INFECTION AND IMMUNITY, Aug. 1989, p. 2447–2451
0019-9567/89/082447-05$02.00/0

Vol. 57, No. 8

Elimination of Vaginal Colonization with *Escherichia coli* by Administration of Indigenous Flora

M. HERTHELIUS,[1,2]* S. L. GORBACH,[3] R. MÖLLBY,[2,4] C. E. NORD,[2,5] L. PETTERSSON,[1] AND J. WINBERG[1]

Department of Pediatrics, Karolinska Institute, Karolinska Hospital,[1] Department of Bacteriology, National Bacteriological Laboratory,[2] and Department of Bacteriology, Karolinska Institute,[4] Stockholm, and Department of Microbiology, Karolinska Institute, Huddinge Hospital, Huddinge,[5] Sweden, and Tufts University School of Medicine, Boston, Massachusetts[3]

Received 5 December 1988/Accepted 17 April 1989

A persistent vaginal colonization with a pyelonephritogenic strain of *Escherichia coli*, induced by administration of amoxicillin, was established in four adult cynomolgus monkeys. This colonization mimicked the one seen in urinary tract infection-prone human females. Attempts to eliminate the *E. coli* colonization and restore normal conditions were made. Either suspensions of lactobacilli or vaginal fluid from a healthy unmanipulated monkey was administered as repeated vaginal flushes for 5 to 9 days. A total elimination of vaginal *E. coli* was observed in two of six experiments with lactobacilli, and a decrease was observed in the other four. A better result was obtained with flushes of vaginal fluid, which eliminated the *E. coli* colonization in eight of eight experiments. In two of these, a single flush was sufficient to obtain a decolonization. The ability of fresh vaginal fluid to eliminate *E. coli* from the vagina could be transferred from one monkey to another. This study demonstrates the role of the normal flora in the defense against genital colonization with potentially pathogenic adhering *E. coli*. The possible clinical relevance of these findings must be further examined.

The periurethral area in women and children with recurrent urinary tract infections (UTI) is often colonized by gram-negative bacteria both during and between infections (2, 5, 8, 16). Also, the anaerobic flora is disturbed (3). We have earlier proposed that antibiotics given to UTI-prone females may facilitate the establishment of such an abnormal genital flora, presumably by disturbing the ecological balance (7).

In recent work, we have been able to establish a pathological vaginal *Escherichia coli* colonization in monkeys, either by simultaneous administration of amoxicillin and *E. coli* (7) or by giving amoxicillin to monkeys who harbored pyelonephritogenic *E. coli* in their fecal flora (M. Herthelius et al., manuscript in preparation). In these studies and in the present one we have used P-fimbriated pyelonephritogenic *E. coli* and cynomolgus monkeys, who carry the alpha-Gal-1-4-beta-Gal receptor moiety for P fimbriae (13). In this way, one important adhesion mechanism was standardized.

The aim of the present study was to further develop our hypothesis and to examine whether the pathological vaginal *E. coli* colonization could be eliminated by administration of indigenous bacteria normally present in the vaginal flora.

MATERIALS AND METHODS

Monkeys. Five healthy adult female cynomolgus monkeys (*Macaca fascicularis*), referred to as monkeys 1 to 5, were used throughout the study period. The animals were kept two per cage or, during certain periods, one per cage. Monkey 5, used only as a source of normal flora, was not manipulated in any way and was kept in a separate cage.

Bacterial strains. The pyelonephritogenic *E. coli* strain DS17 carrying type P and type 1 fimbriae (O6:K5:H−, P^+ and $T1^+$) was used in the colonization experiments. This strain adheres to monkey uroepithelial cells (17), was isolated from a case of pyelonephritis, and has been shown to easily colonize the gastrointestinal tract of newborns and adults (19). The strain is resistant to ampicillin, trimethoprim, sulfonamide, trimethoprim-sulfonamide, and cefalothin. For decolonization experiments, three different strains of lactobacilli were used: *Lactobacillus acidophilus* NCDO 1748, a commercially available strain used in milk products (kindly provided by Arla, Stockholm, Sweden); *L. fermentum* MH1, isolated from the vagina of a healthy adult monkey; and a lactobacillus strain known as GG (not identifiable at the species level) (15), isolated from the gastrointestinal tract of a human volunteer. The strains were identified by biochemical tests and gas-liquid chromatography.

Preparation of bacteria. For *E. coli*, the technique used has been described in a previous paper (7). Briefly, the strain was stored at −20°C and, for colonization experiments, was grown overnight at 37°C on a blood agar or Cled agar plate and then suspended in phosphate-buffered saline (PBS) to a concentration of 10^8 CFU/ml. Lactobacilli were stored at −20°C in MRS-broth (Oxoid Ltd., Hampshire, England). For decolonization experiments, lactobacilli were grown in MRS-broth for 1 to 3 days at 37°C. The bacteria were harvested by centrifugation, washed, and suspended in PBS to a concentration of 10^8 CFU/ml.

Preparation of vaginal fluid used for decolonization experiments. The fluid was obtained from the healthy, unmanipulated animal (monkey 5). Five cotton-tipped swabs were introduced one by one 2 cm into the vagina with the aid of a speculum. The swabs were gently rotated against the vaginal wall and were immediately stirred in 5 ml of PBS. The swabs were removed from the PBS after 30 s, and the fluid was given to the *E. coli*-colonized monkey within 5 min after collection. The aerobic bacterial content of the vaginal fluid from this monkey was examined repeatedly and was dominated by *Corynebacterium* species. Gram-negative bacteria were only occasionally encountered and usually did not exceed 10^2 CFU/ml; sometimes *Staphylococcus* species

* Corresponding author.

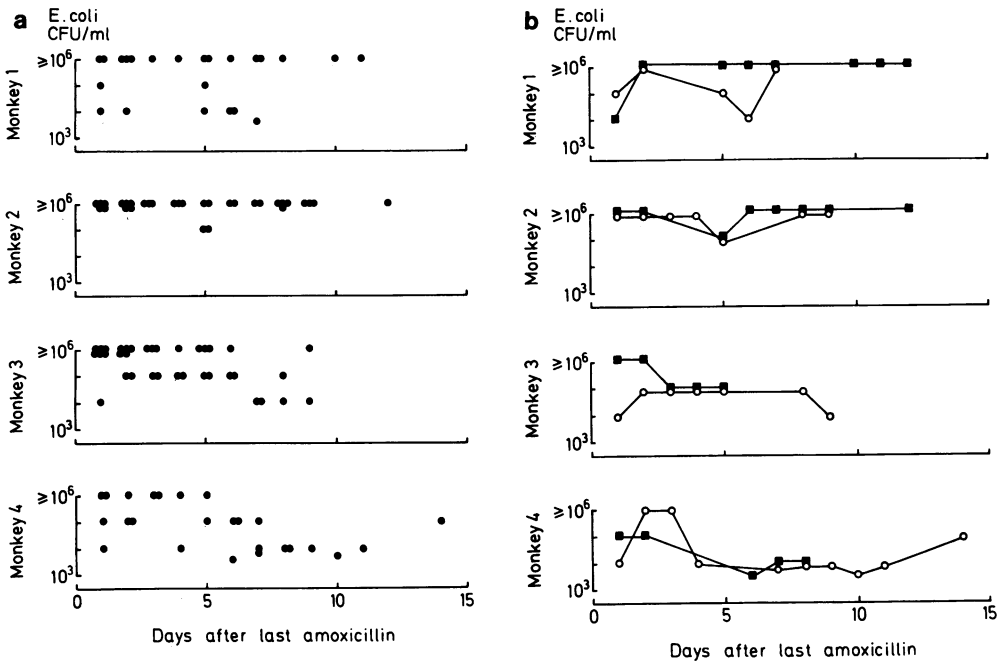

FIG. 1. (a) Persistence of vaginal *E. coli* DS17 following colonization supported by amoxicillin administration. The figure summarizes 20 experiments with four monkeys. (b) Persistence of vaginal *E. coli* DS17 at the beginning (■) and at the end (○) of the study period in four monkeys. The intervals between the experiments shown were 14, 13, 15, and 7 months, respectively, and the numbers of colonization attempts were 2, 2, 4, and 2, respectively. The results are interpreted as not showing any decreased receptivity to *E. coli* DS17.

were recovered. The anaerobic bacterial content was examined on two occasions, and *Eubacterium*, *Propionibacterium*, *Lactobacillus*, *Peptococcus*, and *Bacteroides* species were recovered on both occasions.

Suspensions used in control experiments. Suspensions used in control experiments were as follows: (i) PBS (pH 7.4); (ii) acetic acid diluted in NaCl to two concentrations, 2.1 g/liter (pH 2.98) and 6.0 g/liter (pH 2.76); (iii) lactic acid diluted in NaCl to two concentrations, 13.4 g/liter (pH 2.16) and 27.0 g/liter (pH 2.00); (iv) vaginal fluid (from monkey 5) heated at 80°C for 10 min; and (v) vaginal fluid (from monkey 5) filtered through a 0.45-μm filter (Sartorius, Göttingen, Federal Republic of Germany).

Sampling of vaginal bacteria. Sampling was performed daily (weekends excluded). The samples were cultured aerobically daily and anaerobically at certain intervals. The method has been described previously (7). In summary, a cotton swab was inserted deep into the vagina and rotated against the vaginal wall. The swab was stirred in 1 ml of PBS or prereduced anaerobic substrate, referred to below as vaginal fluid. A 0.1-ml sample was spread and grown on a Cled agar plate and occasionally on a Rogosa agar plate. *E. coli* was semiquantitated and identified by biochemical tests, antibiotic sensitivity, determination of O group, and occasionally biochemical fingerprinting (9). Colonies of different appearance growing on Rogosa agar were stained, and gram-positive rods were further identified by biochemical tests and gas-liquid chromatography.

Colonization of the vagina with *E. coli* DS17. The monkeys were flushed daily with 5 ml of an amoxicillin solution (Imacillin [12.5 mg/ml]; Astra, Södertälje Sweden) for a minimum of 5 days. During this treatment, the monkeys were flushed with 5 ml of an *E. coli* DS17 suspension (10^8 CFU/ml of PBS) as a single dose. The procedure has been described in more detail elsewhere (7). A persistent colonization was defined as $\geq 10^4$ CFU/ml of vaginal fluid for 6 subsequent days or more.

Decolonization experiments. When a persistent colonization with *E. coli* DS17 was established, attempts to decolonize were made in three sets of experiments with (i) suspensions of lactobacilli, (ii) vaginal fluid from a healthy monkey, or (iii) different control fluids. The monkeys were flushed once daily for 5 to 9 days with 5 ml of one of the suspensions as described above. In two instances, vaginal fluid was administered as a single flush.

RESULTS

Persistence of vaginal *E. coli* DS17. In 20 experiments in four monkeys, comprising 117 sampling occasions, the persistence of amoxicillin-induced *E. coli* DS17 colonization was monitored for 6 to 14 days (Fig. 1a). There were only small day-to-day variations, usually within 1 log. No trend in any direction was observed, and no spontaneous decolonization occurred. There were also no signs of a decreased receptivity with increasing number of colonization attempts (Fig. 1b).

Decolonization experiments. Attempts to decolonize were started only when a persistent vaginal colonization with *E. coli* DS17 had been established.

(i) Decolonization with lactobacilli. Six experiments were performed with three monkeys (Fig. 2). Three different lactobacillus species were used and administered as described above. In two instances, the *E. coli* DS17 was eliminated. In the other four experiments, a decrease exceeding 2 logs was observed. Typically, there was a latency period of a few days before an effect could be observed. There was no obvious increase in lactobacillus growth after lactobacillus flush.

(ii) Decolonization with vaginal fluid. Eight experiments were performed with four monkeys. Vaginal fluid obtained

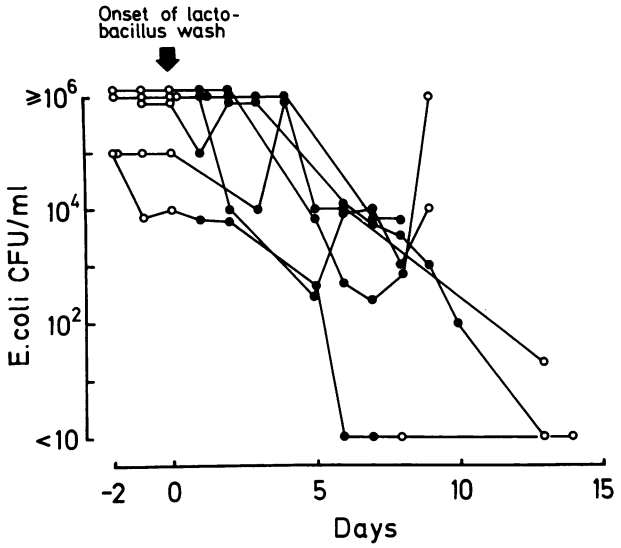

FIG. 2. Vaginal *E. coli* DS17 before (○), during (●), and after (○) vaginal flushing with various solutions of lactobacilli (10^8 CFU/ml) for 5 to 9 days. The figure summarizes six experiments with three monkeys. The findings were interpreted as showing a decolonizing effect of some degree.

from the healthy monkey 5 was given as a vaginal flush once daily for 5 to 6 days in six experiments (Fig. 3) and as a single flush in two experiments (Fig. 4). *E. coli* DS17 was eliminated in all instances, but a minor rebound was observed in one monkey. A latency period of at least 1 day was seen in all experiments.

(iii) Control experiments. Seven experiments were performed with four monkeys (Fig. 5). The mechanical effect of flushing did not induce any obvious change in vaginal *E. coli* DS17 colonization. Two major metabolic products of lactobacilli, acetic acid and lactic acid, were administered as repeated vaginal flushes in two different concentrations.

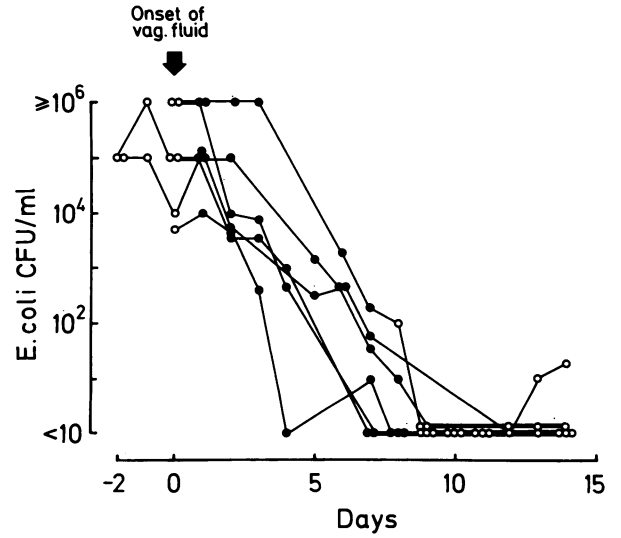

FIG. 3. Vaginal *E. coli* DS17 before (○), during (●), and after (○) vaginal flushing with vaginal (vag.) fluid from a healthy monkey for 5 to 6 days. The figure summarizes six experiments with four monkeys.

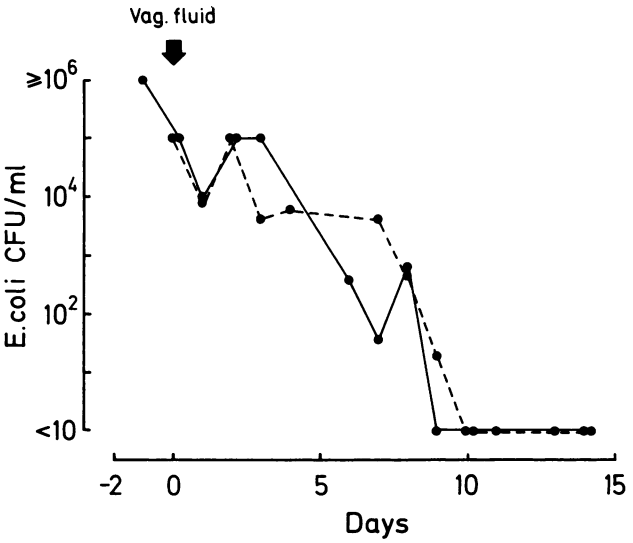

FIG. 4. Vaginal *E. coli* DS17 before and after a single vaginal flush with vaginal (vag.) fluid from a healthy monkey. Shown are results of two experiments with two monkeys. Each dot represents one sampling occasion.

Neither induced any decrease in vaginal *E. coli* DS17 colonization, although the pH of the acid solutions was very low. To examine whether the effect of vaginal fluid was due to microorganisms or to metabolites, both heated and filtered vaginal fluid from the healthy monkey 5 were tested. Neither had any effect on vaginal *E. coli* DS17.

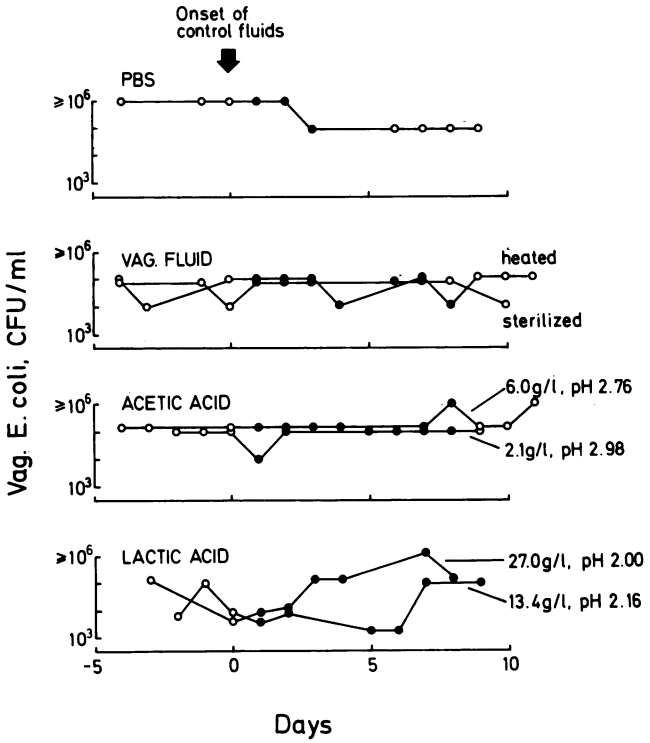

FIG. 5. Persistence of vaginal *E. coli* DS17 before (○), during (●), and after (○) flushing with different control fluids. Shown are results of seven experiments with four monkeys. No effect on *E. coli* DS17 colonization was observed in any experiment. Vaginal (VAG.) fluid was obtained from healthy monkey 5.

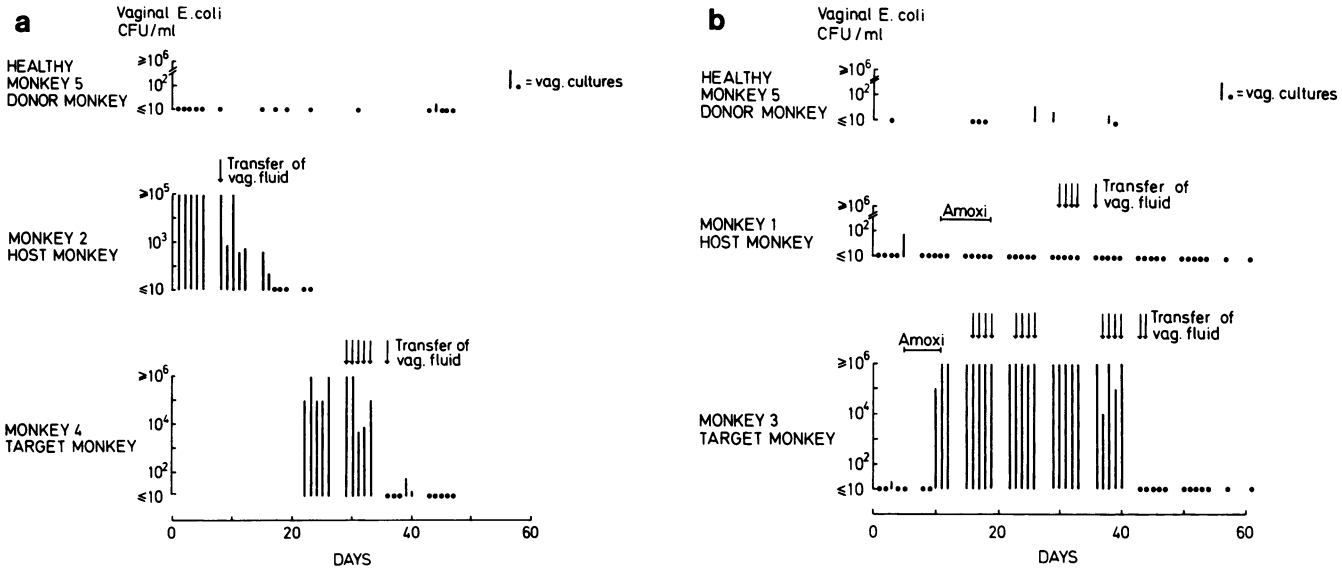

FIG. 6. Transfer of decolonization ability from one monkey to another. (See text for details.) Each vaginal (vag.) sampling occasion is represented by | (indicating growth of *E. coli* DS17) or ● (indicating no growth of *E. coli* DS17).

Transfer of decolonizing property from one monkey to another. Vaginal fluid from the healthy monkey 5 (donor monkey) could eliminate *E. coli* DS17 from the vaginas of four other monkeys, as shown above. This property could be transferred to another monkey. The following experiment (Fig. 6a) was performed to further examine whether the decolonizing property was due to live microorganisms. The decolonizing property was first transferred from the donor monkey to one of the earlier antibiotic-treated and *E. coli*-colonized monkeys as an intermediate host (host monkey). The host monkey was successfully decolonized within 2 weeks after a single flush of vaginal fluid from the donor monkey. The vaginal fluid from the host monkey was then flushed into a third monkey (target monkey), who was heavily colonized with *E. coli* DS17 for 8 weeks. In this case also, *E. coli* DS17 disappeared rapidly. The decolonizing property had thus been transferred from the donor monkey to the host monkey.

To show that the host monkey did not have a decolonizing property before receiving vaginal fluid from the donor monkey, the experiment was repeated with some additions (Fig. 6b). An uncolonized host monkey was treated with amoxicillin for 8 days. Her vaginal fluid was given to a colonized target monkey during and after this amoxicillin treatment. The vaginal fluid transferred during the amoxicillin treatment period contained 1.3, 0, 0, and 1.6 mg of amoxicillin per ml, respectively (days 16 to 19 in Fig. 6b). No effect on the *E. coli* colonization was observed. Thus, the host monkey did not possess the decolonizing property. After the host monkey received vaginal fluid from the donor monkey, the target monkey again received vaginal fluid from the host monkey. A decolonization occurred within 1 week. Thus, the host monkey had acquired the decolonizing property from the donor monkey.

DISCUSSION

Lactobacilli have long had a reputation as a health promoter. They produce acetic and lactic acids and other compounds capable of restricting the growth of members of the family *Enterobacteriacae* (1, 10, 15, 18). With regard to urogenital infections, it was claimed in the preantibiotic era that bladder infusion of lactobacilli could cure severe cystitis (6, 11). Furthermore, Reid et al. later showed in vitro that different species of lactobacilli are able to adhere to and competitively exclude attachment of gram-negative aerobic rods to uroepithelial cells (12).

The monkeys were colonized with *E. coli* DS17 and then flushed with lactobacilli or vaginal fluid. Lactobacilli induced a reduction in vaginal *E. coli* DS17 growth, but only in two instances induced a complete elimination. There was a latency period between administration of lactobacilli or vaginal fluid and effect on vaginal flora. This delay is reminiscent of the incubation time of infectious diseases. A similar finding has been reported in other in vivo studies of bacterial interference (20).

Concerning the mechanisms for the decolonizing effect of lactobacilli, neither lactic acid nor acetic acid infusion influenced the colonization, although the pH of the solutions was very low. Other modes of action of lactobacilli have been suggested, such as production of bacteriocins (1) or competitive adherence (12), but these were not examined in this study.

The observed effect of lactobacillus flushing on vaginal *E. coli* colonization was not correlated with any increase in vaginal lactobacillus growth. Inhibition and elimination of *E. coli* growth was thus not dependent on an extensive vaginal growth of lactobacilli that we could demonstrate with our methods of sampling and culturing. The pertinent question about the persistence of the inoculated lactobacillus strain remains unanswered, not least because of methodological difficulties. Upon testing single strains in two different systems (the API 50 CH system as well as the standard methods used at the Anaerobic Reference Laboratory at the National Bacteriological Laboratory in Stockholm, Sweden), we sometimes obtained different results as to species classification. Even in the same test system phenotypic variations were observed upon repeated testing. Also, variations in colony appearance could be observed. Similar observations have been made by others (C. Lönner, Ph.D. thesis, Lund University, Lund, Sweden, 1988). Few authors seem to have

commented upon such difficulties in studies of bacterial interference.

In the experiments with vaginal fluid directly transferred from a healthy monkey, this fluid successfully eliminated vaginal *E. coli* colonization in all experiments. A reduction was observed 2 to 6 days after onset of flushing, and elimination was completed with 12 days, even in those two instances in which vaginal fluid was flushed only once. The recent report of a case of relapsing *Clostridium difficile*-associated enterocolitis (14) might be a parallel to our findings. The diarrhea remained uninfluenced by six antibiotic treatments and by oral lactobacillus feeding. The patient was finally cured by rectal infusion of normal feces. The difference in effect between lactobacilli and total normal flora in this case as well as in our experiments might indicate that a stable ecological balance is a complex phenomenon which probably requires interaction between several microorganisms or factors. An alternative explanation to the better results obtained with the total normal flora may be a lowered colonizing ability of lactobacillus strains precultured in vitro.

The mechanism for the observed effect of both lactobacilli and vaginal fluid remains uncertain. The available results, however, indicate that the decolonizing ability was due to transfer of living microorganisms, for the following reasons. (i) Monkeys were easily colonized only after treatment with antibiotics (7). (ii) The decolonizing ability of vaginal fluid was eliminated by heating and by sterilizing. (iii) The ability to decolonize other monkeys could be transferred from one monkey to another (Fig. 6). Further support for the hypothesis is the fact that there was a latency period between onset of flushing and elimination. Possibly, the microorganisms need to adapt to the environment before being able to interfere with the pathogens.

Our monkey model shows that amoxicillin can facilitate an abnormal genital colonization with P-fimbriated uropathogenic *E. coli* if these bacteria are administered intravaginally or are present beforehand in the fecal flora (7; Herthelius et al., in preparation). The present study shows that normal conditions can be restored by local administration of lactobacilli or, better still, by fresh vaginal fluid from a healthy donor. The clinical implication is that if the genital flora is severely disturbed because of antibiotic treatment, whether in UTI-prone females or in others, it might be worthwhile to try to restore normal conditions by a bacterial interference approach.

The abnormal gram-negative genital colonization in the monkey model mimics the one seen in human females prone to UTI (2, 5, 8, 16). In UTI-prone girls there is also an abnormal anaerobic genital flora; whether it is induced by antibiotic treatment is unknown (3). How much further the parallel extends is unknown, since the basic defect underlying recurrent urinary tract infections is unknown. Whether it will become feasible in clinical practice to restore a normal ecological balance through bacterial interference therapy (e.g., by the use of single strains or a laboratory-prepared cocktail of strains) and so prevent ascending urinary infections remains unknown. Recently, vaginal lactobacillus inoculation was attempted with some success as a prophylactic method in patients with recurrent UTI (4). However, much further work remains to be done before this can become a prophylactic alternative. Among other things, the mechanisms through which the resident flora exclude an intruder must be better understood. The monkey model may be of some help in investigating this.

ACKNOWLEDGMENTS

We thank Karl-Göran Hedström for generously placing the resources of the Primate Research Center at our disposal, and Ann Lindmark, Ingela Persson, and Catharina Östberg for skillful technical assistance.

This study was supported by the Swedish Medical Research Council (grant no. 16X-0765), the Karolinska Institute, the Magnus Bergwall, the Crownprincess Lovisa's Children's Hospital, and the Ekhaga and the Samariten foundations.

LITERATURE CITED

1. **Barefoot, S. F., and T. R. Klaenhammer.** 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Microbiol. **45**:1808–1815.
2. **Bollgren, I., and J. Winberg.** 1976. The periurethral aerobic flora in girls highly susceptible to urinary tract infections. Acta Paediatr. Scand. **65**:81–87.
3. **Bollgren, I., and J. Winberg.** 1978. The periurethral anaerobic flora in girls highly susceptible to urinary infections. Acta Paediatr. Scand. **65**:74–80.
4. **Bruce, A. W., and G. Reid.** 1988. Intravaginal instillation of lactobacilli for prevention of recurrent urinary tract infections. Can. J. Microbiol. **34**:339–343.
5. **Brumfitt, W., R. A. Gargan, and J. M. T. Hamilton-Miller.** 1987. Periurethral enterobacterial carriage preceding urinary infection. Lancet **ii**:824–826.
6. **Caulk, J. R.** 1915. Incrusted cystitis. Am. J. Urol. Ven. Sex Dis. **11**:1–10.
7. **Herthelius, M., K.-G. Hedström, R. Möllby, C. E. Nord, L. Pettersson, and J. Winberg.** 1988. Pathogenesis of urinary tract infections. Amoxicillin induces genital Escherichia coli colonization. Infection **16**:263–266.
8. **Hofmann, K., W. Marget, and B. Belohradsky.** 1981. Veränderte bakterielle periurethralflora bei jungen mädchen mit chronisch rekurrierenden harnwegsinfektionen. Infection **5**:252–254.
9. **Kühn, I.** 1985. Biochemical fingerprinting of Escherichia coli: a simple method for epidemiological investigation. J. Microbiol. Methods **3**:159–170.
10. **Mårdh, P.-A., and L. V. Soltész.** 1983. In vitro interactions between lactobacilli and other microorganisms occuring in the vaginal flora. Scand. J. Infect. Dis. **40**(Suppl.):47–51.
11. **Newman, D.** 1915. The treatment of cystitis by intravesicular injections of lactic bacillus cultures. Lancet **ii**:330–332.
12. **Reid, G., R. L. Cook, and A. W. Bruce.** 1987. Examination of strains of lactobacilli for properties that may influence bacterial interference in the urinary tract. J. Urol. **138**:330–335.
13. **Robets, J. A., B. Kaack, G. Källenius, R. Möllby, J. Winberg, and S. B. Svenson.** 1984. Receptors for pyelonephritogenic Escherichia coli in primates. J. Urol. **131**:163–168.
14. **Schwan, A., S. Sjölin, U. Trottestam, and B. Aronsson.** 1984. Relapsing Clostridium difficile enterocolitis cured by rectal infusion of normal faeces. Scand. J. Infect. Dis. **16**:211–215.
15. **Silva, M., N. V. Jacobus, C. Deneke, and S. L. Gorbach.** 1987. Antimicrobial substance from a human lactobacillus strain. Antimicrob. Agents Chemother. **31**:1231–1233.
16. **Stamey, T. A., M. Timothy, M. Millar, and G. Mihara.** 1971. Recurrent urinary infections in adult women. The role of introital enterobacteria. Calif. Med. **115**:1–19.
17. **Svenson, S. B., G. Källenius, T. R. Korhonen, R. Möllby, J. A. Roberts, K. Tullus, and J. Winberg.** 1984. Initiation of clinical pyelonephritis—the role of P-fimbriae mediated bacterial adhesion. Contrib. Nephrol. **39**:252–272.
18. **Tramer, J.** 1966. Inhibitory effect of Lactobacillus acidophilus. Nature (London) **211**:204–205.
19. **Tullus, K., K. Hörlin, S. B. Svenson, and G. Källenius.** 1984. Epidemic outbreaks of acute pyelonephritis caused by nosocomial spread of P-fimbriated Escherichia coli in children. J. Infect. Dis. **150**:728–736.
20. **Van der Waij, D.** 1983. The colonization resistance of the digestive tract, p. 7–31. *In* R. N. Grüneberg (ed.), Antibiotic choice: the importance of colonization resistance. Research Studies Press, Letchworth, England.