Antibodies in gonorrhoea; implications for sero-diagnosis and pathogenicity

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ABSTRACT

The factors affecting the sensitivity and specificity of an enzyme linked immunosorbent assay (ELISA) for the detection of antibodies in the sera of patients with gonorrhoea were examined. The test used a crude deoxycholate extract (DOC extract) and this antigen was known to react with antibodies in the sera of patients with meningococcal meningitis.

The outer membrane proteins in the DOC extract were characterised by their behaviour in sodium dodecyl sulphate-polyacrylamide gels. The DOC extract was found to contain the outer membrane proteins Protein I, Protein II and Protein III as well as lipopolysaccharide and other minor proteins. In an attempt to increase the specificity of the ELISA the most abundant protein in the DOC extract, Protein I, was purified. Protein I reacted with IgG antibody in sera from patients with disseminated gonococcal infection or meningococcal infection, but not in those with gonorrhoea.

To help identify an antigen specific to Neisseria gonorrhoeae extracts from N.gonorrhoeae and N.meningitidis were compared. No antigen reacting specifically with antibodies produced in gonorrhoea was identified, but the extracts showed differences in the specificities of antibodies produced in local and systemic neisserial infections.

The technique of immuno-blotting was used to determine which antigen(s) in the DOC extract was reacting with antibodies in sera from

patients with gonorrhoea. Lipopolysaccharide was found to detect IgG antibodies, IgA antibodies may be directed to a different antigen in the DOC extract. Evidence suggested that the important antigen in the DOC-extract was a protein I-lipopolysaccharide complex.

Immuno-blotting and ELISA were used to examine sera for the presence of antibodies reacting specifically with the strain of N.gonorrhoeae causing the infection. The presence of such antibodies would not be detected in an ELISA using an antigen from a single strain of N.gonorrheae. No evidence was found for the presence in patients' sera of antibodies which reacted with determinants specific to their infecting strain.

The possibility of immune complexes in the sera reducing the sensitivity of the ELISA was also examined.

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ABBREVIATIONS

AP - alkaline phosphatase

ASMW - apparent subunit molecular weight

BSA - bovine serum albumin

DEAE - diethylaminoethyl

DGI - disseminated gonococcal infection

DOC - deoxycholate

DTT - dithiothreitol

Ecr - corrected extinction value

EIA - enzyme immunoassay

ELISA - enzyme linked immunosorbent assay

GED-ELISA - gel electrophoresis derived ELISA

GIRA - gel immunoradio assay

HRP - horse radish peroxidase

IIF - indirect immunofluorescence

IS - infecting strain

KDO - 2-keto 3-deoxy octonate

LiAc - lithium acetate

LPS - lipopolysaccharide

MW - molecular weight

P+ - pilated

P- - non-pilated

PI - protein I

PII - protein II

PIII	-	protein III
PIV	-	protein IV
PAS	-	periodic acid - Schiffs
PBS	-	phosphate buffered saline
RIA	-	radioimmunoassay
RIP	-	radioimmuno precipitation
SDS	-	sodium dodecyl sulphate
SDS-PAG	-	sodium dodecyl sulphate polyacrylamide gel
SDS-PAGE	-	sodium dodecylsulphate polyacylamide gel electrophoresis
STD	-	sexually transmitted disease
Tris	-	2-amino-2 (hydroxymethyl) propane - 1,3-diol, (tris)

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1 INTRODUCTION

Infections caused by Neisseria gonorrhoeae are usually confined to the mucous membranes of the genito-urinary tract and are relatively innocuous. However gonorrhoea places a large burden on the health resources of many countries because of both its high incidence and the development of serious complications from genital gonorrhoea e.g. pelvic inflammatory disease, disseminated gonococcal infection.

In 1980 there were 55,784 reported cases of gonorrhoea in England and this number has remained almost static since 1976 (Anon. 1983). The fact that this number has not been decreased despite the availability of effective antibiotics and increased education programmes is worrying, but of greater concern is the increasing number of antibiotic resistant strains of N. gonorrhoeae. The number of penicillinase producing strains of N. gonorrhoeae is increasing at an exponential rate (McCutchan et al. 1982) and there are reports of multiple resistant strains (Easmon et al. 1983).

The search for a suitable vaccine to control gonorrhoea has focussed research on the immunobiology of N. gonorrhoeae. This has included work to characterise the antigenic structure of N. gonorrhoeae and also studies on the host defense mechanisms. Research has been hindered by the lack of a suitable animal model and in particular very little is known about the immune response in gonorrhoea. A specific serological test for gonorrhoea may provide useful information on the humoral response and could also be used to detect infection.

1.1 The possible applications of a serological test for gonorrhoea

1.1.1 Diagnosis of infection

The most satisfactory method of diagnosis of gonococcal infections is the isolation and identification, by biochemical tests of N. gonorrhoeae. However the sensitivity of culture techniques for the isolation of N. gonorrhoeae is influenced by a number of factors, e.g. (1) the number of sites sampled on the patient, (2) the transfer time of the swab to the laboratory and the transport medium used, (3) the prevelance of strains of N. gonorrhoeae sensitive to vancomycin, an antibiotic used in the selective medium for initial isolation. Figures suggest that the sensitivity of culture is between 80 and 90% (Caldwell et al, 1971; Schmale et al, 1969), although it may fall as low as 40% (Johnson et al, 1969). Consequently a serological test would be useful where a gonoccocal infection is suspected but culture has proved negative. Particularly in cases of complicated gonococcal infection, e.g. salpingitis, disseminated gonococcal infection, where sampling sites for culture is more difficult and antibiotic therapy has often begun before the samples are taken. These patients would also often be available for a second blood sample to be taken and diagnosis confirmed by a rising antibody titre.

1.1.2 A screening test for gonorrhoea

A serological test for gonorrhoea could be used to identify infection in people who would not normally be cultured for N. gonorrhoeae, e.g. in ante-natal or family planning clinics. This

may help to identify asymptomatic carriers of infection who may act as a reservoir of infection. Asymptomatic carriage of N. gonorrhoeae is found in both males and females and may play an important role in the spread of the disease (Shapiro, 1963; Handsfield, 1974).

The predictive values of any test are determined not only by the sensitivity and specificity but also independently by the prevelance of the disease in the population studied (Vecchio, 1966). These terms are defined as:

$$\text{Specificity} = \frac{\text{Number of non-infected people with a negative test}}{\text{Total number of non-infected people tested}} \times 100$$

$$\text{Sensitivity} = \frac{\text{Number of infected people with a positive test}}{\text{Total number of infected people tested}} \times 100$$

$$\text{Predictive value of a positive test} = \frac{\text{Number of positive tests in infected people}}{\text{Number of positive tests}} \times 100$$

$$\text{Predictive value of a negative test} = \frac{\text{Number of negative tests in uninfected people}}{\text{Number of negative tests}} \times 100$$

For a test to be useful in screening populations where the prevalence is low its sensitivity and specificity must be greater than 90%. For example Holmes et al (1978) found 3% of women attending an obstetrics and gynaecology clinic were culture positive for gonorrhoea. If a serological test having a specificity and sensitivity of 90% were used to screen this population the predictive value of a positive test would be only 22%. However Glynn and Ison (1981) have

suggested that this might be overcome by the use of two unrelated antigens used in parallel to detect antibody. A positive serum would react with both antigens. For the above example the use of two tests with sensitivities and specificities of 90% would raise the predictive value of a positive test to 70%.

In view of this it seems unlikely that any screening test for gonorrhoea will be cost effective as the prevalence in the general population is much lower than 3% and reinfection is common. However it may prove useful in some high risk populations.

1.1.3 To study the immune response

There is little information available on the antibody response to infections with N. gonorrhoeae. There are two reasons for this, firstly the lack of a suitable animal model and secondly the need for a specific assay for anti-gonococcal antibodies.

Two separate studies have used whole bacteria in indirect immuno-fluorescence tests to study the immune response. Cohen et al (1969) followed the IgG, IgA and IgM antibody levels in the sera of ten experimentally infected male volunteers. IgG levels rose rapidly in the first week and did not fall until three months later, the IgM response was similar but began falling after two weeks. IgA levels rose for 3 to 4 days and fell after two weeks.

McMillan et al (1979) followed the serum antibody response in uncomplicated gonorrhoea. They found the levels of IgM and IgA increased during the first week, followed by a rise in IgG. After two weeks the IgA and IgM levels had fallen almost to normal whilst the IgG levels remained high after four weeks.

Ison and Glynn (1979) used a DOC extract in an ELISA to follow antibody changes in gonorrhoea. Unlike McMillan et al they found no significant changes in female patients. In male patients IgG levels rose during the first week followed by increases in IgA and IgM.

All three studies used relatively crude preparations to detect antibody and more than one antigen may have been involved.

A study of the immune response in gonorrhoea requires well defined antigens which will not detect antibodies produced to N. meningitidis or other comensal Neisseria.

1.1.4 To monitor the response to vaccination

If a suitable vaccine for gonorrhoea was produced serology could be used to monitor the recipients immune response. This would be particularly useful during vaccine trials and if a multi-component vaccine were used it may help to evaluate the relative importance of the different antigens. A serological test could subsequently be used to ensure that vaccination had stimulated the immune system and so confirm protection.

1.2 Problems in the development of a serological test for gonorrhoea

1.2.1 Specificity

The specificity of an antibody test is a reflection of the antigen used, its purity and uniqueness to the organism of interest. The antigenic structures of N. gonorrhoeae and N. meningitidis are similar and low levels of serum antibodies are produced during the naso-pharyngeal carriage of N. meningitidis (Peacock et al, 1965). Five to ten percent of the normal population are colonised with N. mengitidis, but higher rates have been found in patients attending clinics for sexually transmitted diseases, particularly in homosexual men (Turgeon et al, 1981). Consequently the antigen chosen for the detection of anti-gonococcal antibodies must be unique to N. gonorrhoeae and not detect antibodies to N. meningitidis.

The specificity of the test is lowered by the detection of antibodies from previous infections, here the antibody class used for diagnosis may be important. IgM antibodies are usually indicative of a current infection whilst IgG antibodies may persist for weeks after the infection.

1.2.2 Sensitivity

The sensitivity of a serological test for gonorrhoea is influenced by the antigen and the duration of the infection. Unless the antigen is common to all strains of N. gonorrhoeae causing infections in the population under study, the sensitivity will be lowered. Serogrouping and serotyping of gonococcal strains are based on differences in the outer membrane antigens:

Lipopolysaccharides (Maeland, 1971; Apicella, 1976).

Pili (Buchanan, 1975).

Outer membrane proteins (Johnston et al, 1976; Sandström et al, 1982).

The chosen antigen, or an epitope of it, must be present in all these serologically distinct strains so that antibodies produced to them can be detected. Alternatively a mixture of serologically different antigens could be used to detect the antibodies produced during infection.

The sensitivity of an antibody test for gonorrhoea is also affected by the short incubation period of the mucosal infection, during which only small amounts of antibody are produced. In particular many male patients seek medical advice within 1 to 3 days of contracting a gonococcal infection. The antibody levels produced in gonorrhoea are too low to be detected by agglutination or precipitation and consequently more sensitive methods, e.g. (1) complement fixation, (2) agglutination of antigen coated latex particles or red cells, (3) immunoassays using fluorescent, radioisotopes or enzyme labels, have been used. Even with these more sensitive methods the levels of antibody produced may be below the limits of the assay.

1.2.3 Selection of populations for the evaluation of a test

The sensitivity and specificity of new tests are influenced by the populations chosen for their evaluation. Normal/control sera for the evaluation of serological tests for gonorrhoea have been taken from different populations, i.e. (1) nuns, (2) children (3) staff or students, (4) patients, not attending for STD care, (5) patients attending for STD care who are culture negative for N. gonorrhoeae.

Control sera taken from groups 1 or 2 give the highest and group 5 the lowest specificity. This is probably due to the increasing likelihood of the sera being from persons having an undetected infection or a past history of gonorrhoea.

Positive sera can be from patients with or without complications e.g. pelvic inflammatory disease, epididymitis etc., or from patients with different durations of infection. Both of these factors may influence the sensitivity of the test since higher antibody levels are usually found in patients with complications or who have been infected for longer.

1.3 Antigens utilised in serological tests for gonorrhoea

1.3.1 Whole Bacteria

A diagramatic representation of the surface structure of N. gonorrhoeae is shown in Fig. 1.1. Its structure is like that of many other gram-negative bacteria, the outer membrane consisting of a phospholipid bilayer attached on the inside to a thin layer of peptidoglycan. Embedded in the outer membrane are proteins and lipopolysaccharide. Pili (fimbriae) the filamentous, non-flagellar, surface appendages extend out from the outer membrane.

Whole bacteria have been used in a variety of tests for gonococcal antibodies. The advantage of using whole bacteria are that all of the surface antigens, i.e. pili, outer membrane proteins and lipopolysaccharide, may be available to interact with antibodies of different specificities and they are presented in a form most like that in the natural infection. In addition the antigen can be prepared by any laboratory able to grow N. gonorrhoeae. The disadvantage is that the whole bacteria may contain antigens common to other Neisseria, so lowering the specificity of the assay.

Whole gonococci were first used for the detection of serum antibodies by Muller and Oppenheimer (1906) in complement fixation tests. This test has been widely used since then and some of the results are summarised in Table 1.1.

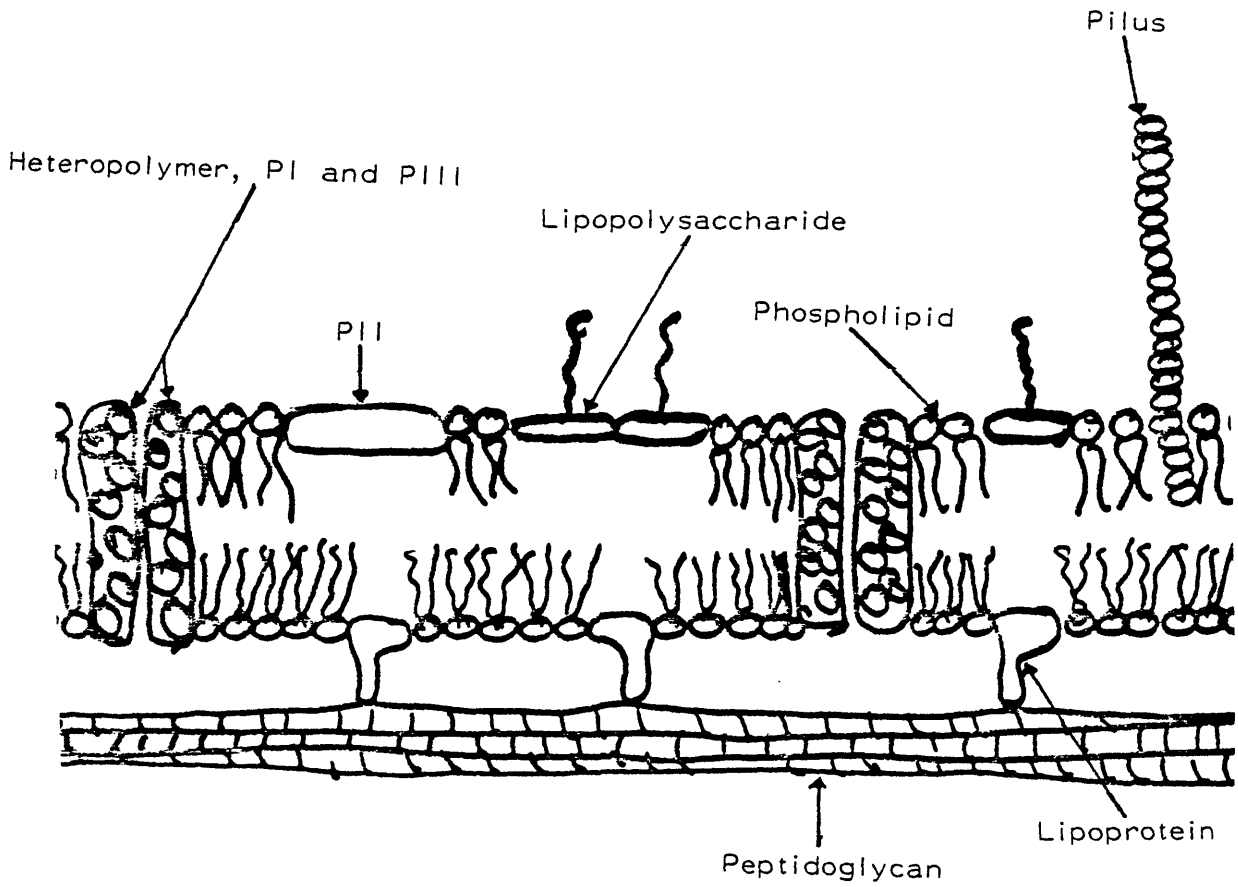


Figure 1.1 Diagramatic representation of the outer membrane of N.gonorrhoeae

The sensitivity of complement fixation tests is relatively high for patients with complicated gonococcal infection, approximately 60%, but is low for uncomplicated infections. The specificity of the test is high even when the control sera are from patients attending clinics for sexually transmitted disease. Due to their low sensitivity complement fixation tests have generally been confined to the diagnosis of complicated infections.

Results of the more sensitive indirect immuno-fluorescent (IIF) test for anti-gonococcal antibodies are summarised in Table 1.2.

N. gonorrhoeae strain 9 was used by Welch and O'Reilly (1973) and McMillan et al (1979) in IIF tests. This strain is reported to have antigens common to most strains of gonococci, but none which cross-react with other strains of Neisseria. However it has not been tested against sera known to contain antibodies to meningococci. McMillan et al (1979) identified the class of antibody produced and their IgG results look particularly promising giving sensitivities of 98 to 100% and specificities of 92 to 94%. The high specificities for all classes of antibody are surprising in sera taken from patients attending a clinic for sexually transmitted disease, but it should be noted that these had been selected to rule out anyone having a gonococcal infection in the previous nine weeks. The authors suggest that the diagnostic value of the IgG results would be reduced if the control sera were from patients with a past history of gonorrhoea.

Antigen	Control Group (No.)	Specificity	Infected Group (No.)		Sensitivity	Reference
20 gonococcal strains	Blood donors and patients attending non-STDC (158)	94	Patients with uncomplicated gonorrhoea	♀(100)	50	Magnusson and Kjellander (1965)
				♂(100)	21	
			Patients with complicated gonococcal infection	♀ (50)	70	
				♂ (50)	60	
"A number of strains"	Culture negative STDC patients (1,164)	94	Culture positive STDC patients	♀(294)	35	Ratnatunga (1971)
				♂(415)	18	
6 gonococcal strains	Blood donors with no past history of STD (100)	95	Culture positive STDC patients	♀ (55)) ♂ (45))	21	Danielsson et al (1972)
			Patients with complicated gonococcal infection	♀ (72)	48	
				♂ (11)	44	
One strain	Culture negative STDC patients (465)	99	Culture positive STDC patients	♀ (86)	9	Reimann et al (1980)
				♂ (98)	11	
One strain (46695)	Culture negative STDC patients (204)	94	Culture positive STDC patients	♀(104)	21	Oranje et al (1983)
				♂(129)	10	
	Blood donors (119)	91	Patients with complicated gonococcal infection)	(11)	64	
	Children (58)	100				

Table 1.1 Complement Fixation Tests for Gonorrhoea

Antigen	Control Group (No.)		Specificity	Infected Group (No.)		Sensitivity	Reference
Strain 9 pilated	Celibate females Culture negative, adolescent females	(50) (120)	96	Culture positive STDC patients	♀(120) ♂(50)	79 56	Welch and O'Reilly, 1973
One strain, a recent isolate	Blood donors Patients attending an antenatal clinic	(34) (148)	100 97	Culture positive STDC patients	♀ (86) ♂(119)	61 20	Wilkinson, 1975
One strain, B370	Children and laboratory personnel	(72)	99	Culture positive STDC patients	♀(244) ♂ (86)	94 86	Gaafar and D'Arcangelis, 1976
Strain 9 pilated	Culture negative STDC patients	(70)	IgA 100 IgG 94 IgM 97	Culture positive STDC patients	♀ (70)	IgA 55 IgG 98 IgM 45	McMillan et al, 1979
		(100)	IgA 100 IgG 92 IgM 97		♂(125)	IgA 51 IgG 100 IgM 56	

Table 1.2 Indirect Immunofluorescent Tests for Gonorrhoea

Antigen	Control Group (no.)		Specificity	Infected Group (No.)		Sensitivity	Reference
N.gonorrhoeae strain HI pilated	Male volunteer medical students	(100)	88	Culture/microscopy positive STDC patients	♀ (100)	56	Ison et al, 1981
					♂ (100)	45	
	Female patients attending non-STDC	(100)	89				

Table 1.3 A whole cell ELISA for gonorrhoea

Ison et al (1981) used poly-L-lysine to attach gonococci to microtitre trays in an enzyme-linked immunosorbent assay (ELISA) to detect IgG serum antibodies. The results are summarised in Table 1.3. Although the sensitivity of this IgG assay is much lower than that obtained in the IIF assay (McMillan et al, 1979), 50% compared with 99%, it does not detect antibodies from previous infections and might therefore have a greater diagnostic potential.

Whole organism assays are useful in serological diagnosis, but they provide little information on the virulence factors of N. gonorrhoeae and any protective antibodies. This requires the use of purified antigens.

1.3.2 Lipopolysaccharides

Lipopolysaccharide is a major constituent of the cell wall of gram-negative bacteria. It is generally composed of three main regions, embedded in the membrane is the lipid-A, attached to this are the core sugars and the 'O' or antigenic sugars which extend out from the membrane. The presence of 'O' or antigenic sugars has not been demonstrated for strains of N.gonorrhoeae (Stead et al, 1975) and therefore gonococcal lipopolysaccharide is classified as rough. The precise structure and chemical composition of gonococcal lipopolysaccharide has not been determined, but it is known to contain 2-keto-3-deoxyoctonate, heptose, glucose, galactose and glucosamine (Perry et al, 1974). These sugars are common to lipopolysaccharides from other species of gram-negative bacteria (Luderitz et al, 1971)

and are very similar to the core sugars found in meningococcal lipopolysaccharide, e.g. all but heptose are found in the lipopolysaccharide from N.meningitidis serogroup B (Linijuco et al, 1978). The similarity in the structure of gonoccocal and meningococcal lipopolysaccharide has been demonstrated with a monoclonal antibody. Apicella et al (1981) produced a monoclonal antibody which reacted with lipopolysaccharides from different serotypes of N.gonorrhoeae and this reaction was partially inhibited by lipopolysaccharide from N.meningitidis. This cross-reaction could lead to a low specificity of any serological test using a lipopolysaccharide antigen (see section 1.2.1).

A second problem in the use of a lipopolysaccharide antigen for serological diagnosis is its antigenic heterogeneity (Glynn & Ward, 1970; Apicella, 1976). Although gonococcal lipopolysaccharide has been shown to contain a common antigen (Tramont et al, 1974; Apicella et al, 1981) antibodies produced during an infection may not be directed to this site but to strain specific sites. If this were the case the use of lipopolysaccharide from one strain of N.gonorrhoeae would give a poor sensitivity.

Some of the results obtained using lipopolysaccharide in gonococcal serology are summarised in Table 1.4. Strain differences are illustrated by the results of Watt et al (1971) and Ward and Glynn (1972). Fletcher et al (1973) used a combination of lipopolysaccharides from ten different strains of N. gonorrhoeae. However the sensitivity of this assay was only equal to that using one strain,

Antigen/Test	Control Group (No.)	Specificity	Infected Group (No.)			Sensitivity	Reference
LPS/Latex agglutination	Laboratory staff and medical students (33)		Culture or smear positive STDC patients	♀(37)	Strain F62	54	Watt et al (1971)
					Strain DA2	89	
	Strain F62	85		♂(80)	Strain F62	46	
	Strain DA2	27			Strain DA2	80	
LPS/Indirect haemagglutination	Blood donors (50)		Culture positive STDC patients)	♀(25)	Strain G1	84	Ward and Glynn (1972)
	Strain G1	98			Strain G2	27	
	Strain G2	90		♂(50)	Strain G1	46	
					Strain G2	33	
LPS's from 10 recent isolates/ indirect haemag-glutination	Blood donors (119)	70	Culture or smear positive STDC patients	♀ (100)		82	Fletcher et al (1973)
	Culture negative STDC patients (138)	70		♂ (100)		51	

Table 1.4 Tests for antibodies to gonococcal lipopolysaccharides

G1, (Ward & Glynn 1972) and the specificity was reduced. These results suggest that the careful selection of one strain may be a more useful approach than the use of several strains.

1.3.3 Pili

Purification methods for the isolation of gonococcal pili have been described by several groups of workers (Brinton et al, 1978; Robertson et al, 1977). Pili are composed of a single repeating peptide unit the molecular weight of which varies between strains from 16 to 20k.

Some of the results obtained using pili in the serological diagnosis of gonorrhoea are summarised in Table 1.5

Buchanan et al (1973) were the first to measure antibodies to gonococcal pili, they used a radioimmunoassay (RIA). The results were promising and the same test was evaluated by Oates et al (1977) for the detection of asymptomatic gonococcal infection in women attending clinics of planned parenthood, family planning and maternity care. The test had a high sensitivity (85%) and specificity (86%) but as the prevelance of gonorrhoea in these populations is around 3% the predictive value of a positive test is low (14%) (Holmes et al, 1978). A major advantage of the RIA method was the quantitation of antibody levels i.e. μg/ml.

Antigen/Test	Control Group (No.)	Specificity	Infected Group (No.)	Sensitivity	Reference
Pili from strain 2686/radio-immunoassay	Patients attending non STDC (133)	100	Culture positive) ♀ (56) STDC patients) ♂ (82)	89 44	Buchanan et al, 1973
	Culture negative STDC patients (600)	86			
Pili from strain 2686/radio-immunoassay	Patients attending non STDC, culture negative (150)	86	Patients attending non-STDC, culture positive asymptomatic ♀ (322)	85	Oates et al, 1977
Pili from strains 82409 and 35209/ indirect haemagg-lutination	Culture negative STDC patients (465)	77	Culture positive) ♀ (86) STDC patients) ♂ (98)	82 59	Reimann et al, 1980
Pili from strain 82409/ELISA	Culture negative STDC patients (444)	88	Culture positive) STDC patients) ♀ (54)	57	Young and Low, 1981
Pili from strain 1443/ELISA	Culture negative STDC patients (204)	74	Culture positive) ♀(104) STDC patients) ♂(129)	45) 32)	
	Blood donors (119)	100		)	Oranje et al, 1983a
Pili from strain 2686/IHA	Culture negative STDC patients (204)	70	Culture positive) ♀(104) STDC patients) ♂(129)	58) 36)	
	Blood donors (119)	100			
Pili from strain 6650	Culture negative STDC patients (28)	71	Culture positive) STDC patients) ♀(104)	69	Oranje et al, 1983b
	Blood donors (119)	92			

Table 1.5 Tests for antibodies to gonococcal pili

In 1975 Buchanan demonstrated that pilus proteins from different strains of gonococci not only varied in molecular weight but were also antigenically distinct. Antisera raised to pili in rabbits only showed about 10% cross-reactivity. Novotony & Cownley (1980) confirmed the antigenic heterogeneity of pili on recent isolates using rabbit antisera in electron microscopy studies. The antigenic heterogeneity of gonococcal pili was shown to be even more complex when Lambden et al (1980) isolated pili variants from a single strain of N. gonorrhoeae (P9). Although these pili variants are structurally similar they vary in their ability to attach to different epithelial cell types and are antigenically distinct (Lambden et al, 1981, Virji et al 1982). These intra-strain variations in pili have also been shown to occur in the natural infection (Duckworth et al, 1983). Monoclonal antibodies, produced to gonococcal pili, demonstrate that common epitopes exist on the pili as well as specific epitopes. The same common epitope also exists on the pili of some meningococci (Virji & Heckels, 1983; Virji et al, 1983).

From the results shown in table 1.5 it appears that during infections antibodies are produced to the common epitopes since a single strain can detect antibody in 80% of patients. As was found with the lipopolysaccharide antigens it seems that the selection of pili from one strain of N. gonorrhoeae which shows a broad cross-reactivity is the most successful approach for the detection of antibodies in humans (Reimann et at, 1982).

1.3.4. Outer Membrane Proteins

The proteins of the gonococcal outer membrane account for approximately 60% of its dry weight (Johnston & Gotschlich, 1974), although they are relatively few in number. Radio-labelling of whole bacteria identified the proteins exposed on the surface of the outer membrane and are thus most likely to be involved in host colonisation. In this way three classes of proteins have been characterised and a fourth proposed (Swanson, 1981). The proteins have been classified on the basis of their occurrence and behaviour in SDS-PAGE.

Table 1.6 gives the names of the proteins following the recommendations on the naming of proteins decided at a workshop on pathogenic Neisseria (Swanson & Heckels, 1980), and the designations previously used in the literature.

Johnson and Gotschlich (1974) first described and purified Protein I (PI), and determined that it accounted for more than 50% of the outer membrane protein. PI is present in all strains of gonococci and has an apparent subunit molecular weight (ASMW) on SDS-PAGE between 32 and 38K. The ASMW of PI is not altered by varying the temperature of solubilisation or by the presence or absence of reducing agents. PI forms a polymer with PIII which spans the membrane to form large aqueous pores.

Gonococcal outer membrane protein	Previous designations	Corresponding Meningo-coccal protein	Characteristics
Protein I (PI)	Major protein (MP) Major outer membrane protein (MOMP) Principle outer membrane protein (POMP) Protein 1	Class 2 and class 3	Most abundant, stable, inter-strain variants in MW but constant within a strain
Protein II (PII)	Minor protein (MP) Opacity associated protein (OAP) Heat modifiable protein (HMP) Protein 2	Class 5	Inter and intra-strain variants in MW, heat modifiable, not always present
Protein III (PIII)	Protein 3	Class 4	Present in all strains, constant MW, MW modified by reducing agents
Protein IV (PIV)		Class 1	

Table 1.6 Gonococcal Outer Membrane Proteins

There is strain to strain variation in the ASMW of PI, but not between colony types of the same strain.

Serotyping methods for N. gonorrhoeae have been based on antigenic differences in PI's. The first was developed by Johnston (1976), he used outer membrane vesicles containing PI, II, III and LPS in agar gel diffusion tests. Two hundred and eighty seven strains were divided into 16 serotypes as a result of antigenic differences in PI. The serotypes were not correlated with pathogenicity or antibiotic resistance. Reproducibility was poor, which may have been due to antigenic variations in other components of the outer membrane vesicles.

A more successful co-agglutination method has been developed by Sandström and Danielsson (1980) with absorbed antisera raised to the serotype strains of Johnston.

In this co-agglutination test 99% of strains causing disseminated gonococcal infection are of serogroup WI and strains of the WII serogroup have an increased resistance to antibiotics. The co-agglutination group is not an absolute predictor of the ASMW of PI suggesting antigenic differences are not directly related to MW.

Swanson (1979) found a high degree of structural homology between the peptide maps of PI's of the same ASMW Peptide maps of PI's having ASMWs of 33 and 34K were different from the lower ASMW 32K PIs. Surface radiolabelling of the bacteria prior to

peptide mapping identified the surface peptides (Judd, 1982, b), some of which were common to all strains but each strain also had unqiue surface peptides. This suggests that the antigenic heterogeneity of PI is expressed on the bacterial surface. Although this has not been confirmed immunologically, peptide mapping of PI's from the three co-agglutination groups, WI, WII and WIII, showed WII and WIII to be similar whilst WI, which are generally of a lower molecular weight, to be different (Sandstrom et al, 1982).

Protein II (PII), when present, is less abundant than PI, it was first described as the leukocyte-association protein (Swanson, 1977). It is largely found in the opaque/dark colony types and is absent, or present in small amounts in transparent/light colony types. The ASMW of PII varies between strains and phenotypes of the same strain. Gonococcal preparations can contain one, two, or no PII(s). The ASMW of PII's is increased when treated at temperatures above 60°, but is not affected by reducing agents.

Peptide mapping of PII's shows structural homology both between strains and between variants within a strain, but no similarity to PI (Swanson, 1980). Each PII has at least one unique surface peptide which may be immunologically important (Heckels, 1981). Diaz and Heckels 1982 used a competitive ELISA to compare five PII variants from a single strain, P9. Less than 5% cross-reactivity was found with antibodies raised in rabbits to outer membranes. Swanson and Barrera (1983) raised antisera to whole gonococci and purified PII's, they used immunoprecipitation and

immunoblotting to study antigenic variations. Surface peptides were unqiue to the PII, but there were common antigenic sites buried in the membrane.

Variations in PII affect the biological properties of N. gonorrhoeae e.g. attachment of N. gonorrhoeae PII variants to epithelial cell types (Lambden et al, 1979). The presence of PII can also increase the serum resistance of gonococci (Lambden & Heckels, 1979). The importance of this as a pathogenic mechanism is suggested by the work of McBride et al (1981) who used the guinea-pig chamber model (Arko, 1972) to study in vivo selection pressures. Initially survivors were transparent, pilated gonococci but these were replaced by opaque, pilated bacteria containing a PII not present in the initial inoculum. Non-pilated strains did not survive and opaque bacteria were killed initially, however as host defences developed the ability of the gonococcus to change its antigenic structure increased the survival. Penn et al (1978) also found that gonococci isolated from guinea-pig chambers were antigenically distinct from those grown in vitro and in addition were more resistant to killing by human phagocytes and resistant to killing by human serum.

Strains isolated from patients with a disseminated gonococcal infection or gonoccal pelvic inflammatory disease are transparent. In local infections the opaque phenotype is usually isolated, suggesting that PII may play a role in evading the local host response.

Protein III (PIII) was first described by McDade and Johnson (1980) as a component of the serotype specific vesicles. PIII is classified on the basis of its change in ASMW in the presence of reducing agents. This protein has been found on all strains and phenotypes of gonococci and its ASMW does not vary between strains. Peptide mapping of PIII's from four strains of gonococci with different PI's, determined they had very similar structures, both on the cell surface and in the outer membrane. (Judd, 1982 a&b). PIII may be responsible for antigenic cross-reactions between gonococcal strains.

Protein IV (PIV) has been proposed as a fourth class of gonococcal outer membrane proteins. The ASMW of 40-44k is not affected by heat or reducing agents PIV is surface radiolabelled to a much lower extent than the other three outer membrane proteins. PIV is very antigenic in rabbits (Swanson 1981).

In summary the outer membrane proteins of N.gonorrhoeae have been shown to display a great deal of antigenic variation. Particularly PII's which like pilus proteins vary not only between strains, but also within a single strain. PI's show variation between, but not within strains. PIII would seem to be the most stable protein being present in the same form in all gonococcal strains and would therefore be the outer membrane protein of choice for serological diagnosis of gonorrhoea.

Antigen/Test	Control Group (No.)	Specificity	Infected Group (No.)	Sensitivity	Reference
DOC extract from strain HI/ ELISA for IgG antibodies	Antenatal clinic patients and medical students (237)	92	Culture or microscopy positive STDC patients ♀(212)	47	Glynn and Ison, 1978
			♂(191)	47	
	Culture negative STDC patients (178)	87			
DOC extract from strain HI/ELISA	Antenatal clinic patients and medical students (237) IgA	90	Culture or micro-) ♀(212) IgA	45	Ison and Glynn, 1979
	IgM	86	scopy positive) IgM	31	
			STDC patients) ♂(191) IgA	43	
			IgM	20	
	Culture negative STDC patients (178) IgA	67			
	IgM	80			
A mixture of equal amounts of LiAC extracts from 8 serotype strains/ELISA for IgG anti- bodies	Family planning clinic patients and laboratory staff (338)	93	Culture positive STDC patients (150)	88	Brodeur et al, 1982
	Culture negative STDC patients (26)	0			

Table 1.7 Tests for antibodies to gonococcal outer membrane proteins

The outer membrane protein preparations used for the serological diagnosis of gonorrhoea are ill defined (Table 1.7). PI or PII or PIII or any combination of these proteins may have been involved in antibody detection.

Glynn and Ison (1978) used an outer membrane antigen (DOC extract) in an ELISA to detect IgG antibodies. The test did not detect antibodies from previous infections. A positive result corresponded to approximately 3 μg/ml of specific IgG in the test serum. This test was subsequently used to determine levels of the immunoglobulin classes, A and M (Ison & Glynn, 1979). There was no significant difference between IgM levels in patients and controls. This may have been due to competition for binding sites by IgG and IgA with the low avidity IgM. IgM detection is usually more successful using an antibody class captive assay (Chantler & Diment, 1981). IgA antibodies persisted after the infection.

Brodeur et al (1982) used a polyvalent antigen composed of outer membrane proteins from eight of the Johnston serotype strains. The use of eight rather than one strain increased the specificity from 54 to 83%. These lithium acetate extracts would contain PI, PII, PIII and lipopolysaccharide, any of these antigens may have been involved in the ELISA. A disadvantage of this test was the detection of antibodies from previous infections.

1.3.5 Summary

In general the use of more purified antigens has not improved the sensitivity or specificity of tests for antibodies in gonorrhoea. Some strains may be better antigens (O'Reilly et al, 1973; Ison et al, 1981; Reimann et al, 1982), but the antigenic heterogeneity of the gonococcus appears to be less important in the human infection than animal studies suggested (Novotony & Cownley, 1980, Buchanan 1975). The sensitivity of tests is often higher for females than males, this is thought to be due to a longer duration of infection in female patients during which higher antibody levels develop.

1.4 Aims of the investigation

The aims of the investigation were to improve the sensitivity and specificity of the ELISA previously used for the serological diagnosis of gonorrhoea (Glynn & Ison, 1978; Ison & Glynn, 1979). In particular to remove the false-positive reactions due to the detection of antibodies to meningococci by indentifying and purifying an antigen unqiue to N. gonorrhoeae. Also to examine some of the factors which may have affected the sensitivity of the ELISA; the production of strain specific antibodies during infection and immune complexes.

2. MATERIAL & METHODS

2.1 Bacteria and culture conditions

2.1.1 Strains

N. gonorrhoeae strain H1 was originally a genital isolate from a female patient attending St. Mary's Hospital. Pilated (P+) colony types have been maintained by selective subculture. Strain H1 has been serogrouped by Dr. E. Sanström and belongs to W II group (Sanström and Danielsson, 1980).

Clinical isolates were obtained from patients attending the clinic for sexually transmitted diseases (STD) at St. Mary's Hospital. When possible strains were subcultured only once before storage.

The N. meningitidis reference strains, NCTC 10025, serogroup A; NCTC 10026 serogroup B and NCTC 8554 serogroup C were obtained from the National collection of Type Cultures, Colindale, England.

2.1.2 Storage

Bacteria were stored as freeze-dried cultures or in liquid nitrogen (Ward & Watt, 1971).

2.1.3 Solid Media

Strains were routinely taken from liquid nitrogen and grown overnight, at 36°C in 7% CO_2, on a G.C. medium. The G.C. medium contained G.C. agar base (Difco) 36g/ℓ and filter sterilised supplements which were added after autoclaving. Supplements were either A, 20 ml/ℓ and B, 2 ml/ℓ, as described by Ison and Glynn (1978), or a solution having the same composition as Isovitalex (BBL), 10 ml/ℓ.

For the isolation of opaque/dark colony types a clear typing medium was used (James & Swanson, 1978).

Colony types were selectively subcultured when viewed on a plate microscope which had sub-stage and incident light.

2.1.4 Liquid Medium

For the batch growth of bacteria the biphasic system of Gerharat and Heden (1960) as modified by Ison and Glynn (1978) was used.

Five hundred millilitre flasks contained a liquid phase of 100 ml proteose peptone (Difco No 3) 15 g/ℓ and 50 ml of a solid agar phase, dextrose starch agar, 65 g/ℓ and Bacto-agar, 10 g/ℓ. The agar was contained in two 150 cm lengths of dialysis tubing, 18/32". Kellogg supplements A (4 ml) and B (0.4 ml) or Isovitalex (1 ml) were added to each flask immediately before use. Each flask was inoculated with the overnight growth of 2 or 3 colonies on G.C. medium. Flasks were shaken for approximately six hours at 36°c. The purity of each flask was checked by Grams stain. Bacteria were harvested by centrifugation, 6,000 g at +4°C for 30 mins and washed twice in 0.1 M phosphate buffered saline, pH 7.2 (PBS pH 7.2). The cell pellet was weighed and stored at -70°C until required.

The yield was approximately 1g wet weight of bacteria per flask. The agar increased the growth of the bacteria which maybe because it releases nutrients into the broth or absorbs toxins from it, or both. The use of agar in dialysis tubing rather than slabs of agar makes harvesting easier and increases the surface area of the agar.

2.2 Preparation of antigens

2.2.1 Triton and deoxycholate extracts

Triton and deoxycholate extracts were prepared as described by Smyth et al (1975). Bacteria were lysed in 0.1M sodium phosphate buffer, pH 7.0, (0.1 g wet weight per ml. of buffer). The mixture was stirred at room temperature for 1.5 hrs and then centrifuged, 40,000 g, at +4°C for 30 mins. The supernatant was discarded and the above procedure repeated on the pellet. The pellet was resuspended in 50 mM Tris-HCl, pH 7.5 (Tris-buffer) (approx. 1g original wet weight per 20 ml) and deoxyribonuclease (Koch-Light) added to a final concentration of 50 μg/ml. After incubation for 1 hr at +37°C the cell walls were harvested, 40,000 g, at +4°C for 30 mins. The pellet was resuspended in Tris-buffer and centrifuged at 1,000 g, at +4°C, for 10 mins to remove any intact gonococci. Cells walls were washed four times in Tris-buffer and stored at -70°C until extracted.

Cell walls were extracted with 4% Triton X-100 (Vol./Vol.) in 10mM Tris-HCl, pH 8.5 (approx, 25 mgs of cell wall protein per ml). After incubation at room temperature for 1 hr the mixture was centrifuged, 40,000 g, at +4°C for 30 min. The Triton X-100 extraction was repeated on the pellet and the two supernatants were designated 1st and 2nd Triton extracts. The pellet was washed once with Tris-buffer and then extracted twice with 2% (wt./vol.) sodium deoxycholate in 0.2 M NaCl, 1mM EDTA, 10mM Tris-HCl buffer, pH

8.0. The supernatants were designated 1st and 2nd DOC extracts, the residue was resuspended in Tris-buffer and designated DOC residue.

The first Triton extract and the first DOC extract were the antigens used in this study.

2.2.2 A Protein II enriched extract

Outer membranes were extracted from strain H1 with 0.2 M lithium acetate, pH 6.0 as described by Heckels (1977). The outer membrane complex was then solubilised with 1% sodium cholate using the method of James and Heckels (1981).

2.2.3 Lipopolysaccharide

Lipopolysaccharide was prepared by the phenol/water method (Westphal et al, 1952). Bacteria were suspended in 45% phenol (wt./vol.) (approx. 1g wet wt. per ml) and heated at 65°C for 15 mins. The aqueous layer was dialysed against running tap water for 48 hrs and then freeze-dried. The white powder was dissolved in saline and centrifuged, 4,000 g, +40°C for 1 hr., to remove insoluble material. Lipopolysaccharide was collected by centrifugation, 100,000 g, +4°C for 2 hr, resuspended in distilled water and assayed for 2-keto-3-deoxyoctonate (KDO) and protein content. The lipopoly

saccharides were approximately 2% KDO and the protein lipopoly-saccharide ration was 1:46.

2.2.4 Pili

Pili were prepared using the ammonium sulphate method of Brinton et al (1978). Type 1 or 2, pilated (P+) colonies, were grown overnight on G.C. medium in 4" petri dishes. The growth from each petri dish was resuspended in 8 ml. of 0.15 M ethanolamine buffer pH 10.5 and blended in a vortex mixer for 60s. The solution was centrifuged, 17,000 g, at +4°C for 15 mins and saturated ammonium sulphate (in 0.15 M ethanolamine buffer, pH 10.5) added to the supernatant to achieve 10% saturation. After mixing the solution was allowed to stand at room temperature for 30 mins. The resulting crystals were collected by centrifugation, 17,000 g, at +4°C for 15 mins, and then dissolved in 0.15M ethanolamine buffer, pH 10.5. After centrifugation, 27,000 g, at +4°C for 45 mins, to remove any outer membrane fragments, the solution was dialysed against 50mM Tris-buffered saline, pH 8.0 for 4 hrs at 4°C. Crystalised pili were collected by centrifugation, 40,000 g, at +4°C for 30 mins.

The presence of pili was confirmed by electron microscopy and the preparation considered pure if only one band was seen on SDS-PAGE.

2.3 Sodium dodecylsulphate - polyacrylamide gel electrophonesis (SDS-PAGE)

Slab gel electrophoresis was performed using either an upright apparatus (Reid & Blieleski, 1968) or on an LKB flat-bed apparatus. The precise composition of the SDS-PAGs is shown in Table 2.1. Each slab was 15 cm x 10 cm x 1 mm and was covered with a 2cm layer of stacking gel (5% acrylamide).

Samples for electrophoresis were taken up in a sampling buffer of 2% SDS, 0.1 M Tris-HClpH 6.8, 10% glycerol, 0.01% bromophenol blue with or without 0.1M dithiothreitol. Samples were heated at +100°C for 5 mins or at +37°C for 2 hours.

The electrophoresis buffer contained in g/ℓ, glycine, 14.4; Tris, 3.0 and SDS, 0.5. Electrophoresis was carried out at a constant current of 30 mA, at +20°C until the tracking dye was approx. 7 cm into the separating gel. Bio-rad low molecular weight standards were used for apparent subunit molecular weight (ASMW) determinations and consist of lysozyme (14.4 K), soybean trypsin inhibitor (21.5 K), carbonic anhydrase (31.0 K), ovalbumin (45.0 k), bovine serum albumin (66.2 K) and phosphorylase B (92.5 K).

2.3.1 SDS-PAG Stains

(a) Gels were fixed in methanol: acetic acid water, 5:1:5 (v/v/v) for 30 mins and stained for protein using 0.1% (w/v)

	5% stacking gel	10%	12.5%	15%
30% acrylamide	1.67	10.0	12.5	15.0
10% bisacrylamide	1.30	3.9	3.1	2.6
IM Tris-HCl	1.25 (pH 6.8)	11.2 (pH 8.6)	11.2 (pH 8.6)	11.2 (pH 8.6)
20% SDS	0.05	0.15	0.15	0.15
H_2O	5.60	4.6	3.0	1.0
TEMED*	0.01	0.02	0.02	0.02
10% Ammonium persulphate	0.15	0.30	0.30	0.30

Table 2.1 The composition of SDS-polyacrylamide gels

*TEMED = N,N,N',N'-tetramethylenediamine

Coomassie Brilliant Blue R-250 (Sigma) in the fixing solution for 2 hr at room temperature. Gels were destained in methanol: acetic acid: water, 10:15:200 (v/v/v).

(b) SDS-PAG's used for anti-sera production were stained with Coomassie Brilliant Blue G-250 (Sigma) by the method of Blakesley and Boezi (1973). As no destaining is required the gels can be washed extensively with water and are not impregnated with acetic acid which may adversely affect the animals.

(c) Gels were stained for carbohydrate with the periodic acid-Schiff (PAS) reagent by the method of Fairbanks et al (1978).

2.4 Protein purification from SDS-polyacrylamide gels

Protein I was purified from SDS-polyacrylamide gels by the method of Hanoaka et al (1979).

Firstly a urea gel was cast in region B-C of a glass tube (Figure 2.1), A-B was filled with 50% sucrose onto which 2ml of the following mixture was layered -: urea, 3.6g; 30% acrylamide, 2ml; 1% bis-acrylamide, 1.6ml; 400mM glycine pH 10.4, 1ml; H_2O, 5.2ml, polymerised with 10% ammonium sulphate, 100µl and TEMED, 10µl. After polymerisation, approx. 2 hr., the gel surfaces were washed with the electrophoresis buffer, 40mM glycine, pH 10.4 (glycine buffer). A to B was filled with glycine buffer and the bottom of the tube sealed with dialysis tubing, held in place with a rubber band.

Proteins were separated in SDS-polyacrylamide gels as described in 2.3., a section was removed and stained with Coomassie Brilliant Blue R-250, the remainder was cut manually into 0.25 cm slices. When slices containing the relevant protein had been identified they were fragmented and layered in 10% glycerol onto the urea gel.

The protein was electrophoresed, 2mA for 16 hrs, from the SDS-polyacrylamide gel, through the urea gel and recovered from section A-B.

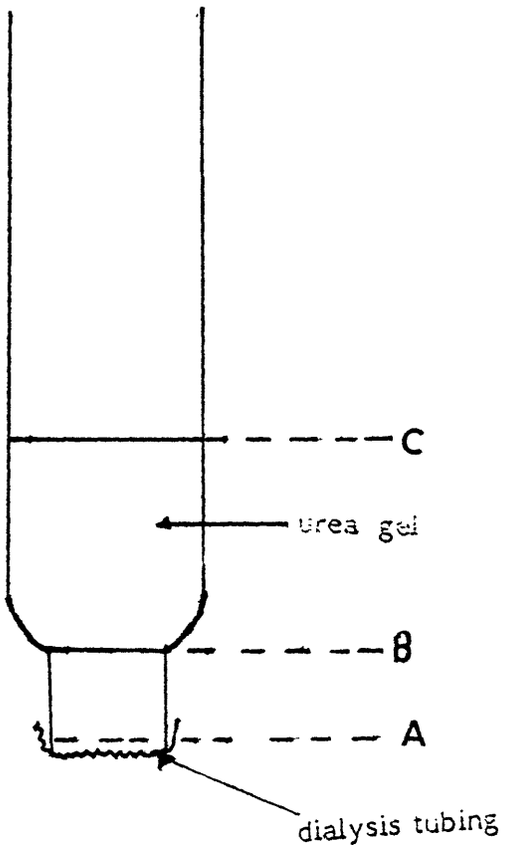


Figure 2.1 Apparatus used for preparative gel electrophoresis

2.5. Enzyme linked immunosorbent assay (ELISA)

An indirect, non-competitive, ELISA was used which consisted of the following steps

(1) Adsorption of antigen to the solid phase.

(2) Addition of the serum, specific antibody attaches to the antigen.

(3) Addition of an enzyme labelled anti-globulin (conjugate).

(4) Addition of the enzyme substrate.

(5) Termination of the reaction and reading of the results.

Between steps 1 and 2, 2 and 3 and 3 and 4 trays were washed to remove unbound sample and to block free sites on the solid phase. The washing buffer was 0.1M phosphate buffered saline, pH 7.4 containing 0.05% Tween-20, PBS-Tween, except for assays using a lipopolysaccharide (LPS) antigen when 0.02M $MgCl_2$ containing 0.5% BSA, $MgCl_2$-BSA, was used. Trays were filled with washing buffer and emptied after three minutes this was repeated three times.

2.5.1. Adsorption of antigen to the solid phase

Flat bottomed polystyrene microtitre plates (Linbro, Flow Labs or Cooke, Dynateck Ltd) were coated with 100µℓ volumes of antigen. Protein and whole bacteria were absorped in a 0.1M carbonate-bicarbonate buffer, pH 9.6 at room temperature for 30 mins, LPS in 0.9% NaCl, 0.02M $MgCl_2$ at 37°C for 1 hr.

The antigen concentration used in the assay was determined by titration of positive and negative pools of sera against increasing amounts of antigen. A working concentration in the plateau range was chosen for each batch of antigen so that small variations in concentration did not affect the test. Protein antigens were used at between 5 and 50µg/ml, LPS at100 µg/ml and whole bacteria at an OD_{550nm} of 0.5.

2.5.2. Addition of the serum

Sera were diluted, in PBS-Tween or for LPS antigen coated trays in $MgCl_2$-BSA, 1 in 1000 to detect IgG and 1 in 100 to detect IgA and IgM. The dilutions were determined by titrations to give the optimum difference between sera from patients and controls (Glynn & Ison, 1978). Each serum was tested in duplicate using 100µℓ of the appropriate dilution in each well. Trays were incubated in moist conditions at 30°C for 1 hr. A positive and negative control serum were tested on each tray so that variations between trays and tests done on different days could be corrected for.

2.5.3. Addition of the conjugate

The class specific antihuman immunoglobulin-enzyme conjugates were usually labelled with horse radish peroxidase, HRP, (DAKO) although alkaline phosphatase, AP, conjugates were also prepared (Voller et al, 1976). Each new batch of conjugate was titrated in the test system to give a low, but readable extinction

value with the negative control serum and a reading of less than 1.0 with the positive control serum. One hundred microlitres of conjugate was dispensed into each well and incubated, in moist conditions, at +30°C for 2 hr.

2.5.4 Enzyme substrates

The substrate used for HRP conjugates was O-phenylenediamine (Sigma), 0.4 g/ℓ in 0.02M phosphate-citrate buffer, pH 5.0, to which 30% hydrogen peroxide, 0.4 ml/ℓ was added just before use. This reaction was stopped by the addition of 50 µℓ of 2.5M H_2SO_4 and the absorbance read at 492 nm. For AP conjugates the substrate was p-nitrophenyl phosphate (Sigma), 1.0 mg/ml in 10% diethanolamine buffer, pH 9.8, the reaction was terminated by the addition of 50 µℓ of 1.0M NaOH and read at 405 nm. Substrate, 100 µℓ, was added to each well and incubated at room temperature for 30 mins, trays were read using a Titertek Multiscan (Flow Labs).

2.6 Gel electrophoresis derived ELISA (GED-ELISA)

The technique of GED-ELISA (Lutz et al, 1979) was used to analyse the antigenic components of complex mixtures. Proteins separated on SDS-PAGs were eluted onto the wells of microtitre trays and used in ELISAs.

SDS-PAGE was performed as described in Section 2.3. Samples were boiled for 5 mins in buffer containing 0.1M dithiothreitol, those containing Triton X-100 were extracted with chloroform to reduce the amount of detergent which distorts the gels (Horikawa & Hiroshi, 1979). Fifteen microgram protein samples were loaded into 3 mm wide wells. After electrophoresis the gel was cut into 3 mm vertical slices and washed three times for 10 mins in distilled water. Slices were stained for protein and carbohydrate and the remainder were cut horizontally into 2.5 mm fractions. Each fraction was placed in an individual flat bottomed well and stored at -70°C until used. Gel fractions were incubated with 100 µl of 0.1M carbonate-bicarbonate buffer, pH 9.6 for 36 hrs at +4°C, during which time protein from the gel became bound to the surface of the well. Gel fractions were removed, the wells washed and the ELISA continued as described in section 2.5, except that sera were diluted 1 in 200 to detect IgG antibodies.

2.7 Immuno-blotting

Immuno (electro or Western) blotting (Towbin, 1979; Burnette, 1981) was used to transfer proteins, separated by SDS-PAGE, onto an immobilising membrane which was then used for ELISAs.

SDS-PAGE was performed as described in section 2.3. The slab gel was covered with a sheet of nitrocellulose membrane (Bio-rad) to which proteins were electrophoretically transferred in a Trans-Blot apparatus (Bio-Rad Laboratories). A current of 200 mA for 2 hours was used to electrophorese proteins from the gel to the nitrocellulose membrane in a buffer of 20mM Tris, 150mM glycine, 20% methanol. The nitrocellulose membrane was washed for 30 min in 0.5% bovine serum albumin (BSA; fraction V, Sigma), 0.9% sodium chloride, 10mM Tris-HCl, pH 7.4 (BSA-buffer). Human sera were diluted 1 in 100 in the BSA-buffer and 10 ml was incubated overnight at +37°C with a strip of nitrocellulose membrane, containing two or three lanes of antigen. After washing, three times for 20 minutes in BSA-buffer, the nitrocellulose membrane was incubated for 4 hours at +37°C with HRPconjugated anti-human IgG (DAKO) diluted 1 in 1000 BSA-buffer. The nitrocellulose was washed as before, the substrate, 3, 4, 3', 4'-tetra-aminobiphenyl hydrochloride (BDH), 0.5 mg/ml in 50mM Tris-HCl, pH 7.4, containing 0.01% hydrogen peroxide, added and incubated at room temperature for 15 minutes. The nitrocellulose membrane was washed in water to terminate the reaction and dried between sheets of filter paper. Controls which

were incubated with conjugate but not sera were also included in the assay.

2.8 Radiolabelling of bacterial cell surface proteins

Surface proteins were radiolabelled with ^{125}I using the Iodogen method (Markwell & Fox, 1978). Twenty millilitre scintillation vials were plated with 100 µg of Iodogen (Pierce) in 250 µl of chloroform by rotation in a well ventilated hood. Vials were stored in a dessicator until used. Bacteria were grown overnight on GC medium and scraped into cold PBS. The absorbance at 550 nm was adjusted to 2.0. Ten millilitres were added to each vial followed by 50 µl of carrier-free $Na^{125}I$ (Radiochemical Centre, Amersham). The mixture was left on ice for 20 min and occasionally shaken. The reaction was stopped by pouring the mixture from the reaction vessel. Ten millilitres of 10mM NaI was added, the cells pelleted by centrifugation and washed four times in PBS. Cells were stored at $-70^{o}C$ until used.

This method has the advantages that no extraneous protein is added and no chemicals are required to stop the reaction unlike the lactoperoxidase method (Marchalonis et al, 1971).

2.9 Production of antisera

Mouse antisera specific for PI, PII and PIII were produced to locate the exact position of these proteins on immunoblots.

Proteins were separated by SDS-PAGE as described section 2.3 and stained using Coomassie Brilliant Blue G, see section 2.3.1. After extensive washing in water the relevant protein bands were cut from the gel and stored at -70°C until used. The slices were suspended in water using a hand tissue homogeniser and approximately 50µg of protein was injected intraperitoneally into each mouse on the first day and again 14 days later. On day 28 antibodies were detectable at a dilution of at least 1 in 10,000 in an ELISA and animals were bled out.

2.10 Source of human sera

Sera from patients who were culture positive for N. gonorrhoeae were obtained from the sexually transmitted diseases (STD) clinic at St. Mary's Hospital, Paddington, as were sera from meningococcal carriers who were mostly homosexuals.

Dr. R.C. Spencer, Barnsley General Hospital, provided sera from patients with meningococcal meningitis or septicaemia or both.

Control sera were from male and female members of staff to whom the purpose of the investigation had been explained. Some sera were also taken from patients attending the ante-natal clinic who were assumed to be culture negative.

2.11 Immunoglobulin fractionation

Immunoglobulins were fractionated by ion exchange chromatography (Mollison, 1975). Human serum, 2.5 ml, was equilibrated with 0.0175M phosphate buffer, pH 6.8 and loaded onto a DEAE sephacel column. Protein was eluted with 0.0175M phosphate buffer, pH 6.8 and the effluent monitored by absorbance at 280 nm. When the first peak had been eluted the buffer was changed to 0.08M phosphate buffer, pH 6.6. The second peak was collected and finally the column was eluted with 0.3M phosphate buffer, pH 6.5.

An ELISA was used to determine the immunoglobulin composition of the fractions. Each was tested for IgG, IgA and IgM content. Fractions were diluted in 0.1M carbonate-bicarbonate buffer, pH 9.6 and incubated at room temperature for 1 hr. in microtitre trays. Immunoglobulins were detected using class specific HRP conjugates (DAKO). A qualitative estimate of the amounts of immunoglobulins was obtained using this method (Figure 2.2).

Previously immunodiffusion has identified the class of immunoglobulin in the three peaks as IgG, IgA and IgM. The more sensitive ELISA detected some IgM in the mainly IgA fractions, peak 2, and IgA in the IgM fractions, peak 3.

Figure 2.2 Analysis by ELISA of the immunoglobulin content of fractions eluted from a DEAE-sephacel column

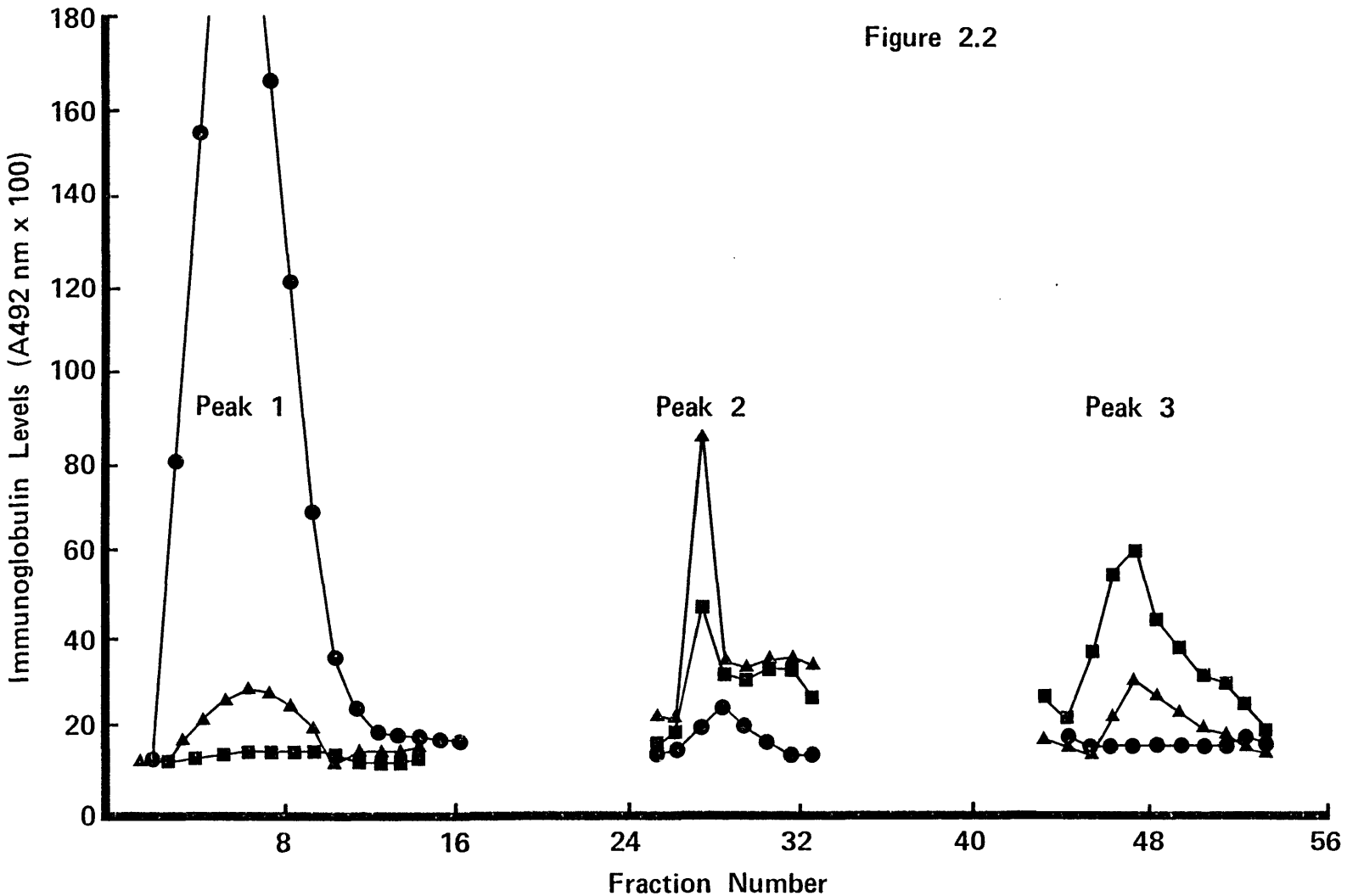
● - IgG

▲ - IgA

■ - IgM

Class specific anti-human conjugates were labelled with horse radish-peroxidase

Figure 2.2


Immunoglobulin Levels (A492 nm x 100)
180
160
140
120
100
80
60
40
20
0
Peak 1
Peak 2
Peak 3
8
16
24
32
40
48
56
Fraction Number

2.12 Chemical determinations

Ketodeoxyoctonate (KDO) was determined by the thiobarbituric acid method (Drödge, et al 1970) using KDO (Sigma) as a standard. Protein concentrations were determined by the method of Lowry, et al (1951) using BSA (Sigma) as a standard.

3. RESULTS

3.1 The Composition of the DOC extract.

A DOC extract from N. gonorrhoeae strain H1, previously used as an antigen in the serological diagnosis of gonorrhoea (Glynn & Ison, 1978; Ison & Glynn, 1979) was known to contain protein which was assumed to be from the outer membrane. Outer membrane proteins of gonococci have been classified on the basis of their occurrence and behaviour on SDS-PAGE by Swanson (1981). He defined protein I (PI) as the major outer membrane protein, the apparent subunit molecular weight of which was not affected by either heat or reducing agents. The apparent subunit molecular weight of protein II (PII) was heat modifiable, whilst that of PIII was changed by reducing agents. The outer membrane protein composition of the DOC extract was examined by SDS-PAGE to determine whether or not it contained any of these proteins.

The DOC extract was prepared in 1% SDS with or without the reducing agent, O.1M dithiothreitol (DTT) at either 100°C for 10 mins or 37°C for 2 hrs. Samples containing 25µg of protein were run on 15% acrylamide gels and were stained either with Coomassie Blue R for protein or with the periodic acid Schiffs stain for carbohydrate.

The major protein band in the DOC extract had an apparent subunit molecular weight of 37K which was not affected by heat or by dithiothreitol and was therefore identified as PI. The apparent subunit molecular weight of one band was changed by dithiothreitol, being 35K or 33K in the presence or absence of dithiothreitol

(compare lane b with lane c in Figure 3.1) so fitting the definitions for PIII. The DOC extract also contained one heat modifiable protein, a PII, whose apparent subunit molecular weight was 26K at 37°C and 30Kat 100°C (compare lane a with lane b in Figure 3.1). The extract also contained undefined minor proteins as well as PI, PII and PIII. An SDS-PAG stained for carbohydrate in a broad band at the aniodic end of the gel (not shown), which was assumed to be lipopolysaccharide as the extract contained 2-keto-3-deoxyoctonate.

SDS-PAGE determined the presence of the three outer membrane proteins of N.gonorrhoeae, as described by Swanson (1981), other minor proteins and lipopolysaccharide in the DOC extract.

As a result of the historical order in which the outer membrane proteins were described i.e. PI, PII and lastly PIII, they are not in numerical order of decreasing apparent subunit molecular weights which is PI,PIII and PII. The heat modifiability of PII is analogous to that shown by the Omp A protein of E.coli which has been shown to be due to the presence of LPS (Schweizer et al, 1978). The change in apparent subunit molecular weight of PIII suggests the presence of a disulphide bond in the polypeptide, the reduction of which leads to an unfolding of the molecule and an apparent increase in its molecular weight.

Any one or combinations of these proteins and lipopolysaccharide may have been important antigens in the serological diagnosis of gonorrhoea using the DOC extract or in the cross-reactions with

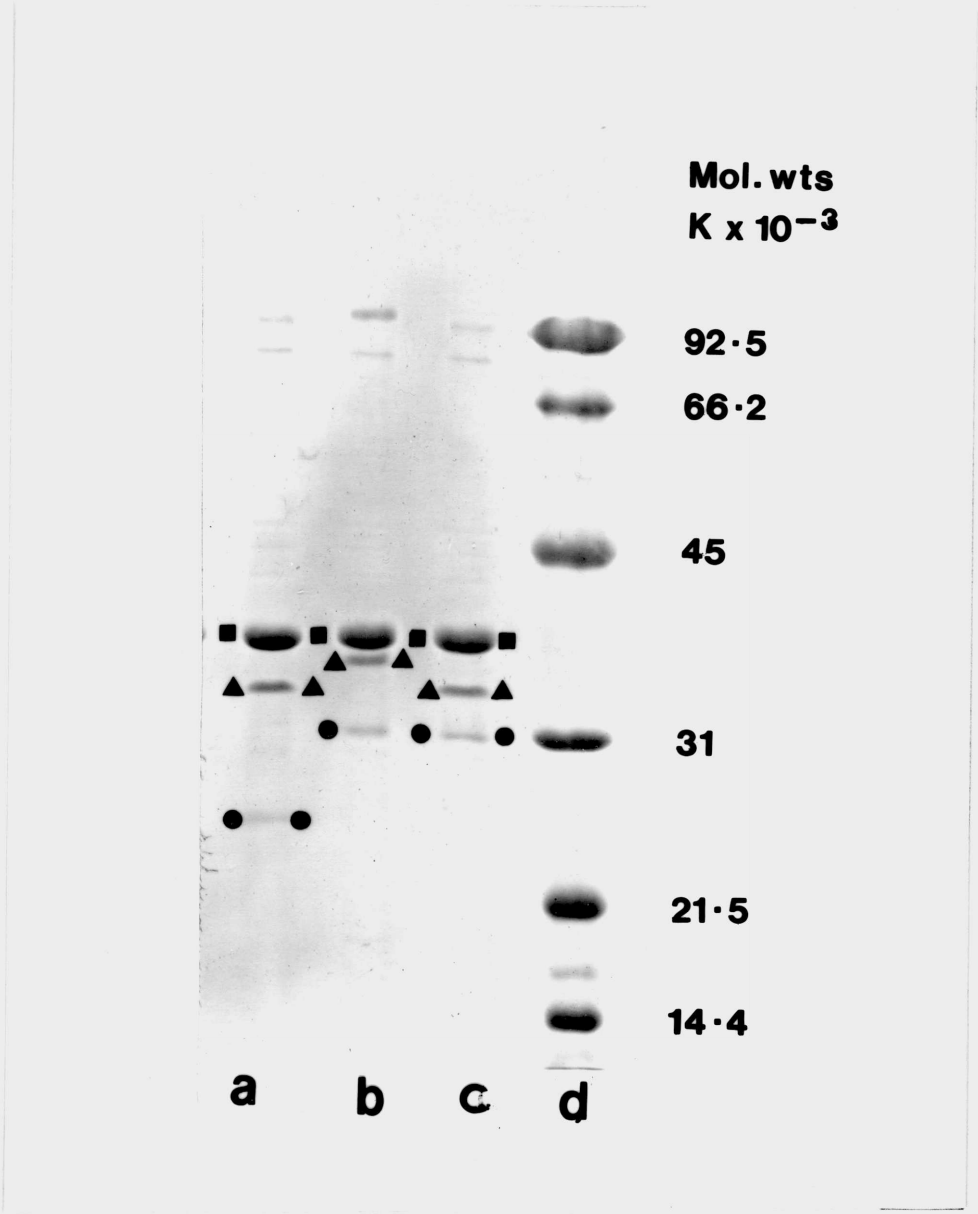


Figure 3.1 SDS-PAGE of the DOC extract from N.gonorrhoeae strain HI

(a) treated at +37°C for 2 hr, without DTT

(b) treated at 100°C for 10 mins in the presence of 0.1M DTT

(c) treated at +100° for 10 mins, without DTT

■ - Protein I

● - Protein II

▲ - Protein III

antibodies to meningococci. As PI was the most abundant protein in the DOC extract it was purified in an attempt to produce an antigen of higher specificity than the DOC extract.

3.1.1 Purification of PI

The method of Hanoaka et al (1979) who purified a biologically active DNA polymerase was used to purify PI from the DOC extract. The DOC extract was prepared in 1% SDS, 0.1M dithiothreitol at 100°C for 10 mins and proteins separated on a 10% polyacrylamide gel. PI was recovered from the relevant gel slice by electrophoresis through a small urea gel as described in sect 2.4.

SDS-PAGE was used to confirm the purity of PI (Figure 3.2). A 99% reduction in SDS concentration was obtained and no lipopolysaccharide (LPS) was detected. LPS was estimated by the thiobarbituric acid method for the detection of 2-keto-3-deoxyoctonate (KDO), the method is sensitive to 0.01 μmoles of KDO, which for gonococci is equivalent to approximately 0.50 μmoles of LPS.

The yield of PI from the DOC extract varied from 3.5 to 14.9% (Table 3.1). The variations were probably due to inaccuracies in locating precisely the position of PI in the unstained gels and loss of protein from the apparatus which was difficult to seal. The average yield of 8% for the purification of PI from the DOC extract is equivalent to a 0.01% yield from the wet weight of bacteria. This purification may have been improved if dithiothreitol had been

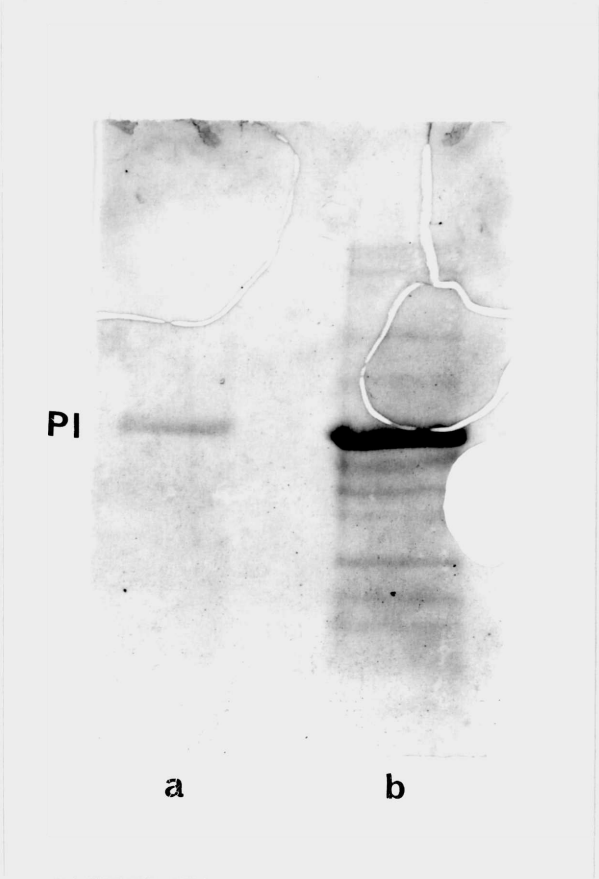


Figure 3.2 SDS-PAGE of (a) Protein I and (b) the DOC extract

Experiment Number	Quantity (mg) of protein applied	Quantity (µg) of P-I recovered	Percentage yield
1	1.17	160	13.7
2	1.40	49	3.5
3	0.75	112	14.9
4	1.30	90	6.9
5	1.20	80	6.7
6	1.40	81	5.8
			($\bar{X}$ = 8.6±4.2)

Table 3.1 Electrophoretic purification of protein I from the DOC extract

omitted from the sampling buffer this would have given a better separation of PI and PIII.

PI purified from the DOC extract was compared with the crude DOC extract in ELISA to determine whether the pure antigen was more specific for the detection of anti-gonococcal antibodies.

3.1.2 Comparison of the DOC extract and PI in ELISAs

PI and the DOC extract were used at a concentration of 10μg of protein per ml. in the ELISA's. Six sera from each of four groups (i) controls, patients with (ii) uncomplicated gonorrhoea (iii) disseminated gonococcal infections, DGI (iv) systemic meningococcal infection, were tested against both antigens.

The DOC extract detected higher IgG antibody levels in sera from all three groups of patients than in sera from controls. The IgG antibody levels detected using PI were no higher in sera from patients with uncomplicated gonorrhoea than in sera from controls. Both groups of patients with systemic infections had anti-PI IgG in their sera. The IgG antibody levels detected in all the patients sera with the DOC extract were reduced by approximately 37% when tested against PI (Table 3.2).

These experiments demonstrate the presence of IgG antibody to PI in the sera from patients with disseminated gonococcal infection or systemic meningococcal infection. The 37% reduction in

Source of sera (6 in each group)	Antibody levels (A405 nm x 100) to DOC extract		Purified PI	
Controls	34±9*		33±8	
Patients with uncomplicated gonorrhoea	63±23	(0.02)+	38±18	(NS)
Patients with DGI	108±37	(0.02)	69±27	(0.05)
Patients with systemic meningococcal infection	105±41	(0.02)	67±28	(0.05)

Table 3.2 Comparison of IgG antibody levels using the DOC extract and PI as antigens in ELISA

* = absorbance A405 x 100 ± S.D.

\+ = P value calculated using Students t-test, comparison of patients sera with controls

antibody levels with PI rather than the DOC extract reduced the IgG antibody level found in sera from patients with uncomplicated gonorrhoea to that of the control sera. This reduction may have been due to the removal of other important antigens present in the DOC extract or possibly the denaturation of PI during its purification.

PI is not a suitable antigen for the serological diagnosis of gonorrhoea for two reasons, firstly IgG antibody to PI is not produced, or is only produced in small amounts in uncomplicated gonorrhoea and secondly PI cross-reacts with antibodies produced to meningococci.

Attempts to purify less abundant proteins than PI from the DOC extract were not practicable due to the poor yields.

As the purification of PI had not produced a specific antigen for the detection of anti-gonococcal antibodies alternative extracts were examined.

3.2 Cross-reacting antigens from N. gonorrhoeae and N. meningitidis.

Triton X-100 (Triton) Na-deoxycholate (DOC) were used sequentially to solubilise antigens from the cell envelope of N.gonorrhoeae strain Hl and N.meningitidis serogroup A, Band C type strains as described in section 2.2.1. The antigenic composition of these extracts was analysed and the extracts were used in ELISA's to detect IgG antibodies in human sera

3.2.1 The Analysis of the DOC and Triton extracts from N.gonorrhoeae strain Hl and N.meningitidis serogroup strains A,B & C.

The composition of the extracts was determined by protein and KDO assays. Extracts were prepared in 1% SDS, 0.1M DTT at 100°C for 10mins for analysis on 10% SDS-polyacrylamide gels. Gels were stained with Coomassie blue R for proteins.

All the DOC and Triton extracts contained protein, but only the DOC extracts had detectable levels of KDO (Table 3.3). The DOC extracts from N.gonorrhoeae strain Hl and N.meningitidis serogroup A and C strains contained similar proteins whilst the DOC extract from N.meningitidis serogroup B strain did not contain a major protein band equivalent to PI (Figure 3.3a). The DOC extract from N.meningitidis serogroup B also contained less KDO than the other three DOC extracts (Table 3.3) The Triton extracts from all four strains contained many similar minor protein bands (Figures 3.3b).

		Protein concentration (mg/ml)	KDO concentration (µg/ml)
N.gonorrhoeae			
HI	(DOC	4.5	65
	(Triton	3.5	0
N.meningitidis			
serogroup A	(DOC	7.2	95
	(Triton	3.7	0
N.meningitidis			
serogroup B	(DOC	1.9	12
	(Triton	2.7	0
N.meningitidis			
serogroup C	(DOC	4.1	88
	(Triton	4.2	0

Table 3.3 Protein and 2-keto-3-deoxyoctonate (KDO) concentrations of DOC and Triton extracts from N.gonorrhoeae strain HI and N.meningitidis serogroups A, B and C

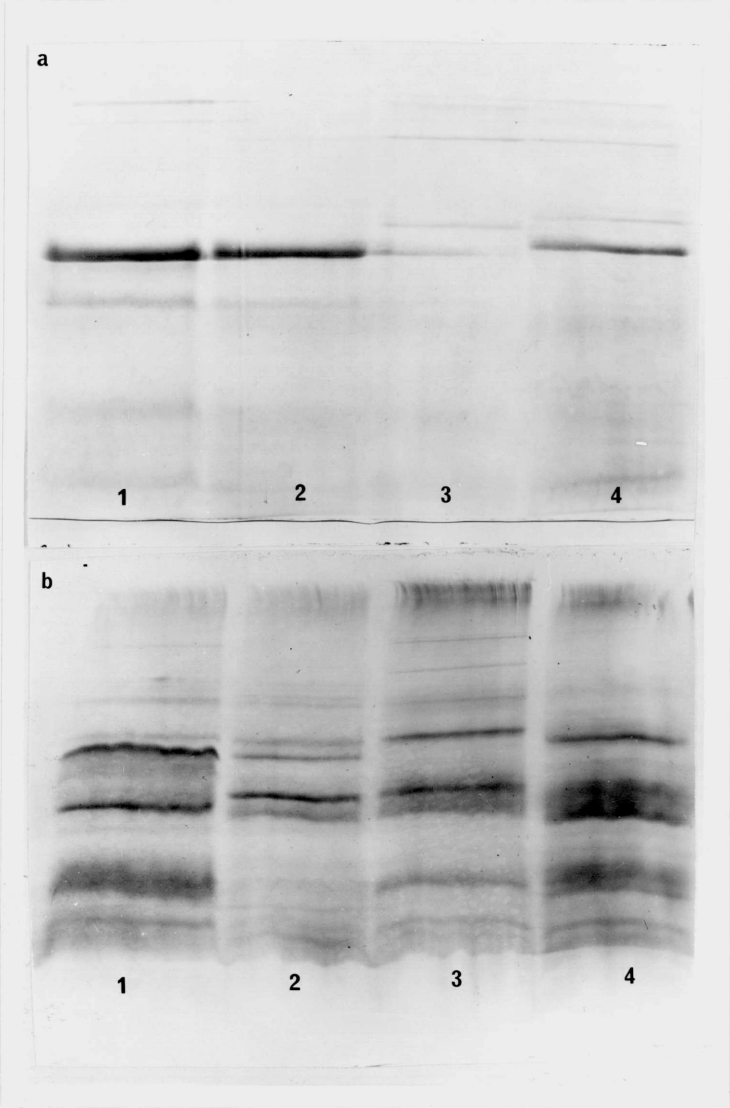


Figure 3.3 SDS-PAGE of (a) Deoxycholate and (b) Triton X-100 extracts from (1) N.gonorrhoeae strain HI and N.meningitidis serogroups (2) A, (3) B and (4) C

The Triton extracts were very much the same, all four containing minor proteins which were probably largely from the cytoplasmic membrane. Smyth et al (1975) analysed Triton extracts from N.gonorrhoeae by crossed immuno-electrophoresis and found they contained enzymes specifically associated with the cytoplasmic membrane. Schnaitman (1971) has also shown that unless EDTA is added Triton X-100 does not solubilise outer membrane components.

The DOC extracts contained both protein and LPS, however the major outer membrane protein of the N.meningitidis serogroup B strain was not solubilised. The DOC extract from N.meningitidis serogroup B contained the lowest concentrations of protein and KDO. The major outer membrane protein of this strain was of a higher apparent subunit molecular weight, 41K, than in the other three strains, 37K. It is possible that the DOC solubility of the major outer membrane protein is related to its molecular weight, although PI is generally less soluble than PII in DOC (Swanson & Heckels, 1980). These extracts were not analysed for heat or DTT modifiable proteins.

3.2.2 The detection of antibodies with the DOC and Triton extracts

The DOC and Triton extracts were used in ELISAs to detect IgG antibody in sera from patients with uncomplicated gonorrhoea or systemic meningococcal infection, to determine the specificities of the antigens.

The DOC extracts were coated onto microtitre trays at concentrations of 10, 20, 30, 40 and 50 µg of protein per ml., Triton extracts at 25,50,75 and 100 µg of protein per ml. These concentrations had previously been determined to be in the plateau ranges for each antigen. The extracts were tested against three pools of sera, each containing four sera, from controls and patients with uncomplicated gonorrhoea or systemic meningococcal infection.

IgG antibody to the DOC extracts from N.gonorrhoeae strain H1 and N.meningitidis serogroups A and C was found in sera from patients with uncomplicated gonorrhoea or systemic meningococcal infection. The DOC extract from N.meningitidis serogroup B did not detect IgG antibody in patients sera (Figure 3.4a). IgG antibody to all four Triton extracts was found in sera from patients with systemic meningococcal infection, but not in the sera from patients with uncomplicated gonorrhoea (Figure 3.4b).

Results using the different DOC extracts suggested that PI or LPS or both were necessary for antibody detection since the DOC extract from N.meningitidis serogroup B contained very little of these two antigens and did not detect antibody. None of the DOC extracts could be used to differentiate anti-gonococcal from anti-meningococcal antibodies. The Triton extracts differentiated the IgG antibodies produced in systemic meningococcal disease from those produced in uncomplicated gonorrhoea. However because the Triton extracts were used at higher protein concentrations than the DOC extracts and the antibody levels detected using the Triton extracts

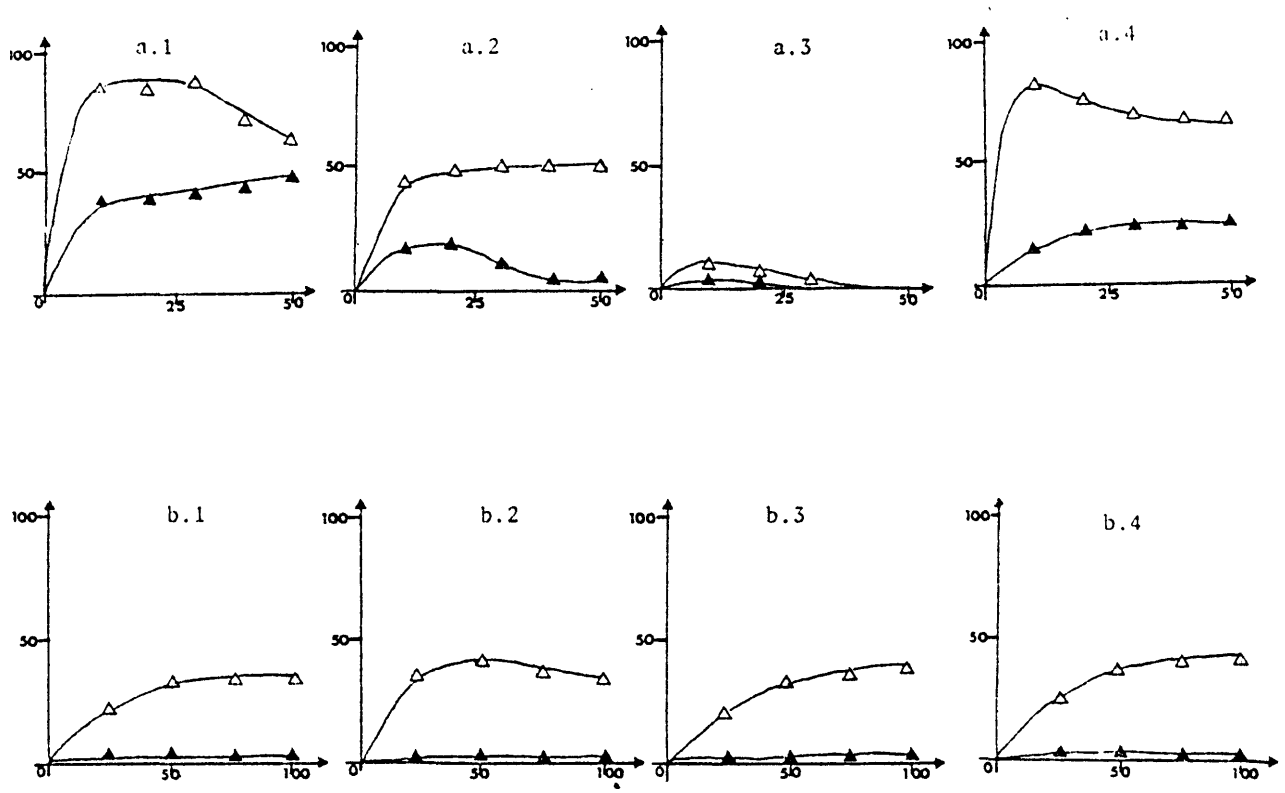


Figure 3.4 Detection of IgG antibodies using ELISA in sera from patients with uncomplicated gonorrhoea (▲) or systemic meningococcal infection (△). Extracts used were (a) DOC and (b) Triton X-100 from (1) N.gonorrhoeae, HI and N.meningitidis serogroups (2) A, (3) B and (4) C.

x axis = protein concentration, µg/ml

y axis = (A_{405} of patients sera) - (A_{405} of control sera) X 100

Conjugate - anti-human IgG/alkaline phosphatase

were lower than with the DOC extracts it was possible that the difference was purely quantitative. The Triton extracts containing the same antigens as the DOC extract but at lower concentrations. To determine whether or not individual antigens in the DOC and Triton extracts were different the technique of gel electrophoresis-derived ELISA was used to identify individual antibody-antigen reactions.

3.3 Analysis of the antigenic components of the DOC and Triton extracts from N.gonorrhoeae strain H1.

3.3.1 Comparison by gel electrophoresis-derived ELISA (GED-ELISA)

GED-ELISA was first described by Lutz et al (1979) who used it to study the specificity of antibodies to feline leukemiavirus. This method was adapted to examine the antibody specificity of IgG in sera from patients with uncomplicated gonorrhoea or systemic meningococcal infection and to identify individual antigens in the DOC and Triton extracts from N.gonorrhoeae strain H1.

DOC and Triton extracts were prepared for SDS-PAGE in 1% SDS, 0.1mM DTT at 100°C for 10 mins. Triton extracts were also treated with chloroform to reduce the Triton concentration which distorts the gels (Horikawa & Hiroshi, 1979). Protein samples (15µg) were separated on 12.5% acrylamide gels and protein bands were subsequently eluted from gel slices into the wells of a microtitre tray as described in section 2.6. Two sera from each of three groups, controls or patients with gonorrhoea or systemic meningococcal infection were tested at a dilution of 1 in 200. Each serum was tested against both the DOC and Triton extract on two separate days. Specifically bound IgG antibody was identified with an anti-human IgG-alkaline phosphatase conjugate and p-nitrophenyl phosphate substrate. Unsliced gel strips were stained for protein by Coomassie blue and carbohydrate by the periodic acid-Schiffs method.

The DOC extract contained PI in gel slices 7 and 8 and LPS in gel slices 12-14, PII and PIII were not identified. No outer membrane proteins or LPS were identified in the Triton extract.

IgG antibody in sera from patients with uncomplicated gonorrhoea reacted with the DOC antigen in the area of the gel corresponding to the presence of LPS and did not react with any antigens in the Triton extract. Sera from patients with systemic meningococcal infection had IgG to PI and LPS in the DOC extract and to many gel slices from the Triton extract (Figures 3.5a and 3.5b).

GED-ELISA experiments confirmed the results obtained previously i.e. IgG antibodies in sera from patients with uncomplicated gonorrhoea react with the DOC extract and from patients with systemic meningococcal infection react with both the DOC and Triton extracts. The IgG antibody in sera from patients with gonorrhoea was specific for the LPS in the DOC extract whilst in sera from patients with systemic meningococcal infection it was specific to both PI and LPS in the DOC extract. The Triton extract may also have contained PI since the IgG in patients sera with systemic meningococcal infection reacted strongly with gel slices 7 and 8 (the position of PI in the DOC extract) of the Triton extract. However other antigens in the Triton extract, not present in the DOC extract, were also involved.

Figures 3.5 a and b

GED-ELISA profiles of the DOC (a) and Triton (b) extracts using sera from patients with uncomplicated gonorrhoea (●) or systemic meningococcal infection (■)

△ - sera from controls

Sera diluted 1 in 200

Conjugate - anti-human IgG/alkaline phosphatase

Figure 3.5b

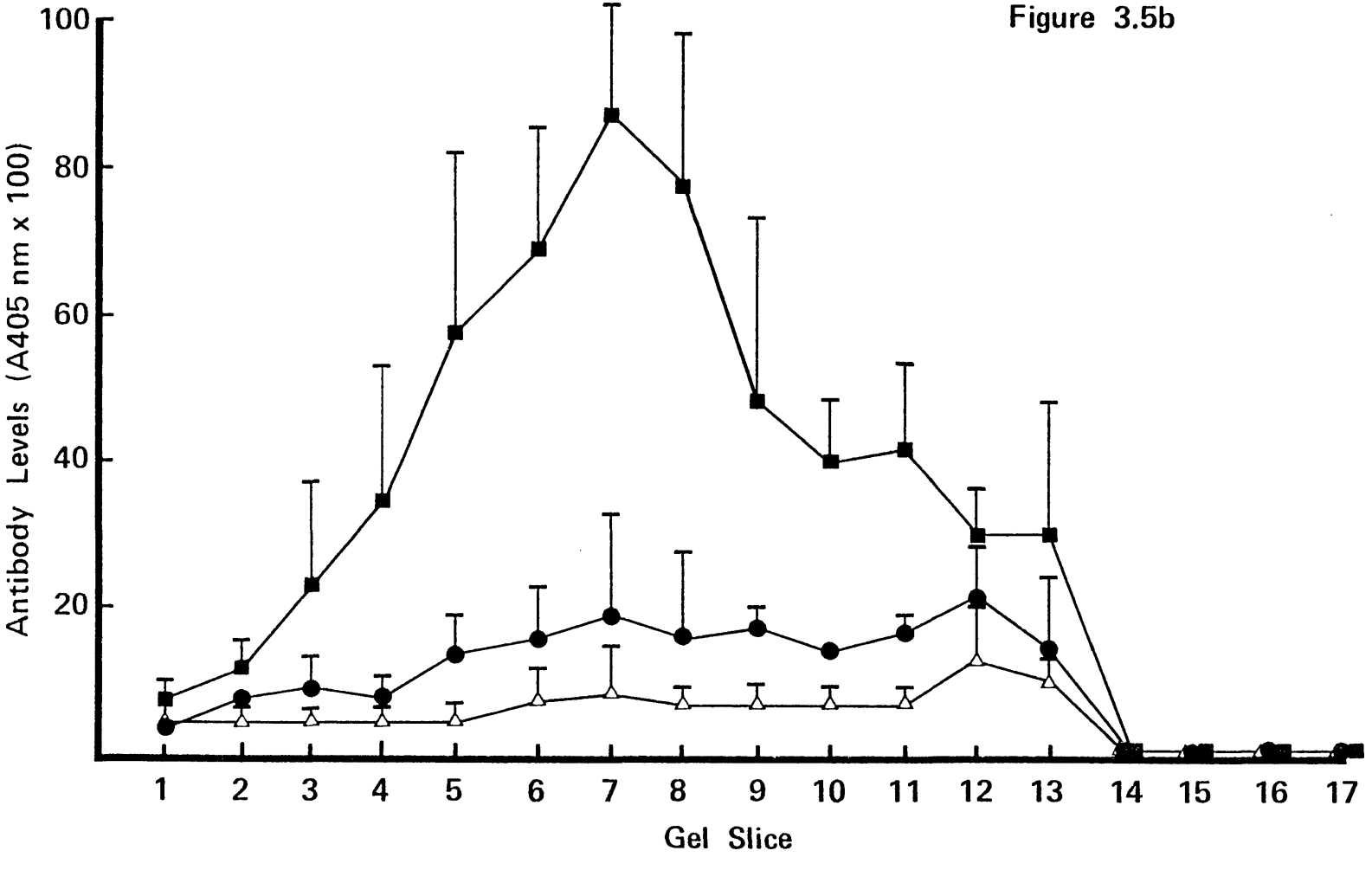
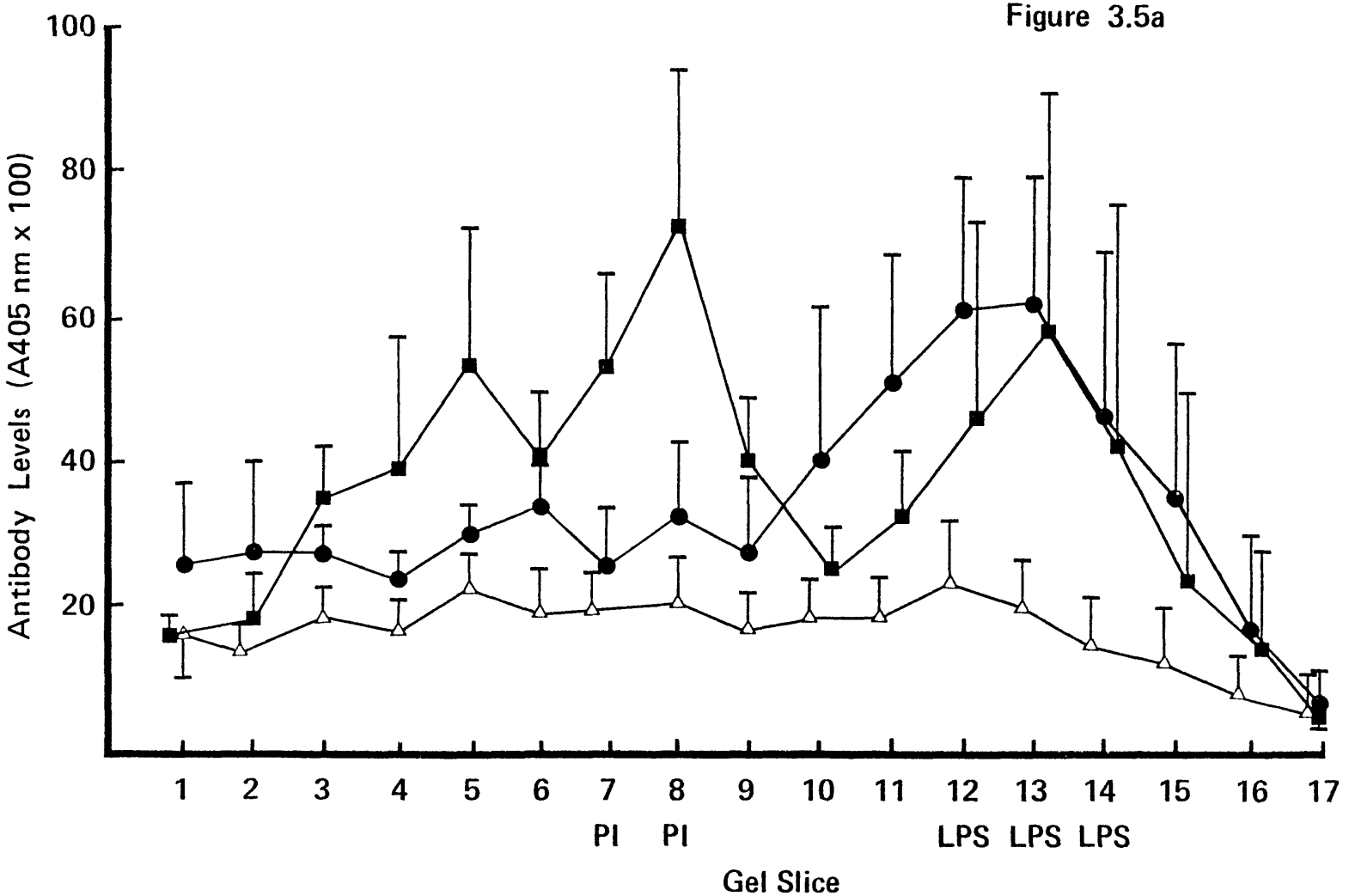

Antibody Levels (A405 nm x 100)
100
80
60
40
20
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Gel Slice

Figure 3.5a


Antibody Levels (A405 nm x 100)
100
80
60
40
20
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
PI
PI
LPS
LPS
LPS
Gel Slice

Gel Slice	Antigen					
	DOC Extract			Triton Extract		
	a	b	c	a	b	c
1					.05	.02
2					.05	
3	.05	.02			.05	
4					.02	.02
5		.05		.05	.01	.02
6	.02	.05			.001	.01
7		.02	.01		.001	.001
8		.01	.02		.001	.01
9		.01		.01	.02	.05
10				.01	.001	.01
11	.02	.02		.01	.01	.02
12	.02				.02	
13	.01	.05			.05	
14	.05					
15						
16						
17						

Table 3.4 Ged-ELISA results. Significance of differences between levels of IgG antibody against gel slices P values calculated using Students T-test

(a) difference between controls and patients with uncomplicated gonococcal infection

(b) difference between controls and patients with meningococcal infection

(c) difference between patients with uncomplicated gonococcal and meningococcal infection

As the antigenic components of the DOC and Triton extracts were different and could be used to differentiate anti-gonococcal from anti-meningococcal IgG, the two extracts were used to try to distinguish antibodies produced in gonorrhoea from antibodies produced to meningococcal carriage.

3.3.2 The detection of antibodies in sera from carriers of meningococci.

In the serological diagnosis of gonorrhoea it is important, because of the high rates of meningococcal carriage in some sexually active populations (Turgeon et al, 1981), to be able to distinguish anti-gonococcal antibodies from antibodies produced during meningococcal carriage. The DOC and Triton extract from N. gonorrhoeae strain H1 were used in parallel in an attempt to differentiate the two types of antibody.

The DOC and Triton extracts were used at protein concentrations of 10 and 50μg respectively to coat the wells of microtitre trays. Sera were collected from patients attending the STD clinic at St. Mary's Hospital who were pharyngeal carriers of meningococci, but did not have a current and had not had a recent (i.e. in the previous 6 months) gonococcal infection. Sera from carriers of meningococci or patients with uncomplicated gonorrhoea or systemic meningococcal infection were tested against both extracts.

The mean antibody level against the DOC extract (Ecr) was lower for the sera from meningococcal carriers than for patients with

	Total No.	DOC ELISA No with ECR $\geqslant$ 20	DOC Extract (A504 x 100) $\bar{x}(\sigma)$	Triton Extract (A405 x 100) $\bar{x}(\sigma)$
Sera from patients with:				
(i) gonorrhoea (duration > 2/7)	15	13	40.4 (22.2)	26.5 (11.7)
			p = .05	
(ii) meningogoccal throat carriage	15	11	27.9 (4.0)	21.8 (4.0)
			p = .001	
(iii) systemic meningococcal infection	15	11	58.8 (49.1)	43.1 (36.1)
			p = N.S.	

Table 3.5 ELISA results using the DOC and Triton extracts from N.gonorrhoeae, HI, to detect IgG in sera from patients with (i) gonorrhoea, (ii) meningococcal throat carriage and (iii) systemic meningococcal infection

P values calculated using Students t-test

Antigen concs, µg of protein/ml, DOC = 10, Triton = 50

Sera dilutions, 1 in 1000, conjugate, Anti-human IgG-alkaline phosphatase

uncomplicated gonorrhoea. However because 11 of the 15 sera from meningococcal carriers were DOC ELISA positive i.e. Ecr $\geq$ 20 (Glynn & Ison, 1978), individual results cannot be differentiated using the DOC extract. The sera from meningococcal carriers did not have higher IgG levels than sera from patients with uncomplicated gonorrhoea when tested against the Triton extract, unlike the sera from patients with systemic meningococcal infection (Table 3.5).

The IgG specificity of sera from meningococcal carriers was DOC positive only, consequently the DOC and Triton extracts could not be used in parallel to distinguish antibodies in gonorrhoea from meningococcal carriers. However this raised the possibility that the Triton extract contained an antigen associated with the increased pathogenicity of some strains of Neisseria. If so antibody to the Triton extract might be produced in systemic but not local infections or carriage. To examine this possibility further sera from patients with a disseminated gonococcal infection were tested against the DOC and Triton extracts from N.gonorrhoeae strain H1.

3.3.3 The detection of antibodies in sera from patients with a disseminated gonococcal infection.

ELISAs were carried out as described in section 3.3.2. The three pools each containing four sera were from controls and patients with uncomplicated gonorrhoea or disseminated gonococcal infection.

		Absorbance 492 nm x 100			
		DOC Extract		Triton Extract	
		$\bar{x}$	σ	$\bar{x}$	σ
(i)	Control pool	23	(11)	14	(5)
	Pool of sera from patients with				
(ii)	gonorrhoea	62	(9)	20	(1)
			p = .01		
(iii)	disseminated gonococcal infection	48	(23)	49	(8)
			NS		

Table 3.6 ELISA results using the DOC and Triton extracts from N.gonorrhoeae, HI, to detect IgG antibodies in pooled sera from (i) a control group and patients with (ii) gonorrhoea, (iii) disseminated gonococcal infection.

$\bar{x}$ = the mean of 3 separate experiments

Antigen concs in μg of progein/ml, DOC = 10, Triton = 50

Sera dilutions 1 in 1000

Conjugate = Anti-human IgG-HRP

P values calculated using Students t-test

Sera from patients with uncomplicated gonorrhoea had higher IgG antibody levels against the DOC extract than did control sera, but levels were no different against the Triton extract. IgG antibody levels in sera from patients with disseminated gonococcal infection were higher than controls against both the DOC and Triton extracts (Table 3.6).

The antibody response in the sera from patients with disseminated gonococcal infection was similar to that found in sera from patients with systemic meningococcal infection. Thus there may be some correlation between the pathogenesis of infection and the IgG reponse. A systemic neisserial infection produces IgG antibody to antigen(s) in the DOC and Triton extracts, local infection or carriage producing antibody to the DOC extract but not the Triton extract. This may be due to the presence in the Triton extract of a surface antigen associated with the increased pathogenesis of systemic infections or possibly to the production of antibodies to proteins from the cytoplasmic membrane in a systemic infection.

To determine whether or not the Triton extract contained surface antigens intact gonococci were surface labelled and then extracted.

3.4 The identification of outer membrane antigens in the DOC and Triton extracts

N.gonorrhoeae strain H1 was surface radiolabelled with ^{125}I and then extracted with DOC and Triton to identify any surface antigens in these extracts, in particular any that may be involved in antibody detection. At the same time the percentage of proteins sticking to the microtitre wells was determined.

Pilated colonies of N.gonorrhoeae strain H1 grown on solid media (section 2.13) were surface radiolabelled with ^{125}I by the Iodogen method (Section 2.8). DOC and Triton extracts from the labelled bacteria were separated on 12.5% acrylamide gels as previously described (section 3.3.1). The gels were sliced, placed in microtitre wells for GED-ELISA and coated in an LKB γ counter. The wells were also counted after removal of the gel slices to determine the percentage of protein sticking to the wells. Two separate experiments were performed for both antigens, in each experiment 8 wells were counted with and without a gel slice. Unsliced gel strips were stained for protein and carbohydrate.

Staining of the gel slices showed the DOC contained PI in gel slices 9 ands 10, PII in slices 11 and 12 and LPS in gel slices 11 to 15. PIII was not identified since in the presence of DTT, PI and PIII are not separated on 12.5% acrylamide gels. These antigens were not easily identifiable by the counting of microtitre wells. However the maximum peak of radioactivity was in gel slices 9 and 10, the

position of PI and the other peak, gel slices 11 and 12 was at the position of PII. Staining of the Triton extract showed pili in this extract prepared from gonococci grown on a solid medium. The pilus protein was ^{125}I labelled and located in gel slice 13. The Triton extract also contained small amounts of PI which were identified by the surface radiolabelling of proteins, but not from stained gels. PI was present in gel slices 8,9 and 10 in the Triton extract, (Figure 3.6).

The average percentage of ^{125}I sticking to the microtitre wells was 16 (σ=6) for proteins in the DOC extract and 9 (σ=4) for the Triton extract. The percentage varied between gel slices but was not significantly different from the mean value. The percentage sticking rapidly declined at the position of the dye front, gel slice 16, (Table 3.7).

Radiolabelling showed that the positions of PII and LPS in the DOC extract overlapped in GED-ELISA experiments. Consequently antibody to the DOC extract previously assumed to be anti-LPS may also have been to PII.

The Triton extract contained pilus protein which was surface radiolabelled. Pilus protein was not usually present in the Triton extract as the bacteria were grown in a biphasic medium, rather than as in these experiments on solid medium. Pili produced in the biphasic growth medium are presumably sheared from the bacteria during shaking of the flasks and centrifugation. The outer membrane

Figure 3.6 Electrophoretic patterns of the (a) DOC and (b) Triton extracts from ^{125}I-surface labelled gonococci

● cpm in gel slices

○ cpm bound to microtitre tray wells

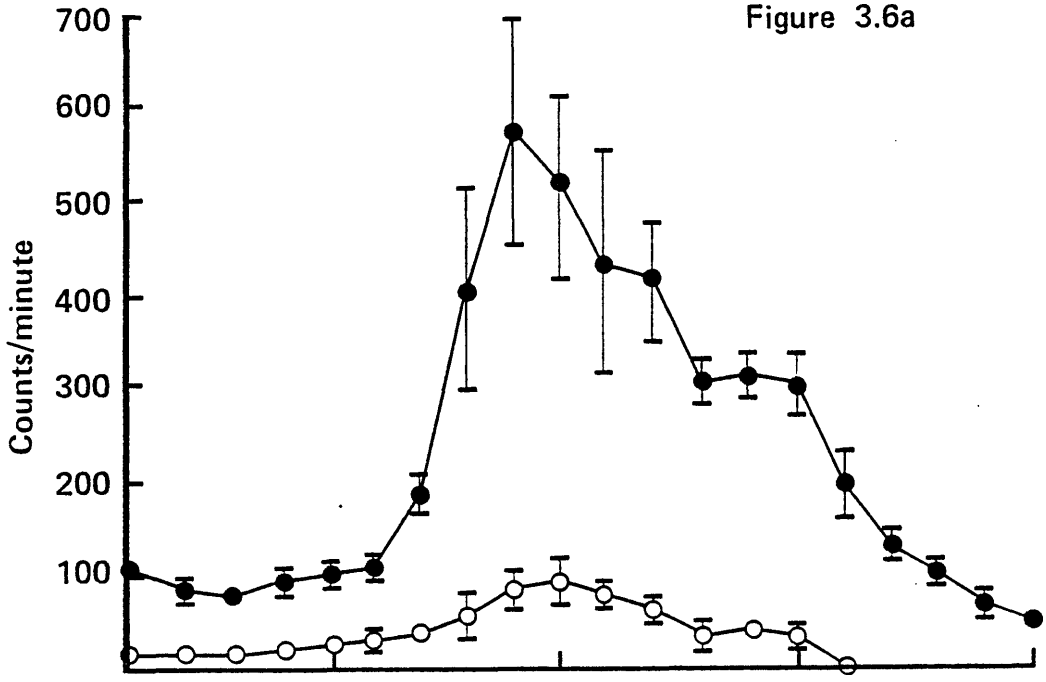


Figure 3.6a

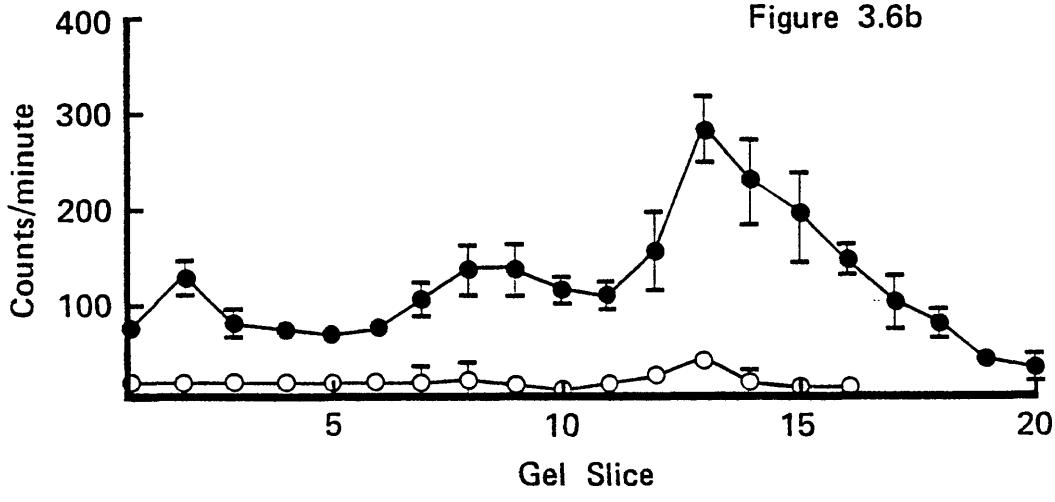


Figure 3.6b

Gel Slice		% of ^{125}I from the gel slice sticking to the microtitre well	
		DOC	TRITON
	1	13.0	6.5
	2	15.5	6.0
	3	20.5	11.5
	4	23.5	12.0
	5	24.0	17.0
	6	27.5	14.0
	7	25.0	8.0
	8	16.0	13.0
	9	15.0	9.0
	10	18.5	4.0
	11	17.5	12.5
	12	14.0	16.0
	13	11.0	13.0
	14	14.5	8.5
	15	10.0	3.0
	16	2.6	1.5
Dye → front	17	-	-
	18	-	-
	19	-	-
	20	-	-
	x (σ)	16.75(6.37)	(9.70)(4.65)

Table 3.7 Percentage of radiolabelled protein from the SDS-PAG slices sticking to microtitre wells

protein PI was also identified in the Triton extract, its apparent subunit molecular weight was slightly higher than in the DOC extract as traces of Triton had distorted the gel . High molecular weight material, gel slice 2, was also labelled in the Triton extract, this was not further identified.

Autoradiography of the SDS-PAGs may be a better way of detecting surface labelled antigens. However this was not used as counting of sliced gels also allowed the separated antigens to be used subsequently for antibody detection.

3.4.1 Identification of surface antigen-antibody reactions by GED-ELISA.

The IgG antibody specificities in sera from patients with disseminated gonococcal infection were compared with sera from patients with uncomplicated gonorrhoea or systemic meningococcal infection in GED-ELISA experiments. The antigens were the ^{125}I-labelled DOC and Triton extracts whose surface components had been located in the gel slices (3.4).

Four pools each containing 4 sera, were from controls and patients with uncomplicated gonorrhoea or disseminated gonococcal infection or systemic meningococcal infection.

Sera from patients with uncomplicated gonorrhoea had IgG antibody to LPS or possibly PII and LPS (PII/LPS). The sera from patients with disseminated gonococcal infection had higher IgG levels

to the gel slices containing PI only, whilst sera from patients with systemic meningococcal infection had IgG to PII/LPS, undefined high molecular weight antigens and possibly PI. Only the IgG antibody in sera from patients with a systemic infection bound to antigens in the Triton extract. PI was probably involved, but there were also other antigens which were not exposed on the surface of N.gonorrhoeae strain H1. (Figures 3.7a and 3.7b).

The IgG antibody in the sera of patients with a systemic infection reacted with PI and other undefined antigens in the Triton extract. The undefined antigens may have been from the cytoplasmic membrane or proteins which are surface exposed on the more pathogenic strains but are not surface exposed on strain H1. Therefore these results may reflect a difference in the surface antigenic make up of strains which produce a serious infection or a difference in the antibody response due to higher antigen levels or prolonged exposure.

With the DOC extract different IgG patterns were found with the sera from all three infected groups. The results confirmed the previous observation that IgG anti-PI was produced in disseminated gonococcal infection but not in uncomplicated gonorrhoea (Section 3.1.2). It was not clear whether anti-LPS alone or anti-LPS and anti-PII IgG were produced in uncomplicated gonorrhoea. Further antigen preparations were made from N.gonorrhoeae strain H1 in an attempt to determine the specificity of IgG in uncomplicated gonorrhoea.

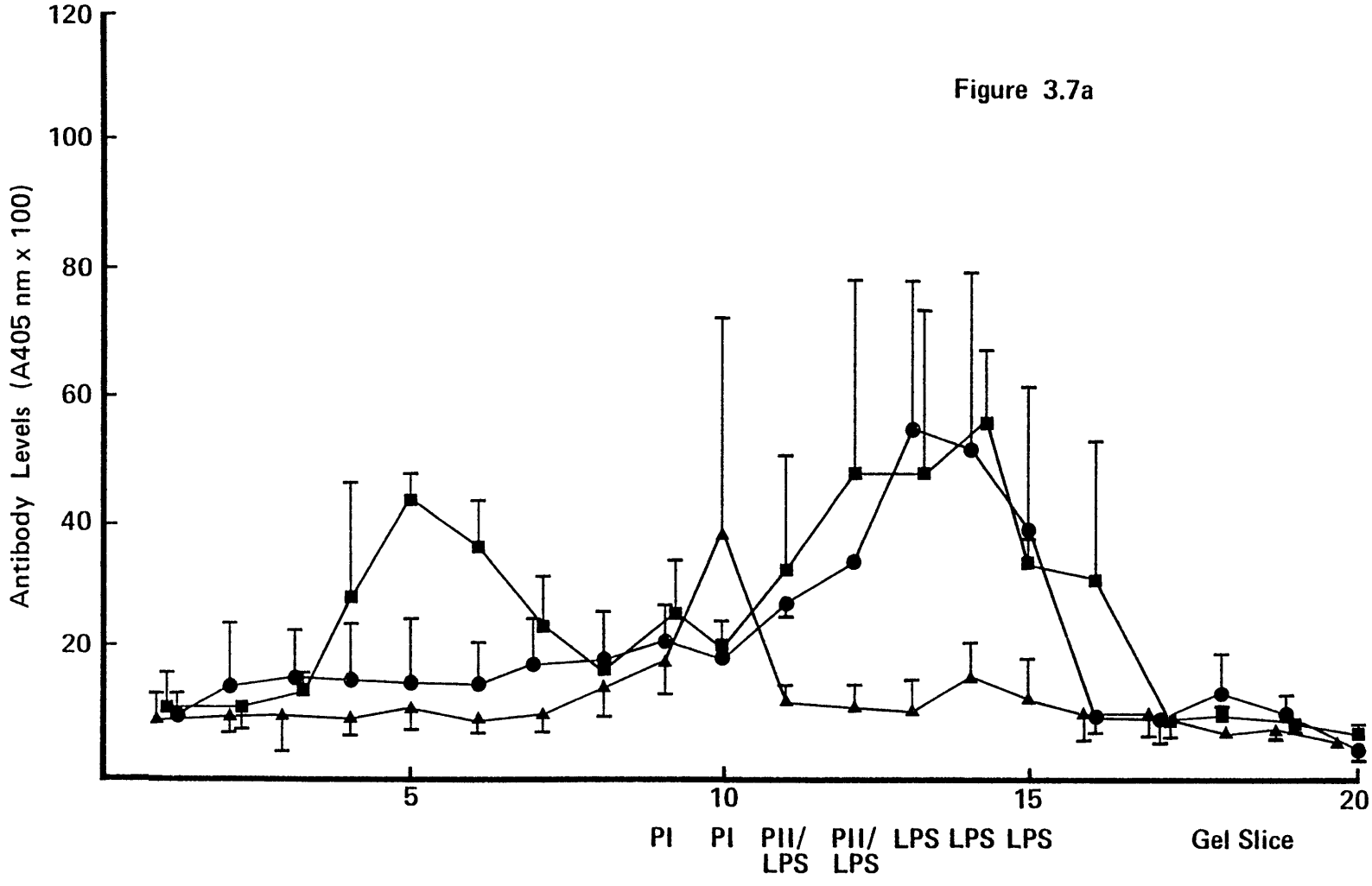
Figures 3.7 a and b

GED-ELISA profiles of the ^{125}I-labelled DOC (a) and Triton (b) extracts using sera from patients with uncomplicated gonorrhoea (●), disseminated gonococcal infection (▲) or systemic meningococcal infection (■).

Sera diluted 1 in 200

Conjugate - anti-human IgG/alkaline phosphatase

Figure 3.7a


120
100
80
60
40
20
Antibody Levels (A405 nm x 100)
5
10
15
20
PI
PI
PII/ LPS
PII/ LPS
LPS
LPS
LPS
Gel Slice

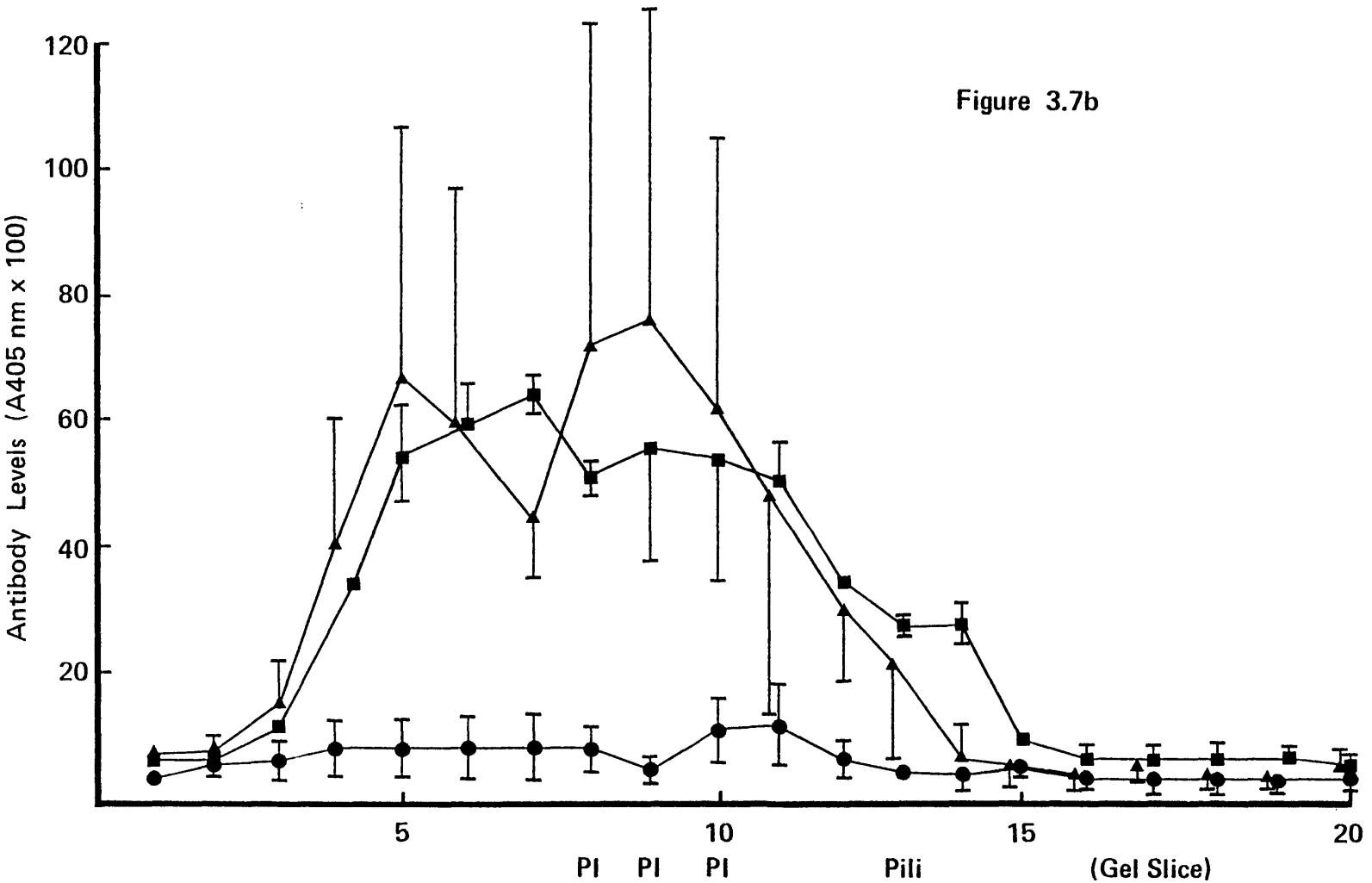

120
100
80
60
40
20
Antibody Levels (A405 nm x 100)
5
10
15
20
PI
PI
PI
Pili
(Gel Slice)

Figure 3.7b

Gel Slice	DOC Extract $\bar{x}$	DOC Extract σ	Triton Extract $\bar{x}$	Triton Extract σ
1	8.5	1.5	2.0	1.0
2	8.5	3.5	4.5	1.5
3	9.0	4.0	7.5	1.5
4	9.5	1.5	7.0	1.0
5	7.5	0.5	6.0	1.0
6	6.5	4.5	5.5	2.5
7	7.5	2.5	5.5	2.5
8	9.5	1.5	5.5	1.5
9	9.5	0.5	5.5	1.5
10	9.5	0.5	6.0	2.0
11	10.5	1.5	6.0	1.0
12	9.5	2.0	6.0	3.0
13	7.6	1.5	6.0	2.0
14	10.0	1.0	6.5	1.5
15	8.5	1.5	5.0	2.0
16	7.0	1.0	6.0	1.0
17	9.0	2.0	5.5	1.5
18	7.5	0.5	5.5	0.5
19	6.5	0.5	6.0	1.0
20	7.0	1.0	7.5	0.5

Table 3.8 Ged-ELISA results for ^{125}I-surface labelled extracts used to detect IgG antibodies in a control group

Gel Slice	ANTIGEN											
	DOC Extract						Triton Extract					
	a	b	c	d	e	f	a	b	c	d	e	f
1												
2												
3												
4										.001	.02	
5				.01		.02				.02	.02	
6				.05		.05				.01	.02	
7								.05	.05	.002	.01	
8				.05						.01	.01	
9												
10				.05						.02		
11	.05		.02							.01	.05	
12	.01		.01							.01	.01	
13										.02	.01	
14				.05		.05					.02	.05
15				.05		.05						
16												
17												
18												
19												
20												

Table 3.9 Ged-ELISA results. Significance of differences between IgG antibody levels against gel slices. P-values calculated using Students 't' test.

(a) Difference between controls and gonorrhoea patients
(b) " " controls and DGI patients
(c) " " gonorrhoea and DGI patients
(d) " " controls and meningitis patients
(e) " " gonorrhoea and meningitis patients
(f) " " DGI and mengitis patients

3.5 The use of other antigen preparations to identify the reactive antigen(s) in the DOC extract.

Three antigen extracts 1, pili; 2, a PII enriched extract and 3, lipopolysaccharide were prepared from N.gonorrhoeae strain H1 and their ability to detect IgG antibodies in patients with uncomplicated gonorrhoea was compared with the DOC extract.

3.5.1 Pili

Surface radiolabelling of N.gonorrhoeae strain H1 had not identified pili in the DOC extract, see Figure 3.6. However it was possible that traces of pili were responsible for all or part of the antigenicity of this extract.

Pili were prepared as described in section 2.2.4. The purity of the pili preparation was checked by SDS-PAGE and the presence of pili confirmed by electron microscopy. Pili were coated onto microtitre trays at a range of concentrations between 1 and 100 μg of protein/ml and were used to detect anti-pili IgG antibodies. Two sera which were DOC-ELISA positive (i.e. Ecr>20) and two control sera were tested.

Electron microscopy showed the presence of pili in the extract attached to small blebs of outer membrane Figure 3.8a. SDS-PAGE of the pili preparation gave one major band at an apparent subunit molecular weight of 20K and a minor band of slightly higher molecular weight. Figure 3.8b. The two protein bands

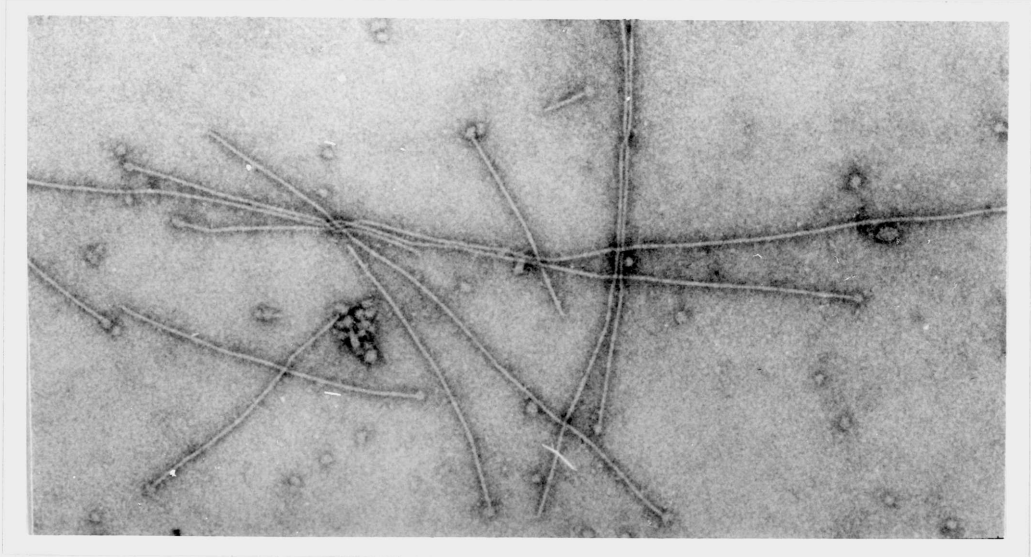


Figure 3.8a Electron micrograph of pili (X 80,000)

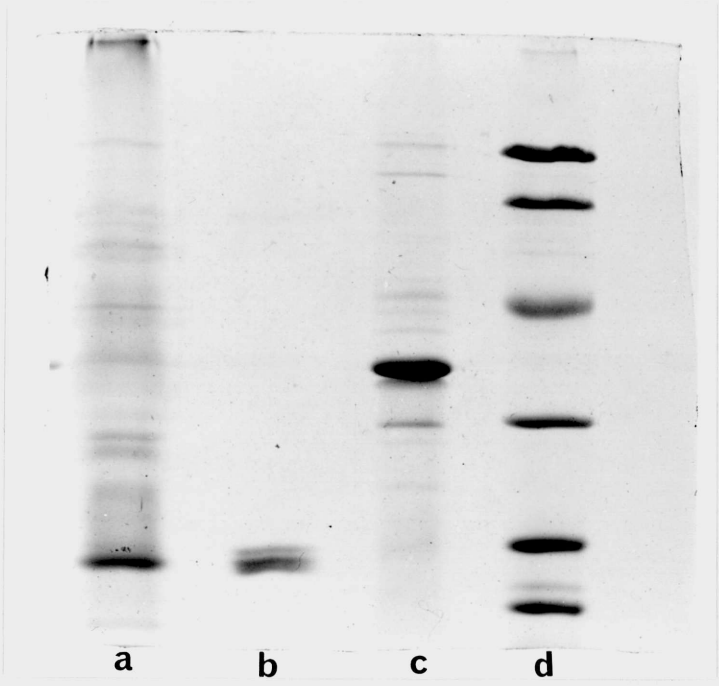


Figure 3.8b SDS-PAGE of Triton extract (a), pili (b), DOC extract (c) and molecular weight standards (d)

on the SDS-PAG are probably due to the presence of pili variants on strain H1, the lower molecular weight variant being the most abundant.

No IgG anti-pilus antibody was detected in DOC positive sera from two patients with uncomplicated gonorrhoea.

This was consistent with previous results which had suggested that anti-pilus antibodies were not being detected by the DOC extract.

3.5.2 A PII enriched extract

Lithium salts are often used to extract outer membrane complexes from Neisseria. James and Heckels (1981) found that by solubilising lithium acetate extracts with deoxycholate a PII enriched fraction was obtained, LiAc-DOC. The remainder was largely PI, LiAc-DOC residue. This procedure was used to prepare PII and PI enriched extracts from N.gonorrhoeae H1 and their antigenicity was compared.

LiAc-DOC and LiAc-DOC residue were prepared from strain H1 and their protein content compared by SDS-PAGE. The presence of LPS in the extracts was determined by KDO analysis. The extracts were coated onto microtitre trays at a range of concentrations between 5 and 50 μg of protein /ml. One DOC-ELISA positive serum from a patient with uncomplicated gonorrhoea and

a control serum were tested. The antigenic components of the two extracts were analysed by GED-ELISA.

An SDS-polyacrylamide gel of the LiAc-DOC and the LiAc-DOC residue is shown in Fig 3.9. The LiAc-DOC contained the outer membrane proteins PI and PII as well as LPS. The LiAc-DOC residue contained PI and a low concentration of LPS, there was no PII visible in the LiAc-DOC residue. Both extracts had similar higher molecular weight protein bands.

The LiAc-DOC detected much higher levels of IgG antibody in the patients serum than the LiAc-DOC residue Figure 3.10. GED-ELISA confirmed that the LiAc-DOC reacted more strongly with the IgG antibodies than the LiAc-DOC residue, Figure 3.11. The antigens in the LiAc-DOC detecting the IgG antibody were in the PII/LPS slices of the gel. PI in both the LiAc-DOC and the LiAc-DOC residue did not detect antibody.

The use of LiAc extracts did not resolve the PII/LPS specificity of the IgG antibodies in patients with uncomplicated gonorrhoea. The low activity of the LiAc-DOC residue may have been due to the presence of some LPS in this extract. To determine whether or not anti-LPS antibodies were being detected LPS was purified from strain HI.

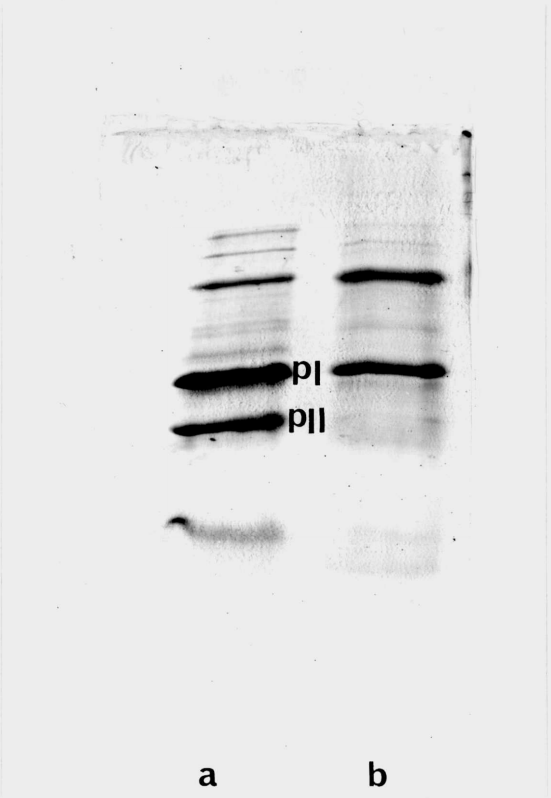


Figure 3.9 SDS-PAGE of the lithium acetate-DOC extract (a) and the lithium acetate DOC residue (b) from N.gonorrhoeae strain HI

Figure 3.10 Antigen titrations of (a) a DOC extract and (b) the DOC-residue from a lithium acetate preparation

●- serum from a patient with uncomplicated gonorrhoeae

○- a control serum

Conjugate - Anti-human IgG/alkaline phosphatase

Figure 3.10a

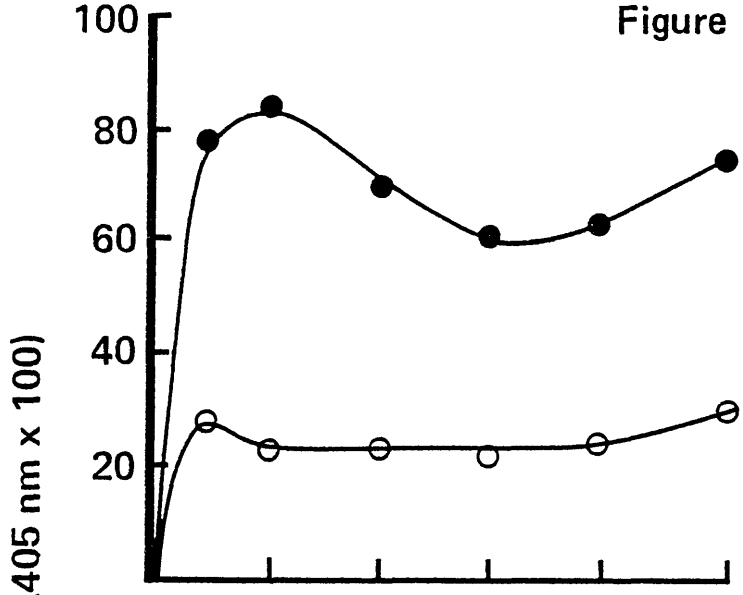


Figure 3.10b

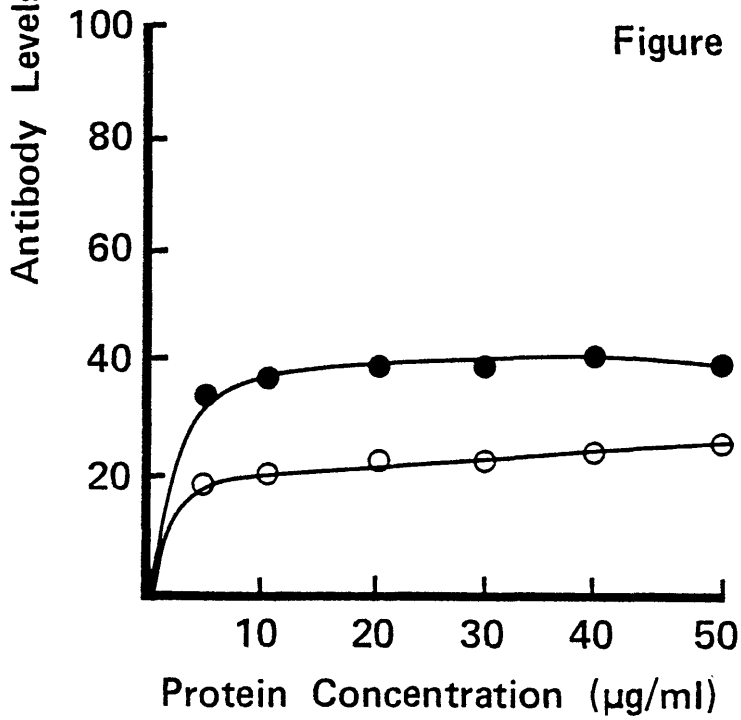


Figure 3.11 GED-ELISA profiles of (a) a DOC extract and (b) the DOC-residue from a lithium acetate preparation

● - serum from a patient with uncomplicated gonorrhoea

○ - a control serum

Conjugate - anti-human IgG/alkaline phosphatase

Figure 3.11a

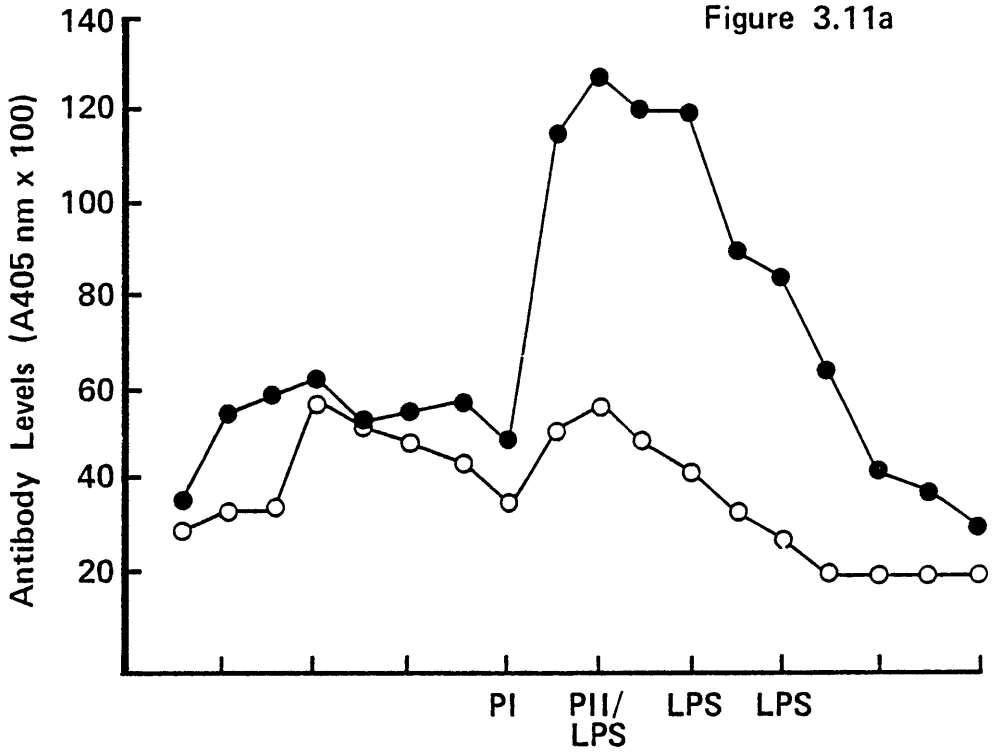
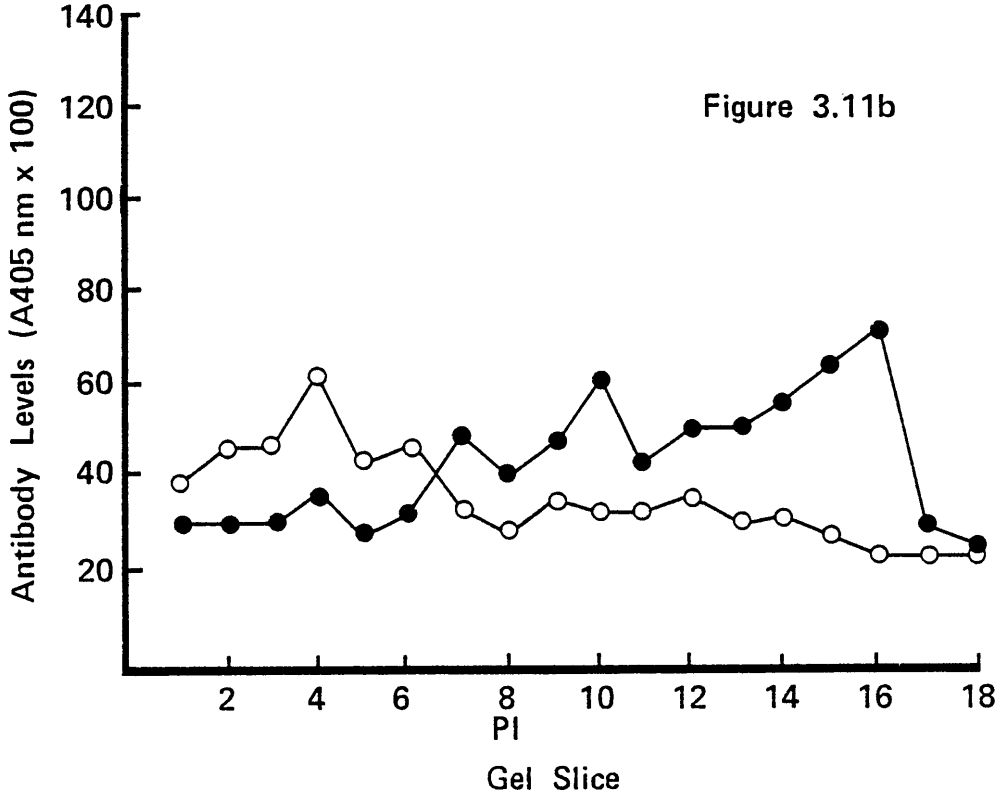


Figure 3.11b



3.5.3 Lipopolysaccharide (LPS)

LPS prepared from N.gonorrhoeae strain HI was attached to microtitre trays by incorporating magnesium ions into the coating buffer (Ito et al, 1980). The concentrations of LPS were varied between 10 and 200 μg of LPS/ml. Two sera from patients with uncomplicated gonorrhoea and a control serum were tested for IgG antibodies.

An LPS antigen titration is shown in Figure 3.12. The LPS detected IgG antibodies in both sera from patients with uncomplicated gonorrhoea.

These results suggested that LPS was an important antigen in the DOC extract, but the presence of anti-PII antibodies could not be ruled out. In order to determine more precisely the specificities of antibodies produced in uncomplicated gonorrhoea the technique of immunoblotting, which gives more accurate localisation of antigens than GED-ELISA was used.

Figure 3.12

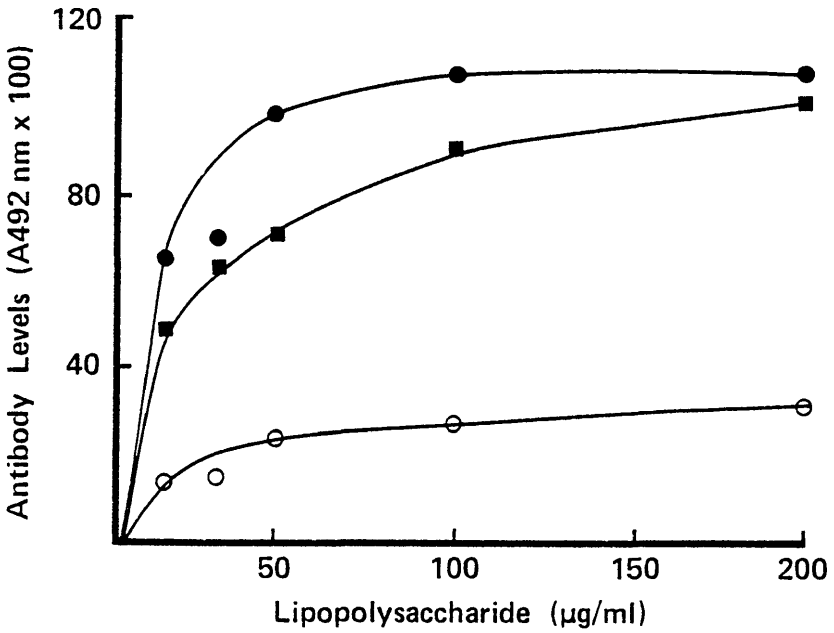


Figure 3.12 Antigen titration of lipopolysaccharide

○ - a control serum

■ (♀) sera from patients with
● (♂) uncomplicated gonorrhoea

sera diluted 1 in 1000

Conjugate - Anti-human IgG/horse radish peroxidase

3.6 The use of immunoblotting to identify the significant antigens in the DOC extract

Western-blotting (Towbin, 1979) is an adaptation of a method first developed by Southern (1975) for transferring DNA fragments to nitrocellulose. Proteins are separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose. The proteins are immobilised on the nitrocellulose and can be detected by staining, autoradiography or by using antibodies, immunoblotting. The antibody specificity in sera from patients with gonorrhoea was examined against separated antigens using the immunoblotting technique.

3.6.1 Protein transfer

Proteins were electrophoretically transferred to the nitrocellulose at a current of 200 mA for 2 h. An estimate of the percentage of protein transferred was obtained by measuring the absorption of Coomassie Blue stained bands before or after transfer.

PIII was transferred more easily (51%) than PI (31%) and PII (28%), Table 3.10.

The efficiency of protein transfer has previously been reported to be directly related to the apparent subunit molecular weight (Burnett, 1981; Lin and Kasamatsu, 1983). The discrepancy found here, i.e. the higher molecular weight PIII being

Protein	Percentage Transfer	
	x	S.D.
PI	31	(±4)
PIII	51	(±12)
PII	28	(±1)

Table 3.10 Immunoblotting. Percentage transfer of protein antigens from the SDS-PAG

transferred more easily than PII, may be due to the co-migration of LPS with PII in the SDS gel, see sect. 3.1. Other workers have used highly purified proteins for these studies.

Monospecific mouse antisera raised to PI, PII and PIII, sect. 2.9, were used to locate the precise positions of these proteins on the nitrocellulose membrane, Figure 3.13. This made the identification of antigens in the blots easier than by comparison with the gels which expanded during staining.

3.6.2 Sensitivity of immunoblotting

An estimate of the sensitivity of immunoblotting for the detection of immunoglobulins was determined using a "spot-test" (Hawkes et al, 1982).

Known quantities of IgG, between 14 and 0.01 ng, and IgA, 29 to 0.2 ng, were dried onto nitrocellulose. Immunoglobulins were detected by an anti-human IgG or IgA linked to horse radish peroxidase and a substrate, tetra-aminobiphenyl-HCl (BDH) which precipitates onto the nitrocellulose when oxidised.

The spot was just visible at 0.2 ng of IgG (Fig. 3.14a, no.8) and at 1.5 ng of IgA (Fig. 3.14b, no.7).

One hundred microlitres of a test serum was diluted in 10 ml of buffer in the immunoblot tests. From these results the serum

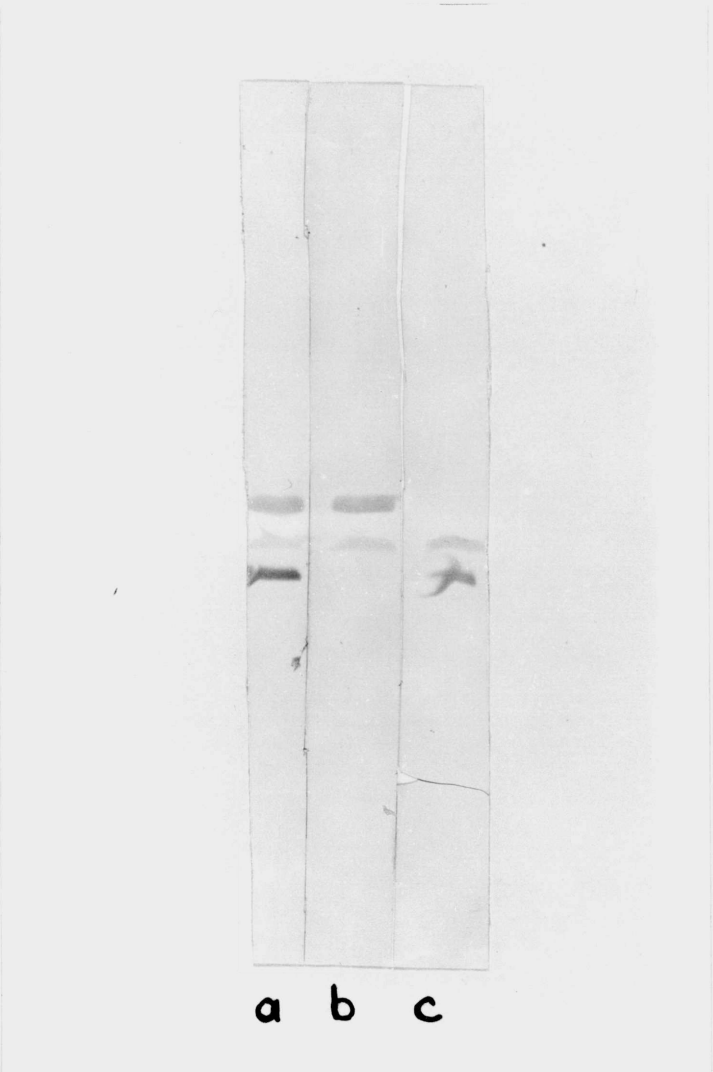


Figure 3.13 Location of proteins I, II and III in the DOC extract by immunoblotting using combinations of specific mouse antisera. (a) Anti-PI, II and III, (b) Anti-PI and III, (c) Anti-PII and III.

Antisera diluted 1 in 10,000

Conjugate - anti-mouse Ig/horse radish peroxidase

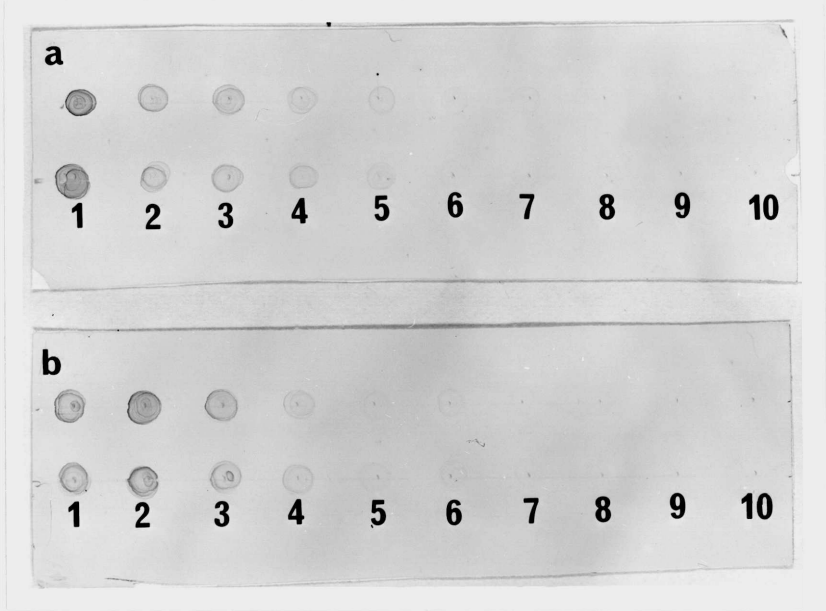


Figure 3.14 Spot test to measure the sensitivity of detection of human immunoglobulins by peroxidase conjugates and the substrate TABH.

(a) Amount of IgG per spot, ng (1) 14, (2) 5, (3) 3, (4) 1.75 (5) 1.3, (6) 0.5, (7) 0.3, (8) 0.2, (9) 0.05, (10) 0.01

(b) Amount of IgA per spot, ng (1) 29, (2) 21, (3) 15, (4) 7, (5) 3, (6) 2, (7) 1.5, (8) 0.7 (9) 0.25, (10) 0.2

must contain a minimum of 2 ng/ml of specific IgG and 30 ng/ml of specific IgA for them to be detected by this method. In order to increase the detection of IgA antibodies 1 ml of serum rather than 100 µl was used whenever a sufficient quantity of serum was available.

The spot-test only gave an estimate of the sensitivity of immunoblotting as under test conditions the immunoglobulins were bound to antigen not to nitrocellulose. Immunogloblins bound to antigen would all be in the same orientation and sticking of the conjugate may be easier or more difficult than when the immunoglobulins have a random orientation as in this assay.

3.6.3 Antibody specificity in sera from patients with uncomplicated gonorrhoea

Antigens in the DOC extract were separated on SDS-polyacrylamide gels. Conditions were exactly the same as for the GED-ELISA experiments with the exception that dithiothreitol was omitted from the sampling buffer to improve the separation of PI and PIII. Antigens were transferred to nitrocellulose and incubated with antibody as described in sect. 2.7. Twenty control sera and twenty sera from patients with uncomplicated gonorrhoea, which were ELISA positive with the DOC antigen, were examined for IgG specificity by the immunoblot method. There were equal numbers of males and females in each group. IgA results were obtained for 28 sera, 13 patients (6 male, 7 female) and 15 controls (7 male, 8 female). One hundred microlitres of serum was used for each assay.

All the patients sera had IgG antibody to one or more of the antigens in the DOC extract. Two examples of IgG immunoblots are shown in Figure 3.15, which have antibodies to PII only (a) and PIII, P40-50K, P60-90K, LPS and minor protein antigens (d). Lines of reaction to one or more of the antigens in the DOC extract were also found in eleven of the control sera. These results are summarised in Tables 3.11a and 3.11b. IgG antibody in patients sera, Nos.1-10 (male) and 11-20 (female) were predominantly directed to LPS, only patients no.2, 3, 7 and 20 did not have antibody to this antigen. Three female, but no male, control sera contained anti-LPS IgG.

Seventeen patients and ten control sera contained IgG antibody to one or more of the protein antigens.

The specificity of the IgA antibodies differed from the IgG (Table 11b). The response to the LPS antigen was reduced whilst the number of sera containing anti-PIII IgA was increased. The specificity of IgG and IgA antibodies were different in individual sera. For example patient No.4 had IgG against LPS and P60-90K and IgA to LPS, PIII and P40-50K. Three sera from patients and nine from controls had no detectable IgA, however the sensitivity of the IgA assay was lower than that for IgG.

The only significant difference in IgG specificity between patients and controls was for LPS, (X^2 test $p = 0.001$). Confirmation of the presence of LPS in the DOC extract was obtained by comparing LPS (phenol-water preparation) with the DOC extract

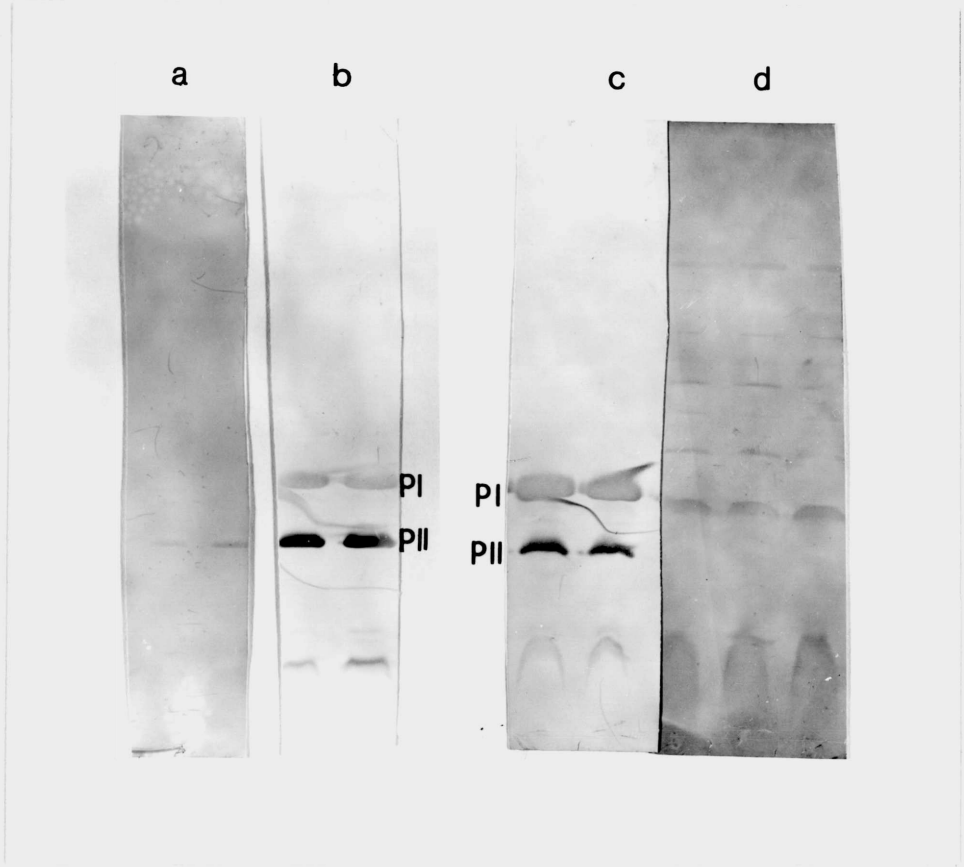


Figure 3.15 Immunoblots of two patients' sera against the DOC extract. (a) showing antibody to PII only; (b and c) mouse antisera to PI and PII; (d) showing antibodies to PIII, a 40-50K protein, a 60-90K protein, LPS and minor protein antigens.

Human sera were diluted 1 in 100

Conjugate, Anti-human IgG/horse radish peroxidase

PATIENTS														CONTROLS													
MALES							FEMALES							MALES							FEMALES						
No.	LPS	PII	PIII	PI	P40-50K	P60-90K	No.	LPS	PII	PIII	PI	P40-50K	P60-90K	No.	LPS	PII	PIII	PI	P40-50K	P60-90K	No.	LPS	PII	PIII	PI	P40-50K	P60-90K
1	+						11	+		+		+	+	21							31					+	
2						+	12	+						22							32						
3		+					13	+				+		23							33						
4	+					+	14	+					+	24							34					+	+
5	+						15	+	+				+	25			+			+	35	+	+				+
6	+		+		+	+	16	+	+					26						+	36						
7						+	17	+	+	+		+		27					+		37						
8	+				+	+	18	+					+	28			+	+			38	+					+
9	+		+	+			19	+		+	+		+	29			+			+	39					+	+
10	+		+		+		20						+	30							40	+					

TABLE 3.11(a) Distribution of specific antibodies in patients and controls IgG antibodies

PATIENTS															CONTROLS													
MALES							FEMALES								MALES							FEMALES						
No.	LPS	PII	PIII	PI	P40-50K	P60-90K	No.	LPS	PII	PIII	PI	P50-50K	P60-90K		No.	LPS	PII	PIII	PI	P40-50K	P60-90K	No.	LPS	PII	PIII	PI	P40-50K	P60-90K
4	+		+		+		13	+							23							31						
6							14						+		25							32					+	
7							15		+						26							33						
8	+		+				17	+		+					27							34						
9			+	+			18								28			+	+			35			+			
10			+				19			+	+				29			+	+		+	38						
							20			+					30							39						+
																						40			+			

Table 3.11(b) Distribution of specific antibodies in patients and controls IgA antibodies

Figure 3.16. The distribution of anti-protein IgG was irregular and antibody to any one protein was not found significantly more frequently among patients than controls, Figure 3.17. Anti-PII IgG was only found in four sera. These results suggest that the LPS of the DOC extract was the antigen responsible for the detection of IgG antibodies in sera from patients with uncomplicated gonorrhoea. IgA antibodies directed against PIII are probably detected by this antigen in the DOC extract.

3.6.4 Antibody specificity of sera from patients with disseminated gonococcal infection

The IgG specificity of two sera from patients with disseminated gonococcal infection was examined by immunoblotting. Both sera were IgG ELISA positive against the DOC extract.

Immunoblotting detected IgG anti-PI in both sera. IgG antibody was also found to other components of the DOC extract. One example is shown in Figure 3.18 this serum contained IgG anti PI, P40-50K, P60-90K and possibly LPS.

These results confirm those previously found using purified PI (sect 3.1) and GED-ELISA experiments (sect. 3.4) that IgG anti-PI is produced in disseminated gonococcal infections.

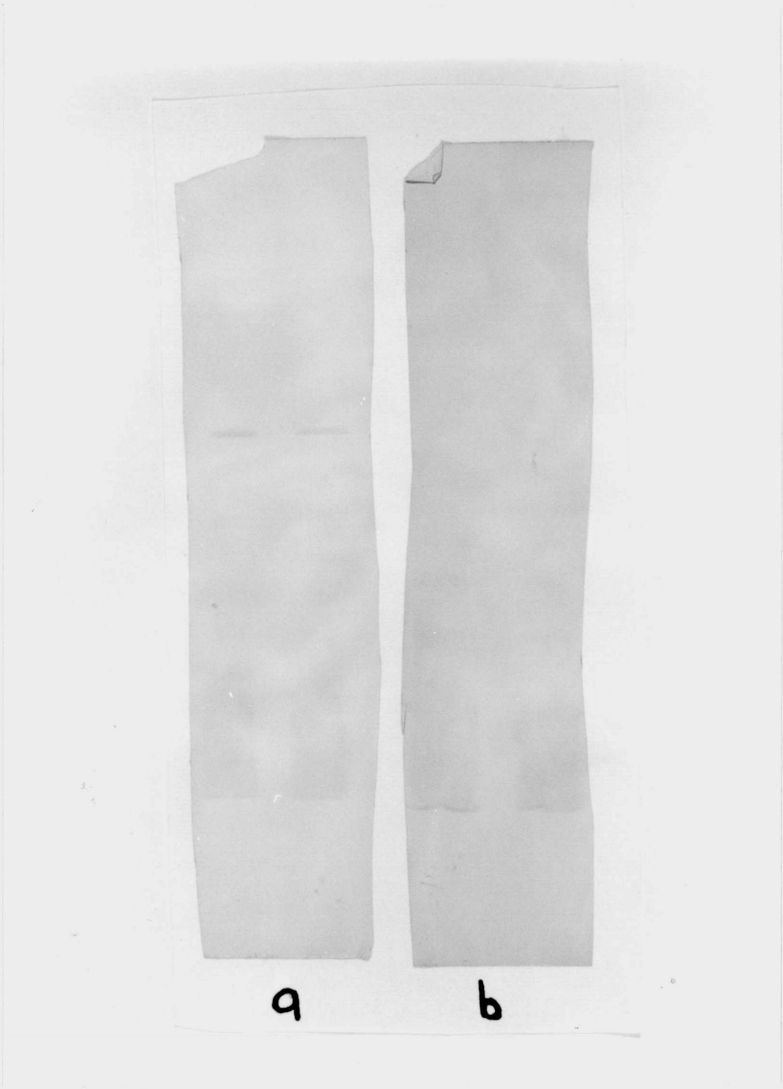


Figure 3.16 Comparison of the DOC extract (a) and lipopoly-saccharide, (b) antigens in immunoblots

Serum from a patient with uncomplicated gonorrhoea

Conjugate - anti-human IgG/horse radish peroxidase

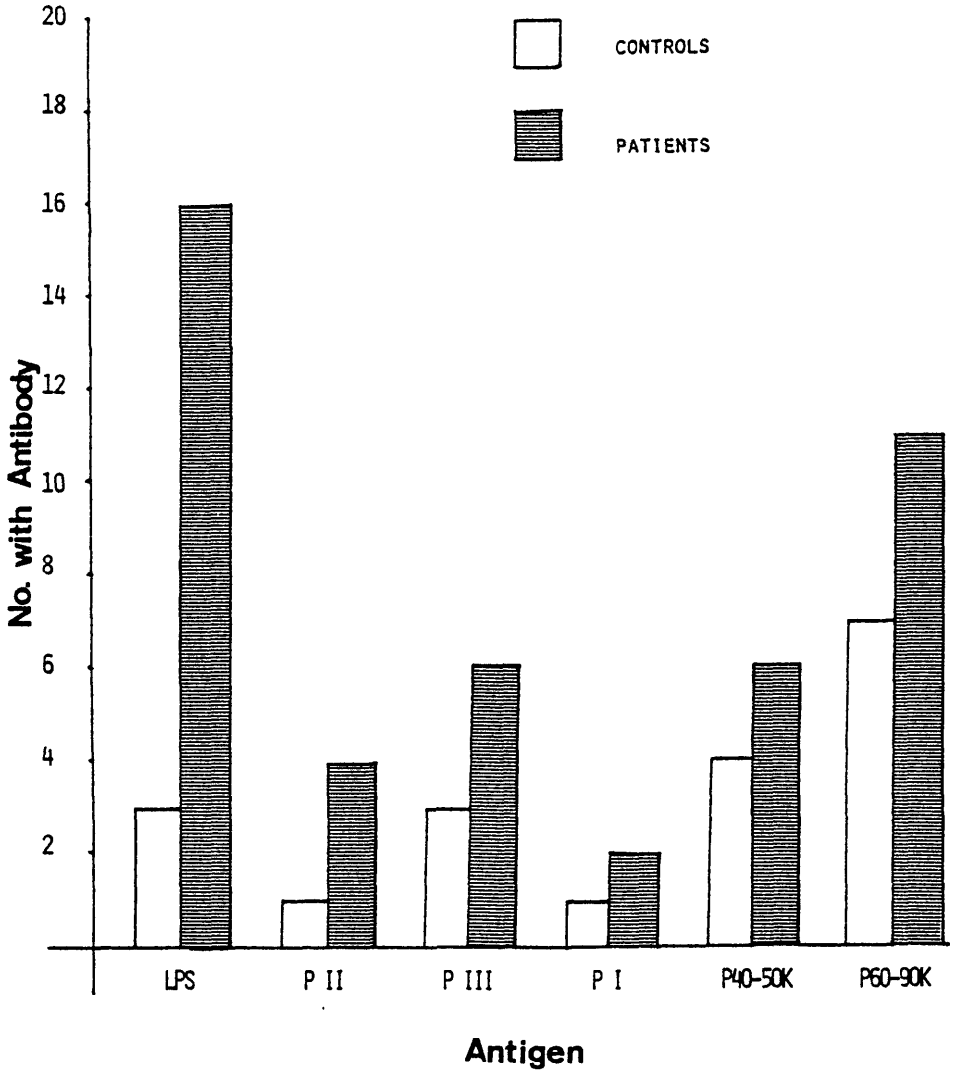


Figure 3.17 Frequencies in patients (■) and controls (□) of IgG antibodies to individual antigens in the DOC extract

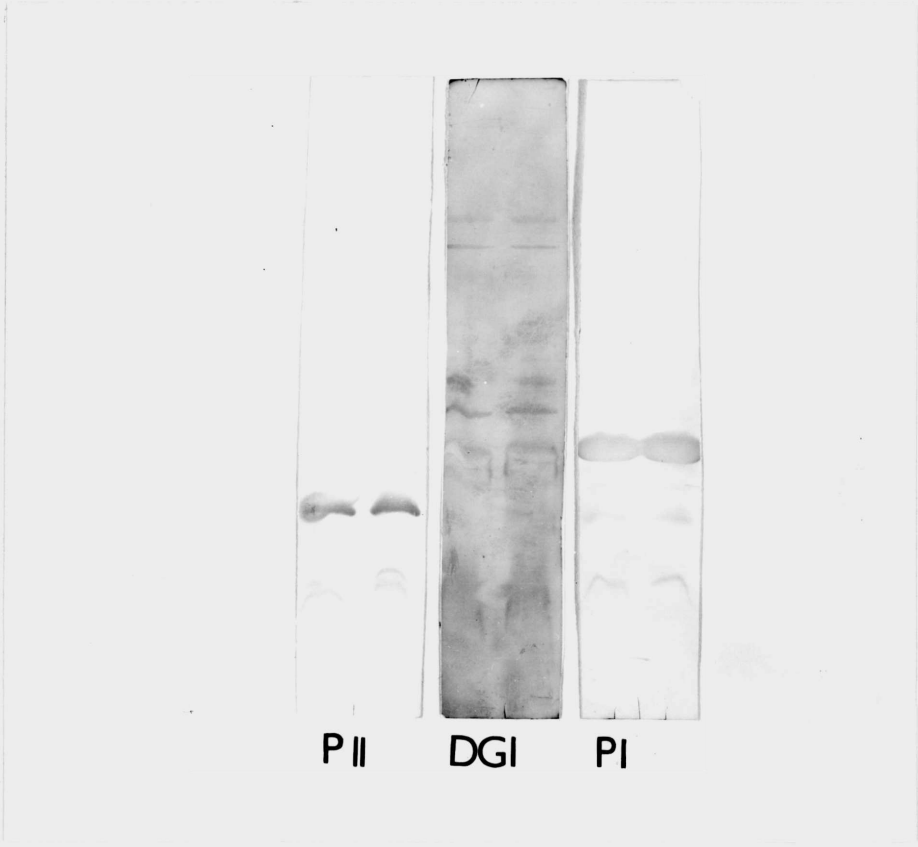


Figure 3.18 Immunoblot of a serum from a patient with a disseminated gonococcal infection (DGI), IgG specificity with the DOC extract. The positions of PI and PII were located with mouse antisera

3.7 Examination of sera from patients with gonorrhoea for the presence of antibody specific to the patients infecting strain

The results obtained using the DOC extract from strain HI suggested that antibodies to protein antigens, particularly outer membrane proteins, were not frequently produced in gonorrhoea. However this may have been due to the production of antibody specific for the outer membrane proteins of the patients infecting strain (IS). Such antibodies would not be detected using strain HI. In order to examine this possibility strains of N.gonorrhoeae isolated from patients were used for antibody detection.

3.7.1 Comparison of strain HI and patients infecting strains by immunoblotting

Whole bacteria were prepared for SDS-PAGE in 1% SDS, without dithiothreitol, by boiling for 5 mins. Ten micrograms of protein were applied to each 3 mm wide gel slot. Separated antigens were transferred to nitrocellulose and incubated with 100 µl or 1 ml of serum, diluted in 10 ml of buffer, to determine IgG and IgA specificity respectively. Twelve sera were tested against opaque, pilated colony types of strain HI and the patients infecting strain (IS). Two additional sera were also tested against opaque, non-pilated colony types of the IS.

The results are summarised in table 3.12 and some examples shown in Figure 3.19.

Antigen	Strain	Sera A	B	C	D	E	F	G	I	J	K	M	N	O	P
P<20K	IS	(+)	( )	+					(+)				+		(+)
	HI	+		+					(+)				+	+	(+)
LPS	IS				+	+	+								
	HI				+	+	+								( )
Pili	IS	+	+	+	(+)	(+)	(+)		+	(+)	(+)		+		
	HI	+		+	(+)	(+)	(+)	+	+	(+)	(+)	+	+	+	
PII	IS	+		+		+			+		(+)		+		
	HI			+		+			+		(+)		+		+
PIII	IS	( )			( )				+				( )		(+)
	HI	(+)			( )				(+)				( )		(+)
PI	IS				( )									+	
	HI	+			( )									+	
P40-60K	IS			(+)	(+)	(+)	+		( )	(+)	(+)	(+)		+	(+)
	HI			(+)	(+)	(+)	+		( )	(+)	(+)	(+)		+	(+)
P61-90K	IS			(+)			(+)	(+)	(+)		(+)	(+)	+	+	
	HI			(+)			(+)	(+)	(+)		(+)	(+)	+	+	

Table 3.12 Immunoblotting results using whole cell antigens.
Distribution of antibodies in patients sera

\+ = IgG antibody

( ) = IgA antibody

(+) = IgG and IgA antibody

IS = patients infecting strain

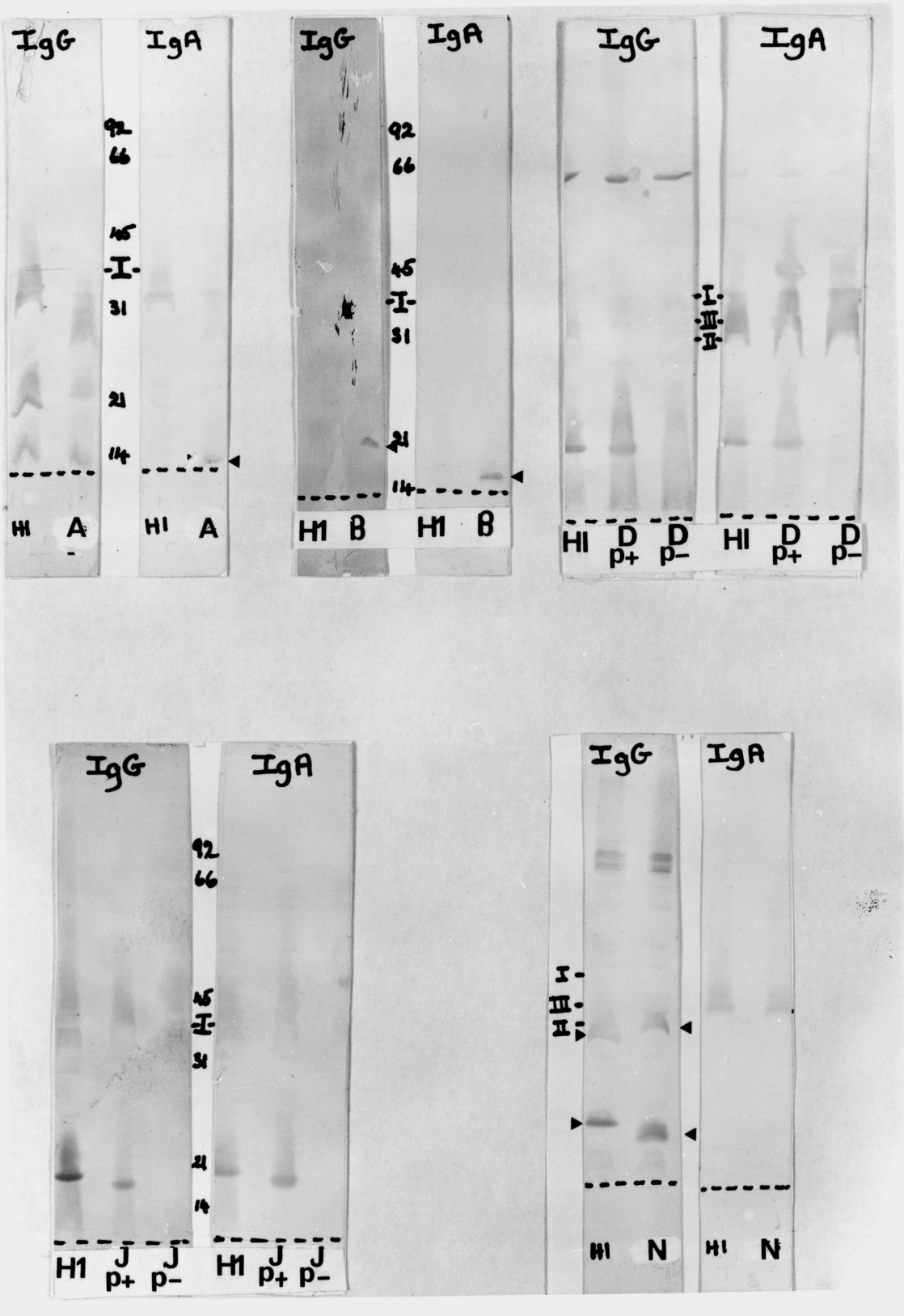
HI = N.gonorrhoeae strain HI

Figure 3.19 Immunoblots comparing strain HI and the patients infecting strain (IS) for the detection of IgG and IgA antibodies.

Serum A has IgA to a P<20K in the IS only

Serum B has IgA to a P<20K and IgG to pili in the IS only

Sera D and J were tested against strain HIP+, ISP+ and ISP-, note the lack of pili antibody in ISP-


IgG
IgA
92
66
45
I
31
21
14
Hl
A
B
D
p+
p−
J
N
II
III

Anti-pilus immunoglobulins

Thirteen of the fourteen sera had anti-pilus IgG and five had anti-pilus IgA. Only in one serum, B, was this specific for the IS. In three sera, G, M and O, IgG to strain HI pili was found but not to pili of the infecting strain. Differences in the apparent subunit molecular weight of the pilus protein in strain HI and the IS did not affect the antibody reaction. For example the subunit molecular weight of pilus protein from strain HI was higher than that of strain J, Figure 3.19, but IgG and IgA antibodies in the serum from patient J reacted with both the pilus antigens.

Anti-outer membrane protein immunoglobulins

IgG or IgG and IgA antibodies were found to undefined proteins of high molecular weight, 40-90K, in 12 of the sera. There were no differences between the IS and strain HI for these antigens. However the results obtained using the DOC extract, 3.6.3, had shown a high frequency of antibodies to these antigens in sera from controls. This suggests these antibodies may not be specific for gonococcal proteins.

Antibodies to an undefined low molecular weight protein, less than 20K, were found in seven sera. Two of these sera, A and B, contained IgA which reacted with this protein in their IS only and one serum, O, contained anti-P@20K IgG which reacted with strain HI but not the IS.

Three sera showed differences in their antibody specificity for the outer membrane proteins, PI, PII and PIII. Serum A contained IgG anti-PI and PIII which reacted only with strain HI and IgG anti-PII specific for the IS. Sera I and P had HI specific anti-PIII IgA and anti-PII IgG respectively.

As was found with the antibodies to pili, differences in the apparent subunit molecular weights of the outer membrane proteins did not affect the reactivity of the antibodies.

Antibodies to lipopolysaccharide (LPS)

Only three sera (approx. 20%) contained anti-LPS IgG compared with 80% of the sera tested against the DOC extract, section 3.6.3. However the sera used in this study were unselected whilst in previous work they were all DOC-ELISA positive which probably selected for sera containing anti-LPS IgG.

Summary

These results gave little evidence for the presence of antibody specific to the patients infecting strain, particularly as six sera contained antibody to antigens in strain HI but not to the same antigens in their IS. However the immunoblot method was not quantitative and therefore higher antibody levels to the IS than to HI would not have been detected and the harsh conditions used to prepare antigens for SDS-PAGE may have destroyed some antigenic

determinants. To examine this possibility whole bacteria were coated onto microtitre trays and were used to detect antibodies in patients sera.

3.7.2 Comparison of strain HI and patients infecting strains by ELISA

Strain HI and the patients infecting strain (IS) in pilated (P+) and non-pilated (P-) opaque colony types were used at an O.D. 550 nm of 0.5 to coat microtitre trays. Ten sera which had been used in the immunoblot experiments, section 3.7.1, were tested for IgG and IgA antibodies. A pool of IgG DOC ELISA positive sera was used to control day to day variations. Each day the pool was tested against strain HI, P+ and P-, to establish a mean value. Antibody levels were multiplied by a factor (F) for that day (F = mean value divided by value on that day x100) to give corrected values (Ecr).

A significantly higher antibody level against IS P+ than HI P+ was interpreted as indicative of the presence of antibody specific for the pili on the IS. IS specific anti-pili immunoglobulin were found in three sera. Serum D had IgA and sera A and F had IgG levels which were higher with IS P+ than HI P+ (Table 3.13a).

A significantly higher antibody level against IS P- than HI P- was interpreted as indicative of the presence of antibody specific for non-pilus, outer membrane antigens on the IS. One serum, J, showed evidence for IS specific IgA to non-pilus antigens (Table 3.13b).

	Ecr for Sera										
	A	B	C	D	E	F	G	I	J	K	
HI P+	32(2)*	19(3)	34(2)	144(15)	39(7)	81(15)	50(16)	98(2)	59(8)	101(7)	IgG Levels
IS P+	40(2)	13(2)	25(3)	137(3)	40(7)	171(3)	28(3)	65(17)	57(2)	124(14)	
Difference #	.01	NS	.01	NS	NS	.001	.01	.05	NS	NS	
HI P+	19(2)	7(2)	105(5)	125(5)	80(5)	43(5)	53(5)	50(7)	50(6)	31(4)	IgA Levels
IS P+	21(2)	8(2)	87(2)	144(7)	72(5)	43(5)	39(3)	40(7)	50(4)	41(4)	
Difference #	NS	NS	.01	.05	NS	NS	.02	NS	NS	NS	

Table 3.13a Comparison of ELISA results using pilated colony types of strain HI or the infecting strain (IS) to detect antibody in patients sera

* = standard deviation

= P value calculated using Students 't' test

	Ecr for Sera										
	A	B	C	D	E	F	G	I	J	K	
HI P-	34(2)	18(2)	34(2)	143(6)	50(4)	129(4)	60(3)	75(3)	54(2)	92(2)	IgG Levels
IS P-	37(4)	15(2)	ND	137(9)	56(5)	129(10)	30(3)	44(11)	37(16)	97(10)	
Difference #	NS	NS	-	NS	NS	NS	.001	.01	NS	NS	
HI P-	16(4)	9(2)	102(4)	122(4)	82(4)	53(2)	65(3)	56(10)	47(6)	44(3)	IgA Levels
IS P-	10(2)	11(2)	ND	85(10)	76(4)	22(4)	51(3)	19(2)	68(4)	34(4)	
Difference	NS	NS	-	.01	NS	.001	NS	.01	.01	.05	

Table 3.13b Comparison of ELISA results using non-pilated colony types of strain HI or the infecting strain (IS) to detect antibody in patients sera

* = standard deviation

= P value calculated using Students 't' test

These results showed little evidence for IS specific antibody, strain HI was more often a better antibody detector than the patients infecting strain.

There was little correlation between the results obtained from immunoblotting and the microtitre tray ELISA. This may have been due to the qualitative nature of immunoblotting and competition between antibodies of different specificities and avidities in the microtitre tray assay.

3.8 The detection of IgM antibody in sera from patients with gonorrhoea

The work of Ison and Glynn (1979) did not show any significant difference between the anti-gonococcal IgM levels in sera from patients and normal controls. This may be representative of the antibody response in gonococcal infections or due to the poor detection of low avidity IgM in a sandwich ELISA method, IgM having to compete with IgG and IgA for binding sites. To establish whether or not IgM antibodies are produced in gonorrhoea IgM was purified from sera and tested in the DOC-ELISA.

Two sera from patients with gonorrhoea were fractionated by ion exchange chromatography and the IgG, IgA and IgM content of the fractions was determined by ELISA (see section 2.11).

The fractions in the third peak contained the highest percentage of IgM (see Figure 2.2) and were concentrated by Amicon filtration to the same volume as the original serum. The IgM fraction was compared with the original serum in a DOC-ELISA, Table 3.14.

The levels obtained in the ELISA with the IgM were lower than were obtained with the whole serum. The lower value is probably due to some denaturation of the immunoglobulin during purification or loss of IgM in the fractions of the second, IgA, peak. The results suggest that competition between other classes of antibody was not playing a significant role in the test and that high levels of IgM are not produced in uncomplicated gonorrhoea.

	Absorbance 492 nm x 100	
	Whole Serum	Purified IgM
1	1.86	1.41
2	1.60	0.81

Table 3.14 ELISA using the DOC antigen to detect IgM antibodies in sera from patients with gonorrhoea

Serum dilution 1 in 100

Conjugate Anti-human IgM-HRP

An antibody capture assay for IgM would be required to confirm these results.

3.9 Immune Complexes in Gonorrhoea

Immune complexes have been reported in sera from patients with disseminated gonococcal infection (Danielsson et al., 1972) but not in sera from patients with uncomplicated gonorrhoea. The object of this study was to determine whether or not circulating immune complexes were decreasing the sensitivity of the DOC-ELISA. Antibody bound in immune complexes would not be available to react with the DOC antigen and sera containing immune complexes would consequently give a negative DOC-ELISA result.

Professor J. Mowbray kindly isolated and characterised the immune complexes from 15 gonococcal and 10 control sera. Immune complexes were precipitated from sera by polyethylene glycol and the antibody class in the precipitate determined by radial diffusion.

Sera numbers 1 to 3 were from patients with disseminated gonococcal infection, numbers 4 to 15 from patients with uncomplicated gonorrhoea and numbers 16 to 25 from patients attending the clinic for STD who had no symptoms of infection and from whom no pathogen was isolated, (Table 3.15). Immune complexes were found in all three patients with disseminated gonococcal infection, four of the 12 sera from patients with uncomplicated gonorrhoea and 3 of the 10 sera from controls. Nine patients sera gave a positive DOC-ELISA result and five of these contained immune complexes. Of the six sera which gave a negative DOC-ELISA result two contained immune complexes.

Patient	Number	Immune Complex Clq	G	M	A	DOC ELISA Result
Disseminated gonococcal infection	1	+	+	+	+	-
	2	+	+	+	+	+
	3	-	+	-	-	+
Uncomplicated gonococcal infection	4	-	-	-	-	+
	5	-	-	-	-	-
	6	+	+	+	+	-
	7	+	-	-	-	+
	8	-	-	-	-	-
	9	-	-	-	-	+
	10	-	+	-	-	+
	11	+	+	+	+	+
	12	-	-	-	-	+
	13	-	-	-	-	+
	14	-	-	-	-	-
	15	-	-	-	-	-
Controls	16	-	+	-	-	ND
	17	-	-	-	-	-
	18	-	-	-	-	-
	19	-	-	-	-	ND
	20	-	-	-	-	ND
	21 *	-	-	-	-	ND
	22	-	-	-	-	ND
	23	-	-	-	-	ND
	24	+	+	+	+	ND
	25	-	-	+	-	ND

Table 3.15 Immune complexes in patients and controls

ND = not done

There was no correlation between patients having immune complexes in their sera and giving a negative DOC-ELISA result. This suggests that circulating immune complexes were not lowering the sensitivity of the DOC-ELISA.

To examine whether the immune complexes contained gonococcal antigens the components of the immune complexes were separated by SDS-PAGE and incubated on immunoblots with mouse anti-PI, PII and PIII. No evidence was found for any of these three gonococcal outer membrane proteins in the immune complexes. Since immune complexes were also found in patients without gonorrhoea it seems unlikely that the immune complexes in sera from patients with uncomplicated gonorrhoea were caused by the gonococcal infection.

For the serological diagnosis of disseminated gonococcal infections both antibody and antigen detection assays may be useful since immune complexes may be formed in excess of either one. The identification of the antigens in the immune complexes formed during disseminated gonococcal infections would indicate the specificity of the antisera most useful for diagnosis.

4. DISCUSSION

4.1 Technical considerations in the immunological analysis of membrane bound antigens

The separation of the outer membrane of gram-negative bacteria from the other cell constituents is relatively easy but the subsequent fractionation of the outer membrane components is more difficult because of the hydrophobic nature of these molecules. The purification of membrane bound antigens is technically difficult and often time consuming because of the poor yields obtained.

Protein I (PI) and pili have been purified from gonococci, but only the latter have been obtained in sufficient quantities for studies on the human antibody response.

Conventional immunoelectrophoretic techniques such as crossed immuno-electrophoresis, do not require pure antigens but the antisera must contain sufficient antibody to precipitate the antigens. These methods have been used to analyse mixtures of antigens from gonococci with animal antisera (Smyth et al, 1976) but are not sensitive enough to detect the low concentrations of antibody produced in gonorrhoea.

One of the more sensitive techniques for the determination of antibody specificity which does not require pure antigens is radio immunoprecipitation. Antigens or whole bacteria labelled with ^{125}I are allowed to interact with antibody. After lysis of whole bacteria the antigen-antibody complexes are precipitated with Protein A-

sepharose. The constituents of the precipitate are separated by SDS-PAGE and subsequent autoradiography identifies the precipitated antigens and therefore the specificity of the antibodies present in the serum. The disadvantages of this technique are: (1) Variations in the activity of ^{125}I makes comparisons between separate experiments difficult. This is due to both batch to batch variations in specific activity and loss of activity because of the relatively short half-life of ^{125}I. (2) Covalently linked antigens will co-precipitate and it may be impossible to determine whether the antibody is directed to both antigens or if to only one which one (Swanson et al, 1982). (3) Only protein antigens are identified. The advantages of radioimmuno precipitation are that the antigens, except for the radiolabel, are in their native state and primary, secondary or higher polypeptide structures may be recognised by the antibodies. Radioimmunoprecipitation has been used with rabbit or monoclonal antibodies to study the surface antigens of the gonococcus (Swanson et al, 1982; Swanson & Barrera, 1983) and by Zak et al (1984) to analyse the specificity of serum antibodies produced in gonorrhoea (see section 4.2).

The methods used in this study to determine antibody specificity were based on the separation of outer membrane antigens by SDS-PAGE followed by immunological analysis. Of the two techniques used, immunoblotting and GED-ELISA, the latter was less good being more difficult technically and less accurate. Another similar technique, gel immunoradioassay (GIRA) has been used by Poolman et al (1983) to study the specificity of antibodies produced in meningococcal infections (see section 4.3). In this technique thin

slices of polyacrylamide gels, in which antigens have been separated, are incubated with sera. ^{125}I-labelled protein A or ^{125}I-labelled anti-human IgG were used to detect bound antibody. This method is more technically demanding than either GED-ELISA or immunoblotting and is very time consuming as the gel requires extensive washing. It is a more accurate method than GED-ELISA. Overall immunoblotting is the most satisfactory method of immunological analysis based on the excellent separation of membrane antigens obtainable by SDS-PAGE. The disadvantages of these methods (GED-ELISA, GIRA and immunoblotting), are that protein antigens can be denatured by both heating and SDS, and only the primary polypeptide structures remain intact. There are two advantages of using GED-ELISA or immunoblotting rather than radioimmunoprecipitation. Firstly there is no change in the activity of the enzyme, unlike ^{125}I, and secondly the class specificity of the antibodies can be identified.

4.2 Immunogenicity of antigens in the DOC extract

4.2.1 Lipopolysaccharide

The results of the GED-ELISA, immunoblotting experiments and the use of purified lipopolysaccharide in microtitre tray ELISAs all suggested that lipopolysaccharide was the most important antigen for the dedetection of IgG antibody in sera from patients with uncomplicated gonorrhoea. However the DOC ELISA was shown by Glynn and Ison (1978) to be dependant on the protein concentration of the DOC extract used to coat the microtitre trays and Ito et al (1980) have demonstrated that magnesium ions are required to stick gonococcal lipopolysaccharide to polystyrene microtitre trays. As no magnesium ions were present in the buffers used in the DOC ELISA these results suggest that the lipopolysaccharide was attached via protein to the microtitre trays. The DOC extract therefore contains a lipopolysaccharide-protein complex rather than free lipopolysaccharide. The fact that the DOC extract from N.meningitidis serogroup B did not contain PI and did not detect antibody in patients sera suggests that the important component of the DOC extract may be a PI-lipopolysaccharide complex.

The use of purified lipopolysaccharide attached to polystyrene microtitre trays by magnesium ions may give a more sensitive antibody test than the methods used previously for the detection of anti-lipopolysaccharide immunoglobulins (see Table 1.4). Lipopolysaccharide may also be a more specific antigen than the

DOC extract for the detection of antibodies to gonococci as a large number of control sera had antibody to undefined proteins in the DOC extract. Inter strain variation in lipopolysaccharides has been shown to be an important determinant of the specificity and sensitivity of serological tests for gonorrhoea (Ward & Glynn, 1972) and N.gonorrhoeae Hl, may not be the most suitable strain.

4.2.2 Protein I

Antibody to purified PI was found in sera from patients with disseminated gonococcal infection and systemic meningococcal infection, but not in uncomplicated gonorrhoea. These results were confirmed by both GED-ELISA and immunoblotting for the gonococcal patients and by GED-ELISA alone for the meningococcal patients. This may be either because PI does not stimulate the host's immune response in the mucosal infection or because the PI present on the more pathogenic strains is more antigenic.

However Zak et al (1984) used radioimmunoprecipitation to analyse the serum antibody response in patients with uncomplicated gonorrhoea and found antibody to PI. Their results are contrary to those found in the present study as antibody to PI was not found in the sera from patients with uncomplicated gonorrhoea by ELISA, GED-ELISA or immunoblotting. The discrepancy between the two sets of results may be due to the different techniques used. If, as proposed in section 4.2.1., PI and LPS are covalently linked, antibodies reacting with lipopolysaccharide would cause the co-precipi-

tation of PI in radioimmunoprecipitation experiments. Whilst in immunoblotting or GED-ELISA PI and lipopolysaccharide are separated by SDS-PAGE before the antibody is added.

It is interesting to note that the figures obtained by Zak et al (1984) for the frequency of anti-PI antibodies in sera from patients with uncomplicated gonorrhoea or controls are very similar to those obtained in this study, by immunoblotting, for the frequency of anti-lipopolysaccharide IgG. They found (80% (12/15) of patients and 20% (3/15) of controls had serum antibody to PI; in the present study 80% (16/20) of patients and 15% (3/20) of controls had serum IgG to lipopolysaccharide. These figures support the view that antibodies to lipopolysaccharide caused the precipitation of PI in the study by Zak et al. An alternative explanation is that the antigenic determinants on PI are destroyed when the protein is broken down into subunits, as in immunoblotting, and patients sera contain both anti-PI and anti-lipopolysaccharide immunoglobulins. The antibodies to lipopolysaccharide were not identified in the radioimmunoprecipitation experiments because only the protein antigens were radiolabelled.

The second explanation seems less likely in view of the fact that anti-PI IgG was found in sera from patients with disseminated gonococcal infection by immunoblotting. In addition Blaser et al (1984) detected antibodies to a major outer membrane protein of Campylobacter jejuni by both immunoblotting and radioimmuno precipitation.

Antibody to PI has been detected in sera from patients with complicated gonococcal infections by Buchanan et al (1980) and Hook et al (1984).

Immunoblotting was used by Hook et al (1984) to determine the specificity of serum IgG produced in patients with complicated gonococcal infection. The sera from four patients with gonococcal salpingitis contained anti-PI IgG and three also contained anti-lipopolysaccharide IgG. Seven of eight patients with disseminated gonococcal infection had serum anti-PI IgG, five also had anti-lipopolysaccharide IgG. The anti-PI IgG reacted with PI's from nine different PI serotype strains of gonococci.

Buchanan et al (1980) used PI's purified 'nearly free of lipopolysaccharide and 85% free of other proteins' in an ELISA. The PI isolated from a serotype strain of N. gonorrhoeae associated with disseminated gonococcal infections detected antibody in sera from 61% of patients with disseminated gonococcal infection, whilst PI isolated from a serotype strain not associated with disseminated infections only detected antibody in 15% of patients.

Strain H1 used in this study belongs to serogroup W II which unlike serogroup W I is not associated with disseminated infections (Sandstrom & Danielsson, 1980) and was isolated from a female with uncomplicated gonorrhoea. This, and the fact that Hook et al (1984) found anti-PI IgG in the sera of patients with disseminated gonorrhoea reacted with PIs from different serotypes, suggests that

the antibody is not specific to antigens on strains of gonococci causing disseminated infections. It seems more likely therefore that of the two serotypes of PI used by Buchanan et al (1980) in ELISAs one was a more suitable antigen for the test system, rather than the sera containing antibody specific to the serotype of PI associated with disseminated infections. This has been found by other workers comparing different strains of gonococci as antigens for antibody detection, see section 4.4.1.

4.2.3 Other Protein Antigens

Immunoblotting of sera from patients with uncomplicated gonorrhoea against the DOC antigen showed the IgA antibodies were most frequently directed against PIII (7/13). Thus the specificities of the IgG and IgA antibodies were different, being directed against lipopolysaccharide or PIII respectively. It was not surprising to find that two different antigens were involved since Ison and Glynn (1979) had shown little correlation between these two classes of antibodies.

Chemical and immunological studies have shown PIIIs from different strains, and variants within a single strain to be identical (Swanson et al, 1982; Judd, 1983 a & b). This suggests PIII would be a good antigen for the detection of anti-gonococcal immunoglobulins, however some control sera (4/13) also contained antibody to PIII.

Immunoblotting showed that antibodies to PII were seldom detected by the DOC extract. A DOC extract prepared from transparent colony types i.e. lacking PII, of strain H1 could have been

used in DOC-ELISAs to confirm PII was not involved in antibody detection. However the results of Mayer (1982) suggest that even a single colony will not be of a pure phenotype and therefore data obtained using pure colony phenotypes should be interpreted with caution.

Antibody to other proteins in the DOC extract, having apparent subunit molecular weights greater than 40K, were found as frequently in control sera as in patients' sera. These proteins may be as yet undefined outer membrane antigens or contaminants from the cytoplasmic membrane. The removal of these less specific antigens from the DOC extract may improve the specificity of the ELISA.

4.3 Cross-reacting antibodies to meningococci

4.3.1 The antibody response in meningococcal infection

The antibody response in meningococcal infections has been studied by Spencer et al (1982) using the DOC extract from N. gonorrhoeae strain H1. The IgG and IgA response varied greatly between patients, but was generally increased in convalescent sera. This general pattern was also found by Poolman and Zanen (1980) and Poolman et al (1983) who used a gel immunoradioassay (GIRA) to study the antibody specificity of sera from patients with meningococcal infection in the acute and convalescent stages. Antibodies to capsular material and low molecular weight antigens were found. The low molecular weight antigens included lipopolysaccharide, heat modifiable proteins, analogous to the PIIs of gonococci, and pili.

The GED-ELISA experiments in this study show the presence of anti-PI IgG in sera from patients with systemic meningococcal infection as well as antibody to low molecular weight antigens. Antibodies to gonococcal PI had not previously been found in meningococcal infections although recently Hook et al (1984) have demonstrated, by immunoblotting, the presence of anti-gonococcal PI IgG in the serum from one patient with a meningococcal infection.

The major outer membrane proteins of meningococci analogous to PI are the class 2 and class 3 proteins. Mandrell and Zollinger (1984) have shown that the antibody binding capacity of the

class 2 proteins is reduced by denaturation in SDS, but this can be restored by immunoblotting in the presence of a zwitterionic detergent. This may explain why Poolman et al (1983) did not detect antibodies to the class 2 or class 3, proteins since in their GIRA the antibodies are incubated with SDS-polyacrylamide gel slices.

IgG in the sera from patients with systemic meningococcal infection gave a strong reaction with antigens in the Triton extract. The antigens in the Triton extract were not identified, but as the IgG in sera from patients with disseminated gonococcal infection also reacted with this extract it was possible that it contained an antigen or antigens associated with increased pathogenicity. Antigens in the Triton extract may have been extracted from the cytoplasmic membrane. Smyth et al (1976) analysed a Triton extract from N.gonorrhoeae by crossed immunoelectrophoresis and found enzymes associated specifically with the cytoplasmic membrane of gonococci. The cross-reactions of the gonococcal antigens, Triton and DOC extracts, with antibodies produced to meningococci illustrates the similarity between the two organisms.

4.3.2 Antibody production during meningococcal carriage

The production of antibody to N. meningitidis or comensal neisseria during carriage of these organisms, has often been proposed as a reason for false-positive results in individuals who did not have gonorrhoea. Rates of meningococcal carriage vary between populations but are generally higher in those attending clinics for sexually

transmitted diseases and are particularly high in homosexual men (Turgeon et al, 1981). As gonococcal antibody tests are usually evaluated in clinics for sexually transmitted diseases meningococcal carriage is an important consideration. Holmes et al (1978) found that meningococcal carriage was a significant factor in the reduction of the specificities of two tests for gonococcal antibodies, one of which used purified pili as the antigen. However Young and Low (1981) used pili in an ELISA for gonococcal antibodies and found that meningococcal carriage did not increase the reactivity of sera from gonococcal culture positive or culture negative patients. Ison & Glynn (unpublished results) found patients with gonorrhoea who also carried meningococci in the throat had higher antibody levels against the DOC extract than patients with only gonorrhoea.

In the present study the antibody levels in sera from patients without gonorrhoea but who were carriers of meningococci showed that cross-reactive antibody was detected using the DOC antigen.

The cut-off point chosen for a positive test may influence the effect of antibody produced during meningococcal carriage on the specificity of antibody tests for gonorrhoea. The results using the DOC antigen showed less antibody was produced in meningococcal carriage than in gonorrhoea, but by using a cut-off point of Ecr$\geq$20, 11 out of 15 sera from carriers of meningococci gave a positive result (Table 3.5). The cut-off point chosen by Young and Low may have been higher, ELISA results are generally expressed in arbitrary absorbance units and not in μg of antibody consequently comparisons

between different laboratories are impossible. An alternative explanation is that the pili from strain 82409, used by Young and Low are a more specific antigen.

Ison et al (1982) have postulated that some throat meningococci may be gonococci which have been transformed by DNA from normal flora enabling them to utilise maltose. They have shown this to be possible by in vitro transformation of gonococci. Although this is unlikely to be true for all strains of N.meningitidis isolated from throat swabs, biochemical evidence suggests that a proportion of strains isolated from the throat are more similar to N.gonorrhoeae than strains isolated from patients with systemic meningococcal infections. This may make the identification and isolation of an antigen from N.gonorrhoeae which does not cross-react with antibody produced during meningococcal carriage very difficult.

4.4 Antigenic variation in gonococci

Studies on the outer membrane constituents of gonococci have shown considerable variations in proteins, both between and within strains. Much of this evidence has been obtained from in vitro studies. More recently Duckworth et al (1983) have shown that such intra-strain variations in pilus phenotype may occur during an infection. Gonococcal isolates from consorts, which had the same auxotype and PI (defined by molecular weight and serology), were examined for variations in pilus type. The pili varied not only between consorts but also between different sites on the same patient i.e. urethra and cervix. They proposed that the ability of the gonococcus to change its surface composition may be an important pathogenic mechanism by which it evades the host's defence mechanisms. Antigenic variation in parasites e.g. the Trypanosomes, and the influenza virus enables these organisms to be successful pathogens.

4.4.1 Specific antibody

The considerable inter-strain variation found in gonococci raised the possibility that antibody produced during an infection may be specific to the strain causing infection, (specific antibody). Particularly in view of the fact that variations in protein structure had been found to be on the surface exposed region of the protein (Heckels, 1981). If this were true the use of a single strain for antibody detection would not be satisfactory. This view was

reinforced by the analysis of antibodies raised in rabbits which showed considerable strain specificity for pili (Buchanan, 1975) and PII (Swanson & Barrera, 1983). In the present study the possibility of patients producing specific antibody was analysed by immunoblotting and by the use of intact bacteria in ELISAs. Neither method produced evidence for specific antibody, both suggesting that some strains were better antibody detectors than others. In particular there were very few sera which contained antibody that would not be detected using strain H1.

Previous work using the serotype strains of Johnston had also shown that some strains were better antibody detectors in an ELISA than others. This was assumed to be due to the prevelance of certain serotypes in the population tested, the patients having produced antibody specific to the strains found to be good antigens (Ison et al 1981). The results from the present study suggest that this was not the case and that strains giving higher antibody levels with patients sera were better antigens rather than the sera containing specific antibody. This was also found by O'Reilly et al (1973) who used nine strains and their matched sera in an indirect immunofluorescent test. The homologous serum and strain did not give the highest titre and one strain was selected which detected antibody in all nine sera, often at a higher titre than was obtained with the homologous strain.

Reimann et al (1982) demonstrated the same to be true using purified gonococcal pili antigens in indirect haemagglutination tests for gonococcal antibodies. Using human sera they demonstrated a

limited antigenic heterogeneity in pili, in contrast to the results obtained using antisera raised in rabbits (Buchanan, 1975). Of twenty two sera which gave a negative antibody result in their test, five were positive when tested against pili from their infecting strain. Pili from one of these strains detected antibody in four of these five patients sera. Incorporation of this pilus type into the test system significantly increased the sensitivity of the test, specificities were not determined. The immunoblotting experiements in the present study support the view that antibody is produced to a common pilus epitope.

Radioimmunoprecipitation was used by Zak et al (1984) to study serum antibody specificities. Variations in the molecular weights of pili and PII were found between isolates from consorts and between isolates from different sites in the same patient. Anti-pilus IgG was found to cross-react with the different pilus species although to different degrees, which suggests there may be some surface specific antibody. Anti-PII IgG was found to be more specific, although not necessarily for the PII species isolated from the patient.

The results of the present study do not rule out the possibility of sera containing some specific antibody. Immunoblotting detects antibody to the primary polypeptide structure and is not quantitative. Consequently the presence of small amounts of specific antibody would not be detected nor would antibody to higher structural orders which may be expressed on the bacterial surface. The use of rabbit antisera to PII in radioimmunoprecipitation

revealed specific antibody whilst immunoblotting showed a large amount of broadly crossreactive antibody, presumably to peptides burried within the membrane rather than surface exposed (Swanson & Barrera, 1983). ELISAs using adsorbed rabbit antisera have shown the presence of specific anti-PII antibodies (Diaz & Heckels, 1982). This type of study may reveal the presence of specific antibody in the human infection, however the results from the present study suggest that even if specific antibody is present it is only a small proportion of the total antibody.

Swanson and Barrera (1983) have found that the method of producing rabbit antisera to PIIs determines its specificity. Antibodies produced to isolated PIIs cross-react with different PII species but antibodies raised to PIIs on intact gonococci are specific for the PII on the strain used for immunisation. This would suggest that in the human infection antibody is produced to released proteins or the orientation of the proteins in the outer membrane is quite different under the growth conditions provided during infection than when bacteria grown in vitro are used to immunise rabbits.

4.5 The Immune Response

The majority of sera (80%) from patients with uncomplicated gonorrhoea which gave a positive DOC-ELISA result were shown to contain anti-lipopolysaccharide (LPS) IgG by immunoblotting. However relatively few (approx. 20%) of the sera tested against the whole cell antigens in immunoblotting contained anti-LPS IgG, but most (90%) contained antibody to pili. Ison and Glynn (1978) have shown that the duration of infection affects the results of the DOC ELISA, positive results are usually from patients with a duration of infection greater than three days. Consequently when DOC-ELISA positive sera were selected they were probably from patients who had a longer duration of infection than in the whole bateria studies when they were unselected. This suggests the initial antibody response may be to pili followed by a rise in antibody to LPS.

The immune response was largely IgG, this was also found by Reimann et al (1980). This makes the distinction between a past and a current infection, which is usually based on the presence of IgM, difficult. In the present study no evidence for anti-gonococcal specific IgM was found. This may be because LPS produces a non-specific IgM response and therefore any naturally occurring anti-LPS IgM in control sera cannot be distinguished from the response in patients with gonorrhoea.

4.6 Immune complexes

The presence of immune complexes in the sera of patients with gonorrhoea did not appear to reduce the sensitivity of the DOC-ELISA for the detection of antibodies to gonococci.

It is however possible that the presence of immune complexes in genital secretions may reduce the sensitivity of antigen detection systems. For example, an enzyme immunoassay, EIA, (Gonozyme, Abbott Laboratories) was shown by Schachter et al (1984) to be more sensitive in detecting N.gonorrhoeae in males than females. If, as is usually the case, the females had a longer duration of infection it is possible that antibody in the genital secretions was bound to the gonococcal antigens leading to a negative EIA result. Whilst the males presented for examination before any local immunity was produced and hence antigen was available for detection.

5. Conclusions

The DOC extract contained outer membrane proteins and lipopolysaccharide. The antigen reacting with IgG antibody in sera from patients with uncomplicated gonorrhoea was lipopolysaccharide. The lipopolysaccharide was attached indirectly to the polystyrene microtitre trays by PI. IgA antibodies reacted with PIII in the DOC extract, but IgA anti-PIII was also found frequently in control sera. The most useful class of antibody for sero-diagnosis of gonorrhoea was IgG and if two antigens were to be used in parallel, as suggested by Glynn and Ison (1981), pili and lipopolysaccharide from carfully selected strains, would be the best choice. The sensitivity of the DOC-ELISA was not reduced by the presence of immune complexes. Antibody specific to the patients infecting strain was, if present, only a small proportion of the total antibody produced to gonococci and would therefore not affect the results of sero-diagnosis using antigen(s) from a single strain.

Cross-reacting antibodies produced to meningococci remain a problem in the sero-diagnosis of gonorrhoea.

Antibodies produced during systemic neisserial infections include anti-PI IgG as well as antibodies directed to antigens extracted from N.gonorrhoea and N.mengitidis by Triton X-100.

The techniques used in the present study have produced some information on the immune response in gonorrhoea. It would be

interesting to extend this study to follow changes in antibody specificity with the duration of infection and to determine whether these antibodies have any functional role, e.g. bactericidal activity, opsonisation of bacteria, both in sera and genital secretions.

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APPENDIX

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Analysis of antibodies in local and disseminated *Neisseria gonorrhoeae* infections by means of gel electrophoresis-derived ELISA

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Summary. Major antigens in *Neisseria gonorrhoeae* were identified by surface labelling the organisms with ^{125}I and electrophoresing extracts in polyacrylamide with sodium dodecyl sulphate. Horizontal slices of the gels were cut out and tested in individual wells against patients' sera using ELISA. Serum from local gonococcal infections reacted with Protein II and, probably, lipopolysaccharide, but not with Protein I in deoxycholate (DOC) extracts and gave no reaction with Triton X-100 extracts. Serum from disseminated gonococcal infections reacted with Protein I in the DOC extract and with pili and a number of undefined possibly cytoplasmic membrane antigens in the Triton X-100 extract. The significance of the results and the potential of the method are discussed.

INTRODUCTION

Previous attempts at developing a serological test for the diagnosis of gonorrhoea (Holmes, Buchanan, Adam & Eschenbach 1978) have been unsuccessful, largely because the antigens used give cross-reactions particularly with sera from patients with meningococcal infection. In an enzyme-linked immunosorbent assay (ELISA) a deoxycholate extract of *Neisseria gonorrhoeae* cell walls detected antibodies in patients with meningococcal meningitis as well as in those with gonorrhoea. A Triton X-100 extract detected antibodies in meningitis only (Hadfield, Ison & Glynn, 1980). It was deduced therefore, that use of these two antigens in parallel would provide a means of distinguishing antibodies from patients with meningococcal from those with gonococcal infection. Further investigation has shown, however, that patients with disseminated rather than local gonococcal infection also have antibodies to the Triton X-100 extract. This makes the Triton X-100 extract useless for diagnosis, but raises the possibility that something in the extract may be involved in the pathogenesis of systemic infection.

The antigenic composition of complex mixtures can be examined by gel electrophoresis derived ELISA (GEDELISA; Lutz, Higgins, Pedersen & Theilen, 1979) without the need for isolating and purifying individual components. Deoxycholate (DOC) and Triton X-100 extracts were electrophoresed on polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS-PAGE) and the individual fractions obtained tested by ELISA against sera from patients with either local or disseminated gonococcal infection.

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MATERIALS AND METHODS

Production of antigen

A single strain, *N. gonorrhoeae* strain H1 was used throughout this study. Opaque piliated colony types

were selected using clear typing medium (Swanson, 1977) and subcultured on to GC medium both containing 1% Isovitalex (BBL). Bacteria harvested from plates, after overnight growth, at 36° in 7% CO_2, were used to inoculate 500 ml flasks containing a biphasic medium (Ison & Glynn, 1978).

Flasks were shaken for 6–7 hr and the bacteria harvested by centrifugation, washed twice in cold PBS (pH 7·4) and stored at −70°. The antigens were prepared as described by Smyth, Friedman-Kein & Salton, 1976. Briefly the bacteria were lysed in 0·1 M phosphate buffer, pH 7·0 (0·1 g ml^{-1} wet weight), cell envelopes were treated with DNAse (50 $\mu g\ ml^{-1}$) and washed four times using 50 mM Tris-HCl pH 7·5. Envelopes were extracted with 4% (w/v) Triton X-100 in 10 mM Tris-HCl pH 8·4 at a protein concentration of 25 mg ml^{-1} for 1 hr. The suspension was centrifuged at 40,000 *g* for 45 min, the supernatant removed and used as the Triton antigen. The Triton extraction was repeated, the pellet washed in 50 mM Tris-HCl pH 7·5, resuspended in a 2% (w/v) sodium deoxycholate buffer containing 10 mM tris-HCl, pH 8·0, 0·2 M NaCl and 1 mM ethylene diamine tetracetic acid (EDTA). After centrifugation at 40,000 *g* for 45 min the supernatant was removed and used as the DOC antigen. Extracts were stored in 100 μl amounts at −70°.

Radiolabelling

In some experiments whole bacteria were radiolabelled before extraction with ^{125}I using the Iodogen method (Markwell & Fox, 1978). Twenty millilitre scintillation vials were plated with 100 μg of Iodogen (Pierce) in 250 μl of chloroform by rotation in a well ventilated hood. Bacteria were grown overnight on GC medium and scraped into cold phosphate-buffered saline (PBS). The absorbance at 550 nm was adjusted to 2·0. Ten millilitres were added to each vial followed by 50 μl of carrier-free Na ^{125}I (Radiochemical Centre, Amersham). The mixture was left on ice for 20 min and occasionally shaken. The reaction was stopped by pouring the mixture from the reaction vessel. Cells were pelleted by centrifugation and washed four times with ice cold PBS. The extraction was then continued as above.

ELISA

The method described previously was used (Glynn & Ison, 1978). Antigen was diluted in carbonate-bicarbonate buffer pH 9·6 and 100 μl added to each well in a flat bottomed microtitre plate (Linbro Flow Labs) and incubated at room temperature for 30 min. Trays were then washed three times with PBS-Tween, 100 μl of serum at a dilution 1 in 1000 was added to the wells and incubated for 1 hr at 30°. After washing, as above, 100 μl of peroxidase-conjugated anti-human IgG (DAKO) was added at an appropriate dilution and incubated at 30° for 2 hr. Trays were washed and 100 μl of substrate added to each well; this consisted of O-phenylenediamine (Sigma) at a concentration of 0·4 g l^{-1} in 0·2 M phosphate-citrate buffer pH 5·0 to which 30% hydrogen peroxide 0·4 ml l^{-1} was added just before use. The reaction was stopped after 30 min by the addition of 50 μl of 2·5 M H_2SO_4, the trays were read using a Titertek Multiscan (Flow Labs) at an absorbance of 492 nm.

Sera

Human sera were from three sources; volunteers as normal controls, patients attending the sexually transmitted diseases (STD) clinic at St Mary's Hospital who were culture positive for *N. gonorrhoeae* and patients at St Mary's Hospital who had disseminated gonococcal infection.

Gel electrophoresis-derived ELISA (GEDELISA)

SDS-PAGE was performed using the method of Laemmli (1970). Five percent stacking gels and 12·5% separate gels were used. Samples were prepared by boiling for 5 min in 0·1 M Tris, 0·1 M Clelands reagent, 2% SDS and 10% glycerol. Samples that contained Triton X-100 were extracted with chloroform to reduce the amount of detergent which distorts the gels (Horikawa & Hiroshi, 1979).

For the GEDELISA 15 μg protein samples of antigen, which had been extracted from ^{125}I-labelled cells were loaded into 3 mm wide wells, additional wells were loaded with molecular weight standards (Biorad, low molecular weight standards). After electrophoresis the gel was cut into 3 mm vertical slices and washed three times for 10 min in water. Slices were stained for protein using Coomassie blue and for carbohydrate using the periodic acid Schiff (PAS) method (Fairbanks, Steck & Wallach 1971). The remaining slices were cut horizontally into 2·5 mm fractions. Each fraction was placed in an individual flat bottomed well (Dynatech), counted in a γ counter (LKB) and stored at −70° until used. Gel fractions were incubated with 100 μl of carbonate-bicarbonate buffer pH 9·6 for 36 hr at 4°, during which time protein from the gel became bound to the surface of the well. Gel fractions were then removed, the wells washed and

the ELISA continued as described above, with the exception that sera were diluted 1 in 200.

RESULTS

Antigen titrations

Figure 1 shows the results obtained when pooled sera from patients with local or disseminated gonococcal infection, four in each group, were titrated against increasing concentrations of DOC and Triton antigens. The DOC detects antibodies in both types of sera, whereas the Triton only detects antibodies in the disseminated cases. Sera from meningococcal meningitis patients also give positive results with both the DOC and Triton antigens (Hadfield *et al.*, 1980).

SDS-PAGE

Figure 2a shows the SDS-PAGE pattern of the proteins in the DOC and Triton extracts. The DOC extract has a major protein band at the position of protein I [P-I (mol. wt 37,000)], it also contains protein II [P-II (mol. wt 24,000)] and stains for carbohydrates at the position of the dye front. The Triton antigen contains pili (mol. wt 21,000) and other minor proteins. From counts of slices of SDS-PAGE gels the surface antigens in the extracts could be identified further (Fig. 2b). Small amounts of P-I were detectable in the Triton extract.

GEDELISA

Figure 3 shows the results obtained when the proteins separated by SDS-PAGE are coated onto wells and tested against serum from patients with (a) local and (b) disseminated gonococcal infection. The serum from the local gonococcal infection, as would be expected from the antigen titration results, reacts only with the DOC antigen in the region which corresponds to the positions of P-II and carbohydrate antigens in this extract. The serum from the disseminated patient reacts with both DOC and Triton extracts. With the DOC antigen it reacts only at the position P-I. With the Triton antigen this serum reacts at the positions of P-I, pili and of higher molecular weight antigens which are not surface antigens since they are not radiolabelled.

GEDELISAS were also performed using 5, 10, 15 and 20 μg protein samples of the antigens on the gel and although the individual values varied the peaks remained constant in number and position.

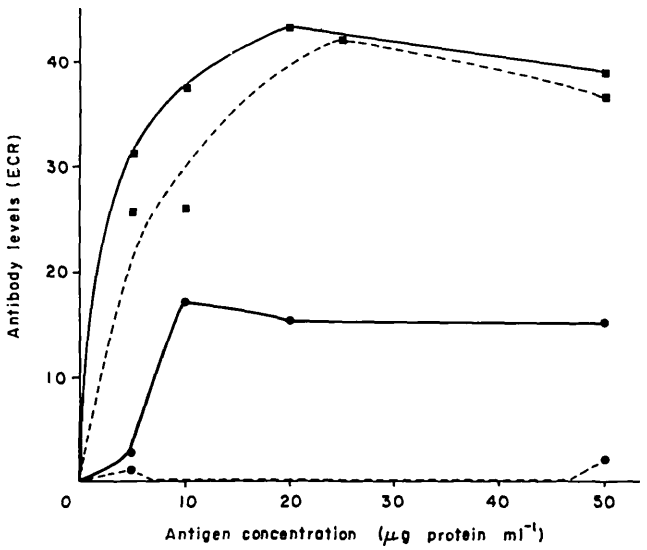


Figure 1. Detection of IgG antibodies in serum from disseminated and local gonococcal infections. Disseminated (■); local (●); DOC antigen (——); Triton antigen (– – – –).

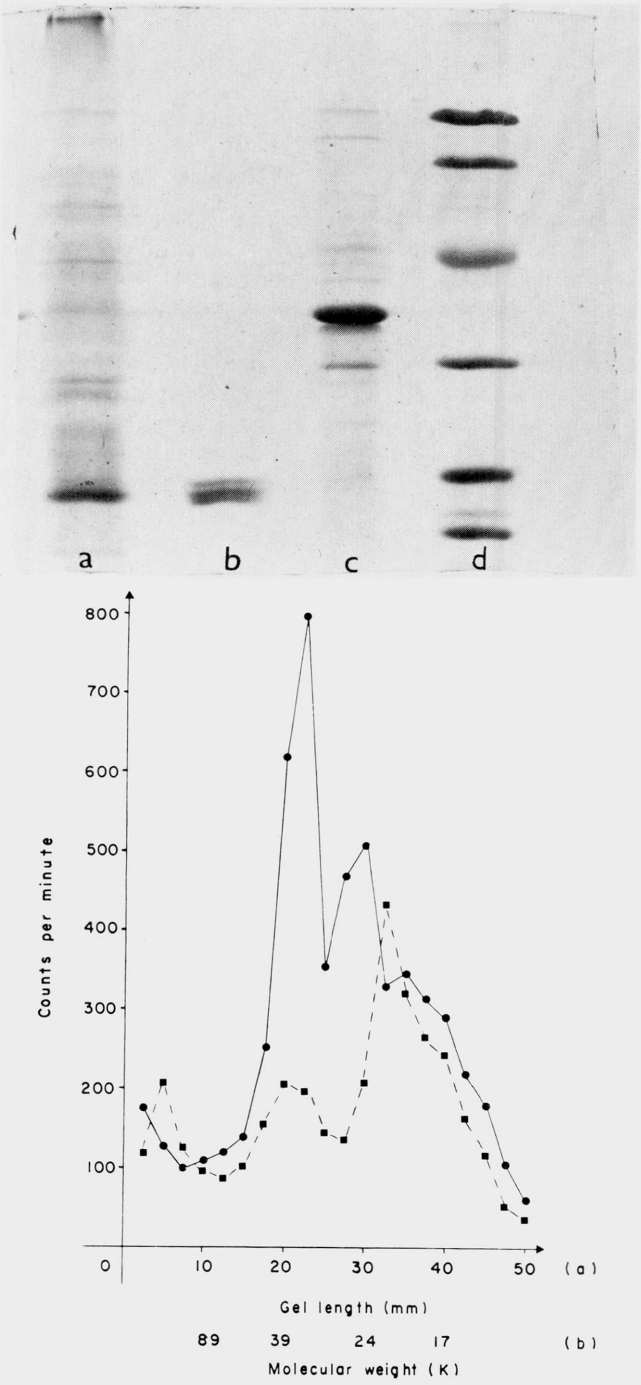


Figure 2. (a) Sodium dodecyl sulphate polyacrylamide gel electrophoresis of, a, Triton; b, gonococcal pili; c, DOC antigen; d, protein molecular weight standards: Phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme.

(b) Electrophoretic patterns of the DOC (●) and Triton (■) antigens extracted from ^{125}I-surface labelled gonococci.

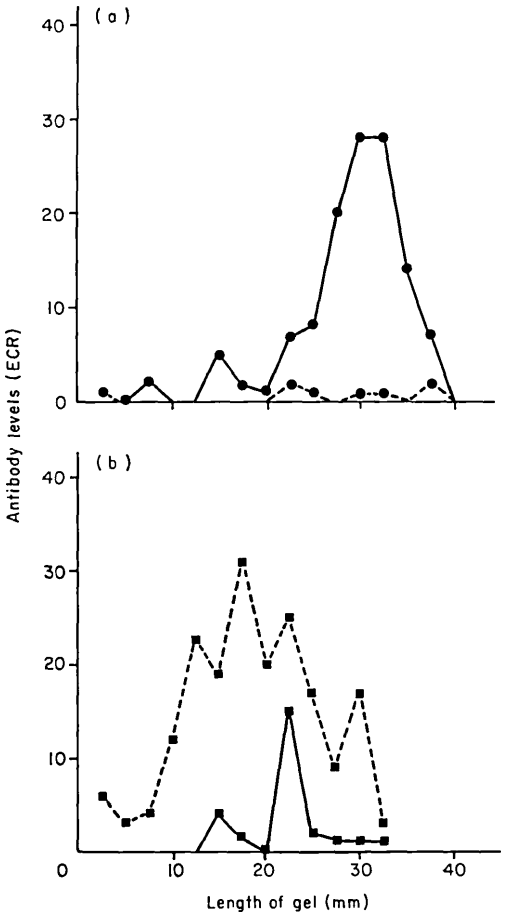


Figure 3. GEDELISA profiles of the DOC and Triton antigens using serum from a patient with (a) local and (b) disseminated gonococcal infections. DOC antigen (——); Triton antigen (– – – –).

DISCUSSION

The DOC and Triton antigens illustrate differences in the antibody response in local and disseminated gonococcal infection. The major detectable antibody in the serum from a local gonococcal infection appeared to be directed against P-II or carbohydrate, possibly lipopolysaccharide, or both. No antibodies to P-I were found in sera from patients with local gonococcal infections. When serum from a patient with a disseminated gonococcal infection was used in a GEDELISA with a DOC antigen, there were no antibodies to LPS, but there were antibodies to P-I. The use of Triton as an antigen with this serum shows the presence of more antibodies some of which are again directed against P-I and also against pili. The others represent antigens which are not on the surface of the bacterial strain used in these experiments. These antigens may be from the inner surface of the outer membrane or from the cytoplasmic membrane. It is also possible that in gonococcal strains that cause disseminated infection these antigens are on the surface either because they are related to invasiveness or because they are exposed when the bacteria break down *in vivo*. The antibodies directed against the antigens in the Triton extract may also be related to the duration of the infection.

Antibody tests for gonorrhoea have often used crude extracts and the unsatisfactory results may be a reflection of the number of antigens in such mixtures. Purification of antigens for use in antibody tests has not increased the reliability of results and involves far more work. Using techniques such as GEDELISA we can identify antigens of potential use and their purification may provide more meaningful results and a greater insight into the antibody response during gonococcal infections.

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Application of immuno-blotting to the differentiation of specific antibodies in gonorrhoea

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Summary. The technique of immuno-blotting was used to examine the specificity of serum antibodies in patients with gonorrhoea and to define the significant antigenic determinant present in the desoxycholate extracts of gonococcal cell walls used previously for serological diagnosis. IgG but not IgA antibody was directed predominantly against lipopolysaccharide. Antibodies to outer membrane proteins were not found as frequently as expected.

There was a suggestion from the results that antibodies to antigen PI was more common in systemic than in local infections.

INTRODUCTION

Studies on the specificity of antibodies produced in gonorrhoea could provide useful information on the pathogenicity of *Neisseria gonorrhoeae* and for the development of a serological test for gonococcal infection. Many such tests have been described using a variety of crude or purified antigens ranging from whole bacteria (Gafaar & D'Arcangelis, 1976) to pili (Buchanan *et al.*, 1973). The deoxycholate (DOC) extract of gonococcal outer membranes used in an ELISA for the detection of anti-gonococcal immunoglobulins (Glynn & Ison, 1978; Ison & Glynn, 1979) contains three well defined gonococcal outer membrane protein antigens, protein I (PI), protein II (PII)—a heat-modifiable protein—and protein III (PIII), a protein modified by reducing agents (Swanson, 1981). The DOC extract also contains lipopolysaccharide (LPS) and other minor protein antigens.

The technique of gel electrophoresis-derived ELISA has previously been used to analyse the specificity of IgG antibodies detected using the DOC antigen (Hadfield & Glynn, 1982). However the technique was not sufficiently accurate to determine whether IgG in patients with uncomplicated gonorrhoea was directed to PII or LPS, or both. In this study we have used immuno-blotting (Burnette, 1981) to analyse the specificities of serum antibodies to components of the DOC extract.

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MATERIALS AND METHODS

Antigen

The antigen used was a DOC extract of *N. gonorrhoeae* strain H1, prepared as previously described (Hadfield & Glynn, 1982). Strain H1 has been serotyped by Dr E. Sanström and belongs to group WII (Sandström & Danielsson, 1980).

Immuno-blotting

Proteins in the DOC antigen were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method described previously, except that Clelands reagent was omitted from the sampling buffer to give a better separation of PI and PIII (Hadfield & Glynn, 1982). Fifteen microgram samples of protein were applied to 5 mm wide slots and electrophoresed on a 12·5% acrylamide gel until the dye front was approximately 7 cm into the gel. The slab gel was covered with a sheet of nitrocellulose membrane to which proteins were electrophoretically transferred in a Trans-Blot apparatus (Bio-Rad Laboratories). A current of 200 mA for 2 hr was used to electrophorese proteins from the gel to the nitrocellulose membrane in a buffer of 20 mM Tris, 150 mM glycine, 20% methanol. The nitrocellulose membrane was washed for 30 min in 0·5% bovine serum albumin (BSA; fraction V, Sigma), 0·9% sodium chloride, 10 mM Tris-HCl, pH 7·4 (BSA-buffer). Human sera were diluted 1 in 100 in the BSA-buffer and 10 ml was incubated overnight at 37° with a strip of nitrocellulose membrane, containing two or three lanes of antigen. After washing, three times for 20 min in BSA-buffer, the nitrocellulose membrane was incubated for 4 hr at 37° with peroxidase conjugated anti-human IgG (DAKO) diluted 1 in 1000 in BSA-buffer. The nitrocellulose was washed as before, the substrate, 3, 4, 3′, 4′-tetra-aminobiphenyl hydrochloride (BDH), 0·5 mg/ml in 50 mM Tris-HCl, pH 7·4, containing 0·01% hydrogen peroxide, added and incubated at room temperature for 15 min. The nitrocellulose membrane was washed in water to terminate the reaction and dried between sheets of filter paper. Controls which were incubated with conjugate but not sera were also included in the assay.

SDS-PAG lanes containing separated antigen were stained using 0·1% Coomassie Brilliant Blue R (Sigma) before or after blotting. The absorption of the protein bands was measured using a gel scanner and the amount of protein estimated from the area under the absorption peak.

Sensitivity

The quantity of human IgG and IgA detectable was estimated by a spot-test (Hawkes, Niday & Gordon, 1982). Ig standards were diluted in 0·9% sodium chloride to concentrations between 3·0 μg/ml and 2·0 ng/ml, IgG and 145 μg/ml to 1 μg/ml IgA. Five microlitres of each dilution was spotted, in duplicate, on to nitrocellulose membrane and allowed to dry at room temperature for 1 hr. The assay for the Igs was continued using the same conditions, i.e. washing, conjugate dilution and substrate, as for the immunoblotting.

Production of mouse antisera

Mouse antisera specific for PI, PII and PIII were produced to locate the exact position of these proteins on the nitrocellulose membrane. Proteins were separated by SDS-PAGE as described and stained using Coomassie Brilliant BLue G (Sigma) (Blakesley & Boezi, 1973). After extensive washing in water the relevant protein bands were cut from the gel and

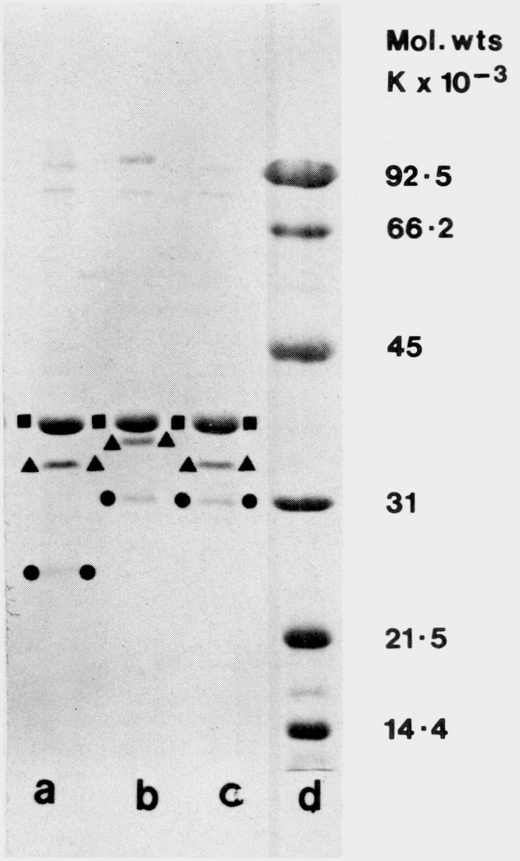


Figure 1. SDS-PAGE of DOC antigen of *N. gonorrhoeae* strain H1.
(a) Incubated 37° 2 hr without reducing agent;
(b) 100° 10 min with dithiothreitol;
(c) 100° 10 min without dithiothreitol.
(d) Molecular weight standards. (■) Protein I, (▲) protein III, (●) protein II.

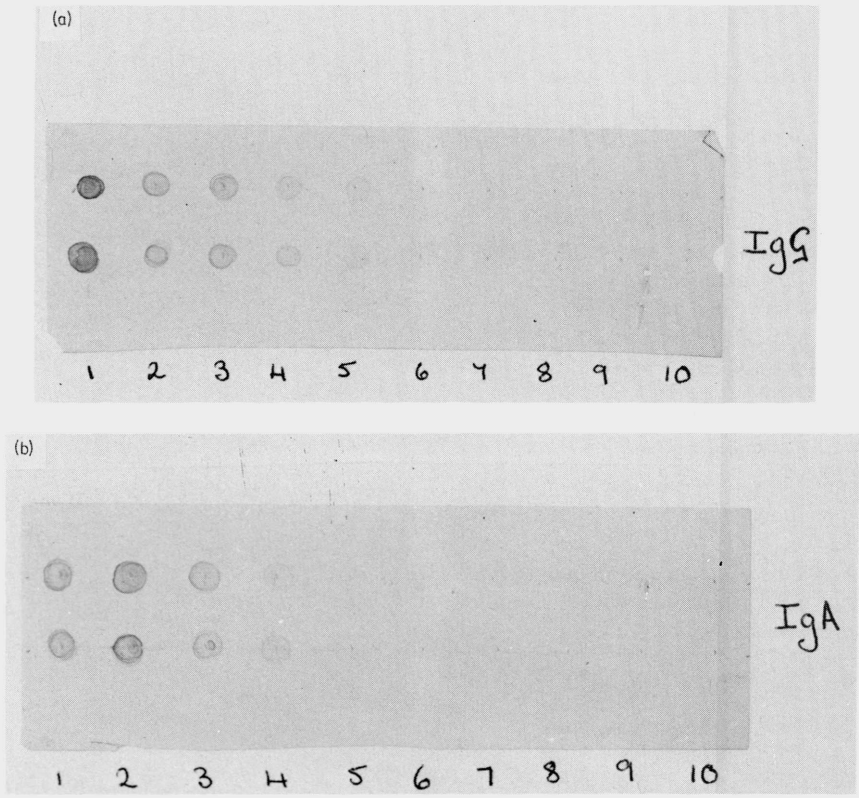


Figure 2. Spot test to measure sensitivity of detection of human IgG by peronedase-labelled mouse antiserum.
(a) Amount of IgG per spot in ng: (1) 14, (2) 5, (3) 3, (4) 1·5, (5) 1·0, (6) 0·5, (7) 0·3, (8) 0·2, (9) 0·05, (10) 0·01.
(b) Amount of IgA per spot in ng: (1) 29, (2) 21, (3) 15, (4) 7, (5) 3, (6) 2, (7) 1·5, (8) 0·7, (9) 0·25, (10) ·02.

stored at −70° until used. The slices were suspended in water using a hand tissue homogenizer, and approximately 50 μg of protein was injected intraperitoneally into each mouse on the first day and again 14 days later. On day 28 antibodies were detectable at a dilution of at least 1 in 10,000 in an ELISA and animals were bled out. Mouse antisera were used in the immuno-blotting at a dilution of 1 in 10,000 and a peroxidase-labelled anti-mouse conjugate (DAKO) was used at a dilution of 1 in 2000.

Source of human sera

Positive sera were obtained from heterosexual patients attending the sexually transmitted diseases clinic at St Mary's Hospital, Paddington, who were culture-positive for gonorrhoea and had detectable levels of IgG antibodies in an ELISA using the DOC antigen (Glynn & Ison, 1978). Control sera were from male and female members of staff to whom the purpose of the investigation had been explained. Some sera were also taken from patients attending the ante-natal clinic who were assumed to be culture negative.

LPS preparation

LPS was prepared from *N. gonorrhoeae* strain H1 using the phenol/water method of Westphal, Lüderitz & Bister (1952).

Chemical determination

Ketodeoxyoctonate (KDO) was determined by the thiobarbituric acid method (Dröge *et al.*, 1970) using KDO (Sigma) as a standard. Protein concentrations were determined by the method of Lowry *et al.* (1951) using BSA (Sigma) as a standard.

RESULTS

The DOC antigen contains the three major protein antigens PI, PIII and PII (Fig. 1). The apparent subunit molecular weight of PIII is increased in the presence of a reducing agent, dithiothrietol (Fig. 1b, c), whilst that of PII is heat modifiable (Fig. 1a, c), so fitting the definitions given by Swanson (1981).

Antigens were transferred to a nitrocellulose membrane using the method described and the percentage protein transferred was estimated by measuring the absorption of stained protein bands before or after transfer. PIII is transferred more easily (51%) than PI (31%) and PII (28%).

The minimum quantity of IgG detectable by this method was determined using a spot test. Quantities of IgG ranging from 14 μg to 14 pg were dried on to a nitrocellulose membrane (Fig. 2a). At 0·2 ng of IgG (No. 8) the spot was just visible. This would correspond to a test serum containing a minimum of 4·0 ng/ml of specific IgG for detection. The amounts of IgA used in the spot test ranged from 29 ng to 0·2 ng (Fig. 2b). The spot was just visible at 1·5 ng (No. 7). A test serum must contain a minimum of 30 ng of specific IgA for detection using this method.

Mouse antisera

Mouse antisera raised to PI, PII and PIII was used to locate the precise position of these proteins on the nitrocellulose membrane (Fig. 3). Strip (a) was incubated with anti-PI, II and III, (b) with anti-PI and III, (c) with anti-PII and III.

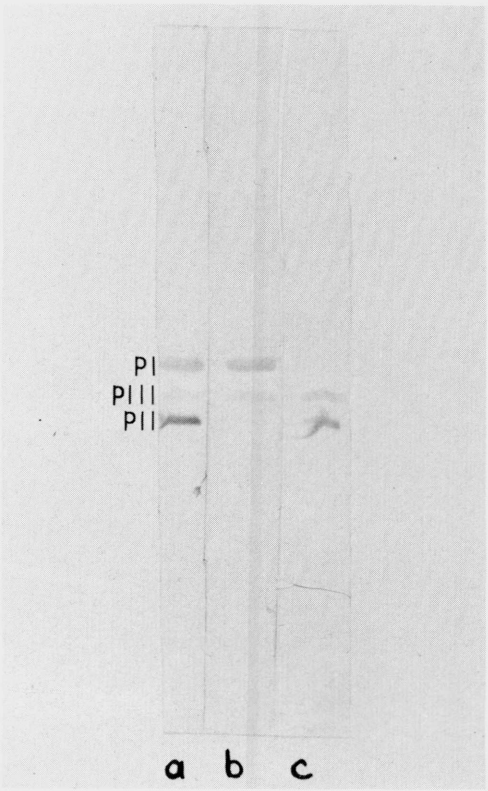


Figure 3. Location of proteins I, II and II in DOC antigen by Immuno-blotting using combinations of specific mouse antisera. (a) Anti-PI, II and III. (b) Anti-PI and III. (c) Anti-PII and III.

Human sera

IgG antibodies. Twenty sera from patients with gonorrhoea, which were ELISA-positive with the DOC antigen, and 20 sera from controls were examined for anti-gonococcal IgG specificity by the Western Blot method. There were equal numbers of males and females in both groups. All the patients had detectable levels of IgG antibody to one or more of the antigens in the DOC extract. Two examples are shown in Fig. 4 which have antibodies to PII only (a) and PIII, P40–50K, P60–90K, LPS and minor protein antigens (d). Lines of reaction to one or more of the antigens were also found in eleven of the control sera. Antibodies in patients Nos 1–10 (male) and 11–20 (female) were predominantly directed to the LPS, only patients Nos 2, 3, 7 and 20 were negative for this antigen. Three female but no male control sera had antibody to LPS. Sera in which antibodies were detected to LPS in the DOC antigen also had antibodies to LPS, purified from strain H1 by the phenol/water method.

Seventeen patients and 10 controls had antibody to one or more of the protein antigens. The only significant difference in IgG specificity between patients and controls is for LPS (χ^2 test $P = 0{\cdot}001$).

IgA antibodies. Twenty-eight of the 40 sera were also tested for IgA specific antibodies, 13 patients (six male, seven female) and 15 controls (seven male, eight female). There was insufficient sera from the 12 remaining specimens to test for IgA.

The IgA results differed from the IgG, the response to the LPS antigen was reduced in patients and controls. The percentage of patients and controls with IgA to PIII was increased.

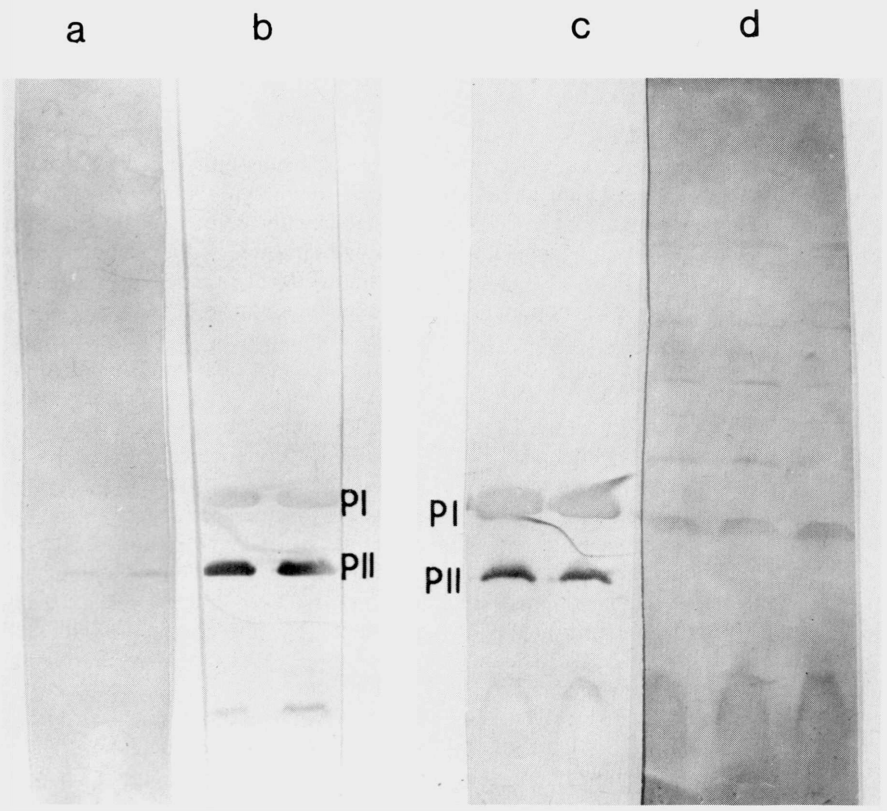


Figure 4. Immuno-blots of two patients' sera against DOC gonococcal antigen. (a) Showing antibody to PII only. (b, c) Standard mouse antisera to PI and PII. (d) Showing antibodies to PIII, a 40–50K protein, a 60–90K protein, LPS and minor protein antigens.

An example of the difference in IgG and IgA responses is seen in patient No. 4. IgG was detected to LPS and P60–90K, IgA to LPS, PIII and P40–50K. Anti-PIII IgA was found in sera from three patients, 4, 8 and 20, in which no IgG anti PIII was detected, and in two controls, 35 and 40.

Three sera from patients and nine from controls had no detectable IgA; however the results on the sensitivity of this assay suggest that a 10-fold increase in the amount of serum used for IgA detection would be necessary for the sensitivity to equal that of the IgG assay.

Antibody response in disseminated gonococcal infection (DGI)

Two sera from patients with DGI were IgG ELISA-positive against the DOC antigen (Ecr >20). Immuno-blotting of the DOC antigen detected IgG anti-PI in both sera. IgG was also found to other components of the DOC with these sera. For example, one serum contains IgG anti-PI, P40–50K, P60–90K and, possibly, LPS.

DISCUSSION

The immuno-blot method has been applied to the detection of antibodies in gonorrhoea. Outer membrane proteins and LPS were transferred from SDS-polyacrylamide gels on to nitrocellulose membrane and used for the detection of specific IgG. The relative efficiencies of outer membrane protein transfer were not directly related to the apparent sub unit molecular weight as has been reported for other proteins (Burnette, 1981; Lin & Kasamatsu, 1983).

Immuno-blotting was used in preference to immunoprecipitation (Swanson, Mayer & Tam, 1982) since it avoids the need to radiolabel antigens. Moreover studies with monoclonal antibodies have shown there may be co-precipitation of antigens. In immunoblotting there is the possible drawback that antigens could be denatured by treatment with sodium dodecyl sulphate before they can react with antibody. However, this is not known to cause significant changes in specificity.

The major antigen to which IgG is produced in mucosal infections appears to be LPS. These results suggest that the antigen found using the GED-ELISA technique previously described was LPS not PII. If LPS is the major antigenic determinant in the DOC antigen it is probably associated with protein in the conventional ELISA test. Since magnesium ions are required to attach pure LPS to polystyrene microtitre trays (Ito *et al.*, 1980), this would explain the dependence of this assay on protein concentration.

Preliminary results suggest that the IgA response to gonococcal antigens may be different from the IgG. The correlation between IgG and IgA levels in patients was found to be poor using the DOC antigen in ELISA (Ison & Glynn, 1979); if two antigens are involved this may explain the lack of correlation.

Antibodies to LPS were present in 15% of the control sera (Fig. 5), a similar proportion to that found previously by Watt, Ward & Glynn (1971), using agglutination of LPS-coated latex particles. Antibodies were detected not only to proteins I, II and III, but to several undefined proteins of high molecular weight. It is possible that the latter are not surface antigens. The distribution of these antibodies among patients was irregular and unlike anti-LPS they were not significantly more frequent than among controls in the small sample tested. This paucity of antibodies to proteins may reflect the antigenic diversity of gonococcal outer membrane proteins, both between and within strains (Johnston, 1980; Diaz & Heckels, 1982) or the denaturing conditions used in SDS-PAGE or both. Although outer membrane proteins look unpromising as antigens in serodiagnosis, it is possible that the use of a different protein serotype strain (Ison, Hadfield & Glynn, 1981), or a combination of serotype protein antigens (Brodeur, Ashton & Diena, 1982) may improve the specificity. However, this was not found to be true for the detection of antibodies to pili (Reimann, Oranje & Michel, 1982). Thus while sera produced by immunizing rabbits or guinea-pigs showed marked heterogeneity among pili (Buchanan, 1975; Novotny & Cownley, 1980), sera from naturally infected humans cross-react widely with pili from different strains (Brinton *et al.*, 1978; Lind & Reimann, 1980). By analogy with serological tests for syphilis it has been argued (Glynn & Ison, 1981) that two distinct antigens used in separate tests should give better diagnostic specificity than one alone. An infected patient would be expected to react in both tests, whereas false positive results are more likely to occur in one only if the two antigens used have nothing in common. The results presented here suggest that some care will be needed in the selection of the two antigens. The most promising looking combination is LPS and pili.

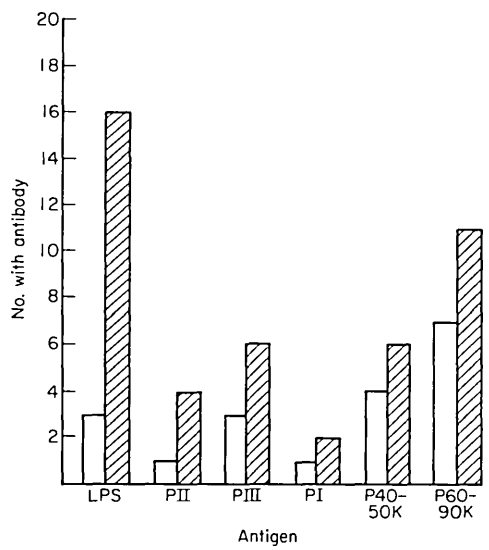


Figure 5. Frequencies in patients (■) and controls (□) of antibodies to individual antigens.

Although insufficient material has yet been studied, it looks too as if anti-PI IgG is produced in systemic rather than uncomplicated gonococcal infection. This was also found using the GED-ELISA technique for antibody analysis (Hadfield & Glynn, 1982). *N. gonorrhoeae* strain H1 is serogroup WII which is not strongly associated with DGI, as is WI (Sandström & Danielsson, 1980). It therefore seems unlikely that the PI of strain H1 will only detect antibodies in DGI.

These preliminary results suggest that the immunoblotting technique because of its ability to discriminate between antibody specificities without necessitating complex antigen purifications merits wider application.

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