Identification of *Neisseria gonorrhoeae* by Means of Fluorescent Antibodies. (24925)

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One of the more important applications of fluorescent antibody methods is pathogen identification in smears, without use of prior culture procedures(1,2,3,4,5). In the present work an attempt was made to develop similar technics for *Neisseria gonorrhoeae* identification because of their possible usefulness in control programs. Wilson(6,7) reviewed and studied antigenic relationships of gonococcus and meningococcus. It was demonstrated that 8 heat-stable antigens are shared by these species and that freshly isolated gonococcus cultures are frequently inagglutinable, but behave in a normal manner after being heated at 100°C for 30 minutes. In the same report it was also suggested that the "factor" responsible for inagglutination in freshly isolated cultures could be a substance similar to Vi antigen of *Salmonella typhi*. The antigenic properties of this "factor," however, were not demonstrated since rabbits inoculated with living inagglutinable cultures produced antibodies identical to those which received heat-killed cultures (Wilson, 1957, personal communication). Since surface antigens such as Vi(8) and the Kantigens of the Escherichia group(9) frequently control serologic activity of the bacterium, it is conceivable that the "factor" responsible for inagglutinable gonococcus cultures could be the most important diagnostic element of the *N. gonorrhoeae* cell. Identification of this material therefore has been of primary importance in the present work.

Materials and methods. Cultures of *N. gonorrhoeae* and other *Neisseria* were obtained from laboratory stocks and other laboratories.* Freshly isolated strains and smears were made available from the Division of Venereal Disease Control, Fulton Co. Health Dept., Atlanta, Ga. Cultures were grown on Eugon Agar(BBL)† plus 0.2% corn starch, and on GC Agar Base plus hemoglobin and Supplement B(Difco),‡ and were incubated in an atmosphere of 8-10% CO_2 gas at 35°C for 12-16 hours. Cultures were harvested in phosphate buffered saline pH 7.2 (BS) and killed by heating at 100°C for 30 minutes, 120°C for 2½ hours, and with 3% formalin for 30 minutes. Killed suspensions were washed 3 times with BS, adjusted to a density of either McFarland No. 10 or 10X McFarland No. 10. All suspensions were preserved by addition of 0.3% formalin. Live cultures were suspended in BS and used at density of approximately 20X No. 10 McFarland immediately after preparation. Antiserums were produced in healthy, young rabbits weighing approximately 5 lb each. Killed suspensions (McFarland No. 10 density) were injected *via* ear vein (IV) according to following schedule: 0.5 ml followed by 1 ml every 3-4 days for 2 weeks, one week rest followed by another 2 week injection schedule. One week after last injection, animals were bled from heart. Formalin-killed *N. gonorrhoeae* antigens were used in combination with Bacto-Adjuvant Complete (Difco). In this case, 5 ml of the antigen 5 X McFarland No. 10 was mixed with 5 ml of adjuvant. The mixture was administered by subcutaneous injection into the back of each animal. After one month these rabbits received additional IV injections as previously described, and were then bled. Antiserums were separated from blood clots in usual manner and stored at −20°C. Antigen suspensions for use in the slide agglutination tests were adjusted to density of 20X McFarland No. 10. Equal portions of undiluted serum and antigen were mixed on 1″ x 3″ microscope slides, hand-tilted or rotated, and observed against a dark

* Dr. Sara E. Branham's collection, Nat. Inst. Health, Bethesda, Md. Dr. Michael J. Pelczar, Jr., Univ. of Md., Baltimore, Md. Am. Type Culture Collection, Washington, D.C.

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‡ Difco Labs., Detroit, Mich.

background for clumping. Normal rabbit serums and BS were used as controls. Rabbit serum globulin was obtained by precipitation with ammonium sulfate. Fluorescein isothiocyanate (BBL) was employed in preparing globulin conjugates using a modification of the Goldman and Carver technic(10). Fluorescein isothiocyanate powder was added to chilled (4°C) globulin solution buffered at pH 9.0 (sodium bicarbonate-carbonate buffer), in the amount of .05 mg/mg protein. The mixture was shaken 6 hours in the cold, then dialyzed in BS to remove unreacted fluorescein. Where absorption of serums or conjugates is reported, packed cells were used in amount equal to volume of material to be treated. Cells were obtained and treated as described for antigens. Absorptions were carried out for 4 hours at 50°C. Cells were removed by centrifugation at 12,800 x G for one hour in the cold. Smears prepared for microscopic observation were made from antigens (prepared as previously described) and from 16 hour living cultures. Smear fixation was accomplished by air drying or by light heat. Undiluted fluorescein conjugates (1 g % protein) were used for staining. Unabsorbed conjugates were applied to smears for 30 minutes at 37°C, using rotation as described earlier(11). Staining times were frequently increased to one hour, as necessary, when absorbed conjugates were used. After staining, smears were washed in running BS and allowed to stand in same solution for 30 minutes. Slides were finally blotted lightly and a small drop of mounting mixture (9 parts C.P. glycerine plus 1 part BS) was placed on each smear. A cover glass placed upon the mounting mixture completed the slide preparation. Observations were made using Leitz or Reichert microscopes equipped with appropriate filters and ultraviolet light assemblies. Photomicrographs were recorded on 35 mm Super Anscochrome, daylight film. Fluorescent antibody controls included normal rabbit globulin conjugates and inhibition of specific staining by means of unlabeled globulins.

Results. Agglutination findings (Table I) confirm the observations of Wilson that gonococcal cultures may be inagglutinable in homologous antiserums. Since formalin-killed cultures, represented by GC(F), demonstrate inagglutinability like that of the living culture, GC(L), it appears that formalin preserved the characteristic of the substance or "factor" responsible for inagglutination. Since the antiserum produced against formalin-killed antigen GC(F) agglutinates all antigens (L, F, 100° and 120°) while GC (100°) serum clearly agglutinates only antigens GC (100°) and (120°), it would seem that an antigen has been preserved by formalin treatment, making GC(F) similar to GC(L). However, since all reactivity was removed from GC(F) serum by absorption with antigen GC(100°), it appears that this antigen must also contain some of the same "factor." If this "factor" is similar to the K antigens of the Escherichia group, then the reaction noted would indicate that it is of the B type (agglutination characteristics destroyed by 100°C, absorption ability, unaltered). This antigen, however, is apparently destroyed at 120°C because antiserum produced by GC(F) after being absorbed with antigen GC(120°) contains agglutinins for L and F antigens, but does not contain agglutinins for 120°

TABLE I. Slide Agglutinations Employing *N. gonorrhoeae* Antigens and Antiserums.

	Serums undiluted					
Antigens*	GC (F)	GC (100°)	GC (120°)	GC (F) absorbed with GC (120°)	GC (100°) absorbed with GC (120°)	GC (F) absorbed with GC (100°)
GC (L)	+	±	—	+	±	—
" (F)	+	±	—	+	±	—
" (100°)	+	+	+	+	+	—
" (120°)	+	+	+	—	—	—

* Representing 5 strains.

L = Living culture; F = Formalin killed; 100° = Heat killed; 120° = Heat killed; —, ±, + = Degrees of agglutination.

TABLE II. Neisseria Reactions with *N. gonorrhoeae*—GC (F) Fluorescein Labeled Antiserum.

Antigens	Unabsorbed	Single Absorption with serogroup A (100°)	Single Absorption with serogroup A (F)
Gonococcus			
GC (L) (3 strains)	*3+ s oc 4+ s	3+ s oc 4+ s	3+ s oc 4+ s
(100°)	3+ oc 4+	3+ oc 4+	3+ oc 4+
Urethral smears (25 individuals)	4+ s	4+ s	4+ s
Meningococcus			
Serogroup A (L)	1+ oc 4+ s	— oc 3+	—
(100°)	1+ oc 4+ s	1+	—
Serogroup B (L)	1+ oc 4+ s	1+	—
(100°)	1+ oc 3+ s	1+	—
Serogroup C (L)	1+	1+	1+
(100°)	2+	1+	—
N. catarrhalis (L)	1+	1+	1+
(100°)	1+	1+	1+
N. perflava (L)	—	—	—
(100°)	—	—	—
N. sicca (L)	—	—	—
(100°)	—	—	—

* Degrees of fluorescents: —, 1+, 2+, 3+, 4+ (1+ barely visible). s = Solid stain. oc = Occasional.

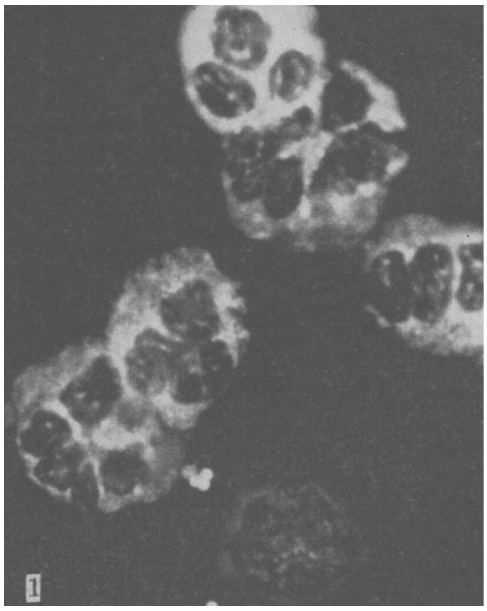
antigens. Thus it would appear that the 120° antigen represents a somatic complex, or the O antigens comprised by the gonococcus. The K-like antigen of the gonococcus will hereafter be referred to as the GC-K(B).

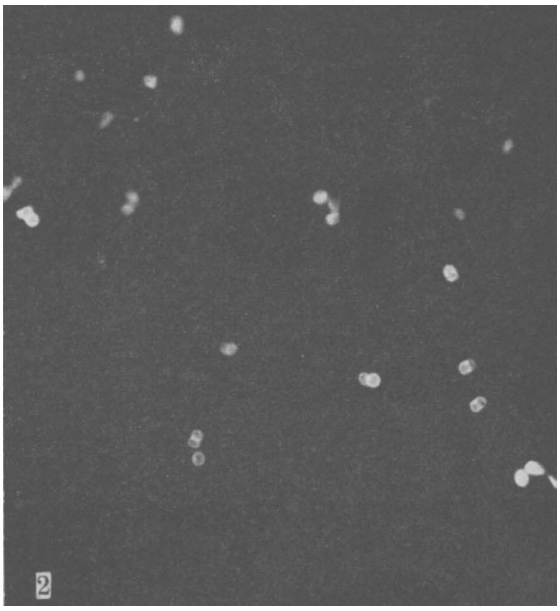
Table II gives fluorescent antibody reactions obtained by staining various *Neisseria* antigens with absorbed and unabsorbed GC (F) fluorescein labeled antiserum. Strong cross-reactions or staining occurred when unabsorbed conjugate was used to stain meningococcal Serogroups A and B. However, since the somatic antigens of gonococcus and meningococcus are shared(7,8), the fluorescent antibody reactions were interpreted as resulting from this relationship. Minor reactions will also be noted for Serogroup C and *N. catarrhalis*. A single absorption with Serogroup A (100°) and A (F) antigens removed all cross-reactions of a troublesome nature, yet leaves an adequate supply of specific antibody for gonococcus identification in smear preparations.

Typical gonococcus fluorescent reactions are illustrated in Figs. 1, 2 and 3. Organisms in urethral smears are brilliant and solid in appearance, while young inagglutinable cultures lose some of the solid staining character. Older cultures, Fig. 3, show poor staining, indicating K antigen loss.

Discussion. Our evidence appears to indicate that *N. gonorrhoeae* possesses a heretofore unrecognized antigenic component similar in character to Vi antigen of *S. typhi* or K antigen of the Escherichia group. The new antigen appears to be species specific and may be recognized by slide agglutination tests employing living or formalin-killed cultures and by fluorescent antibody technics. The fluorescent antibody method for *Neisseria* study allows detection of serologic reactions which are not apparent by the usual agglutination tests. It appears to be an extremely valuable tool in determining development and/or loss of particular antigens of the gonococcus in relation to nutritional and physical growth conditions. It is evident that further studies are justified in respect to production of species specific antiserums of high titer, of determining additional *Neisseria* relationships based on absorption technics and demonstration of strain or type antigenic differences in *N. gonorrhoeae* cultures.

Summary. 1. A species specific antigen, associated with freshly isolated, inagglutinable gonococcus cultures, is recognized. 2. The new antigen appears to possess characteristics similar to Vi antigen of *S. typhi* or the K an-





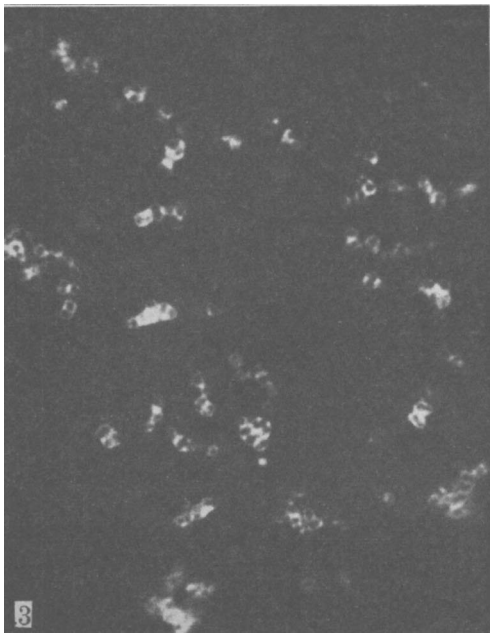


FIG. 1. Urethral (male) smear stained with GC(F), fluorescein labeled antiserum. Solid staining of gonococci indicates fully developed GC-K (B) antigen.

FIG. 2. Young culture (12-16 hr) stained as previously described. GC-K(B) antigen well developed but not as complete as in Fig. 1.

FIG. 3. Older culture (30 hr) illustrating extreme lability of GC-K(B) antigen. Some cells show only a remaining trace of the substance.

tigens of the Escherichia group, and is fully developed only in freshly isolated cultures or in infectious exudate. 3. Antiserums containing GC-K(B) antibodies may be labeled with fluorescein and used as a means of identifying *N. gonorrhoeae* in smear preparations.

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