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The specificity of serum and local antibodies in female gonorrhoea

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SUMMARY

Immunoblotting has been used to compare the specificity of serum and local IgG and IgA antibodies in 13 women with gonorrhoea and in 13 controls. The technique allowed the simultaneous detection of antibodies to the major outer membrane proteins I, II, and III, pili and lipopolysaccharide; antibodies to another antigen which is probably a 'carbohydrate' were also detected. Serum and local IgG and IgA were found to be produced to several antigens during gonococcal infections, although the quantity of antibody was greater in serum. There was little change in the specificity of serum antibodies whereas the local response to LPS and pili increased over the two week study period. Serum antibody to LPS was more often IgG than IgA. Sera contained antibodies to 'carbohydrate', pili and lipopolysaccharide (LPS) whilst the local response was largely to the latter two antigens. Antibody to the outer membrane proteins was rarely detected. Control sera, but not vaginal washings, contained IgG and IgA to the major antigens but the staining of the immunoblots was less intense than those from patient's sera suggesting quantitative differences.

Keywords specificity antibodies gonorrhoea

INTRODUCTION

Gonorrhoea is initially at least an infection of mucosal surfaces. Systemic (Ison & Glynn, 1979; Buchanan *et al.*, 1973) and local (O'Reilly, Lee & Welch, 1976; McMillan *et al.*, 1979; Tramont *et al.*, 1980) immune responses have been demonstrated although the part either plays in modifying the course of the infection is only poorly understood. The specificity of serum antibodies has been examined by gel-electrophoresis-derived enzyme-linked immunosorbent assay (GED-ELISA) (Hadfield & Glynn, 1982), immunoblotting (Hadfield & Glynn, 1984) and immunoprecipitation (Zak *et al.*, 1984). Functional aspects of the local antibody response were studied by adsorption of genital secretions with purified antigens, and a reduction demonstrated in their ability to inhibit the attachment of gonococci to epithelial cells (Tramont *et al.*, 1980).

Our aim in this study was to examine the host response to gonorrhoea in females using immunoblotting. We have examined and compared the detailed specificity of antibodies to the patients' own infecting strain in serum and vaginal washings.

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MATERIALS AND METHODS

Patient selection. All patients studied were attending the Praed Street Clinic for sexually transmitted diseases in London and had not received antibiotic therapy in the preceding 2 weeks. Thirteen women with gonorrhoea diagnosed by culture at the initial visit were asked to reattend for two further visits at weekly intervals. Control specimens were obtained at one visit only from 13 women who were asymptomatic and had a normal vaginal discharge on examination. The age, presence of symptoms and past history of gonorrhoea for the patients with gonorrhoea are shown in Table 1. There was no significant difference in the age distribution of women with (mean = 23 years, range 17–41) and without gonorrhoea (mean 25 years, range 21–52) and none of the controls had a past history of gonorrhoea.

Specimens collected. The following specimens were collected from each patient at every visit.

During the vaginal examination, urethral and cervical specimens were taken and immediately placed on neisseria isolation medium and stored at 36°C in 7% CO_2 until transported to the laboratory. Vaginal washings were collected by introducing isotonic saline (2 ml) into the vagina through the speculum. The saline was then aspirated by a syringe into a sterile universal container (Sterilin, Feltham, Middlesex). Secretions were not taken during menses. Blood samples were also taken and all specimens transported to the laboratory within 2 h.

Storage of specimens. Vaginal washings were stored in screw cap plastic ampoules (Sterilin) and placed at −70°C as soon as possible to preserve them and in particular prevent degradation of IgA by proteases. Serum was stored in aliquots of 200 μl at −70°C.

Culture and storage of isolates. N. gonorrhoeae was isolated on neisseria medium containing GC agar base (Difco, Becton Dickinson, Cowley, Oxford), 36 g/l + 1% IsoVitalex (BBL, East Moseley, Surrey, UK) made selective by the addition of vancomycin, colistin, trimethroprim and amphotericin. After arrival in the laboratory specimens were incubated for up to 48 h in 7% CO_2 at 36°C. *N. gonorrhoeae* were identified as oxidase positive, Gram negative cocci, that utilized glucose but not maltose and sucrose and did not produce β-galactosidase.

After primary isolation all strains of *N. gonorrhoeae* were maintained on the above medium without the addition of antibiotics. Colonies that were piliated (p^+) were selectively subcultured, suspended in 10% glycerol broth and stored at −70°C.

Table 1. Patient information.

Patient No.	Age	Days since LSI*	Symptoms†	Past History No. attacks (time since last attack in months)
1	17	4	C	0
2	18	6	C	0
3	19	6	C	1 (12/12)
4	19	14	S	0
5	19	3	C	0
6	19	3	C	0
7	19	180	S	0
8	21	2	C	0
9	21	8	S	3 (3/12)
10	22	4	C	0
11	23	7	C	0
12	33	7	S	0
13	35	7	C	1 (12/12)

* (LSI) last sexual intercourse.

† (C) asymptomatic contact of gonorrhoea; (S) symptomatic.

Estimation of total immunoglobulin. Levels of total IgG and IgA were determined on vaginal washings only. The samples were thawed, centrifuged at 1000 ***g*** to deposit cells and bacteria and the supernatant used in a capture enzyme-linked immunoassay. Washings were returned to −70°C to await immunoblotting.

The assay has four steps. (1) Antihuman Ig (Dako Patts, High Wycombe, Bucks, UK), 100 μg/ml, was coated on to a microtitre tray (Flow laboratories, Cruine, Scotland) in carbonate-bicarbonate buffer pH 9·6, at room temperature for 1 h. (2) Vaginal washings and standards were diluted into the tray in phosphate buffered saline pH 7·4+0·05% Tween-20 (PBS-T) (BDH, Dagenham, Essex, UK), and incubated at 30°C for 1 h. (3) Antihuman Ig linked to horse radish peroxidase (Dako) was diluted in PBS-T and added to the appropriate wells for 2 h at 30°C. (4) Substrate, (0·4 mg/ml *o*-phenylene diamine (Sigma Chemical Co., Poole, Dorset, UK) in 0·2 M phosphate citrate buffer pH 5·0+4 μl 3% (w/v) H_2O_2), was added and left at room temperature for 30 min before the reaction was stopped by the addition of 50 μl 2·5 M sulphuric acid. The working volume was 100 μl and between each step the trays were emptied and washed three times with PBS-T.

The standard curves used were for IgG, 0·005–1·0 μg/ml, and for IgA, 0·01–5·0 μg/ml. Vaginal washings were diluted in a range 1:500–10,000 for IgG and 1:100–2,000 for IgA. All class specific antihuman Ig sera conjugated to horse radish peroxidase were used at a dilution of 1:500. Results were read at 492 nm, compared to the standard curve and expressed as μg/ml Ig.

Preparation of whole cell antigen. The isolate of *N. gonorrhoeae* (p^+) from each patient and laboratory strain H1 were grown for 24 h as described above. Growth was suspended in saline, the protein estimated (Lowry *et al.*, 1951) and the suspension diluted to 1 mg/ml protein in sampling buffer and boiled at 100°C for 10 min (Hadfield & Glynn 1982).

Immunoblotting. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and antibody detected as previously described (Hadfield & Glynn, 1984).

The sera and vaginal washings from each patient with gonorrhoea were tested against the patient's own isolate of *N. gonorrhoeae*. The samples from controls were tested against *N. gonorrhoeae (H1)* used in previous antibody studies (Ison & Glynn, 1979). The whole cell antigen, (5 μg protein), was placed in duplicate tracks for each sample tested. To each gel, standards and two tracks of antigen were added for control purposes. One track was stained for protein only and the remaining track tested with mouse antisera against PI, PII and PIII (Hadfield & Glynn, 1984).

Vaginal washings were made up to a volume of 10 ml before testing. This represents a mean dilution of 1:7 both for patients with gonorrhoea (range 1:6–11) and controls (range 1:5–9). There was no significant difference between the dilution of washings collected at visit 1, 2 or 3. Antihuman IgG, IgA and antimouse Ig linked to horse radish peroxidase were all used at a dilution of 1:1000, IgM was not determined as there was no total IgM present in previous unpublished studies.

Statistical analysis. Differences between quantitative date were determined using Student's *t*-test and between qualitative date using Chi squared.

RESULTS

Culture for N. gonorrhoeae

All patients with gonorrhoea were culture negative at each follow up attendance. All controls were culture negative. Specimens were not taken for Chlamydia.

Total immunoglobulin levels in vaginal washing

The levels of total IgG and IgA are shown in Table 2. The concentration of IgG was twice that of IgA. The mean levels of IgG and IgA in patients with gonorrhoea did not differ significantly between visits. The IgA but not IgG level in controls was significantly lower than in patients (Student's *t*-test, $P < 0{\cdot}05$).

Serum antibody response in patients with gonorrhoea

All patients produced IgG and IgA antibodies to their own infecting strain as detected by

Table 2. Total immunoglobulin levels in vaginal washings.

	Patients visit:			
	1	2	3	Controls
IgG (μg/ml)				
mean	64	42	43	9
range	<1–500	6–170	4–100	9–22
IgA (μg/ml)				
mean	27*	20	21	5*
range	1–146	6–74	3–50	1–20

* Student's *t*-test, $P < 0{\cdot}05$.

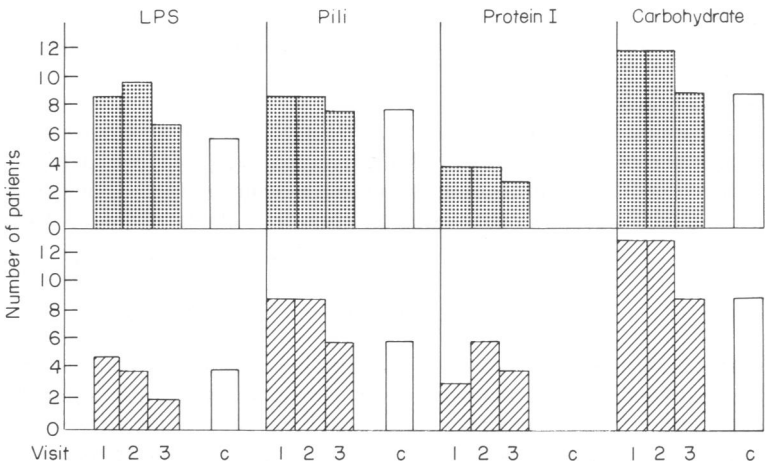

Fig. 1. The frequency and specificity of serum IgG (▨) and IgA (■) in patients and controls (□, C). Only nine patients attended at visit 3.

immunoblotting. The frequency of IgG and IgA antibody detected to individual antigens at each visit is shown in Fig. 1. IgG and IgA were produced to lipopolysaccharide (LPS), pili, protein I, and 'carbohydrate', which appeared as a streak between apparent subunit molecular weight 60–40 K. There was no significant difference in the frequency of these antibodies. However, there was a significantly greater number of patients with IgG than IgA to lipopolysaccharide (χ^2, $P < 0{\cdot}05$). Antibody could be detected in sera at each visit in all patients. Antibody against additional antigens was detected at subsequent visits in a total of 10 patients, four for IgG and four patients for IgA at the second visit and a further two patients for IgG alone at the third visit. The major change in specificity was an increase in antibody to protein antigens between the first and second visit. Antibody was not detected on a subsequent visit on two occasions, once it was directed against LPS and the second occasion a protein.

Antibody to other minor proteins was detected in four patients but only two patients had antibody directed against Protein II.

Vaginal washings

All patients produced local IgG or IgA or both to their own strain, two patients had only IgG and one patient IgA alone. The frequency of antibody, both IgG and IgA, and specificity of the different classes to individual antigens is shown in Fig. 2. There was no significant difference between the

specificity of the local IgG and IgA response to individual antigens. Six patients showing additional antibody at the second visit. Antibody directed against pili was present in an increasing percentage of patients at each subsequent visit, i.e. 46% (6/13) at visit one, 62% (8/13) at visit two and 89% (8/9) at visit three whereas antibody to LPS and 'carbohydrate' increased between visits one and two but was present in less patients at visit three (Fig. 2). Antibody to Protein I was found in only one patient. No antibody to other proteins was detected.

Comparison of serum and local immune response

The local antibody response was of a lower intensity and present in significantly lower number of patients although local antibody was produced to the major antigens LPS, pili, Protein I and 'carbohydrate'. In vaginal washings but not in serum antibody to pili increased with time. In contrast, antibody to Protein I increased with time in serum rather than vaginal washings.

Effect of clinical history on immune response

All symptomatic patients had a serum response, either IgG or IgA, to Protein I, in addition to other antigens. Although the numbers are small this showed a significant increase compared to asymptomatic patients (χ^2, $P < 0{\cdot}05$). The local response did not differ. Only three patients had a past history of gonorrhoea and all showed a strong reaction to one or more major antigens.

Antibody response in control patients

IgG and IgA was found to *N. gonorrhoeae* strain HI in the sera of all control patients tested, however the intensity of staining was markedly reduced except in two patients suggesting quantitative differences. The specificity of the serum antibody in controls is shown in Fig. 1. There was little or no antibody detected in vaginal washings (Fig. 2). Examples of immunoblots are shown in Fig. 3.

DISCUSSION

Individual purified antigens of *N. gonorrhoeae* have been used in attempts to increase the specificity and sensitivity of antibody tests particularly for screening populations. The techniques of GED-ELISA, immunoblotting and immunoprecipitation have been used to study the immune response to multiple antigens at the same time. Serum from patients with disseminated infection reacts with Protein I (Hadfield & Glynn, 1982; Hook, Olsen & Buchanan, 1984), LPS (Hook *et al.*, 1984) and pili (Hadfield & Glynn, 1982).

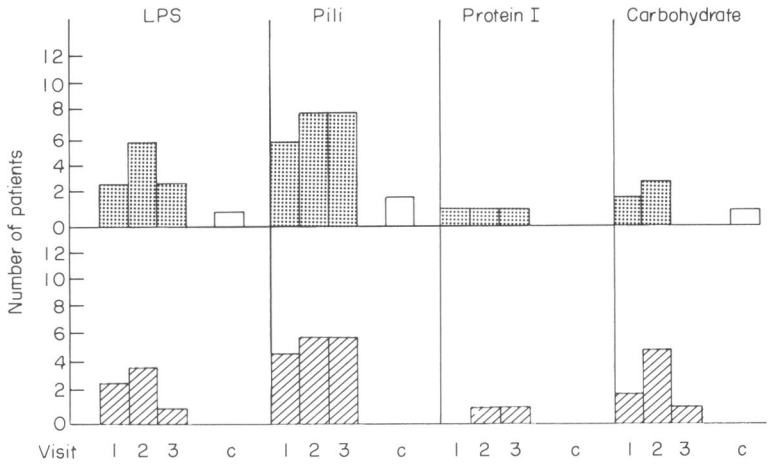

Fig. 2. The frequency and specificity of local IgG (□) and IgA (■) in patients and controls (□, C). Only nine patients attended at visit 3.

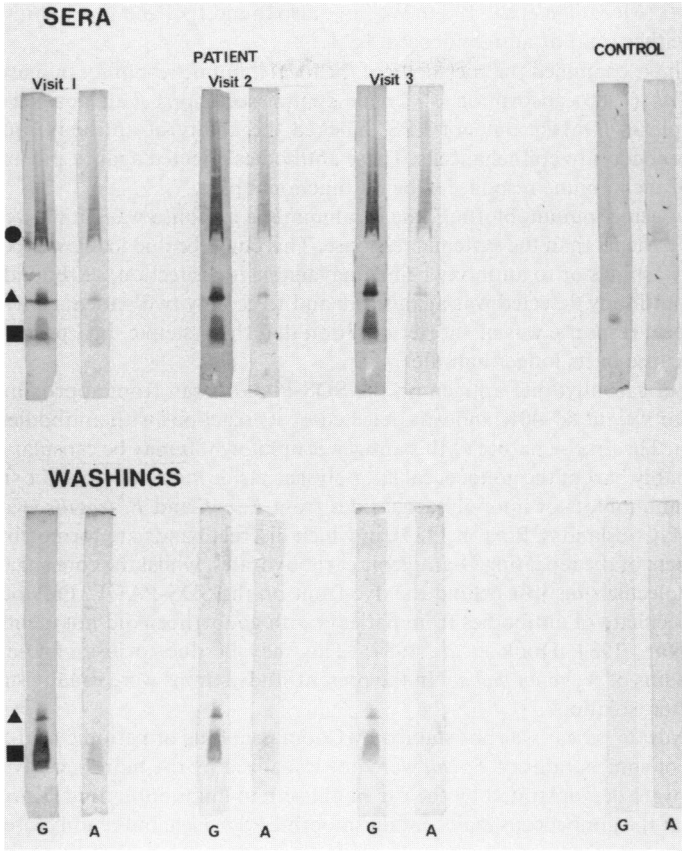

Fig. 3. Examples of immunoblots of sera and vaginal washings from a patient and control. (●)'Carbohydrate'; (▲) pili; (■) LPS.

Some patients with mucosal infection have serum antibody to Protein II and LPS (Zak *et al.*, 1984; Hadfield & Glynn, 1984). However, antibody to Protein I was detected by immunoprecipitation (Zak *et al.*, 1984) but not by immunoblotting (Hadfield & Glynn, 1984). In female mucosal infection we have found antibody directed against pili and LPS in the serum of most patients. The antibody to LPS is more often IgG than IgA as previously demonstrated using outer membrane preparations (Hadfield & Glynn, 1984). Protein I antibody, IgG and IgA, was detected more often in symptomatic than in asymptomatic patients. The numbers tested were small, but this lends support to the view that some variations in the host's response result from changes in antigens presented at the surface of the gonococci in different stages of infection. Protein II antibody was only found in two patients and antibody to Protein III in none.

The local immune response of the female genital tract to infection is poorly understood. However, in gonorrhoea which is primarily an infection of the cervical epithelium it is possible that local antibody may prevent or modify colonization. Plasma cells which preferentially secrete IgA are found mainly in the lamina propria of the endocervix (Rebello, Green & Fox, 1975). Immunoglobulins, IgG and IgA were demonstrated in cervical secretions in a ratio of 2:1 (Tjokronegoro & Sirisinha, 1975) and in the same ratio in this study. The concentrations were similar to those found by McMillan *et al.* (1979), but two- to seven-fold less than O'Reilly *et al.* (1976).

Local antigonococcal antibody was detected by indirect immunofluorescence (McMillan *et al.*, 1979, O'Reilly *et al.*, 1976) and by inhibiting the attachment of gonococci to epithelial cells (Tramont *et al.*, 1977). The class of antibody was IgG and IgA in $>90\%$ and IgM in 39% of

untreated patients (McMillan *et al.*, 1979). We have also found IgG and IgA but have not detected the presence of either total or antigonococcal IgM.

Few studies have examined the specificity of the local immune response. In one patient studied by Tramont *et al.* (1980) adsorption of female genital secretions with purified pili and outer membrane complexes (OMC), but not LPS, blocked the ability of antibody present to inhibit attachment of gonococci to epithelial cells. These antibodies directed against pili and OMC's only represent part of the immune response, albeit a functional part.

In this study, using immunoblotting, the local immune response was of a lower intensity and present in fewer patients than the systemic response. This could be due to a lower concentration of antibody in the washings or to antibody being unavailable for detection, i.e. bound up in immune complexes. The antibody detected was against pili and LPS, only two patients having antibody to Protein I. The local response was of shorter duration that the systemic. IgG persisted longer than IgA possibly because of its longer half life.

The 'smear' of carbohydrate antigen on the SDS-PAGE's ran from approximately apparent subunit molecular weight 60–40K and was detected by its reactions with antibodies from patients with gonorrhoea. The precise nature of this antigen is unknown. It may be capsular polysaccharide but is more probably part of the gonococcal lipopolysaccharide molecule since the smear resembles those seen in immunoblots of lipopolysaccharides from *E. coli* and *B. fragilis* (Karch, Leying & Opferkuch, 1984; Cousland & Poxton 1983), in which discreet bands are formed by progressively increasing numbers of the repeating O-antigenic carbohydrates, whilst the core sugars and the lipid A of the LPS molecule runs just behind the dye-front on the SDS-PAGE. Previous immunoblot studies of the specificity of antibodies from patients with gonorrhoea did not identify this antigen (Hadfield & Glynn, 1984; Hook *et al.*, 1984). This may be due to loss of O-antigens during subculture of strains of *N. gonorrhoeae*. In the present study strains were usually subcultured only once or twice after isolation.

The 'carbohydrate' smear was not stained by Coomassie Blue or periodic acid-Schiffs reagent. The O-antigens of some strains of *E. coli* were not visualized by the more sensitive periodic acid-silver stain, but were demonstrated by the use of antisera in immunoblotting (Karch *et al.*, 1984). This suggests that the gonococcus may contain smooth LPS *in vivo*, but readily loses O-antigen on subculture.

Local and systemic antibody responses to individual antigens varied between samples from any one patient. This may be due to differences in the concentration of antibody between visits. However, all patients were treated successfully with antibiotics at their initial visit, so that subsequent changes in antibody may be responses to degraded bacteria. The gonococcus may expose different antigens on its surface during the course of an infection or antigens may be released on lysis. Confirmation by studying untreated patients over a period of time is not ethically permissible.

Antibody responses to individual antigens can be distinguished by the immunoblot method both in serum and local secretions though quantitation is still unsatisfactory. In local secretions specific antibodies were found only in patients with known gonorrhoea. The number of controls with no evidence of present or past gonorrhoea but with serum antibodies is disturbing. The most likely explanation is that they result from cross-reacting neisserial antigens.

Further work perhaps using more sensitive methods is needed to clarify the antibody responses to the gamut of gonococcal antigens in the varied clinical manifestations of gonorrhoea.

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