Fluorescent Antibody Test for the Serological Diagnosis of Gonorrhea

HASSAN A. GAAFAR* AND DORA C. D'ARCANGELIS

Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

Received for publication 15 October 1975

An indirect fluorescent antibody technique has been developed for the serological diagnosis of gonorrhea. The selected strain(s) of *Neisseria gonorrhoeae* possesses a heat-labile surface antigen (L-antigen). Sera are diluted 1:10, and an aliquot is heat inactivated at 59 C for 30 min. The treated and untreated aliquots are then examined for human immunoglobulin G anti-L-antigen. In a preliminary study of 495 sera, 95% of those from women with a bacteriologically confirmed diagnosis of gonorrhea and 87% of those from male patients were reactive in this test, whereas only 1.4% of the sera from presumably normal individuals were reactive.

Gonococcal disease is currently a major public health problem throughout the world (6, 21). Whereas a number of factors are responsible for the current epidemic, the inability to detect the asymptomatic carrier remains perhaps the most important element in our failure to control gonococcal disease (9, 13, 14). This asymptomatic carrier population comprises the reservoir out of which the majority of disseminated infections arise.

Whereas bacterial confirmation is likely to remain the principal diagnostic test for gonorrhea, a serological test to aid in the detection of asymptomatic patients would be an invaluable adjunct. Several such tests have been reported, but they have lacked either sufficient specificity or sensitivity (11, 16, 18, 19, 23). The indirect fluorescent antibody method has been a useful diagnostic tool in syphilis and has been used by Hess et al. (9) to diagnose uncomplicated gonorrhea. Peacock et al. (15), however, found it unsuitable because of the presence of cross-reacting antibodies. The varying degrees of success reported are most probably due to the common *Neisseria* antigens (25) and interspecies types (4, 12, 17, 20). Our success in identifying an antigen of *Neisseria gonorrhoeae* that reacts with antibodies present in patients' heat-treated sera (H. A. Gaafar and D. D'Arcangelis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, M206, p. 114) prompted us to investigate a serological test for the diagnosis of gonorrhea. This communication reports the methodology of an indirect fluorescent antibody technique developed in our laboratory and the results obtained with a selected population.

(This work was presented in part at the 72nd

Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., 1972.)

MATERIALS AND METHODS

Bacterial cultures. The cultures of *N. gonorrhoeae* were from the Division of Laboratories and Research (DLR) Culture Collection. Strain B370 was isolated originally from the urethral discharge of a 28-year-old male and was submitted for confirmation studies. One week after positive identification, this isolate was lyophilized as a prototype strain of *N. gonorrhoeae*. When tested with known positive and negative control sera, it appeared to be both stable and serologically reactive. Two other strains, B585 and B1094, were submitted on antibiotic-containing agar for isolation from a 38-year-old male and a 17-year-old female, respectively. These two strains appeared to have the same heat-labile, trypsin-sensitive antigen (L-antigen) and were lyophilized for future use. While in use, all strains were maintained in semisolid agar with a mineral oil overlay and were transferred monthly (22).

Prototype strains of *N. meningitidis* and *N. catarrhalis* from the DLR Culture Collection were grown under standard conditions and used in the same manner as described for *N. gonorrhoeae*.

Preparation of antigen slides. Lyophilized cultures were reconstituted with beef infusion broth or physiological saline and inoculated into semisolid medium and onto fresh rabbit blood chocolate agar (22). After 48 h the colonies were transferred to chocolate agar slants. After one or two additional transfers, 18- to 24-h-old cells were used to prepare the antigen slides. All cultures were grown and maintained in an atmosphere of 5% CO₂ at 37 C.

Growth from each 18- to 24-h culture was harvested into a small amount of physiological saline, the clumps were gently disrupted, and the density of the suspension was adjusted to 3×10^6 to 4×10^6 cells/ml. One drop (approximately 40 μ l) of suspen-

sion was placed on each end of an alcohol-cleaned fluorescent antibody slide and smeared with a loop. The slide was allowed to air-dry and then was fixed in 3% buffered formalin for 10 min, washed by dipping into distilled water 10 times, drained, blotted dry, and stored in a closed container at -20°C . Slides were prepared as needed and were rarely stored for more than 2 weeks. However, slides stored at -20°C for up to 1 month were also found to be satisfactory. Slides allowed to remain at room temperature for more than 1 h showed evidence of antigen deterioration and were discarded.

Controls. A strongly reactive positive control serum, obtained from a bacteriologically positive patient, was dispensed in small aliquots and stored at -20°C . This serum was diluted to give 2+ and 4+ fluorescence and was used to titrate new lots of conjugates and positive controls.

Test sera. Three separate groups of sera from bacteriologically positive patients were used to determine specificity (see Table 4). The first group of 161 sera (130 males, 31 females) were obtained from patients visiting the Albany County Venereal Disease Clinic. Sera were selected from clinically apparent cases of gonococcal disease by Roy Shaffer, and bacterial confirmation was obtained at an independent laboratory (Bender Hygienic Laboratory, Albany).

The second group of 134 sera (39 males and 95 females) and the third group of 128 sera (30 males and 98 females) both consisted of specimens obtained from patients who were diagnosed as having active gonorrhea on the basis of cultures examined in our own laboratory. Sera were generally received within 1 week of the bacteriological diagnosis, but the interval between diagnosis and receipt of serum ranged from less than 1 week to as long as 6 weeks.

Normal control sera were obtained from presumably uninfected individuals (laboratory personnel with no history of past or current infection) and children aged 8 to 15 admitted to Children's Hospital in Buffalo.

Fluorescent antisera. Fluorescein-conjugated rabbit anti-human immunoglobulin G (IgG) (H-chain-specific) were purchased from Behring Diagnostic, Woodbury, N.J. The working dilutions of the antiserum lots varied from 1:80 to 1:320 and were determined by titration with controls.

Fluorescent microscopy and grading of fluorescence. Stained slides were examined with Leitz ultraviolet microscopes, each fitted with an HB-200 light source, a 3-mm BG-12 exciter filter, a BG-38 red excluding filter, a K530 ocular filter, and a dark-field condenser. The slides were examined under an X100 oil immersion objective ($\times 1,000$).

Several fields were examined for a peripheral homogeneous fluorescence of single cells. Irregular or patchy fluorescence and whole-cell fluorescence were not scored. The following subjective scoring system was devised: negative, cells visible only under white light; shadows, cells visible under ultraviolet but no fluorescence; 1+, diffuse, weak green fluorescence, indistinct cells; 2+, definite but subdued peripheral fluorescence; 3+, intense peripheral

fluorescence, cells more distinct; 4+, bright peripheral fluorescence, very distinct cells. Typical 2+ and 4+ smears are shown in Fig. 1.

RESULTS

Preliminary experiments were undertaken to determine the distribution of antibody to *N. gonorrhoeae* antigen (L-antigen) (Gaafar and D'Arcangelis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, M206, p. 114) in three classes of immunoglobulins, IgG, IgA, and IgM. Twenty-eight sera, 15 from presumably normal volunteers and 13 from bacteriologically positive patients, were diluted in twofold increments from 1:10 to 1:160. These sera were not heat treated. The titer for each class was defined as the highest dilution giving a 2+ or higher fluorescence. The results showed IgG antibodies present in 100% of the patients (Table 1). A similar study with sera from three patients and three controls

FIG. 1. Typical 4+ (top) and 2+ (bottom) fluorescence of aliquots of sera positive for *N. gonorrhoeae* by the indirect immunofluorescent antibody test. $\times 100$.

showed no correlation between antibody titer and Ig class (Table 2). Nevertheless, this experiment suggested that a test for IgG antibodies might provide a suitable screening procedure.

The validity of such a test appeared to be compromised, however, by the finding of a significant IgG antibody titer (≥ 10) in 60% of uninfected controls (Table 1). Since Cohen (1) had reported that natural IgG antibodies are heat labile, whereas immune antibodies are heat-stable, a follow-up study was designed to determine the heat sensitivity of the IgG antibodies encountered in both patients and controls. A total of 25 sera, 14 from culture-negative and 11 from culture-positive patients, were examined at different temperatures and dilutions. Antibodies in culture-positive patients' sera were heat resistant, whereas only three of the sera from negative patients contained heat-resistant antibodies ($P > 0.001$). Two of these three had histories of previous infection with gonorrhea.

In a typical experiment, four sera from patients and four from uninfected controls were serially diluted in phosphate-buffered saline in

twofold increments from 1:5 to 1:80. The diluted sera were incubated for up to 30 min at temperatures from 10 to 70 C. Incubation at 70 C for 10 min or more resulted in complete loss of activity (Table 3), but incubation at 59 C for 30 min distinguished between patient and normal sera. This treatment eliminated the fluorescence observed at a 1:10 dilution in some of the normal sera. The same treatment of patient sera did not result in a drop in the titer, but a minimal reduction in the degree of fluorescence was observed. However, the antibodies in these sera were markedly reduced by heating to 65 C and were completely inactivated at 70 C.

These experiments indicated that a 2+ fluorescence or more at a 1:10 dilution of heat-inactivated serum might be a sensitive and specific indication of infection. To test this possibility, 495 sera (423 from patients with bacteriologically confirmed diagnosis of gonorrhea and 72 controls; see Materials and Methods) were examined as follows.

One milliliter of a 1:10 dilution of the test serum was prepared in physiological saline, and a 0.5-ml portion was heated in a water bath for 30 min at 59 ± 1 C. One drop of the heated, diluted serum was applied to one end of the antigen slide, and 1 drop of the unheated material was applied to the other end. The slide was allowed to stand for 20 min at room temperature in a moist chamber. It was then dipped rapidly 10 times and washed for 10 min in phosphate-buffered saline, pH 7.6, dipped 10 more times in Millipore water, and gently blotted dry. The smears were then counterstained with 1 drop of the working dilution of the anti-human IgG conjugate, incubated for an additional 20 min, and rinsed and dried as described above. One drop of glycerol diluted 1:10 in 0.5 M carbonate-bicarbonate buffer, pH 9.0, was

TABLE 1. Distribution of *N. gonorrhoeae* antibodies in three Ig classes

Population (no. tested)	Sera with titer ≥ 10 (%)		
	IgG	IgA	IgM
Patients (13)	100	46	54
Presumed normal controls (15)	60	27	0

TABLE 2. Titration of *N. gonorrhoeae* antibodies in three Ig classes

Serum ^a	Dilution	IgG	IgM	IgA
Patients with positive cultures				
31057	1:10	4+	2+	3+
	1:20	3+	±	3+
	1:40	2+	±	2+
67031	1:10	4+	2+	±
	1:20	3+	2+	±
	1:40	2+	±	±
69540	1:10	4+	4+	±
	1:20	±	4+	±
	1:40	—	2+	±
Presumably noninfected controls				
GCS-25	1:10	3+	1+	3+
	1:20	2+	±	3+
	1:40	±	±	2+
GCS-32	1:10	4+	±	±
	1:20	2+	±	±
	1:40	±	±	±
GCS-39	1:10	3+	1+	±
	1:20	2+	1+	±
	1:40	±	±	±

^a Unheated samples only.

TABLE 3. Effect of temperature on the titer of *N. gonorrhoeae* antibodies^a

Serum	Titer after incubation at:					
	70 C	65 C	59 C	56 C	25 C	10-14 C
Patients						
1	Neg	40	40	40	40	40
2	Neg	40	80	80	80	80
3	Neg	ND	20	10	10	10
4	Neg	20	40	40	ND	40
Controls						
1	Neg	Neg	Neg	Neg	10	10
2	Neg	Neg	Neg	Neg	5	5
3	Neg	Neg	5	ND	ND	20
4	Neg	ND	5	10	ND	10

^a Incubation time, 30 min. The titer was defined as the highest dilution of serum giving 2+ fluorescence or higher. ND, Not done; Neg, negative.

placed on a smear, and a cover slip was applied. Finally, the stained slides were examined, and the fluorescence of the two smears on each slide was measured. Serum specimens that showed 2+ fluorescence or more, with no or insignificant reduction after heating, were considered positive.

The results are summarized in Table 4. Only one (1.4%) of the sera from normal controls had heat-stable *N. gonorrhoeae* antibodies. This serum was from a young girl admitted to Children's Hospital in Buffalo, and no bacteriological studies were done. In contrast, antibodies were demonstrated in 212 (94.6%) of the sera from infected women and 173 (86.9%) of the sera from infected men.

DISCUSSION

The indirect immunofluorescent technique has been used successfully in the diagnosis of several bacterial, viral, and parasitic diseases. Hess et al. (9) used it satisfactorily to demonstrate *N. gonorrhoeae* antibodies in sera from patients with gonococcal arthritis, and Cohen (1) used it to study the Ig class distribution of these antibodies as well as to monitor the immune response to experimental gonorrhea in male volunteers (2). All of these investigators, however, examined sera from chronic or complicated cases of gonorrhea, which usually show a high antibody titer. Welch and O'Reilly (24), on the other hand, used a selected group of

patients with uncomplicated gonorrhea and observed a much lower mean titer. Our identification of the L-antigen of *N. gonorrhoeae* enabled us to reevaluate the indirect immunofluorescent test as a potential screening test to aid in the diagnosis of gonorrhea.

The difference in heat stability between the natural and immune antibodies could be either an inherent characteristic (1) or, more probably, a function of the starting titer and temperature (10). Heat inactivation of antibodies has been reported (3, 5, 7), and Muschel and Trefers (10) observed that heating the serum to 56 C for as little as 4 min reduced the antibody titer, especially with low-titered sera.

The comparison between the heat-inactivated and unheated serum specimens is important to differentiate between natural and immune antibodies, especially at borderline levels, i.e., a fluorescence between 1+ and 2+ in the heated smear. Generally the heated and unheated aliquots of a given sample varied only slightly (e.g., 4+ to 3 to 4+) or no more than 1+ (e.g., 4+ to 3+). Samples that fluoresced 3+ or more in both aliquots were reported as "positive." When fluorescence was 2+ in both aliquots or 1 to 2+ in both aliquots, the report was "borderline reactive" or "suspicious." Most of these borderline specimens were retested once or twice before they were reported as such. If the unheated aliquot was 2+ and the heated was 1 to 2+ or 1+, the sample was reported as "negative."

Our results confirm the feasibility of using the indirect immunofluorescence technique for the diagnosis of gonorrhea (12, 24), but this test appears to be more sensitive, detecting 94.6% of infected women and 86.9% of infected men, compared to 79 and 56% in the test described by Welch and O'Reilly (24). The higher sensitivity may be attributable to the antigen used in our studies, as well as to the fact that the serum specimens were examined at a lower dilution. The increased sensitivity was achieved without loss of specificity, as only 1.4% of the negative controls were reactive, compared to the 4.0% false positivity rate observed by Welch and O'Reilly. The specificity may result from the use of IgG-specific antibodies, which would eliminate reactions due to IgA antibodies, and from the use of unheated versus heated grading of the fluorescence to differentiate natural and immune antibodies.

The lower detection rate of the test with male patients is to be expected from the nature of the disease. The male patient is usually symptomatic and may seek treatment within 3 to 7 days after exposure, whereas the asymptomatic

TABLE 4. Results of preliminary assay

Group tested	No. tested	No. re-active	% Re-active
Patients with positive cultures			
Group 1 (ACVD ^a)			
Male	130	109	83.8
Female	31	27	86.0
Group 2 (statewide)			
Male	39	37	94.9
Female	95	92	96.8
Group 3 (statewide)			
Male	30	27	90.0
Female	98	93	94.9
Combined results			
Male	199	173	86.9
Female	244	212	94.6
Total	423	385	91.0
Presumed negative controls			
Children			
Male	19	0	0
Female	25	1	4.0
Adults			
Male	20	0	0
Female	8	0	0
Combined results			
Male	39	0	0
Female	33	1	3.3
Total	72	1	1.4

^a Albany County Venereal Disease Clinic.

female may harbor the infection considerably longer before diagnosis.

The lag period between infection and the appearance of antibodies detectable by this method is not known, but Cohen et al. (2) have reported a twofold increase in IgG antibodies in 80% of the men in their test group 5 to 10 days after experimental infection. This is comparable to the 86% reactivity in our population and suggests that our method may be sensitive enough to detect infection 1 to 2 weeks after exposure. These encouraging results have been obtained with selected sera. A field evaluation is currently in progress in our laboratory to evaluate the sensitivity and specificity of the test for the general population, the effect of previous infection, and the effect of treatment.

ACKNOWLEDGMENTS

We acknowledge the cooperation of Erwin Neter, Buffalo, N.Y.; the physicians in private practice; and the staffs of the Albany and Orange county health departments in supplying the sera and necessary information for this study.

LITERATURE CITED

- Cohen, I. R. 1967. Natural and immune human antibodies reactive with antigens of virulent *Neisseria gonorrhoeae*: immunoglobulins G, M, and A. *J. Bacteriol.* 94:141-148.
- Cohen, I. R., D. S. Kellogg, Jr., and L. C. Norins. 1969. Serum antibody response in experimental human gonorrhoea: immunoglobulins G, A, and M. *Br. J. Vener. Dis.* 45:325-327.
- Cohn, M., and A. M. Pappenheimer, Jr. 1949. A quantitative study of diphtheria toxin-antitoxin reaction in the sera of various species including man. *J. Immunol.* 63:291-312.
- Danielsson, D. 1965. The demonstration of *Neisseria gonorrhoeae* with the aid of fluorescent antibodies. 3. Studies by immunofluorescence and double diffusion-in-gel technique on the antigenic relationship between strains of *N. gonorrhoeae*. *Acta Pathol. Microbiol. Scand.* 64:243-266.
- Felton, L. D., and G. H. Bailey. 1926. The immunological characteristics of a water-insoluble protein in type I anti-pneumococcus sera. *J. Immunol.* 11:197-214.
- Fiumara, N. J. 1972. The diagnosis and treatment of gonorrhea. *Med. Clin. North Am.* 56:1105-1113.
- Gump, D. W., P. Tarr, C. A. Phillips, and B. R. Forsyth. 1971. Bactericidal antibodies to *Haemophilus influenzae*. *Proc. Soc. Exp. Biol. Med.* 138:76-80.
- Handsfield, H. H., T. O. Lipman, J. P. Harnisch, E. Tronca, and K. K. Holmes. 1974. Asymptomatic gonorrhea in men: diagnosis, natural course, prevalence, and significance. *N. Engl. J. Med.* 290:117-123.
- Hess, E. V., D. K. Hunter, and M. Ziff. 1965. Gonococcal antibodies in acute arthritis. *J. Am. Med. Assoc.* 191:531-534.
- Muschel, L. H., and H. P. Treffers. 1956. Quantitative studies on the bactericidal actions of serum and complement. II. Some implications for mechanisms of the bactericidal reaction. *J. Immunol.* 76:11-19.
- Organon, Inc. 1973. Gonosticon Dri-Dot—a two-minute serologic slide test for gonorrhea. Organon, Inc., West Orange, N.J.
- O'Reilly, R. J., B. G. Welch, and D. S. Kellogg, Jr. 1973. An indirect fluorescent-antibody technique for study of uncomplicated gonorrhea. II. Selection and characterization of the strain of *Neisseria gonorrhoeae* used as antigen. *J. Infect. Dis.* 127:77-83.
- Pariser, H. 1972. Asymptomatic gonorrhea. *Med. Clin. North Am.* 56:1127-1132.
- Pariser, H., and A. F. Marino. 1970. Gonorrhea—frequently unrecognized reservoirs. *South. Med. J.* 63:198-201.
- Peacock, W. E., Jr., J. E. Martin, Jr., J. D. Thayer, and A. L. Schroeter. 1965. Immunofluorescent detection of serum antibodies to the gonococcus among meningococcal carriers, p. 649-651. *Antimicrob. Agents Chemother.* 1964.
- Ratnatunga, C. S. 1971. Evaluation of the gonococcal complement-fixation test. *Br. J. Vener. Dis.* 47:279-288.
- Reyn, A. 1949. Serological studies on gonococci. II. Cross-absorption experiments and factor serum determinations. *Acta Pathol. Microbiol. Scand.* 26:234-251.
- Reyn, A. 1969. Recent developments in the laboratory diagnosis of gonococcal infections. *Bull. W.H.O.* 40:245-255.
- Reyn, A. 1972. Serological tests for gonorrhoea. 1972. *Br. Med. J.* 1:584.
- Torrey, J. D., and G. T. Buckell. 1922. A serological study of the gonococcus group. *J. Immunol.* 7:305-359.
- U.S. Public Health Service. 1972. Reported incidence of notifiable diseases in the United States, 1972. *Morbidity Mortal. Week. Rep. Annu. Suppl.* 21(53):14.
- Wadsworth, A. B. 1947. Standard methods of the Division of Laboratories and Research of the New York State Department of Health, 3rd ed., p. 205. The Williams & Wilkins Co., Baltimore.
- Watt, P. J., M. E. Ward, and A. A. Glynn. 1971. A comparison of serological tests for the diagnosis of gonorrhoea. *Br. J. Vener. Dis.* 47:448-451.
- Welch, B. G., and R. J. O'Reilly. 1973. An indirect fluorescent-antibody technique for study of uncomplicated gonorrhea. I. Methodology. *J. Infect. Dis.* 127:69-76.
- Wilson, J. F. 1954. A serological study of *Neisseria gonorrhoeae*. *J. Pathol. Bacteriol.* 68:495-510.