Inhibition of Neisseria gonorrhoeae by a Factor Produced by Candida albicans

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When Candida albicans is present on Transgrow specimens, Neisseria gonorrhoeae is detected less frequently or else can be seen in Gram stains but cannot be readily cultured. When C. albicans and N. gonorrhoeae are grown together on Transgrow, the gonococcal cells die off much more readily than N. gonorrhoeae grown on Transgrow alone. By use of a cross-streaking technique on agar plates, it has been demonstrated that C. albicans produces a soluble substance inhibitory to N. gonorrhoeae, although not to other microorganisms tested. Preliminary results indicate that this inhibitory factor can be extracted by the use of tertiary butanol. Since approximately one-third of the Transgrow specimens with growth contains yeasts, of which C. albicans is by far the most frequent, this factor presents an important complication in the diagnosis of gonorrhoea in women.

Vaginal and cervical specimens taken for the detection of gonorrhoea frequently contain the yeast Candida albicans, which is found in the vagina either as a commensal or, under certain conditions, as the cause of vaginitis (9). Transgrow (6) is a culture medium and transport system used to maintain Neisseria gonorrhoeae until it reaches the laboratory. Our experience in the New York State gonorrhoea screening program has been that, despite its antibiotic content, Transgrow does not effectively inhibit the growth of C. albicans and other contaminating yeasts. It was noted that, when C. albicans is present, either N. gonorrhoeae is observed less frequently or else it is seen in the Gram stains but cannot be cultured readily from the original growth. We could find no explanation for this inhibition in the literature. There have been reports of C. albicans producing autoantibiotics (5), as well as some enzymes and toxins which may be important in the invasion of animal cells (2), but there is no reference to its producing any substance active in inhibiting bacteria.

We present here our findings of a soluble factor produced by C. albicans that inhibits the growth of the majority of N. gonorrhoeae isolates, and we discuss the clinical significance of this finding for the diagnosis of gonorrhoea.

MATERIALS AND METHODS

Culture and identification of organisms. Isolates of C. albicans and N. gonorrhoeae were cultured from Transgrow specimens sent to the Division of Laboratories and Research, New York State Department of Health, for diagnosis. The N. gonorrhoeae isolates were grown and maintained either on Pfizer solid medium (7) or on Difco GC medium base plus 1% Iso-Vitalex (BBL) at 37 °C in a CO2 incubator. For long-term storage, the cultures were put into GC broth (GC medium base without agar) containing 10% dimethyl sulfoxide and stored in liquid nitrogen (8). The growth in the Transgrow bottles was washed off into GC broth, and this suspension was used for subsequent isolation and identification of N. gonorrhoeae and yeasts. N. gonorrhoeae isolates were identified by standard methods: presumptive identification by oxidase reagent (N,N-dimethyl-p-phenylene diamine monohydrochloride) and Gram stain, confirmation by direct fluorescent staining, and sugar fermentations.

The C. albicans isolates were identified by chlamydospore production on corn meal agar containing 1% Tween 80 and by sugar fermentation patterns (1). The other yeasts were kindly identified by Helen Buckley of the Harvard School of Public Health, who also provided samples of serotypes A and B of C. albicans. After isolation, the yeast isolates were maintained on Sabouraud agar slants.

Flip-flop technique. To test for inhibition of N. gonorrhoeae by various yeasts we employed a flip-flop technique (Fig. 1) similar to that of Kekeisy and Piquet (3). The yeast was inoculated onto an agar plate in a streak about 2 cm wide and allowed to grow at 37 °C for 18 to 24 h. The agar was then loosened and inverted into the lid of the petri dish, and the organisms to be tested were streaked onto the unused side of the agar, perpendicular to the yeast streak. The plate was incubated for 18 to 24 h at 37 °C in a CO2 incubator. Inhibition of N. gonorrhoeae was
measured by its lack of growth over the _C. albicans_ streak; its degree was determined by the distance from the edge of the yeast streak to the gonococcal growth. This was more readily visualized if the gonococci were stained with oxidase reagent.

**Extraction of inhibitory factor.** The yeast was inoculated heavily on GC base agar plates and grown at 37°C for 24 h. The agar and yeast growth was then extracted using a solution of 85 g of tertiary butyl alcohol (t-BuOH) in 100 ml of distilled water, as suggested by D. S. Berns of the Division of Laboratories and Research, New York State Department of Health. Ten milliliters of this solution was used for each petri dish, including an uninoculated agar plate as a control, and the extraction was carried out for 18 h in a water bath at 37°C. The cells and agar were then spun down at 27,000 × g for 15 min. The t-BuOH supernatant was dialyzed for 2 days against frequent changes of distilled water and then lyophilized. Before testing, the lyophilized material was dissolved in 0.85% NaCl and filtered through a 0.45-µm membrane filter (Millipore Corp.) to remove any remaining yeast cells.

**RESULTS**

Of 62,261 samples of Transgrow received in 1972 in this laboratory by mail and hand carrier, 2,051 (3.3%) were positive for _N. gonorrhoeae_. Of the negatives, 44,499 (71%) had growth other than _N. gonorrhoeae_; about one-third of these negative samples contained yeast. We were impressed by the observation that a substantially smaller number than one-third of the specimens positive for _N. gonorrhoeae_ contained yeast. To investigate this observation we examined 3,422 random vaginal and/or cervical specimens for the presence of both gonococci and yeast. Initial examination of the data indicated no correlation between presence of yeast and either number of days in transit or lag period (between receipt of sample and growth of positive samples). All specimens included in this study were received during relatively cold winter weather. Male specimens were excluded because yeast contamination is encountered only in about 4% and a combination of gonococci and yeast is extremely rare. Of the specimens examined, 1,244 (36%) had no growth. The remaining 2,178 showing growth were further divided into those that contained yeast (32%) and those that did not (68%) (Table 1).

In the group having yeast, the gonorrhea positivity rate was 2.0%, as opposed to 0.6% in the group where no yeast was present. When yeast and gonococcal cells were found together, the gonococci frequently did not grow on subsequent subculture. The rate of confirmation when both were present was only 28.5%, as opposed to 76.5% when gonorrhea organisms were found either alone or in the presence of contaminating organisms other than yeast.

This apparent effect of yeast on _N. gonorrhoeae_ was investigated by the flip-flop technique (Fig. 1). Thirty samples of yeasts were isolated at random from specimens submitted on Transgrow. Five species were identified (Table 2), of which _C. albicans_ was by far the most frequently encountered, comprising 80% of

![Fig. 1. Flip-flop plate of _C. albicans_ and _N. gonorrhoeae_. White lines are two _C. albicans_ strains as visualized through the agar. 1, 3: sensitive _N. gonorrhoeae_ strains; 2, 4: resistant _N. gonorrhoeae_ strains. The plate was flooded with oxidase reagent prior to photography.](http://aem.asm.org/)

**Table 1. Correlation of the presence of yeast with the gonorrhea positivity rate of 2,178 Transgrow specimens showing growth**

| Yeast | Total samples | Positives | Presumptive | Confirmed |
|-------|---------------|-----------|-------------|-----------|
|       | No. | %  | No. | %  | No. | %  |
| Present | 710 | 32 | 14 | 2.0 | 4 | 28.5 |
| Absent  | 1,468 | 68 | 89 | 6.0 | 68 | 76.5 |

**Table 2. Identification and inhibitory effect of 30 random yeast isolates from specimens on Transgrow**

| Yeast                  | No. of specimens | Inhibition of _N. gonorrhoeae_ |
|-----------------------|-------------------|--------------------------------|
| _Candida albicans_    | 24                | +                              |
| _Candida tropicalis_  | 1                 | —                              |
| _Candida parapsilosis_| 1                 | —                              |
| _Torulopsis glabrata_ | 3                 | —                              |
| _Trichosporon cutaneum_| 1               | —                              |
this sample group, and the only one found to inhibit gonococcal growth.

We have so far tested a total of 51 C. albicans isolates by this flip-flop method, and all have shown some inhibitory activity against N. gonorrhoeae. The effectiveness of each C. albicans isolate was measured by the width of the zone of inhibition of gonococcal growth on each side of the yeast streak. The size of this zone was taken as being directly proportional to the ability of a given C. albicans to produce inhibitor. Of all C. albicans tested, one isolate, number 4244, that produced a wide zone of inhibition was chosen for further study and tested against a variety of N. gonorrhoeae isolates freshly obtained from Transgrow specimens. Of 30 such isolates tested, 19 (63%) were inhibited to varying degrees.

Table 3 illustrates this variation typical of C. albicans strains, as well as the varying susceptibility of eight typical N. gonorrhoeae isolates. In this test the inhibiting effect of C. albicans 4244 was compared to that of the two known serotypes of C. albicans, A and B. The gonococcal isolates represented in this table were selected to show that all degrees of reactivity are encountered, ranging from resistance to at least 1.5-cm inhibition on either side of the yeast streak.

It was possible that this variation in gonococcal susceptibility to C. albicans might be related to the different colonial types (4) present in the samples. Gonococci change on subculture from virulent (T1) to nonvirulent (T4) forms, which are associated with obvious colonial variations. To investigate this possibility, six isolates containing both T1 and T4 colonies were studied. A single colony of each type was streaked over C. albicans on a flip-flop plate. Both types of colonies from two isolates proved resistant to the yeast factor and grew over the yeast. With the other four isolates, both the T1 and T4 populations were sensitive. These results indicate no correlation of these colonial variations with susceptibility to inhibition by the candida factor.

Since the results of the flip-flop tests suggested that C. albicans produces a soluble, diffusible factor that may inhibit gonococcal isolates, the effect of a mixed culture of C. albicans 4244 and a sensitive isolate of N. gonorrhoeae was investigated. A known number of cells of both organisms were grown together for 1 and 2 days on petri plates containing Transgrow media. Each day all the cells were washed off paired plates. The gonococci were then counted on Transgrow containing 100 units of nystatin per ml to kill the yeast cells; the yeast were counted on blood agar plates incubated in the absence of CO₂. This experiment was carried out in duplicate in three separate trials using standard plate counting methods. In all cases, although the original number of cells varied, the results were essentially the same. The results of one typical trial are given in Table 4.

These experiments showed that, after 2 days of growing with yeast on Transgrow, practically all of the initial inoculum of N. gonorrhoeae cells were dead. In contrast, N. gonorrhoeae cells inoculated onto Transgrow alone increased substantially by day 1, and by day 2, even with natural death rates, they still contained more than the original inoculum. The experiment in Table 4 represents a starting ratio of yeast to

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Table 3. Three Candida albicans strains tested by flip-flop technique against selected Neisseria gonorrhoeae isolates

| N. gonorrhoeae isolates | C. albicans* | 4244 |
|-------------------------|-------------|------|
|                         | A           | B    |     |
| 18397                   | R           | R    | R   |
| 18398                   | S (0.5)     | S (1.0) | S (1.2) |
| 18595                   | R           | S    | S   |
| 18596                   | S (0.5)     | S (1.0) | S (1.0) |
| 18437                   | R           | S    | S   |
| 19795                   | S (0.5)     | S (1.5) | S (1.2) |
| 8853                    | S (0.8)     | S (1.2) | S (1.5) |
| 18083                   | R           | S (0) | S (0.2) |

* Abbreviations: S, gonococci sensitive to inhibition by C. albicans; S-, gonococci very weakly sensitive; R, gonococci grow over C. albicans. Numbers in parentheses indicate centimeters of inhibition from edge of C. albicans streak.

Table 4. Effect of Candida albicans on the growth of Neisseria gonorrhoeae in Transgrow system

| Assay time | Growth of N. gonorrhoeae only | Growth of N. gonorrhoeae and C. albicans together |
|------------|-------------------------------|-----------------------------------------------|
|            | N. gonorrhoeae* | C. albicans* |
| Start      | 4.0 x 10⁴       | 4.0 x 10⁴   | 4.6 x 10⁴   |
| 1 Day      | 1.0 x 10⁵       | 1.5 x 10⁵   | 2.0 x 10⁵   |
| 2 Day      | 2.0 x 10⁷       | <1.0 x 10⁷  | 2.0 x 10⁵   |

* Counted on Transgrow and 100 U of nystatin per ml.
* Counted on blood agar plates; incubated with no CO₂.
gonococcal of 10:1 with a resulting loss of
>99.9% of the _N. gonorrhoeae_ cells when _C. albicans_ is present.

In the second experiment the starting ratio of yeast to gonococcal cells was 3:1 and in the third experiment it was 1:5. The percentage of gonococcal cells lost in each experiment was >98% and 98.8%, respectively.

To determine whether other organisms are inhibited in the same manner as _N. gonorrhoeae_ by _C. albicans_, nine different microorganisms were tested by the flip-flop technique using _C. albicans_ 4244. Included were isolates of _Escherichia coli_, _Bacillus subtilis_, _Staphylococcus_, _Alcaligenes_, _Proteus_, _Pseudomonas_, _Saccharomyces_, _Candida albicans_ A and B, and _Neisseria meningitidis_. None of these organisms were affected.

To test whether this inhibition by _C. albicans_ is due to a substance that could be extracted from cells or simply to alterations of the environment necessary for gonococcal growth, _C. albicans_ 4244 cells were treated with t-BuOH, a relatively mild method for the extraction of proteins. The ability of this butanol extract to inhibit gonococcal growth was tested by placing a drop of extract (lyophilized material in NaCl, 10 mg/ml) onto GC base plates that had been streaked with _N. gonorrhoeae_. Four sensitive gonococcal isolates, numbers 18398, 18596, 19735, and 8833 (Table 3), were tested and were all completely inhibited. The butanol control extract of an uninoculated plate showed no inhibition. A plate inoculated with _N. meningitidis_ (resistant to all _C. albicans_ tested by the flip-flop technique) was not inhibited by the butanol extract of _C. albicans_ 4244. These preliminary results were encouraging, and purification of this material is now under way.

**DISCUSSION**

Yeast contamination of Transgrow medium has proved to be a troublesome problem. Yeasts are present in at least 32% of our specimens that show growth; of these, 80% can be expected to be _C. albicans_. The studies of clinical specimens received on Transgrow suggest that when _C. albicans_ is present, gonorrhea may be going undetected, since only 14 of 103 total samples (13%) presumed positive for _N. gonorrhoeae_ contained yeast (Table 1). The alternate possibility, that _N. gonorrhoeae_ kills yeast, appears untenable, since none of our experiments support this possibility. Rather, the laboratory experiments presented here indicate that _C. albicans_ produces a factor inhibitory to _N. gonorrhoeae_ and that if these two organisms are present together on Transgrow, the ability to detect and confirm gonorrhea infections may be seriously impaired.

Another important aspect of these studies is that this is apparently the first time that a substance inhibitory to bacteria has been isolated from _C. albicans_. Studies are now being conducted into the chemical and physical nature of this factor. It appears to be a water-soluble, nondialyzable substance that can be extracted from the cells and lyophilized. This retention by dialysis tubing would suggest a molecular size of at least 9,000. These initial findings indicate that the inhibition is not due simply to the depletion of media constituents, change of pH, or other environmental changes but rather is due to a substance produced and liberated by the yeast.

Further purification of this substance will also allow us to investigate the resistance that was shown by 11 of the 30 gonococcal strains tested against the growing _C. albicans_ cells. It is possible that this resistance was simply a function of the effective concentration of inhibitory substance that could be achieved by growth and diffusion by the flip-flop technique.

The fact that _C. albicans_ appears inhibitory only to _N. gonorrhoeae_ is especially significant, since these microorganisms are frequently encountered together in vaginal samples. This selective inhibitory action of _N. gonorrhoeae_ poses an interesting question with regard to women with vaginal _C. albicans_ infections. Do these women not contract gonorrhea as readily as other females, due to the inhibitory effect of this yeast in vivo? Or do they in fact have gonorrhea infections that are not readily detectable because so many cells are killed off in transport, leaving too few for a positive diagnosis to be made? At any rate, it seems apparent that the greater the number of _C. albicans_ cells present in the vagina, the less likely one is to be able to diagnose gonorrhea.

The important fact to be considered here is that _C. albicans_ does inhibit, in vitro and possibly in vivo, the growth of _N. gonorrhoeae_. This has serious clinical implications for the diagnosis of gonorrhea. If the inhibition occurs only in vitro, an appropriate transport system is required. A growth medium high in nystatin will inhibit the yeast and allow the gonococci to grow. We are presently studying this possibility. However, until further evidence is gained, we would suggest that failure to culture _N. gonorrhoeae_ from suspected cases of gonorrhea in women should be considered as a possible false negative if the woman is known to have vaginitis due to _C. albicans_.

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_C. Albicans Inhibition of N. Gonorrhoeae_
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