Master Copy

STUDIES ON THE ANTIGENIC VARIATION OF PILI AND OUTER MEMBRANE PROTEINS FROM NEISSERIA GONORRHOEAE

submitted

by

JOSE-LUIS DIAZ

for the degree of Master of Philosophy

Faculty of Medicine

University of Southampton

Microbiology
Faculty of Medicine
University of Southampton

Acknowledgements

I wish to thank Dr. J.E. Heckels for his excellent supervision and advice, Mrs. Mitchell for her accurate typing and my wife Roma for her constant support and endless supply of cups of tea whilst I was preparing this thesis.

The work presented in this thesis has provided material for the following publications:

Diaz J-L and Heckels J.E. (1982) Antigenic variation of outer membrane protein II in colonial variants of Neisseria gonorrhoeae p9. Journal of General Microbiology, 128, 585-591

Zak K., Diaz J-L, Jackson D. and Heckels J.E. (1984) Antigenic variation during gonococcal infection: detection of antibodies to surface proteins in sera of patients with gonorrhoea. Journal of Infectious Diseases, 149, 166-174

Diaz J-L, Virji M. and Heckels J.E. (1984) Structural and antigenic differences between two types of meningococcal pili, FEMS Microbiology Letters, 5, 263-265

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

MICROBIOLOGY

Master of Philosophy

STUDIES ON THE ANTIGENIC VARIATION OF PILI AND OUTER MEMBRANE PROTEINS FROM NEISSERIA GONORRHOEAE

by Jose-Luis Diaz

Rabbits were immunised with outer membranes from colonial opacity variants of Neisseria gonorrhoeae P9 and antibodies were detected by an enzyme-linked immunosorbent assay (ELISA). ELISA-inhibition experiments with purified antigens revealed approximately equal proportions of antibodies directed against each of the three major surface antigens: lipopolysaccharide, protein I and protein II. Inhibition experiments with intact gonococci showed considerable surface antigenic diversity which correlated with differences between the molecular species of protein II present. Proteins II from colonial variants of the same strain showed little cross-reactivity with specific anti-protein II sera, demonstrating antigenic variation in the surface exposed region of the protein.

Gonococci were cultured from the urethra of male patients and from the cervix and urethra of female contacts. Isolates from within a patient group were of the same strain, but showed differences in molecular weight of protein II and pili. Radioimmune precipitation assay showed that most patients produced serum antibodies directed against protein I. Anti pilus antibodies, when present, showed cross-reactivity with pili from homologous and heterologous strains. Antibodies to protein II were found to be highly specific. These data suggest that the host immune response may be an important factor in causing antigenic shift during infection. Several sera also contained antibodies to a common surface protein of 43,000 molecular weight present in all strains tested.

Sera from patients convalescing from meningococcal infections had antibodies to the main outer membrane proteins which cross-reacted with gonococcal protein I and the 43,000 molecular weight protein. Anti-meningococcal protein II antibodies were highly specific. Strains of pilated meningococci could be divided into two groups on the basis of antigenicity and sub-unit molecular weight. Strains from group 1 reacted with monoclonal antibodies directed against gonococcal pili, had pili with sub-unit molecular weight similar to that of gonococci, and could be detected by radioimmune precipitation or electroblotting. Strains from group 2 failed to react with the monoclonal antibodies, had pili with lower sub-unit molecular weight and could only be detected by radioimmune precipitation using polyclonal anti-pilus antiserum but not by electroblotting.

CONTENTS

Abbreviations

OM - Outer membrane

LPS - Lipopolysaccharide

SDS-PAGE - Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

ELISA - Enzyme Linked Immunosorbant Assay

RIP - Radioimmuno-precipitation Assay

MOMP - Major outer membrane protein

INTRODUCTION

Epidemiology

Neisseria gonorrhoeae, the causative agent of gonorrhoea was first described by Albert Neisser in 1879 and cultivated three years later by Leistikow . . . . . (1882). Sulphonamides provided the first effective therapy, but resistance became common. In 1943 with the advent of penicillin, sulphonamides were replaced and gonorrhoea became easy and cheap to treat.

Effective antibiotic treatment did however not reduce the incidence of gonorrhoea, which suffered a sharp rise during the latter part of the 1960's. The increase continued during the 1970's in many countries, till it probably became the most prevalent transmissible bacterial disease in man, with a total figure of approximately 200 million cases reported annually (W.H.O. Annual Report, 1978). In the United Kingdom the disease is gradually being brought under control with a fall in new cases reported, from 65,963 in 1977 to 58,301 in 1981, although overall there has been a marked rise in numbers of sexually transmitted diseases in the same period (Social Trends, Central Statistics Office, 1983). The fall can generally be attributed to good contact tracing methods, and the high percentage of people with infections who ultimately receive treatment.

Gonorrhoea in Britain could pose a new problem with the increased isolation of β-lactamase producing gonococci. These strains were first reported in the U.K. in 1976 and have been increasing in numbers rapidly. In the past few years the number of penicillin-resistant cases have been doubling annually; 211 in 1980, 443 in 1981 and 613 in the July to December period of 1982 compared with 247 cases in the same period of 1981 (Social Trends No. 14, Central Statistical Office, 1983). Significantly more and more of these cases are contracted in the U.K. (62% in 1981, 72% in 1982), endemic transmission is now the major cause of increase.

The problem in developing countries is more serious. In Gambia 16,000 cases of gonorrhoea are reported per annum for males, out of a total population of 600,000 half of which is under 15 years old (W.H.O. Annual Statistical Report, 1980). This represents approximately 10% of the population, without taking into account

the sizeable number who may not be attending clinics (Dr. Mabey, personal communication); an incidence 20 to 50 times greater than in the U.K. Similarly the level of gonorrhoea in Zaire is 4%, and 5% in the Lagos and Ibadan regions of Nigeria (W.H.O. Annual Statistical Report, 1980).

Taking into consideration the low level of per capita expenditure in health and the relative scarcity of penicillin, allied to the high incidence of penicillinase producing gonococcal isolates in West Africa, the scale of the problem becomes apparent, particularly when the further consequences of the infection are considered. A high percentage of women with gonorrhoea give birth to babies with gonococcal ophthalmia. Women may also develop pelvic inflammatory disease (PID), a complication leading to sterility if the fallopian tubes are damaged. Thus 5% of all females in rural areas of Gambia are infertile and 14% develop secondary infertility (defined as failure to bear children after the age of 30) perhaps correlating to the high incidence of PID in the area (Billewicz and McGregor, 1981), a serious occurrence in a society where infertility is considered a great stigma. Where PID does not cause infertility, partial occlusion of the fallopian tubes may occur giving rise to subsequent ectopic pregnancies.

When penicillin is ineffective due to β-lactamase production, spectinomycin is the recommended alternative, but gonococcal strains with multiple resistance are already beginning to appear. In 1981 the Centre for Disease Control, Atlanta reported the first known infection caused by spectinomycin-resistant-β-lactamase producing gonococci, originating from the Philipines (Morbidity and Mortality Weekly Report, 1981, 30 No.19). In 1983 seven cases of spectinomycin resistance were reported in U.K. alone. The uncontrolled levels of the disease in developing countries, and the increase in the number of infections due to antibiotic resistance in the developed and the "Third" World including the recent appearance of multiple resistant strains, emphasises the importance of a prophylactic alternative to chemotherapy. Development of a vaccine however, requires understanding of the pathobiology and immuno-chemistry of gonorrhoea. These aspects of the gonococcal infection are discussed in the following sections.

Pathology

Gonorrhoea is a disease of non-squamous epithelial surfaces, invading the columnar epithelium of the urethra, endocervix, fallopian tube, rectum and pharynx as well as the conjunctiva (Harkness, 1948). Electron microscopic studies of mucosal cells obtained from urethra of men with gonorrhoea showed gonococci attached and partially embedded in the surface of epithelial and mucus secreting cells (Ward and Watt, 1972). Studies on experimentally infected human fallopian tube organ cultures showed that the initial interaction of gonococci is with microvillous projections from the host cell surface (Ward and Watt, 1975). Once the gonococcus is bound to the epithelial surface by high avidity binding a proportion of the organisms enter the epithelial cells (Watt et al., 1978).

The sequence of events during infection as envisaged by Ward et al. (1974) from studies on fallopian tube model are the following: the gonococci lie in membrane bound vesicles within the epithelial cells, multiply and pass through the epithelial cell layer, leaving by a process of exocytosis and entering the sub-epithelial connective tissue. By day three to four there is a patchy destruction of the mucosal surface, an abrupt onset of dysuria and a purulent exudate which demonstrates polymorphonuclear leukocytes with intracellular gonococci.

It has been suggested that the close proximity of the gonococcal cell to the host cell surface may initiate phagocytosis which is preceeded by sequential, circumferential interactions between gonococcal ligands and receptors on the host cell membrane, creating a "zipper mechanism" which ultimately results in ingestion of the gonococci within an invaginated host cell membrane (Watt et al., 1978).

Prior to antibiotics therapy symptomatic cases usually resolved within 8 weeks and 95% of patients were free of symptoms within six months. Healing following acute inflammation occurs by fibrosis and may result in the formation of strictures in the male urethra or partial occlusion of the fallopian tubes. Acute anterior urethritis is the most common manifestation of gonococcal infection in the male, but local spread may result in prostatitis, seminal vesiculitis, epididymitis etc. although all of these syndromes have become uncommon since the introduction of antibiotic therapy. In

women the primary site of urogenital infection is the columnar epithelium of the endocervix, but it has been estimated that 10% to 17% of women with gonorrhoea develop complicated infections (Eschenbach and Holmes, 1975). These include gonococcal salpingitis (swelling of the fallopian tubes) an ascention of the infection from the cervix along the surface of the endometrium to the fallopian tubal mucosa (Rees and Annels, 1969). Monthly shedding of the endometrium implies that endometritis is only transient and thus an extensive tubo-ovarian infection may appear in association with a normal uterus. Primary destruction of the mucosa frequently leads to partial or complete occlusion of the fallopian tubes often with resultant subinfertility or infertility. Purulent exudate may spread locally giving rise to pelvic peritonitis and abscess. Sub-acute gonococcal salpingitis, because of the delay in diagnosis and treatment may present a more serious hazard to fertility.

Some gonococcal infections may present no symptoms. Gonococci from asymptomatic patients do not induce an acute inflammatory response since they fail to generate chemotaxins on interaction with normal human serum, induce only a weak antibody response (Rice and Goldenberg, 1981), and if untreated may subsequently give rise to disseminated gonococcal (DGI) infections. The dissemination of gonococci in the bloodstream can produce fever and pustular skin lesions, located primarily on the extremities, from which gonococci may be isolated (Handfield, 1975). Gonococcal arthritis of the knees, ankles and joints of the feet may be followed by synovitis with gonococci and pus in the synovial fluid. Gonococcal endocarditis and meningitis are uncommon manifestations of DGI since the advent of antibiotics. Gonococci isolated from DGI appear to possess several characteristic traits, these are discussed more fully in a later section (see page 20).

Gonococcal adhesion to mucosal surfaces and invasion of epithelial cells must be mediated by molecules on the surface of the gonococci, knowledge of the structure and immunochemistry of these components is therefore essential in order to understand the pathogenesis of the disease. The structure of pili, outer membrane proteins and lipopolysaccharide are thus discussed below.

Structure of gonococcal surface antigens

Early electron microscopy studies revealed that the cell envelope of Gram-negative bacteria is composed of two distinct membranes, the inner cytoplasmic membrane and the outer membrane which have a peptidoglycan layer located between them. Most information on Gram-negative cell membranes has resulted from studies involving E. coli (for reviews see Di Rienzo et al., 1978; Osborn and Wu, 1981). The cytoplasmic membrane houses the systems for active transport; oxidative phosphorylation and biosynthesis of various macromolecules. The outer membrane though morphologically similar, contains less phospholipids, fewer proteins and a unique carbohydrate component, lipopolysaccharide (LPS). The outer membrane is also functionally distinct, acting as a diffusion barrier against compounds such as antibiotics, toxic chemicals and nutrients. It contains a small variety of proteins which appear to impart on the membrane functions such as non-specific passive diffusion pores that allow the passage of low molecular weight substrates, as well as specific uptake systems for nutrients such as iron, vitamins and carbohydrates (Fig. 1)

a) Outer membrane proteins

Material for studies on outer membrane of enteric bacteria were generally obtained by isopycnic centrifugation of lysozyme-ethylene-diamine tetraacetic acid (EDTA) prepared spheroplasts, lysed by sonification (Osborn et al., 1972). Johnston and Gotschlich (1974) used a similar procedure for gonococci, but found it difficult to isolate sufficient material for detailed serological analysis. Johnston et al. (1976) developed a method in which small vesicles containing LPS, one major protein and a characteristic secondary protein, were released when gonococcal cells were shaken in lithium acetate.

On chemical analysis and sucrose density centrifugation, the vesicles obtained were found to be representative of the outer membrane; this method is now used in many laboratories.

Studies of the gonococcal outer membrane have shown the presence of three different major proteins: proteins I, II and III. Protein I has been observed in all gonococci examined so far; its apparent molecular weight varies from strain to strain but falls

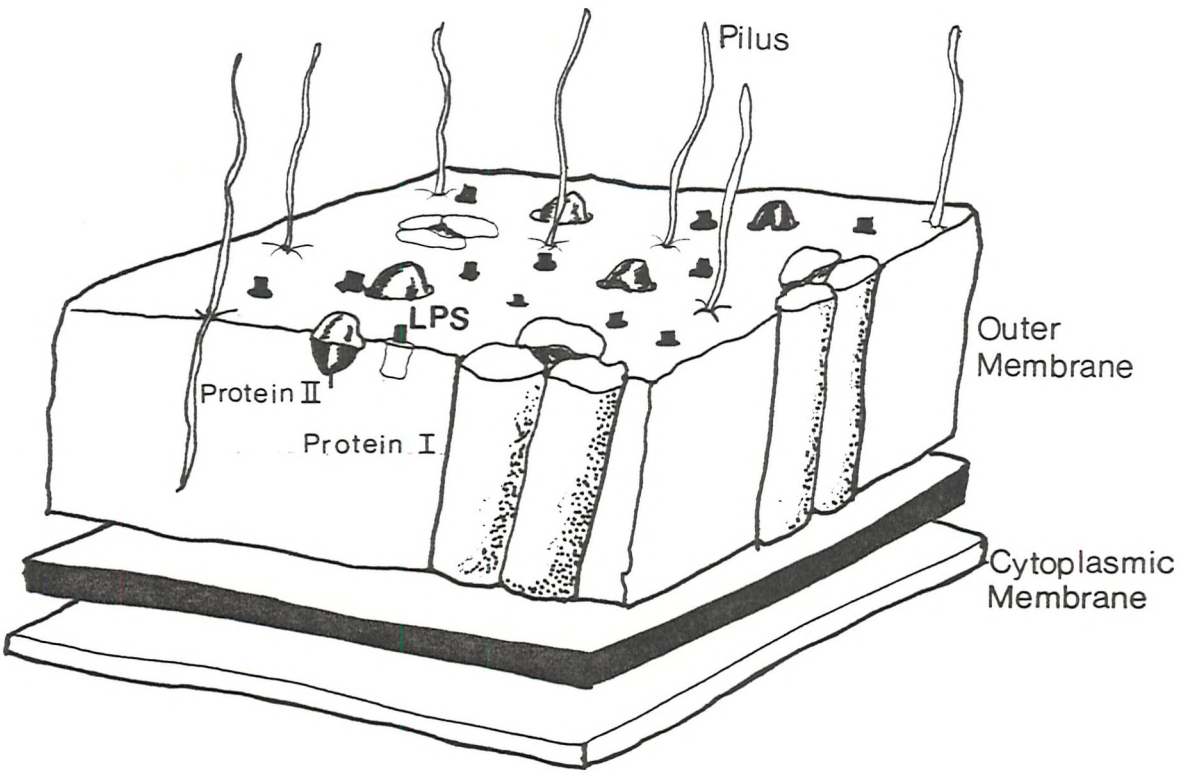


Figure 1.

Schematic diagram of the organisation of the gonococcal outer surface. (Courtesy of Dr. J.E. Heckels, Microbiology, Faculty of Medicine, University of Southampton).

within the range of 32,000 to 40,000 (32K - 40K). This protein is readily labelled using lactoperoxidase (Heckels, 1978; Swanson, 1978) a non-permeant system for radio-iodination, implying it is exposed on the cell surface. Recent investigations have shown that protein I can be cross-linked to the peptidoglycan layer (Heckels, 1979), suggesting that the protein spans the outer membrane.

Cross-linking analysis of the gonococcal outer membrane proteins, followed by two dimensional sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) shows protein I to be cross-linked primarily to itself to form a trimeric complex (Newhall et al., 1980). "Near neighbour analysis" techniques have shown the major outer membrane proteins of Escherichia coli to be trimeric as well (Palva and Randall, 1978). Similar trimeric units in Salmonella typhimurium can function as diffusion channels in artificial vesicles (Tokunaga et al., 1976). Matrix proteins from E. coli outer membranes can be integrated into planar lipid bilayers (Schindler and Rosenbusch, 1978) to form aqueous channels of uniform size across the membranes, allowing free diffusion of small uncharged molecules. This in addition to evidence on the permeability of artificial membranes containing gonococcal protein I (Douglas et al., 1981) suggests that its role is that of a porin incorporated into the lipid bilayer as a trimer, creating channels to allow the passage of ions and small hydrophilic molecules.

Although it has long been known that different gonococci grown on clear solid medium may produce transparent or opaque colonies (Sparling and Yobs, 1967), it has since been shown that the situation is more complex and that a single gonococcal strain may produce a number of colonial variants exhibiting different colonial opacities (Swanson, 1978; Lambden and Heckels, 1979). Comparisons using SDS-PAGE carried out on whole solubilised gonococci showed that the transparent colony variant in each case had simple cell wall patterns, whereas the opaque colonies had more complex banding patterns with one or more additional prominent proteins, varying in molecular weight from 24K to 30K (protein II)(Swanson, 1978). Protein II species can be identified on SDS-PAGE due to their characteristic heat modifiability; when dissociated by boiling the apparent molecular weight is greater than when dissociated by incubating at 37°C.

Detailed work carried out using a single strain N.gonorrhoeae

strain P9, has demonstrated the complexity of the situation: selection of colonies with different opacities produced a series of variants with different protein II species (II, II_a, II_b, II_c, II_d, II_e, II_f, Table 1) of varying molecular weights (29,000-27,500). No single variant has yet been isolated which possess more than two protein II species (Lambden and Heckels, 1979). Lactoperoxidase-^{125}I labelling studies (Heckels, 1978; Swanson, 1978) have shown protein II to be exposed on the cell surface, and may therefore be of importance in interactions with host cells, indeed alterations in the content of these proteins have been correlated with differences in virulence properties of isogenic variants from a single strain (Lambden et al., 1979, to be discussed in a subsequent section).

A third protein, protein III, is present in all gonococcal strains (McDade and Johnston, 1980), and characterised by an increase in apparent molecular weight when solubilised in the presence of 2-mercaptoethanol. The apparent molecular weight of the unreduced (30K) and the reduced (31K) forms of protein III are the same in all strains and intrastrain phenotypes studied to date (Judd, 1982a). It was shown that protein III from four gonococcal strains with protein I of different molecular weights, all had very similar apparent structures on the basis of peptide mapping. This protein probably occurs in close association with protein I. The protein III described here must not be confused with a 60K protein described and isolated by Heckels and Everson (1978) then termed protein III and subsequently found to be loosely associated with the internal surface of the gonococcal outer membrane, and not expressed on the external surface.

Studies by Swanson (1979) used ^{125}I-peptide mapping techniques to investigate the structure of three different protein I's of different molecular weight isolated from ten gonococcal strains, a high degree of homology was found between all the protein I's. Sandström et al. (1982) have however reported differences in outer membrane protein I molecules which are mutually exclusive and represent two different antigens used in serotyping. Similar techniques show that protein II of different strains and colonial variants of the same strain also show structural similarities (Swanson, 1980). The structure of protein II in strain P9 was studied by Heckels (1981) using whole cells labelled with ^{125}I-lactoperoxidase, followed by tryptic digestion. Autoradiographs revealed a considerable

degree of structural homology, but also showed major differences in the most hydrophilic peptides which corresponded to the most intense on the autoradiographs. The evidence suggested that protein II species form a family with a homologous region embedded in the membrane, and a variable region of the polypeptide which is expressed on the gonococcal cell surface and capable of variations within a single strain. This model of surface variations may have important implications on the antigenic cross-reactivity of protein II species.

b) Lipopolysaccharide (LPS)

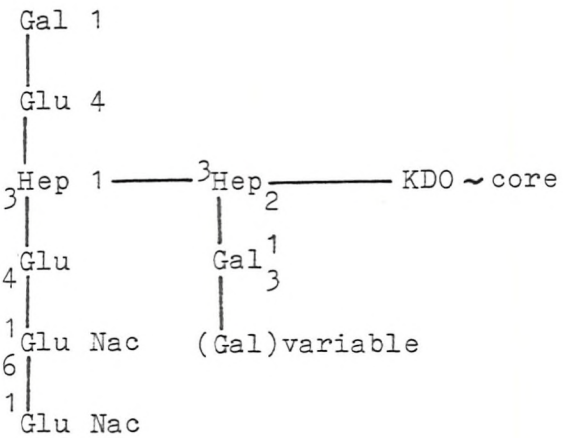
Outer membranes of Gram-negative bacteria contain LPS responsible for O-antigen activity in Salmonella and E.coli for example, the LPS consists of a polysaccharide lipid A core and repeating O-antigen chains. LPS was isolated from cells of Neisseria gonorrhoeae by Tauber and Garson (1959), using the aqueous phenol extraction procedure described by Westphal et al. (1952). The preparation was toxic for mice and was shown to be composed of lipid, carbohydrate and a small peptide component. Chemical characteristics of gonococcal LPS reported by Perry et al. (1975), Stead et al. (1975), Perry et al. (1978) and Wiseman and Laird (1977) agreed on a structure consisting of Lipid A attached through 2-keto-3 deoxyoctonic acid (KDO) to an oligosaccharide containing heptose, D-glucose, glucosamine and D-galactose, present in a substituted or unsubstituted form. The amounts of galactose, glucose, heptose and glucosamine were respectively, 81, 32, 8 and 19 µg/mg of LPS (Wolf-Watz et al., 1976). The five sugars listed are widely distributed in LPS from gonococcal strains, and are also constituents of LPS from Enterobacteriaceae (Lüderitz et al., 1966).

The lipid moiety consists of a hydrophobic lipid A core with endotoxic properties, embedded in the outer membrane; the attached oligosaccharide section being exposed on the surface (Figure 2). Analysis of gonococcal lipid A from five strains found all contained glucosamine joined to phosphate groups and fatty acids, principally hydroxy-acids thought to be amide linked (Stead et al., 1975). The composition was comparable to that found in commensal neisseria (Adams, 1971) and salmonellae (Rietschel et al., 1972).

There is however conflicting evidence as to whether gonococci are capable of producing "smooth" (S) type LPS with repeating

Figure 2

The chemical structure of gonococcal lipopolysaccharide



(as reported by M.B. Perry at the ASM meeting: Immunobiology of Neisseria gonorrhoeae Washington, D.C., Jannuary 1978)

O-antigen side chains. Stead et al. (1975) detected no difference between LPS from virulent pilated (P^+) and a virulent non-pilated (P^-) variants. In contrast, Perry et al. (1975) concluded that virulent (P^+) colonies produced an "S" type LPS with O-polysaccharides containing sugars such as galactosamine, rhamnose, xylose, or fucose depending on the strain; whereas avirulent (P^-) cells produced a common "R" type LPS. A more recent study reported analyses of glycose and fatty acid contents of LPS extracted from 38 strains of gonococci, indicating that all polysaccharides usually associated with the presence of O-antigen. Virulent (P^+) colony types in contrast to avirulent strains and several non-pathogenic species N.sicca and N.lactamica contained no rhamnose (Wiseman and Laird, 1977). Fucose which was characteristic of non-pathogenic species was not detected in gonococci. A subsequent report by Perry et al. (1978) was inconsistant with their earlier studies, and noted that "S" LPS could only be detected in minor amounts. Unpublished data from SDS-PAGE gels of LPS run in our laboratory, and unpublished observations from others, lead us to conclude that most gonococci grown under standard laboratory conditions produce "R" type LPS. lacking O-polysaccharide repeating chains.

The antigenic structure of gonococcal LPS is a complex of at least three major antigenic determinants which are contained on the

carbohydrate moiety. These determinants consist of a specific serotype antigen and up to two common antigens (Apicella and Gagliardi, 1979). Six antigenically distinct gonococcal serotype antigens have been described (Apicella and Gagliardi, 1979), in addition phenotypic variation can occur within isogenic strains of gonococci, producing a distinct LPS with different antigenic characteristics (Morse and Apicella, 1982). Recent evidence however suggests that LPS from different strains show considerable structural homology (Apicella et al., 1981). A monoclonal antibody was used to identify a common epitope on gonococcal LPS; meningococcal LPS was also found to contain a portion of the site. The antibody failed to recognise the site on LPS isolated from E.coli and Salmonella minnesota. Inhibition studies with a variety of sugars indicated that the chemical structure of the determinant was D-galactosamine-galactose-glucose, with the galactose and glucose in a (1-4) pyranosyl linkage (Apicella et al., 1981).

c) Pili

Pili are a diverse group of non-flagellar proteins occurring as filamentous appendages of a wide range of Gram-negative bacteria, especially the Enterobacteriaceae. Two groups of pili have been distinguished. The first, conjugative or sex pili, are encoded by self transmissible plasmids, and promote infection by certain pilus-specific bacteriophages. They play an important role in the process of bacterial mating. The second group, non-conjugative pili, is of more general occurrence, and are unrelated to donor function in bacterial conjugation. Gonococcal pili belong to this second category.

Pili were first reported on gonococci in 1971 (Jephcott et al; Swanson et al., 1971) when they were observed by electron microscopy in negatively stained preparations of freshly isolated gonococci. They were seen to extend 2 microns from the bacterial surface and to have a diameter of approximately 8 nm. Considerable interest developed in gonococcal pili because of their consistant presence on virulent colony types and absence in avirulent forms, suggesting a relationship between the pili and the pathogenic potential of the organisms (Swanson et al., 1971).

Development of methods for isolation of pili was spurred by

the association of pilation with gonococcal virulence, thus facilitating the study of their immunochemistry and their role in pathogenesis. Much of the early work of preparation and purification of pili was carried out by Brinton (1965) on E.coli, but Buchanan et al. (1973) were the first to report purified gonococcal pili, this they did by sharing of pili from broth grown organisms, aggregating them by adjusting to pH 4 and then harvesting by slow speed centrifugation. The pili were purified by subjecting them to three cycles of disaggregation and aggregation, using 0.01M Tris pH 10.3 and adjusting to pH 4 respectively. This procedure however gave a low yield (15 µg pili/l of liquid culture). Robertson et al.(1977) isolated purified gonococcal pili by selective disaggregation in sucrose and purification by CsCl density gradient centrifugation. High yields of pure pili have since been obtained (Brinton et al., 1978) by repeated cycles of suspensions in ethanolamine buffer pH 10.5 to disaggregate pili and removal of cell debris by centrifuging, followed by precipitation in 10% ammonium sulphate causing aggregation of the filaments to form bifringent crystals.

Studies on purified gonococcal pili revealed each pilus to be an assembly of multiple units of a single pilin protein, with an aggregate molecular weight of 1-20 million (Buchanan et al., 1973; Robertson et al., 1977; Brinton et al., 1978). The molecular weight of the sub-units also varied from strain to strain, with similar but consistant variations shown by iso-electric focussing, isopycnic centrifugation and amino acid analysis. Analytical studies of such preparations (Robertson et al., 1977) revealed the presence of both phosphate and carbohydrate groups corresponding to 1-2 and 2 moieties respectively, per pilin subunit. It is unknown however, whether this is due to the presence of covalently bound sugars,or a non-covalent association of pili with contaminating material as is the case with pili of E.coli (Armstrong et al., 1981).

Pilin subunits contain some 200 amino acid residues, and have molecular weights in the range of 17-21.5K depending on the different isolates. These molecular weight differences may account for the antigenic variation in pili; antiserum raised in rabbits against one pilus type for example, revealed little shared antigenicity with pili from other strains (Buchanan, 1978).

Despite the antigenic heterogeneity of gonococcal pili, there are similarities in the percentage composition of amino acids

(Robertson et al., 1977). There exists a high percentage of hydrophobic amino acids, indeed the first 24 residues from the N-terminal region are all hydrophobic, with the exception of threonine and glutamic acid at positions 2 and 5 (Hermodson et al., 1978) perhaps reflecting its role as a surface protein. The N-terminal amino acid sequence through the 49th residue was determined for two serologically unrelated gonococcal pili (Schoolnik et al., 1982) confirming previous observations. The sequence was identical for the two strains studied and for the pili from the four strains sequenced to the 29th residue by Hermodson et al. (1978). It was also highly homologous with N-terminal acid sequences of pili from Moraxella nonliquefaciens (Froholm and Sletten, 1977) and Pseudomonas aeruginosa (Paranchych et al., 1978), but entirely different from the analogous region of E.coli common pili (Hermodson et al., 1978). The N-terminus of all the above pili, except that of E.coli, is an unusual amino acid, N-methyl-phenylalanine, not yet detected in other pili. The high degree of structural conservation of this region, implies it has an important role in the function of the protein. The highly hydrophic nature of the region makes it likely that it is located in one of two sites in the protein structure: buried deep inside the pilin polypeptide, maintaining subunit structure, or, at a point where it could be involved in subunit-subunit interaction, maintaining the polymeric structure.

Cyanogen bromide cleavage at the two methionine residues contained by gonococcal pili resulted in three fragments which could be purified by gel filtration (Schoolnik et al., 1982; Buchanan et al., 1982). A small peptide derived from the N-terminus and two larger peptides. One of the larger fragments encompassed a highly conserved region which mediated binding to erythrocytes, whereas the other contained a variable region which determined type-specific antigenicity. A common pilus determinant was contained within the fragment with the erythrocyte binding domain, but was not immunogenic in the intact pili, only becoming immunodominant when the fragment was cleaved from the subunit. Antibodies to this fragment bound to heterologous gonococcal pili. The above data suggest gonococcal pili have a constant common molecular structure responsible for adherence, and an immunodominant antigenic determinant capable of structural modification.

Extensive studies with a single strain of gonococcus, P9,

showed that variation in pilus types can occur not only amongst different strains but also within isogenic variants of the same strain. Originally two different types of pili, designated α and β were isolated from strain P9 (Lambden et al, 1980, 1981b). More recently two further pilus types, γ and δ, were found in variants of strain P9 recovered from guinea pig subcutaneous chambers (Lambden et al., 1981a). Thus pili with four different sub-unit molecular weights (21K, 20.5K, 19.5K and 18.5K) have been isolated from variants of strain P9. The four types of pili differ in sub-unit isoelectric point, buoyant density and amino acid composition. There does however exist structural homologies in peptide maps of tryptic/chymotryptic digests of pili with several major peptides apparently common to all four pilus types, although some peptides were unique to a particular type (Lambden, 1982). Despite the close structural similarity, antiserum raised against each pilus type showed only about 10% cross-reactivity with the heterologous types. Differences have also been observed between pili from opaque and transparent colonies of several strains (Salit et al., 1980). The ability to produce different types of pili implies that the gonococcus is capable of varying the properties of its surface, suggesting it may play an important role in the virulence of the organism.

d) Capsule

In meningococci a polysaccharide capsule is prominent and plays an important role in virulence (Roberts, 1967; Craven and Frasch, 1978), blocking the binding of antibodies to the LPS core, thus protecting the organism from opsonisation and phagocytosis. Three groups of investigators published morphological evidence for the presence of a capsule on gonococci (Hendly et al., 1977; James and Swanson, 1977; Richardson and Sadoff, 1977). Encapsulation was noted on freshly isolated strains (Richardson and Sadoff, 1977). Subsequent morphological evidence has been offered for the existence of a capsule by Demarco de Hormache et al. (1978) using Alcian blue stained preparations of gonococcal strain gc40 viewed by electron microscopy. A recent study using gel precipitation techniques (Demarco de Hormache et al., 1983) also suggested the presence of an antigen in the virulent encapsulated in vivo grown variant of strain gc40, absent in the non-virulent non-capsulated variant. This antigen was found in the saline extracts and in isolated LPS from the virulent variant and seemed to be involved in the reaction with wheat germ agglutinin.

Considering the importance of the capsule in the pathogenesis of meningococci, and the relative success of meningococcal vaccines to capsular polysaccharides, the existence of a gonococcal capsule could have important implications. However, until capsular material is isolated and characterised, we can only speculate on its possible significance to the pathogenesis of gonorrhoea.

Role of surface components in virulence

a) Attachment of gonococci

The surface components of gonococci play an important role in the pathogenesis of the disease, acting as major antigens and as mediators of attachment to host cell surfaces. The ability to adhere to mucosal surfaces is fundamental for colonisation if the gonococcus is to overcome the "flushing" effects of urine and mucus secretions. Electron microscopic studies of urethral mucosal cells from patients with gonorrhoea, demonstrate gonococci firmly attached to host mucosal surfaces (Ward and Watt, 1972). It has been proposed that attachment may involve two separate stages: first the initial

attachment by pili, followed by close contact of the bacterial and host cell surfaces (Swanson, 1973).

i) The role of pili.

There is strong evidence for pili having an important role in attachment to a range of human cells. Punsalang and Sawyer (1973) demonstrated enhanced adhesion of pilated gonococci to rabbit, guinea pig, sheep, chicken and human blood group O, Rh^+ erythrocytes. Increased attachment was also seen to spermatozoa (James-Holmquest et al., 1974), fallopian tube epithelium (Ward et al., 1974), vaginal epithelial cells (Mardh and Westrom, 1976) and tissue culture cells (Swanson, 1973; Swanson et al., 1975). The possibility that an anti-pilus vaccine might induce antibodies which inhibit attachment and invasion of epithelial cell surfaces, has stimulated interest in the mechanism of adhesion.

The gonococcus and the host mucosal cell surfaces both bear an overall net negative charge producing an initial electrostatic repulsive force which the gonococcus must overcome in order to attach (Heckels et al., 1976). Once the two cells are sufficiently close Van der Vaal's attractive forces may cause the gonococcus to be held in a stable position. The role of pili in attachment may be to penetrate the barrier of electrostatic repulsion. A study using chemically modified gonococci with altered surface charges (Heckels et al., 1976) showed that blocking amino groups increased the surface net negative charge (pI 4.0) and reduced binding of gonococci to tissue culture cells. Blocking carboxyl groups (pI 8.2) reversed the usual negative surface charge (pI 5.6) on the gonococcus and doubled the mean number of gonococci attached per cell. Increased attachment was not simply due to electrostatic attraction to the altered positively charged surface since adherence was also enhanced when both amino and carboxyl groups were blocked. Pili did not promote adhesion of gonococci when the electrostatic repulsive barrier was reduced, conferring advantage only when gonococci had an overall negative charge. It was suggested that the length of the pili permits initial interaction with the host cell membrane at distances where electrostatic repulsive forces between the two cell bodies would be negligible, thus increasing the probability of a closer approach by the two cell surfaces.

A specific receptor for gonococcal pili has not yet been found on host cell surfaces. Adhesion of certain E.coli pili to

erythrocytes can be selectively inhibited by D-mannose and methyl mannoside (Ottow, 1975) suggesting the pilus attaches to a specific carbohydrate on the membrane; haemagglutination by pilated gonococci is not inhibited by mannose (Karansky et al., 1975). Simple sugars also failed to inhibit attachment of gonococcal pili to buccal epithelial cells, the adhesion was however markedly inhibited by treatment of the buccals with a neuraminidase-exoglycosidase mixture (Trust et al., 1980). The pilus receptor might thus be a polysaccharide with a specific glycosidic linkage. Pilated strains also demonstrated enhanced aggregation in the presence of a ganglioside indicating a possible attachment role for structurally similar oligosaccharides present in a surface glycolipid (Trust et al., 1980). Binding studies of ^{125}I-labelled purified pili to a range of human cell types led Buchanan et al. (1978) to similar conclusions when they found that attachment to buccal cells was inhibited by gangliosides. The peptides obtained from cyanogen bromide cleavage of pili were tested for their capacity to inhibit the agglutination of human erythrocytes by intact pili (Schoolnik et al., 1982). It was found that only the fragment containing the common pilus region was capable of competing with uncleaved pili and attaching to erythrocytes. The conserved nature of the region with the erythrocyte binding domain, probably reflects the importance of the ability to adhere for normal pilus function.

Attempts to establish the molecular mechanism of pilus attachment are complicated by the variety of host cell and pilus structure encountered. Gonococci are found colonising human anatomical sites with different cell types and environmental conditions. Gonococcal adhesion to vaginal epithelial cells, for example, was found to vary during the course of the menstrual cycle (James and Swanson, 1978). Furthermore, differences in adhesive properties are not only found between pilated and non-pilated gonococci, but also between isogenic variants of the same strain possessing distinct pilus types. Lambden et al. (1980, 1981) found that of two pili types isolated from strain P9, only one attached to buccal epithelial cells. All four pilus types isolated from this strain show differential cell specificity in adhesion experiments with a range of cells (Heckels, 1982).

ii) The role of outer membrane proteins.

The mechanism of the second stage of attachment involves structures other than pili since non-pilated organisms have the ability to attach to cells, though with reduced efficiency (Lambden et al., 1979). In this study it was found that variants of strain P9, each with a distinct protein II, attached more readily to buccal cells than the variant containing only protein I. Further buccal attachment experiments using ^{125}I-labelled P9 outer membranes were performed (Heckels and James, 1980). As with intact cells, the presence of a protein II conferred a three-fold advantage in attachment which could not be inhibited by addition of a series of simple sugars. However treatment of the buccals with exoglycosidases or treatment of the gonococcal outer membranes with trypsin, reduced the attachment of outer membranes possessing protein II to the levels of those lacking protein II. This protein is known to be sensitive to proteolytic enzymes, which cleave it into fragments which are not retained in the membrane (Blake et al., 1981). These observations suggest that protein II interacts with oligosaccharides on the cell surface, but indicates that protein I is not involved in adhesion.

Buccal cells, which are dead, may not be the ideal cell line for the study of attachment, but protein II has been seen to mediate attachment to other eukaryotic cells. Variants with protein II show increased attachment to Chang conjunctival cells (Heckels, 1982), and tissue culture cells such as Hela and Flow 2000 cells (James et al., 1980). Recently a highly specific monoclonal antibody directed against protein II was used in attachment assays and inhibited the adhesion of non-pilated gonococci to Hela cells (Sugasawara et al., 1983).

Opacity variants from the same strain with distinct protein II have been shown to have different eukaryotic cell attachment specificities (Heckels, 1982). One variant from strain P9 with a 28.5K molecular weight protein II for example, had optimal attachment with buccal cells, whilst another with 28K protein II had increased adhesion to Chang conjunctival cells. Differences in outer membrane protein structure may be important in enhancing attachment to mucosal cells at different anatomical sites.

b) Resistance to phagocytosis

Phagocytosis of most bacteria by normal human polymorphonuclear leukocytes (PMN) leads to death of micro-organisms. Although it was originally thought that gonococci seen in association with PMN on smears of urethral exudate were intracellular, several studies demonstrated that many of the bacteria were in fact attached to the cell surface with the organism remaining viable and apparently immune to microbicidal attack by PMN. This evasion of phagocytosis was considered to be important as a virulence enhancing property and was examined *in vitro* by several groups (Ofek *et al.*, 1974; Thongai and Sawyer, 1973; Dilworth *et al.*, 1975). It was determined that non-pilated organisms were phagocytosed at a higher rate than the pilated gonococci, which were mostly found bound to the outside of the cell.

The reduction of gonococcal phagocytosis has been attributed to pili, but outer membrane protein II has been implied as the primary determinant of leukocyte association (Blake and Swanson, 1975; Lambden *et al* , 1979). The inability of normal PMN to kill attached pilated gonococci appears to be the consequence of inadequate release of enzymes from the PMN primary granules, and the failure of PMN to incorporate pilated gonococci into a closed phagocytic vacuole. The mechanism by which virulent pilated gonococci escape ingestion is unknown, but may relate to a decrease in phagocytic membrane fluidity as a result of its interaction with gonococcal pili (Densen and Mandell, 1978).

c) Relation between gonococcal serotype and virulence

Many of the serotyping schemes for gonococci, whether using whole cells or crude antigenic fractions, are based on antigenic differences in protein I. Johnston *et al.* (1976) found at least sixteen different serotypes using a system involving double-diffusion analysis resulting in a characteristic and distinguishing precipitin arc. Wang *et al.* (1977) applied the micro-immunofluorescence (Micro-IF) method with absorbed mouse antisera against gonococcal antigens, and found 175 of 180 isolates were classified into three immunotypes. Using co-agglutination (COA) Danielsson and Sandström (1980) divided all strains examined into only three distinct groups. Buchanan and Hildebrandt (1981) recognised nine serotypes using

Enzyme Linked Immunosorbent Assay (ELISA) inhibition assay and purified protein I.

Recent investigations have suggested that different clinical syndromes may be associated with particular protein I serotypes. In one study (Buchanan et al., 1980) 72% of strains causing salpingitis belonged either to serotype 1 or 2 described by Buchanan and Hildebrandt (1981). Strains from patients with DGI also tend to share several other traits found less frequently among strains from patients with the uncomplicated disease. These include sensitivity to penicillin (Wiesnen, 1973), resistance to bactericidal action of normal human serum (Brooks et al., 1981; Schoolnik, 1976) and unusual nutritional requirements (Catlin, 1973). There is however little evidence regarding the mechanism by which some, or all of these characteristics might contribute to their virulence.

Resistance to killing by normal human serum and complement is exhibited by most strains of gonococci that cause disseminated infections. Rice and Kasper (1982) showed that complement dependent killing of human serum resistant gonococci could be blocked by naturally occurring human IgG antibodies, which impair access of lytic antibody and complement to LPS targets due to steric hindrance. It was suggested that antigenic differences in the outer membrane proteins of strains could be important in the recognition and avidity of these natural blocking antibodies.

Despite the fact that limited numbers of serotypes may be responsible for most DGI, suggesting that protein I may have a role in the pathogenesis of DGI, it has been demonstrated that the genetic locus affecting susceptibility to bactericidal activity of normal human serum, designated sac 1, is closely linked but discrete from a second locus (nmp 2) that affects protein I (Cannon et al., 1981).

d) Iron binding

Pili may have influences on virulence other than attachment and inhibition of phagocytosis. It has been reported that pilated gonococci bind iron more avidly than non-pilated (Payne and Finkelstein, 1975). The same group also observed increased virulence of gonococci in the presence of iron, and suggested that the greater virulence of pilated gonococci might be due partly to binding of iron and other cations by pili.

e) Role of LPS in virulence

Purified gonococcal LPS is an endotoxin and gonococci can release large quantities of the endotoxin bleb into the surrounding medium under laboratory growth conditions (Stead et al., 1975). Release of endotoxin is not a determinant of virulence as it is also seen in non-pathogenic neisseriae (Russell, 1976), though it has been suggested that released LPS is responsible for sloughing off and death of ciliated epithelial cells (Melly et al., 1981). The role of LPS in virulence of gonococci in the human infection is unclear, antibodies to LPS however are protective in a number of model laboratory infections (Diena et al., 1978; Robertson, 1979, see pages 21 and 22).

Host immune response

Infections caused by Neisseria gonorrhoeae have been shown to evoke an antibody response in which IgG, IgA and IgM are all produced (Cohen et al., 1969), and can be found both in the circulation and in genital tract mucosal secretions (Tramont, 1977). The antibodies detected have been directed to the major gonococcal surface components such as outer membrane proteins (Glynn and Ison, 1978), pili (Buchanan et al., 1973) and LPS (Glynn and Ward, 1970).

a) Protective effect of antigonococcal antibodies

Antigonococcal antibodies, of both IgG and IgA classes, found in genital secretions of men and women with gonorrhoea, showed inhibition of pilated gonococcal adhesion to epithelial cells only with homologous strains (Tramont, 1977). Recently volunteers given a gonococcal pilus vaccine produced an antibody capable of blocking the attachment of the homologous gonococcal strain to buccal epithelial cells (Tramont et al., 1981). The stimulated antibodies cross-reacted with isolated pili of heterologous gonococcal strains and blocked the attachment of heterologous gonococci at a much lower but significant level. The antiphagocytic role of pili is also impaired by antipilus antibodies raised in rabbits (Jones et al., 1980).

Animal models have also shown anti-LPS antibodies to have a potential protective role. Intra-peritoneal immunisation of mice with LPS had a protective effect when they were challenged with

heterologous strains (Diena et al., 1975). Similarly chick embryos from hens pre-immunised with LPS were protected against subsequent challenge with three different strains (Diena et al., 1978). Antisera raised in rabbits against whole outer membrane and LPS respectively, were also protective for 11-day chick embryos challenged with a known lethal dose of gonococci (Robertson, 1979). When rabbit antisera raised against gonococcal LPS, pili and outer membrane proteins were tested, LPS was found to be the most effective antigen at inducing a bactericidal response to both homologous and heterologous strains (Ward et al., 1978).

b) Limited immunity

Despite the data suggesting the capability of antigonococcal antibodies to inhibit the virulence properties of gonococcal antigens in laboratory conditions, immunity to gonorrhoea is incomplete and repeated infections are common. Lack of immunity may be due to several factors, such as the existence of high variability within antigenic structures giving rise to antibodies which fail to protect from infections with heterologous strains. The antibodies induced by the infection may be directed to cell surface antigens not important for virulence, or in the first part of an acute attack may be of a class, such as IgM, ineffective at the mucosal cell surface.

Brinton et al. (1978) determined that the mean infectious dose needed to establish gonorrhoea in man was 500 organisms for a laboratory grown pilated strain. Indeed only 4.0×10^2 to 1.8×10^7 gonococci were recovered in a study of cervical washings from infected females (Lowe and Kraus, 1976). These numbers of gonococci represent a small antigenic mass, perhaps insufficient to engender a considerable immune response with a single attack.

Extensive studies by Mahoney et al. (1946) revealed that of those volunteers examined who had complement fixing antibodies to gonococci, only 14% developed an infection on challenge, compared with 37% of the group lacking detectable antibodies. Tests of sera from patients exposed but not infected with gonococci, of patients with the disease and of non-infected controls, revealed bactericidal antibodies were present in less than 31% of the men and women with uncomplicated gonococcal infection. Prolonged mucosal infection (i.e. 33 days) correlated with the presence of bactericidal antibodies (Kasper et al., 1977), but these were absent in 95% of specimens of

acute-phase serum from women with pelvic inflammatory disease.

A protease which cleaves proline-threonine bands in the hinge region of the heavy chain of human IgA1 (Kornfeld and Plant, 1981) has been purified from broth cultures of gonococci (Blake and Swanson, 1978). It is possible that IgA proteases represent a microbial adaptation which allows protection from immunological attack, although there is currently no evidence to support this hypothesis. Indeed IgA2 which is enzyme resistant may be the dominant form of IgA in human secretions (Gray et al., 1968).

Seminal plasma has a marked inhibitory effect on phagocyte mediated bacteriocidal and opsonic actions against gonococci. Inhibition appears to occur in the early phase, probably complement activation rather than ingestion, and seems to be mediated by a low molecular weight protease or protease inhibitor (Brooks et al., 1981). The protective activity of complement and antibodies in the female genital tract might be blocked by seminal plasma, thus enhancing the pathogenesis of gonococci transmitted during intercourse.

c) Cell mediated immunity

Cell mediated immunity to gonorrhoea is most likely to be significant in cases of chronic or complicated disease, in the case of acute uncomplicated infections it is weak and short lived, offering no protection against subsequent mucosal infections. Lymphocyte blastogenesis induced by gonococcal antigens was only marginally greater than controls on first infection, but significantly greater with multiple attacks (Kearns et al., 1973). Peripheral leukocyte migration inhibition tests using gonococcal proteins demonstrated significant migration inhibition tests using gonococcal proteins demonstrated significant migration inhibition in only 16 of 30 patients with uncomplicated gonococcal infection, but a correlation was found between the number of previous infection and increased migration inhibition (Landolfo et al., 1981).

Experimental Vaccines

Early efforts at producing a vaccine with whole lysed gonococci were a failure (Greenberg et al., 1974), the test vaccine gave no protection, but sensitised the recipients to subsequent gonococcal

infection. Subsequently some purified surface components have been used in vaccine trials. Pili isolated and purified have been shown to be safe and antigenic in a prototype gonococcal pilus vaccine injected subcutaneously or intramuscularly into human volunteers (Tramont et al., 1981). The most common complaint was that of a sore arm correlating with the volume of material injected. Six of 64 volunteers receiving the larger doses also complained of malaise. It was shown in this study that a minute amount of LPS was present in the vaccine perhaps accounting for the slight adverse reactions. All of the volunteers developed a serum antibody response in the three principal immunoglobulin classes as measured by solid-phase radioimmuno assay. The antibody rise to the homologous pili generally correlated to the amount of antigen given. IgG antibody persisted at approximately one-third of the peak value when tested 30 weeks post vaccination but the IgA and IgM levels had fallen to near pre-immunisation values. A booster vaccine raised the antibody level of the low responders, but did not boost the antibody levels of those with good initial response.

A subsequent study (McChesney et al., 1982) showed that the above parenteral gonococcal pilus vaccine (Tramont et al., 1981) also resulted in production of specific local genital antibody. All three major antibody classes were present in local secretions.

Similar findings have been reported by Siegel et al. (1982) in trials where 39 volunteers received 100 or 200 μg of pili in aluminium phosphate adjuvant subcutaneously. Volunteers had a significant antibody response to the vaccine with peak mean responses one to three weeks after the booster. In vitro, post-immune sera enhanced phagocytosis of the homologous pilated gonococci by human polymorph nuclear leukocytes. Pre-absorbtion of sera with the homologous purified pili blocked this activity. The vaccine was thus seen to be immunogenic and lead to production of functional serum antibodies with respect to the homologous gonococci.

The antibodies raised by the vaccine of Tramont et al. (1981) were capable of blocking the attachment of gonococci to epithelial cells. The stimulated antibodies cross-reacted with isolated pili of heterologous strains but blocked the attachment of heterologous gonococci with much reduced efficiency. Absorbtion of immune sera by a heterologous pilus reduced the inhibition of attachment to the pre-immune level, suggesting that the immune response was directed

at a common pilus determinant. Recent field trials of a pili vaccine carried out in South-East Asia have however proven to be ineffective in providing any protection to gonorrhoea (Heckels personal communication).

Aims of this Study

While the above human trials have demonstrated the potential of a gonococcal vaccine involving purified surface components specifically pili, there obviously exists a problem of antigenic variability. The development of an effective vaccine demands the understanding of the pathogenic mechanisms involved in causing gonorrhoea, the nature of the immune-response and the definition of the major surface components involved in pathogenesis.

The aims of this study were to investigate the antigenic nature of pili and outer membrane proteins; establish their variability in vitro and during the course of the infection; and to investigate the immune response to surface antigens in patients suffering from gonorrhoea.

MATERIALS AND METHODS

Growth and Storage of Bacteria

Neisseria gonorrhoeae strains were grown at 36°C for 16 h in an atmosphere of 5% (v/v) CO_2 with high humidity on 8.5 cm diameter plates of solid clear typing medium (CT medium) of the following composition:-

	g/l
Agar (Oxoid no.1)	10
Proteose peptone no.3 (Difco)	10
Soluble starch (BDH)	1
$K_2HPO_4(3H_2O)$	4 (5.24)
KH_2PO_4	1
NaCl	5

The medium was enriched with a supplement similar to commercial Isovitalex (BBL). The supplement was prepared as two separate solutions, sterilised by filtration through a 0.22 μ filter and stored at -20°C. The solutions contained the following:-

Supplement A	g/l
D-glucose	100
L-glutamine	10
NAD (DPN)	0.25
Co-carboxylase	0.10
Thiamine-HCl	0.003
Vitamin B_{12}	0.01
$Fe(NO_3)_3$	0.02
p-aminobenzoic acid	0.013

Supplement B	g/l
Adenine	5
Guanine-HCl	0.15
L-cysteine-HCl	26
Uracil	0.008
Hypoxanthine	0.0032

Supplement A (5 ml) and Supplement B (1 ml) were added to each 500 ml bottle of molten typing medium (50°C) before pouring.

Colonies were examined using a stereomicroscope (Carl Zeiss, Jena) equipped with a plane polished substage reflector, adjusted to differentiate between opaque and transparent colonies (Swanson, 1978). Homogeneous cultures of the colonial variants were obtained by picking and subculturing several identical colonies of the appropriate morphology from plates of stock cultures previously purified and stored in liquid nitrogen (Lambden and Heckels, 1979).

Stock cultures of fresh clinical isolates were prepared by Mr. K. Zak (Zak et al., 1983) from samples obtained from patients attending the Genito-Urinary Medicine Clinic at Basingstoke District Hospital, who were suspected of having gonorrhoea. Samples were taken with a cotton wool swab from the urethra of males and from the urethra and cervix of their female partners. The samples were plated directly on CT medium and grown at 37°C for 16 h. Isolates were examined for growth of gonococci and stored in liquid nitrogen if pure; if contaminated the colonies were picked, subcultured then frozen. All isolates were subsequently confirmed as gonococci by Gram staining, positive oxidase reaction and production of acid from fermentation of glucose, but not maltose or fructose.

For large scale isolation of outer membranes (OM), bacteria were grown on stainless steel trays (27 x 37 cm) using the same medium. All bacterial strains were preserved in 1% (w/v) proteose peptone no.3 (Difco) containing 10% (v/v) glycerol as a cryoprotective agent, and stored in a liquid nitrogen refrigerator (Ward and Watt, 1971).

Meningococci were obtained as fresh clinical isolates from the Public Health Laboratory, Southampton General Hospital and from Dr. R.J. Fallon, Ruckhill Hospital, Glasgow and grown under the same conditions as gonococci

Protein Determinations (Lowry et al., 1951)

Reagents:-

1) Alkaline copper reagent was prepared by mixing 1% (w/v) copper sulphate (0.5 ml) with 2% (w/v) potassium tartrate (0.5 ml) and adding the mixture to 2% (w/v) sodium carbonate in 0.1 N sodium hydroxide (50 ml).

2) Folin-Ciocalteus phenol reagent (BDH) was diluted to be equivalent to 1N Acid.

Method:-

Alkaline copper reagent (1 ml) was added to samples (0.1 ml) containing 0-80 µg of protein, and incubated at room temperature for 15 min. Folin reagent (100 µl) was then added and mixed immediately, left for 30 min and read against a reagent blank at 750 nm in a spectrophotometer (Unicam SP1800 Ultraviolet Spectrophotometer). The amount of protein present was estimated from a standard curve obtained using Bovine Serum Albumin (BSA) similarly treated.

Biorad Protein Assay (Bradford, 1976)

Reagents:-

1) Dye reagent was prepared by dissolving Coomassie brilliant blue G250 (100 mg) in 95% (v/v) ethanol (50 ml), adding 85% (w/v) phosphoric acid (100 ml) to this solution, and diluting to 1 litre.

Method:-

Dye reagent (0.5 ml) was added to samples (0.1 ml) containing 10 to 100 µg of protein, incubated for 5 min at room temperature and read against a reagent blank at 595 nm in a spectrophotometer. The protein present in the unknown sample was estimated from a standard curve using BSA.

Carbocyanine Dye Assay for LPS (Janda & Work, 1971)

Reagents:-

1) Carbocyanine dye reagent was prepared by dissolving 10 mg of carbocyanine dye, 1-ethyl-2(3-(1-ethylnaptho-(1,2d)-thiazolin-2-ylidene-2 methylpropenyl)naptho (1,2d) thiazolium bromide (Eastman, Kodak) in 1,4 dioxan (10 ml) and 0.03M sodium acetate buffer, pH 4.05 (10 ml), once dissolved the solution was further diluted with 0.03M sodium acetate buffer (80 ml).

2) 0.1M ascorbic acid (170 mg/10 ml)

Method:-

To the carbocyanine dye reagent (3 ml) was added 0.03M sodium acetate buffer (2 ml), water (4 ml) and just before use 0.1M ascorbic acid (60 µl). A volume of the resultant mixture (1 ml) was added to samples containing 1 to 10 µg of LPS in water (0.1 ml), and allowed to stand in the dark at room temperature for 5-10 min before reading against a reagent blank at 472 nm in a spectrophotometer. The concentration of LPS was determined from a standard curve using purified gonococcal LPS similarly treated.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Heckels, 1981)

Reagents:-

1) Acrylamide solution containing 50% (w/v) acrylamide and 1.3% bis-acrylamide.
2) Two percent (w/v) SDS solution.
3) Separating gel buffer, 1.2M Tris-HCl pH 8.8.
4) Stacking gel buffer, 0.25M Tris-HCl pH 6.8 containing 80 µl N,N,N',N'-tetramethylenediamine (TEMED) per 100 ml of buffer.
5) Running buffer, 0.05M Tris containing 0.192M glycine and 0.1% SDS (pH 8.3).
6) Ammonium persulphate solution, 10 mg/ml.
7) Dissociating buffer, 0.125M Tris-HCl pH 6.8 containing 4% SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.002% (w/v) bromophenol blue.

Method:-

SDS-PAGE was carried out using the discontinous buffer system of Laemmli (1970) in a slab gel cast in a mould made by two glass plates (20x14 cm) separated by plastic spacers 1.5 mm thick. The gel incorporated a linear gradient of 10 to 25% (w/v) acrylamide generated using a triple channel peristaltic pump to mix two solutions (16 ml of each) of the following composition:-

Reagents	25% (w/v) solution	10% (w/v) solution
50% (w/v) acrylamide	10 ml	4 ml
separating buffer	5 ml	5 ml
glycerol	2 ml	-
2% (w/v) SDS	1 ml	1 ml
ammonium persulphate	0.2 ml	0.4 ml
water	1.8 ml	9.6 ml
TEMED	0.001 ml	0.001 ml

The gel solutions were deaerated under reduced pressure before the addition of TEMED. The gel mould was kept in a circulating water bath below 15°C to dissipate the heat produced by the exothermic polymerisation reaction, thus ensuring even setting. The gel was allowed to set covered with a thin layer of isobutanol. Once set the layer of butanol was removed and wells for the loading of samples were cast in a 6% (w/v) acrylamide stacking gel of the following composition by use of a 24 toothed spacer:-

50% (w/v) acrylamide	0.8 ml
2% (w/v) SDS	0.5 ml
ammonium persulphate	1.0 ml
water	2.8 ml
stacking gel buffer	5.0 ml

Samples to be run were mixed with an equal volume of dissociating buffer, heated at 100°C for 5 min and 50 µl (containing approximately 5 to 25 µg of protein) was loaded into each well. The buffer reservoirs of the gel running apparatus were filled with running buffer and subjected to electrophoresis at 200V at 4°C for 20 h. Standard proteins of known molecular weight were run in parallel.

In some cases thin gels were cast and run, these were made in the same manner described above but using spacers 0.75 mm thick and half the volume of all gel solutions.

Protein Staining (Fairbanks et al., 1971)

The gels were fixed and stained simultaneously, 0.1% (w/v) Kenacid Blue R 2500 was dissolved in 10% (v/v) acetic acid and 20% (v/v) iso-propanol (500 ml) and filtered before use. The gels were stained overnight at 37°C. Excess stain was removed by placing the

gel in 10% (v/v) acetic acid, 10% (v/v) isopropanol for 4 h followed by several changes of 10% (v/v) acetic acid until the background was colourless.

Silver Staining of Proteins (Merril et al, 1981)

Reagents:-

1) 0.0034m potassium dichromate in 0.0032N nitric acid
2) 0.012M silver nitrate
3) 0.28M disodium carbonate (Na_2CO_3) containing 0.5 ml formalin per litre

Method:-

All operations were carried out wearing rinsed gloves, using acid washed glass trays and double distilled water for all solutions. The gel was fixed in 50% (v/v) methanol, 12% (v/v) acetic acid for at least 30 min. The gel was then washed three times for 10 min in 10% (v/v) ethanol, 5% acetic acid (200 ml) before soaking in the potassium dichromate reagent for 5 min. After four 30 s washes in water the gel was placed in silver nitrate solution for 30 min, washed twice for 10 s in sodium carbonate reagent (300 mls) and developed by placing in the same solution (300 ml) for approximately 5 min. Once the gel was stained to intensity required the reaction was stopped in 1% (v/v) acetic acid.

Drying of Gels

Gels destined for autoradiography were shrunk in 50% (v/v) methanol and 10% (v/v) glycerol in water for four to five hours. The gel was placed on filter paper covered with cling film, placed on a drying apparatus (Raven, Suffolk) in a plastic bag and dried by attaching to a vacuum line and placing the board on a photographic plate heater (50°C).

Autoradiography

Radioactively labelled proteins present on gels were visualised by exposing the dried gel to Kodak X-Omat AR film at -70°C for 16-48 h in a metal cassette with an X-Omatic regular intensifying screen.

Preparation of Gonococcal Antigens

(a) Outer membrane complex (Heckels, 1981)

Bacteria from thirty-two stainless steel trays were scraped into cold phosphate buffered saline (PBS) pH 7.4 and centrifuged at 15,000 g for 20 min. The bacterial pellet (30 g) was resuspended by gentle homogenisation in 0.2M lithium acetate (240 ml, pH 6.0) and shaken for 2.5 h at 45°C in a water bath. After incubation, the mixture was homogenised for 1 min in a Vortex Mixer (P. Silver, Hampton, England) with a blade clearance of 0.0025 mm. Bacteria were removed by centrifuging at 15,000 g for 20 min. Traces of cell debris were removed by three further centrifugations of the supernatant solution at 15,000 g for 20 min. The clear supernatant solution was then centrifuged at 100,000 g for 2.5 h to obtain a translucent crude outer membrane pellet (Heckels, 1977) and washed twice in 0.2M sodium acetate (pH 6.0).

The pellet was further purified by suspending in 6M urea in 0.2M sodium acetate buffer (10 mls) at a concentration of 3 mg/ml. After 30 min incubation at 25°C, the suspension was diluted with an equal volume of sodium acetate buffer and centrifuged at 100,000 g for 3 h. The supernatant solution containing loosely associated non-outer membrane proteins, was discarded. The pure outer membrane complex was washed three times with water and the final pellet was suspended in water (5 ml) and stored at -20°C. The purity of the antigen was assessed by SDS-PAGE.

(b) Pili (Brinton et al., 1978)

Pilated variants were grown on trays as above, and the growth was harvested by scraping off into a dry beaker. Ice cold ethanolamine/HCl 0.15M pH 10.2 (800 ml) was added and the cells were subjected to shearing for 2 min in a Vortex Mixer with a blade clearance of 0.0025 mm. The cells were centrifuged at 20,000 g for 30 min and the supernatant solution decanted. The pili were precipitated by addition of saturated ammonium sulphate (SAS) (88 ml) to give a final saturation of 10%; the addition of SAS was made dropwise using a peristaltic pump at a rate of 6 mls/min. The solution was stirred at room temperature for an hour and the bifringent

pili crystals were harvested by centrifuging at 15,000 g for 30 min. The crude pili were re-solubilised in 70 ml ethanolamine buffer by stirring for 10 min, and separated from insoluble particular impurities, such as cell wall debris, by centrifuging at 25,000 g for 30 min. After two further cycles of disaggregation in ethanolamine and precipitation in SAS, the pili were washed in 1M NaCl (40 ml), centrifuged at 25,000 g for 30 min and resuspended in a small volume of 1M NaCl containing 0.05M azide and stored at 4°C. The purity of the pili was tested by running on an SDS-polyacrylamide gel.

c) Lipopolysaccharide (Lambden and Heckels, 1982)

Cells (100 g wet weight) were suspended in hot water (200 ml, 70°C) and an equal volume of pre-warmed 90% (w/w) phenol was added. The suspension was homogenised for 15 min with a Vortex Mixer at 70°C in a water bath, and centrifuged at 1,000 g for 30 min at 4°C. The upper aqueous phase was removed and the remaining material was re-extracted with an equal volume of water at 70°C for 15 min. After centrifugation the aqueous phases were bulked and dialysed against running tap water for two days. EDTA (sodium salt) was then added to give 5mM final concentration and the material was further dialysed for 20 h against distilled water. A few crystals of $MgCl_2$ were added to the non-diffusable fraction followed by two volumes of cold acetone, and the mixture was allowed to stand at 4°C overnight. The precipitated LPS was spun down, washed with 60% (v/v) acetone and placed under vacuum for 30 min to remove excess acetone.

The crude LPS was homogenised in 0.025M Tris acetate pH 7.2 (100 ml) using a sonicator. RNA ase (Sigma) (10 mg, 700 units), DNA ase (Sigma) (2 mg, 100 units) and 0.1M MgCl (1 ml) solution were added, and this solution was incubated at 37°C for 4 hours then transferred to a dialysis bag and dialysed at 37°C for 16 h against the same buffer.

The insoluble debris was removed by centrifuging at 1,000 g for 15 min, and the LPS recovered from the supernatant by centrifuging at 100,000 g for 2 h, then washed once in water. The LPS pellet was dissolved in water and transferred to a Sartorius SM16530 electro-dialyser fitted with 0.22 μ membranes and electrodialysed against running deionised water at a constant 100V for 4 h. The LPS which precipitated as the insoluble free acid was washed with water and

dissolved by addition of 1% (v/v) triethylamine solution, to give a clear non-viscous solution (pH 7.0) (Galanos and Luderitz, 1975). This solution had no protein or nucleic acid contaminant detectable when scanned at 280 nm and 260 nm respectively. Pure LPS was recovered by freeze drying.

Production of Hyper-immune Antisera

Antisera to OM and pili were raised in Half-lop rabbits (Froxfield Rabbit Company, Froxfield, Hampshire). Suspensions of antigens (100 µg in 1 ml water) were emulsified in equal volumes of Freund's complete adjuvant (Difco) and injected subcutaneously into eight sites on the back and hind legs. Booster doses of the antigens in Freund's incomplete adjuvant were given in a similar manner on days fourteen, thirty-five and seventy; on day eighty, blood (25 ml) was taken from a marginal ear vein. After clotting, sera were separated and stored at -20°C.

Preparation of Protein II Specific Antisera

Antisera directed against P9-13 outer membranes (OM-13) containing antibodies to protein I, protein II (28.5K) and LPS (table 1) were absorbed with whole cells of P9-1 (containing protein I and LPS). Heat treated serum (56°C, 30 min to inactivate complement) was diluted 1:200 in PBS, mixed with an equal volume of a suspension of P9-1 of A_{550} 10.0 ($2x10^9$ cfu/ml) and incubated at 37°C for 3h. The bacteria were removed by centrifugation at 10,000 g for 5 min and a second absorption was carried out in a similar manner. The serum was stored at -20°C and diluted to the final working concentration before use. The specificity of the anti-sera for protein II (28.5K) was tested by enzyme linked immunosorbent assay (ELISA) which demonstrated reactivity with OM13 but not OM-1. Antisera specific for protein II (28K) were prepared in a similar manner from anti OM-16 sera.

Enzyme Linked Immunosorbant Assay (ELISA)

Reagents:-

1) Coupling buffer: 0.1M $NaHCO_3$ pH9.5 including 0.05% azide
2) Phosphate buffered saline pH 7.4 containing 0.05% (v/v) Tween 20 (PBST)

TABLE 1.

Variants of N. gonorrhoeae P9 used and their OM protein and pili composition. (Molecular weight x 10^{-3} = K). To conform to recent recommendation for standardised nomenclature of gonococcal outer membrane proteins (Proceedings of 1980 EMBO Symposium on Genetics & Immunobiology of Pathogenic Neisseria, University of Umea) protein II species in study have been denoted by their subunit molecular weight.

Variant	Previous Protein II nomenclature	Protein II Molecular weight	Pili nomenclature	Pili Molecular weight
P9-1	-	-	-	-
P9-2		-	α	19.5K
P9-9	IIa + IIe	28.5K + 27.8K	-	-
P9-11	IIa + IId	28.5K + 28.85K	-	-
P9-13	IIa	28.5K	-	-
P9-16	IIb	28K	-	-
P9-32	IIf	28.3K	-	-
P9-39	IIe	27.8K	-	-
P9-40	IId	28.85K	-	-

3) Goat anti-rabbit IgG peroxidase conjugate (Miles, Stoke Poges, U.K.) diluted 1:2,000 in PBST

4) Substrate: made by dissolving 10 mg of O-phenylenediamine dihydrochloride in 1 ml warm methanol, adding 99 ml water plus 10 µl 30% H_2O_2

Method:-

Antibodies to surface antigen components were detected by enzyme linked immunosorbent assay (ELISA) based on a method previously described by Buchanan (1978). The purified antigen was suspended in coupling buffer pH 9.6 at the optimal concentration (1 µg/ml) established by preliminary experiments. Individual wells of flat-bottomed polystyrene microtitre trays (Dynatech, Billingshurst, Kent) were coated with the antigen suspension (200 µl) at 37°C for 16 h. The antigen was then discarded and wells were washed three times with PBST. Multiple dilutions of the test sera in PBST were added in 200 µl portions to the antigen coated wells and to uncoated control wells, and incubated at 37°C for 1 h. The wells were then emptied and washed three times with PBST, anti-globulin conjugate (200 µl) was added and incubated at 37°C for 1 h. After washing three times with PBST enzyme substrate (200 µl) was added to each well and incubated at room temperature for 30 min. The reaction was terminated by the addition of 1M H_2SO_4 (50 µl), and the A_{490} was determined in a Dynatech MR580 Micro Elisa Auto Reader.

ELISA procedure for detection of anti-LPS antibodies was modified slightly to minimise loss of antigen during the washing procedures (Ito et al., 1980). Trays were coated at an LPS concentration of 5 ug/ml coating buffer containing 0.02M $MgCl_2$. The wells were incubated with 0.5% (w/v) bovine serum albumin (BSA), 0.02M $MgCl_2$ in PBS for 1 h at 37°C before adding the sera and continuing the assay as previously described. All washings were carried out using PBST containing 0.02M $MgCl_2$.

Radioiodination of Whole Cells

Reagents:-

1) Iodogen reagent; prepared by dissolving 1 mg 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril(Iodogen, Pierce Chemical Company, Rockford, Illinois, U.S.A.) in 1 ml of chloroform
2) Carrier free $Na^{125}I$ (1 mCi/10 ml)
3) RIP buffer consisting of 0.3% (w/v) Empigen BB (Albright & Wilson Limited, Whitehaven), 0.1 (w/v) SDS, 0.05% (w/v) sodium azide, 0.1mM p-toluene sulphonyl fluoride in PBS

Method:-

Cells were labelled following a modification of the procedure used by Swanson (1981). Glass test tubes, previously acid washed and rinsed in deionised water followed by chloroform, were coated with 10 μg of Iodogen (10 μl) in chloroform. After the chloroform had evaporated off the tubes were capped and stored under vacuum in a desiccator at room temperature.

Bacteria were scraped from agar plates into Dulbecco's complete phosphate buffer (PBSB, 4°C) centrifuged at 10,000 g for 2 min and resuspended gently in PBSB (1 ml). A sample of the bacterial suspension was diluted 1 in 100 and its A_{550} recorded, this was then used to calculate the A_{550} of the bacterial suspension which was then adjusted to give an $A_{550}$of 1.0 (corresponding to 1.7×10^8 cfu/ml). A sample (0.6 ml) of the adjusted suspension was centrifuged, the pellet was resuspended in 45 μl PBSB containing 2×10^{-6}M KI and transferred to the Iodogen coated tubes. Na-^{125}I (200 μCi) was added and the tubes were incubated at 15°C for 10 min with occasional shaking. The contents of the tube were diluted with cold PBSB (750 μl) centrifuged at 10,000 g for 2 min, and washed twice with PBSB. The washed pellet was resuspended in PBSB (1 ml, 4°C) to be used immediately in whole cell RIP assay, or solubilised by incubating in RIP buffer at 40°C for 1 h before removing non soluble cell debris by centrifugation at 100,000 g for 2 h depending on RIP method to be used.

Immunoblotting (Towbin et al., 1979)

Reagents:-

1) Blotting buffer: 20mM Tris, 150mM glycine and 20% methanol
2) Blocking buffer: 3% BSA in 10mM Tris-HCl (pH 7.4) containing 0.9% NaCl
3) Gelatin buffer: 150mM NaCl, 5mM EDTA, 50mM Tris-HCl (pH 7.4), 0.25% gelatin and 0.05% NP40
4) ^{125}I-Protein A: ^{125}I-labelled protein A from S. aureus

Method:-

Whole cell bacterial lysates were subjected to SDS-PAGE on thin (0.75mm) 10-25% (w/v) acrylamide gels described above. The gels were applied to nitrocellulose sheets (BA85, Schleicher and Schull, Dassel, West Germany) and proteins were transferred electrophoretically at 50V for 16 h in blotting buffer. The nitrocellulose was incubated with blocking buffer at 37°C for 1 h in order to prevent non-specific protein binding, then excess BSA was rinsed off the sheet with gelatin buffer. The nitrocellulose was incubated with the appropriate dilution of sera or monoclonal antibodies in gelatin buffer at room temperature for 1 h, the unbound antibodies were rinsed off and the nitrocellulose sheet was washed three times for 5 min in gelatin buffer. ^{125}I-Protein A diluted in the same buffer to give a final activity of 10^6 cpm/ml was added to the nitrocellulose. After 1 h incubation at room temperature the sheet was washed extensively for 1 h with frequent changes of buffer, rinsed with water and air dried. Immunological reactions were detected by autoradiography.

EXPERIMENTAL AND RESULTS

I. Antigenic Variation of Outer Membrane Proteins in Colonial Variants of Strain P9

Detection of Antibodies Directed Against Gonococcal Outer Membranes by ELISA

Antisera to outer membranes from opacity variants of strain P9 (Table 1) were raised in rabbits. Initial experiments showed that antibodies present could be readily detected in an ELISA system (page 36) using wells coated with the homologous outer membrane preparation. Trials using different concentrations of outer membranes (50 ng, 500 ng, 1 ug and 5 μg/ml) demonstrated 1 μg/ml to be the optimal concentration at which to coat the wells. A logarithmic plot of A_{490} against serum dilution (Fig. 3) showed an initial absorbance plateau where antibody was in excess, with a linear relationship on further dilution where the antibody was the limiting factor.

ELISA for detection of anti-LPS antibodies were found to give optimum absorbances at an increased well coating concentration of 5 μg/ml. Furthermore, initial problems of non-specific antibody binding were solved by the pre-incubation of the wells with BSA before the addition of the serum. The presence of $MgCl_2$ in the washing buffer prevented the LPS detaching from the wells on washing (Ito et al., 1980).

Quantitation of Antibodies to Specific Outer Membrane Components by ELISA Inhibition

The relative proportions of antibodies directed against individual outer membrane components (LPS, protein I, protein II), were determined by adsorption experiments in which the antiserum was pre-incubated with increasing concentrations (0, 1, 2.5, 5, 10 and 20 μg/ml) of either LPS, OM1 (containing LPS and protein I) or the homologous OM (LPS, pI and pII).

To avoid an antibody excess, preliminary titration was carried out to determine the concentration of serum which gave an A_{490} of approximately 75% of the maximum value when tested by ELISA. Using antiserum raised against OM-13, 25% of the total antibodies present could be absorbed by pre-incubation with LPS alone, 65% with OM-1 (LPS + pI)

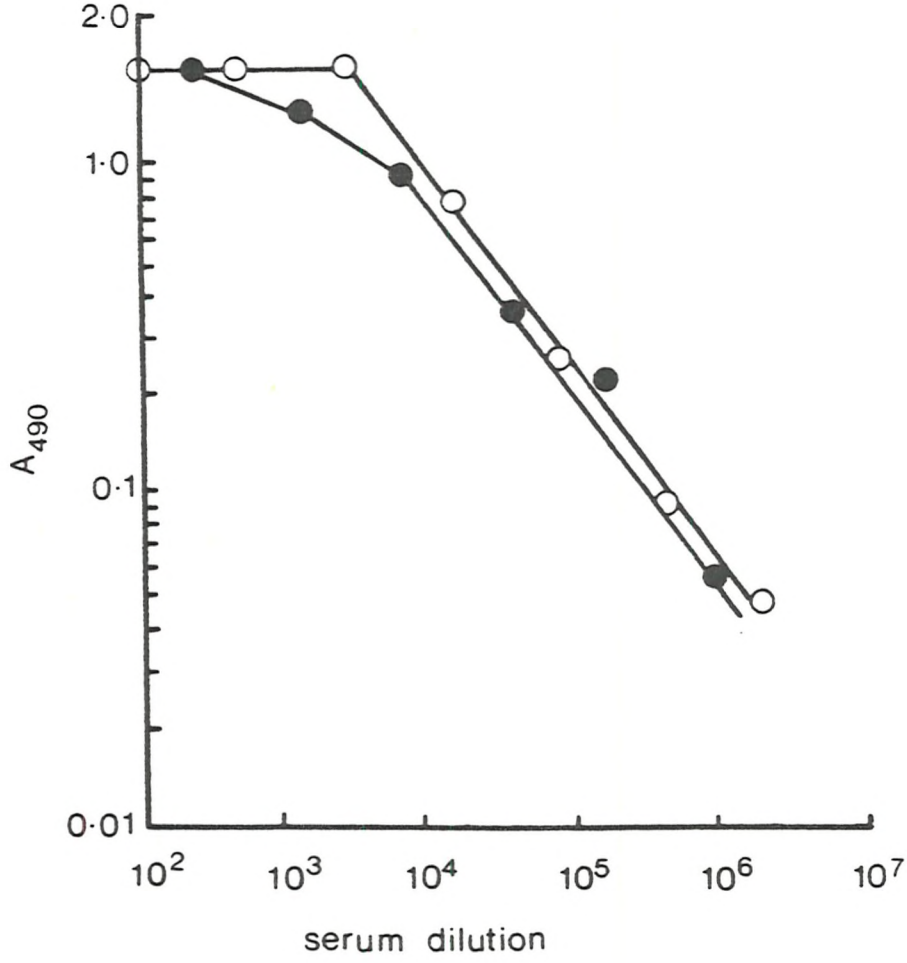


Figure 3.

Detection of anti-OM antibodies by ELISA. Increasing dilutions of anti-OM 13 (o) and anti-OM 16 (●) sera were tested in the standard ELISA system using wells coated with the homologous OM preparations.

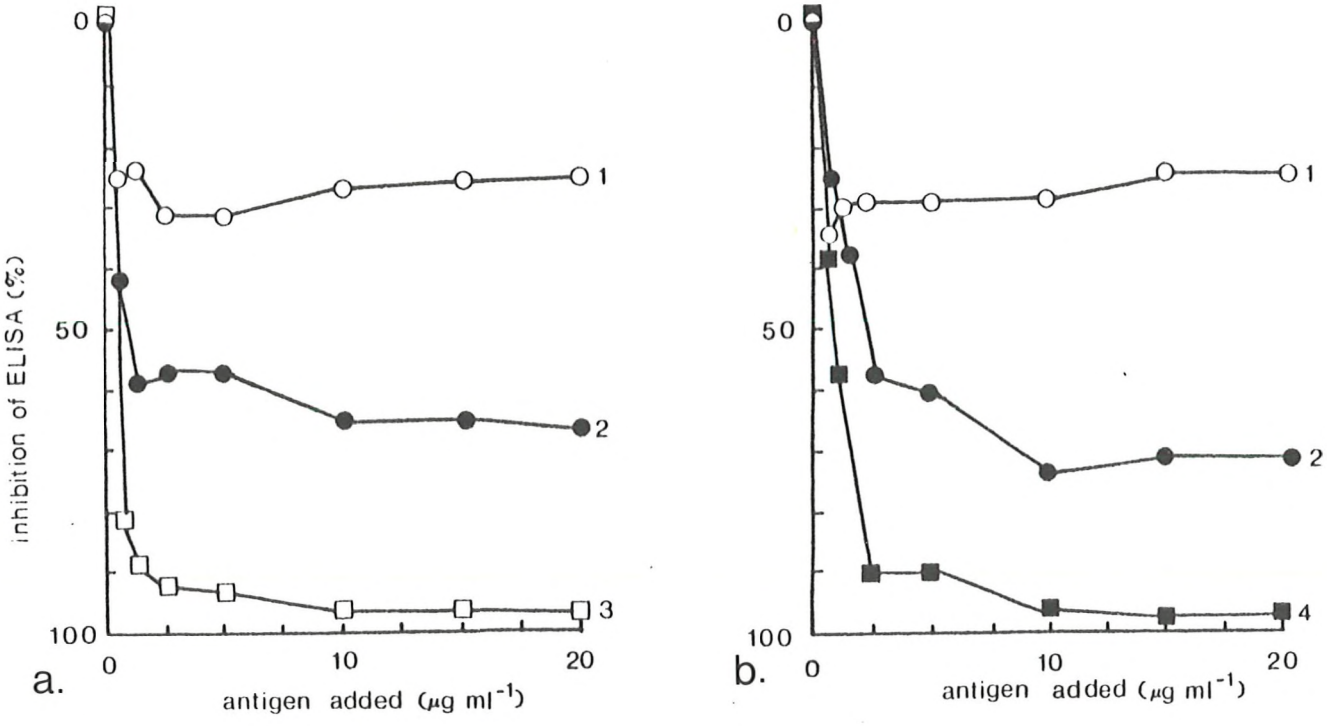


Figure 4. Inhibition of ELISA by defined antigens. Anti-OM 13 (a) and anti-OM 16 (b) sera were incubated with soluble antigens before reaction with homologous OM. Antigens used were (1) LPS, (2) OM-1, (3) OM-13 and (4) OM-16.

and 97% with OM-13 (LPS + pI + pII) (Fig. 4a). Thus the proportions of antibodies directed against each individual antigen were found to be: LPS 25% , protein I 40% and protein II (28.5K) 32%. Similarly in serum raised against OM-16 the proportions of antibodies were found to be: LPS 23%, protein I 47% and protein II (28K) 27% (Fig. 4b).

Cross Reactivity of Outer Membrane Proteins of Variants of Strain P9

a) In intact gonococci

The degree of surface antigen cross-reactivity of different P9 variants was measured by the ability of intact gonococci to inhibit the ELISA reaction of an antiserum with its homologous OM. Each variant to be tested was harvested, washed in PBSB (pH 7.4, 4°C) and resuspended in the same buffer to give an A_{550} of 1.0. These suspensions were added in increasing concentration across rows of a 96-well microtitre tray (Dynatech, U-shaped wells) to final concentrations of $0-10^7$ cfu in 100 µl PBSB. Heat inactivated antiserum (100 µl) was then added to each well and the microtitre tray was incubated at 37°C for 30 min on an orbital shaker. The tray was centrifuged at 1,000 g for 15 min in a centrifuge equipped with Dynatech Micro-ELISA plate carriers, in order to pellet the bacteria. A sample (100 µl) of the supernatant solution was transferred from each well to an analogous well in a flat-welled tray pre-coated with outer membrane antigen (1 µg/ml), already containing 100 µl of PBST. The serum thus absorbed was assayed by ELISA (Fig. 5).

Cells of P9-13 absorbed all activity from anti-OM 13 serum showing that the antigenic determinants reactive in the OM-ELISA system are those which are expressed on the surface of the gonococcus and not those exposed on the surface of inside out outer membrane vesicles. Variants P9-9 (containing pII 28.5K + 27.8K) and P9-11 (pII 28.5K + 28.85K) also completely inhibited the assay. In contrast pre-incubation of anti OM-13 serum with P9-1, lacking any protein II, reduced the activity of the assay by only 65%, a figure comparable to that obtained using isolated outer membrane, reflecting the relative proportion of the antibodies directed against protein II within the assay. Pre-incubation with P9-16 (pII 28K), P9-39 (pII 27.8K) and P9-40 (pII 28.85K) showed only marginally greater inhibition than P9-1. Thus only variants with homologous protein II of 28.5K were able to completely inhibit the

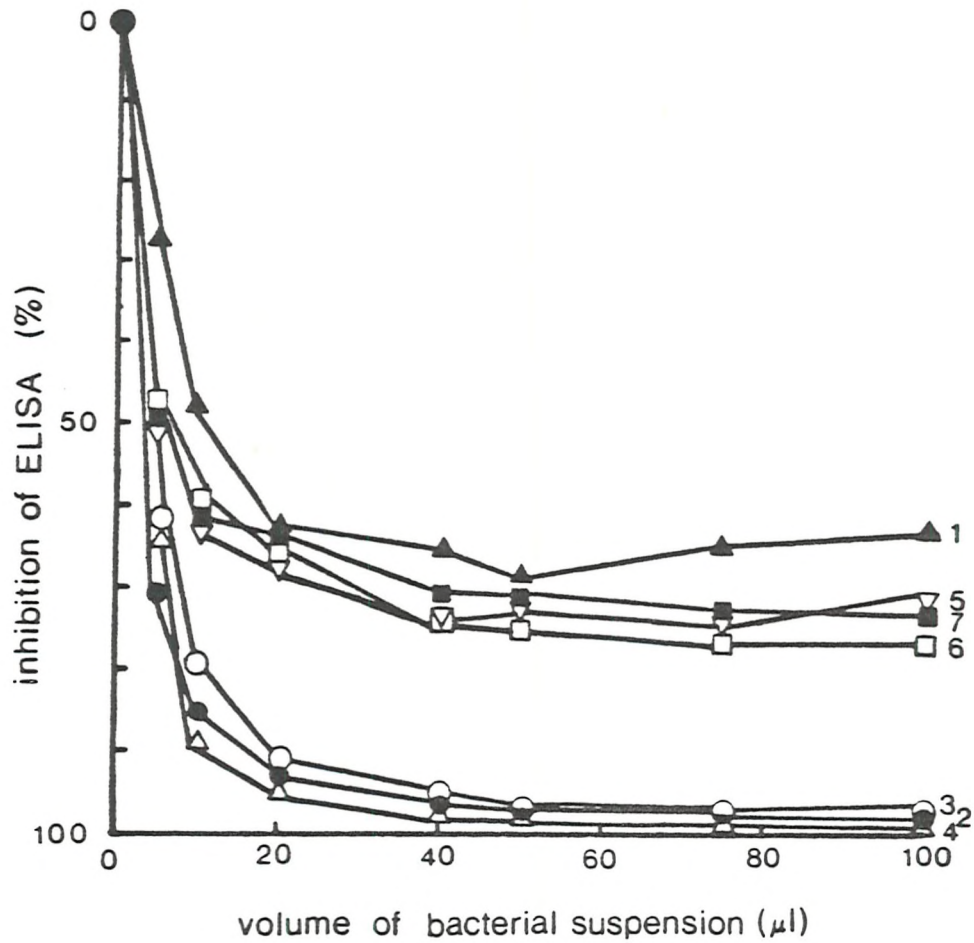


Figure 5. Inhibition of ELISA by intact gonococci. Anti-OM-13 sera incubated with increasing concentrations of (1) P9-1, (2) P9-9, (3) P9-11, (4) P9-13, (5) P9-16, (6) P9-39 and (7) P9-40, before reacting with wells coated with OM-13 in an ELISA system.

ELISA, suggesting that there is little, if any, cross-reactivity between this and the other protein II species.

b) Specific anti-protein II sera

In order to examine the contribution made by the protein II species alone to the antigenic cross-reactivity of the gonococcal surface, anti-outer membrane serum was first made specific for protein II by absorption with whole cells of P9-1, thus avoiding the possibility of undetected variations in LPS or protein I structure masking partial cross-reactivity of the protein II species.

Sera adsorbed with intact cells of P9-1 (page 34) showed no reactivity on ELISA with OM-1 but retained activity with the homologous OM. Anti-P9-13 and anti-P9-16 sera treated and tested in this manner were used in inhibition experiments to determine the cross reactivity of protein II species (Fig. 6). The activity of anti-protein II (28.5K) serum was completely inhibited by pre-incubation with P9-9, P9-11 and P9-13 all of which contained the homologous protein II (28.5K). In contrast, P9-16, P9-39 and P9-40, all containing heterologous protein II species, only inhibited ELISA activity by 12%, 3% and 9% respectively. This small amount of cross-reactivity is of doubtful significance since a control pre-incubation of the serum with P9-1 also inhibited the ELISA activity by 3%.

Specific anti-protein II (28K) serum was similarly completely inhibited only by the homologous P9-16, but not by any of the other variants containing different protein II species (Fig. 6b), though P9-9 and P9-13 did show a small amount of inhibiting activity (9% and 13% respectively). The results confirmed the low degree of antigenic cross-reactivity between protein II species within the strain P9.

c) Quantitation of protein II cross-reactivity

The degree of cross-reactivity of the heterologous protein II species was quantified by using specific anti-protein-II antisera. Anti-protein II (28.5K) and anti-protein II (28K) sera were reacted with individual OM on microtitre trays, including OM-1 (lacking pII) and the homologous OM as controls. The results shown in Table 2 are the percentage cross-reactivity defined as:

$$\frac{[A_{490}\ (\text{heterologous OM}) - A_{490}\ (\text{OM-1})]}{[A_{490}\ (\text{homologous OM}) - A_{490}\ (\text{OM-1})]} \times 100$$

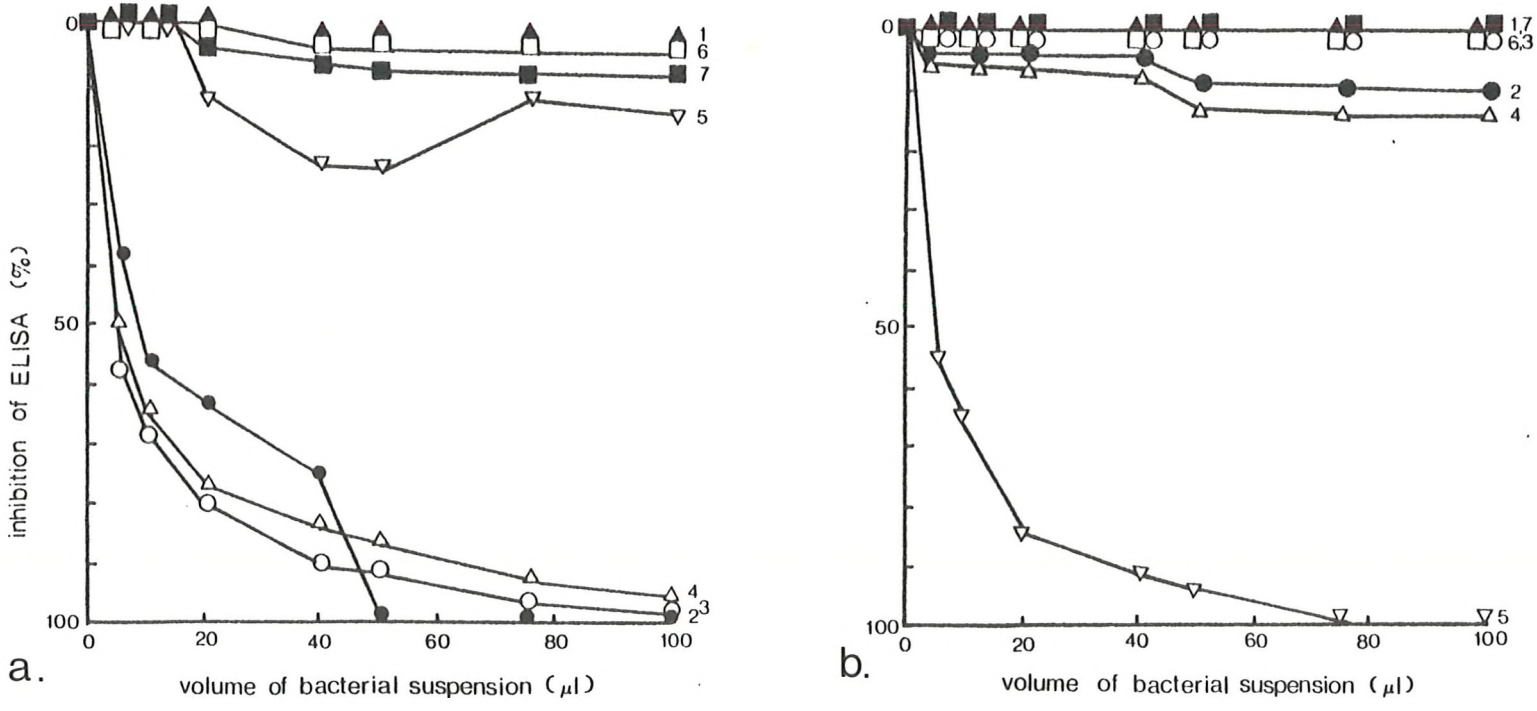


Figure 6. Cross-reaction with specific anti-protein II serum. (a) Anti-protein II (28.5K) and (b) Anti-protein II (28K) sera incubated with an increasing concentration of colonial variants before ELISA with homologous OM. Variants used were 1) P9-1, (2) P9-9, (3) P9-11, (4) P9-13, (5) P9-16, (6) P9-39 and (7) P9-40.

The results were calculated from three to five separate experiments. In all cases cross-reactivity of specific anti-protein II serum with membranes containing only heterologous protein II species was 5% or less.

TABLE 2.

Cross-reactivity of specific anti-protein II sera with OM from variants of strain P9. (Figures in parentheses show standard deviations).

OM from P9 variant	Protein	Anti-II (28.5K)	Anti-II (28K)
1	-	0	0
13	28.5K	100	2.6 (±0.8)
16	28K	5.3 (±2.9)	100
32	28.3K	0.03 (±0.02)	3.5 (±1.8)
39	27.8K	2.5 (±1.0)	1.5 (±1.2)
40	28.85K	3.1 (±0.2)	3.3 (±0.9)

II. Detection of Antibodies to Surface Proteins in Sera of Patients with Gonorrhoea

The experiments above on the cross-reactivity of strain P9 showed antigenic differences exist between different protein II species expressed on the surface of variants of the same strain. Similar variations occurring during the course of the natural infection could play an important role in the pathogenesis of gonorrhoea. The immune response of patients with gonorrhoea to outer membrane proteins and their cross-reactivity was therefore investigated. Since pili also show similar variations in variants of strain P9 (Lambden, 1979) grown in laboratory culture, they were also included in this investigation. The ELISA method used above was not suitable for the detection of specific antibodies in small amounts using large numbers of clinical strains, therefore a radio-immuno-precipitation (RIP) assay was developed. Initial experiments were carried out using strain P9.

Iodogen Labelling of Gonococci

Gonococci labelled with ^{125}I using Iodogen (page 36) run on SDS-PAGE and autoradiographed, revealed only a small number of radio-active proteins per sample. The most intensely labelled proteins with molecular weights of 36K, 27-32K and 17-19.5K, corresponded to protein I, protein II and pili. Lesser amounts of radioactivity were present in minor bands at 31K (protein III), 43K and 51K. The labelling was apparently specific to the surface components and produced a clear pattern without high background.

Radio-immunoprecipitation (RIP)

The assay was initially carried out using the method of Swanson (1981). This system involves the reaction of intact ^{125}I-surface labelled cells with antiserum followed by solubilisation of the cells in detergents. Experiments to find a suitable detergent were carried out. Portions of the whole ^{125}I-labelled cell suspensions (100 µl, approximately 0.5 µCi) were mixed with the sera to be tested (40 µl, heat inactivated) and incubated in an ice bath for 20 min with occasional mixing, the cells were then washed twice with cold PBS (1 ml) to remove excess antibodies. The resultant pellet was suspended

TABLE 3 Detergents used for solubilisation of gonococci in radio-immune precipitation experiments.

1.	1% (w/v) Octyl glucoside in PBS
2.	1% (w/v) Zwittergent in PBS
3.	1% (v/v) Empigen in PBS
4.	1% (v/v) Empigen + 0.1% (w/v) SDS in PBS containing 0.05% (w/v) sodium azide and 0.1 mM p-toluene sulphonyl fluoride (RIP buffer)
5.	1% (v/v) NP-40 + 1% (w/v) Sodium deoxycholate + 0.1% (w/v) SDS + 1mM EDTA in 0.1M Tris pH 7.4

in 205 µl of detergent (Table 3) and solubilised at 40°C for 1 h. The insoluble cell debris was pelleted by centrifugation at 100,000 g for 15 min (Beckman, Airfuge, High Wycombe, Bucks) and the soluble fraction was transferred to a sealed 1.5 ml microfuge tube containing a suspension of Protein A-Sepharose CL4B (Sigma, 50 µl of a 100 mg/ml suspension in PBS). The tube was then incubated at 4°C for 1 h with end over end rotation. The sepharose beads were centrifuged and washed five times with the detergent tested (1 ml), transferred to a clean tube and boiled in dissociating buffer to solubilise precipitated antigen-antibody complexes. The beads were removed by centrifugation, and a sample of each of the supernatant solutions were subjected to SDS-PAGE. Radioactive proteins were detected by autoradiography.

Rabbit anti-OM-13 serum in this system was capable of precipitating protein I and protein II (28.5K) from P9-13 (Fig. 7a). Whilst all detergents seemed suitable for use in RIP assays where only outer membrane proteins were of interest, 1% (v/v) Empigen BB + 0.1% (w/v) SDS in PBS containing 0.05% (w/v) sodium azide and 0.1mM p-toluene sulphonyl fluoride (RIP buffer) was the only combination capable of solubilising pili sufficiently to be seen on autoradiography. Though the serum used had been raised by injecting the rabbits with purified pili, the antiserum also contained antibodies active against protein I, control experiments using pre-immunisation serum from the same rabbit failed to precipitate any surface labelled antigens suggesting that the anti-protein I antibodies present are due to contaminating protein I in the purified pili used for immunisation of the rabbits.

Subsequent experiments performed using a range of serum dilutions (1/1 to $1/10^4$) established that 1/100 dilution of hyperimmune antiserum and 0.5 µCi of homologous labelled cells were adequate to precipitate sufficient amounts of protein to be visible on autoradiography.

Pre-solubilised Antigen in RIP System

The RIP system using whole cells had a major disadvantage in that it necessitated the iodination of cells each time the experiment was performed, therefore a system using pre-solubilised antigen was devised. Gonococci were radiolabelled as before but the final washed pellet was resuspended in RIP buffer (1 ml) incubated at 40°C for 1 h and centrifuged at 100,000 g for 2 h to remove insoluble material. The supernatant solution was stored at 4°C and used as required. The

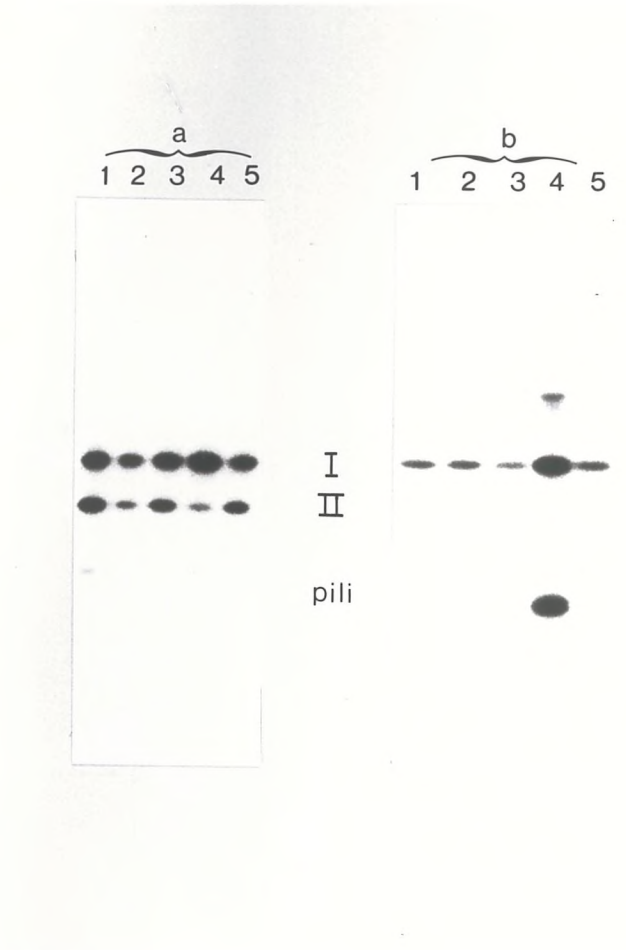


Figure 7. Autoradiography of SDS PAGE following radioimmune precipitation of a) P9-13 with rabbit anti-OM 13 serum; b) P9-2 with rabbit anti-α pilus serum; carried out using different detergents: track 1, octyl glucoside; track 2, Zwittergent; track 3, Empigen; track 4, Empigen + SDS; track 5, NP-40 + deoxycholate + EDTA (Table 3).

specific activity of the solubilised protein was approximately 0.1-0.2 μCi/mg protein.

Samples of the solubilised antigens (0.5 μCi in 100 μl of RIP buffer) were mixed with sera (40 μl of 1/100 dilution) and a suspension of protein-A-Sepharose (50 μl) in a sealed microfuge tube and incubated at 4°C for 1.5 h with end over end rotation. The beads were pelleted by centrifugation then washed, and the precipitated antigens dissociated from the beads and subjected to SDS-PAGE. Figure 8 shows variant P9-13 and variant P9-2 immunoprecipitated by their homologous sera in both pre-solubilised antigen and whole cell RIP methods. Each procedure gave identical results; the proteins I and II of P9-13 and the protein I and α pili of P9-2 were precipitated in both methods. Control tests using pre-immune rabbit serum showed no non-specific precipitation in either case. It was therefore decided to carry out all future RIP experiments using the pre-solubilised system.

Cross-reactivity of P9-Variants

RIP assays were carried out using anti-OM-13 and anti-OM-16 sera incubated with P9-13, P9-16, P9-39 and P9-40. The antisera exhibited a high degree of specificity (Fig. 9). Each antiserum precipitated an intense band of protein II only with the homologous variant. Anti-OM-13 serum cross-reacted with no other protein II to any extent, although anti-OM-16 serum showed very slight cross-reaction with a heterologous protein II (27.8K) from P9-39. Other ^{125}I-labelled gonococcal proteins were precipitated from all variants; protein I by anti-OM-13 serum and proteins I and III by anti-OM-16 serum. These results confirmed conclusions made using ELISA inhibition techniques about the antigenic heterogeneity of protein II from variants within strain P9, and showed that RIP was a sensitive and convenient method for testing patient serum.

Isolation of Gonococci from Patients

In order to examine the effect of antigenic variation during the course of the natural infection, gonococcal isolates were obtained from patients and their sexual partners. Complete sets of isolates were obtained from seven groups of consorts (Table 4) and no further isolates from their sexual contacts could be obtained. In each case the colonial type was assessed after growth on CT medium and the predominant colonial

Figure 8. Comparison of two radioimmune precipitation systems, using i) intact cells; ii) pre-solubilised antigen. Both methods tested using a) P9-13 and rabbit anti-OM 13 serum; b) P9-2 and rabbit anti-α pilus serum.

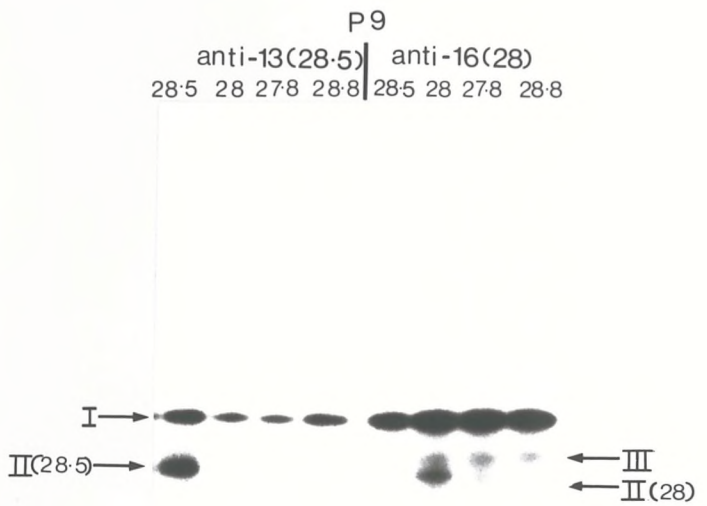


Figure 9. Radioimmune precipitation of P9 variants with differing protein II species using rabbit anti-OM 13 serum and anti-OM 16 serum. The numbers in the brackets indicate the molecular weight (in thousands) of the protein II present in the variant (Table 1).

type (> 90%) from each site was used for further studies. The above selection of colonial types was carried out by Mr. K. Zak.

To exclude the possibility that variations between isolates from different sites were due to concurrent infection with more than one strain, isolates within each group were compared for auxotype, outer membrane protein I molecular weight, and serotype using the method of Tam et al. (1983) with reagents kindly donated by Dr. E. Sandström. All isolates from within a patient group were confirmed as belonging to the same strain by these criteria.

Surface antigen preparations of all isolates were made by subjecting gonococcal suspensions to mild shearing forces. Gonococci were suspended in PBS and passed rapidly through a 21G needle approximately 20 times (Lambden and Heckels, 1979), the resulting suspension was centrifuged at 150 g for 5 min. The supernatant solution containing outer membrane vesicles and pili was centrifuged at 100,000 g for 2 h and the pellet was resuspended in PBS to a final concentration of about 1 mg protein/ml. When surface antigens prepared as above were run on SDS-PAGE, isolates of the same strain from within a patient group showed considerable differences in pili and protein II (Fig. 10 and Table 4). For example, in group 4, the male urethral isolate contained two protein II species of 30.7K and 27.2K molecular weight, the female urethral isolate contained protein IIs of 28.5K and 27K whilst the cervical isolate of the same patient only expressed one protein II of 27.7K. Similarly each variant produced pili of distinct sub-unit molecular weight (Fig. 10). Such variations in pili and protein II were seen in all groups examined, with some variants possessing more than one pilus sub-unit. All isolates obtained contained at least one protein II even when transparent in colony phenotype. There was no obvious correlation between molecular weight and site of isolation.

Detection of Antibodies to Gonococcal Surface Proteins in Patient Sera

Serum from each patient was initially screened by RIP assay against a pre-solubilised antigenic mixture containing all the gonococcal variants isolated from within their group of contacts. Normal serum from blood donors and laboratory personnel was used as a control.

Several factors may complicate rigorous interpretation of results obtained using RIP assay, the main one being ambiguities due to arbitrary selection of autoradiography exposure time which can

Footnote to Table 4

* Site of isolation:- M, male; F, female, (1) and (2) indicate two different patients in group; Ur indicates urethral isolate and Cx cervical isolate; O^+ and O^- denote separate opaque and transparent colonial types isolated from a single site.

† Auxotype:- A, arginine requiring; H, hypoxanthine; U, uracil; O, prototrophic.

‡ Serotype determined as described by Tam et al. (1982) with reagents supplied by Dr. E. Sandstrom.

§ Molecular weights:- Protein II were identified by difference in mobility on derivatization at 100 C and 37 C. Figures in parenthesis denote minor bands detected on over-loaded gels.

TABLE 4 PROPERTIES OF GONOCOCCI ISOLATED AT DIFFERENT SITES IN GROUPS OF CONTACTS

Group	Site of Isolation*	Auxotype†	Protein I Serotype Reactivity‡				Apparent Molecular Weight ($\times 10^{-3}$)§		
			2F12	4A12	2H1	3B10	PI	PII	PIIi
	MUr	AHU	-	-	-	-	34.5	29.5;29	(18.5);17
1	FCx	AHU	-	-	-	-	34.5	29.5;28.7	18;(17.3);17
	FUr	AHU	-	-	-	-	34.5	29.5	17.3;(17)
	MUr	AH	-	-	+	-	36.0	32; 28	18.7
2	FCx	AH	-	-	+	-	36.0	30; 28	18.5
	FUr	AH	-	-	+	-	36.0	28	18.0
	MUr	AH	+	+	-	-	36.3	32	17.5
3	F(1)Cx	AH	+	+	-	-	36.3	29.5	18.3
	F(1)Ur	AH	+	+	-	-	36.3	(31);29.5	17
	F(2)Cx	AH	+	+	-	-	36.3	31;29	19;18.5
	MUr	0	-	-	+	-	35.7	(30.7);27.2	(19.2)(17.5)
4	FCx	0	-	-	+	-	35.7	27.7	19.2;18.5
	FUr	0	-	-	+	-	35.7	(28.5);27.0	19.2;18.5;18
	MUr	0	-	-	-	-	35.0	28.2	(19);18.3
5	FCx	0	-	-	-	-	35.0	30.7;(28)	18.5
	FUr	0	-	-	-	-	35.0	30.7;28.2	19.5;19
	MUr	0	-	-	+	-	35.8	28	19.5;18
6	FCx	0	-	-	+	-	35.8	30;28	19.5;18.7
	FUr	0	-	-	+	-	35.8	28	19.5;18.7
7	MUr	AHU	-	-	+	-	35.8	31;27.2	18.5
	F(1)Cx-0$^+$	AHU	-	-	+	-	35.8	31;27.2	18.5
	F(1)Cx-0$^-$	AHU	-	-	+	-	35.8	30	18.2
	F(1)Ur-0$^+$	AHU	-	-	+	-	35.8	29.3;28.3	17.8
	F(1)Ur-0$^-$	AHU	-	-	+	-	35.8	31;(30);29.3	17.8
	F(2)Cx	AHU	-	-	+	-	35.8	30.2	17.5

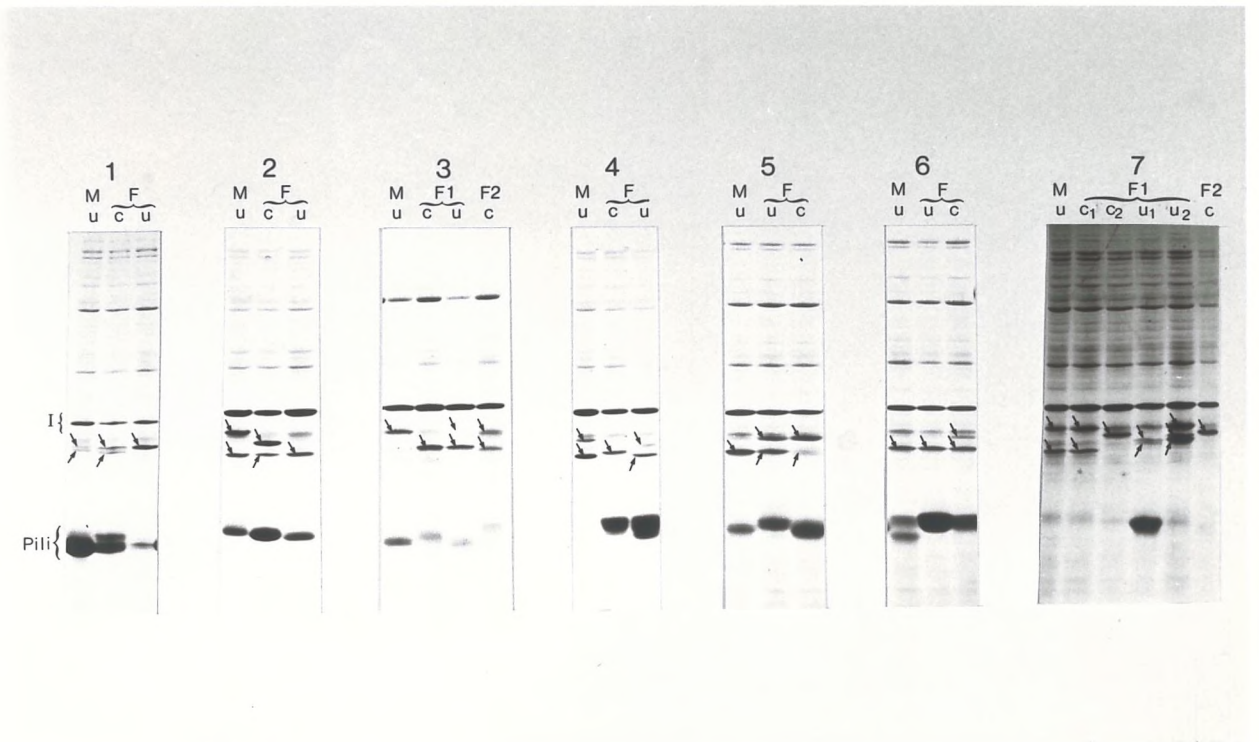


Figure 10. SDS PAGE of surface antigen preparations of gonococci isolated from the different sites in each patient group. (Gel Coomassie Blue stained.)
M, male; F, female; C, cervix; U, urethra.
When more than one colony type was isolated from a single set C_1 denotes the majority type and C_2 the minority population. Arrows indicate protein II.

alter visibility of some very weak bands which may or may not be significant, and thus affect interpretation of autoradiographs. In an attempt to minimise variations inherent in the technique, autoradiographs to be compared were exposed for the same period of time and a method was introduced to make judgement on the significance of antibody levels more objective. In the case of protein I for example, immunoprecipitated protein I bands were cut out of gels and their radioactivity was measured in a γ-counter. Background radioactivity in the assay was calculated by counting analogous areas of gel in the track with normal serum control of eight different gels and calculating the mean. Antibody levels were considered significant if radioactivity in immunoprecipitated band was greater than twice the mean of the background.

Eighty percent (12/15) of patient sera tested contained varying levels of antibodies to protein I (Fig. 11). Antibodies to other surface components were detected more infrequently, 30% of sera contained antibodies to at least one other surface antigen. In contrast only 20% (2/10) of control sera showed low levels of antibodies against protein I and all failed to react with any of the other surface antigens.

All patient sera exhibiting significant levels of antibodies to the homologous strain were also assayed in a similar manner against antigenic mixtures from each of the other groups to test for antigenic cross-reactivity.

i) Protein I

Table 5 shows cross-reactions of protein I when serum from each of the patients previously seen to have protein I antibodies (Fig. 11) was tested against all other patient strains. As well as antibodies against the homologous protein I, each of the sera had widely differing levels of cross-reactivity with the protein I of other strains. Some, such as sera from the male of group 2 (2M) and sera from 3M, 5M, 5F and 6M immunoprecipitated protein I of all strains. The rest of the sera were cross-reactive only with a limited number of heterologous strains, indicating an immune response that was serotype specific. There was no apparent correlation between the cross-reactivity of protein I in RIP assay and its reactivity profile using the monoclonal antibodies supplied by Dr. Sandström.

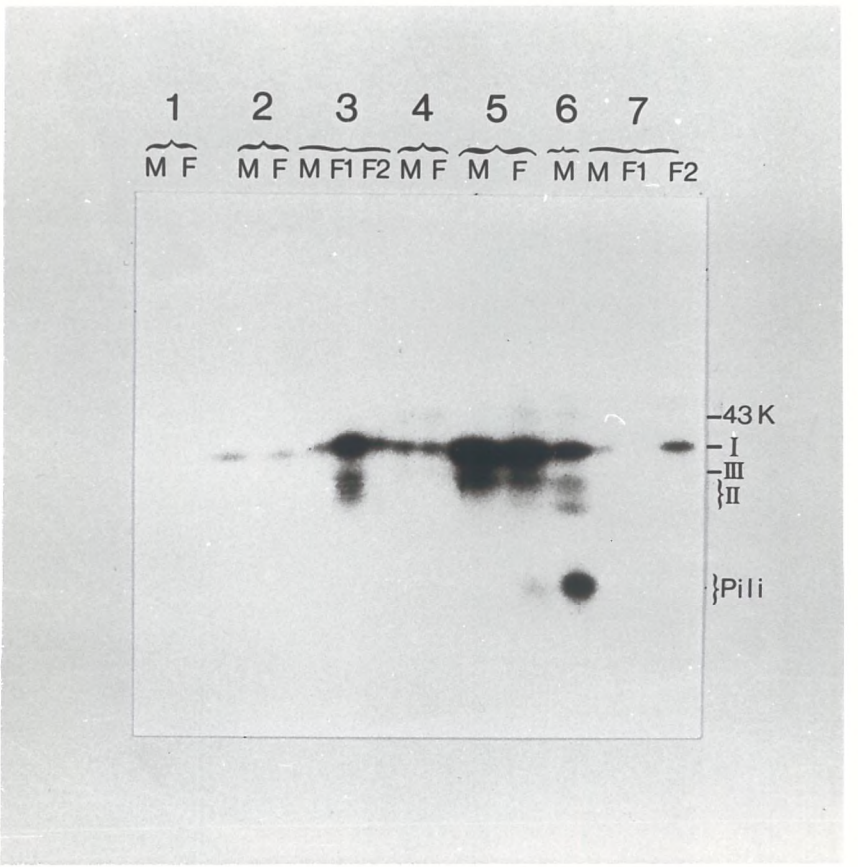


Figure 11. Radioimmune precipitation with mixed antigen preparations. Autoradiographs of SDS PAGE following RIP assay of each of the patient's sera with the antigen mixtures containing all the variants isolated from within their group of contacts. Numbers denote patient group; M, male; F, female.

TABLE 5

Summary of Protein I cross-reactions. (Parentheses indicate homologous reaction).

		Antigen (Strain No.)						
		1	2	3	4	5	6	7
Antiserum	2M	+	(+)	+	+	+	+	+
	2F	-	(+)	+	+	+	+	+
	3M	+	+	(+)	+	+	+	+
	$3F_1$	+	-	(+)	+	+	-	+
	$3F_2$	+	-	(+)	-	+	-	+
	4M	-	-	+	(+)	+	+	+
	4F	-	-	+	(+)	+	-	-
	5M	+	+	+	+	(+)	+	+
	5F	+	+	+	+	(+)	+	+
	6M	+	+	+	+	+	(+)	+
	7M	-	-	-	+	-	-	(+)
	$7F_2$	-	+	+	+	+	+	(+)

ii) Protein III

Three sera (5M, 5F and 6M) precipitated protein III in the homologous and all the heterologous strains, in each case however the intensity of the protein III band was apparently related to the intensity of the protein I band immunoprecipitated. Therefore the possibility exists that protein III was being precipitated not by anti-protein III specific antibodies but by anti-protein I antibodies, as part of a complex with protein I which is not dissociated by the Empigen-SDS combination used in RIP buffer.

In order to determine whether protein III was reacting specifically, or was precipitated as part of a complex, immunoblotting was performed. Whole cell lysates of variants from different groups were subjected to SDS-PAGE and transferred electrophoretically to nitro-cellulose sheets. The blots were reacted with serum 5M diluted 1:100 in gelatin buffer, washed and reacted with ^{125}I-protein A (see Methods, page 38). Autoradiography showed that the serum reacted with both proteins I and III, confirming the presence of antibodies specifically directed against protein III (Fig.12). Protein III on the blots appeared as two close but discrete bands, a phenomenon which has previously been described by Swanson et al. (1982) and may result from the action of the Iodogen reagent during radio-iodination.

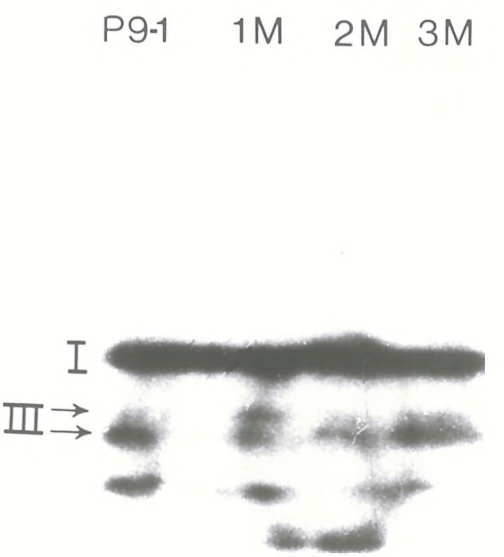


Figure 12. Detection of protein III specific antibodies in patient serum. Immunoblot of serum from patient 5M reacted against P9-1 and isolates from three heterologous groups.

iii) Pili

Homologous anti-pilus antibodies were detected in two sera (5F, 6M, Fig. 11). The sera were assayed by RIP against each of the separate variants from all the groups. Sera from both patients cross-reacted with all pili with variable intensity (Fig. 13). Serum 5F for example, reacted equally with pili from each of the homologous isolates (Fig.13a). Serum 6M also reacted with pili from all variants of the homologous strain but in contrast showed greater reactivity with the isolate from the cervix and urethra of the female partner than with the homologous isolate from the male urethra (Fig.13b). The reciprocal reaction however produced similar results, the serum from patient 6M reacted equally with pili from all variants of group 5 (Fig.13b), and serum 5F reacted more weakly with pili from the male urethral isolate than from the other two variants. Similar patterns appeared in other cases, both sera immunoprecipitated bands of comparable intensity for each pilus type. The intensity of the reaction does not therefore seem dependent on the specificity of the sera but rather appears to be a reflection on the radiolabelling properties of the pili, or the level of pili production of the variant.

iv) Protein II

The initial experiment showed that two sera 3F, and 6M, had significant levels of anti-protein II antibodies (Fig.11). These sera were screened for cross-reactivity against each of the mixtures of strain variants, the results are summarised in Table 6 . One patient ($3F_1$) reacted only with the homologous strain, the other (6M) immunoprecipitated a protein II not only from the homologous strain but also from two heterologous groups (3 and 4).

Sera $3F_1$ and 6M were then assayed by RIP against each of the individual variants of the homologous and cross-reactive strains. Serum from the male patient 6M, when reacted with individual isolates from the homologous group was found to immunoprecipitate a protein II of 28K molecular weight, present in all three variants, but did not have antibodies to a 30K protein II present only in the isolate from the female cervix (Fig.14b). When tested against the separate variants of group 3 serum 6M precipitated a 29.5K protein II present in the cervical and urethral isolates from one female ($3F_1$) and a 29K protein II present in the cervical isolate of the other female contact ($3F_2$)

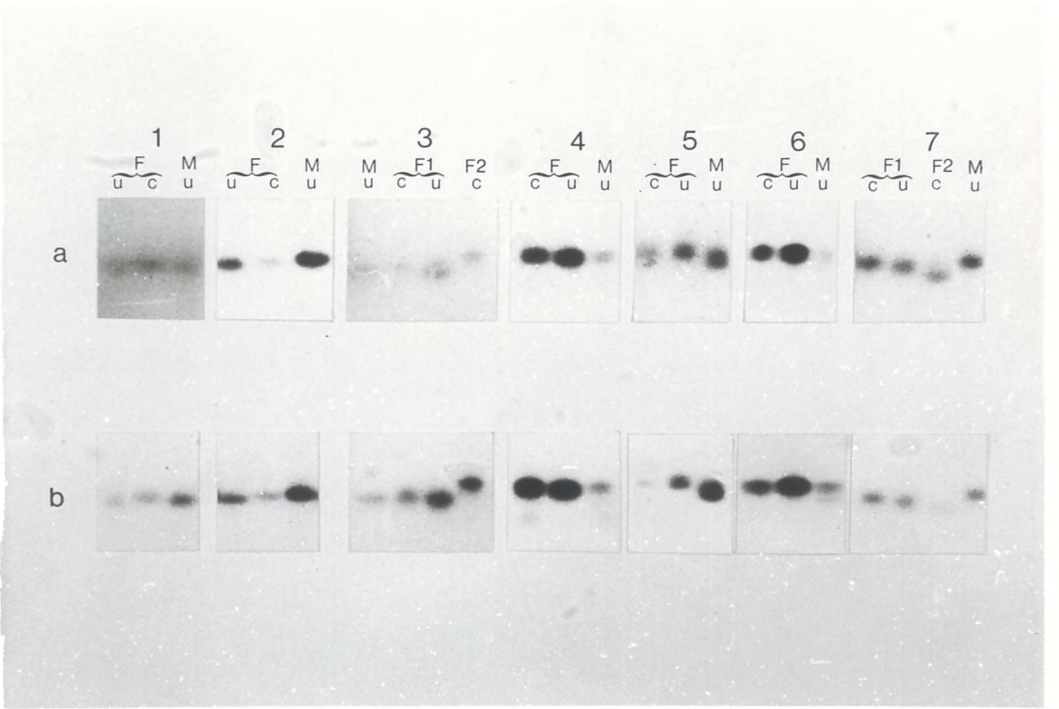


Figure 13. SDS PAGE following RIP assay showing cross-reactivity of a) serum 5F and b) serum 6M, with pili from isolates of all patient groups.

TABLE 6.

Summary of protein II cross-reactions. (Parentheses indicate homologous reaction).

		Antigen (Strain No.)						
		1	2	3	4	5	6	7
Antiserum	2M	-	(-)	-	-	-	-	-
	2F	-	(-)	-	-	-	-	-
	3M	-	-	(-)	-	-	-	-
	$3F_1$	-	-	(+)	-	-	-	-
	$3F_2$	-	-	(-)	-	-	-	-
	4M	-	-	-	(-)	-	-	-
	4F	-	-	-	(-)	-	-	-
	5M	-	-	-	-	(-)	-	-
	5F	-	-	-	-	(-)	-	-
	6M	-	-	+	+	-	(+)	-
	7M	-	-	-	-	-	-	(-)
	$7F_2$	-	-	-	-	-	-	(-)

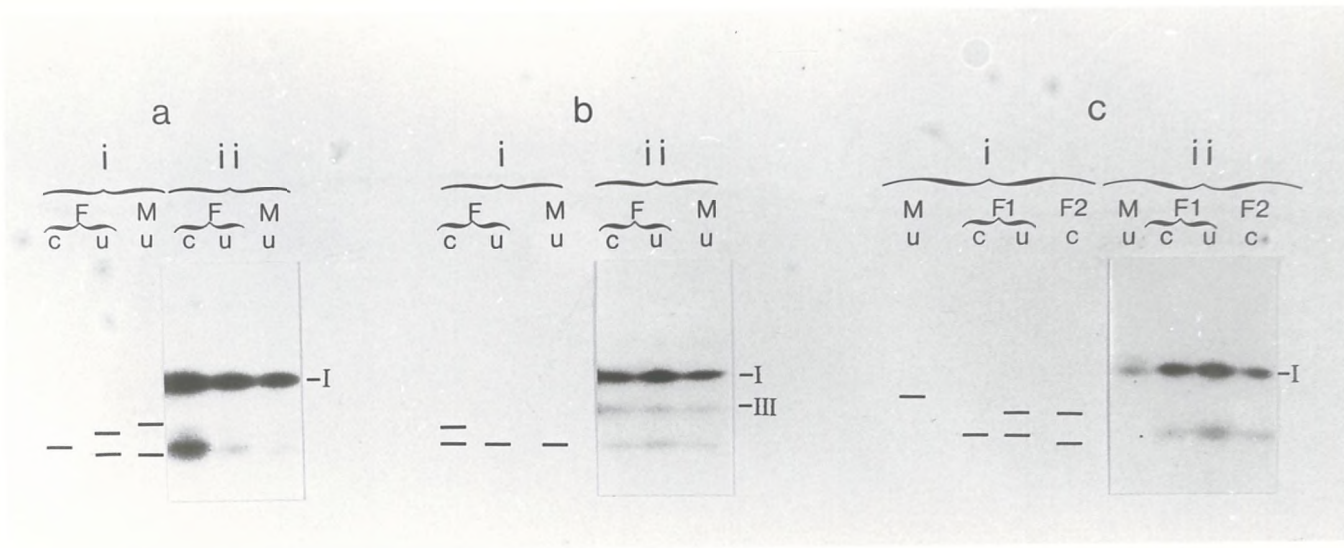


Figure 14. Radioimmune precipitation showing protein II cross-reactivity between serum 6M and each of the variants isolated from groups a) 4; b) 6; c) 3; i, schematic diagram of protein II species present in variant(Table 4 and Figure 10); ii, auto-radiography of SDS PAGE.

(Fig.14c). It did not however cross-react with the 31K protein II found in the urethral and cervical isolates of patients $3F_1$ and $3F_2$ respectively, or the 32K protein II found in the male contact. Serum 6M showed similar specificity for protein II species of variants of group 4 (Fig.14a). This group consisted of a male with protein II species of 30.7K and 27.2K molecular weight in the urethral isolate, and a female contact with a single protein II (27.7K) in the cervical isolate and two protein II (28.5K + 27K) species in the urethral variant. Serum 6M immunoprecipitated the 27.7K protein II from the cervical isolate as an intense band, and the 27.2K and 27K protein II species, from the male and female urethral samples respectively only as very weak bands.

In contrast serum from patient $3F_1$ failed to cross-react with protein II species from any other strains, but showed specificity similar to serum 6M with respect to protein II in the homologous strain (group 3), consisting of one male and two female contacts (3M, $3F_1$ and $3F_2$, Fig.10). The serum from $3F_1$ reacted with a 31K protein II but not the 29.5K protein II present in her urethral isolate (Fig.15). No activity was detected against the single 29.5K protein II present in the cervical isolate of the same patient, or against the 32K protein II present in the isolate from the male partner. The serum also reacted against two proteins II (29K + 31K) in $3F_2$ despite the absence of the 29K protein in either of the isolates from patient $3F_1$.

The results demonstrated the high degree of specificity of anti-protein II antibodies produced during the course of gonococcal infection.

v) Protein IV

In addition to antibodies against known surface proteins, four sera from the groups of consorts (4F, 5F, 6M and $7F_2$, Fig.11) precipitated low but significant levels of a protein of 43K molecular weight in all strains tested. Further investigation showed that serum from one patient for whom no isolate from the partner was available, contained high levels of antibodies against this protein (protein IV). When the serum was tested against the homologous isolate and a panel of heterologous strains including P9, it was found to react equally with protein IV from each (Fig. 16).

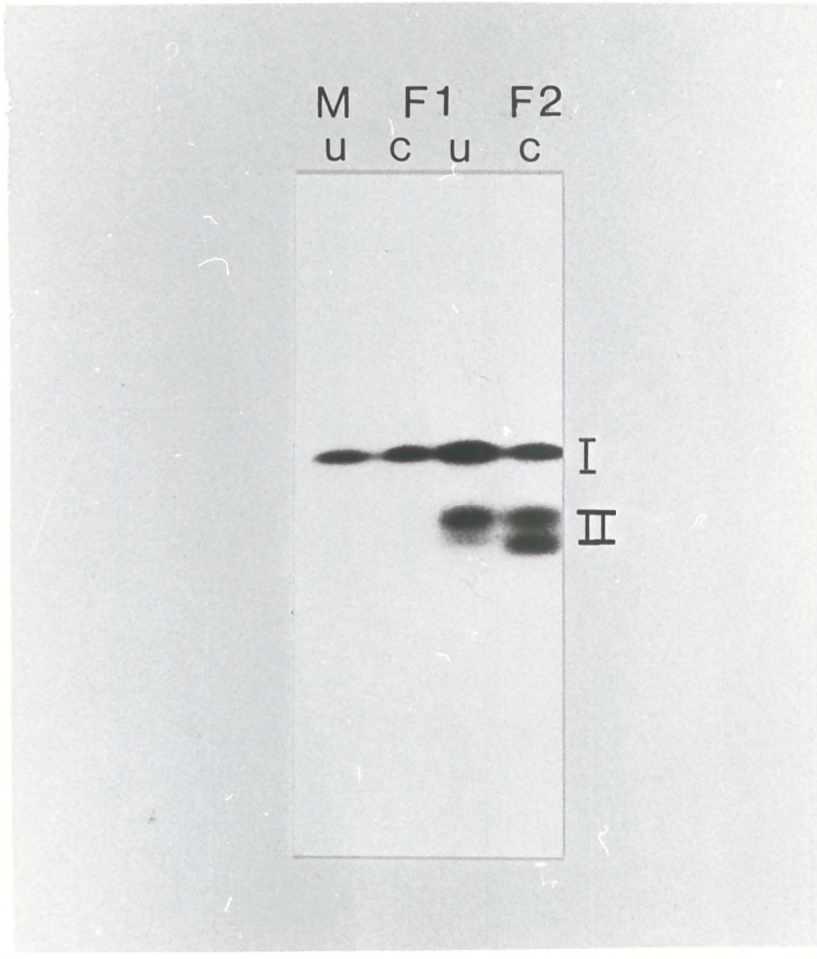


Figure 15. Radioimmune precipitation with individual antigen preparations. SDS PAGE following RIP assay of sera $3F_1$ with each of the variants isolated from group 3.

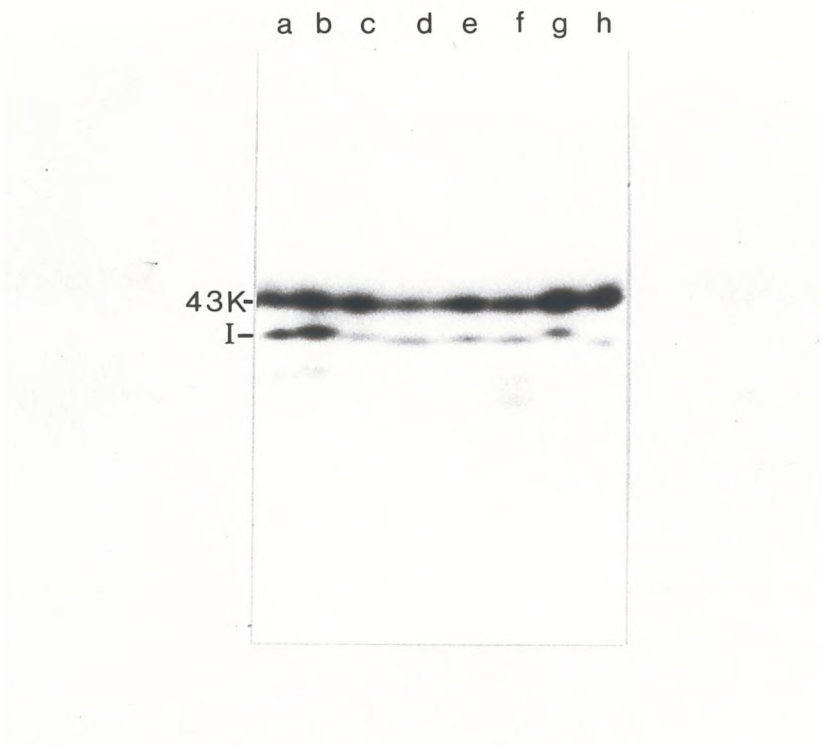


Figure 16. Radioimmune precipitation showing cross reactivity of a 43K protein. Serum from one patient was reacted inRIP with labelled antigen from eight different strains; a) strain P9; b) M group 3; c) FU group 4; d) FU group 5; e) FC group 6; f) homologous isolate from patient; g) F_2C group 3; h) M group 1.

Protein IV was visible as a weak band on autoradiography after SDS-PAGE of whole cells surface labelled with ^{125}I using Iodogen, but could not be detected in whole cells stained with Coomassie blue or using the silver staining method of Merril (Page 31). The protein was not detected on autoradiographs of outer membrane preparations radiolabelled using Iodogen.

Screening of a further 60 sera for high levels of antibody to this protein, revealed that approximately 40% had significant levels; but none had high enough levels to be used for the isolation of useful quantities of this protein by immunoprecipitation.

III Antigenic Diversity of Meningococcal Outer Membrane Proteins and Pili

Protein surface components in meningococci appear to correspond to similar proteins present in Neisseria gonorrhoeae outer membranes (Table 7). Both for example have principle outer membrane proteins (POMP, protein I in gonococci) which elicit serotype specific antibodies; heat modifiable proteins (protein II) of similar molecular weight range; enzyme resistant constant molecular weight protein (protein III) present in all strains and pili. Experiments were therefore carried out to investigate antigenic variations in meningococci and possible cross-reactivity with corresponding gonococcal antigens.

Detection of Anti-meningococcal Antibodies in Patients

Sera from six patients convalescing after meningococcal meningitis were tested in a RIP assay for anti-meningococcal antibodies using three different ^{125}I-surface labelled meningococci (SMC12, SMC16 and SMC21, serogroup A, B and C respectively). All the sera showed differing levels of antibody activity against the high molecular weight major outer membrane proteins (MOMP) and the 34K protein (corresponding to gonococcal protein III) (Fig. 17). The majority of sera (4/6) also immunoprecipitated a 46K molecular weight protein present in all isolates. Only one serum had antibodies to heat modifiable low molecular weight proteins (protein II, 25K-32K), these were highly specific for a 28K protein species present only in one meningococcal isolate (SMC21). None of the patients showed any anti-pilus activity.

When the same six sera were tested by RIP assay against a gonococcal strain, the majority (5/6) immunoprecipitated protein I and several (4/6) also precipitated gonococcal protein IV (Fig. 17). The serum which immunoprecipitated an intense band of the meningococcal 46K protein also precipitated the 43K gonococcal protein IV band with equal intensity.

Cross-reactivity of Anti-gonococcal Sera with Meningococcal Antigens

Eleven meningococcal isolates were tested for reactivity in RIP assays with serum from a patient suffering gonorrhoea. The serum

TABLE 7.

Molecular weights of corresponding outer membrane proteins in *Neisseria gonorrhoeae* and *N. meningitidis* (as presented by C. Frasch at the 3rd International Symposium on Pathogenic Neisseria, Montreal, 1982). Nomenclature of meningococci as suggested by C. Frasch.

Corresponding proteins

Gonococcal		Meningococcal	
Protein I	34 - 38K	Class 2 and 3	38 - 42K
Protein II	24 - 32K	Class 5	38K (± 2K)
Protein III	30 - 31K	Class 4	33K (± 1K)
Protein IV	40 - 44K	Class 1	46K (± 2K)

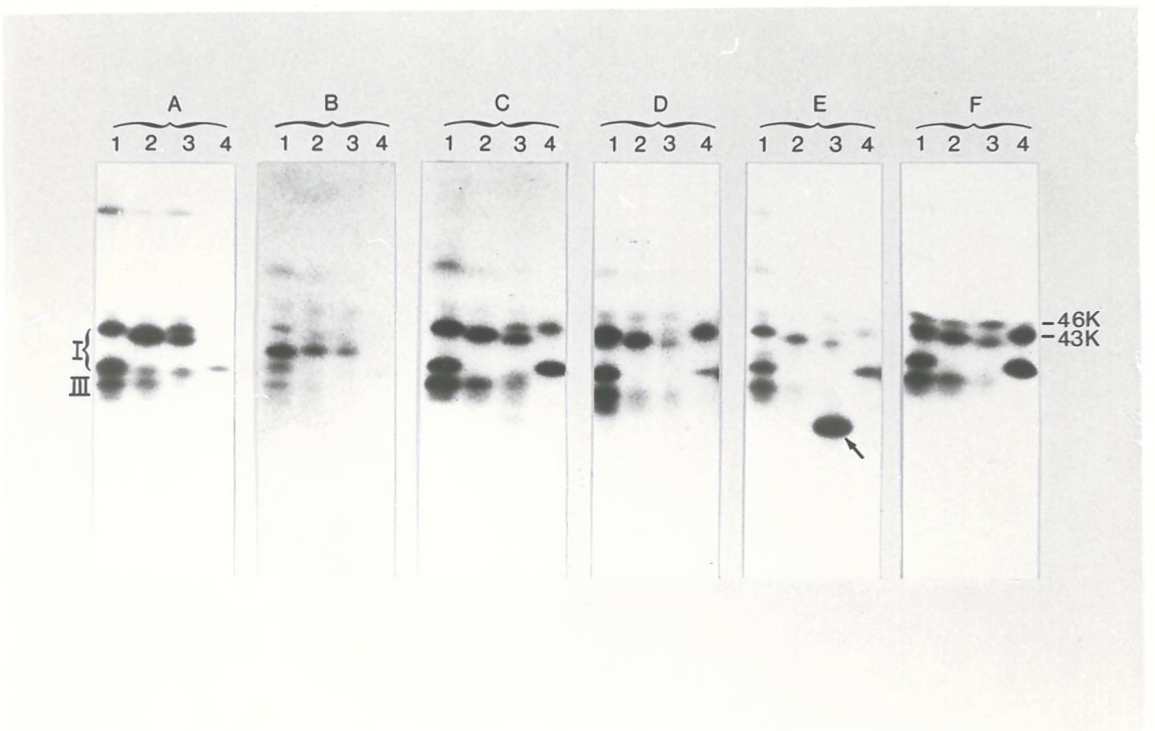


Figure 17. Radioimmune precipitation of meningococci and gonococci with sera from six patients convalescing after meningococcal meningitis. Track 1, Neisseria meningitidis strain SMC12; track 2, SMC16; track 3, SMC21; track 4, Neisseria gonorrhoeae from patient 3M.

($7F_2$) known to have antibodies against the homologous proteins I and to a lesser extent protein IV (Fig. 11) also cross-reacted with high molecular weight MOMP from all meningococci tested (Fig. 18) and immunoprecipitated the 46K protein present in all. This in addition to the above results from the reciprocal assay, shows that protein IV in gonococci corresponds to the 46K protein in meningococci.

Although the serum lacked antibodies to the homologous gonococcal protein II species, it precipitated a meningococcal protein II. This cross-reaction however, was highly specific for the 28.5K protein present in SMC22. No protein II cross-reactions were detected when meningococci were tested with a gonococcal patient serum ($3F_1$) known to have high levels of antibodies against the homologous gonococcal protein II.

The heterogeneity of meningococcal protein II species was tested further by reacting the panel of meningococci against two rabbit hyperimmune sera. Anti-OM-13 serum only cross-reacted with the MOMP from all the isolates (Fig. 19a) whilst anti-OM-16 serum also cross-reacted weakly with a 28K protein species in SMC12 and the 28K protein in SMC21 (Fig. 19b).

Antigenic Relationship between Gonococcal and Meningococcal Pili

The above experiments showed no cross-reaction of gonococcal serum with meningococcal pili although studies with a monoclonal antibody have shown that gonococcal and meningococcal pili share antigenic determinants (Virji and Heckels, 1983). When the meningococcal strains used here were tested with the same monoclonal antibody, SM1, approximately half reacted. Electron microscopy however, showed that the majority of non-reacting strains were pilated (Virji and Heckels, personal communication). The meningococcal strains were therefore tested by RIP assay for reactivity with the cross-reacting monoclonal antibodies SM1 and SM2. Of the fifteen meningococcal isolates assayed by RIP with antibody SM1, six strains (group 1) produced a single pilus band on SDS-PAGE in the molecular weight range 18.8K-19.4K; the rest failed to react. SM2 antibodies did not react with any pili in this system, failing to immunoprecipitate even the homologous α -pilus from gonococcal strain P9. (Fig. 20a).

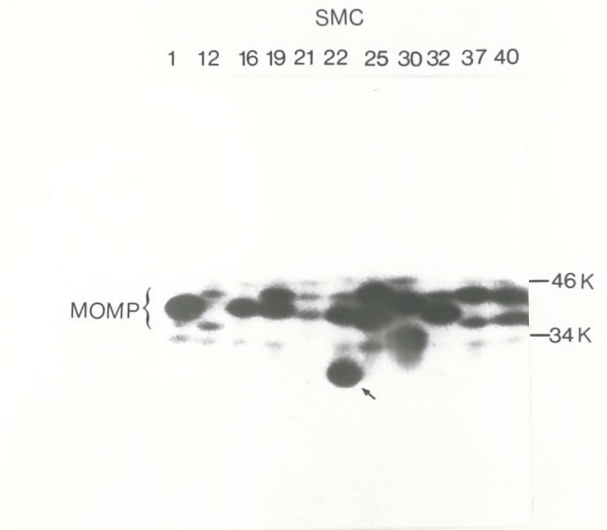


Figure 18. Radioimmune precipitation of meningococcal isolates with serum from a patient suffering from gonorrhoea ($7F_2$). Arrow indicates protein II.

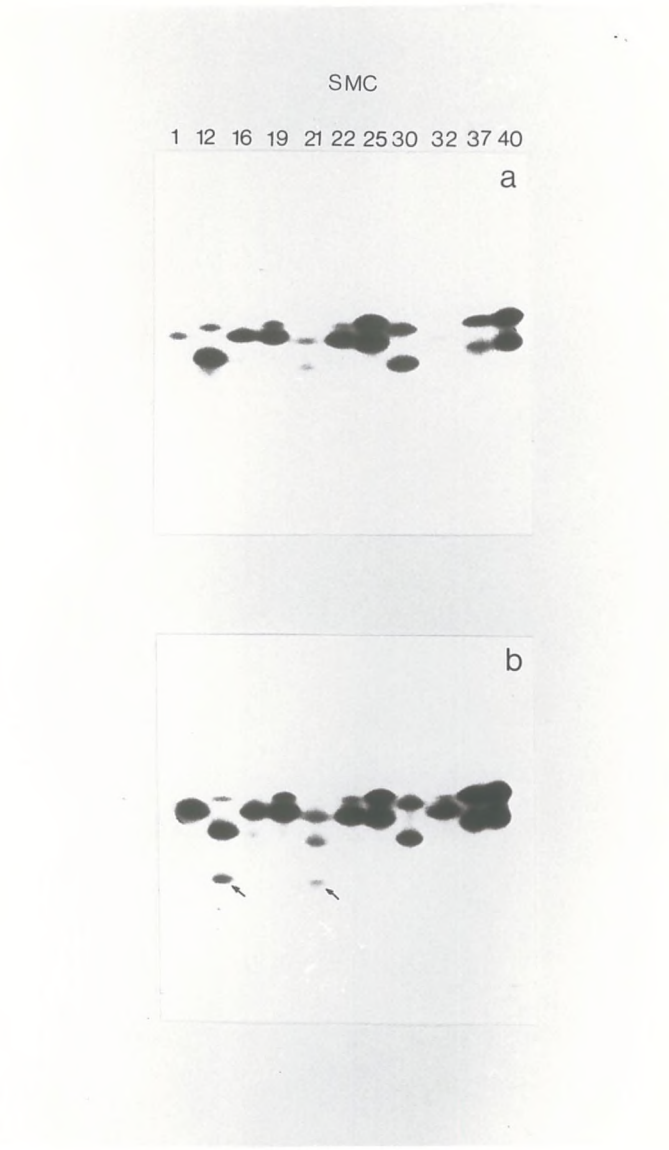


Figure 19. Radioimmune precipitation of menigococcal isolates with hyperimmune anti-gonococcal rabbit sera raised against a) OM 13, b) OM 16. Arrows indicate protein II.

The RIP assay was repeated using a polyclonal rabbit antiserum which had been raised against purified α-pili from strain P9. The serum cross-reacted with pili from each meningococcal strain in group 1, but in addition also immunoprecipitated five of the other strains (group 2). In each case the pili from group 2 were of significantly lower molecular weight (15.7-16.2K) and one strain produced an additional band at 13.0K (Fig. 20b). The four remaining strains failed to cross-react with polyclonal serum.

The anti α-pili polyclonal serum and monoclonal antibodies SM1 and SM2, were also studied by immunoblotting against SDS-PAGE gels of whole cell lysates of the same meningococcal isolates used above in RIP assays. The polyclonal serum and SM1 reacted with pili from group 1 (Fig.20a&b) but although readily precipitated by polyclonal serum in RIP, the pili from group 2 were not detected by immunoblotting (Fig. 20c). Therefore implying that following SDS-PAGE the pili of group 2 lost the ability to react with polyclonal serum. Antibody SM2 in contrast, though not precipitating pili in RIP, reacted with group 1 pili on blotting, reflecting the effect of the detergents not on the antigen, but on the antibody. Radioimmuno assay experiments with SM2 have shown that the binding of this low avidity antibody is probably sensitive to the presence of detergents (M. Virji, personal communication).

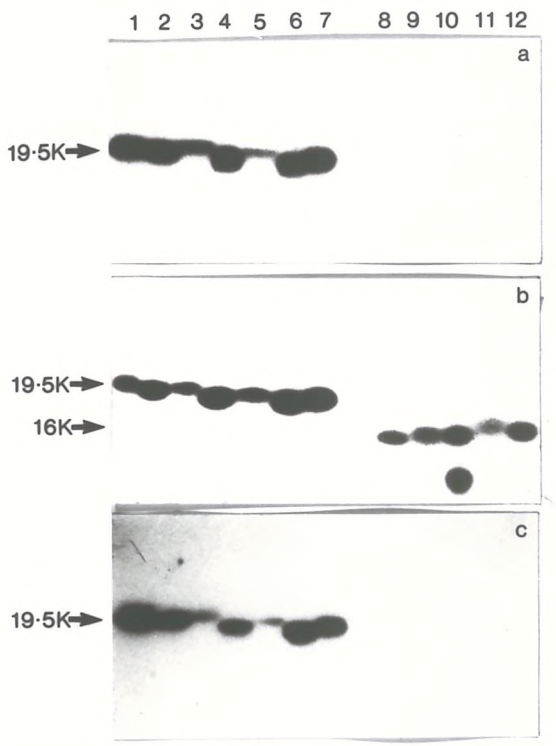


Figure 20. Reactivity of meningococci with anti-pilus antibodies. (a) Radioimmune precipitation with monoclonal antibody SM1; (b) radioimmune precipitation using polyclonal rabbit antiserum; (c) immunoblot of SDS PAGE gel of meningococcal lysates reacted with polyclonal antiserum. Track 1, N. gonorrhoeae; tracks 2-7 isolates from group 1; tracks 8-12 isolates from group 2. (Immunoblots of SM1 SM2 identical to (c)).

DISCUSSION

Protein II Heterogeneity in Strain P9

Initial experiments using outer membrane preparations showed that ELISA provided a convenient system for the detection of antibodies, but could give no indication as to the relative proportions directed against individual components (LPS, protein I, protein II). Quantitation would be possible using purified antigens. Isolation of protein I and II outer membrane, however, is a complex procedure entailing sequential extraction with detergents followed by chromatography on Sephadex gels (Heckels, 1977; James and Heckels, 1981); an alternative approach was therefore adopted. A series of adsorption experiments were carried out by pre-incubating the sera with LPS, OM (containing LPS and pI) and OM from the homologous variant (LPS, pI and pII). Using this method, anti-OM antisera raised in rabbits were found to contain antibodies to LPS, protein I and protein II.

ELISA adsorption experiments using whole gonococci instead of isolated LPS and outer membranes demonstrated that variants from a single strain, P9, cross-reacted due to the common LPS and protein I components. In addition protein II was shown to be immunogenic, exposed on the surface of the intact gonococci, but not cross-reactive with other protein II species from variants of the same strain. The ELISA inhibition experiments were also performed using specific anti-protein II sera made by adsorbtion of anti-outer membrane serum with whole cells of P9-1. Thus ensuring that only the antigenic cross-reactivity of the protein II species was measured, avoiding the possibility of variations in other known or unknown surface components masking partial protein II cross-reactivity. The different protein II species produced by strain P9 were antigenically distinct with respect to the region of the protein exposed on the surface of the intact gonococci. Indeed quantitation studies using the specific anti-protein II antisera gave a figure of less than 5% cross-reactivity with each of the other protein II species produced by this strain.

The above findings demonstrate clear differences in the immunochemical properties of protein II, and are in accordance with previous structural studies carried out on these proteins (Heckels, 1981) which showed that protein II species form a family with a homologous

region embedded in the membrane, and a variable region which is expressed on the surface. Clearly this surface exposed region is the immunodominant portion of the protein II molecule, hence antibodies to protein II show little cross-reaction.

The surface variations in gonococci are also associated with altered biological properties such as leukocyte association (King and Swanson, 1978; Lambden et al., 1979), and attachment to epithelial cells (Lambden et al., 1979). The variant of strain P9 which differs only in the possession of the 28.5K protein II, for example, shows a sevenfold increase in leukocyte association compared to the variant which contains the 28K protein II (Lambden et al., 1979). Virji and Everson (1981) reported that the ability of strain P9 variants to damage Chang conjunctival epithelial cells grown in tissue culture was influenced by the presence of particular pili types or protein II species. Protein II variations have also been seen to play a part in resistance of strain P9 to host defences. When chambers subcutaneously implanted into guinea pigs (McBride et al., 1981) were infected with P9 variants, only transparent variants survived. These however were eventually replaced by opaque pilated forms, which in the majority of cases contained protein II species not present in the original inoculum. It may be that diversity of surface antigens confer particular tissue tropism, permitting individual variants preferential colonisation of different mucosal surfaces encountered. The results discussed above suggest that another selective pressure influencing antigenic variation may be the specific immune response. Surface variations similar to those described for laboratory strains, occurring during the course of the gonococcal infection in humans could play an important part in the pathogenesis of gonorrhoea.

Antigenic Variation in the Human Infection

Only limited evidence exists for gonococcal antigenic variation in the human infection. James and Swanson (1978) found opacity characteristics of gonococcal isolates from patients varied with the sex of the subject, and during the course of the menstrual cycle of the female patients. Immune electron microscopy has been used to detect serological differences between pili expressed on isolates from

different sites on some patients (Novotny and Cownley, 1980). In another study when two consorts were re-infected 34 to 41 days later with apparently the same gonococcal strain, antigenic differences of pili were noted by solid-phase radio-immunoassay, and a difference in pili molecular weight was detected (Tramont et al., 1979). More recently Duckworth et al. (1983) studied and compared the surface protein profile of gonococcal isolates of a strain found at different sites in the same patient, and of isolates obtained from their consorts. In each case the pili expressed by the isolates differed in sub-unit molecular weight.

The data presented in this study extends the observations of Duckworth et al. (1983) using a larger number of groups. In each group considerable variations were seen in the molecular weight of both protein II and pili in isolates of the same strain. Investigation of the cross-reactivity and antigenicity of the protein II and pili, in such a large number of samples was not however possible using the ELISA inhibition techniques employed for strain P9. The ELISA system demands large scale grow ups, purification of gonococcal outer membranes and pili, and a substantial volume of each of the sera to be tested. The RIP system was therefore developed, enabling a large number of subjects to be tested using small amounts of sera. Furthermore by running immunoprecipitates on SDS-PAGE followed by autoradiography, the exact target antigen could be seen and characterised with respect to molecular weight. The method of Swanson (1981) using intact labelled cells ensures that only proteins exposed on the surface are available for interaction with antibodies in the sera. Since excess antibodies are washed off before lysing the gonococci with detergents, at no point are internal antigens able to combine with unbound antibodies. This technique however demands that gonococci are grown and iodinated before each experiment. The development of a RIP method using pre-solubilised antigens overcame this difficulty, and one iodination was sufficient for many experiments. Although in this technique the antibodies were in contact with antigens not normally exposed on the gonococcal surface, internal proteins were not iodinated and therefore not detected if subsequently immuno-precipitated. Comparison of the two RIP methods, reacting whole and pre-solubilised gonococci respectively with homologous serum, showed that identical surface components (protein I, etc.) were

immunoprecipitated in both systems.

Lack of immunological cross-reactivity between protein II species in strain P9 variants observed using ELISA inhibition, was also demonstrated by RIP using pre-solubilised antigen. Rabbit hyperimmune sera precipitated protein I of all variants, but reacted only with the homologous protein II. RIP studies applied to human sera revealed similar specificity in the human immune response to gonococcal infection. Sera from patients were initially screened by RIP assay against an antigenic mixture containing all the gonococcal variants isolated from within the homologous group of contacts. Those sera with detectable levels of antibodies to the homologous antigens were then tested against similar antigenic mixtures from each of the heterologous groups. Where significant levels of anti-pili or protein II antibodies were detected, the serum was assayed further by RIP against each of the separate isolates to identify which particular variant within a group was reacting.

Antibodies to protein I were present in the majority of sera from patients, cross-reacting equally with protein I from all isolates within the homologous group, and to a variable extent with some from the other strains. In contrast antibodies to pili and protein II were detected less frequently.

Antibodies to pili, when present, also cross-reacted with pili from all other variants whether from the homologous or heterologous groups. This may seem surprising taking into account the high degree of antigenic variability found in pili and the observations that when rabbits are immunised with gonococcal pili, the antibodies raised show less than 10% cross-reactivity with other strains (Buchanan, 1978). Similar heterogeneity is also found between variant pili produced by a single strain P9 (Lambden *et al.*, 1982). However, anti-pilus antibodies detected in human volunteers immunised with purified pili (Brinton *et al.*, 1978), and in infected patients (Lind and Reimann, 1980) show greater cross-reactivity than those raised in laboratory animals, suggesting that the human immune response may be directed towards shared antigenic determinants to a greater extent. This apparent anomaly may be explained by consideration of the pilus structure proposed by Buchanan *et al.* (1982) and Schoolnik *et al.* (1982) in which pili contain a conserved and a variable region (see Introduction, page 13). It may be that

rabbits immunised with pili responded to the variable region, whereas the human response may be directed more towards the common amino acid sequence.

In contrast to pili, antibodies to protein II, when detected showed a very narrow range of cross-reactivity, with affinity only for specific molecular species. Of particular interest was the observation that serum from female $3F_1$ was seen to have antibodies which reacted with protein II present in isolates from other individuals within a group of contacts, but not with the patient's own isolates. This suggests that protein II antigenic variation may represent a mechanism which enables gonococci to evade the effects of the host immune response. An infecting gonococcus producing one of several variant protein II species, if subsequently subjected to an immune response to that protein would be eliminated. The high rate of transition associated with protein II production *in vitro* ($0.2\text{-}4 \times 10^{-3}$ per colony forming unit per generation, Mayer, 1982) would however always ensure the presence of a minority population with different protein II profiles which would overgrow the original variant. Indeed, even the most homogenous isolates contained a small number of colonies (1-5%) on primary culture, which differed from the majority colony type.

Other selection factors must operate on gonococci during the course of the disease, influencing antigenic variation. James and Swanson (1978) found cervical isolates obtained at or about the time of menstruation, had increased occurrence of transparent phenotype. This they correlated with greater resistance in variants lacking protein II to the proteolytic enzymes present in cervical mucus and menstrual blood; known to increase during the luteal phase of the menstrual cycle (Beller, 1971; Schumacher, 1975). The same studies also noted an increased isolation rate of transparent colonial types from women taking oral contraceptives, suggesting an hormonal influence on antigenic expression. Although data on the menstrual history of the females in our study was not available, most were taking oral contraceptives. The colonial variations described above however, cannot be attributed solely to hormonal influence since differences were found consistently between isolates taken at the same time from urethra and cervix. Furthermore, although in laboratory cultures lack of colonial opacity has been associated with lack of protein II, as is the case

in strain P9, all isolates collected in these studies contained at least one protein II species even when transparent in phenotype.

Antibodies to minor non-variable surface antigens were common, three sera immunoprecipitated protein III from all strains and four immunoprecipitated protein IV. Protein I and protein III, thought to exist as a complex on the gonococcal surface, are not dissociated in some detergents (Swanson, 1981) and could be co-precipitated though only anti-protein I antibodies were present. In this study however, serum 5M precipitating proteins I and III in RIP, also reacted with both components on immunoblotting after separation of the proteins by SDS-PAGE. The work described in this thesis shows that protein III is expressed on the gonococcal surface, and is immunogenic during the course of an infection, producing antibodies which cross-react between strains.

The precipitation of protein IV on RIP assay was independent of the intensity of the protein I band. One serum reacted intensely with protein IV from all strains, though it immunoprecipitated the different protein I's to a varying degree. Like protein III this newly described protein is apparently conserved in all gonococcal strains.

Surface components other than proteins, such as LPS could also be precipitated by RIP, but their inability to be radio-iodinated meant they were not detected in this study. The technique adopted in this study to detect specific antibodies is selective for serum IgG. The most important antibodies encountered by gonococci are likely to be those present in genital secretions. Serum immunoglobulin responses however presumably reflect local antibody response, and indeed since IgG is the predominant antibody class in cervical mucus, with lower levels of IgA and no IgM (Schumacher and Yang, 1977), the RIP experiments give a fair representation of the host immune response influencing gonococcal infection. In addition recent studies have shown that protein A is capable of binding a significant proportion of both IgM and IgA (Brunda et al., 1979).

Meningococcal Antigens

a) Antibody response during meningococcal infection

Neisseria gonorrhoeae and Neisseria meningitidis, it is generally agreed, have major outer membrane proteins (MOMP) with

similar characteristics (presented by C. Frasch at the 3rd International Symposium on Pathogenic Neisseria, Montreal, 1982). The MOMP of meningococci with molecular weights between 38K and 42K is present in all strains and corresponds to gonococcal protein I. Lower molecular weight protein (26K - 30 K) show greater variability in molecular weight, are heat modifiable and may be absent in some strains. These proteins correspond to gonococcal protein II. A survey was therefore carried out to investigate the possibility of antigen variability during the course of meningococcal infection.

Radio immunoprecipitation experiments carried out using meningococcal sera showed that in general, antibodies to the outer membrane proteins were widely cross-reactive. This cross-reactivity extended to the stable antigens (protein I, protein III and protein IV) of gonococci. Similar experiments using gonococcal patient sera showed that antibodies to gonococcal protein I and IV were also cross-reactive with meningococcal proteins. In contrast antibodies to meningococcal protein II were seen to be highly specific to particular protein II species.

Antibodies to meningococcal pili were not seen in any of the sera tested. Conclusions to be drawn from the above results are limited by the fact that meningococcal sera and isolates came from different sets of patients, the results could not therefore be compared with a homologous sera-isolate reaction, but suggest that protein II variability may play an important role in the immune response to meningococcal infection.

When similar studies were recently carried out using gel-immunoradio assays (GIRA, Poolman et al., 1983), human sera tested gave the general impression that meningococcal protein I's were scarcely immunogenic. However, it may be that the SDS-denaturation of protein before reacting with the sera is responsible for the relatively poor human antibody activity. Recent results using non-denaturing SDS-PAGE support this conclusion (Poolman and Buchanan, unpublished observations, quoted in Poolman et al., 1983). Poolman et al. (1983) found that all patients tested showed antibody activity to protein II, in agreement with our results the activity was specific. Such specificity suggests the existence of protein II antigenic shift during the course of the infection. The same study also found that pili induced a strong, cross-reactive antibody response. The pili-specific antibodies however, were detected by

^{125}I-labelled protein A, but not ^{125}I-labelled antihuman IgG (γ chain specific). The authors suggested that this reflected an IgA response, and noted that protein A may bind some human IgA subclasses (Brunda, 1979).

b) Structural relationship between meningococcal and gonococcal pili

Previous studies have revealed considerable homology between meningococcal and gonococcal pili. Primary sequence data obtained from fragments generated on cyanogen bromide cleavage of pili (Buchanan et al., 1982; Schoolnik et al., 1982), showed that complete homology existed between the short CNBr-1 fragment generated from the first seven N-terminal amino-acids of gonococci and meningococci. The large fragment with the erythrocyte binding activity (CNBr-2), also showed considerable homology between gonococci and meningococci (Hermodson et al., 1978).

In the case of pili from strain P9 gonococci, the conserved epitopes detected with two monoclonal antibodies, SM1 and SM2 (Virji et al., 1983), are shared with all other gonococci tested. The binding of antibody SM1 is inhibited by antisera specific for CNBr-2 fragment, suggesting that the epitope recognised by SM1 is located in the common peptide. Virji and Heckels (1983) showed by colony blotting that this epitope was present on all gonococci and in some strains of meningococci, but not in others.

Data from experiments described in this thesis illustrated the difference in reactivity of meningococcal pili with monoclonal antibodies SM1 and SM2 . This difference did not correlate with any difference in morphology of meningococcal pili as observed by EM (Devoe and Gilchrist, 1978), or with the ability of pilated meningococci to haemagglutinate (Trust et al., 1983). Pili from group 2 meningococci which failed to react with SM1 or SM2 antibodies in RIP assay and immunoblots, were however detected in RIP using polyclonal rabbit serum. Group 2 pili were consistantly seen to be of a significantly lower molecular weight, implying that at least a portion of the conserved region containing the monoclonal binding site was absent.

The difference in reactivity of polyclonal serum with pili from group 2, depending whether RIP or immunoblotting technique is used, suggests a structural difference between the antigenic determinants in the two groups of pili. The epitope recognised in group 1 by the monoclonal antibodies, and by at least a proportion

of antibodies in the polyclonal serum, still reacts after SDS-PAGE, suggesting that it is a primary sequence. In contrast the epitope(s) in group 2 pili, probably form part of the tertiary structure as they are denatured by SDS and 2-mercaptoethanol treatment.

Since pili are associated with epithelial cell adhesion, the existence of two groups of meningococcal pili may have considerable implications in the understanding of meningococcal infection and carriage. At present the factors determining attachment of meningococci predominantly to the naso-pharynx mucosa rather than to other mucosal surfaces, and the factors that result in asymptomatic carriage rather than the disease are poorly understood. Structural differences between the two types of meningococcal pili might correlate with cell specificity and asymptomatic carriage. Certainly a recent study has demonstrated that attachment of meningococcal pili to buccal cells may be inhibited by glycoconjugates in some strains but not in others (Trust et al., 1983). It is tempting to speculate that the two types of pili may correlate with the structural differences described here. Clearly further work would be required to determine if this is the case.

Genetic Mechanism of Antigenic Variation

Pili and protein II variations are observed in laboratory cultures, and the alteration of gene expression is stably inherited by the majority of progeny. Although tissue tropism, immune response and other host factors may influence selection of particular antigenic variants in a population of gonococci, the most likely assumption is that the alterations occur spontaneously at a low frequency during infections and not as a direct response to host factors.

The high rate of change indicates that the variations are not controlled by random point mutation and reversion. Norlander et al. (1979) have reported that the process of colony opacity changes was DNAase sensitive, and concluded that some genetic exchanges were mediated by high levels of transformation involving extracellular DNA. Subsequent information contradicts this conclusion: pilated (P^+) transformable gonococci, and non-pilated (P^-) non-transformable cells; cells in optimal ($Mg^{++} + Ca^{++}$) and cells in poor (EDTA) conditions for transformation; cells in the presence of DNA and

and cells in the presence of DNAase, all had identical rates of transition (Mayer, 1982). Furthermore, Sparling (1977) had already demonstrated in a mixture of cells that drug resistance markers could be transferred to P^+ but not to P^- gonococci. If it is assumed that transformation of colony morphology genes and drug resistance genes occur by the same mechanism, then the identical rates of transition for isogenic P^+ and P^- gonococci excludes transformation as an explanation.

Recently cloned pilus protein genes have been used as a probe in Southern hybridization experiments, and have demonstrated that the conversion of pilus genes from the expressed to the non-expressed state involves rearrangement of segments of the gonococcal chromosomal DNA (Meyer et al., 1982). The experiment showed that the switch from transparent (P^+O^-) to opaque (P^+O^+) also involved chromosomal re-organisation.

Many studies have divided all colony opacity forms into just opaque (O^+) and transparent (O^-), but this is an oversimplification since intermediate colony types exist (Lambden et al., 1979; Swanson, 1982), and as many as six protein II and four pili species have been observed in a single strain in this laboratory. Multiple antigenic shifts have also been seen to occur in the surface proteins of Trypanosoma brucei which expresses sequentially a large number of varying surface glycoprotein antigens (VSG) during infection. It has been established that a separate basic (silent) copy gene exists for each VSG (Bernards et al., 1981) and that the expression of VSG is accompanied by the appearance of an expression-linked copy of the gene. It is the expression-linked copy and not the basic copy that is used for VSG mRNA synthesis. It has been suggested that production of different pili protein II species in gonococci could operate in a similar fashion, with a number of different "cassettes" available which could be inserted into the expression site.

Gonococcal Surface Proteins as Vaccines

The studies described in this thesis have implications in the design of a possible future gonococcal vaccine. Anti-pilus antibodies in patients having contracted gonorrhoea have been found to be cross-reactive. Vaccine trials on humans have shown however,

that the problem of pilus antigenic heterogeneity has not yet been solved since the protective effect is restricted to challenge with the homologous strain (Tramont et al., 1981). If pili are to prove an effective vaccine the cross-reactive antibody response will have to be increased perhaps by selection of a pilus type which induces a good response to the common pilus sequence; by increasing the immunogenicity of pilus preparations through combination with the non-toxic LPS moeity; or by the use of the purified CNBr-2 fragment containing the common antigenic site. The common fragment although immunorecessive in intact pili, becomes immunodominant when cleaved from the pilus sub-unit; the induced antibody binds gonococcal pili from diverse strains (Schoolnik et al., 1982).

Protein II due to its lack of cross-reactivity, high rate of antigenic transition and its total absence from some variants, does not seem a likely choice for the sole component of an anti-gonococcal vaccine. It does however have a role in gonococcal adhesion to certain cell types.

As a component of a combined vaccine also including pili protein II may induce antibodies that could enhance inhibition of gonococcal adhesion to epithelial cell surfaces by the anti-pilus antibodies.

The high degree of variability of the gonococcal surface antigens and the problem this presents for the development of an effective vaccine, suggests that greater attention should be paid to antigens shared between strains. Although protein I's differ between strains we have seen the presence of cross-reactive antibodies, this antigen therefore seems a possible candidate for a vaccine since it can be highly immunogenic and cross-reactive. The presence of anti-protein I antibodies has been correlated with protection against salpingitis but not uncomplicated gonorrhoea (Buchanan et al., 1980). Protein III is another common antigen to which antibodies have been detected. It has been identified in all gonococci investigated in this study, and in strains used by other groups (Judd, 1982a and b). In all cases it has been observed to be of the same molecular weight, as measured by mobility in SDS-PAGE, furthermore it has a homologous structure irrespective of strain (Judd, 1982a and b). Indeed our own observations point to the extensive cross-reactivity of protein III. This protein however, although clearly labelled with ^{125}I using Iodogen as the catalyst,

is not readily seen on gonococci when surface labelled with ^{125}I and lactoperoxidase. . . . . This may be attributed to protein III having less surface exposure and access to surface reactive agents than proteins I and II. Limited exposure could inhibit the antigenicity of protein III and its usefulness as a possible vaccine, although further investigations will be required.

In view of the problems discussed above identification of the 43K surface protein, which we have termed protein IV, may have a particular significance in the context of vaccine development. The protein is found in all strains and variants tested, and has been seen to be immunogenic inducing antibodies which are totally cross-reactive between protein IV of all strains tested. Future studies on protein IV to determine its structure and role in pathogenesis, will neccessitate its isolation, though this might prove difficult since it cannot be observed in SDS-Gels by Coomassie blue or silver staining, and is therefore difficult to characterise. The solution may lie in the development and use of a protein IV-specific monoclonal antibody which is currently under investigation in this laboratory.

REFERENCES

Adams, G.A. (1971). Structural investigations on a cell wall lipopolysaccharide from Neisseria sicca. Canadian Journal of Biochemistry, 49, 243-250.

Apicella, M.A. and Gagliardi, N.C. (1979). Antigenic heterogeneity of the non-serogroup antigen structure of Neisseria gonorrhoeae lipopolysaccharide. Infection and Immunity, 26, 870-874.

Apicella, M.A., Bennet, K.M., Hermerath, C.A. and Roberts, D.E. (1981). Monoclonal Antibody Analysis of Lipopolysaccharide from Neisseria gonorrhoeae and Neisseria meningitidis. Infection and Immunity, 34, 751-756.

Armstrong, G.D., Frost, L.S., Vogel, H.J. and Paranchych, W. (1981). Nature of the Carbohydrate and Phosphate Associated with ColB2 and EDP208 pilin. Journal of Bacteriology, 145, 1167-1176.

Beller, F.K.. (1971). Observations on the clotting of menstrual blood and clot formation. American Journal of Obstetrics and Gynecology, 111, 535-546.

Bernards, A., Van der Ploeg, L.H.T., Frasch, A.C.C. and Borst, P. (1981). Activation of Trypanosome Surface Glycoprotein Genes Involves a Duplication-Transposition Leading to an Altered 3'End. Cell, 27, 497-505.

Billewicz, W.Z. and McGregor, I.A. (1981). The demography of two West African (Gambian) villages 1951 1975. Journal of Biosocial Science, 13, 219-240.

Blake, M.S. and Swanson, J. (1975). Studies on gonococcus infection IX. In vitro decreased association of pilated gonococci with mouse peritoneal macrophages. Infection and Immunity, 11, 1402-1404.

Blake, M.S., Gotschlich, E.C. and Swanson, J. (1981). Effects of Proteolytic Enzymes on the Outer Membrane proteins of Neisseria gonorrhoeae. Infection and Immunity, 33, 212-222.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-Dye binding. Analytical Biochemistry, 72, 248-254.

Brinton, C.C. (1965). The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram negative bacteria. Transactions of the New York Academy of Science, 27, 1003-1054.

Brinton, C.C., Bryan, J., Dillon, J.A., Guerina, N., Jacobson, L.S., Kraus, S., Labik, A., Lee, S., Levene, A., Lim, S., McMichael, J., Polen, S., Rogers, K., To, A.C.C. and To, S.C.M. (1978). Uses of pili in gonorrhoea control. Role of pili in disease, purification and properties of gonococcal pilus vaccine for gonorrhoea. In Immunobiology of Neisseria gonorrhoeae, Ed. Brooks, G.F., Gotschlich, E.C., Holmes, K.K., Sawyer, W.D., Young, F.E. American Society for Microbiology, Washington, D.C. pp. 155-178.

Brooks, G.F., Lammel, C.J., Peterson, B.H. and Stiles, D.P. (1981). Human seminal plasma inhibition of antibody complement-mediated killing and opsonisation of Neisseria gonorrhoeae and other gram-negative organisms. Journal of Clinical Investigation, 67, 1523-1531.

Brunda, M.J., Minden, P. and Grey, H.M. (1979). Heterogeneity of binding of human IgA subclasses to protein A. Journal of Immunology, 123, 1457-1461.

Buchanan, T.M., Swanson, J., Holmes, K.K., Kraus, S.J. and Gotschlich, E.C. (1973). Quantitative determination of antibody to gonococcal pili. Changes in antibody levels with gonococcal infection. Journal of Clinical Investigation, 52, 2896-2909.

Buchanan, T.M. (1978). Antigen-specific serotyping of Neisseria gonorrhoeae. I. Use of an Enzyme-Linked Immunosorbent Assay to quantitate pilus antigens on gonococci.
Journal of Infectious Diseases, 138, 319-325.

Buchanan, T.M., Eschenbach, D.A., Knapp, J.S. and Holmes, K.K. (1980). Gonococcal salpingitis is less likely to recur with Neisseria gonorrhoeae of the same principle outer membrane protein antigenic type. In "Genetics and immunobiology of Pathogenic Neisseria" p.266. EMBO Workshop June 16-19, 1980, edited by Dan Danielsson.

Buchanan, T.M. and Hildebrandt, J.F. (1981). Antigenic-specific serotyping of Neisseria gonorrhoeae: characterisation based upon principle outer membrane protein. Infection and Immunity, 32, 985-994.

Buchanan, T.M., Siegel, M.S., Chen, K.C.S. and Pearce, W.A. (1982). Development of a vaccine to prevent gonorrhoea. In: Seminars in Infectious Disease, Vol.IV, Bacterial Vaccines, pp.160-164. Edited by L.Weinstein, B.N. Fields, J.B. Robbins, J.C. Hill and G. Sadoff. New York: Thieme-Stratton.

Cannon, J.G., Lee, T.J., Guymon, L.F. and Sparling, P.F. (1981). Genetics of Serum Resistance in Neisseria gonorrhoeae: The sac-1 genetic locus. Infection and Immunity, 32, 547-552.

Catlin, B.W. (1973). Nutritional profiles of Neisseria gonorrhoeae, Neisseria meningitidis, and Neisseria lactamica in chemically defined media and the use of growth requirements for gonococcal typing. Journal of Infectious Diseases, 128, 178-194.

Cohen, I.R., Kellog, D.S. and Norins, L.C. (1969). Serum antibody response in experimental human gonorrhoea. British Journal of Venereal Diseases, 45, 325-327.

Craven, D.E. and Frasch, C.E. (1978). Pili mediated and non-mediated adherence of Neisseria meningitidis and its relationship to invasive disease. In: Immunobiology of Neisseria gonorrhoeae, (Brooks, G.F., Gotschlich, E.C., Holmes, K.K., Sawyer, W.D. and Young, F.E. eds.) 250-252, American Society for Microbiology, Washington, D.C.

Danielsson, D. and Sandstrom, E. (1980). Serology of Neisseria gonorrhoeae. Demonstration by co-agglutination and immunoelectrophoresis of antigenic differences associated with colour/opacity colonial variants. Acta Path.Microbiol. Scand., 88, 39-46.

Demarco de Hormaeche, R., Thornley, M.J. and Glauert, A.M. (1978). Demonstration by light and electron microscopy of capsules on gonococci recently in vivo. Journal of General Microbiology, 106, 81-91.

Demarco de Hormaeche, R., Thornley, M.J. and Holmes, A. (1983). Surface antigens of gonococci: correlation with virulence and serum resistance. Journal of General Microbiology, 129, 1559-1567.

Densen, D. and Mandell, G.L. (1978). Gonococcal interactions with polymorphonuclear neutrophils. Journal of Clinical Investigation, 62, 1161-1171.

De Voe, I.W. and Gilchrist, J.E. (1978). Piliation and Colonial morphology among laboratory strains of meningococci. Journal of Clinical Microbiology, 7, 379-384.

Diena, B.B., Ryan, A., Ashton, F.E., Wallace, R., Perry, M.B. and Daoust, V. (1975). A mouse intracerebral infection with Neisseria gonorrhoeae immunising properties. British Journal of Venereal Diseases, 51, 22-24.

Diena, B.B., Ashton, F.E., Ryan, A., Wallace, R. and Perry, M.B. (1978). The lipopolysaccharide (R type) as a common antigen of Neisseria gonorrhoeae immunizing properties. Canadian Journal of Microbiology, 24, 117-123.

Dilworth, J.A., Hendley, J.O. and Mandell, G.L. (1975). Attachment and ingestion of gonococci by human neutrophils. Infection and Immunity, 11, 512-516.

DiRienzo, J.M., Nakamura, K. and Inouye, M. (1978). The outer membrane proteins of Gram negative bacteria: biosynthesis, assembly and functions. Annual Review of Biochemistry, 47, 481-532.

Douglas, J.T., Lee, M.D. and Nikaido, H. (1981). Protein I of Neisseria gonorrhoeae outer membrane is a porin. FEMS Microbiology Letters, 12, 305-309.

Duckworth, M., Jackson, D., Zak, K. and Heckels, J.E. (1983). Structural variations in pili expressed during gonococcal infection. Journal of General Microbiology, 1929, 1593-1596.

Eschenbach, D.A. and Holmes, K.K. (1975). Acute pelvic inflammatory disease. Clinical Obstetrics and Gynecology, 18, 35-56.

Fairbanks, G., Steck, T.L. and Wallack, D.F.H. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry, 10, 2606-2617.

Froholm, L.D. and Sletten, K. (1977). Purification and N-terminal sequence of a fimbrial protein from Moraxella nonliquefasciens. FEBS letters, 73, 29-32.

Galanos, C. and Lüdentz, O. (1975). Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. European Journal of Biochemistry, 59, 603-610.

Glynn, A.A. and Ison, C. (1978). Serological diagnosis of gonorrhoea by an enzyme-linked immunosorbent assay (ELISA). British Journal of Venereal Diseases, 54, 97-102.

Glynn, A.A. and Ward, M.E. (1970). Nature and heterogeneity of the antigens of Neisseria gonorrhoeae involved in the serum bactericidal reaction. Infection and Immunity, 2, 162-168.

Gray, H.M., Abel, C.A. Yount, W.J. and Capra, J.D. (1968). A subclass of human A globulins (A2) which lacks the disulfide bonds linking heavy and light chanin. Journal of Experimental Medicine, 128, 1223-1236.

Greenberg, L., Diena, B.B., Ashton, F.A., Wallis, R., Kenny, C.P., Znamirowski, R., Ferrari, H. and Atkinson, J. (1974). Gonococcal vaccine studies in Inuvik. Canadian Journal of Public Health, 65, 29-33.

Handfield, H.H. (1975). Disseminated gonococcal infection. Clinical Obstetrics and Gynecology, 18, 131-142.

Harkness, A.H. (1948). The pathology of gonorrhoea. British Journal of Venereal Diseases, 51, 137-147.

Heckels, J.E., Blackett, B., Everson, J.S. and Ward, M.E. (1976). The influence of surface charge on the attachment of Neisseria gonorrhoeae to human cells. Journal of General Microbiology, 96, 359-364.

Heckels, J.E. (1977). The surface properties of Neisseria gonorrhoeae: isolation of the major components of the outer membrane. Journal of General Microbiology, 99, 333-341.

Heckels, J.E. (1978). The surface properties of Neisseria gonorrhoeae: topographical distribution of the outer membrane protein antigens. Journal of General Microbiology, 108, 213-219.

Heckels, J.E. and Everson, J.S. (1978). The isolation of a new outer membrane protein from the parent strain of Neisseria gonorrhoeae P9. Journal of General Microbiology, 106, 179-182.

Heckels, J.E. (1979). The outer membrane of Neisseria gonorrhoeae: Evidence that protein I is a transmembrane protein. FEMS. Microbiology Letters, 6, 325-327.

Heckels, J.E. and James, L.T. (1980). The structural organisation of the gonococcal cell envelope and its influence on pathogenesis. In: Genetics in Immunobiology of Pathogenic Neisseria, Eds., Danielson, D., Normark, S. University of Umea, Umea. pp. 25-28.

Heckels, J.E. (1981). Structural comparison of Neisseria gonorrhoeae outer membrane proteins. Journal of Bacteriology, 145, 736-742.

Heckels, J.E. (1982). Role of surface proteins in the adhesion of Neisseria gonorrhoeae. In: Microbiology 1982, Ed., Schlessinger, D. American Society for Microbiology, Washington, (In press).

Hendley, J.O., Powell, K., Rodewald, R., Holzgrefe, H. and Lyles, R. (1977). Demonstration of a capsule on Neisseria gonorrhoeae. New England Journal of Medicine, 296, 608-611.

Hermodson, M.A., Chen, K.C.S. and Buchanan, T.M. (1978). Neisseria pili proteins: amino-terminal amino acid sequences and identification of an unusual amino acid. Biochemistry, 17, 442-445.

Ito, J.I.Jr., Wunderlich, A.C., Lyons, J., Davis, C.E., Guiney, D.G. and Braude, A.I. (1980). Role of magnesium in the Enzyme-Linked Immunosorbent Assay for Lipopolysaccharides of rough Escherichia coli strain T5 and Neisseria gonorrhoeae. Journal of Infectious Diseases, 142, 532-537.

James-Holmquest, A.N., Swanson, J., Buchanan, T.M., Wende, R.D. and Williams, R.P. (1974). Differential attachment of pilated and non-pilated Neisseria gonorrhoeae to human sperm. Infection and Immunity, 9, 897-902.

James, J.F. and Swanson, J. (1977). The capsule of the gonococcus. Journal of Experimental Medicine, 145, 1082-1086.

James, J.F. and Swanson, J. (1978). Studies of gonococcus infection. XIII. Occurrence of colour/opacity colonial variants in clinical culture. Infection and Immunity, 19, 332-340.

James, J.F., Lammel, C.J., Draper, D.L. and Brooks, G.F. (1980). Attachment of Neisseria gonorrhoeae colony phenotype variants to eukaryotic cells and tissues. In: Genetics and Immunobiology of pathogenic Neisseria, EDS., Danielson, D., Normark, S. University of Umea, Umea, pp.213-216.

James, L.T. and Heckels, J.E. (1981). An improved method for the isolation of the major protein of the gonococcal outer membrane in an antigenically reactive form. Journal of Immunological Methods, 42, 223-228.

Janda, J. and Work, E. (1971). A colourimetric estimation of lipopolysaccharide. FEBS letters, 16, 343-345.

Jephcott, A.E., Reyn, A., Birch, C.J. and Anderson, A. (1971). Brief report: Neisseria gonorrhoeae III. Demonstration of presumed appendages to cells from different colony types. Acta Pathol. Microbiol. Scand. Sect. B. 79, 437-439.

Johnston, K.H. and Gotschlich, E.C. (1974). Isolation and characterisation of outer membrane of gonococci. Journal of Bacteriology, 119, 250-257.

Johnston, K.H., Holmes, K.K. and Gotschlich, E.C. (1976). The serological classification of Neisseria gonorrhoeae. I. Isolation of the major outer membrane complex responsible for serotypic specificity. Journal of Experimental Medicine, 143, 741-758.

Jones, R.B., Newland, J.C., Olsen, D.A. and Buchanan, T.M. (1980). Immuno-enhanced phagocytosis of Neisseria gonorrhoeae by macrophages. Journal of General Microbiology, 121, 365-372.

Judd, R.C. (1982a). Surface peptide mapping of protein I and protein III of four strains of Neisseria gonorrhoeae. Infection and Immunity, 37, 632-641.

Judd, R.C. (1982b). ^{125}I-Peptide mapping of protein III isolated from four strains of Neisseria gonorrhoeae. Infection and Immunity, 37, 622-631.

Kasper, D.L., Rice, P.A. and McCormack, W.M. (1977). Bactericidal antibody in genital infection due to Neisseria gonorrhoeae. Journal of Infectious Diseases, 135, 243-251.

Kearns, D.H., Siebert, B.G., O'Reilly, R., Lee, L. and Logan, L. (1973). Paradox of the immune responce to uncomplicated gonococcal urethritis. New England Journal of Medicine, 289, 1170-1174.

King, G.J. and Swanson, J. (1978). Studies in gonococcus infection. XV. Identification of surface proteins of Neisseria gonorrhoeae correlated with leucocyte association. Infection and Immunity, 21, 575-584.

Koransky, J.R., Scales, R.W. and Kraus, S.J. (1976). Bacterial hemagglutination by Neisseria gonorrhoeae. Infection and Immunity, 12, 495-498.

Kornfeld, S.J. and Plant, A.G. (1981). Secretory Immunity and the bacterial IgA proteases. Review of Infectious Diseases, 3, 521-534.

Leistikow,. (1882) Uber Backterien bei den venerischen Krankheiten. Charité Ann, 7, 750-772

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 237,680-685.

Lambden, P.R. (1979). Biochemical comparison of pili from variants of Neisseria gonorrhoeae P9. Journal of General Microbiology, 114, 305-312.

Lambden, P.R., Heckels, J.E., James, L.T. and Watt, P.J. (1979). Variation in surface protein composition associated with virulence properties in opacity types of Neisseria gonorrhoeae. Journal of General Microbiology, 114, 305-312.

Lambden, P.R. and Heckels, J.E. (1979). Outer membrane protein composition and colonial morphology of Neisseria gonorrhoeae strain P9. FEMS Microbiology Letters, 5, 263-265.

Lambden, P.R., Robertson, J.N. and Watt, P.J. (1980). Biological properties of two distinct pilus types produced by isogenic variants of Neisseria gonorrhoeae P9. Journal of Bacteriology, 141, 393-396.

Lambden, P.R., Heckels, J.E., McBride, H. and Watt, P.J. (1981a). The identification and isolation of novel pilus types produced by variants of Neisseria gonorrhoeae P9 following selection in vivo. FEMS Microbiology Letters, 10, 339-341.

Lambden, P.R., Heckels, J.E., McBride, M. and Watt, P.J. (1981b). The identification and isolation of novel pilus types produced by variants of Neisseria gonorrhoeae P9. following selection in vivo. FEMS Microbiology Letters, 10, 339-341.

Lambden, P.R., Heckels, J.E. and Watt, P.J. (1982). Effect of anti-pilus antibodies on survival of gonococci within guinea pig subcutaneous chambers. Infection and Immunity, 38, 27-30.

Lambden, P.R. and Heckels, J.E. (1982). Synthesis of immunogenic oligosaccharide-protein conjugates from the lipopolysaccharide of Neisseria gonorrhoeae P9. Journal of Immunological Methods, 48, 233-240.

Landolf, P.J., Marrie, T.J., Nelson, N.A.and Ronald, A.R. (1981). Cell mediated immune response in gonococcal infection. Canadian Journal of Microbiology, 27, 76-80.

Lind, I. and Reimann, K. (1980). Studies of the antigenic heterogeneity of Neisseria gonorrhoeae pilus antigens. In: Genetics and Immunobiology of pathogenic Neisseria, Eds., Normark, S., Danielsson, D. University of Umea, Umea pp. 95-97.

Lowe, T.L. and Kraus, S.J. (1976). Quantitation of Neisseria gonorrhoeae from women with gonorrhoea. Journal of Infectious Diseases, 133, 621-626.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurements with the Folin reagent. Journal of Biological Chemistry, 193, 265-275.

Lüderitz, O., Staub, A.M. and Westphal, O. (1966). Immunochemistry of O and R antigens of Salmonella and related Enterobactiriaceae. Bacteriological Reviews, 30, 192-255.

McBride, H., Lambden, P.R., Heckels, J.E. and Watt, P.J. (1981). The role of outer membrane proteins in the survival of Neisseria gonorrhoeae P9 within quinea pig subcutaneous chambers. Journal of General Microbiology, 126, 63-67.

McChesney, D., Tramont, E.C., Boslego, J.W., Ciak, J., Sadoff, J. and Brinton, C.C. (1982). Genital antibody response to a parenteral pilus vaccine. Infection and Immunity, 36, 1006-1012.

McDade, R.L. and Johnston, K.H. (1980). Characterization of serologically dominant outer membrane proteins of Neisseria gonorrhoeae. Journal of Bacteriology, 141, 1183-1191.

Mahoney, J.F., van Slyke, C.J., Cutler, J.C. and Blum, H.L. (1946). Experimental gonococcal urethritis in human volunteers. American Journal of Syphilis, Gonorrhoea and Venereal Diseases, 30, 1-19.

Mårdh, A.A. and Weström, L. (1976). Adherence of bacteria to vaginal epithelial cells. Infection and Immunity, 13, 661-666.

Mayer, L.W. (1982). Rates of in vitro changes of gonococcal colony opacity phenotypes. Infection and Immunity, 37, 481-485.

Melly, M.A., Gregg, G.R. and McGee, Z.A. (1981). Studies of toxicity of Neisseria gonorrhoeae for human fallopian tube mucosa. Journal of Infectious Diseases, 14, 423-431.

Merril, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science, 211, 1437-1438.

Meyer, T.F., Meawer, N. and So, M. (1982). Pilus expression in Neisseria gonorrhoeae involves chromosomal rearrangement. Cell, 40, 45-52.

Morse, S.A. and Apicella, M.A. (1982). Isolation of a lipopolysaccharide mutant of Neisseria gonorrhoeae: An analysis of the antigenic and biologic differences. Journal of Infectious Diseases, 145, 206-216.

Newhall, W.J., Sawyer, W.D. and Haak, R.A. (1980). Cross-linking analysis of the outer membrane proteins of Neisseria gonorrhoeae. Infection and Immunity, 28, 785-791.

Norlander, L., Davies, J. and Normark, S. (1979). Genetic exchange mechanisms in Neisseria gonorrhoeae. Journal of Bacteriology, 138, 756-761.

Novotny, P. and Cownley, K. (1980). Investigation of serological cross-reactivity between gonococcal pili. In: Normark, S. and Danielsson, D. eds., Genetics and immunobiology of pathogenic Neisseria. Umea, Sweden: University of Umea, 91-94.

Ofek, I., Beachey, E.H. and Bisno, A. (1974). Resistance of Neisseria gonorrhoeae phagocytosis: Relationship to colonial morphology and surface pili. Journal of Infectious Diseases, 123, 310-316.

Osborn, M.J. and Wu, H.C.P. (1980). Proteins of the outer membrane of Gram negative bacteria. Annual Review of Microbiology, 34, 369-422.

Osborn, M.J., Gander, J.E., Parisi, E. and Carson, J. (1972). Mechanism of assembly of the outer membrane of Salmonella typhimurium. Journal of Biological Chemistry, 247, 3962-3972.

Palva, E.T. and Randall, L.L. (1978). Arrangement of Protein I in Escherichia coli outer membrane: Cross linking study. Journal of Bacteriology, 133, 279-286.

Paranchych, W., Frost, L.S. and Carpenter, M. (1978). N-terminal amino acid sequence of pilin isolated from Pseudomonas aeruginosa. Journal of Bacteriology, 134, 1179-1181.

Payne, S.M. and Finkelstein, R.A. (1975). Pathogenesis and immunology of experimental gonococcal infection: role of iron in virulence. Infection and Immunity, 12, 1313-1318.

Perry, M.B., Daoust, V., Diena, B.B., Ashton, F.E. and Wallace, R. (1975). Lipopolysaccharides of Neisseria gonorrhoeae colony types 1 and 4. Canadian Journal of Biochemistry, 53, 623-629.

Perry, M.B., Daoust, V., Johnson, K.G., Diena, B.B. and Ashton, F.E. (1978). In: Brooks, G.F., Gotschlich, E.C., Holmes, K.K., Sawyer, W.D., and Young, F.E. eds. Immunobiology of Neisseria gonorrhoeae, Washington, DC., American Society for Microbiology, pp. 101-107.

Poolman, J.T., Hopman, C.T.P. and Zanen, H.C. (1983). Immunogenicity of meningococcal antigens as detected in patient sera. Infection and Immunicity, 40, 398-406.

Punsalang, A.P. and Sawyer, W.D. (1973). Role of pili in the virulence of Neisseria gonorrhoeae. Infection and Immunity, 8, 255-263.

Rees, E. and Annels, E.H. (1969). Gonococcal salpingitis. British Journal of Venereal Diseases, 45, 205-215.

Rice, P.A. and Goldenberg, D.L. (1981). Clinical Manifestations of Disseminated Infection Caused by Neisseria gonorrhoeae are linked to Differences in Bactericidal Reactivity of Infecting Strains. Annals of Internal Medicine, 95, 175-178.

Rice, P.A. and Kasper, D.L. (1982). Characterisation of Serum Resistance of Neisseria gonorrhoeae that disseminate. Journal of Clinical Investigation, 70, 157-167.

Richardson, W.P. and Sadoff, J.C. (1977). Production of a capsule by Neisseria gonorrhoeae. Infection and Immunity, 15, 663-664.

Rietschel, E.T., Gottert, H. and Lüdentz, O. (1972). Nature and linkages of the fatty acids present in the lipid A component of Salmonella lipopolysaccharides. European Journal of Biochemistry, 28, 166-173.

Roberts, R.B. (1967). The interaction in vitro between group B meningococci and rabbit polymorphonuclear leukocytes: demonstration of type specific opsonins and bactericidins. Journal of Experimental Medicine, 126, 795-818.

Robertson, J.N., Vincent, R. and Ward, M.E. (1977). The preparation and properties of gonococcal pili. Journal of General Microbiology, 102, 169-177.

Robertson, J.N. (1979). Protection by monospecific gonococcal antisera of the chicken embryo challenged with Neisseria gonorrhoeae. Journal of Medical Microbiology, 12, 283-289.

Russell, R.R.B. (1976). Free endotoxin - a review. Microbiol. letters, 2, 125-135.

Salit, I.E., Blake, M. and Gotschlich, E.C. (1980). Intrastrain heterogeneity of gonococcal pili is related to opacity colony variance. Journal of Experimental Medicine, 151, 716-725.

Sandstrom, E.G., Chen, K.C.S. and Buchanan, T.M. (1982). Serology of Neisseria gonorrhoeae: coagglutination serogroups WI and WII/WIII correspond to different outer membrane protein I molecules. Infection and Immunity, 38, 462-470.

Schlindler, H. and Rosenbusch, J.P. (1978). Matrix protein from Escherichia coli outer membranes form voltage-controlled channels in lipid bilayers. PNAS, 75, 3751-3755.

Schoolnik, G.K., Buchanan, T.M. and Holmes, K.K. (1976). Gonococci causing DGI are resistant to the bactericidal action of normal human sera. Journal of Clinical Investigation, 58, 1163-1173.

Schoolnik, G.K., Tai, J-Y. and Gotschlich, E.C. (1982). The human erythrocyte binding domain of gonococcal pili. In: Seminars in Infectious Disease, vol. IV, Bacterial vaccines, pp. 172-180. Edited by L. Weinstein, B.N. Fields, J.B. Robbins, J.C. Mill and J. Sadoff. New York: Thieme-Stratton.

Schumacher, G.F.B. and Yang, S.L. (1977). Cyclic changes of immunoglobulins and specific antibodies in human and rhesus monkey cervical mucus. In: Insler, C., Bettendorf, G. eds: The uterine cervix in reproduction. Stuttgart, F.R.G. Georg Thieme, 187-203.

Siegel, M.S., Olsen, D.A., Critchlow, K. and Buchanan, T.M. (1982). Gonococcal pili: safety, immunogenecity in human and in vitro antibody function. Journal of Infectious Diseases, 145, 300-310.

Sparling, P.F. and Yobs, A.R. (1967). Colonial morphology of Neisseria gonorrhoeae isolated from males and females. Journal of Bacteriology, 93, 513-519.

Sparling, P.F. (1977). Antibiotic resistance in the gonococcus, pp111-136. In: R.B. Roberts (ed.). The gonococcus. John Wiley and Sons, New York.

Stead, A., Main, S., Ward, M.E. and Watt, P.J. (1975). Studies on lipopolysaccharides isolated from strains of Neisseria gonorrhoeae. Journal of General Microbiology, 88, 123-131.

Swanson, J.S., Krause, S.J. and Gotschlich, E.C. (1971). Studies on gonococcus infection I. Pili and zones of adhesion: their relation to gonococcal growth patterns. Journal of Experimental Medicine, 134, 886-906.

Swanson, J. (1973). Studies on gonococcus infection IV. Pili: their role in attachment of gonococci to tissue culture cells. Journal of Experimental Medicine, 137, 571-589.

Swanson, J., King, G. and Zeligs, B. (1975). Studies on gonococcus infection. VIII. ^{125}Iodine labelling of gonococci and studies of their interactions with eukaryotic cells in vitro. Infection and Immunity, 11, 453.

Swanson, J. (1978). Studies on gonococcus infection. XII. Colony color and opacity variants of gonococci. Infection and Immunity, 19, 320-331.

Swanson, J. (1978). Studies on gonococcus infection. XIV. Cell wall protein differences among color/opacity colony variants of Neisseria gonorrhoeae. Infection and Immunity, 21, 292-302.

Swanson, J. (1979). Studies on gonococcus infection. XVIII. ^{125}I-labelled peptide mapping of the major protein of the gonococcal cell wall outer membrane. Infection and Immunity, 23, 799-810.

Swanson, J. (1980). ^{125}I-labelled peptide mapping of some heat-modifiable proteins of the gonococcal outer membrane. Infection and Immunity, 28, 54-64.

Swanson, J. (1981). Surface exposed protein antigens of the gonococcal outer membrane. Infection and Immunity, 34, 804-816.

Swanson, J. (1982). Colony opacity and protein II composition of gonococci. Infection and Immunity, 37, 359-368.

Swanson, J., Mayer, L.W. and Tam, M.R. (1982). Antigenicity of Neisseria gonorrhoeae outer membrane protein(s) III detected by immunoprecipitation and Western blot transfer with a monoclonal antibody. Infection and Immunity, 38, 668-672.

Tam, M.R., Buchanan, T.M., Sandstrom, E.G., Holmes, K.K., Knapp, J.S., Siadak, A.W. and Nowinski, R.C. (1982). Serological classification of Neisseria gonorrhoeae with monoclonal antibodies. Infection and Immunity, 36, 1042-1053.

Tauber, H. and Garson, W. (1959). Isolation of lipopolysaccharide endotoxin. Journal of Biological Chemistry, 234, 1391-1393.

Thongai, C.F. and Sawyer, W.D. (1973). Studies on the virulance of Neisseria gonorrhoeae. I. Relation of colonial morphology and resistance to phagocytosis by polymorphonuclear leukocytes. Infection and Immunity, 7, 373-379.

Tokunaga, M., Tokunaga, H., Okajima, Y. and Nakae, T. (1979). Characterisation of porins from the outer mambrane of Salmonella typhimurium. European Journal of Biochemistry, 95, 441-448.

Towbin, H., Staehlin, T. and Gordon J. (1979). Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences of the United States of America, 76, 4350-4354.

Tramont, E.C. (1977). Inhibition of adherence of Neisseria gonorrhoeae by human genital secretions. Journal of Clinical Investigations, 59, 117-124,

Tramont, E.C., Hodge, W.C., Gilbreath, M.J. and Ciak, J. (1979). Differences in attachment antigens of gonococci in re-infection. Journal of Laboratory and Clinical Medicine, 93, 730-735.

Tramont, E.C., Sadoff, J.C., Boslego, J.W., Ciak, J., McChesney, D., Brinton, C.C., Woods, S. and Takafuji, E. (1981). Gonococcal pilus vaccine: studies of antigenicity and inhibition of attachment. Journal of Clinical Investigation, 68, 881-888.

Trust, T.J.., Lambden, P.R. and Watt, P.J. (1980). The cohesive properties of variants of Neisseria gonorrhoeae strain P9: Specific pilus-mediated and non-specific interactions.
Journal of General Microbiology, 119, 179-187.

Trust, T.J., Gillespie, R.M., Bhatti, A.R. and White, L.A. (1983) Differences in the adhesion properties of Neisseria meningitidis for human buccal epithelial cells and erythrocytes.
Infection and Immunity, 41, 106-113.

Virji, M. and Everson, J.S. (1981). Comparative virulence of opacity variants of Neisseria gonorrhoeae strain P9. Infection and Immunity, 31, 965-970.

Virji, M. and Heckels, J.E. (1983). Antigenic cross-reactivity of Neisseria pili: Investigation with type- and species-specific monoclonal antibodies. Journal of General Microbiology, 129, 2761-2768.

Virji, M., Heckels, J.E. and Watt, P.J. (1983). Monoclonal antibodies to gonococcal pili: Studies on antigenic determinants on pili from variants of strain P9. Journal of General Microbiology, 129, 1965-1973.

Wang, S.P., Holmes, K.K., Knapp, J.S., Ott, S. and Kyzer, D.D. (1977). Immunogenic classification of Neisseria gonorrhoeae with microimmunofluorescence. Journal of Immunology, 119, 795-803.

Ward, M.E. and Watt, P.J. (1971). The preservation of gonococci in liquid nitrogen. Journal of Clinical Pathology, 24, 122-123.

Ward, M.E. and Watt, P.J. (1972). Adherence of Neisseria gonorrhoeae to urethral mucosal cells: an electron microscope study of human gonorrhoea. Journal of Infectious Diseases, 126, 601-605.

Ward, M.E., Watt, P.J. and Robertson, J.N. (1974). The human fallopian tube: a laboratory model for gonococcal infection. Journal of Infectious Disease, 129, 650-659.

Ward, M.E. and Watt, P.J. (1975). Studies on the cell biology of gonorrhoea. In: Genital Infections and their Complications. (Eds. Danielsson, D., Juhlin, L. and Mardh, P) pp. 229-242. Almqvist and Wiksell International, Stockholm, Sweden.

Ward, M.E., Lambden, P.R., Heckels, J.E. and Watt, P.J. (1978). The surface properties of Neisseria gonorrhoeae: determinants of susceptibility to antibody complement killing. Journal of General Microbiology, 108, 205-212.

Watt, P.J., Ward, M.E., Heckels, J.E. and Trust, T. (1978). Surface properties of Neisseria gonorrhoeae: Attachment to and invasion of mucosal surfaces. In: "Immunobiology of Neisseria gonorrhoeae", pp. 253-257. Edited by G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer and F.E. Young. Washington, D.C: American Society for Microbiology.

Westphal, O., Lüderitz, O. and Bister, F. (1952). Uber die Extraktion von Bakterien mit Phenol/Wasser. Zeitschrift fur Naturforschung, 76, 148-155.

Wiesner, P.J., Handsfield, H.H. and Holmes, K.K. (1973). Low antibiotic resistance of gonococci causing disseminated infection. New England Journal of Medicine, 228, 1221-1222.

Wiseman, G.M. and Caird, J.D. (1977). Composition of the lipopolysaccharide of Neisseria gonorrhoeae. Infection and Immunity, 16, 550-556.

Wolf-Watz, H., Elmros, T., Normak, S. and Bloom, G.D. (1976). Cell envelope of Neisseria gonorrhoeae. A comparitive study with Escherichia coli. British Journal of Venereal Diseases, 52,142-145.

Zak, K., Diaz, J-L, Jackson, D. and Heckels, J.E. (1983). Antigenic variation during infection with Neisseria gonorrhoeae. Detection of antibodies to surface proteins in sera of patients with gonorrhoea. Journal of Infectious Diseases, 149, 166-173.