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Inhibition of *Neisseria gonorrhoeae* by Aerobic and Facultatively Anaerobic Components of the Endocervical Flora: Evidence for a Protective Effect Against Infection

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The ability of aerobic and facultatively anaerobic endocervical flora to inhibit the growth of *Neisseria gonorrhoeae* in vitro was assayed. Factors influencing the occurrence of inhibitory components of the flora in vivo were evaluated. Endocervical swabs were obtained from 229 women at a local venereal disease clinic. Endocervical flora and *N. gonorrhoeae* were isolated and identified, and the ability of the flora to inhibit the growth of *N. gonorrhoeae* was determined by an agar overlay assay. Results revealed the most active inhibitors to be streptococci, staphylococci, and lactobacilli, in that order. Among only those women harboring inhibitory endocervical flora, inhibitory lactobacilli were recovered from fewer women infected with *N. gonorrhoeae* than uninfected women ($P < 0.05$). Among women having contact with an infected partner, those who subsequently developed gonorrhea were less likely to have inhibitory lactobacilli than those who did not become infected ($P < 0.05$). No other significant differences in the composition of the inhibitory flora were noted between infected and uninfected women. During the 2 weeks following menses, recovery of inhibitory lactobacilli on culture was highest, whereas recovery of *N. gonorrhoeae* was lowest. These observations suggest that the presence of certain lactobacilli may reduce risk of acquisition of *N. gonorrhoeae* following exposure to infected partners and that the potential protective effect may be greatest during the 2 weeks after menses.

Despite the development of effective chemotherapeutic agents and recent intense efforts to control gonorrhea, it has become one of the most prevalent of bacterial infections. Very little is known about factors which influence acquisition of *Neisseria gonorrhoeae* by females. Various factors, such as method of contraception (2, 8, 14) and host immunity (4, 6, 7, 21) have been hypothesized to play a role in susceptibility or resistance to gonorrhea. In addition, it is possible that fluctuations in the composition of the endocervical flora associated with the menstrual cycle may influence acquisition rates.

Many investigators have studied bacterial antagonisms occurring between the normal flora and potential human pathogens in vitro. Studies at other body sites have suggested that antagonistic components of the indigenous flora may play a role in resistance to gram-negative bacillary colonization of the pharynx (32), group A streptococcal colonization of the throat (9, 29), and staphylococcal infections in the newborn (31). To date, however, the possible role of the endocervical flora in resistance to gonococcal infections has not been extensively investigated. Therefore, the present study was designed to (i) determine the ability of aerobic and facultatively anaerobic components of the endocervical flora to antagonize the growth of *N. gonorrhoeae* in vitro and (ii) examine various factors which might influence the occurrence of these organisms in vivo.

MATERIALS AND METHODS

Study design. Endocervical swabs were taken from 229 women reporting to a local "free" clinic for gynecological reasons. Components of the endocervical flora were isolated in pure culture and tested for inhibitory activity against *N. gonorrhoeae* by an agar overlay technique. Cultures for *N. gonorrhoeae* were performed on Thayer-Martin medium. To evaluate host factors that might influence the presence or absence of inhibitory endocervical flora and to minimize possible bias, pertinent history was obtained by only one investigator (J.H.S.). For data analysis, the women were subgrouped according to three parameters: (i) presence or absence of *N. gonorrhoeae* in the endocervix, (ii) week during the menstrual cycle at which the endocervical culture was obtained, and (iii) use of oral contraceptives. Data relating to the menstrual cycle were not available for 22 women. No pregnant women were included in the study population. All tests of statistical significance were performed with chi-square analysis for a 2×2 contingency table with Yates correction factor.

Endocervical cultures. Endocervical cultures

were obtained with sterile calcium-alginate swabs (Calgiswab, Inolex Corp.). Swabs were immediately placed in 1 ml of Eugon broth (Difco) and vortexed for 3 min. Then 0.01 ml of the broth was placed on the surface of a dextrose starch agar (DSA) plate (Difco) supplemented with 5% sheep blood and streaked for isolation of colonies in the four-quadrant fashion. A second 0.01-ml portion of the broth was similarly inoculated onto Thayer-Martin medium (33) for the selective recovery of *N. gonorrhoeae*. Cultures were read after incubation for 24 and 48 h at 37°C in 10% CO_2 in air. Thus only aerobic and facultatively anaerobic components of the flora were evaluated.

Isolation and identification of organisms. Each morphologically distinct colony on the DSA plate was isolated in pure culture and identified. Staphylococci, streptococci, diphtheroids, yeasts, *Corynebacterium vaginalis*, and other less-commonly encountered organisms were identified on the basis of colonial morphology, Gram stain characteristics, catalase production, and standard biochemical tests, where applicable (5). Streptococci were separated further on several bases. Beta hemolytic streptococci sensitive to bacitracin (A disk, Difco) were presumptively identified as group A, and those giving a positive CAMP reaction (12) were presumptively identified as group B. Streptococci capable of growth in broth containing 6.5% NaCl were considered enterococci. Thayer-Martin plates were examined for the presence of oxidase (tetramethyl-*p*-phenylenediamine dihydrochloride)-positive, gram-negative diplococci. To confirm the organism as *N. gonorrhoeae*, sugar fermentation tests were performed in cysteine trypticase agar (Baltimore Biological Laboratories) to which 1% of the appropriate carbohydrate was added prior to sterilization.

Agar overlay technique. This technique was developed to determine the inhibitory activity of endocervical flora against *N. gonorrhoeae* and was a modification of the assay method employed by Crowe and associates (9). Each endocervical organism to be tested was inoculated onto a 1-in^2 (ca. 25.4-mm^2) area of a DSA plate so as to yield confluent growth, and the plate was incubated until macroscopic growth was obtained. The surface of the place was then overlayed with 7.5 ml of molten GC agar base (Baltimore Biological Laboratories) plus 1% Kellogg defined supplement (22) and allowed to solidify. A standardized suspension of *N. gonorrhoeae* sufficient to yield a confluent lawn of growth was placed on the surface of the plate. The plate was incubated overnight at 37°C in 10% CO_2 in air and then examined for areas of inhibition of the growth of *N. gonorrhoeae*. As shown in Fig. 1, the endocervical organism in the top left quadrant of the assay plate inhibited the growth of *N. gonorrhoeae*, whereas the organisms in the right top and bottom quadrants were non-inhibitory. Preliminary studies with this procedure indicated that the colonial type of the test strain of *N. gonorrhoeae* did not influence results. Components of the endocervical flora that were antagonistic to colonial types 3 and 4 were also antagonistic to colonial types 1 and 2. Endocervical organisms that did not inhibit the growth of colonial types 3 and 4 were also non-inhibitory for colonial types 1 and 2. Overlay assays on flora from infected women were performed in duplicate with the infecting strain of *N. gonorrhoeae* and one other strain of *N. gonorrhoeae*. All results of assays for inhibition were identical regardless of the target strain used. Therefore, results reported below are those obtained with a single clinical isolate of *N. gonorrhoeae*. A conscientious effort was made to select and test each morphologically distinct colony by this procedure. However, an organism present in very small numbers (one to two small colonies) may not have been recognized on the DSA plate and tested. For this reason, all results were subjected to statistical analysis (chi square with Yates correction) to ensure that the differences observed were significant and therefore unlikely to be due to this small, potentially inherent error in the assay.

RESULTS

The inhibitory activity of 477 endocervical organisms was determined by the agar overlay technique (Table 1). The highest percentage of inhibitory isolates occurred among the streptococci, followed by staphylococci and lactobacilli. Very few diphtheroids, yeasts, and miscellaneous other organisms showed inhibitory activity in the agar overlays. Among the streptococci tested, the majority (39 of 48) were nonhemolytic, non-enterococcal isolates. Of these, 25 (64%) were inhibitory. Eight isolates were enterococci, seven (88%) of which were inhibitory. The remaining inhibitory isolate was beta-hemolytic, not group A, B, or D. No group A or B isolates were recovered on the cultures.

Influence of host factors on the prevalence of *N. gonorrhoeae* in the study population. The highest percentage of women infected with *N. gonorrhoeae* was observed among those cultured during the week preceding and the week of menses (Table 2). Use of oral contraceptives had no significant influence on the prevalence of *N. gonorrhoeae*. The percentage of women infected with *N. gonorrhoeae* was the same among users (27 of 125) and nonusers (23 of 104) of oral contraceptives.

Factors influencing inhibitory flora in the endocervix. Recovery rates of inhibitory endocervical organisms within the study population were examined first. Inhibitory endocervical organisms were recovered from approximately one-half of both uninfected women (97 of 179) and women infected with *N. gonorrhoeae* (26 of 50). When the study population was grouped by the week of the menstrual cycle at time of culture, inhibitory endocervical organisms were recovered from 55% (24 of 44) of women cultured during week 1, 49% (32 of 65) of women cultured during week 2, 51% (27 of 53) of women cultured during week 3, and 67% (30 of 45) of women cultured during and after week 4. None of these differences were statistically significant. Inhibitory flora was recovered

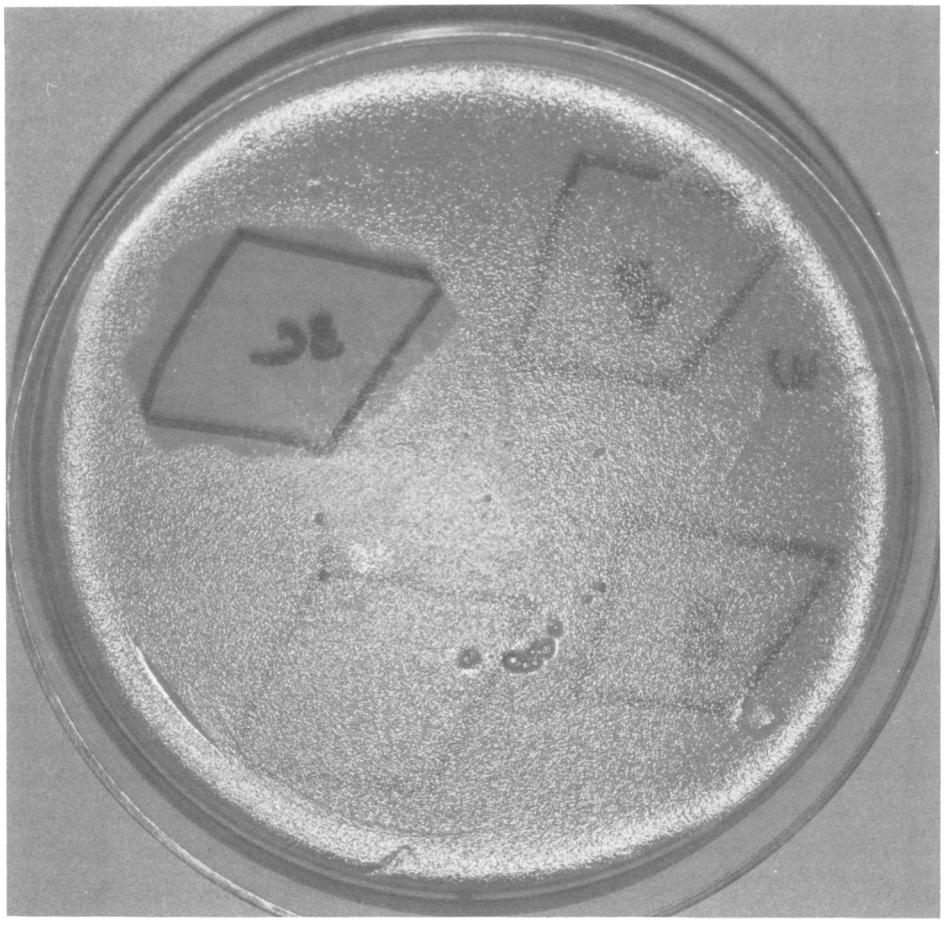

FIG. 1. *Agar overlay assay for the detection of the antagonism of N. gonorrhoeae by endocervical organisms. Organism in top left quadrant completely inhibited growth of N. gonorrhoeae on overlay layer. Organisms in right top and bottom quadrants are non-inhibitory. Bottom left quadrant was not inoculated with endocervical organism.*

from a similar percentage of cultures from users (50%) and nonusers (57%) of oral contraceptives.

The influence of these same factors on the qualitative composition (i.e., types of organisms) of the inhibitory flora was examined next. For these analyses, only results from the 123 women from whom inhibitory flora had been recovered were used. The composition of the inhibitory flora was found to vary significantly between women infected with *N. gonorrhoeae* and uninfected women (Table 3). Inhibitory lactobacilli were recovered from more uninfected women than infected women ($P < 0.05$). Although inhibitory streptococci and staphylococci were recovered from more infected women, these differences were not significant. Use of oral contraceptives had no significant influence on the composition of the inhibitory flora. Inhibitory lactobacilli were recovered from approximately 70% of both users (38 of 52) and nonusers (50 of 71); inhibitory staphylococci were recovered from 19% of users and 28% of nonusers; and inhibitory streptococci were recovered from 25% of both groups.

The phase of the menstrual cycle appeared to influence the composition of the inhibitory flora (Fig. 2, hashed bars). Significantly fewer women had lactobacilli as a component of their inhibitory flora during the week of menses than during the 2 weeks following menses ($P < 0.05$). Although the prevalence of inhibitory lactobacilli appeared to be high among women cultured during and after week 4, it was not significantly different from week 1. Conversely, the recovery of inhibitory streptococci decreased during the 2 weeks following menses and then began to increase thereafter. The difference between results obtained in weeks 1 and 3 was significant

($P < 0.05$). The recovery of inhibitory staphylococci, although highest during week 1, did not vary significantly during the 4 weeks. Due to the small number of women with inhibitory organisms of other genera, similar comparisons could not be made.

To determine if these week-dependent variations were restricted to the inhibitory components of the endocervical flora, the recovery of each organism—without regard to its inhibitory activity—was evaluated (Fig. 2, solid bars). Fluctuations in the recovery rates of streptococci and staphylococci were similar. Rates were highest during menses and the week preceding menses. Lactobacilli were recovered from the majority of women cultured during each week; however, the lowest recovery rate occurred during the week of menses. None of the differences were significant, indicating that in this subpopulation of 123 women, the likelihood of recovering each organism was similar regardless of the week during the menstrual cycle at which the culture was taken. Thus the week-dependent fluctuations in the inhibitory activity of several organisms could not have been due to differences in overall recovery rates.

TABLE 1. *Inhibitory activity of various endocervical organisms against N. gonorrhoeae in agar overlay assays*

Endocervical organism	No. of isolates tested	No. (%) inhibitory
Streptococci	48	33 (69)[a]
Staphylococci	72	32 (44)[b]
Lactobacilli	242	104 (43)[b]
C. vaginalis	52	6 (12)
Diphtheroids	43	1 (2)
Yeast	12	0 (0)
Miscellaneous	8	0 (0)

[a] Significantly higher than every other group; $P < 0.05$.

[b] Significantly higher than *C. vaginalis*, diphtheroids, yeast, or miscellaneous; $P < 0.05$.

TABLE 2. *Recovery of N. gonorrhoeae from women cultured during different weeks of the menstrual cycle*

Week cultured	No. of women cultured	No. (%) with positive cultures for *N. gonorrhoeae*
1 (menses)	44	10 (23)
2	65	10 (15)
3	53	10 (19)
≥4	45	16 (36)[a]

[a] Significantly higher than week 2 ($\chi^2 = 4.98$; $p < 0.05$).

For comparative purposes, the recovery of *N. gonorrhoeae* among women with inhibitory flora was calculated for each of the four weeks (Fig. 2). As was observed for the entire study population, recovery of *N. gonorrhoeae* was highest among women cultured during the week preceding or the week of menses. The difference between recovery during week 2 and during and after week 4 was significant ($P < 0.05$).

Influence of inhibitory flora on acquisition of *N. gonorrhoeae*. The influence of the presence of inhibitory flora on the acquisition of *N. gonorrhoeae* was examined from data obtained from 61 women with documented exposure to an infected partner. These included (i) the 50 infected women and (ii) 11 uninfected women who came to the clinic because they had been named as contacts by an infected partner or because they wished to report that contact had occurred. The percentage of women with inhibitory lactobacilli was significantly smaller among the 50 who became infected than among the 11 who did not (Table 4; $P < 0.05$). Although a higher percentage of infected women had inhibitory streptococci or staphylococci, these differences were not significant.

DISCUSSION

Several studies have indicated that antagonistic interactions between components of the normal flora and potential pathogens may play a role in host resistance to infection. To date, the role of endocervical flora in resistance to gonococcal infections has not been examined extensively. The present study was performed to determine the inhibitory activity of endocervical flora against *N. gonorrhoeae* in vitro and to evaluate factors which might influence occurrence of these organisms in vivo.

In this study, the inhibitory activity of the different components of the endocervical flora against *N. gonorrhoeae* in agar overlay assays was found to be variable. The largest percentage of strains of streptococci were inhibitory, fol-

TABLE 3. *Recovery of inhibitory endocervical organisms from uninfected women and women infected with N. gonorrhoeae*

Women	No. with inhibitory flora	No. (%) with inhibitory *Lactobacilli*	No. (%) with inhibitory *Streptococci*	No. (%) with inhibitory *Staphylococci*
Uninfected	97	74 (76)[a]	23 (24)	20 (21)
Infected	26	14 (54)	8 (31)	9 (35)

[a] Significantly higher than culture-positive women ($\chi^2 = 4.02$; $P < 0.05$).

lowed by staphylococci and lactobacilli. Other studies using a variety of techniques have demonstrated the in vitro antagonism of *N. gonorrhoeae* by *Candida albicans,* staphylococci, *Corynebacteria,* streptococci, other species of *Neisseria,* Enterobacteriaceae, and *Pseudomonas aeruginosa* (10, 17, 20, 24, 25, 27, 30, 34). However, very few of these studies provided direct evidence for a protective effect in vivo. Hipp and associates reported the antagonism of *N. gonorrhoeae* by *C. albicans* and speculated that this interaction may be responsible for falsely negative cultures for *N. gonorrhoeae* in vitro and may possibly provide protection from infection in vivo (17). Studies by Wallen and Gnarpe demonstrated an association between the presence of *C. albicans* and absence of *N. gonorrhoeae* in females (35). However, this association could not be demonstrated among females with known exposure to an infected partner. Other studies on the clinical significance of in vitro antagonisms of *N. gonorrhoeae* have been limited to studies in males (24, 34). From three males who did not contract gonorrhea following contact with infected females, urethral isolates of staphylococci from two and *N. meningitidis* from one were found to inhibit the growth of *N. gonorrhoeae* in vitro.

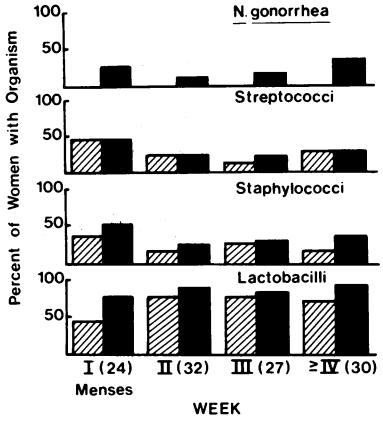

FIG. 2. *Weekly fluctuations in the endocervical flora among women harboring inhibitory flora. Hashed bars represent percentage of women with inhibitory strain of designated organism. Solid bars represent percentage of women with designated organism. Numbers in parentheses indicate number of women with inhibitory flora that were cultured during each week.*

The importance of simultaneous evaluation of in vitro antagonisms and potential clinical significance was apparent in this study. Results suggested that not all in vitro antagonisms may be indicative of in vivo resistance to infection. For example, although streptococci were found to be the most frequent inhibitors of *N. gonorrhoeae* in vitro, their presence in vivo was not associated with absence of infection. A similar lack of association between in vitro and in vivo results were observed with the staphylococci. However, from comparisons between women infected with *N. gonorrhoeae* and uninfected women, inhibitory lactobacilli were found to be more prevalent not only in all uninfected women studied but also in women who did not become infected following known exposure to infected consorts. These data suggest that certain lactobacilli may play an active role in resistance to gonococcal infection of the endocervix or that recovery of these organisms correlates with the presence of other, yet unidentified, host resistance factors. Since lactobacilli were more likely to be an active component of the inhibitory endocervical flora during the 2 weeks following menses, this protective effect may be maximal during this period. Conversely, during menses, this protection may be minimal due to the low prevalence of inhibitory lactobacilli. Support for this hypothesis was provided by the inverse relationship observed between the prevalence of *N. gonorrhoeae* and inhibitory lactobacilli when assayed for each of the weeks of the menstrual cycle. The flora of women during and after week 4 may reflect a transition phase. Although the prevalence of inhibitory lactobacilli was high in this group, it was on the decline from weeks 2 and 3, and the prevalence of *N. gonorrhoeae* was increasing.

The relationship between the phases of the menstrual cycle and recovery of *N. gonorrhoeae* remains controversial (11, 13, 19, 23). The higher prevalence of *N. gonorrhoeae* in women cul-

TABLE 4. *Influence of inhibitory endocervical flora on acquisition of infection in women following exposure to N. gonorrhoeae*

Women	Total no. of women	No. (%) with inhibitory *Lactobacilli*	No. (%) with inhibitory *Streptococci*	No. (%) with inhibitory *Staphylococci*
Acquiring infection	50	14 (28)	8 (16)	9 (18)
Not acquiring infection	11	8 (73)[a]	1 (9)	0

[a] Significantly higher ($\chi^2 = 5.97$; $P < 0.05$) than respective value for women acquiring infection.

tured during the week preceding and the week of menses reported here has also been observed by other investigators (19, 23). However, variations in procedures used to determine and/or define the phase of the menstrual cycle may in part explain the disagreement between the various published studies.

Since other investigators have suggested that gonorrhea may be more prevalent in women who use oral contraceptives (16), the effect of this variable was evaluated in this study and was found not to be significant. Other authors have failed to find an association between use of oral contraceptives and occurrence of gonorrhea (3, 15).

Due to the small number of infected women cultured during this study, many differences were significant only between the extremes observed, and several aspects concerning the role of the endocervical flora in resistance to gonorrhea could not be evaluated. Since many women infected with the gonococcus are asymptomatic, the influence of inhibitory flora, not only on the acquisition of the organism, but also on development of symptoms, needs to be evaluated. It is possible that inhibitory flora in some women may be insufficient to prevent infection but adequate to prevent rapid replication and invasion of tissues. Furthermore, certain women acquiring the pathogen during the weeks following menses may remain asymptomatic until the suppressive effect of the flora decreases with onset of menses. Such cycle-dependent symptomatic disease has been well documented among women chronically infected with *Candida* and *Trichomonas* (1, 26, 28). Evidence for a similar situation among some women harboring *N. gonorrhoeae* has been suggested by several observations. First, in a study of disseminated gonococcal infections by Holmes and associates, a significant number of women were found to have had asymptomatic infections which disseminated later during menstruation (18). Also, the reportedly higher incidence of gonorrhea during menses may not be due solely to higher rates of acquisition but may simply reflect a greater risk of developing symptoms for which medical attention would then be sought. Further studies on a large population of infected women, with and without symptoms, will be necessary to answer these and other questions concerning possible interactions between the endocervical flora and the gonococcus. Also, an attempt should be made to design an assay that would permit evaluation of the inhibitory activity, if any, of viable obligate anaerobes against gonococci.

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