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ENZYME-LINKED IMMUNOSORBENT ASSAY WITH POLYVALENT GONOCOCCAL ANTIGEN

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SUMMARY. An indirect enzyme-linked immunosorbent assay (ELISA) with a mixture of eight different gonococcal outer-membrane proteins (OMP) as coating antigen was evaluated for detection of gonococcal antibody in 507 sera obtained from patients selected from high-risk and low-risk populations. The indirect ELISA method was more specific and sensitive when the polyvalent antigen was used than when OMP from only one serotype was used. Past episodes of gonorrhoea had a significant influence on the seropositivity of the test. In a selected low-risk population the specificity of the assay was 94% and in a selected high-risk population the sensitivity was 78%. When sera from 24 asymptomatic individuals were tested the sensitivity was 83%. The ELISA polyvalent-antigen test should be useful as an aid for the detection of gonorrhoea in populations with a low prevalence.

INTRODUCTION

The available methods for epidemiological study, diagnosis and treatment are inadequate to control the present pandemic of gonorrhoea (*World Health Organization*, 1978). A major problem is the identification and treatment of male and female asymptomatic carriers in a population with a low incidence of gonorrhoea. The use of traditional culture techniques presents several difficulties. There are the trouble and expense of screening large numbers of patients to detect a few carriers. Screening requires a pelvic examination in the female and direct urethral scraping in the male. Even with the sampling of many sites and direct inoculation of material onto selective media, the isolation rate of *Neisseria gonorrhoeae* in overtly infected patients will not exceed 85%.

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and in epidemiological screening of asymptomatic individuals may be as low as 16% (Gaafar, 1979). There is clearly a need for a reliable serological test for gonococcal carriage.

During recent years, several methods for detection of antibody have been described, and of these radioimmunoassay (RIA), with purified pili as antigen (Oates *et al.*, 1977), and enzyme-linked immunosorbent assay (ELISA), with cell-surface proteins as antigen (Glynn and Ison, 1978; Gaafar, 1979), appear most promising. Brodeur, Ashton and Diena (1978) used ELISA to detect antibodies to a complex of gonococcal outer-membrane proteins (OMP). Cross reactivity was not observed with OMP extracted from non-pathogenic *Neisseria* spp. and very low levels of cross reactivity were recorded with OMP extracted from *N. meningitidis* serogroup A. The reaction of immune sera against OMP was not inhibited by lipopolysaccharide (Brodeur *et al.*, 1978; Johnston, 1980).

We here describe an indirect ELISA method that uses as antigen a mixture of OMP from eight prevailing serotypes of *N. gonorrhoeae*, and preliminary results of its use in population screening.

MATERIALS AND METHODS

Bacterial strains. Strains of *N. gonorrhoeae* serotypes A, B, D, E, S, T, W and X were obtained from Dr K. H. Johnston (University of Texas, Health Science Center, Dallas, Texas, USA). The bacteria were routinely grown at 37°C, in an atmosphere containing 5–10% CO₂, for 20 h on solid medium (GC Agar Base, Oxoid) supplemented with 1% IsoVitalex (Baltimore Biological Laboratories) and 0.4% yeast extract (Oxoid), as previously described (Brodeur *et al.*, 1977). Identification of each strain of *N. gonorrhoeae* was confirmed by positive oxidase reaction, typical appearance after gram stain, and fermentation of glucose but failure to ferment maltose or sucrose.

Preparation of antigen. Serotype-specific OMP were extracted from bacteria by the method of Johnston, Holmes and Gotschlich (1976) with slight modifications. Briefly, 15 g (wet weight) of whole cells were suspended in 100 ml of 300mM lithium acetate buffer, pH 5.8, containing 20mM sodium chloride and 10mM sodium ethylenediaminetetracetate (EDTA). The bacteria were shaken with 6-mm glass beads at 300 rpm for 2 h at 45°C. The spheroplasts were removed by centrifugation at $3 \times 15\,000\,g$ for 20 min and the supernate was acidified to pH 4.0 with glacial acetic acid. After 18 h at 4°C the precipitate was collected by centrifugation at 40 000 g for 1 h and washed in 100mM tris (hydroxymethyl) aminomethane-hydrochloride, pH 8.5, with 10mM lithium chloride and 10mM EDTA (tris buffer). After centrifugation at 40 000 g for 60 min, the pellet was suspended in 100mM glycine-NaOH buffer, pH 8.5, with 10mM EDTA and 2% sodium cholate, and incubated at 22°C for 30 min. The suspension was concentrated to 3 ml by pressure dialysis, on an Amicon PM-30 membrane, washed with 50 ml of tris-buffer and reconcentrated to 3 ml. The concentrated solution was applied to a 25 × 450-mm column of Sepharose-6B equilibrated with tris buffer. The separation was achieved at a flow of 32 ml cm²/h and 8-ml fractions were collected. The eluant was monitored at 280 nm. Fractions eluting at the void volume (74 ml) of the column were concentrated eightfold by pressure dialysis (Amicon PM-30) and used as antigens.

The protein content of the fractions was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Mississauga, Ontario).

ELISA procedure. Antibodies to gonococcal OMP were measured by the ELISA technique described by Brodeur *et al.* (1978) with antihuman IgG conjugated with alkaline phosphatase (Miles Laboratories, Elkhart, Indiana, USA). The optimal concentration of antigen, antiserum and conjugate used in these experiments was determined by chequerboard titration to obtain

maximum differences between positive and negative sera. Usually, 0.2 ml of a solution of antigen 2.5 mg/L in 0.05M carbonate buffer, pH 9.6, was dispensed to each well in a flat-bottomed microtitration plate (Micro-ELISA Immulon plate, Dynatech Laboratories, Alexandria, Va, USA) and absorbed for 18 h at 22°C. The antigen solution was aspirated and the microplate was washed once for 3 min with PBS containing 0.05% Tween-20 (Sigma Chemical, St Louis, Mo, USA), and 0.2 ml of 0.1% bovine serum albumin (BSA) was added to each well. After 1 h at 37°C the BSA was discarded and the plate was washed twice more. The test serum and a standard serum were always diluted 1 in 200 in PBS, containing 0.05% Tween-20 and 0.4% BSA, and 0.2 ml of each dilution was transferred to each well of the antigen-coated plate. The plate was incubated for 1 h at 37°C in a humidified atmosphere. The serum was removed and the plate was washed thrice more as before. Alkaline-phosphatase-conjugated goat anti-human immunoglobulin was diluted 1 in 4000 in PBS containing 0.05% Tween-20 and 4% BSA, and 0.2 ml of this solution was placed in each well. After incubation for 1 h at 37°C in a humidity chamber, the plate was washed as above and 0.2 ml of a solution of enzyme substrate, p-nitrophenylphosphate (Sigma Chemical Co., St Louis, Mo), 1 g/L in 10% diethanolamine buffer, pH 9.8, containing 0.001M MgCl₂, was added. The enzyme reaction was performed at 22°C and read several times from 25 to 30 min of reaction with a Titertek

TABLE I
Anti-outer-membrane-protein antibody activity of sera from different groups

Origin	Group*	Cultures for <i>N. gonorrhoeae</i>	Previous history of gonorrhoea	Number of sera	Percentage of sera with ELISA > 0.14
Family-planning clinic	Selected low-risk	Negative	No	312	6
Laboratory and hospital staff	Unselected low-risk	Not done (presumably negative)	No	26	7
Venereal-diseases clinic	Selected high-risk	Positive	No	101	78
		Positive	Yes	49	97
		Negative	Yes	19	100

* See *Materials and methods*.

Multiskan Plate Reader (Flow Laboratories, Mississauga, Ontario, Canada). When the standard serum reached an absorbance value of 0.7 ± 0.025 the data were recorded immediately, avoiding the need to stop the reaction. In each experiment duplicate assays were carried out and the average absorbance value (at 400 nm) was recorded.

Sera and patient populations. This study involved 507 sera obtained from the Edmonton Provincial Laboratory, the Ottawa venereal-disease clinic and from laboratory and hospital staff. Of these sera, 80% were part of a group collected from June 1976 to June 1977 for a project in which the Fisher gonococcal screening test and the Fluorescent Gonorrhea Test-Heated (Fisher Lederle Laboratories) were tested.

The sera were divided into three groups (see table I). 1) 312 selected low-risk sera from patients attending a family-planning clinic; 2) 26 unselected low-risk sera from laboratory or hospital volunteers; 3) 169 selected high-risk sera were obtained from venereal-disease clinics; this group included sera from 24 asymptomatic carriers who were contact referrals. There were no specimens from patients with disseminated gonococcal infection. From each of the patients in groups 1 and 3, but not from the volunteers in group 2, urethral and one or more of cervical, rectal and pharyngeal swabs (Toschach *et al.*, 1979) were taken for culture for *N. gonorrhoeae*.

All the sera were stored in 1-ml portions at -70°C. There was no significant variation of the extinction value of the standard positive sera during a period of 12 months.

RESULTS

Comparison of monovalent and polyvalent gonococcal antigens

To overcome the problem of heterogeneity among strains of *N. gonorrhoeae* and because there is little cross reactivity among certain antigenic variants of gonococcal outer-membrane proteins, we decided to evaluate, as coating antigen, a mixture of equal amounts of OMP from eight serotypes. From preliminary results on the serotyping of Canadian strains of *N. gonorrhoeae*, the serotypes A,B,D,E,S,T,W and X described by Johnston *et al.* (1976) were selected and combined in equivalent amounts to form the polyvalent antigen. In all cases studied, the binding curves obtained with the polyvalent antigen were either superior or at least equal to the binding curves obtained with a single OMP antigen extracted from *N. gonorrhoeae* serotype B. Furthermore, when 37 sera from low-risk patients were tested against both antigens, the antigen from serotype B gave 17 (46%) false-positive reactions, while the polyvalent antigen gave only 5 (13%) false-positive reactions. These selected low-risk patients were individuals presenting themselves at the family planning clinics, were culture negative for *N. gonorrhoeae* and had no previous history of gonococcal infection.

Correlation of gonococcal culture with anti-OMP antibody activity

From patients attending venereal-disease clinics, 169 sera, classified as high-risk, were obtained. In all, 150 patients had a culture positive for *N. gonorrhoeae*, and of these only 101 had no previous gonococcal infection or treatment (table I). The selected low-risk sera were collected from patients attending a family-planning clinic and comprised 312 sera from patients who had negative cultures for *N. gonorrhoeae* and no previous history of gonococcal infection, urethritis, vaginitis, cystitis, dysuria or pelvic inflammatory disease. The lowest extinction value that allowed distinction between seropositive culture-positive and seronegative culture-negative patients was 0.14 degrees of absorbance (at 400 nm). This ELISA value was accurately reproducible each time the enzyme reaction was read when the mean of the standard positive antiserum was 0.7 ± 0.025 . The distribution of serological results for the culture-positive and culture-negative groups is represented in fig. 1. In the culture-positive group, 54% of the patients had an extinction value greater than 0.23 as against <1% in the culture-negative group. From culture-positive individuals, 22 sera had undetectable antibody activity (<0.14) and 20 sera from the culture negative group gave a false-positive reaction.

In addition, 26 sera were collected from laboratory and hospital staff and represent the unselected low-risk group with no history of gonococcal or meningococcal infection. Material from the people in this group was not cultured for *N. gonorrhoeae* but was presumed to be negative. In this group, the percentage of those having antibody giving an ELISA value greater than 0.14 was 7% (table I). The two positive sera had ELISA values of 0.18 and 0.20.

Effect of previous gonococcal infection on serological results

The influence of a previous gonococcal infection on serological testing by ELISA is shown in fig. 2. From patients with a past history of gonorrhoea and currently either positive or negative culture of *N. gonorrhoeae*, 68 sera were selected from the high-risk group. Only one individual of the 68 (one in the group with positive cultures currently) was serologically negative. The mean ELISA value of culture-positive and culture-negative groups was higher among those who had a previous history of gonococcal infection. It is interesting to note that in the group of culture-negative patients with no previous history of gonorrhoea only three had an ELISA value greater than 0.23 while 95% of the culture-negative patients with past history of infection had greater antibody activity. Even though the number of sera tested in the culture-negative seropositive group was small, it seems that the persistence of OMP antibodies due to previous infection might be a limiting factor in the use of a serological test in a high-risk population in which recurrent infection is often noticed.

FIG. 1.—Distribution of antibody activity to gonococcal OMP by culture results in patients with no history of previous gonococcal infection.

FIG. 2.—Distribution of antibody activity to gonococcal OMP by culture results in patients with a history of previous gonococcal infection.

FIG. 3.—Serological results in asymptomatic carriers.

TABLE II
Sensitivity and specificity of ELISA polyvalent-antigen test

Group of patients*	Number of sera	Sensitivity per cent	Specificity per cent
Selected low-risk	312	...	94
Unselected low-risk	26	...	92
Selected high-risk	101	...	—
(Asymptomatic)	24	83	(...)

* See *Materials and methods*.

Antibody activity in sera from asymptomatic carriers

Twenty-four patients in the high-risk group had a positive culture for *N. gonorrhoeae* and were asymptomatic. These patients attended a venereal-disease clinic as contact referrals. As shown in fig. 3, these asymptomatic carriers had approximately the same distribution of antibody activity as patients with symptomatic gonorrhoea. From culture-positive patients, 4 (16%) sera had undetectable antibody activity. The same 24 sera were also tested with the gonorrhoea screening test or the fluorescent gonorrhoea test-heated and these gave respectively 29% and 45% false-negative results.

Sensitivity and specificity of ELISA/polyvalent antigen test

The correlation between patients with or without disease and serology in different populations is better expressed in terms of sensitivity and specificity in table II. Specificity is defined as the percentage of seronegative results among people who do not have the disease, i.e., were culture negative for *N. gonorrhoeae*. Sensitivity is defined as the percentage of seropositive results in patients with the disease, i.e., who were culture positive for *N. gonorrhoeae*. As shown in table II, the sensitivity for the selected high-risk population was 78%; but more interestingly, although the number of sera was small, was the sensitivity of 83% for the asymptomatic carriers. The specificity of the test in the two populations was 94% and 92% respectively.

DISCUSSION

A reliable and inexpensive serodiagnostic test is needed for screening populations with a low incidence of gonorrhoea. To date, most serodiagnostic tests have been done with antigens extracted from single strains of *N. gonorrhoeae*. Because recent findings have demonstrated extensive antigenic polymorphism among strains of *N. gonorrhoeae* (Johnston *et al.*, 1979; Wong *et al.*, 1976; Wang *et al.*, 1977; Armstrong *et al.*, 1979; Johnston, 1980) the use of an antigen from a single gonococcal strain would presumably limit the

sensitivity of an assay. Therefore, a polyvalent coating antigen was fabricated with equivalent amounts of outer-membrane proteins from eight different serotypes. When the polyvalent antigen was tested with sera from *N. gonorrhoeae* culture-positive patients, the antigen-antibody reactions or extinction values observed with the polyvalent antigens were always equivalent to or higher than the values obtained with monovalent antigen, thus indicating improved sensitivity with the polyvalent antigen.

In addition, with negative control sera, false-positive reactions due to cross reacting antibodies to other species of *Neisseria* or to other organisms sharing antigens with the gonococcus were significantly lower when compared with single outer-membrane protein antigen. The minimal protein concentration of a single antigen required to give a reproducible antigen-antibody reaction or ELISA value is approximately 1 mg/L (Brodeur *et al.*, 1978; Johnston, 1980). By using protein at 0.125 mg/L for each antigen in the polyvalent antigen we are working with a subminimal antigenic concentration for a single antigen that effectively eliminated cross reactivity of antibodies to that particular strain of *N. gonorrhoeae* with other organisms. Thus, while the specificity of the test with the polyvalent antigen was improved, the sensitivity was not altered because OMP of different gonococcal strains share common antigenic determinants (Johnston, 1980).

Because the incidence of gonorrhoea in the population under investigation will influence the serological results, we selected sera from different populations to estimate the sensitivity and specificity of the ELISA/polyvalent antigens system. The number of negative reactions in low-risk patients with known negative culture and no previous history of infection did not differ significantly from the number reported by Toshach *et al.* (1979) who used the same sera in the indirect fluorescent-antibody test (IFA) or the fluorescent gonorrhea test-heated (FGT-H). In this selected population the specificity of ELISA/polyvalent-antigen system, IFA and FGT-H were respectively 94%, 96% and 94%. Glynn and Ison (1978) and Gaafar (1979) have also reported similar specificities using either crude outer-membrane extracts or purified L antigen as coating preparations in their ELISA test. In addition, Oates *et al.* (1977) and Holmes *et al.* (1978), using sera from patients attending a family-planning clinic as negative controls, found a specificity of 74% and 86% respectively for the pili-RIA.

The percentages of seropositivity in the group of sera from culture-positive patients with no previous gonococcal infection and especially in the asymptomatic group were similar to the percentages published by Buchanan *et al.* (1973), Oates *et al.* (1977), and Gaafar (1979), who used RIA or ELISA with a monovalent antigen preparation. In contrast, the level of sensitivity reported by Glynn and Ison (1978) was significantly lower for a high-risk population. This variance could possibly be explained by the fact that their sera were collected at an early stage of infection. The serological data reported in this paper and in several other publications for *N. gonorrhoeae* culture-positive or culture-negative individuals who had a past history of gonococcal infection clearly suggest that in high-risk populations the ELISA/polyvalent-antigen system as well as other tests should not be used because antibodies from

previous infections would make the result ambiguous. However, because of its high degree of specificity and objectivity when compared to IFA or FGT-H, as well as its chemical stability, ease of performance and lower cost as opposed to RIA, the ELISA polyvalent-antigen test could possibly be used for screening low-incidence populations where the prevalence of gonorrhoea is in the range 1–5%.

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