

# Quantitative Determination of Antibody to Gonococcal Pili

## CHANGES IN ANTIBODY LEVELS WITH GONOCOCCAL INFECTION

THOMAS M. BUCHANAN, JOHN SWANSON, KING K. HOLMES, STEPHEN J. KRAUS, and  
EMIL C. GOTSCHLICH

*From The Rockefeller University, New York 10021, the Department of  
Pathology, University of Utah School of Medicine, Salt Lake City, Utah 84112,  
the Division of Infectious Diseases, University of Washington School of  
Medicine, Seattle, Washington 98105, and the Venereal Disease Research  
Section, Center for Disease Control, Atlanta, Georgia 30333*

**ABSTRACT** Gonococcal pili, pure by the criteria of electron microscopic examination and polyacrylamide gel electrophoresis in sodium dodecyl sulfate, have been prepared by repeated cycles of precipitation with 0.1 M  $\text{MgCl}_2$ , followed by dissolution in 0.01 M Tris pH 8, 0.01 M  $\text{NaN}_3$ . Using a fluorescein-conjugated antibody prepared to pili from a single strain, pili antigen(s) was found to be present in each of 18 strains of gonococci tested, and absent from strains of pilated meningococci, nonpathogenic *Neisseria sp.*, and *Escherichia coli*. Purified pili, labeled with  $^{125}\text{I}$  were used in an antigen binding assay to quantitatively measure antibody to pili in rabbit sera and in 561 human sera. The range of antibody activity for 133 persons unlikely to have experienced gonorrhea was 0.1–1.6  $\mu\text{g/ml}$  with a geometric mean of 0.5  $\mu\text{g/ml}$ . This geometric mean antibody activity was significantly lower than the geometric mean for asymptotically infected males (1.0  $\mu\text{g/ml}$ ,  $P < 0.002$ ), males with symptomatic gonococcal anterior urethritis (1.6  $\mu\text{g/ml}$ ,  $P < 0.001$ ), or asymptotically infected females (4.2  $\mu\text{g/ml}$ ,  $P < 0.001$ ). Antibody appeared in elevated levels ( $> 1.6 \mu\text{g/ml}$ ) 2–3 wk after infection and returned toward control levels 1 or more months after treatment. Antibody levels higher than 1.6  $\mu\text{g/ml}$  were found in 26 (50%) of 52 males with gonococcal anterior urethritis, in 10 (33%) of 30 males with asymptomatic urethral infection and in 50 (89%)

of 56 asymptotically infected females. In a high-risk group of 103 females for whom culture results and antibody to pili were compared, 58 (57%) had elevated antibody levels to pili and 86% of the infected females were within this seropositive group. The antigen binding assay may provide a means to detect asymptomatic gonococcal infection in women.

## INTRODUCTION

Gonococcal pili are filamentous structures present on the surface of colonial variants T1 and T2 of *Neisseria gonorrhoeae* (1, 2). Variants T1 and T2 have produced experimental infection in human volunteers, whereas colonial variants T3 and T4 devoid of pili have not (3, 4). This same correlation between colonial type and infectivity has been observed in the chick embryo animal model (5).

Gonococcal pili have been purified and studies of their immunochemistry have been performed (6). This report describes the quantitative measurement of antibodies to gonococcal pili in human and rabbit serum by means of a radioactive antigen binding assay (7), utilizing a double antibody technique (8–10).

## METHODS

**Purification of gonococcal pili.** Type 2 gonococci, strain 2868 of Kellogg et al. (3), were grown in 60 liter quantities in a Biogen fermenter (American Sterilizer Co., Erie, Pa.) using a defined meningococcal medium (11) supplemented with 0.5% dextrose and 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) (2). The culture was allowed to complete log phase growth (approximately  $5 \times 10^8$  or-

This work was presented in part to the general session of the American Society for Clinical Investigation, Atlantic City, N. J., 1 May 1972.

Received for publication 2 April 1973 and in revised form 9 July 1973.

ganisms per milliliter, pH approximately 5.8). Organisms were sedimented using a Sharples continuous-flow centrifuge (Sharples Div., Pennwalt Corp., Philadelphia, Pa.). Pili, which were sheared from the gonococci during centrifugation, were precipitated from the supernate by adjusting the pH to 4.0 with HCl. This precipitate was collected by Sharples centrifugation and was redissolved with stirring at 4°C in 0.01 M Tris pH 7.5 and 0.01 M NaN<sub>3</sub>. This suspension was centrifuged at 12,000 *g* for 10 min and the supernate was dialyzed overnight at 4°C against distilled H<sub>2</sub>O. Magnesium chloride was then added to the dialyzed supernate to a concentration of 0.1 M and placed at 4°C overnight, analogous to the method developed by Brinton for purifying type 1 pili of *E. coli* (41). The magnesium-treated preparation was centrifuged at 12,000 *g* for 10 min and the precipitate suspended in 100 cc of 0.01 M Tris pH 8, 0.01 M NaN<sub>3</sub> and dialyzed for 3 days at 4°C against 12 liters of the same buffer. The suspension was then centrifuged at 12,000 *g* for 10 min and the supernate was dialyzed overnight distilled water. The supernate was then adjusted to 0.1 M MgCl<sub>2</sub> and placed at 4°C overnight. After three cycles of precipitation with MgCl<sub>2</sub> and dissolution in 0.01 M Tris buffer pH 8 containing 0.01 M NaN<sub>3</sub>, the final product was ready for use in the antigen binding assay.

**Chemical methods.** Pili were electrophoresed in 10% polyacrylamide gels with 0.30% bisacrylamide, 0.1% sodium dodecyl sulfate to assess purity of the product and the molecular weight of the pili subunit (12-15). Approximately 1 µg of pili or molecular weight marker labeled with <sup>125</sup>I by the chloramine T method (16), was boiled for 1 min in 1% SDS, 1% 2-mercaptoethanol prior to applying to the gel. After completion of the electrophoresis, the gels were sliced in 1 mm segments and assessed for radioactivity.

Protein determinations on purified pili were performed by Folin-Ciocalteu method using rabbit Cohn fraction II as a standard (17, 18).

**Electron microscopy.** Gonococci of a particular colony type, nonpathogenic *Neisseria sp.*, meningococci, and *Escherichia coli* were visualized by negative staining of buffer suspensions of agar cultures. Purified pili preparations and whole organisms were examined utilizing either 1% potassium phosphotungstate (pH 6.8) or 2% aqueous uranyl acetate (pH 3.5) as negative stains (1). Carbon-coated, collodion-covered grids were used and examinations were made with an AEI EM801 microscope (AEI Scientific Apparatus, Inc., White Plains, N. Y.) operating at 90 kV. (3). Freeze-fracture, freeze-etch studies were done using a Balzers 360 M apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) (19).

**Culture methods for isolating gonococci.** Cultures were obtained by a platinum wire loop or sterile cotton swab from urethral, cervical, throat, or anal sites and streaked directly onto warm Thayer-Martin culture media (20). The culture plates were incubated at 36°C for 24 h in a candle extinction jar, and colonies were identified as gonococci by oxidase reaction, Gram's stain, and sugar fermentations (21).

**Preparation of rabbit antiserum to gonococcal pili.** A total of 50 µg in 2 ml volume per rabbit of purified pili in complete (0.1 mg/ml *Mycobacterium butyricum*) Freund's adjuvant was injected intramuscularly and into the footpads of several adult New Zealand Red rabbits and the same regimen was repeated after 1 mo. The gamma globulin fraction of antiserum obtained 10 days after the second immunization from the rabbit was conjugated with fluores-

cein isothiocyanate (22) at a fluorescein to antibody protein ratio of 2.5:1 and reacted with gonococci or pilated organisms of several bacterial species. Dilutions of IgG isolated by DEAE chromatography from such antisera were used to standardize the antigen binding assay.

**Antigen binding assay.** Purified gonococcal pili were stored at 4°C at pH 7 in 0.01 M Tris buffer containing 0.01 M NaN<sub>3</sub> until needed for the assay. After dialysis against water to remove the azide, 40 µg of pili were labeled using 50 µg chloramine T and 200 µCi of <sup>125</sup>I by a modification of the method of Hunter (16). The labeled pili with a specific activity of approximately 2,000 cpm/ng were diluted to a concentration of 0.5 µg/ml in 1% bovine serum albumin in phosphate-buffered saline pH 7.2. 1 µCi of <sup>22</sup>Na was added per milliliter of antigen and used as a volume marker (23). 20 µl of antigen was mixed with 10 µl of a 1:31 dilution of the heat-inactivated test serum, diluted with phosphate-buffered saline pH 7.2 containing 1% bovine serum albumin. After equilibration at 4°C overnight, antibodies in the test serum were precipitated by goat anti-human immunoglobulin or anti-rabbit immunoglobulin antiserum. In each instance, the amount of second antibody utilized was twice that needed to totally precipitate <sup>125</sup>I-labeled immunoglobulin preparations added in minute amounts to test sera under conditions identical to those used in the assay except for the omission of labeled pili. This amount of goat antiserum was chosen because it was found that the twofold excess did not significantly diminish the precipitation of human immunoglobulin, and because it insured that complete precipitation would occur in human sera with elevated immunoglobulin levels. After centrifugation at 10,000 rpm for 5 min, nearly all of the supernate was removed without disturbing the precipitate. This precipitate with a variable volume of supernate overlying it was counted in a Packard model 3022 two-channel gamma spectrometer with one channel set to count <sup>22</sup>Na and the other <sup>125</sup>I. The iodine count represented the total amount of antigen left in the tube, including that bound to antibody and precipitated and that remaining in the supernate. The sodium count indicated the volume of supernate left. With these data it was possible to calculate the percent of added antigen bound to antibody (23). All sera were tested in duplicate and if duplicate binding results varied from each other by more than 5%, the serum was retested.

Volumetric measurements were done with Eppendorf pipettes (Brinkmann Instruments, Inc., Westbury, N. Y.), and Hamilton microsyringes (Hamilton Co., Whittier, Calif.). The test was done in disposable Microfuge tubes which were centrifuged in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.). All sera were heat-inactivated at 56°C for 30 min prior to testing.

**Quantitation of precipitating antibody to gonococcal pili in IgG isolated from rabbit antiserum.** Quantitative micro-precipitin tests utilizing purified pili and IgG isolated by DEAE chromatography from rabbit antiserum were performed. The rabbit antiserum contained 15.6 mg/ml of IgG as determined by Mancini radial immunodiffusion (24), and the IgG isolated from it was free of other immunoglobulin classes or protein contamination when tested by immunoelectrophoresis against goat anti-rabbit whole serum, goat anti-rabbit immunoglobulin, and goat anti-rabbit IgG antisera. Pili labeled with <sup>125</sup>I and IgG labeled with <sup>131</sup>I were added in minute amounts to each precipitin reaction. The washed precipitates were counted in a two-channel gamma counter with one channel set to read <sup>131</sup>I and the other <sup>125</sup>I. By measuring the amount of <sup>131</sup>I and <sup>125</sup>I disintegrations in each precipitate and comparing it with the

TABLE I  
Variation in Percent Binding and Calculated Antibody  
Concentration with Repeat Testing of Three  
Specimens by Antigen Binding Assay\*

	Serum sample 1	Serum sample 2	Serum sample 3
Range			
% Binding	37.7-41.0	57.3-60.9	50.4-54.5
µg/ml Ab	0.21-0.29	1.67-2.45	0.80-1.24
Mean, µg/ml	0.25	2.12	1.02
Standard deviation	0.03	0.20	0.13
Standard error, µg/ml	0.008	0.058	0.038

\* All serum samples tested at 1:31 dilution; six separate dilutions and 12 tests performed on each sample.

number of counts added to each test, it was possible to calculate the percentage of total antigen and IgG precipitated at each point. The percentage of total antibody precipitated at a concentration of antibody which precipitated 80% of the antigen (P-80) was accepted as the percentage of the IgG preparation which represented specific antibody to pili (25). 16% of the IgG was in the precipitate at the P-80 and thus the amount of specific antibody to pili was calculated as 16% of the IgG preparation. Dilutions of this IgG preparation in rabbit Cohn fraction II absorbed with pilated gonococci were used to standardize all tests of human and rabbit sera tested by antigen binding assay. The binding activity of a human serum was expressed in terms of the amount of rabbit IgG precipitating antibody required to produce the same level of antigen binding.

When three samples were tested in duplicate, six different times, the standard error of repeat testing was less than 4% (Table I).

*Study populations.* A total of 561 human sera were tested with the antigen binding assay (Table II). Human sera were obtained from the following groups: (a) 133 persons unlikely to have experienced gonococcal infection; (b) 88 persons recovering from unrelated acute illnesses, meningococcal meningitis, or who were nasopharyngeal carriers of meningococci; (c) heterosexual men and women examined for gonorrhea in a venereal disease clinic (245 patients, 279 sera); (d) seven patients with acute gonococcal arthritis and eight females with asymptomatic gonorrhea who were examined serially for change in titer (69 sera, Table II). Of the 473 patients tested, 252 were male. Half (44 persons) of the patients tested for crossreactive antibody and 80 of the persons unlikely to have experienced gonorrhea were less than 14 yr of age (Table II).

Of the 133 persons unlikely to have experienced gonorrhea, cultures were obtained only from the 11 men who denied any sexual intercourse (Table II). Cultures of the anterior urethra of these men did not grow *N. gonorrhoeae* at the time when their serum specimens were obtained. The healthy laboratory personnel specifically denied any history of gonococcal infection. Neither the nuns nor the children were questioned regarding gonorrhea.

Of the 88 patients whose sera were tested for cross reactive antibody to gonococcal pili, 13 were recovering from nonspecific urethritis, viral respiratory syndromes, rheumatic fever, or an exacerbation of rheumatoid arthritis (Table II). The other 75 patients were selected because their sera had high levels of antibody to Group A or C meningococcal polysaccharide as determined by Farr assay (26), or because they had been infected with Group B meningococci for 2 or more weeks (Table II).

TABLE II A  
Serum Specimens Tested for Antibody to Gonococcal Pili

Patient group	No. patients	Age	Sex		No. sera
			M	F	
<i>yr</i>					
Unlikely to ever have had gc*					
Healthy children	80	6 mo.–13	41	39	80
Nuns	29	16–60	0	29	29
Adult male virgins	11	18–28	11	0	11
Healthy laboratory personnel	13	20–50	6	7	13
		<14    ≥14			
Total	133	80    53	58	75	133
Tested for crossreactive antibody					
Recovering from unrelated acute illness	13	10–30	7	6	13
Nasopharyngeal carriers of Gr.‡ A (16), B (5), or C (5) meningococci	26	1–55	12	14	26
Recovering from Gr. A (25), B (8), or C (16) meningococcal meningitis	49	3 mo.–67	30	19	49
		<14    ≥14			
Total	88	44    44	49	39	88

Numbers in parentheses indicate number of subjects.

\* gc, gonorrhea.

‡ Gr., group.

TABLE II B  
*Serum Specimens Tested for Antibody to Gonococcal Pili*

Patient group examined for gonorrhea	No. patients	Age	Sex		No. sera
			M	F	
		yr			
Girls confined in reformatory					
With asymptomatic gonorrhea*	27	11-15		27	59
Without gonorrhea	32	11-15		32	32
Females examined in VD clinic					
With asymptomatic gonorrhea	29	17-32		31	50
Without gonorrhea	15	17-25		13	28
Heterosexual males examined in VD clinic					
With symptomatic gonorrhea	52	15-45	52		52
With asymptomatic gonorrhea	30	16-45	30		30
Without gonorrhea	60	16-45	60		60
		<15 >15			
Total	245	60 185	142	103	311

\* Eight females with asymptomatic infection who were examined serially after treatment were drawn from the group confined in reformatory.

TABLE II C  
*Serum Specimens Tested for Antibody to Gonococcal Pili*

Patient group examined serially for change in antibody	No. patients	Age	Sex		No. sera
			M	F	
		yr			
Humans with gonococcal arthritis	7	19-42	3	4	29
Human females with asymptomatic gc*	8	11-15		8	40
Total	15		3	12	69

\* Eight females with asymptomatic infection who were examined serially after treatment were drawn from the group confined in reformatory. gc, gonorrhea.

Of the 245 heterosexual men and women who were examined for gonorrhea, those with positive cultures were comparable to those with negative cultures with respect to age, sex, race, and employment status (Table II). Two endocervical, one pharyngeal, and one anal canal culture were obtained from each of the 59 girls confined in a reformatory in Atlanta, Ga. at the time the serum specimen was obtained. The other 44 asymptomatic heterosexual women were examined in a venereal disease clinic in Seattle (Table II). They gave written informed consent to undergo weekly endocervical, anal canal, and pharyngeal culture examinations for gonococci for 4 wk, and to forego sexual intercourse, and to defer any antibiotic treatment until the end of the 3 wk period of followup. Sera were obtained at the first and final visit and patients were treated with the appropriate antibiotics after the study period if *N. gonorrhoeae* had been isolated on any culture. Of the asymptomatic women whose cultures were negative for gonococci, 27% had a previous history of gonorrhea. Of the 142 heterosexual men examined in venereal disease clinics in Seattle and New York, urethral exudate was obtained for culture if present, or if not a swab was inserted 4 cm into the anterior urethra and cultured (Table II). Those with symptomatic anterior urethritis had an exudate

and usually dysuria. Those without exudates or genitourinary symptoms but whose swab cultures grew gonococci were classified as having asymptomatic gonorrhea. Of the asymptomatic men with negative urethral cultures, 28% had a history of previous gonococcal infection.

The time-course of the antibody response to gonococcal pili was studied in the following groups: (a) seven patients with acute disseminated gonococcal infection and arthritis, and (b) eight of the asymptotically infected females confined to a reformatory who were serially bled and cultured following treatment (Table II).

## RESULTS

*Antigen preparation, assessment of purity and yield.* Pili are present on the surface of type 2 gonococci as demonstrated in freeze-etch, freeze-fracture preparations (Fig. 1a) or after negative staining (Fig. 1b). In the former method the pili appear to adhere to the surfaces of gonococci whereas in the negatively stained gonococci, pili radiate from their surfaces, as previously noted (19, 2).

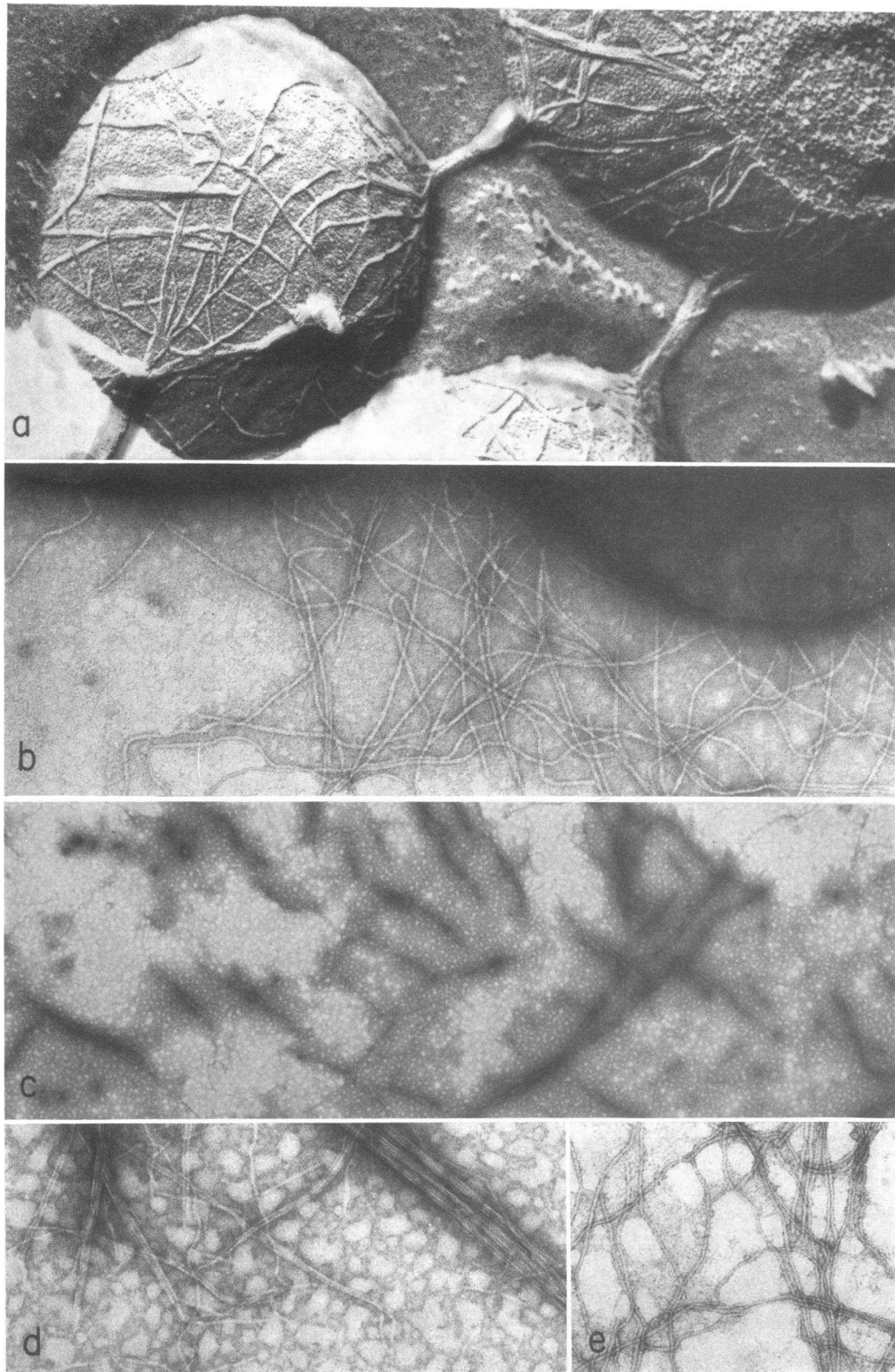


FIGURE 1 Gonococci and gonococcal pili. (a) Freeze-fracture, freeze-etch prepared gonococci with pili on surface ( $\times 80,000$ ). (b) Negatively stained gonococcus with pili radiating from

Specimens were examined by negative staining at each step during isolation and purification of pili. The major non-pilar component found in specimens from early preparative steps were membrane-limited vesicular structures varying in size from 150 to 500 Å in diameter. These represent fragments of disrupted membranes that comprised the protoplasmic and cell wall membranes of once-intact gonococci. As can be seen in the low magnification micrograph (Fig. 1c) these contaminating membrane vesicles are virtually absent in preparations obtained after the several purification steps outlined in Methods. The majority of pili are arranged as laterally adherent aggregates when negatively stained with uranyl acetate (Figs. 1c and 1d). The low pH of the negative stain (pH = 3.5) causes isoelectric precipitation of the pili (pI approximately 4.0). Phosphotungstate negative staining (pH 6.8), by contrast, results in pili which are dispersed on the specimen grid (Fig. 1e).

Figures 2a and 2b summarize the results obtained with  $^{125}\text{I}$ -labeled gonococcal pili or molecular weight markers when submitted to electrophoresis in sodium dodecyl sulfate polyacrylamide gels. Purified pili produce a single major peak (Fig. 2a) with a mobility corresponding to a subunit molecular weight of approximately 24,000 (Fig. 2b).

Approximately 15  $\mu\text{g}$  of purified pili were obtained per liter of liquid culture. Assuming  $5 \times 10^8$  gonococci per milliliter of liquid culture, and using Avogadro's number, and a molecular weight of 20 million for the pilus calculated from its dimensions, periodicity, and subunit size, approximately one pilus was isolated from each gonococcus. The average gonococcus grown in liquid culture has 10 pili according to our electron microscopic observations (2) and those of others (1), and thus we estimated a 10% yield for the purification procedure.

**Reactivity of fluorescein-conjugated anti-pili globulin with *Neisseria* sp. and *Escherichia coli*.** The fluorescein-conjugated rabbit anti-pili globulin preparation was reacted with pilated and non-pilated gonococci, and with pilated *N. meningitidis* (Groups B and C), and one strain each of pilated *N. catarrhalis*, *N. flavescens*, and *E. coli* to determine the specificity of the antibody for gonococcal pili. The surface of pilated gonococci fluoresced brightly producing a doughnut-shaped staining pattern. This staining was not observed with non-pilated gonococci or with pilated meningococci, *N. catarrhalis*, *N. flavescens*, or *E. coli*. These differences in staining between pilated gonococci and pilated non-gonococcal organisms were present over a 10-fold

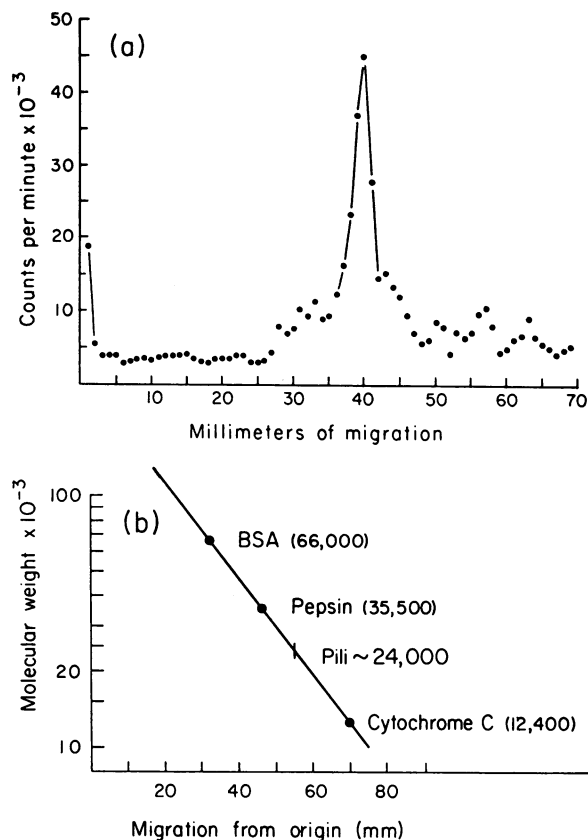


FIGURE 2 (a) SDS polyacrylamide gel of  $^{125}\text{I}$  purified pili. (b) Migration of gonococcal pili and molecular weight markers in SDS polyacrylamide gels.

range in antibody concentration (1:20–1:200 dilution of the fluorescein-conjugated globulin preparation). At a 1:10 dilution of the globulin preparation weak staining was observed with pilated meningococci and non-pilated gonococci, but not with other *Neisseriae* or *E. coli*. To seek antigenic variation among pili of different strains of gonococci, pilated organisms from 18 gonococcal strains were reacted with the fluorescein-conjugated globulin preparation. No significant differences in staining were observed; all strains fluoresced brightly with the anti-pili preparation produced against pili from a single strain.

**Antigen binding assay.** Gonococcal pili after labeling with  $^{125}\text{I}$  by the chloramine T method (16) were indistinguishable from unlabeled pili when examined by electron microscopy. Fig. 3 summarizes the pattern of antigen binding when 10 ng of labeled pili were reacted in each assay with varying amounts of rabbit IgG antibody to pili. 8 or more ng of rabbit IgG anti-

surface (uranyl acetate,  $\times 70,500$ ). (c) Purified pili, low power ( $\times 12,500$ ) with uranyl acetate stain. (d) High power ( $\times 86,000$ ) of bundles of pili from 1c, uranyl acetate stain. (e) High power ( $\times 90,000$ ) of purified pili, phosphotungstate stain.

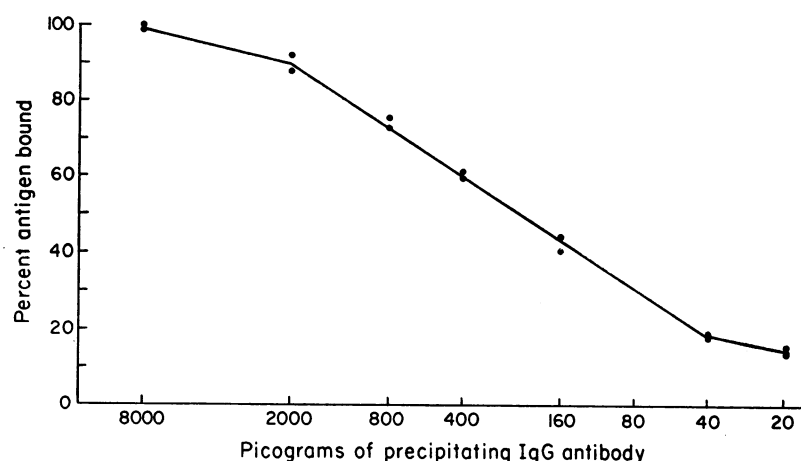


FIGURE 3 Percent antigen bound by rabbit precipitating IgG antibody to pili.

body produced 100% binding of the labeled antigen. Between approximately 20 and 90% antigen binding, there was a linear relationship between the percent antigen bound and the logarithm of the rabbit antibody concentration (Fig. 3). No antigen binding occurred using human IgG or IgA myeloma protein or human macroglobulin and precipitating with goat anti-human immunoglobulin antiserum.

*Serologic response in patient groups and controls.* Fig. 4 summarizes the antibody response to pili of 138 patients with gonococcal infection and 133 persons unlikely to have experienced gonorrhea (Table II). The geometric mean antibody activity to pili of the latter group was 0.5  $\mu\text{g/ml}$ . The 53 persons older than 13 yr of age within this group had a geometric mean antibody level of 0.6  $\mu\text{g/ml}$ , and the distribution of their

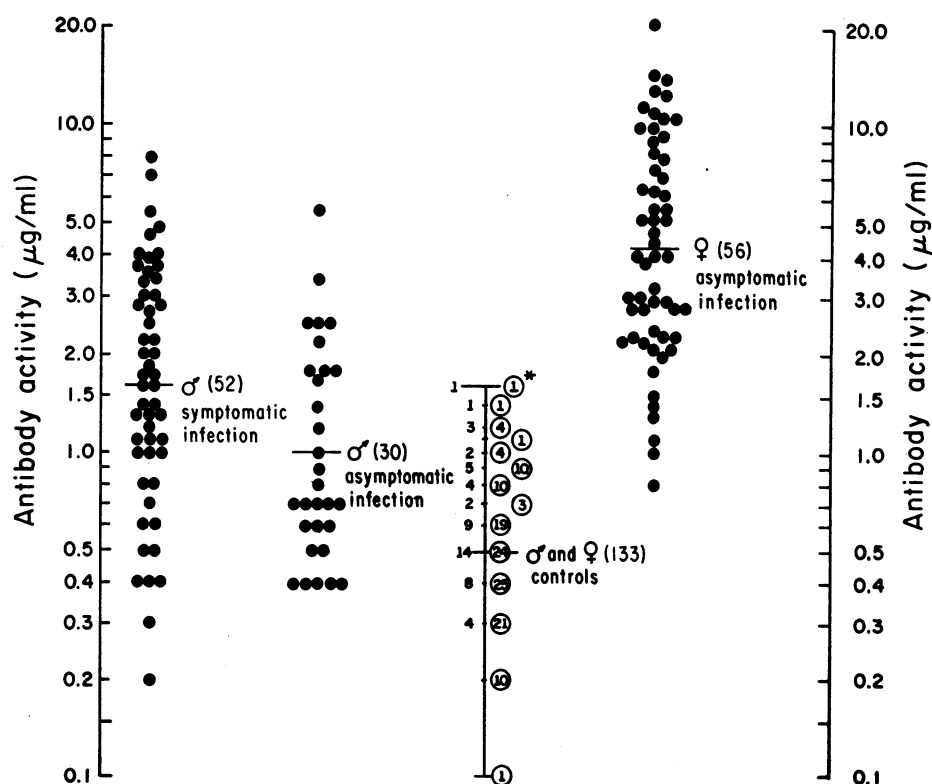


FIGURE 4 Human antibody to pili. \*For the 133 controls unlikely to have experienced gonorrhea, the circled numbers represent the total group (geometric mean 0.5  $\mu\text{g/ml}$ ) and the uncircled numbers the adults within that group (geometric mean 0.6  $\mu\text{g/ml}$ ).

levels is indicated to the left of the control line in Fig. 4. The range of antibody activity for the entire group of 133 persons unlikely to have experienced gonorrhea was 0.1–1.6  $\mu\text{g/ml}$  (Fig. 4). The geometric mean antibody activity for the 88 sera tested for crossreactivity was 0.66  $\mu\text{g/ml}$ . Three of the 88 persons whose sera were tested for crossreactivity had more than 1.6  $\mu\text{g/ml}$  antibody activity to pili. Each of these persons was more than 15 yr old (Table II).

The geometric mean antibody activity in serum from 52 males with acute symptomatic gonorrhea was 1.6  $\mu\text{g/ml}$ , a level which was significantly higher than the level for the adults within the group of 133 persons unlikely to have experienced gonorrhea ( $P < 0.001$ , Fig. 4). Also 26 (50%) of the 52 males with gonococcal urethritis had more antibody to pili than any of the controls. The geometric mean antibody for the 30 asymptotically infected males was 1.0  $\mu\text{g/ml}$ . This level was significantly higher than the mean for the adults within the control group ( $P < 0.002$ ), and 10 of the 30 had more than 1.6  $\mu\text{g/ml}$  antibody activity (Fig. 4). Asymptotically infected females had the highest antibody levels, with a geometric mean antibody activity to pili of 4.2  $\mu\text{g/ml}$  for the group. One serum had 20.6  $\mu\text{g/ml}$  antibody activity and 50 of the 56 (89%) had more antibody to pili than any of the controls ( $> 1.6 \mu\text{g/ml}$ , Fig. 4).

Among sera from the group of 133 persons unlikely to have experienced gonorrhea, antibody levels to pili increased with age for a given population. The geometric mean antibody level of sera from 26 healthy children 6 mo to 2 yr of age was 0.35  $\mu\text{g/ml}$ , as compared with a level of 0.56  $\mu\text{g/ml}$  for the 23 3–5 yr olds from the same group ( $P < 0.002$ ). Similarly, for a group of 12 Rockefeller University clinic patients from whom sera had been collected over a period of

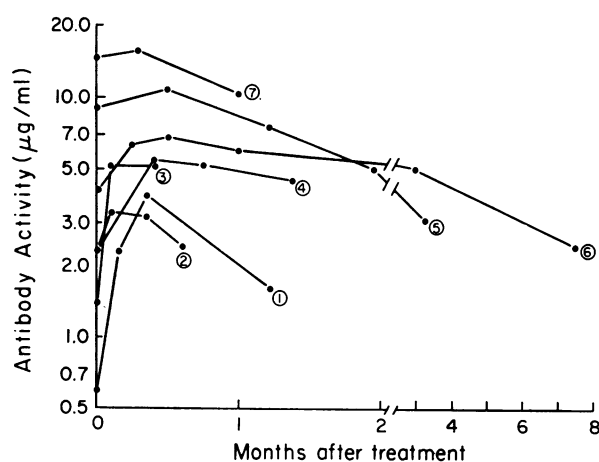


FIGURE 5 Changes in antibody after treatment for systemic gonococcal infection.

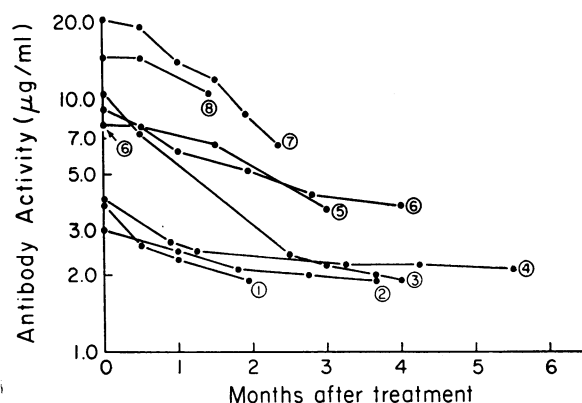


FIGURE 6 Decline in antibody after treatment for asymptomatic gonococcal infection.

years, the geometric mean antibody level to pili rose from 0.3  $\mu\text{g/ml}$  at ages 5–8 to 0.5  $\mu\text{g/ml}$  for the same persons at 9–12 yr of age.

Each of the seven patients with systemic gonococcal infection showed an early rise in antibody during the first 2 wk of disease (Fig. 5). Two of the seven (patients nos. 1 and 3) had initial antibody levels which were comparable to persons who had not experienced gonorrhea (Fig. 5). Patient no. 1 had skin lesions, arthritis, a positive throat culture, and serum antibody to pili of 0.6  $\mu\text{g/ml}$  8 days after well documented exposure to gonorrhea. 9 days later she had an antibody activity of 3.8  $\mu\text{g/ml}$  which after 1 mo declined to levels within the control range (1.6  $\mu\text{g/ml}$ , Fig. 5).

Cervical, pharyngeal, and anal canal cultures for *N. gonorrhoeae* remained negative in each of the eight asymptotically infected females after treatment, and antibody declined for most patients with a half-life of approximately 45–60 days (Fig. 6). Patients 1, 2, and 4 whose initial serum contained 3.0–4.0  $\mu\text{g/ml}$  antibody activity declined to plateau levels of antibody between 2.0 and 2.3  $\mu\text{g/ml}$  within 2–3 mo after treatment. Antibody levels for the other patients which were initially 8.0–20.6  $\mu\text{g/ml}$  declined to plateau levels within 4 mo (patient no. 3) or did not reach plateau levels (patients nos. 5–8) after observation periods of 1½–4 mo (Fig. 6).

Fig. 7 plots the antibody activity to pili of serum from 142 heterosexual men who were cultured for gonococci in venereal disease clinics. 82 had urethral infection with *N. gonorrhoeae* and 60 had negative rectal, urethral, and oropharyngeal cultures for gonococci. Men with symptoms and positive cultures had a significantly higher geometric mean antibody activity (1.6  $\mu\text{g/ml}$ ) than those with negative cultures ( $P < 0.05$ , Fig. 7). Of the eight men with negative cultures and antibody levels greater than 1.6  $\mu\text{g/ml}$ , six (75%) admitted a previous history of gonorrhea (Fig. 7). The



two men who denied a history of gonorrhea had antibody levels of 2.0  $\mu\text{g/ml}$  and 2.2  $\mu\text{g/ml}$ . 11 (21%) of the 52 men with negative cultures and antibody levels of 1.6  $\mu\text{g/ml}$  or lower had a previous history of gonorrhea. The geometric mean antibody level for the asymptotically infected men (1.0  $\mu\text{g/ml}$ ) was not significantly different from the level for symptomatically infected or for culture negative men (Fig. 7), but was significantly lower than the level for asymptotically infected women (Fig. 4,  $P < 0.01$ ).

Fig. 8 summarizes the culture results and serum antibody to pili for 59 girls confined in a reform school, and 45 women examined in a venereal disease clinic. The girls in the reform school or women in the venereal disease clinic whose cultures grew gonococci were comparable in age, sex, race, and employment status to those in their respective group with negative cultures (Table II). Of the 103 females, those with negative cultures at all sites had a lower geometric mean antibody activity (1.1  $\mu\text{g/ml}$ ) than those with positive cultures (4.2  $\mu\text{g/ml}$ ,  $P < 0.001$ ). 50 (86%) of the 58 females with more than 1.6  $\mu\text{g/ml}$  antibody activity had cultures which grew gonococci, as compared to eight (18%) of 45 for those with 1.6  $\mu\text{g/ml}$  or less. Six of the eight asymptotically infected females

whose antibody activity was less than 1.6  $\mu\text{g/ml}$  were women examined in a venereal disease clinic and two were girls in a reform school. Followup sera were available from seven of these eight females, and three of the seven developed elevated antibody levels to pili, suggesting that these three patients had contracted infection too recently to develop antibodies at the time they were first tested. The eighth asymptotically infected female with antibody activity less than 1.7  $\mu\text{g/ml}$  had been cultured 10 days previously. Her cultures at that time did not grow gonococci, suggesting that she had acquired her infection within 10 days of when her serum plotted in Fig. 8 was obtained. Each of the eight females with negative cultures for gonococci and elevated antibody levels to pili ( $> 1.6 \mu\text{g/ml}$ ) were from the group of girls confined in a reform school (Table II, Fig. 8). These girls were cultured only once, and of the five culture-negative girls whose serum contained 2.1  $\mu\text{g/ml}$  or more antibody to pili, one gave a history of previous treatment for gonorrhea and three had another venereal infection at the time of culturing. For the total group of 103 females, if one had obtained cultures only from the 56% of the group with elevated antibody activity to pili ( $> 1.6 \mu\text{g/ml}$ ), one would have identified 86% of the infected persons (Fig. 8).

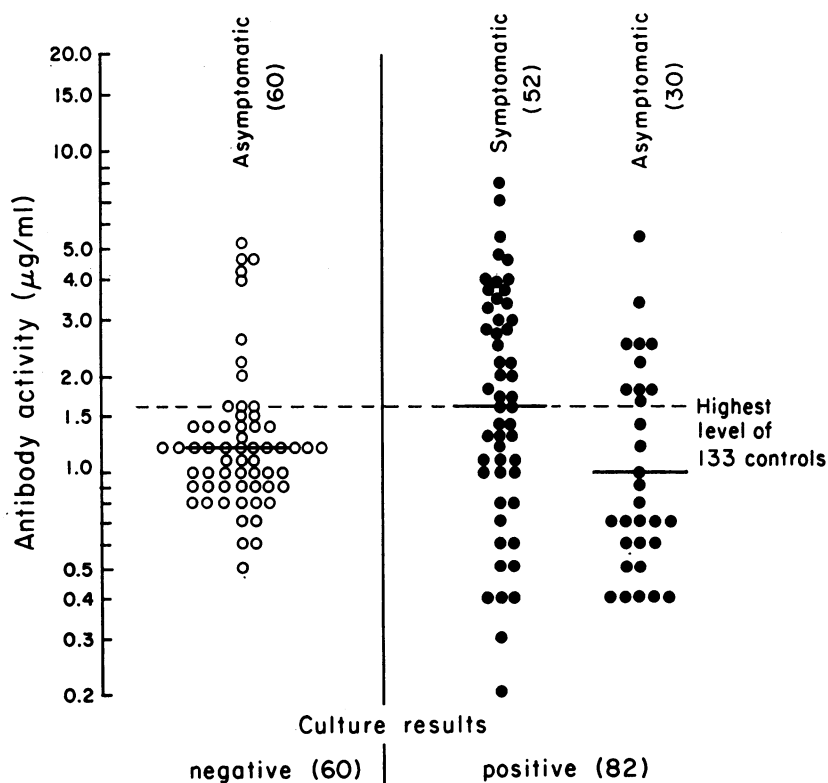


FIGURE 7 Culture results and antibody activity to gonococcal pili for 142 heterosexual men.

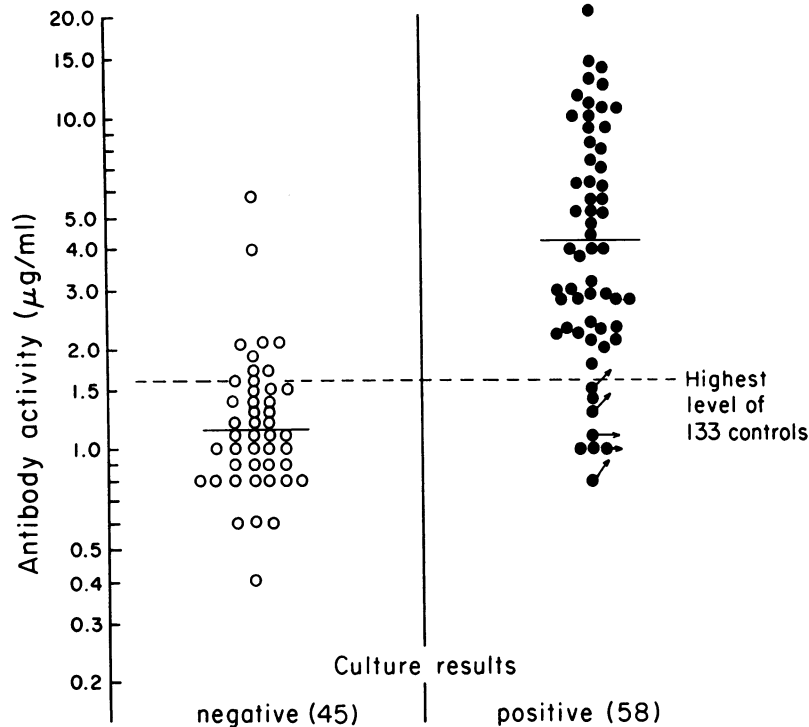


FIGURE 8 Culture results and antibody activity to gonococcal pili for 103 asymptomatic heterosexual women. Arrows indicate direction of change in antibody level for followup serum specimen obtained 1-4 wk later.

## DISCUSSION

Studies of the human humoral immune response to gonococcal infection have been complicated by the use of impure antigens and test systems incapable of reproducibly detecting small differences in antibody levels between patients and controls (27-40, 43-54). The use of a pure antigen preparation decreases the chance of detecting crossreacting antibody responses to organisms other than gonococci, and is a prerequisite to quantitating the antibody response to a single antigen. A few examples will illustrate the complexity of gonococcal antigens which have been employed for diagnostic serologic analysis.

Magnusson and Kjellander used a suspension of gonococci heated to 60° as antigens in a complement fixation test (27). Watt, Ward, and Glynn (30) and Ratnatunga (43) also used heated whole organisms in a complement-fixation test system. Danielsson, Schmale, Peacock, and Thayer purified gonococcal protoplasm antigens by gel filtration and ion exchange chromatography (28, 29). These antigens were from the supernate of gonococci disrupted in a Ribi cell fractionator following centrifugation at 36,000 *g* for 30 min, and contained approximately equal amounts of carbohydrate and protein (29). More than one protein was present

in this preparation as evidenced by polyacrylamide gel electrophoresis and nucleic acids were also present (30). Multiple precipitin bands were produced in agar gel diffusion using these antigens and hyperimmune rabbit serum (29). Some of these antigens have been shown by double diffusion in agar to be also present in the protoplasm of *N. meningitidis*, *N. flava*, and *N. sicca* (31). Tauber et al. and Maeland studied the immunochemistry of *N. gonorrhoeae* endotoxin prepared by several methods (32-39). Endotoxin was obtained from gonococci by extraction with phenol-water (32-35), alkali (35), trichloroacetic acid (35), heat (35), or aqueous ether methods (35-39). The endotoxin was shown to be present in the gonococcal cell wall (37) and depending upon the method of extraction was found to be composed of either lipopolysaccharide alone or a combination of lipopolysaccharide and protein (32-39). Heterogeneity of the endotoxin was demonstrated by Pevikon block electrophoresis (36) and indirect hemagglutination inhibition tests (35, 39). The protein antigen of endotoxin was common to meningococci and gonococci, and crossreactivity was observed between lipopolysaccharide antigens of gonococcal endotoxin and some nonpathogenic *Neisseriae* and meningococci (39). Wallace, Diena, Yugi, and Greenberg (40) utilized antigens isolated from the phenol phase of a

phenol-water extract of gonococci. These antigens were 65–80% protein and 5–10% carbohydrate by weight. They observed crossreactivity between this antigen and immune sera produced against *N. catarrhalis* but not with antisera to meningococci.

Gonococcal pili, as isolated in this study, are pure when examined by electron microscopy (Figs. 1c–1e) and are aggregates of a single protein subunit as demonstrated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Figs. 2a, 2b). Fluorescein-conjugated anti-pili antibody, over a 10-fold range in antibody concentration, stained only pilated gonococci and did not stain pilated meningococci or pilated nonpathogenic *N. catarrhalis*, *N. flavescens* or pilated *E. coli*. Since gonococci possess few pili than the strains of nonpathogenic *Neisseria* and *E. coli* tested, no staining of the latter organisms indicates that their pili are antigenically different from gonococci. Furthermore, the pili of gonococci are morphologically distinguishable from those of *E. coli* and nonpathogenic *Neisseria* (2, 41, 42). However, the lack of staining of meningococci with the fluorescein-conjugated antigonococcal pili antibody could result from the fact that meningococci are much more sparsely pilated than gonococci. Whatever the reason, persons infected with meningococci do not, in general, produce antibodies which crossreact with gonococcal pili. In this study, 72 sera containing high levels of antibody to meningococcal group-specific polysaccharides, obtained from patients with meningococcal infections, possessed antibody levels to gonococcal pili which were within the range of 133 persons unlikely to have experienced gonorrhea (Fig. 4). Sera from three patients with meningococcal infections contained elevated antibody levels to gonococcal pili ( $>1.6 \mu\text{g/ml}$ ). These three persons were a 37-yr old male, a 26-yr old female, and a 25-yr old female who were not questioned regarding their sexual activity or whether they had experienced gonorrhea, and their antibody levels to pili were 19.2  $\mu\text{g/ml}$ , 2.3  $\mu\text{g/ml}$ , and 1.8  $\mu\text{g/ml}$ , respectively.

Gonococcal pili described in this report were from a single strain, strain 2868 of *N. gonorrhoeae*. Since fluorescein-conjugated antibody to pili of this strain brightly stained pilated organisms of 18 different strains, it is unlikely that major antigenic differences exist between gonococcal pili from different strains. These considerations suggest that gonococcal pili isolated by the methods described above are a pure antigen common to all gonococcal strains and specific to *N. gonorrhoeae*.

The antigen binding assay using  $^{125}\text{I}$ -labeled gonococcal pili is capable of detecting as little as 100 pg of antibody (Fig. 3). This represents a considerable increase in sensitivity over methods used previously

to detect antibodies to gonococcal antigens (55). This sensitivity allows detection of antibody to pili in as little as 10  $\mu\text{l}$  of serum from patients with gonorrhea, a disease which is usually a localized infection (56, 57). However, it is likely that specificity and reproducibility are more important determinants of whether an assay can accurately separate infected persons from those who are not infected with gonococci. Patients infected with gonococci produce antibody to pili in concentrations of 1–20  $\mu\text{g/ml}$  (Fig. 4), a range detectable by conventional serologic tests. Since antibody levels to some gonococcal antigens are as little as 0.5  $\mu\text{g/ml}$  higher in infected as compared with noninfected persons (Fig. 4), the reproducibility of an assay upon repeat testing may be the principal factor which determines its usefulness for any large scale testing of sera. Typically, repeat testing results are within 10–20% for the antigen binding assay (26, Table I), as compared with variations of up to two twofold dilutions (400%) for conventional serologic tests may account for some of the difficulties with previously published methods of serologic testing for gonorrhea.

The time course of the immune response to gonococcal pili is similar to the response to some other gonococcal antigens. Each of the seven persons with gonococcal arthritis in this study showed a rise in antibody to pili within 1 wk of the onset of their symptoms (Fig. 5). Five had elevated levels to gonococcal pili when they first presented with systemic disease (nos. 2, 4, 5–7) and the other two (nos. 1 and 3) developed elevated levels within the first week of illness. Magnusson and Kjellander (27), Chacko, and Nair (50), and Genner, Anton, and Boas (62) also found that elevations in antibody were detectable after 7–10 days of illness. Hess, Hunter, and Ziff, using an indirect fluorescent antibody method, observed antibody to gonococci in 80% of sera obtained from patients during the first week of arthritis, and in 100% of sera obtained 8–21 days after the arthritis began (63). The time required for antibody to decline to normal levels following treatment was found by Hess to be 1–9 mo (63), and by Magnusson and Kjellander to be 1 mo to 1 yr (27). Genner observed elevated antibody levels for 6 mo to 2 yr in untreated patients (62). Antibody levels to gonococcal pili declined to 2.2  $\mu\text{g/ml}$  or less within 1 mo after treatment in one patient with gonococcal arthritis (no. 1, Fig. 5) and within 3 mo after treatment in four asymptotically infected females (nos. 1–4, Fig. 6). It was observed that more time was required to decline to an antibody level of 2.2  $\mu\text{g/ml}$  if the initial serum antibody level to pili was very high (Figs. 5 and 6). The rate of different immunoglobulin classes (64, 65) in the rate of decline of this antibody remains to be determined.

Our observation that more females than males infected with gonococci have elevated antibody levels to gonococcal pili is consistent with nearly every previous report of serologic tests for gonorrhea (27, 30, 40, 43-46, 48) and may be related to the duration of infection in each sex. Most men develop dysuria and urethral discharge within 5 days after infection and seek diagnosis or treatment. Consequently, their infection persists for a sufficient duration to develop elevated antibody levels. However, this is unlikely to be the only reason for the higher antibody levels seen in women because in some instances even prolonged asymptomatic gonococcal infection of males may not provide sufficient antigenic stimulus to produce elevated serum antibody levels to pili. Three of five asymptotically infected men who had urethral cultures which remained positive for 2-3 wk while treatment was withheld failed to develop elevated antibody levels to gonococcal pili. The gonococci isolated from the urethra of one of these asymptotically infected men was examined by electron microscopy and found to be pilated. It is therefore unlikely that normal antibody levels to pili in these persons reflect infection with non-pilated gonococci. The sex difference in antibody levels may be related to other factors such as the general tendency of females to produce more antibody to a number of antigens (66-68) or to local factors such as extent of infection and lymphatic drainage.

One of the principal reasons that gonorrhea is the most common reported infectious disease in the United States today is that the reservoir of asymptotically infected females capable of transmitting the disease (69, 70) is not being detected. Many women infected with gonococci never develop iatrogenic symptoms, and it is estimated that there were approximately 800,000 of these women in the United States in 1972 (71, 72). In this study, the antigen binding assay detected elevated antibody levels to gonococcal pili in 86% of asymptotically infected females (Fig. 4). Cultures of the endocervix are useful as a screening method for populations with a high prevalence of gonorrhea. However, cultural methods are too expensive and logistically impractical for screening low prevalence groups. It is conceivable that mass screening of low risk female populations with the antigen binding assay could provide a means to detect and eliminate many asymptomatic gonococcal infections in women. The low level of antibody response to pili in asymptotically infected men, combined with the lack of antibody rises in many men with symptomatic urethritis who are treated within 10 days of becoming infected, indicates that the antigen binding assay may be of limited usefulness for detection of localized gonococcal infection in males (Fig. 7). However, most men with gonococcal infection develop

symptomatic urethritis and are treated (71, 72). Therefore one could hypothesize that a significant reduction in the number of asymptotically infected women, in concert with cultural detection and treatment of asymptotically infected males, would lead to a general reduction in the prevalence of the disease. Since it would be more feasible to screen large numbers of women by serologic test than by endocervical culture, the antigen binding assay may prove a useful method for detecting the reservoir of asymptotically infected women in the United States.

## ACKNOWLEDGMENTS

The authors wish to thank Lawrence R. Schulman for his excellent technical assistance, Dr. H. Hunter Handsfield, Dr. James P. Harnish, and Dr. Paul J. Wiesner for their considerable help with collecting clinical information and serum from the study patients, and Mrs. Roberta Poole for typing the manuscript.

This work was supported in part by Center for Disease Control Grant CC00593, CDC Contract HSM 21-73-502, U. S. Army R & D Contract DADA-17-72-C-2023, U. S. Public Health Service (National Institute of Allergy and Infectious Diseases) Grants AI00045, AI50525, and AI1061502, and a grant from The Rockefeller Foundation.

## REFERENCES

1. Jephcott, A. E., A. Reyn, and A. Birch-Andersen. 1971. Brief report: *Neisseria gonorrhoeae*. III. Demonstration of presumed appendages to cells from different colony types. *Acta Pathol. Microbiol. Scand. Sect. B* 79: 437.
2. Swanson, J., S. J. Kraus, and E. C. Gotschlich. 1971. Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. *J. Exp. Med.* 134: 886.
3. Kellogg, D. S., Jr., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bacteriol.* 85: 1274.
4. Kellogg, D. S., Jr., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising. 1968. *Neisseria gonorrhoeae*. II. Clonal variations and pathogenicity during 35 months in vitro. *J. Bacteriol.* 96: 596.
5. Buchanan, T. M., and E. C. Gotschlich. 1973. Studies on gonococcus infection. III. Correlation of gonococcal colony morphology with infectivity for the chick embryo. *J. Exp. Med.* 137: 196.
6. Buchanan, T. M., J. Swanson, and E. C. Gotschlich. 1972. Antibody response to gonococcal pili in patients with gonorrhea. *J. Clin. Invest.* 51: 17a.
7. Farr, R. S. 1958. A quantitative immunochemical measure of the primary interaction between I\*BSA and antibody. *J. Infect. Dis.* 103: 239.
8. Skom, J. H., and D. W. Talmadge. 1958. Nonprecipitating insulin antibodies. *J. Clin. Invest.* 37: 783.
9. Morgan, C. R., and A. Lazarow. 1962. Immunoassay of insulin using a two-antibody system. *Proc. Soc. Exp. Biol. Med.* 110: 29.
10. Wiktor, T. J., H. Koprowski, and F. Dixon. 1972. Radioimmunoassay procedures for rabies binding antibodies. *J. Immunol.* 109: 464.
11. Frantz, I. D., Jr. 1942. Growth requirements of the meningococcus. *J. Bacteriol.* 43: 757.

12. Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. *Ann. N. Y. Acad. Sci.* 121: 321.
13. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* 121: 404.
14. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* 244: 4406.
15. Dunker, A. K., and R. R. Rueckert. 1969. Observations on molecular weight determinations on polyacrylamide gel. *J. Biol. Chem.* 244: 5074.
16. Hunter, W. M. 1967. The preparation of radioiodinated proteins of high activity. In *Handbook of Experimental Immunology*. D. M. Weir, editor. 608.
17. Kabat, E. A., and M. M. Mayer. 1961. *Experimental Immunochemistry*. Charles C Thomas, Springfield, Ill. 556.
18. Williams, C. A., and M. W. Chase, editors. 1968. *Methods in Immunology and Immunochemistry*. Vol. II. Physical and Chemical Methods. Academic Press, Inc., New York. 272.
19. Swanson, J. 1972. Studies on gonococcus infection. II. Freeze-fracture, freeze-etch studies on gonococci. *J. Exp. Med.* 136: 1258.
20. Thayer, J. D., and J. E. Martin, Jr. 1964. A selective medium for the cultivation of *N. gonorrhoeae* and *N. meningitidis*. *Public Health Rep.* 79: 49.
21. Bodily, H. L., E. L. Updyke, and J. W. Mason, editors. 1970. *Diagnostic Procedures for Bacterial, Mycotic, and Parasitic Infections*. American Public Health Association, New York. 5th edition. 296.
22. Holborow, E. J., and G. D. Johnson. 1967. Immunofluorescence. In *Handbook of Experimental Immunology*. D. M. Weir, editor. 571.
23. Gotschlich, E. C. 1971. A simplification of the radioactive antigen-binding test by a double label technique. *J. Immunol.* 107: 910.
24. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry*. 2: 235.
25. Talmage, D. W., and P. H. Maurer. 1953. I<sup>125</sup>-labelled antigen preparation as a measure of quantity and quality of antibody. *J. Infect. Dis.* 92: 288.
26. Gotschlich, E. C., M. Rey, R. Triau, and K. J. Sparks. 1972. Quantitative determination of the human immune response to immunization with meningococcal vaccine. *J. Clin. Invest.* 51: 89.
27. Magnusson, B., and J. Kjellander. 1965. Gonococcal complement-fixation test in complicated and uncomplicated gonorrhea. *Br. J. Vener. Dis.* 41: 127.
28. Danielsson, D. G., J. D. Schmale, W. L. Peacock, Jr., and J. D. Thayer. 1969. Antigens of *Neisseria gonorrhoeae*: characterization by gel filtration, complement fixation, and agar-gel diffusion of antigens of gonococcal protoplasm. *J. Bacteriol.* 97: 1012.
29. Schmale, J. D., D. G. Danielsson, F. J. Smith, L. Lee, and W. L. Peacock, Jr. 1969. Isolation of an antigen of *Neisseria gonorrhoeae* involved in the human immune response to gonococcal infection. *J. Bacteriol.* 99: 469.
30. Watt, P. J., M. E. Ward, and A. A. Glynn. 1971. A comparison of serological tests for the diagnosis of gonorrhea. *Br. J. Vener. Dis.* 47: 448.
31. Danielsson, D. 1965. The demonstration of *N. gonorrhoeae* with the aid of fluorescent antibodies. IV. Studies by immunofluorescence and double diffusion-in-gel technique on the antigenic relationship between *N. gonorrhoeae* and other *Neisseria* strains. *Acta Pathol. Microbiol. Scand.* 64: 267.
32. Tauber, H., and W. Garson. 1957. Preparation and some properties of *Neisseria gonorrhoeae* endotoxin. *Proc. Soc. Exp. Biol. Med.* 95: 669.
33. Tauber, H., and W. Garson. 1959. Isolation of lipopolysaccharide endotoxin. *J. Biol. Chem.* 234: 1391.
34. Tauber, H., and H. Russell. 1960. Amino compounds in lipopolysaccharides. *J. Biol. Chem.* 235: 961.
35. Maeland, J. A. 1968. Antigenic properties of various preparations of *Neisseria gonorrhoeae* endotoxin. *Acta Pathol. Microbiol. Scand.* 73: 413.
36. Maeland, J. A. 1969. Antigenic determinants of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae*. *Acta Pathol. Microbiol. Scand.* 76: 475.
37. Maeland, J. A. 1969. Immunochemical characterization of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae*. *Acta Pathol. Microbiol. Scand.* 76: 484.
38. Maeland, J. A. 1969. Serological properties of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae*. *Acta Pathol. Microbiol. Scand.* 77: 495.
39. Maeland, J. A. 1969. Serological cross-reactions of aqueous ether extracted endotoxin from *Neisseria gonorrhoeae* strains. *Acta Pathol. Microbiol. Scand.* 77: 505.
40. Wallace, R., B. B. Diena, H. Yugi, and L. Greenberg. 1970. The bentonite flocculation test in the assay of *Neisseria* antibody. *Can. J. Microbiol.* 16: 655.
41. Brinton, C. C., Jr. 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. *Trans. N. Y. Acad. Sci.* 27: 1003.
42. Wistreich, G. A., and R. F. Baker. 1971. The presence of fimbriae (pili) in three species of *Neisseria*. *J. Gen. Microbiol.* 65: 167.
43. Ratnatunga, C. S. 1971. Evaluation of the gonococcal complement-fixation test. *Br. J. Vener. Dis.* 47: 279.
44. Reising, G., J. D. Schmale, D. G. Danielsson, and J. D. Thayer. 1969. Reactivity of two selected antigens of *Neisseria gonorrhoeae*. *Appl. Microbiol.* 18: 337.
45. Lee, L., and J. D. Schmale. 1970. Identification of a gonococcal antigen important in the human immune response. *Infect. Immun.* 1: 207.
46. Logan, L. C., P. M. Cox, and L. C. Norins. 1970. Reactivity of two gonococcal antigens in an automated microhemagglutination procedure. *Appl. Microbiol.* 20: 907.
47. Maeland, J. A., and B. Larsen. 1971. Human serum antibodies reacting with endotoxin from *Neisseria gonorrhoeae*. *Br. J. Vener. Dis.* 47: 269.
48. Ward, M. E., and A. A. Glynn. 1972. Human antibody response to lipopolysaccharides from *Neisseria gonorrhoeae*. *J. Clin. Pathol.* 25: 56.
49. Reising, G., and D. S. Kellogg, Jr. 1965. Detection of gonococcal antibody. *Proc. Soc. Exp. Biol. Med.* 120: 660.
50. Chacko, C. W., and G. M. Nair. 1969. Sero-diagnosis of gonorrhea with a microprecipitin test using a lipopolysaccharide antigen from *N. gonorrhoeae*. *Br. J. Vener. Dis.* 45: 33.
51. Glynn, A. A., and M. E. Ward. 1970. Nature and heterogeneity of the antigens of *Neisseria gonorrhoeae* involved in the serum bactericidal reaction. *Infect. Immun.* 2: 162.
52. Cohen, I. R., D. S. Kellogg, Jr., and L. C. Norins. 1969. Serum antibody response in experimental human

- gonorrhea. Immunoglobulins G, A, and M. *Br. J. Vener. Dis.* **45**: 325.
53. Cohen, I. R. 1967. Natural and immune human antibodies reactive with antigens of virulent *Neisseria gonorrhoeae*: immunoglobulins G, M, and A. *J. Bacteriol.* **94**: 141.
  54. Serological Tests for Gonorrhea. 1972. *Br. Med. J.* **1**: 584.
  55. Kwapinski, J. B. 1965. In *Methods of Serologic Research*. John Wiley & Sons, New York. 126.
  56. Barr, J., and D. Danielsson. 1971. Septic gonococcal dermatitis. *Br. Med. J.* **1**: 482.
  57. Holmes, K. K., P. J. Weisner, and A. H. B. Pedersen. 1971. The gonococcal arthritis-dermatitis syndrome. *Ann. Intern. Med.* **75**: 470.
  58. Danielsson, D. 1965. The demonstration of *N. gonorrhoeae* with the aid of fluorescent antibodies. III. Studies by immunofluorescence and double diffusion-in-gel technique on the antigenic relationship between strains of *N. gonorrhoeae*. *Acta Pathol. Microbiol. Scand.* **64**: 243.
  59. Reyn, A. 1949. Serological studies on gonococci. I. Technique, gono-reaction of "normal" rabbits. Serological relation between gonococci and pasteurellae. *Acta Pathol. Microbiol. Scand.* **26**: 51.
  60. Reyn, A. 1949. Serological studies on gonococci. II. Crossabsorption experiments and factor serum determinations. *Acta Pathol. Microbiol. Scand.* **26**: 234.
  61. Reyn, A. 1949. Serological studies on gonococci. III. Thermostability and biochemical nature of the gonococcus antigens. Durability of the factor sera. Discussion. *Acta Pathol. Microbiol. Scand.* **26**: 252.
  62. Genner, V., K. Antons, and H. Boas. 1932. Course of the complement fixation reaction in gonorrhea in men. *Hospitalstidende.* **75**: 299.
  63. Hess, E. V., D. K. Hunter, and M. Ziff. 1965. Gonococcal antibodies in acute arthritis. *J. Am. Med. Assoc.* **191**: 531.
  64. Svehag, S., and B. Mandel. 1964. The formation and properties of poliovirus-neutralizing antibody. I. 19S and 7S antibody formation: differences in kinetics and antigen dose requirement for induction. *J. Exp. Med.* **119**: 1.
  65. Svehag, S., and B. Mandel. 1964. The formation and properties of poliovirus-neutralizing antibody. II. 19S and 7S antibody formation: differences in antigen dose requirement for sustained synthesis, anamnesis, and sensitivity to X-irradiation. *J. Exp. Med.* **119**: 21.
  66. Michaels, R. H., and K. D. Rogers. 1971. A sex difference in immunologic responsiveness. *Pediatrics.* **47**: 120.
  67. Ainbender, E., R. B. Weisinger, M. Hevizi, and H. L. Hodes. 1968. Difference in the immunoglobulin class of polioantibody in the serum of men and women. *J. Immunol.* **101**: 92.
  68. Stoop, J. W., B. J. M. Zegers, P. C. Sander, and R. E. Ballieux. 1969. Serum immunoglobulin levels in healthy children and adults. *Clin. Exp. Immunol.* **4**: 101.
  69. Brown, L., B. C. Brown, M. J. Walsh, and C. I. Pirkle. 1963. Urethritis in males produced by *Neisseria gonorrhoeae* from asymptomatic females. *J. Am. Med. Assoc.* **186**: 153.
  70. Pariser, H. 1971. Asymptomatic gonorrhea. The VD Crisis. International Venereal Disease Symposium, St. Louis, Mo. 26.
  71. Pariser, H. 1972. Asymptomatic gonorrhea. Symposium on Venereal Diseases. *Med. Clin. North Am.* **56**: 1127.
  72. Lucas, J. B. 1972. The National Venereal Disease Problem. Symposium on Venereal Diseases. *Med. Clin. North Am.* **56**: 1073.