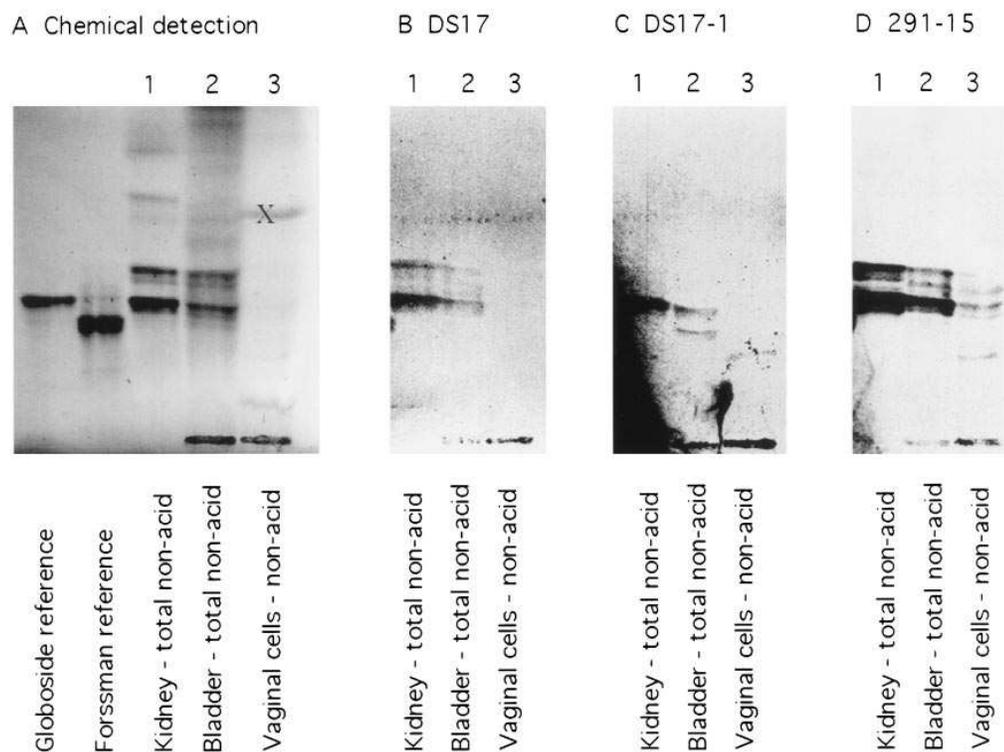
Fig. 4 demonstrates selected binding results from a number of bacterial overlays with  $^{35}$ S-labeled HB101/pPil 291-15, overexpressing P-fimbriae with a class II PapG adhesin, on thin-layer chromatography plates containing glycolipid preparations from monkey kidney, ureter, bladder, and vaginal wall tissues. Similar results were obtained with  $^{35}$ S-labeled DS17 (Fig. 5 B) showing binding to nonacid glycosphingolipids from kidney and bladder. Overlay with  $^{35}$ S-labeled DS17-8 lacking the class II PapG adhesin did not give rise to any detectable binding (data not shown), suggesting that binding was specific for Gal $\alpha$ (1-4)Gal-containing glycosphingolipids. The class switch mutant DS17-1 bound to globoside, and to a minor compound migrating in the pentaglycosyl ceramide region in

kidney and bladder (Fig. 5 C). It has been shown previously that bacteria with Forssman antigen-binding adhesins (class III) cannot interact with globoside in natural cell membranes, even though binding is obtained to globoside immobilized on thin-layer chromatograms (18). Furthermore, there was no binding of a monoclonal antibody directed against the Forssman epitope, to the glycosphingolipids isolated from bladder and kidney (data not shown), demonstrating the absence of the Forssman glycosphingolipid in these tissues.

The patterns of isoreceptors were distinctly different between the upper and lower urinary tract. Thus, the binding pattern to glycosphingolipids extracted from cynomolgus monkey kidney was similar to that of human kidney (34), and was dominated by globoside, with lower amounts of galabiosa-sylceramide, globotriaosylceramide, and only trace amounts of slower migrating glycosphingolipids, implicating that the Forssman antigen is not present in the kidney of cynomolgus monkeys. Tissue extracts of the ureters (Fig. 4, lanes 5-7), bladder (Fig. 4, lanes 8 and 9), vaginal wall tissue (Fig. 4, lanes 10-12), and extracts of vaginal epithelial cells (not shown) were also tested for PapG-specific receptor-binding activity. These tissues were similar to one another, and contained, in addition to binding-active glycosphingolipids migrating as globotriaosylceramide and globoside, three slow-moving binding active compounds. The relative amount of globoside seemed to be less in the bladder than in the kidney.

**Discussion**

Clinical experience suggests that a protective immune response may develop upon repeated infections of the urinary



*Figure 5.* Comparison of the glycosphingolipid-binding patterns of  $^{35}$ S-labeled DS17 (B), DS17-1 (C), and HB101/pPil 291-15 (D). The chromatogram in A was stained with anisaldehyde, while B-D are autoradiograms obtained after binding of radiolabeled bacteria as described in Methods. The lanes on the chromatogram were: 1, nonacid glycosphingolipids from monkey kidney, 40  $\mu$ g; 2, nonacid glycosphingolipids from monkey bladder, 40  $\mu$ g; 3, nonacid glycosphingolipids from monkey vaginal cells, 40  $\mu$ g. The mobilities of reference globoside and Forssman glycosphingolipid are shown in A. The band marked with an X is a nonglycolipid contaminant. DS17 bound to compounds with mobility as globotriaosylceramide and globoside (B) while the class switch mutant DS17-1 bound to globoside and to a compound migrating in the pentaglycosyl ceramide

region (C). Although this binding is obtained on thin layer chromatograms, bacteria with Forssman antigen-binding adhesins (class III) cannot interact with globoside in natural cell membranes (18).

tract. The nature of this protection, however, remains unknown, and available data are contradictory (4, 5). Immunity to experimental animal UTI have mainly focused on protection against pyelonephritis in rats and monkeys. Immunity studies during bladder infections have mainly concerned demonstration of antibody response and not protection. Available data are difficult to interpret, but it seems clear that the urinary tract is immunologically competent in the sense that immunoglobulins can be present at various levels (4, 5). To what extent these antibodies are protective remains unknown. In rats, however, both bladder and vaginal immunization were followed by a rapid bladder immune response on subsequent bladder challenge with live *E. coli* (35–37). Furthermore, individuals with immunodeficiency syndromes do not seem to have an increased risk of attracting UTI, and patients with recurrent UTI do not seem to be especially prone to other types of infections (3), adding to the confusion about the role of immunity in the defense against lower UTI.

Level diagnosis in UTI can be difficult. The following observations suggest that in the present study, the infections did not involve the kidneys. First, the infections were usually spontaneously cured, and others could be eliminated by an intravesical flush of ciprofloxacin, a method used to distinguish between lower and upper UTI in humans (38). Second, we have shown earlier that DS17-8 is unable to cause pyelonephritis (16). Since in this and in previous studies there were no observed differences between initial infections caused by the wild strain and those caused by the mutants with regard to duration of infection, it seems probable that the infections were all limited to the same level, i.e., the bladder. Third, when temperature was measured, it did not seem to be raised. Fourth, the monkeys never appeared to be ill. Fifth, the white blood cell count was not raised to the extent one would expect had the kidneys been involved.

We show here that cystitis caused by the P-fimbriated clinical isolate DS17 can generate long-lasting (> 150 d) protection against subsequent infections with the same strain. In both experimental bladder infections and in vaginal colonizations with strain DS17, the organism also spontaneously colonized the intestine for a substantial amount of time. We do not know if the protection elicited by bladder infections emanated from the bladder, or from colonization of the intestine. Repeated colonizations of the intestinal tract after vaginal colonizations with DS17, however, did not provide protection in the gut. Furthermore, preliminary studies in the monkey model suggest that prior vaginal and intestinal colonizations do not elicit protection against DS17 infection of the bladder. We therefore suggest that the bladder itself is an immunocompetent organ. This conclusion is reinforced by the findings of secretory IgA antibodies in the urine after DS17 infections. The fact that such antibodies were not found in monkeys infected with DS17-1, for which the Forssman epitope receptor is not available in the bladder, suggests that a specific receptor binding is important for the antibody response. It is unknown whether the secretory IgA antibodies contribute to the protection induced by DS17 infection.

An unexpected finding in this study was that the PapG adhesin positioned at the tip of the P-pilus fiber was required to generate protection against subsequent challenge. Since protection was also evident against the mutant DS17-8 lacking the tip adhesin, the role of the adhesin is not to act as a specific antigen generating humoral or local antibodies. Rather, our data

suggest that the globoside-binding class II PapG adhesin acts as an adjuvant during infection to enhance a specific response against other bacterial antigens such as LPS. An analogy to this finding might be the demonstration that other carbohydrate-binding proteins such as the B subunit of cholera toxin, which binds with high affinity to the ganglioside GM<sub>1</sub>, enhance mucosal response to the antigens bound to them (39).

The finding that neither P-fimbriated *E. coli* carrying the related class III PapG adhesin (DS17-1) nor the knock-out mutant DS17-8 provide protection in the experimental cystitis model used here, suggests that the specific receptor-binding properties of the adhesin may be important for the adjuvant effect. Glycolipid extractions from the bladder wall of cynomolgus monkey reveal the presence of globoside, the preferred isoreceptor for the class II adhesin, but no Forssman antigen (the preferred isoreceptor for the class III adhesin) as shown by the failure of anti-Forssman monoclonal antibodies to bind to glycolipids isolated from the bladder wall. The receptors for the class II adhesin may occur only in limited numbers on epithelial bladder cells, since DS17 did not adhere to bladder mucosa. Alternatively, it may be that the globoside receptor is confined to a small area in the bladder such as the trigone, or is present only on subepithelial tissues. It may also be that PapG receptors become available to the microbe, only as a consequence of the inflammation. An alternative explanation to the differential protective effect of the three strains would be that type 1 fimbriae (which are thought to be implicated in the pathogenesis of cystitis [40]) were better expressed in the wild strain than in the mutants. We could not, however, find any evidence for this possibility in systemic *in vitro* cultures of the three strains in broth and urine. Another possibility would be that the three strains caused infections of different duration, which was not the case. In addition, in earlier studies (16, 17) the median duration was 10, 9, and > 10 d for DS17, DS17-1, and DS17-8, respectively.

The antibody response in serum appeared to be similar upon infections caused by DS17 and DS17-1. The data on antibody response in urine, however, support the hypothesis that the class II adhesin of P-fimbriae may have at least local adjuvant effect.

If the proposed adjuvant effect is due to an interaction between the adhesin and the host cell, it can be caused by a signaling effect invoked on the host cells by the adhering microorganism. For example, *E. coli* expressing functional P-fimbriae elicited an increased inflammatory response as compared to strains lacking P-fimbriae expression (41). Alternatively, bacteria adhering to the Gal $\alpha$ (1–4)Gal receptor may become altered as compared to nonadhering ones, leading to an upregulation of protective antigenic epitopes. It was demonstrated recently that a novel sensory regulatory protein is induced when strain DS17 interacts with its cognate receptor. This transmembrane protein seems to link attachment to an adaptive response to low iron conditions, including an upregulation of iron siderophore receptors in the outer membrane, allowing growth in urine (42). The information in the present study may be useful in future vaccine constructions.

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