Asymptomatic Females: Detection of Antibody Activity to Gonococcal Pili Antigen by Radioimmunoassay

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A gonococcal pili antigen preparation was used to detect antibody activity in sera obtained from 322 culture-positive asymptomatic females and 150 negative controls. Pili were obtained from a culture of type 2 *Neisseria gonorrhoeae* (strain 2686) and labeled with ^{125}I for use in a double-antibody radioimmunoassay test system. Of the 322 sera obtained from culture-positive, asymptomatic females, 276 (85.7%) showed antibody activity greater than or equal to 1.8 $\mu\text{g}/\text{ml}$. Negative controls were obtained from three different groups of individuals, and 130 (86.7%) had undetectable antibody activity. Sera from asymptomatic, culture-positive females were absorbed with three different strains of *N. gonorrhoeae*, one of these strains being the organism used for pili antigen preparations. The absorbed sera were tested for antibody activity, and in each case the activity in the absorbed sera dropped to an undetectable level. When the same sera were absorbed with *N. meningitidis*, *N. catarrhalis*, *N. perflava*, *Escherichia coli*, *Herellea vaginicola*, *Mima polymorpha*, *Staphylococcus aureus*, and *Candida albicans*, little, if any, decline in the level of anti-pili antibody activity was observed.

A wide variety of antigen preparations and test systems have been proposed for use in a serological test for the detection of antibody to *Neisseria gonorrhoeae* (4, 5, 10, 11, 13, 16-18). To develop a serological test that would detect small amounts of antibody and decrease the number of false positives, the trend in research shifted toward the development of a more sensitive test system combined with the utilization of a homogeneous antigen preparation, specifically, the development of a radioimmunoassay using a gonococcal pili antigen preparation. Gonococcal pili were first isolated and purified by Buchanan et al. (2), who later reported the quantitative measurement of antibody activity to gonococcal pili in human sera by using a radioimmunoassay (3). Their technique was utilized in this study to measure the levels of gonococcal antibody activity in asymptomatic, gonococcal infected females.

MATERIALS AND METHODS

Preparation and purification of gonococcal ^{125}I -labeled pili. A culture of type 2 gonococci strain 2686 of Kellogg et al. (9) was used for the preparation of pili antigen. The growth of the organisms, preparation of pili antigen, and purification and external labeling of the pili with ^{125}I were done by the procedure of Buchanan et al. (3) with the following modifications.

At the height of log-phase growth, the liquid culture was sheared in a Waring blender at the high setting for 10 s. Organisms and large debris were removed from the sheared culture by centrifugation at $20,000 \times g$ for 30 min. The supernatant was centrifuged at $150,000 \times g$ for 12 to 16 h. The pellet was resuspended in 0.01 M tris(hydroxymethyl)aminomethane-0.01 M sodium azide (pH 7.5) at a concentration of 200- to 250-fold. The resuspended pellet was centrifuged at $50,000 \times g$ for 2 h, and the supernatant was mixed with an equal volume of 30% saturated $(\text{NH}_4)_2\text{SO}_4$ and left at 4°C overnight. The 15% saturated suspension was centrifuged at $30,000 \times g$ for 30 min. After centrifugation, the precipitate was redissolved in 0.01 M phosphate-buffered saline (pH 7.2) to a volume equal to $1/5$ of the volume of ammonium sulfate used in the preceding step. This suspension was then sonically treated at 30 W for 5 min, distributed into 0.1-ml amounts, and stored at -70°C until the time of labeling.

Approximately 50 μg of purified pili protein was labeled, using 50 μg of chloramine T and 1.0 mCi of ^{125}I . The reaction was stopped with the addition of 50 μg of sodium metabisulfite. Radioiodinated pili were separated from free iodine by passing the reaction mixture through a Sephadex G-50 column.

Physical and chemical analysis of pili preparation. Pili preparations were examined with an electron microscope, using 1% potassium phosphotungstate (pH 6.8) as the negative stain. This procedure was used to establish the presence of pili.

Protein purity was determined by electrophoresis

ing 75 μ g of pili protein in a 10% polyacrylamide disc gel with 0.1% sodium dodecyl sulfate. The gels were fixed in 25% isopropanol and 10% glacial acetic acid and stained with Coomassie blue. The gels were destained in 10% methanol and 7% glacial acetic acid. The pili preparations used in these studies produced one protein band.

Radiochemical purity of the iodinated pili preparations was determined in a 10% polyacrylamide disc gel with 0.1% sodium dodecyl sulfate. Each gel was loaded with approximately 1.5×10^6 cpm. After electrophoresis, the gels were sliced in 1-mm sections and counted in a gamma counter. The disc gel profiles showed one major peak of radioactivity, which contained approximately 80% of all of the radioactivity in the sample. It has not been determined whether the other approximately 20% bound radioactivity represents the presence of cell wall antigen.

Radioimmunoassay for the detection of anti-pili activity. The labeled pili, with a specific activity of approximately 10 μ Ci/ μ g, were diluted to a concentration of 0.1 μ g/ml in 1% bovine serum albumin in 0.01 M phosphate-buffered saline (pH 7.2). One μ Ci of 22 Na was added per ml of the antigen preparation and used as a volume marker. The radioimmunoassay procedure was performed as described by Buchanan et al. (3). A group of control sera with a known quantity of antibody activity was used to quantitate antibody activity in the test sera. The range of antibody activity for human sera was 1.8 to 28.0 μ g/ml.

Collection of human sera and cervical specimens. Specimens from asymptomatic females (collected over a period of 12 months extending from June 1974 through May 1975) were obtained from clinics providing services in the areas of planned parenthood, family planning, and maternity care. These clinics are under the supervision of the Department of Health and Mental Hygiene, State of Maryland. Cervical specimens for the isolation of *N. gonorrhoeae* were delivered to the laboratory on the same day as were the tubes of clotted blood from the same females.

Cervical specimens were plated at the clinic on selective Transgrow medium (Granite Diagnostic Inc., Burlington, N.C.), delivered to the Gonorrhea Screening Unit (Department of Health and Mental Hygiene), and incubated at $36 \pm 0.5^\circ\text{C}$ for 48 h in a CO_2 incubator. The sera were considered to be from culture-positive individuals based on the following protocol. Cervical specimens upon culturing for *N. gonorrhoeae* revealed: (i) typical colonies, (ii) a positive oxidase reaction with dimethyl-*p*-phenylenediamine, and (iii) typical gram-negative diplococcal morphology. Absence of the above criteria indicated a negative cervical specimen, and sera from these individuals were considered negative. The tubes of clotted blood from these patients were centrifuged at $600 \times g$ for 8 min, and the sera were placed in stoppered glass tubes and stored at -20°C until the time of testing.

Sera used as negative controls were obtained from three different groups of individuals: (i) 70 sera from a family planning clinic sponsored by a local health department, (ii) 20 sera from healthy laboratory

personnel, and (iii) 60 sera from children between the ages of 2 and 12. Groups (ii) and (iii) were not cultured for *N. gonorrhoeae*, but were presumed to be negative.

Preparation of microorganisms for absorption studies. Cultures of *Candida albicans* (no. 15064), *Staphylococcus aureus* (no. 25923), *N. meningitidis* (group C no. 48512), *Escherichia coli* (no. 05585), *Mima polymorpha* (no. 46085), and *Herellea vagincola* (no. 48080) were obtained from the Laboratories Administration, Maryland State Department of Health and Mental Hygiene. Cultures of *N. perflava* (no. 0799) and *N. catarrhalis* (no. 5) were provided by the laboratory of Michael Pelczar, University of Maryland, College Park, Md.

Cells of *S. aureus*, *E. coli*, *M. polymorpha*, and *H. vagincola* were grown in 5 ml of Trypticase soy broth at 37°C for 24 h, and 2 ml of the growth suspension was overlaid onto 100 ml of Trypticase soy agar in 32-ounce (ca. 0.946-liter) vaccine bottles. *N. meningitidis*, *N. catarrhalis*, and *N. perflava* were inoculated onto chocolate agar, plated, and incubated at 37°C for 24 h. The cells were suspended in 5 ml of sterile saline, and 2 ml of this suspension was overlaid onto 100 ml of chocolate agar in 32-ounce vaccine bottles. Cells of *C. albicans* were inoculated onto Sabouraud agar and incubated at 25°C for 24 h. The cells were suspended in 5 ml of sterile saline, and 2 ml of this suspension was overlaid onto 100 ml of Sabouraud agar in 32-ounce bottles. All vaccine bottles were incubated at 37°C for 24 h, except for those containing *C. albicans* which were incubated at room temperature. All cells were then washed off the agar and suspended in sterile saline (0.85%). Cells of *N. gonorrhoeae* were inoculated onto Thayer-Martin plates (Bio-Medical Products, Ft. Lauderdale, Fla.) and incubated at 37°C for 48 h in a CO_2 incubator. The cells were similarly washed off the agar and resuspended in sterile saline.

Each suspension was washed three times, resuspended in 40 ml of sterile saline containing 0.1% merthiolate, heat inactivated in a 65°C water bath for 1 h, and subsequently checked for purity by Gram stain and viability by plating on the growth medium. The suspensions were stored at 4°C until the time of sera absorption studies.

Absorption of human sera with microbial suspensions. Absorption of the sera was by the procedure of Gaines and Landy (7). Briefly, the microbial suspensions were centrifuged at $800 \times g$ for 30 min, washed twice with isotonic saline, and diluted so that the final centrifugation would yield approximately 0.1 ml of packed cells. The sera (0.6 ml) were mixed with the cells and incubated at 37°C for 1 h, followed by 4°C overnight. After centrifugation at $800 \times g$ for 30 min, the absorbed sera were removed and tested for anti-pili antibody activity in duplicate.

RESULTS

Anti-pili antibody activity of sera from culture-positive females. The sera obtained from culture-positive females were acquired from local clinics that provide planned parenthood, family planning, and maternity services. The prevalence of culture positives in these clinics

was 1.8, 4.5, and 5.1%, respectively.

A total of 276 sera obtained from culture-positive females had antibody activity greater than or equal to 1.8 $\mu\text{g/ml}$ (Fig. 1). This represents 85.7% of the 322 culture-positive sera tested. Forty-six of the sera obtained from culture-positive individuals had undetectable antibody activity (<1.8 $\mu\text{g/ml}$), representing 14.3% of the culture-positive sera tested.

A total of 130 of the sera used as negative controls had undetectable antibody activity (Fig. 1). This represents 86.7% of the negative control sera tested. If each of the three groups is evaluated separately, the percentage of those displaying antibody activity less than 1.8 $\mu\text{g/ml}$ was as follows: family planning clinic, 74.3%; laboratory personnel, 100%; and children, 96.7% (Table 1). For the two groups who would

TABLE 1. Anti-pili antibody activity of sera used as negative controls

Source of sera used as negative controls	Total no. of sera	No. of sera displaying <1.8 $\mu\text{g/ml}$	% of total
Family planning	70	52	74.3
Laboratory personnel	20	20	100.0
Children	60	58	96.7
Total	150	130	86.7

be most unlikely to have experienced gonorrhea, selected laboratory personnel and children, the percentage of those having antibody activity less than 1.8 $\mu\text{g/ml}$ was 97.5%.

The entire range of antibody activity (1.8 to 28.0 $\mu\text{g/ml}$) was divided into equal intervals (Table 2). The mean of the antibody concentration of the positive sera was 7.4 ± 5.2 $\mu\text{g/ml}$. A total of 60% of the sera from culture-positive females had antibody activity in the range of 1.8 to 8.3 $\mu\text{g/ml}$, with 3% of the culture-positive sera showing greater than 28.0 $\mu\text{g/ml}$. The majority (65%) of activity observed in the negative control individuals was in the lowest interval (1.8 to 5.0 $\mu\text{g/ml}$).

Absorption of human sera. The antibody activity in the sera of four individuals (DA, ER, BM, and LF) was determined in the radioimmunoassay with the labeled pili antigen preparation to be 20, 20, 3, and 18 $\mu\text{g/ml}$, respectively. Portions of these sera were then absorbed with three different strains of *N. gonorrhoeae*, including the homologous strain used for pili antigen preparation. The absorbed sera showed a marked decrease in the level of antibody activity as measured in the radioimmunoassay (Table 3). After absorption with each of the three gonococcal organisms, activity dropped to an undetectable level (<1.8 $\mu\text{g/ml}$). It should be noted that the absorption procedure resulted in very little, if any, dilution of the sera.

To test for nonspecific reactivity of the antibody detected in the radioimmunoassay and also to test the specificity of the absorption procedure, sera from two of the above individuals (DA and ER) were also absorbed with each of the following organisms: *N. catarrhalis*, *N. meningitidis*, *N. perflava*, *E. coli*, *S. aureus*, *M. polymorpha*, *H. vaginalis*, and *C. albicans*. The results of the antibody activity of these absorbed sera in the radioimmunoassay (Table 4) revealed little, if any, decline in the level of antibody activity to the pili antigen

FIG. 1. Anti-pili antibody activity of sera from culture-positive, asymptomatic females and individuals used as negative controls.

TABLE 2. Numbers of culture-positive and negative control sera displaying activity in each interval of increasing antibody concentrations

Antibody activity interval ($\mu\text{g/ml}$)	Culture-positive sera		Negative control sera	
	No. of sera	%	No. of sera	%
<1.8	46	14.3	130	86.6
1.8-5.0	120	37.3	13	8.6
5.1-8.3	71	22.0	1	0.6
8.4-11.6	29	9.0	1	0.6
11.7-14.9	19	5.9	2	1.5
15.0-18.2	14	4.3	0	0
18.3-21.5	8	2.5	0	0
21.6-24.8	1	0.4	1	0.6
24.8-28.0	5	1.6	0	0
>28.0	9	2.7	2	1.5
Total	322	100.0	150	100.0

TABLE 3. Anti-pili antibody activity of human sera untreated and absorbed with three different strains of *N. gonorrhoeae*

Sera absorbed with:	Anti-pili antibody activity ($\mu\text{g/ml}$) with sera designation:			
	DA	ER	BM	LF
Untreated	20	20	3	18
<i>N. gonorrhoeae</i> no. 2686	— ^a	—	—	—
<i>N. gonorrhoeae</i> no. 3531	—	—	—	—
<i>N. gonorrhoeae</i> no. 3564	—	—	—	—

^a — indicates <1.8 $\mu\text{g/ml}$.

preparation after absorption with any of the eight microorganisms.

DISCUSSION

Asymptomatic females were chosen for evaluation in this study as these individuals constitute one of the major factors in the uncontrollable spread of gonorrhea (14). The clinics that participated in this study provide planned parenthood, family planning, and maternity services. It is the policy of these clinics that if a female presents with symptoms of gonorrhea, she is immediately sent to the venereal disease clinic for diagnosis and treatment. None of the sera used in these studies was obtained from venereal disease clinics.

The 85.7% positivity of sera from culture-positive females is as high as that previously reported with other test systems. Buchanan et al. (3) reported a similar sensitivity, using a gonococcal pili antigen preparation. The sera used in their study were obtained from clinics in the New York City and Seattle areas, whereas the sera tested in this study were ob-

tained from clinics in the Baltimore area. Since the percent detection of culture positives obtained in these two studies was similar (86% in New York City and Seattle and 85.7% in Baltimore), it would appear that gonococcal pili preparations from one particular strain (no. 2686) are effective in detecting antibody activity in sera from patients residing in three different geographical regions. This is in contrast to the proposal of Novotny and Turner (12) that different strains of *N. gonorrhoeae* predominate in different regions and for a pili antigen preparation to be effective in a serological test it should be prepared from several different strains of *N. gonorrhoeae*. If a different strain or strains of *N. gonorrhoeae* predominate in different geographic areas, the pili on these strains appear to share antigenic determinants. In fact, in previous studies in which the investigators quantitated antigenic differences between pili from different gonococcal strains, a small degree of common antigenicity was found (1, 15). This may account for the ability of a pili preparation from a single strain to detect antibody activity.

The culture-positive, seronegative individuals represented 14.3% of the individuals tested in this study. The single criterion used to determine whether the serum sample was taken from a gonococcal infected individual was the isolation of *N. gonorrhoeae* from the cervix of the individual by the culture method. The incubation period for *N. gonorrhoeae* is approximately 3 to 5 days (6). A person who has been exposed to gonorrhea and who is cultured 3 to 5 days later may give a positive culture. However, due to the kinetics of the immune response system, a serum specimen taken on this visit may display antibody activity less than 1.8 $\mu\text{g/ml}$, and hence a culture-positive female would appear seronegative by the radioimmu-

TABLE 4. Anti-pili antibody activity of human sera untreated and absorbed with indicated microorganisms

Sera absorbed with:	Anti-pili antibody activity ($\mu\text{g/ml}$) with sera designation:	
	DA	ER
Untreated	20	20
<i>N. catarrhalis</i>	21	18
<i>N. meningitidis</i>	20	16
<i>N. perflava</i>	28	18
<i>E. coli</i>	20	18
<i>S. aureus</i>	21	20
<i>M. polymorpha</i>	23	18
<i>H. vaginalis</i>	20	20
<i>C. albicans</i>	18	18

noassay. If a second serum specimen, taken 14 to 21 days later, could have been obtained, it is possible that antibody activity would have been detected. Because it was not possible to obtain a convalescent serum specimen in this study, it is not known whether the culture-positive, seronegative females would have later converted to seropositive (3).

Of the 150 sera used as negative controls, 13.3% had antibody activity $>1.8 \mu\text{g/ml}$. A total of 70 of these sera were obtained from a family planning clinic, 18 of which displayed antibody activity greater than or equal to $1.8 \mu\text{g/ml}$. These individuals were not questioned regarding any previous history of gonorrhea or previous contact with a person who had been diagnosed as having gonorrhea. The validity of requesting information from a patient as to any previous history is questionable because gonorrhea involves moral and ethical issues and a patient often responds with unreliable information. Medical records obtained from the clinic showed no previous history of gonorrhea for the 18 culture-negative females; if a previous diagnosis of gonorrhea had been made in another clinic, this information was not available. This group of culture-negative seropositives accounted for 12% of the sera used as negative controls. The inability to isolate gonococci from all patients who are harboring the organism is presumed to play a role in culture-negative seropositive individuals.

Absorption of sera with three gonococcal isolates removed all antibody activity to an undetectable level. Thus, it appears that these three different clinical isolates share common pili antigenic determinants. The usefulness of this test as a diagnostic tool depends upon its ability to detect antibody reactive to pili sharing antigenic determinants.

It has long been known that many organisms undergo reversible mutation between the piliated and the nonpiliated phase (8). More recent evidence (12) has indicated that even though pili may not be observed radiating from the cell wall, the pili proteins may be located within the cell wall and under certain conditions give rise to the pili structure. It was observed that antisera prepared to a pili antigen when absorbed with a nonpiliated type 3 organism removed preabsorption antibody activity (12). As the three gonococcal strains in this study removed antibody from the sera, it was assumed they had pili antigens. The use of fresh clinical isolates in the absorption studies suggested the use of piliated organisms; however, they may not have been piliated.

The radioimmunoassay with a labeled pili

antigen may be a useful tool in the serodiagnosis of gonorrhea. Its use in horizontal studies of gonococcal infected individuals and individuals with multiple infections and studies with better defined negative controls will allow a greater understanding of the immune response during gonococcal infection and the value and limitations of this test in diagnosing asymptomatic gonorrhea.

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