Host-parasite interactions with the pathogenic *Neisseria* species

by

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To my parents and C.F.T.

CONTENTS

INDEX OF TABLES

INDEX OF FIGURES

ABBREVIATIONS GLOSSARY

B	Dulbecco's supplement B
DPBS	Dulbecco's Phosphate Buffered Saline
EGTA	Ethylene glycol bis (B - amino ethyl ether) N, N, N', N' - tetracetic acid
FHS	Fresh Human serum
HIS	Heat Inactivated Serum
KDO	2-Keto-3-deoxyoctulosonic acid
LPS	lipopolysaccharide
MNYC	Modified New York City Medium
NHS	Normal Human Serum
PBS	Phosphate Buffered Saline
PMN	Polymorphonuclear Leukocyte (neutrophil)
SDS-PAGE	Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
Tris	Tris (hydroxymethyl) amino methane
Glu	Glucose
Gal	Galactose
GluN	Glucosamine
GalN	Galactosamine
GluNAc	N-acetyl glucosamine
GalNAc	N acetyl galactosamine
Hep	Heptose

DECLARATION

This thesis is my own composition. Some of the experimental work was done in collaboration with members of the Department of Microbiology, University of Birmingham and their contributions acknowledged in the text.

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Summary

Epidemiological evidence suggests that individuals of blood group B are more susceptible to infection by Neisseria gonorrhoeae than individuals of other blood groups. This report tests the hypothesis that this susceptibility might be due to a comparative deficiency in 'natural' bactericidal antibodies reactive with the gonococcus in individuals of blood group B. Titration of sera of blood groups A and B for 'natural' bactericidal antibodies reactive with a panel of gonococcal strains showed that differences in the titres obtained depended on the gonococcal strain used in the tests rather than the blood group of the serum. The hypothesis was found to be true only with certain strains. Some strains were found to be resistant to all the sera with which they were tested.

A correlation between serum-resistance and resistance to rod-type (R-type) pyocins of Kageyama's class R1 is described. Strains of N. gonorrhoeae were classified into two groups on the basis of pyocin sensitivity; Group I strains were resistant to R1 pyocins and Group II strains were sensitive to R1 pyocins. Both groups were sensitive to pyocins of Kageyama's class R5. Group II strains were generally found to be killed by normal human serum, whereas all Group I strains were serum-resistant.

Since pyocins and 'natural' bactericidal antibodies react with the lipopolysaccharide (LPS) molecule of the gonococcus, it was proposed that physical, chemical and antigenic differences would exist between the LPS molecules of Group I and Group II strains. Examination of the LPS isolated from strain P280 (Group II) and its serum-resistant, R1 pyocin-resistant, isogenic mutant, P280μ (Group I), confirmed this proposal.

The hypothesis proposing that induction of phenotypic serum-resistance in N. gonorrhoeae is associated with the loss of sensitivity to R1 pyocins was tested. When treated with the inducing factor, N. gonorrhoeae strain BS4 (agar) lost sensitivity to class R1 pyocins. This result was in agreement with the hypothesis.

Cross reactions between Group I and Group II strains were demonstrated using bactericidal immune mouse sera. It was proposed that similar cross-reacting bactericidal antibodies reactive with Group I gonococcal strains would be formed during human infection. An examination of the sera of 22 patients with gonorrhoea failed to support this proposal.

Non-serogroupable strains of N. meningitidis have also been shown to express the same pyocin sensitivity patterns as gonococcal strains of Groups I and II. The hypothesis that a similar relation between serum-sensitivity and pyocin-type exhibited by gonococci was shown in non-serogroupable meningococci was examined. The experiments refuted this proposal.

In a short appendix to this report, a system to determine the oxidative metabolism of glass-adherent mononuclear phagocytes stimulated by N. gonorrhoeae is described. When stimulated by N. gonorrhoeae, the responses of monocytes taken from four individuals, one from each ABO blood group were heterogeneous. A tentative speculation that the poor response observed with monocytes of blood group B might be a factor associated with the observed increase in susceptibility of individuals of this blood group to gonorrhoea is made.

ABSTRACT

Individuals of blood group B have been shown to be more susceptible to infection by Neisseria gonorrhoeae than those of other ABO blood groups. Isohaemagglutinins have been shown to opsonise and kill bacteria possessing antigens with ABO blood group specificity. The principal gonococcal antigen reactive with 'natural' bactericidal antibodies, the lipopolysaccharide (LPS) molecule contains terminal galactose residues. Galactose is the immunodominant sugar of B blood group substance. This report examined the hypothesis that the increased susceptibility of blood group B individuals was associated with a lower bactericidal capacity of B serum against N. gonorrhoeae due to the absence of anti-B isohaemagglutinins which are present in sera of blood groups A and O. A comparison of the bactericidal activity of 16 group A sera and 16 group B sera against 14 strains of N. gonorrhoeae isolated from localised infection and 2 strains isolated from disseminated infection was made. These strains were distributed into two groups, Group I and Group II, on the basis of LPS type, as determined by sensitivity to a panel of rod-type pyocins isolated from strains of Pseudomonas aeruginosa. Group A sera gave higher titres than Group B sera against 2/4 serum-sensitive strains ($P < 0.005$). Absorption of isohaemagglutinins did not reduce the titre of bactericidal antibody. It was removed, however, by absorption with other Neisseriae species. Serum sensitivity was related to pyocin type for the strains isolated from localised infections: Group I strains were serum-resistant; Group II strains were serum-sensitive. The strains isolated from disseminated infection were serum-resistant, irrespective of pyocin type. The LPS of a serum-sensitive strain, P280 (Group II), and an isogenic serum-resistant mutant, P280μ (Group I) were compared by physical, chemical and biological criteria and were found to differ in all three respects. Induction of N. gonorrhoeae strain 8S4 (agar) to phenotypic serum resistance by incubation with an ultrafiltrate from guinea pig serum was found to be associated with changes in the pyocin type which suggests that alterations occur within the structure of the LPS molecule during this treatment.

Cross-reactivity of N. gonorrhoeae strains of Group I and Group II was determined in a bactericidal test using immune mouse serum. It was proposed that bactericidal antibody reactive with Group I

strains of gonococci would form during infection. An examination of sera from 22 patients (mainly female) with gonorrhoea did not detect bactericidal antibodies against Group I strains in 19/22 sera.

The susceptibility of strains of N. meningitidis to bactericidal antibodies in serum from normal individuals and patients with gonorrhoea was compared with that observed with strains of N. gonorrhoeae. In contrast to the results obtained with N. gonorrhoeae, pyocin type did not influence susceptibility of the non-serogroupable strains to serum. No significant differences in titre was observed between group A and group B sera. Whereas normal sera demonstrated moderate titres of bactericidal antibody against a strain of serogroup A (geometric mean titres - group A serum, 10.5; group B serum 24.7) small or undetectable amounts of bactericidal antibody against strains of serogroups A, B and C (geometric mean titres, 3.4; 2.6 and 4.0 respectively) were observed in 18 sera from patients with gonorrhoea. The possibility that this absence of bactericidal activity might predispose patients with gonorrhoea to meningococcal disease is discussed.

A short appendix to this report is the development of a system to determine the oxidative metabolic response of glass-adherent human peripheral blood monocytes to stimulation with N. gonorrhoeae. The test used nitroblue tetrazolium (NBT) reduction as qualitative and semi-quantitative measure of superoxide generation by individual monocytes. A comparison of the response of monocytes from donors of blood groups A, B, AB and O to two strains of N. gonorrhoeae suggests that there was variation in the degree of response which was dependent on the presence of both reactive antibody and complement. The intensity of response was less for monocytes of blood group B than for monocytes of blood groups A, AB and O.

GENERAL INTRODUCTION

The major infectious diseases that once threatened mankind have been controlled by preventive practices such as improvements in sanitation, development of immunisation programmes and the use of antibiotic therapy and prophylaxis. There are still, however, infectious diseases that continue to evade concerted efforts to control them; two of these infections are meningococcal disease and gonorrhoea.

Both of these infections are caused by members of the genus Neisseria. In spite of the development of antibiotics and some vaccines, disease due to Neisseria meningitidis is still a world-wide medical problem, particularly of the developing countries in Africa and South America. Medical advances have also been slow to halt the spread of gonorrhoea, a disease caused by Neisseria gonorrhoeae.

Gonococcal conjunctivitis in the newborn (opthalmia neonatorum), once a major cause of blindness, has been greatly reduced due to the use of silver nitrate eye drops. (Rein, 1977). In contrast to neonatal infections, gonorrhoea, especially in young adults, is one of the most common notifiable infectious diseases in the United Kingdom, and is a major burden on the financial resources of many nations. In 1979 the cost of medical care for the women in America who developed salpingitis as a consequence of gonorrhoea was 1.25 billion dollars. (May, 1981).

Individuals are not equally susceptible to infectious diseases. An examination of the mechanisms underlying the differential susceptibility within a population may lead to a fuller understanding of the complex interactions between host and parasite. This information, though not essential for the development of effective vaccines, is a necessary basis for the scientific development of gonococcal vaccines.

This report examines the interaction between 'natural' bactericidal antibodies, present in the serum of normal individuals, and organisms of the pathogenic Neisseria species, N. gonorrhoeae and N.meningitidis. In the subsequent introductory chapter I will briefly outline the nature and occurrence of diseases due to these micro organisms, and then discuss at greater length the role of their surface components in the pathogenesis of the disease states that they cause and the interaction of the immune systems of normal individuals and patients with gonorrhoea with these organisms. The two species, N. gonorrhoeae and N. meningitidis, are compared with regard to these points, but throughout, the main emphasis is on the gonococcus.

1. DISEASES CAUSED BY N.GONORRHOEAE and N.MENINGITIDIS

1.1 A Brief Description of the Diseases

Infections with Neisseria gonorrhoeae and Neisseria meningitidis are limited to humans; there is no other natural host. When restricted to the mucosa, the infection may be asymptomatic, or, as in the case of gonorrhoea, result in distressing, but not life threatening, symptoms. Bacteraemic spread from the submucosal tissues is associated with much more serious conditions which can rapidly lead to death of the host.

1.1.1 Gonorrhoea

Gonorrhoea results from infection with N.gonorrhoeae. This disease is mainly transmitted by sexual intercourse and, consequently, is principally an infection of the genito-urinary tract. Infections of the oral and rectal mucosa are also frequently observed. Generally, the organism is restricted to the mucosa at the site of infection, but it may spread to adjacent tissues. In a small percentage of cases bacteraemia results in disseminated gonococcal infection (DGI); which may involve infection of the skin, joints and major organs of the body.

1.1.2 Clinical features of uncomplicated gonococcal infection

1.1.2.1 Genital Infection

The majority of men (approx. 85%) with genital gonorrhoea develop a purulent urethral discharge. This contrasts with infection in females, where about 70% of infections are asymptomatic. Female genito-urinary infection occurs predominantly in the cervical (85-90%) and urethral (65-75%) mucosa (Robertson, McMillan and Young, 1980). In both sexes the infection may spread from the mucosa of the genito-urinary tract leading to abscess formation in the peri- and para-urethral glands. Infection of the prostate, seminal vesicles

and epididymis can occur as a local complication of infection in the male. Similarly,infection in the female, especially when untreated, can result in ascending spread of the infection to include the uterus and fallopian tubes (salpingitis). This results in a condition termed pelvic inflammatory disease (PID) and is found in approximately 10 per cent of women with untreated gonococcal infection.

1.1.2.2 Oropharyngeal Infection

Infection of the pharynx results from oro-genital sex. It is only associated with symptoms in about one fifth of cases. The main clinical feature is a sore throat, perhaps with referred pain in the ear (Robertson, McMillan and Young, 1980).

1.1.2.3 Ano-rectal Infection

Ano-rectal infection with N.gonorrhoeae is generally asymptomatic. In symptomatic cases (about 30%) anal pruritus and a purulent discharge are common features. Histological examination shows epithelial degeneration and sub-epithelial infiltration with granulocytes in only a small proportion (3/57) of cases (McMillan, et al, 1983).

1.1.2.4 Gonococcal conjunctivitis

Gonococcal conjunctivitis is a purulent infection which, if left untreated may result in the whole eye becoming infected. This may lead to blindness. Although gonococcal conjunctivitis of the newborn (opthalmia neonatorum) was once the largest single cause of blindness, accounting for one third of all cases entering schools for the blind, no cases of blindness have occurred in the United Kingdom due to this infection since 1955. (Robertson, McMillan and Young, 1980).

1.1.3 Disseminated gonococcal infection (DGI)

Haematogenous spread of N.gonorrhoeae from the primary site of infection is a rare event. It is found in less than one per cent of cases of gonococcal infection, and occurs most frequently in women and homosexual men in whom the primary infection was either asymptomatic or untreated. Patients with DGI usually have the following symptoms; fever, rash, and painful joints. In this form of the disease the organism can usually be isolated from the blood, but not from the synovial fluid of affected joints. In another form, which may be consecutive to the former one, the arthralgia is localised to one joint. Examination of the synovial fluid shows a purulent infiltrate of neutrophils. N.gonorrhoeae can be isolated from the synovial fluid in this presentation.

Skin lesions may be associated with DGI. Two forms have been described; haemorrhagic lesions and vesiculo-papular lesions. Histological examination of these lesions has demonstrated infiltration of granulocytes and mononuclear cells. Gonococcal antigens may be detected using immunofluorescent techniques. (Robertson, McMillan and Young, 1980) and circulating immune complexes have been detected in a patient with gonococcal bacteraemia (Danielsson, Norberg and Svanbom, 1975).

Rare complications of DGI include meningitis, endo- and peri-carditis and hepatitis.

1.2 Meningococcal Disease

Infection of the oropharyngeal mucosa with *N.meningitidis* usually results in an asymptomatic carrier state. Meningococcal disease, which results from bacteraemic spread of the organisms from the mucosa, is a rare event. A peak incidence of about 38 cases per 100,000 individuals has been recorded during a severe epidemic (De Voe, 1982). Herrick (1919) described three stages of invasion by *N.meningitidis* :

1. A meningococcal bacteraemic phase resulting from penetration of the oropharyngeal mucosa;
2. Involvement of the skin, eyes, joints, heart, adrenals and meninges. 90% of cases are associated with inflammation of the pia and arachnoid, and half of these also have petechial and large purpura;
3. Eruption of skin lesions. Eruption of skin lesions is one of the most consistent features of meningococcal disease (De Voe, 1982).

The clinical response of the host to meningococcal disease can vary from a benign meningitis to a fulminant meningococcaemia in which haemorrhage and capillary thromboses result in shock and a rapid death.

1.3 Epidemiology

1.3.1 Gonorrhoea

Gonorrhoea has a world-wide distribution and is a major health problem in many countries. Man is the only natural host for Neisseria gonorrhoeae and Rein (1977) has attributed the success and historical persistence of this pathogen to "the prodigious efforts of humanity on its behalf".

During and after the Second World War the population of the United Kingdom made considerable efforts on behalf of the gonococcus, resulting in a peak incidence of 47,343 cases in 1946. The incidence then dropped rapidly up to 1954, when only 17,536 cases were reported. (Venereal Diseases in England and Wales, 1959). This drop may, in part, be attributable to the death of young people during the war, since gonorrhoea is a disease of young adults. Most cases occur among the 20-24 year olds. Women tend to get gonorrhoea at an earlier age than men, and the second highest incidence is in the 15-19 year olds, primarily due to the female contribution. (Rein, 1977; Robertson, McMillan and Young, 1980).

The 1960s saw a steady rise in incidence of gonorrhoea in most countries in Western Europe that has continued up to recent times. The United States and Sweden experienced a more explosive increase in incidence of gonorrhoea, the peak incidence being about 400 cases per 100,000 inhabitants in both countries (Kellings and Moberg, 1977). This rise in incidence is due to an increase in promiscuity and a change in the pattern of contraceptive use. The use of barrier methods of contraception, such as the condom, can provide effective prophylaxis against genital gonorrhoea. Such protection is absent for those using oral contraceptives or intra-uterine devices. (Rein, 1977).

Thirty years ago epidemiological reports showed great disparity between the sexes vis à vis the incidence of gonorrhoea. In 1946 the ratio of male infections to female infections was 3.5 : 1 and in 1954 it was 3.9 : 1. (Venereal Diseases in England and Wales, 1959). Nowadays this ratio is approaching unity. (Robertson, McMillan and Young, 1980). This is probably due mainly to improved methods of diagnosing gonorrhoea in the asymptomatic female, rather than any radical change in patterns of sexual behaviour. (Rein, 1977).

1.3.2.1 Meningococcal Disease

Epidemic meningitis has been prevalent in many countries for several centuries. In recent times an area with a high incidence of meningococcal disease has been reported in equatorial Africa; this is the meningitis belt. The reason for this geographical link with disease due to N.meningitidis is not known, although the association of perennial epidemics with the end of the season of dust storms (the harmattan) and the beginning of the annual rainy season implicates climatic and environmental factors.

There is no other endemic region comparable to the meningitis belt. Epidemics occur less frequently in other parts of the world. Prior to 1940, large epidemics occurred in all parts of the world, with a local inter-epidemic period of about 10 years. (Peltola, 1983) Exceptionally large epidemics were associated with the Second World War. In spite of the successes in vaccine development and the widespread availability and use of anti-microbial drugs, meningococcal disease is "still with us". In the 1970's epidemics of meningococcal disease were seen in at least thirty countries. (Peltola, 1983).

1.3.2.2 Age-Distribution

During non-epidemic conditions cases of meningococcal disease are seen most frequently in children less than five years old.

In a study of meningococcal disease in the Netherlands during the period 1971 to 1980, over half of the 202 cases were children in the age group 0-4 years old and one fifth of the patients were in the age range 10-19 years old. (Zanen, 1980). During epidemic conditions a statistically significant shift in the age distribution towards the older groups has been observed (Peltola, 1983). Peltola, Kataja and Mäkelä (1982) suggested that this shift in age distribution might be predictive of an imminent epidemic.

1.3.2.3 Sex Distribution

Although meningococcal disease affects both sexes, a higher incidence among males is generally reported. (Peltola, 1983). This has been partly due to the large numbers of cases seen in male institutions such as military camps and lodging houses for male chronic alcoholics. With the information available it is difficult to form any final conclusions on the influence of gender on susceptibility to meningococcal disease.

1.3.2.4 Meningococcal Disease in Military Institutions

The incidence of meningococcal disease among military recruits is ten times higher than in the general population (Peltola, 1983). The risk of disease is greatest during the first few weeks of training, but not later. Bristow, van Peenen and Volk (1965) described an epidemic within a Naval Training Centre in which 80% of cases were recruits who had been at the Centre for less than 5 weeks. Permanent military staff are not a special risk group.

1.3.2.5 Meningococcal Serogroup Distribution

At least eight serogroups of N.meningitidis have been recognised (A,B,C,X,Y,Z,W135 and 29E (or Z')) on the basis of the immunochemical characteristics of the capsular polysaccharide. (See section 1.5.6.1) Groups, A,B and C account for over 90% of all

cases of meningococcal disease (Peltola, 1983). Recently there has been an increase in the isolation of meningococci of serogroups X,Y, Z, W135 and 29E (or Z'). Data from the Centre for Disease Control in Atlanta, USA, shows that in 1978 infections due to serogroups C and Y were approximately equal in number, wherease in 1971-2 group C organisms accounted for 63% of cases and group Y, 8.6%. (Counts and Petersdorf, 1980). The reasons for such a change in frequency of the serogroups causing disease are not completely defined. Since meningococcal disease principally affects children, and the resistance of older people is due in part to serum antibodies reactive with the capsular antigens (Goldschneider, Gotschlich and Artenstein, 1968; See section 1.8.4.1), these changes in prevalence of serogroup are probably reflections of the state of anti-meningococcal herd immunity within a population.

1.4 Some Host Factors Associated with susceptibility to infection with *Neisseria gonorrhoeae* or *Neisseria meningitidis*

All people are susceptible to infection by the pathogenic *Neisseria* species, but some are more susceptible than others. The differential susceptibility of individuals may arise from many factors. Social conditions and behavioural patterns frequently determine the risk of meeting an infected individual in circumstances which are favourable to the successful transmission of the pathogen. This is especially true of gonorrhoea, where the disease is almost exclusively restricted to sexually active people. Other factors which may be associated with increased susceptibility to infections with the pathogenic *Neisseria* species include the genetic make-up of the individual, HLA type; absence of 'natural antibodies', and the presence of underlying pathologies.

1.4.1 Genetic Factors

Congenital absence of the terminal components of the complement pathway predisposes to recurrent bacteraemias, especially with *N.gonorrhoeae* and *N.meningitidis* (Lee *et al*, 1978; Lee *et al*; 1979; Nicholson and Lepow, 1979; See sections 1.7.2.3 and 1.7.3).

Certain haplotypes of the human major histocompatibility gene complex have been associated with an increased susceptibility to disease. For example, the haplotype B27 is found in a high proportion of patients with ankylosing spondylitis. Little work has been done in trying to find associations between human genotypes and susceptibility to gonorrhoea. One report, however, shows that in the Chinese population in Singapore, HLA-B17 is found more frequently (26%) in prostitutes suffering from both gonorrhoea and syphilis than those in the normal, uninfected population (7%). Furthermore, the HLA-B17 haplotype was associated with a poor blastogenic response of lymphocytes to gono coccal and treponemal antigens (Chan and Rajan,1982)

1.4.1.1 ABO Blood Group

Epidemiological evidence has demonstrated the association of several infectious diseases, including gonorrhoea, with ABO blood group (Kinane et al; 1982; Foster and Labrum 1976; Kinane et al 1983 b). In a study of 565 patients attending a sexually transmitted diseases clinic in Edinburgh, Kinane and colleagues (1983 b) found the proportion of blood group B in all patients was 13.2%; similar to the incidence of blood group B in that locality (10.7%). After segregation of the patients on the basis of diagnosis, the incidence of blood group B in those patients with gonorrhoea was 21.1%. This is higher than the incidence in patients without gonorrhoea (12%) but the difference is not statistically significant. It is, however, a statistically significant increase in incidence of blood group B compared with the control population.

Foster and Labrum (1976) proposed that the anti-B isohaemagglutinin, which is absent in individuals of blood groups B and AB might be a better opsonic antibody than the anti-A isohamagglutinin. This, they suggested, might provide an immunological basis for the observed increased susceptibility of individuals of blood group B. The data does not allow comment on the susceptibility of people with the AB blood group, since their numbers were too small.

This project, in tandem with one conducted principally by Dr D F Kinane, sought to examine the biological effects of A and B isohaemagglutinins on N.gonorrhoeae. The binding of gonococci to human mononuclear phagocytes has been shown to be enhanced by isohaemagglutinins. In addition, the degree of binding is greatest for monocytes from blood group B individuals compared with cells taken from individuals of the other ABO blood groups (Kinane et al, 1983 a). This evidence contradicts the proposal of Foster and Labrum (1976), yet, when viewed in the light of a scheme for the pathogenesis of gonorrhoea proposed by Novotny et al, (1977)it indicates a

mechanism by which the susceptibility of individuals of blood group B may be increased. This is discussed in detail in Section 1.7.2.1.1 *et seq.*

A hypothesis relating the potential bactericidal activities of anti - B isohaemagglutinins to increased host resistance to gonorrhoea is stated in Section 1.10.1.

1.4.2 'Natural' antibodies

Susceptibility to meningococcal disease has been shown to correlate with the absence of 'natural' antibodies which act together with the complement system to kill *N.meningitidis* (Goldschneider, Gotschlich and Artenstein, 1969; Goldschneider, Gotschlich and Artenstein, 1969 b).

'Natural' antibodies reactive with the somatic antigens of gram-negative bacteria, such as *N.gonorrhoeae* and *N.meningitidis* are principally of the IgM class (Michael and Rosen, 1963). Severe IgM deficiency, which was thought to be familial, has been described in patients dying from fulminating meningococcal septicaemia. (Hobbs, Milner and Watt, 1967).

1.4.3 The influence of infection with *N.gonorrhoeae* on oropharyngeal infection with *N.meningitidis*

The incidence of meningococcal colonisation of the pharnyx has been shown to be higher in patients with gonorrhea than in those without. Young,Harris and Robertson (1979) studied over 3000 patients with gonorrhoea and found that the meningococcus was isolated 3.4 times more frequently from men and twice as frequently from women with genital gonorrhoea than in those without. They suggested that the preponderance of meningococcal isolates from individuals with gonorrhoea may be more a reflection of behavioural patterns than of any difference in host suscepitibility. Similar

results were obtained from a smaller sample by Ødegaard and Gedde-Dahl (1979).

Young and colleagues (1983) have continued their studies on this phenomenon; analysis of the serogroup of the meningococcal strains isolated from the oropharynx of patients with gonorrhoea revealed that women with gonorrhoea were more likely to be colonised by serogroupable meningococci than were women without gonorrhoea. There were no significant differences in the frequency of colonisation with non-serogroupable meningococci between patients with gonorrhoea and controls without gonorrhoea. No such differences were noted for male patients.

1.5 Anatomy and Physiology of the pathogenic Neisseria species

1.5.1 Introduction

N. gonorrhoeae and N.meningitidis are, in common with other members of the genus Neisseria, gram negative cocci. They usually occur in pairs with the long axes parallel and opposing surfaces showing slight concavity. All members of the genus can oxidise dimethyl - or tetramethyl paraphenylene diamine (TMPD), and this, together with typical staining and microscopic morphology, form the main distinguishing features used by medical microbiologists in their identification of the Neisseria spp. Differentiation of the Neisseria species is accomplished by sugar utilisation tests. (Table I).

1.5.1.1 Colonial morphology

When grown on solid medium both N.gonorrhoeae and N.meningitidis exhibit a variety of colonial forms. Kellogg et al (1963) described four colony types for N.gonorrhoeae; T1 to T4. Colony types T1 and T2 predominate in fresh isolates, but indiscriminate subculture results in a shift to the T3 and T4 colony types. Each colony type can be maintained by daily selective subculture, and the shift in colony type from T1/T2 to T3/T4 can be reversed by isolation and selective subculture of revertants. After prolonged selective subculture in vitro of the four colony types of N.gonorrhoeae strain F62, Kellogg et al (1968) found that only colony types T1 and T2 gave rise to infection after urethral inoculation of human volunteers. Two additional gonococcal colony types have been described; T5 (Jephcott and Reyn, 1971) and T1' (Chan and Wiseman, 1975). Colony types T1, T2 and T1' are capable of infecting embryonated hens eggs whereas types T3, T4 and T5 are not (Chan and Wiseman, 1975). Electron microscopy has revealed that pili are present in large numbers of organisms from colonies of

TABLE I

Carbohydrate Utilisation of *Neisseria* species

		glucose	lactose	maltose	fructose	sucrose
N.gonorrhoeae		+	-	-	-	-
N.meningitidis		+	-	+	-	-
N.lactamica		+	+	+	-	-
N.subflava	)	+	-	+	-	-
N.flava	)	+	-	+	+	-
N.perflava	) *N.pharyngis*	+	-	+	+	+
N.sicca	)	+	-	+	+	+

types T1 and T2, but sparse or absent in those of T3 and T4 (Jephcott, Reyn and Birch-Anderson, 1971).

Penn, Veale and Smith (1977) developed a simple lighting system which allows the colonies to be characterised by the number of highlights observed. Organisms from double-highlight (DH) colonies are similar to those in Kellogg's groups T1 and T2. Organisms from single-highlight (SH) and no-highlight (NH) colonies have fewer pili and are similar to those found in Kellogg's T3 and T4 colonies (Kellogg et al, 1963).

N.meningitidis also exhibits variation in colonial morphology and pilation. DeVoe and Gilchrist (1978) have described six different colony types, M1 to M6. Most strains in their study elaborated only an M1 colony type, that was similar in appearance to T4 colonies of N.gonorrhoeae. Meningococci resemble gonococci in possessing pili upon primary isolation and (usually) in subsequent loss of pilus expression after subculture. However, there is no correlation between pilation and colony morphology such as that observed with gonococci. DeVoe and Gilchrist (1978) observed several combinations of colonial morphology and pilation in their meningococcal strains.

The question of a causal relationship between degree of pilation and colonial morphology has been addressed by McGee et al (1977). They found no consistent relationship and concluded that unless the pili of N.gonorrhoeae are unique among Neisseria species in their ability to influence colonial morphology, other factors, in addition to pilation are responsible for the colonial morphology of T1 and T2 gonococci.

The colouration and opacity of gonococcal colonies has also been noted to vary. (Swanson, 1978a). Swanson found that these optical properties of gonococcal colonies were independent of pilation (Swanson, 1978a) and appeared to correlate with differences in cell surface proteins that he termed 'colony opacity - associated proteins' (Swanson, 1978b). Opaque colonies are predominant in isolates from

male patients whereas transparent colonies are more typical of female isolates, particularly if the woman is menstruating. This is thought to be due to the susceptibility of the opaque colonial type to proteolytic enzymes such as trypsin and, possibly, those occurring in cervical mucus and menstrual blood. (James and Swanson, 1978).

1.5.2 The Cell Envelope

The cell envelope of the pathogenic Neisseria species conforms to the general characteristics of gram-negative cells. Electron-microscopic examination of thin sections of N.gonorrhoeae reveals an undulating outer membrane, periplasmic space and peptidoglycan layer, with zones of adhesion between the outer membrane and peptidoglycan occurring at frequent intervals. (Fitz-James et al, 1964). A similar picture is observed with meningococci (Swanson and Goldschneider, 1969). Both N.gonorrhoeae and N.meningitidis release vesicles, or 'blebs', of outer membrane during rapid growth, (Stead et al, 1975; DeVoe and Gilchrist, 1973). Pili are observed on both species, but the evidence for possession of capsular material is only clear-cut for certain strains of N.meningitidis.

The components of the cell envelope will be discussed with regard to their role in host-parasite interactions.

1.5.3. Pili

Pili have been observed on both pathogenic and non-pathogenic Neisseria species. (Jephcott, Reyn and Birch-Anderson, 1971; McGee et al, 1977). McGee et al, (1977) described two types of pilus: short pili (175-210nm in length) found only on non-pathogenic species, and longer pili (up to 4,300nm) found on both pathogenic and non-pathogenic species. Pili are polymers of protein subunits. Recent work has shown that a single strain of gonococcus, strain P9, is capable of expressing one of four pilus subunit types :

α (Mr 19.5k), β (Mr 20.5k), γ (Mr 21k) and δ (Mr 18.5k) (Lambden et al, 1981). These pilus variants are antigenically distinct (Virji, Heckels and Watt, 1983) yet monoclonal antibody analysis has shown a common epitope which has also been found on pili from fresh meningococcal isolates. (Virji and Heckels, 1983). These workers also found meningococcal pili that did not have this common epitope and suggested that there may be greater antigenic diversity among meningococcal pilus subunits. The complete amino acid sequence analysis for a gonococcal pilus (Mr 17,497) from strain MS11 has recently been elucidated (Schoolnik et al, 1984)

Bacterial pili have been demonstrated as adhesins in other genera (Ottow, 1975) and studies on both the gonococcus and the meningococcus have demonstrated that pili enhance the degree of binding to cells of many types. Studies with the gonococcus show enhanced binding to red blood cells (Punsalang and Sawyer, 1973; Koransky, Scales and Kraus, 1975), human vaginal epithelium (Mardh and Westrom, 1976),human fallopian tube (Watt and Ward, 1977) and sperm cells (James-Holmquest et al, 1974). Care must be taken in extrapolating these data to the invivo situation since the cell type used in binding studies is not always that involved in pathogenesis.

Binding studies have shown pili to enhance attachment of meningococci to nasopharyngeal and buccal cells (Stephens and McGee, 1981). Two possible methods by which pili might facilitate attachment have been postulated (Watt and Ward, 1977). The small diameter of the pilus might allow it to penetrate the repulsive charge barrier between the surfaces of the bacterium and the epithelial cells so as to facilitate binding by hydrophobic bonding. Alternatively the pilus might act by binding to specific receptor(s) on the cell surface. They suggested that this receptor might be a saccharide moiety since this had been described for haemagglutinating pili of other species (Ottow, 1975).

The hypothesis that there is a saccharide pilus receptor on the cell surface has gained impetus from the work of Buchanan and colleagues (Buchanan, Pearce and Chen, 1978) who found that binding of purified, radiolabelled gonococcal pili could be inhibited from binding to a variety of human cell types by gangliosides (especially GT2), heparin (a sulphated mucopolysaccharide) and pre-treatment of the cell surface with glycosidases. This receptor is thought to be an oligosaccharide since binding is unaffected by the constituent simple sugars of the cell surface. However, small oligosaccharides can disperse aggregated pili (Watt and Ward, 1980).

Gonococcal pili of the four variant types have been shown to exhibit different binding characteristics (Heckels, 1982), but with most cell types their effect is to enhance binding. However, with PMN the gonococcal pilus appears to hinder cell-bacterium interactions and prevent phagocytosis (Thongthai and Sawyer, 1973; Ofek, Beachey and Bisno, 1974; Dilworth, Hendley and Mandell, 1975), possibly by reducing membrane fluidity around the forming phagosome (Densen and Mandell, 1978). Swanson (1977) suggested that the major gonococcal determinant influencing binding to PMN is a gonococcal surface protein which he has termed 'leukocyte association factor' (LAF).

In addition to their role in promoting adhesion to cell surfaces, gonococcal pili have been reported to enhance the microbe's ability to acquire iron <u>in vivo</u> (Payne and Finkelstein, 1975). Iron has been shown to increase the virulence of many pathogenic species (Weinberg, 1978) including <u>N.gonorrhoeae</u> (Payne and Finkelstein, 1975). Pili also appear to increase the frequency of genetic transformation of gonococci (Sparling, 1966), so increasing the chances of acquiring new genes coding for such things as antibiotic resistance.

Despite the wealth of evidence suggesting that pili have many roles to play in the infective process and enhancement of virulence of pathogenic Neisseria it is paradoxical to note that gonococcal pili have been rarely observed in vivo (Watt and Ward 1977) and so some caution is required when attempting to relate results from in vitro experiments to the infective process in vivo.

1.5.4 Major Outer Membrane Proteins

Analysis of the outer membrane proteins of N.gonorrhoeae shows a relatively simple pattern. There are three major proteins designated Proteins I, II and III (Swanson and Heckels, 1980).

Protein I, or the Major (Principal) outer membrane protein M(P)OMP, as it was described in early reports, was first recognised by Johnston and Gotschlich (1974) as a protein of Mr 34.5k that accounted for over 60% of the outer membrane protein in strain 2686 (colony type 4). A later study by Johnson, Holmes and Gotschlich (1976) showed that there was considerable variation of Protein I between strains. They described molecules with a Mr ranging from 32k to 39k.

Cross-linking experiments using strain 2686 suggest that Protein I exists as a trimer (Newhall, Sawyer and Haak, 1980) and it is also closely associated with another membrane protein (Mr 28k). They suggested that these protein aggregates might act as water-filled diffusion channels across the outer membrane, though they did not refer to the evidence already presented by Heckels (1979), demonstrating that Protein I was a transmembrane protein. This hypothesis has now been confirmed (Heckels and James, 1980; Douglas, Lee and Nikaido, 1981).

In addition to Protein I, the opaque colonial forms of gonococci contain a protein with Mr ranging from 24k to 30k. This family of proteins is classed as Protein II. (Swanson 1978b). Their apparent molecular weight on SDS-PAGE varies dependent on the temperature

of solublisation, the so-called heat-modifiability of Protein II (Heckels, 1977).

Both Proteins I and II were radiolabelled when whole cells were exposed to an I^{125} - lactoperoxidase - H_2O_2 system, whereas Protein III was poorly labelled, suggesting that it is not exposed at the cell surface. (Heckels, 1978) Protein III (Mr 31-34k) appears to be closely associated with Protein I since they are found in similar proportions in immunoprecipitation experiments (Swanson, 1981) and antiserum produced against electrophoretically purified Protein I immunoprecipitated both Proteins I and III,yet reacted only with Protein I (McDade and Johnston, 1980).

N.meningitidis appears to possess a larger mosaic of major outer membrane proteins than N.gonorrhoeae. Peptide mapping of the major outer membrane proteins of Group B strains have demonstrated five structural classes of proteins which correlate with the apparent molecular weights (46 $\pm$ 1k, 41 $\pm$ 1k, 38 $\pm$ 1K, 33 $\pm$ 1k and 28 $\pm$ 1k). Tsai, Frasch and Mocca, 1981). Cell surface labelling experiments suggest that proteins of classes 1, 2,4 and 5 are exposed at the cell surface.

1.5.4.1 Role of outer membrane proteins in pathogenesis of gonorrhoea

A number of studies have indicated that the protein II molecules found in the opaque phenotype may be associated with increased virulence of these strains of N.gonorrhoeae. Lambden et al (1979) described opaque variants of a transparent parent strain, P9, that showed increased adhesion to buccal epithelial cells and were more resistant to killing by NHS than the parent strain . This strain, however, has been reported to be relatively resistant to killing by human serum (Stead et al 1975). They also confirmed the reports of Swanson and colleagues (Swanson, 1977) that outer membrane proteins in the molecular weight range 27-29k have a

greater influence on the degree of interaction with PMN than pili. However, in the case of bacteria bearing Protein IIa* (Mr 28.5k) the influence was to increase binding, whilst bacteria bearing Protein IIb (Mr 28.0k) exhibited decreased binding compared with the prototype strain lacking Protein II. The ability of Protein II to enhance binding to epithelial cells has been reported by other workers, including Virji and Everson (1981), who used Chang conjunctival cells. This is an improvement on the use of buccal cells and erythrocytes, since N.gonorrhoeae does infect the eye.

Serological studies of intact gonococci have demonstrated considerable antigenic diversity related to the Protein II species present. (Diaz and Heckels, 1982). This paper also cites a communication from P. Lambden suggesting that the antigenic variants of Protein II had considerable structural homology. Since antibody specific for protein II has been shown to inhibit binding on non-pilate gonococci to Hela cells (Sugasawara et al, 1983), the ability to alter antigenicity whilst maintaining structure and, presumably, the functional capacity of this protein appears to be of advantage to the microbe in evading the host immune response.

In contrast, the transparent phenotype of N.meningitidis is most frequently associated with the disease state whilst opaque colonies are most frequently isolated from the nasopharnyx of asymptomatic carriers. The transparent colony phenotype, associated with heat modifiable membrane proteins of Mr 26-32K, is more resistant to NHS than the opaque phenotype. However, the increased binding of opaque pilate meningococci to human buccal cells compared with transparent variants is a point of similarity with the gonococcus. (Stephens and McGee, 1983).

1.5.5 Lipopolysaccharide (LPS)

Most of our information concerning the chemical nature and biological effects of lipopolysaccharide (LPS) have come from studies of LPSs produced by members of the Enterobacteriaceae. The classical structure of an LPS molecule consists of three distinct regions : a hydrophobic lipid called Lipid A; a core oligosaccharide composed of a variety of sugars, but usually containing some heptose molecules and 2-keto-3 deoxy-octulosonic acid (KDO); a series of repeating subunits, usually composed of a small number of sugar molecules. It is these repeating subunits that confer serological specificity to the LPS molecule. Spontaneous mutation leading to loss or inhibition of an enzyme such as a transferase or synthetase associated with the formation of these repeating subunits can result in bacteria expressing an incomplete LPS molecule.

Since this imparts different surface characteristics to colonies of the bacterium, this variation has been termed Smooth - Rough variation, and the LPS molecules associated with each type are called S-type LPS and R-type LPS respectively. (Davis et al, 1980) LPS is found at the microbial cell surface and a number of Neisseria species have been shown to release LPS into culture medium. This may be free LPS (Johnson, Perry and McDonald, 1976) or LPS associated with other outer membrane components - outer membrane complexes (De Voe and Gilchrist 1973; Stead et al, 1975).

1.5.5.1 Lipopolysaccharide - Chemical and Structural Analyses

Several groups have undertaken chemical analyses of gonococcal LPS extracted from the cells by a procedure employing phenol and water. An early analysis by paper chromatography of the sugar components of gonococcal LPS isolated from strains grown in liquid medium showed that it contained glucosamine, glucose, galactose, a heptose and KDO linked to lipid in the following ratio 4:1:2:2:3.

(Stead et al, 1975). They examined two strains in the T1 colonial form (P9 and F62) and three strains in the T4 form (G2, G4 and G50) and reported that there were no significant differences between LPSs of 'virulent' and 'avirulent' gonococci, i.e. between strains of T1 and T4 colony types. It is curious that they did not examine LPS isolated from colonial variants of the same strain. The absence of deoxy-, dideoxy or N- acetylated sugars, typical components of the repeating subunits in LPS of the enterobacteria, led Stead and colleagues to propose that gonococcal LPS occurs as the R-type.

Perry and colleagues (1975) analysed LPSs isolated from strains grown on solid medium in both T1 and T4 colony types. They observed differences in LPS composition associated with the colony type of four strains, (In 31, 188, G9, GC6). The LPS isolated from T4 colonies of these strains had lipid, aldoheptose and KDO concentrations consistent with those found in typical R-type LPS. Sialic acid, deoxy-and dideoxy-glycoses were not observed. In contrast, the LPS isolated from T1 colonies of the same strains exhibited compositions comparible to those of S-type LPS preparations from other gram-negative organisms. This work was later re-investigated and the opinion of Perry and colleagues in 1978 (Perry et al, 1978) was that the S-type LPS had not been satisfactorily determined in N.gonorrhoeae . Wiseman and Caird (1977) undertook compositional studies of LPSs extracted from 38 strains of N.gonorrhoea with examples of colonial types T1 to T5. They grew the strains in liquid medium, extracted LPS with phenol and water, and analysed the carbohydrate fraction by gas-liquid chromatography of trimethyl silyl derivatives of hydrolysed LPS. They identified the following sugars in all colony types : glucose, galactose, mannose, N-acetyl neuraminic acid (sialic acid), 2-keto-3-deoxyoctulosonic acid (KDO), glucosamine and galactosamine (probably in the N-acetylated form) were also invariably present. In contrast to the findings of Perry et al (1975) they found rhamnose in the avirulent colony types (T3 to T5) and in N.sicca and N.lactamica (colonial morphology not defined), but not in

T1 and T2 variants of the same strains. (Two exceptions being strains 3256 and KC, in which small amounts of rhamnose were observed in LPS isolated from T2 colonies). Fucose was not found in the gonococcal strains, but was present in N.sicca and N.lactamica.

Variants in colonial forms T1 and T2 generally contained a greater total glycose concentration with higher ratios of mannose: KDO glucose: KDO and galactose: KDO, compared with T3 to T5 variants of the same strain. This suggested to Wiseman and Caird that these sugars might form part of a repeating unit typical of S-type LPS. They concluded that the LPSs of virulent (T1) and avirulent (T4) gonococci do not differ qualitatively (except, of course, for the absence of rhamnose in the T1 colonial form), but they do differ in the concentration of their respective constituents. Perry and colleagues, in their later paper on analysis of gonoccal LPS, (Perry et al, 1978), described LPSs which had the same mobility on SDS-PAGE irrespective of the colony type from which they were isolated. Carbohydrate analyses were typical of those expected for all R-type LPS, viz. glucosamine, glucose, galactose, L-glycero-D-manno heptose and 3-deoxy-D-manno-octulosonic acid. Gel filtration of the oligosaccharide portions liberated from the LPS by mild acid hydrolysis revealed two core oligosaccharide types in the same LPS preparation. These were found to have the same structure, except one core type (the faster moving fraction on gel filtration) had an additional 1-3 linked D-galactose unit. Both core oligosaccharide types had two D-galactopyranose and one D-glucosamine units present as non-reducing end groups (which tend to be immunodominant (Kabat 1968)). Enzymatic degredation with snail intestinal juices (rich in β-D-galactosidase) and further structural analysis suggested that one of the D-galactose end residues is part of a 4-O β-D-galactopyranosyl-D-glucose (lactosyl) unit. The other terminal galactose, which was not removed by the snail intestinal juice, was thought to be linked via an α-linkage.

Investigations of a variety of Neisseria species have demonstrated variation in LPS composition dependent on the growth conditions. McDonald and Adams (1971) demonstrated the influence of growth rate on the hexosamine concentration of LPS extracted from N.sicca. When grown at a fast rate the galactosamine: glucosamine and hexosamine : KDO ratios were higher than when the organisms grew at a slower rate. In addition,greater quantities of LPS could be isolated from cells which were growing at the faster rate. In these experiments the growth rate was determined by the degree of aeration of the culture. Morse et al (1983) used nutrient-limitation to alter the growth rate of N.gonorrhoeae strain FA 171 and found a growth-rate-dependent change in the quantity of LPS that could be isolated from the cells. Cells growing at a high dilution rate ($0.56\ h^{-1}$) contained approximately eight times more extractable LPS than those growing at a lower dilution rate ($0.12\ h^{-1}$). The type of LPS isolated differed in band pattern in SDS-PAGE, and the cells growing at the higher dilution rate had less hydrophobic surfaces, as determined by hexadecane binding, than those grown at the lower dilution rate. Nutrients have also been observed to alter the LPS of N.gonorrhoeae. Norrod et al (1983) observed changes in LPS structure of strain F62 resulting from growth in medium containing pyruvate and a high cysteine: cystine ratio. They were unable to define a mechanism for this change.

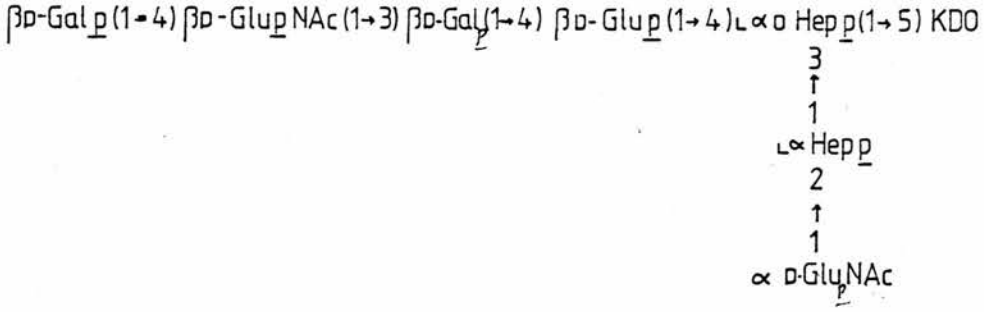
The LPS of N.meningitidis has been studied extensively by Jennings and colleagues (Jennings et al, 1973; Jennings et al, 1980; Jennings, Johnson and Kenne, 1983) who have found LPS isolated from strains of different capsular serogroups to be R-type LPS. Recent electrophoretic studies are in agreement with this and show that they have similar migration patterns to gonococcal LPS. (Tsai, Boykins and Frasch; 1983; Mintz, Apicella and Morse, 1984). At least 11 immunotypes of meningococcal LPS have been found and, in general, there is no correlation between LPS immunotype and capsular serogroups. The exceptions are immunotypes 10 and 11, which have only been observed

in strains of serogroup A. (Zollinger and Mandrell, 1980). Different immunotypes can be simultaneously observed in many strains, and electrophoretic separation of LPS from strains of different immunotypes has shown that most contain two predominant components, (Tsai, Boykins and Frash, 1983). The different mobilities of these components probably reflects compositional differences. The relative quantities of each component have been found to alter in response to changes in growth rate (controlled by the degree of aeration of the culture). At fast growth rates the lower molecular weight species increases in quantity, and the proportion of galactose in the LPS decreases. This suggests that the lower molecular weight component contains less galactose than the larger molecular species. (Tsai, Boykins and Frasch, 1983). Irrespective of growth rate, qualitative analysis of the core oligosacchoride from meningococcal LPS has yielded glucose, galactose, glucosamine, heptose and KDO. (Jennings et al, 1973; Tsai, Boykins and Frasch, 1983; Jennings, Johnson and Kenne, 1983).

Structural analysis of a core oligosaccharide bearing determinants for the L3, L7 and L9 serotypes has yielded the structure shown in Figure 1 (Jennings, Johnson and Kenne, 1983).

FIGURE 1

The structure of a core oligosaccharide from meningococcal LPS (Jennings, Johnson and Kenne, 1983)



1.5.5.2 Lipopolysaccharide - Immunochemical Characterisation

Purified gonococcal LPS is a poor immunogen for many animals, including rabbits. However, it does provoke a substantial immune response in hens (Perry, Diena and Ashton, 1977). Hen antiserum to purified R-type LPS has been shown to agglutinate both T1 and T4 colonial forms of gonococcus, but not strains of meningococcus (serogroups A,B and C), N.lactamica, or Pseudomonas aeruginosa. This suggests a high degree of structural homology among gonococcal LPSs from different strains. Cross-reactivity did occur with some streptococcus strains, especially type A and G. (Wallace et al, 1978)

In gel and quantitative precipitation studies, lactose and its structural analogue 3-O-β-D-galactopyranosyl-D-arabinose have been shown to inhibit precipitate formation by hen anti-LPS serum and gonococcal LPS (Perry, Diena and Ashton, 1977; Perry et al, 1978), suggesting an immunodominant role for the lactosyl residue on gonococcal LPS described by Perry et al (1978)(See Section 1.5.5.1).

In addition to common determinants, gonococcal LPS has been shown to exhibit a high degree of antigenic heterogeneity - presumably indicative of structural heterogeneity. Apicella and Gagliardi (1979) describe how gonococcal LPS contains one of six serogroup determinants (GC1 to GC6) and at least two sets of non-serogroup determinants ; one which is common to all six serogroups, and another found only on serogroups GC1, GC3 and GC4.

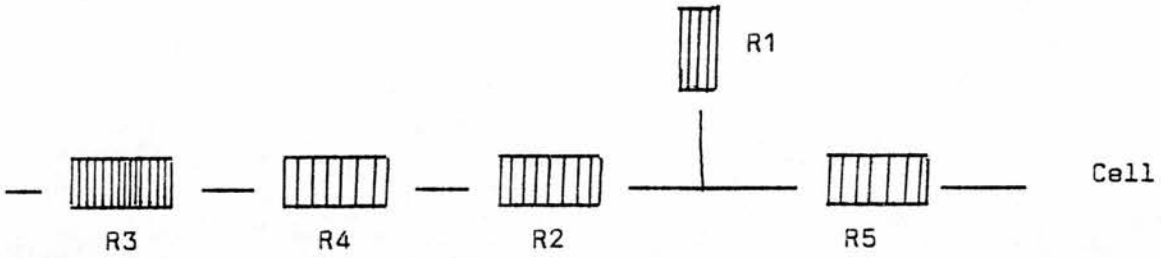
Structural analysis of a common determinant of gonococcal LPS has been attempted by Apicella and colleagues (1981) using sugars to inhibit binding of a monoclonal antibody reactive with this determinant. They suggested that it reacted with the following structure: D-galactosamine-O-D-galactopyranosyl-(1-4)-D-glucopyranose. The 1-4 linkage was suggested since glucose and galactose, alone or in combination, failed to inhibit binding of the antibody whereas α and β lactose did.

Meningococcal LPS also appears to be capable of binding this monoclonal antibody, but to a lesser degree than gonococcal LPS. Apicella and colleagues (1981) suggested that the meningococcal LPS might only contain a portion of the putative binding site for this monoclonal antibody. They proposed that the receptor in meningococcal LPS for this antibody might be a galactopyranosyl (1-4) D-glucopyranose moiety. This has been shown to be present from the structural analysis done by Jennings and coworkers. (Jennings, Johnson and Kenne, 1983). (Figure 1).

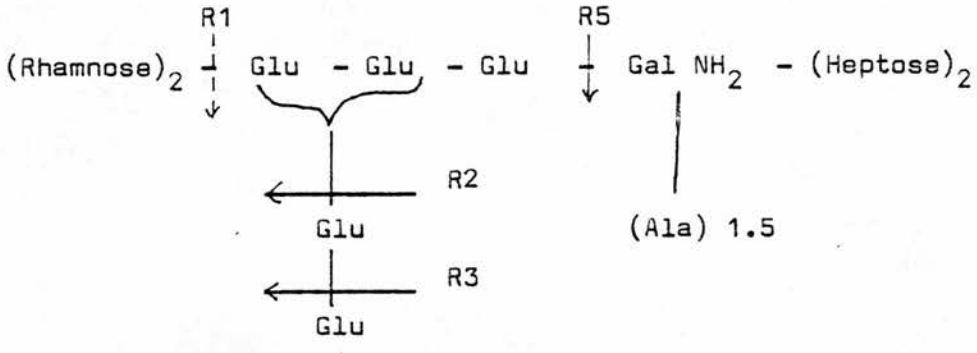
1.5.5.3 Lipopolysaccharide - role as a receptor for Rod type pyocins of Pseudomonas aeruginosa

Rod-type (R-type) pyocins are a class of bacteriocins produced by Pseudomomas aeruginosa. The receptor site in Ps aeruginosa for these bacteriocins has been shown to be the LPS (Ikeda and Egami, 1969; Govan, 1974). Bacteriocins are usually only active against species closely related to the producing species. R-type pyocins, however, have been shown to inhibit growth of gonococci, (Morse et al, 1976) meningococci (Blackwell and Law, 1981) and thermophilic Campylobacters (Blackwell, Winstanley and Telfer Brunton, 1982). The LPS molecule has been shown to be the gonococcal receptor for R-type pyocins (Sadoff, Zollinger and Sidberry, 1978) and this molecule is presumed to have a similar function in meningococci (Blackwell and Law, 1981).

R-type pyocins have been classified in 5 groups R1 to R5 based on the location of their binding site on the LPS molecule of Pseudomonas. (Kageyama, 1975) and a diagramatic representation of the relationship between the LPS molecule and these binding sites is given below :



(Taken from Kageyama, 1975)



(Taken from Meadow and Wells, 1978)

Exposure of gonococci and meningococci (especially non-serogroupable strains) to a panel of pyocins with representatives from groups R1, R2, R3 and R5 has shown several patterns of sensitivity and this has been proposed as the basis for an epidemiological typing scheme. (Sidberry and Sadoff, 1977; Blackwell, Young and Anderson, 1979; Blackwell and Law, 1981). Mutations from pyocin sensitivity to resistance have been shown to correlate with compositional changes in LPS. Sadoff, Zollinger and Sidberry (1978) described pyocin-resistant mutants of gonococci which had higher glucose: galactose ratios in the LPS (1.6) than the pyocin sensitive parent strain (0.97). Connelly and Allen (1983) demonstrated loss of LPS antigen reactivity associated with mutational loss of pyocin sensitivity. These data suggest that non-reactivity with an R-type pyocin is due to lack of a receptor determinant in the LPS.

Reports concerning the stability of pyocin sensitivity vary. Guymon, Esser and Shafer (1982) gave an approximate mutation rate of strain FA19 to resistance to pyocins of Ps.aeruginosa strains 1 and 103 as 10^{-6}. Blackwell, Young and Anderson (1979), however, describe strains maintaining their pyocin sensitivity spectrum over more than 100 subcultures. There have been no reports of pyocin-resistant gonococci developing pyocin-sensitivity, suggesting that the mutation to resistance is a permanent step.

1.5.5.4 Lipopolysaccharide : Reactivity with lectins

Lectins are proteins or glycoproteins that are able to selectively bind to cell surfaces or to biopolymers via specific oligosaccharide determinants. (Sharon and Lis, 1972), many of which have been structurally defined. By use of a panel of lectins of defined binding specificity, it is possible to derive chemical information about the occurrence of specific sugars, their location and configuration in complex carbohydrates such as those found in LPS molecules. Investigation of the lectin-binding of 54 strains of N.gonorrhoeae by Allen, Conelly and Apicella (1980) suggested the following structural features are common in gonococcal LPS :

1. multiple β-linked N-acetyl-D-glucosamine and β-D-galactosyl units.
2. α N-acetyl-D-galactosamine and β-D-galactose linked to N-acetyl galactosamine and/or N-acetyl glucosamine.
   Reference to the meningococcal core oligosaccharide structure in Figure I shows a component like (1) above to be present.

1.5.5.5 Lipopolysaccharide : Toxic Properties

LPS forms the major part of endotoxin that is released in large quantity from rapidly growing gonococci and meningococci (Stead et al, 1975; De Voe and Gilchrist, 1973).

Human fallopian tube mucosa is extensively damaged by LPS released from growing gonococci (Melly, Gregg and McGee, 1981) and by LPS extracted with phenol and water. (Gregg et al, 1981) The haemorrhagic spots on the skin in meningococcal disease are thought to be due to the potent LPS - containing endotoxin released from N.meningitidis (Davis and Arnold, 1974)

The toxicity of gonococcal LPS for embryonated eggs is abrogated by presence of anti-LPS antibodies (Diena et al, 1978) suggesting that immunity to the LPS might afford protection against tissue damage.

1.5.6 Capsule

N.meningitidis can produce capsules with a great diversity of compositions, many of which have been elucidated to the level of their chemical structure (Table II). The evidence for capsule production by N.gonorrhoeae is much more controversial. Few workers have observed them. (Hendley et al, 1977; Richardson and Sadoff, 1977; Demarco de Hormaeche, Thornley and Glauert, 1978) Richardson and Sadoff (1977) could only observe them on gonococci co-cultivated with viridans streptococci.

N.meningitidis has been isolated from the genitourinary tracts of males and females (Faur, Weisburd and Wilson, 1975, Blackwell Young and Bain, 1978). It is curious to note that the capsulate gonococci described by Demarco de Hormaeche and colleagues (1978) retained the low, planoconvex T3/T4 colony type after growth for up to 80 days in subcutaneous chambers implanted in guinea pigs, in contrast to the T1/T2 colony type observed in other gonococcal strains under similar conditions (Penn et al, 1976).

These T3/T4 colony forms are similar to the M1 colony type elaborated by most strains of N.meningitidis (De Voe and Gilchrist,

1978). Melly et al (1979) believe that the observation of apparent gonococcal capsules is due to peeling off of the lower membrane or the presence of the organism within an isolated phagocytic vacuole.

1.5.6.1 Capsule : Serotypes and Chemical Structures of Meningococcal Capsules

The serotypes and structures of meningococcal capsules are given in Table II

TABLE II

Capsular polysaccharides of the meningococci

(Taken from DeVoe, 1982)

Serogroup	Components[a]	Structural repeating unit
A (homopolymer)	ManNAc, phosphate, NAc and OAc	ManNAc-(1-P $\xrightarrow{\alpha}$ 6)--- 3 ! ! OAc[b]
B (homopolymer)	NeuNAc	NeuNAc-(2 $\xrightarrow{\alpha}$ 8)---
C (homopolymer)	NeuNAc, OAc	NeuNAc-(2 $\xrightarrow{\alpha}$ 9)--- 7 8 ! \ ! \ OAc OAc[c]
W-135 (disaccharide repeating unit)	Gal, NeuNAc	6-D-Gal(1 $\xrightarrow{\alpha}$ 4)-NeuNAc(2 $\xrightarrow{\alpha}$ 6)---
X	GlcNAc, phosphate	DOGlcNAc(1-P $\xrightarrow{\alpha}$ 4)---
Y (Bo) (disaccharide repeating unit)	Glc, NeuNAc, OAc	6-D-Glc(1 $\xrightarrow{\alpha}$ 4)-NeuNAc(2 $\xrightarrow{\alpha}$ 6) ! O-Ac[d]
Z (monosaccharide-glycerol repeating unit)	GalNAc, glycerol phosphate	D-GalNAc, (1 $\xrightarrow{\alpha}$ 1′)-glycerol-(3′-P $\rightarrow$ 4)---
29-e (disaccharide repeating unit)	GalNAc, KDO, OAc	D-GalNAc(1 $\xrightarrow{\beta}$ 7)-KDO(2 $\xrightarrow{\alpha}$ 3)--- 4,5 ! ! O-Ac

[a] The abbreviations used are: Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine (2-acetamido-2-deoxy-D-glucose); KDO, 3-deoxy-D-manno-octulosonic acid; ManNAc, *N*-acetylmannosamine (2-acetamido-2-deoxy-D-mannose); NeuNAc, *N*-acetyl neuraminic acid (sialic acid); OAc, *O*-acetylated; NAc, *N*-acetylated; phosphate, phosphodiester linkage.

[b] Group A is substituted with *O*-acetyl at C_3 on ca. 70% of the ManNAc-P residues.

[c] Group C is substituted on C7 or C8 with 1 mol of *O*-acetyl per mol of sialic acid. One-quarter of the sialyl residues are not acetylated. Some di-*O*-acetylated (C7 and C8) may exist.

[d] The Y polysaccharide contains 1.3 mol of *O*-acetyl per NeuNAc residue. The most probable site for acetylation is C3, C4, or C7 (26). ^{13}C nuclear magnetic resonance studies have shown that the serogroup By polysaccharide is identical to serogroup Y although Bo contains 1.8 mol of *O*-acetyl per mol of NeuNAc

1.5.6.2 Capsule : The biological properties of meningococcal capsules

Capsules have been shown to confer increased virulence on bacteria compared with non-capsulate variant strains. In the case of Streptococcus pneumoniae the increase in virulence is due to the capsule hindering phagocytosis of the bacteria by leucocytes. This hinderence is not observed when anti-capsule antibodies are present. (Mims, 1982). Capsulate strains of meningococcus can be isolated from patients and healthy carriers. However, an association between capsulation and the virulence of meningococci is suggested by the higher frequency of isolation of capsulate strains from patients than from carriers (Poolman, Hopman and Zanen, 1980) and the high frequency of isolation of non-serogroupable (many of which are non-capsulate) strains from healthy carriers. (Devine, Rhode and Hagerman, 1972). This proposition has received some backing from estimates of the lethal dose of capsulate strains of serogroup B N.meningitidis compared with non-capsulate variants of the same strains. Masson and colleagues (Masson, Holbein and Ashton, 1982) found that the capsulate strains had a 10^4 fold lower LD50 compared with the non-capsulate variants. The evidence for the role of capsules in the natural human infection comes from the great success in prevention of meningococcal disease achieved by use of capsular polysaccharide vaccines. (Gotschlich et al, 1978).

It is interesting to note that capsules reduce the enhacement of epithelial cell binding conferred by pili. (Stephens and McGee, 1981), an effect presumably resulting from an increase in repulsive forces due to an increase in bacterial negative charge brought about by the capsule.

1.5.7 Peptidoglycan

Gonococcal peptidoglycan is a polymer of muramic acid, glucosamine, alanine, glutamic acid and diaminopimelic acid in the molar ratios 1:1:2:1:1. There appears to be no covalently linked lipoprotein, such as that found in *Escherichia coli*, which could account for the loose association of peptidoglycan and outer membrane seen in electron micrographs (Wolf-Watz *et al*, 1975). However, the outer membrane protein, Protein I, appears to be linked to the peptidoglycan. (Heckels, 1979). The observation of a high degree of *O*-acetylation of peptidoglycan is gonococcal strains by Swim *et al*, (1983) and others is of interest since this modification results in increased resistance to the peptidoglycan-degrading enzyme lysozyme. The indigestibility of this modified peptidoglycan is thought to result in relatively large peptidoglycan oligomers remaining after gonococci have been killed by serum components and/or leukocytes. These complexes are more able to induce an imflammatory response than monomeric peptidoglycan (Rosenthal quoted in Swim *et al* 1983).

1.6 Microbial products associated with virulence

1.6.1 Iron-binding proteins (Siderophores)

Availability of free iron has been shown to influence the rate of growth and degree of virulence of many pathogenic organisms (Payne and Finkelstein, 1978). The addition of free iron enhances the growth and virulence for embryonated hen's eggs of both gonococci and meningococci (Payne and Finkelstein, 1975). Since the availability of free iron in body fluids is severely restricted by specific iron binding proteins, such as transferrin and lactoferrin, the mode of iron-acquisition by the pathogenic Neisseria spp has been the subject of many investigations. Both gonococci and meningococci have been shown to elaborate iron-binding proteins, called siderophores; these appear to be related to virulence, since T1 (virulent) gonococci produce larger quantities than T3 (avirulent) organisms. (Yancey and Finkelstein, 1981).

In contrast to the commensal Neisseria species, meningococci and gonococci have been shown to be capable of scavanging iron from transferrin. (Mickelsen and Sparling, 1981) and lactoferrin (Mickelsen, Blackman and Sparling, 1982) by a mechanism that appears to require the interaction of transferrin with the bacterial cell surface. However, the role of siderophores in this type of iron acquisition is still to be elucidated.

1.6.2 Proteolytic enzymes that may contribute to pathogenesis

IgA is the predominant class of immunoglobin present in the secretions bathing mucous membranes. Two sub-classes of IgA occur, IgA1 and IgA2. Whereas in serum the ratio of IgA1 to IgA2 is 4:1, in the mucous secretions, equal proportions of each subclass are found. The role of this antibody is to protect mucous membranes from colonisation by micro organisms *via* interference with the function of molecules such as microbial adhesins.

The pathogenic Neisseria species have been shown to elaborate a proteolytic enzyme that cleaves IgA. It is only active against the IgA1 subclass. Possession of this IgA - protease is a characteristic feature of the pathogenic Neisseria species, N.meningitidis and N.gonorrhoeae, (Plaut et al, 1975) and, in the study of Mulks and Plaut (1978) was not found in eight commensal species. This enzyme could, presumably, reduce the efficiency of the protection the mucosal surfaces derive from IgA1. The IgA protease cleaves a prolyl-threonyl bond, present to the hinge region of IgA, but absent together with 11 other amino acids, as a block deletion, in IgA2.

Proteases with other specificities have been observed in culture supernates from both gonococci and meningococci (Chen and Buchanan, 1980). The endopeptidase Gonocosin cleaves the alanyl-alanine peptide bond, found frequently in elastin, and may contribute to gonococcal invasiveness in opthalmic neonatorum. Chen and Buchanan (1980) also described an asparaginase produced by the pathogenic Neisseria spp. and suggested that its activity may interfere with the immune response, since asparaginase has been shown to inhibit lymphocyte blastogenesis (Ohno and Hersh, 1970). This inhibition is probably due to asparagine starvation in the presence of the enzyme.

1.7 Pathogenesis of diseases due to Neisseria gonorrhoeae and Neisseria meningitidis

A micro-organism must successfuly take many sequential steps to cause disease. In the case of disease due to the pathogenic Neisseria species the initial steps include attachment and colonisation of the mucosal surfaces followed by penetration of the normally protective mucosa into the sub-mucosal spaces. Here the organisms must avoid or survive phagocytosis and resist the lethal effects of antibody and complement. Having survived they cross from the blood stream into other compartments such as the synovial cavities, in the case of disseminated gonococcal infection, and the cerebrospinal fluid, in the case of meningococcal meningitis.

This section will focus on the ultrastructural events and possible molecular mechanisms of the steps leading to infection by N.gonorrhoeae and N.meningitidis.

1.7.1 Attachment and transgression of the mucosal epithelium

After deposition onto a mucous membrane, bacteria must adhere rapidly to the underlying cell surface if they are not going to be washed away by mucus and, in the case of the urethra, the flow of urine. It has been suggested that gonococci can adhere rapidly and strongly to the urethral epithelium since micturition following intercourse does not protect against infection. (Bernfield, 1972). However, shearing forces, such as may occur at the urethral mucosal surface during micturition, may enhance binding rather than reduce it. (Brooks and Trust, 1983).

The bacterial surface components thought to first interact with the cell surfaces, thereby mediating attachment, are the pili (described in Section 1.5.3). Both gonococci and meningococci, when first isolated from clinical specimens, are covered in large numbers of pili, (Jephcott, Reyn and Birch-Andersen, 1971; Stephens and

McGee, 1981) and strains expressing pili have been shown to bind in greater numbers to mucosal epithelial cells than those without (Mårdh and Weström, 1976; Stephens and McGee, 1981; Section 1.5.3) Scanning electron micrographs of human fallopian tube organ cultures infected with N.gonorrhoeae show the bacteria tethered to non-ciliated mucosal microvillar surface by bundles of pili (Ward, Watt and Robertson, 1974) (McGee, Johnson and Taylor-Robinson, 1981). The importance of pili as gonococcal adhesins in vivo is still in doubt due to the curious infrequency with which pili are observed in electron microscopy studies on fresh clinical material. (Ward and Watt, 1972; Watt and Ward, 1977; Ovčinnikov and Delektorskij,1971).

Other surface structures which may mediate bacterium-mucosa adherence have been described. Protein II is actually a small family of heat modifiable outer-membrane proteins. Gonococci that have protein II tend to adhere to human cells with greater avidity than gonococci lacking protein II (Lambden, et al, 1979; Section 1.5.4.1), but the association between meningococcal protein II and adherence to host cells has not been investigated.

As an adjunct to the adhesion mediated by pili and/or outer membrane proteins, reduction of the mucus flow by damage to the epithelial cilia assists the colonisation process. Ciliarostasis is mediated by lipopolysaccharide, which is liberated in copious amounts by rapidly growing gonococci and meningococci (see Section 1.5.5), and monomeric peptidoglycan, which is released during degradation of peptidoglycan by PMN lysosmal enzymes (Melly, McGee and Rosenthal, 1984).

Subsequent to the attachment phase, gonococci and meningococci migrate through to the sub-mucosal tissues. The elegant experiments of McGee and colleagues (1981) demonstrated the events associated with gonococcal transgression of the human fallopian tube mucosa in infected organ cultures. After attachment to the microvilli of non-ciliated mucosa, gonococci entered the cells. Coincident with

the attachment and invasion of the non-ciliated epithelium, there was desquamation of ciliated cells. By 44 hours post-infection with T1 gonococci, ciliated cells accounted for less than 10% of the cells in the epithelial cell surface. The internalised gonococci migrated to the basal aspect of the non-ciliated cells through the cells. No bacteria were seen in the inter-cellular spaces. About 40 hours after infection gonococci began to be released into the sub-mucosal tissues. This progressed so that by 60 hours post-infection and thereafter, there was focal disruption of the epithelium and an increasing number of gonococci in the sub-epithelial tissues. In these experiments both T1 and T4 gonococci attached to, damaged and traversed the fallopian tube mucosa, though the rate of infection and invasion was much slower for the T4 organisms.

Similar studies using organ culture have been undertaken by Stephens, Hoffman and McGee (1983) to examine the interaction of N.meningitidis with human nasopharyngeal mucosa. The course of events observed with this model closely resembled the events which take place when gonococci infect fallopian tube mucosa in organ culture. The meningococci appeared to adhere solely to the microvilli of non-ciliated cells of the epithelium. The microvilli then appeared to become restructured and surrounded the micro organisms. Simultaneous with this attachment phase (up to 12 hours post-infection) a rapid decrease in ciliary activity together with sloughing off of ciliate epithelial cells was observed. The meningococci attached to the non-ciliate cells were engulfed within phagocytic vesicles. Despite the apparent absence of endocytic vacuoles toward the basal aspects of the cells, meningococci were seen in the subepithelial tissues 18-24 hours post-infection. No bacteria were observed penetrating the apical junctions and intercellular spaces.

In summary, both gonococci and meningococci appear to gain access to the submucosal tissues by traversing through the epithelial cells. This process involves endocytosis of the bacterium by the

epithelial cell. The exact mechanism for this is not fully understood, though it is probably stimulated by a ligand-receptor interaction at the epithelial cell surface. (Stephens, Hoffman and McGee, 1983). The stimulating factor is probably not pili since T4 gonococci are endocytosed at the same rate as T1 gonococci. (McGee, Johnson and Taylor-Robinson, 1981).

1.7.2 Gonorrhoea

1.7.2.1 Symptomatic Localised Infection

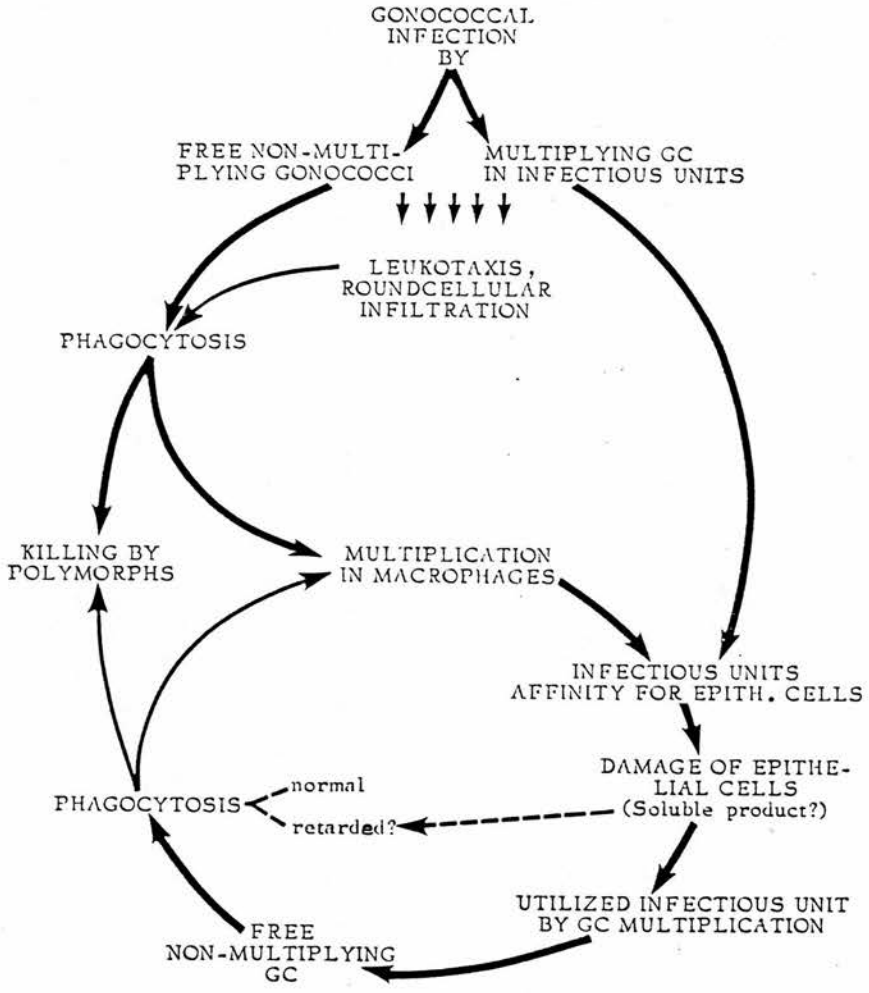
Current ideas concerning the pathogenesis of localised gonococcal infection are, for the main part, derived from microscopic examination of urethral pus. Such studies show clusters of gonococci either free, or in association with epithelial or phagocytic cells (Ovčinnikov and Delektoskij, 1977; Novotny and Short, 1977). The gonococcal clusters on epithelial cells are located at the basal aspect of the cells, and so are typical of those observed by McGee Johnson and Taylor-Robinson (1981) in the fallopian-tube mucosal cells. Moreover, these clusters of bacteria are regularly arranged and have intact division planes, suggesting that they are healthy and viable.

Symptomatic localised gonococcal infection is characterised by a purulent exudate containing many PMNs. These cells may be attracted to the site of gonococcal invasion by bacterial products such as formyl-methionyl peptides, but experiments by Watt and Medlen (1978) using infected fallopian tube organ cultures suggested that gonococci do not produce chemotactic substances. Their experiments suggested that the predominant chemotactic component in gonococcal infection is the complement fragment C5a which was produced by activation of the alternative pathway. However, experiments by James and Williams (1978) and Rank and Holmes (1984) suggest that many, but not all, clinical isolates of N.gonorrhoeae produce low molecular weight substances which are chemotactic for PMNs.

Electron-microscopy has shown many gonococci within PMN phagocytic vacuoles.(Ovčinnikov and Delektorskij, 1971; Ovčinnikov and Delektorskij, 1977). In many phagosomes the bacteria appear to be undergoing lysis. However, in some phagosomes the gonococci appear healthy and intact. Novotny, Short and Walker, (1975) described regular arrays of gonococci within membraneous packages covered with granules which appeared to be the remnants of phagocytes. Later studies showed this material to be reactive with anti-phagocyte serum. (Novotny et al, 1977). The type of phagocyte could not be determined using this serum, but examination of the granules and the occasional nucleus which was seen in association with these membraneous packages suggested that they were remnats of macrophages. These packages have been termed 'infectious units' since gonococci multiply within them, and appear to be protected from antibody and phagocytes. According to Novotny et al, (1977) they are only derived from mononuclear phagocytes, not from PMN, nor epithelial cells. Novotny and colleagues (1977) suggested that'infections units' were not formed in PMN. PMN can ingest large numbers of gonococci, even to self-destructive levels; however, the appearance of gonococci released from PMN is dissimilar to that of 'infectious units' due to the lack of a dense granular coat. Novotny and colleagues (Novotny et al, 1977; Novotny and Short, 1977) have outlined a scenario for the pathogenesis of gonorrhoeae which is summarised below : Figure 2

FIGURE 2

Summary of the gonococcal infectious process (From Novotny et al., 1977)



1.7.2.1.1 Survival of N.gonorrhoeae within phagocytes

Evidence that gonococci can survive within human phagocytes has come from several workers. However, many reports are difficult to interpret since there is uncertainty regarding the location of the surviving bacteria. Were they really phagocytosed, or were they just adherent to the phagocyte surface? By treating a mixture of gonococci and phagocytes with an antibiotic such as peniciliin or spectinomycin it is possible to kill the bacteria outside the phagocyte, whilst not interfering with the fate of the intracellular organisms. Subsequent lysis and viability counts will give information on the survival of only those bacteria which have been phagocytosed. These in vitro experiments have shown that gonococci can survive and multiply within human PMN (Witt, Veale and Smith, 1976; Veale et al, 1979; Casey, Veale and Smith, 1979) and mononuclear phagocytes (Veale et al, 1976) Using spectinomycin to kill non-adherent and surface-associated gonococci Casey, Veale and Smith (1980) have examined the theory of intracellular survival within urethral exudate cells proposed by the electron-microscopy studies (Ovčinnikov and Delektorskij, 1971; Novotny, Short and Walker, 1975). Half the gonococci seen in urethral exudates from acute cases of gonorrhoea are associated with PMN (Veale et al, 1979) and from the experiments of Casey, Veale and Smith (1980) these organisms are within the phagocytes, viable and, in some cases, growing. These experiments provide support for one of the ideas proposed by Novotny and colleagues (1977), viz. that gonococci can survive within phagocytes and so possibly avoid the immune response.

The nature of the gonococcal determinants conferring the capacity for residence within phagocytes has been investigated by Parsons and colleagues (Parsons et al, 1982). They associated three non-pilus proteins, present in outer membrane vesicles, with this capacity.

1.7.2.1.2 Opsonisation of N.gonorrhoeae by ABO isohaemagglutinins

ABO blood group has been shown to be a factor influencing susceptibility to gonorrhoea (Foster and Labrum, 1976; Kinane et al, 1983b; Section 1.4.1.1). Studies comparing the relative degrees with which mononuclear phagocytes of the four ABO blood groups (A,B,AB and O) in the presence of autologous serum associate i.e. bind and/or ingest, with N.gonorrhoeae demonstrate that cells from blood group B individuals bind more gonococci than cells of groups A, AB and O. (Kinane et al, 1983a). Furthermore, these experiments showed that anti-A and anti-B isohaemagglutinings enhanced binding of gonococci to monocytes since absorption of the isohaemagglutinins reduced the opsonising capacity of the serum, reducing the degree of bacterial binding to that observed in serum-free conditions.

Earlier experiments by Blackwell and colleagues (1983) suggest that opsonisation by isohaemagglutinins is not involved in the association of gonococci to PMN. When viewed in the light of the schema proposed by Novotny and colleagues (1977), the increased association of gonococci with mononuclear phagocytes of blood group B compared with those of other ABO blood groups could be a factor increasing the susceptibility of group B individuals to gonorrhoea.

1.7.2.2 Asymptomatic localised Infection

Asymptomatic localised gonococcal infection in women has been recognised for a long time and recently the high incidence of asymptomatic infections in males has been appreciated. Studies in Denmark in 1975 showed that 57% of infections of females were devoid of symptoms, whilst the figure for infections of males was 15%. (Nielsen, Søndergaard and Ullman, 1975). These asymptomatic individuals probably constitute a major reservoir of infection since N.gonorrhoeae causing asymptomatic infection are virulent and transmissible. (Brown et al, 1963)

The reasons for asymptomatic carriage are not clear; however, gonococci isolated from these infections tend to have auxotypes that are different from those isolated from symptomatic localised infection, but similar to those strains isolated from disseminated infections (Rein, 1977). Since strains causing asymptomatic infection in one host cause symptomatic infections in other hosts (Brown et al, 1963) the reasons for the lack of symptoms are probably complex. The degree of bacterial invasion of the mucosa and the propensity of the organism to activate complement are likely to be factors influencing the development of symptoms. The similarity of strains associated with asymptomatic infection to those isolated from disseminated infections has been noted. These strains from disseminated infections are slower to stimulate complement-mediated neutrophil chemotaxis in vitro than strains isolated from localised symptomatic infections (Densen, Mackeen and Clark, 1982).

1.7.2.3 Disseminated Gonococcal Infection (DGI)

Disseminated gonococcal infection (DGI) is a disease complex resulting from haematogenous extension of the gonococcus from the mucosa and submucosal tissues at the site of infection. DGI is predominant in women. In an American study 80% of DGIs were found to occur in women, and this is thought to be associated with the asymptomatic nature of gonorrhoea in most women (Al-Suleiman, Grimes and Jonas, 1983). The consequences of dissemination include migratory polyarthralgias and tenosynovitis. There is either positive blood culture, or positive synovial fluid culture. These appear to be mutually exclusive. Skin lesions similar to those observed in Schwartzman reactions are also observed (O'Brien, Goldenberg and Rice, 1983).

Several factors have been proposed that are thought to influence the dissemination of gonococci from the site of infection.

The increased susceptibility of women has already been mentioned. Among women with gonorrhoea, pregnancy or menses can predispose to dissemination (Rein, 1977). Genetic absence of the terminal components of the complement cascade, C6, C7 and/or C8, increases the probability that dissemination will occur during infection. (Petersen, Graham and Brooks, 1976; Lee et al, 1978) Common characteristics have been observed for strains associated with DGI. These include; various auxotypes, predominantly $Arg^{-}Hyx^{-}Ura^{-}$; transparent colony phenotype (therefore absence of Protein II); a particular coagglutination serogroup, WI, ; very low MIC to penicillin (< 0.03 μg/ml); resistance to the bactericidal activity of normal human serum. (O'Brien, Goldenberg and Rice, 1983). Resistance to Killing by human serum presumably assists dissemination through the blood. In a study of 89 patients with DGI the gonococcus was cultured from the blood in 19 cases. (Tronca et al, 1974). It is also interesting to note that examination of the white blood cells in the buffy coat from individuals for whom blood cultures were negative demonstrated intracellular gonococci in four out of eight cases. (Tronca et al, 1974). These bacteria were identified by a fluorescent antibody technique, so their viability was not assessed. However, this observation provides in vivo evidence supporting the concept of gonococcal spread and transmission whilst resident within phagocytes proposed by Novotny and colleagues (1977).

1.7.3 Meningococcal disease

Meningococcal disease has a variety of clinical manifestations ranging from chronic meningococcaemia to fulminant meningococcaemia associated with coagulopathy. Although N.meningitidis is normally a harmless commensal bacterium it is one of the swiftest-killing human pathogens. The natural habitat of the meningococcus is the human oropharynx, where it lives in and around the mucous membranes. Individuals harbouring meningococci are usually asymptomatic

and the frequency of carriage in the normal population ranges from 5 to 30%, but during epidemics this can approach 100%. (De Voe, 1982).

The incidence of meningococcal disease is not simply related to the carriage rate. The rarity of meningococcal disease (up to 37.4 per 100,000) compared to the rate of carriage of meningococci (up to 100,000 per 100,000) (De Voe, 1982) suggests that host factors are very important in determining susceptibility to meningococcaemia.

Immunity to meningococcal disease has been shown to correlate with the presence of bactericidal antibodies in serum reactive with the infecting strain (Goldschneider, Gotschlich and Artenstein, 1969; Section 1.8.4.1). The importance of the lytic components of the complement system, C6-C8, in preventing meningococcaemia is highlighted by the susceptibility of individuals genetically deficient in these components to repeated meningococcal bacteraemias (Lee et al, 1978; Lee et al., 1979). Antibodies reactive with meningococci are stimulated by carriage of meningococci (Reller, McGregor and Beatty, 1973) and by other commensal organisms such as enteric bacteria (Robbins et al., 1972). The bactericidal activity of IgM and IgG can be blocked by IgA specific for the same antigens. A high level of serum IgA reactive with meningococcal surface antigens is thought to increase susceptibility to meningococcal disease by allowing the development of a bacteraemic phase (Griffiss, 1977).

The colonisation and transgression of the mucosa has been described in Section 1.7.1. If the organism enters the bloodstream unchecked by bactericidal antibodies, one of the several facets of meningococcal disease will develop. Concomitant with the bacteraemia, the meningococcus is thought to metastasise to the skin, eyes, joints, heart, adrenal glands or meninges. 90% of all cases of meningococcal disease have inflammation of the meninges, giving rise to meningitis, and half of these cases will have dermal lesions (De Voe, 1982).

The dermal lesions are part of a generalised Shwartzman reaction. They result from vascular damage and intravascular coaggulation. However, unlike experimentally induced Schwartzman reactions in animals, histological examination early in the development of these skin lesions does not show high concentrations of inflammatory exudate cells. PMNs only infiltrate the area as the vascular endothelium becomes damaged.

Death in fulminant meningococcaemic is due to acute endotoxaemia and shock. Meningococci release copious amounts of endotoxin during growth (De Voe and Gilchrist, 1973) and it is thought that meningococcal endotoxin may act directly on the central nervous system to produce remote pathological effects (Ducker and Simmons, 1968) such as haemorrhagic pulmonary oedema, visceral congestion and adrenal haemorrhage. The consequences of this action would be a rapid death.

Meningococcal meningitis is associated with a severe inflammatory process. In this way it is distinct from the development of skin lesions and similar to symptomatic localised gonorrhoea. The brain becomes oedematous and there are small haemorrhages, suggesting the action of endotoxin (De Voe, 1982). As with gonorrhoea, the meningococci are observed both inside and outside the neutrophils in purulent cerebrospinal fluid. (De Voe, 1982). The inflammation of the meninges is thought to be due to meningococcal endotoxin.

The ability of meningococci to survive within human phagocytes has yet to be fully examined. However, studies on the interactions between the meningococcus and PMNs suggest that they are killed (De Voe, 1980), degraded and then egested by a specific exocytosis mechanism (De Voe, 1976). This egestate has been found to enhance the coagulopathic effect of meningococcal endotoxin in rabbits (De Voe and Gilka, 1976, The mechanism for this is not certain, but DeVoe (1980) thinks that the egestate stimulates release

of PMNs from the bone marrow which accumulate in the liver and the lungs. There they compromise the main degraders of endotoxin, the macrophages (Filkins, 1971), thereby seriously affecting the body's capacity to cope with even small amounts of endotoxin. This endotoxin could then stimulate thromboplastin production by leukocytes and platelets, leading to intravascular coaggulation, the hallmark of meningococcal disease (Østerud and Flaegstad, 1983; DeVoe, 1980)

1.7.4 Summary

The gonococcus and meningococcus cause radically different disease states. They exhibit similarities in adherence and penetration of mucous membranes. Restriction to the mucous membranes and sub-mucosal tissues appears to depend on effective serum bactericidal activity for both organisms. The inability of phagocytic cells to kill and degrade these bacteria is a significant factor contributing to pathogenesis. The severity of systemic disease is greater for meningococcal infection. This is, in part, due to the pharmacological effects mediated by the profusely liberated endotoxin.

1.8 NATURAL IMMUNITY

1.8.1 The protective role of 'natural' antibodies

Natural antibodies are immunoglobins which react with antigens present on cells, such as pathogenic microbes and red blood cells, with which the immune system has not been previously challenged. They are cross-reacting antibodies. They develop in the classical manner, as a response to antigenic stimulation. The stimulating antigens are usually present in an innocuous form, such as in the food we eat and in the micro organisms colonising the skin and gut, rather than as components of virulent pathogenic bacteria. These natural antibodies are identical to immune antibodies in their biological activity and hence can be protective against pathogens with which they fortuitously cross-react.

1.8.2 The role of natural antibodies in protection against Neisserial infection

1.8.2.1 Natural antibodies reactive with N.gonorrhoeae

Sera from normal children and adults have been shown to contain IgM, IgG and IgA antibodies which react with N.gonorrhoeae. By a fluorescent antibody technique, Cohen (1967) found that IgG and IgM antibodies in NHS reacted with heat-labile (protein) and heat-stable (carbohydrate) gonococcal antigens. IgA was found to react only with the heat-stable antigens, and the titre was very low. A study of the development of antigonococcal antibodies with similar techniques found that the IgG antibodies are present in the newborn at a similar concentration to that in maternal sera. This titre was found to drop in titre for the first 3-4 months of life and then return to adult levels. This pattern follows that observed for serum IgG concentration during the first year of life and is due to the ability of maternal IgG to cross the placenta. IgM and IgA do not have this property. Antigonococcal IgM was found in trace amounts in

the newborn, but was found to increase in titre with increasing age. Reactive IgA was not detectable in the first 4 months of life, and the titres of older children never exceeded 1:2 (Cohen and Norrins, 1968).

1.8.3 The biological activities of natural antibodies

1.8.3.1 Bactericidal Effects

Normal human serum has been shown to kill N.gonorrhoeae (Glynn and Ward, 1970) by means of an antibody-complement bactericidal reaction. This is thought to be due to IgM antibodies since treatment of bactericidal serum with 2-mercapto-ethanol severely diminishes this capacity. Further evidence obtained from the use of purified immunoglobin fractions shows IgM to be bactericidal but IgG has negligible activity (Schoolnik, Ochs and Buchanan, 1979).

The antigenic determinant(s) with which this natural bactericidal antibody reacts has been shown to be predominantly in the lipopolysaccharide molecule. (Glynn and Ward, 1970; Tramont, Sadoff and Wilson, 1977) though in experiments with immune serum raised in rabbits, outer membrane proteins can also be involved in bactericidal reactions (Tramont, Sadoff and Artenstein, 1974).

Sera vary in their capacity to kill N.gonorrhoeae (Glynn and Ward, 1970) but a greater source of variability appears to reside in the bacterium itself. Ward, Watt and Glynn (1970) investigated the serum sensitivity of gonococci in urethral pus. They found the organisms to be resistant to the patient's serum and NHS; however, after subculture most strains became sensitive to these same sera. Other variations in susceptibility to NHS have been observed for strains cultured in vitro. Not all strains isolated from localised infection are serum-sensitive after subculture. Some maintain their resistance (Glynn and Ward, 1970). However, in a study of the sensitivity to NHS of 39 strains of gonococcus isolated from disseminated infection,

38 (97%) were resistant. (Schoolnik, Buchanan and Holmes, 1976). It appears that strains associated with localised infection exhibit a resistance to serum which is expressed in the environment of the urethral pus but not in laboratory media, whereas strains associated with disseminated infections exhibit a serum resistance which is independent of the environment.

1.8.3.2 Inducible serum resistance

This variable serum resistance exhibited by strains isolated from localised infections is also seen in strains adapted to growth with subcutaneously implanted chambers in guinea pigs. (Penn et al., 1976). Since this resistance was acquired and lost at a faster rate than if it had occurred by mutation or selection, it was thought to be phenotypic (Rittenberg et al, 1977). Analysis of the factor(s) responsible for inducing this phenotypic serum resistance in a laboratory strain BS4 (agar) indicate that a low molecular weight (< 12000) fraction of guinea pig serum contains the inducing principle. (Veale, Penn and Smith, 1981). In a study to determine the influence this agent has on serum sensitivity of recent clinical isolates, Martin et al (1983) described the induction of resistance to a 1:4 concentration of serum in 13/20 isolates from males and 9/10 isolates from females. The eight isolates that did not exhibit resistance to the 1:4 concentration of serum were found to be significantly resistant to lower concentrations of serum compared to the level of serum sensitivity before induction.

Human serum and genital secretions have been shown to induce a similar phenotypic serum resistance. In a survey of the capacity of 47 sera to induce serum resistance in strain BS4 (agar), only 13 positive sera were found (Martin et al, 1981). The nature of the inducing agent appeared to be similar to that observed in guinea pig serum, but it was less potent. Human genital secretions also induce strain BS4 (agar) to serum resistance. 5/11 samples of cervical secretions converted $> 50\%$ of approximately 10^4 gonococci

to serum resistance. A higher proportion of seminal plasmas, 9/10, had the same effect (Martin et al., 1982).

The clinical significance of the capacity of human serum to induce phenotypic serum resistance in gonococci has been investigated. (Martin et al., 1982b). They suggest that this factor does not affect susceptibility to the disease. Absence of the inducing factor was, paradoxically, associated with the development of salpingitis and with females who had successive infections. However, those sera from men with a previous history of infection and women with their first infection were similar to control sera (Martin et al, 1982b; Martin et al., 1984).

1.8.3.3 Genetically determined serum resistance

The gonococcal determinants of constitutive serum resistance are thought to be influenced by two genetic loci: sac-1, and sac-3. (Shafer, Glymon and Sparling, 1982) sac-1 is closely linked to a locus nmp-2 which influences Protein I. This led to a misinterpretation of the role of protein I in determining serum sensitivity. (Hildebrandt et al., 1978; Cannon et al, 1981). Expression of these sac genes is thought to influence the gonococcal surface so as to vary the degree of serum sensitivity.

Schneider et al., (1982) have examined the potential role of factors in both the serum and the gonococcus in an attempt to define the immunological basis for serum resistance. They concluded that neither intrinsic resistance of the gonococcal outer membrane to the lytic action of complement, nor inaccessibility of the membrane to $\overline{C5b}$ were involved in gonococcal serum resistance. It results from absence of antigenic loci on the LPS molecule to which bactericidal antibody binds. Consequently, the sac loci may influence the composition and/or structure of the LPS, since serum-resistant strains have LPS molecules which are immunologically distinct from serum sensitive strains (Glynn and Ward, 1970).

1.8.3.4 Disseminated Gonococcal Infection (DGI)

Strains isolated from disseminated gonococcal infections are constitutively resistant to the bactericidal effect of normal human serum, though they can be killed by convalescent serum. (Schoolnik, Buchanan and Holmes, 1976). Joiner *et al* (1983) suggest that the lytic complex of complement is deposited on the surface of DGI strains incubated in NHS to the same extent as that deposited on serum-sensitive strains. However, the lytic complexes $\overline{(C5b-9)}$are aggregated on the membranes of the DGI strains and fail to penetrate the membrane. The bactericidal antibody present in convalescent serum is thought to allow insertion of the lytic complexes into the membrane, resulting in cell death.

1.8.3.5 Does the complement mediated bactericidal activity of Normal Human Serum confer protection against Gonococcal Infection?

The bactericidal activity in normal human serum does prevent dissemination of serum-sensitive gonococci from the mucous membranes. The evidence for this comes from the predisposition of individuals with deficiencies in the terminal components of the complement system to gonococcaemia with serum-sensitive organisms. Replacement of the complement components *in vitro* restores the bactericidal capacity of the patient's serum for the infecting strain. (Petersen, Graham and Brooks, 1976) Additional evidence is provided by the serum-resistant nature of the great majority (97%) of strains isolated from DGI in contrast to the smaller proportion (42%) that are isolated from localised infections (Schoolnik, Buchanan and Holmes, 1976). However, this also indicates that factors in addition to serum-resistance may also influence dissemination. The role of bactericidal immunity in protecting the mucosa is, however, more uncertain than its role in preventing bacteraemia.

1.8.3.6 Opsonic effects

IgG antibodies present in normal human serum have been shown to promote phagocytosis of gonococci by human neutrophils. (Schiller, Friedman and Roberts, 1979). This opsonic effect is augmented by the presence of complement, but complement alone was not opsonic. The antigenic specificity for this natural opsonising antibody resides in the Protein I molecule in the gonococcal outer membrane (Sarafian, Tam and Morse, 1983). Serum also enhances the degree of binding of gonococci to human mononuclear phagocytes (Kinane et al, 1983a). This opsonic activity can be removed by absorption of the isohaemagglutinins. Absorption of isohaemagglutinins by N.gonorrhoeae has been previously reported. (Miler et al., 1977).

1.8.4. Meningococcal Disease

1.8.4.1 Natural antibodies reactive with N.meningitidis

Normal human serum has bactericidal (Matsunami and Kolmer, 1918) and opsonising (Kolmer, Toyama and Matsunami, 1918) activities against N.meningitidis. A study of the development of bactericidal antibody (Goldschneider, Gotschlich and Artenstein, 1969) has shown an age-related pattern similar to that described for anti-gonococcal antibody development (Cohen and Norrins, 1968; Section 1.8.2.1). An inverse relationship exists between the development of bactericidal immunity and the risk of meningococcal disease. (Goldschneider, Gotschlich and Artenstein, 1969). Neonates have a low incidence of meningococcal disease; this is probably due to anti-meningococcal IgG obtained from the mother by placental transfer. Young children who have low levels of bactericidal antibody, are at greatest risk from meningococcal disease. The development of natural bactericidal antibodies reactive with serogroupable meningococci confers the increased resistance observed to develop in later childhood. These antibodies are thought to develop as a consequence of antigenic stimulation by commensal enteric bacteria (Robbins et al, 1972), and asymptomatic carriage of N.meningitidis (especially non -

sero-groupable strains) and N.lactamica in the nasopharynx. (Reller, McGregor and Beatty, 1973; Gold et al., 1978). Gold and colleagues (1978) found that carriage and meningococci was low ($< 1\%$) for the first two years of life, but rose in later childhood and adolesence. In contrast, carriage of N.lactamica was high in infants, reaching 24% in 2 year old children but declined after the fourth year. They found that 40% of children who carried N.lactamica developed increased titres of bactericidal antibody reactive with meningococci of serogroups A,B and C.

The role of antibody-complement mediated bactericidal immunity in host defense against meningococcal disease is highlighted by the frequency with which meningococcaemia is observed in individuals deficient in the terminal components of the complement system. (Lee et al, 1978; Lee et al., 1979). In a recent survey of 13 people who suffered from recurrent meningococcaemia, 4 had genetic defects in the complement system (Merino et al., 1983). Investigations by Nicholson and Lepow (1979) determined that neutrophil phagocytosis and intracellular killing of meningococci was unaffected by serum deficiency of the complement component C8. The bactericidal function of the serum was absent in the C8 deficient serum, but could be restored by the addition of purified C8.

Natural resistance to meningococcal disease, therefore, appears to be more dependent on bactericidal immunity than does natural resistance to gonorrhoea.

In both diseases bactericidal immunity is a major defense against haematogenous spread of the bacteria from the sites of mucosal colonisation. However, gonococcal colonisation of mucous membranes is generally more pathogenic than carriage of N.meningitidis. The relationship between localised gonococcal infection and serum bactericidal activity may be comparable with the relationship between nasopharyngeal carriage of meningococi, and the bactericidal activity of the serum. Serum bactericidal activity against the meningococus does reduce the rate of nasopharyngeal carriage, but it does not prevent nasopharyngeal infection. (Brooks and Ingwer,1978; Gotschlich, Goldschneider and Artenstein, 1969).

1.9 IMMUNE RESPONSE TO DISEASES DUE TO PATHOGENIC NEISSERIA SPECIES

1.9.1 Humoural Response

1.9.1.1 Gonorrhoea

In a study of the immune response to experimental human infection, Cohen, Kellogg and Norins (1969) determined the levels of serum IgA, IgG and IgM reactive with heat-labile and heat-stable gonococcal antigens over a 140 day period by an immunofluorescence technique. They observed an increase in IgG, IgA and IgM activities against the heat-labile (protein) antigens. The only immunoglobin class to increase in titre against the heat-stable (lipopolysaccharide) antigens was IgA, However, IgG and IgM antibodies reactive with the heat-stable antigens were present in the sera.

In a similar investigation of natural gonococcal infection McMillan et al., (1979) correlated the distribution of titres of IgA, IgG and IgM antigonococcal antibodies with the duration of infection. IgM and IgA antibody titres were found to be raised in the early period of infection (up to 14 days) but then declined to the levels observed in uninfected individuals. IgG antibodies were retained at high titre for a longer period. Treatment of the infection resulted in a rapid decline in levels of IgM and IgA, followed by a more gentle drop in titre of IgG. This study, however, did not ascertain the antigenic specificity of these antibodies.

An examination of the antigonococcal antibodies in urethral exudates of 132 men with gonorrhoea demonstrated reactive antibodies in 129 cases. IgA and IgG were predominant, being observed in 129 (98%) and 119 (90%) cases, respecively. IgM was observed in only 64 (49%) specimens. After treatment, the levels of IgA and IgM declined rapidly whereas IgG levels dropped at a slower rate. This pattern was similar to that observed with antigonococcal antibodies in serum. (McMillan, McNeillage and Young, 1979). These authors noted that three men in their study were asymptomatically infected with N.gonorrhoeae yet they elaborated a similar immune response as those men who showed symptoms of infection.

The functional aspects of IgG and IgA antigonococcal antibodies in genital secretions have been examined by Tramont (1977). He found that the secretions could inhibit gonococcal attachment to epithelial cells, but the activity was relatively specific for the antibody-inducing (i.e. infecting) strain. Bactericidal antibody has been shown to increase in approximately one third of patients with localised infection (Brooks and Ingwer, 1978) and patients with disseminated infections. In particular, those with suppurative joint infections tended to develop antibodies that killed the infecting strain (Rice, McCormack and Kasper, 1980): Opsonisation of DG I strains has been reported to occur only with sera bactericidal for the test strain (Brooks, Israel and Petersen, 1976).

Patients with gonorrhoea have been shown to develop elevated isohaemagglutinin titres (Miler et al., 1977). Individuals of blood group O developed raised titres against red cells of Groups A, B and AB. Group A individuals only showed an increase in reactivity with AB cells. Group B individuals, however, showed significantly lower titres against AB than control values. No simple relationship between the agglutinin titres and the duration of infection was observed. Other gram-negative organisms have been shown to induce isohaemagglutinin formation (Springer, 1970). The isohaemagglutinins induced by N.gonorrhoeae are IgM antibodies, and they can be present in high titres ($\geqslant$ 1:512). The absence of high titres of IgM reactive with gonococci in the sera of patients with gonorrhoea is a paradox (Cohen, Kellogg and Norins, 1969; McMillan et al., 1979). An equally curious observation is the failure to gonococcal immunisation to induce high titres of isohaemagglutinins in rodents and primates (Miler et al. 1977).

1.9.1.2 Meningococcal Infection

Meningococcal disease, if untreated, has a case fatality rate in excess of 80%. Appropriate antibiotic therapy reduces this figure to less than 10%. During meningococcal disease the antibody

response in adults and children over 2 years old is both rapid and intense for the three major classes of immunoglobin (Zollinger, Pennington and Artenstoin, 1974). Antibodies to membrane proteins, lipopolysaccharide and the group-specific capsular polysaccharide are formed (Artenstein et al., 1971). The group-specific polysaccharide and lipopolysaccharide antigens of group B meningococci, however, are poor stimulators of antibody (Zollinger, Pennington and Artenstein, 1974) whilst the antibody response to the protein antigens is comparable to that observed with other serogroups. Infants under 1 year old do not respond to systemic disease in the same way as adults. They are unable to elaborate a bactericidal antibody response (Baltimore and Hammerschlag, 1977), yet they appear to be capable of developing cross-reactive antibodies which are bactericidal for the meningococcus as a result of carriage of N.lactamica (Gold et al., 1978). The observation by Griffiss (1975) that convalescent sera contain IgA capable of blocking immune lysis probably accounts for the diminished bactericidal activity in acute serum of patients with meningococcal disease. Since bactericidal antibody is the main host defense against systematic meningococcal disease, this observation provides a caveat for the designers of meningococcal vaccines.

1.9.1.3 Immune response to meningococcal vaccines

Following the observation by Goldschneider, Gotschlich and Artenstein (1969b) that the major antigen involved in the immune response to serogroup C meningococci was the group-specific polysaccharide capsule, vaccines containing groups A and C polysaccharides have been developed and widely used. Group B polysaccharide does not appear to stimulate an immune response in man and attempts are being made to develop vaccines for group B meningococci with outer membrane proteins associated with serogroup B (proteins 2 and 9) (Lepow and Gold, 1981).

Intracutaneous immunisation of a vaccine of group C polysaccharide antigen results in increased levels of specific IgM, IgG and IgA. (Artenstein et al., 1970). This vaccination appears to

stimulate mucosal immunity since the carriage rate in the vaccinated subjects is reduced in comparison to unvaccinated controls. (Gotschlich, Goldschneider and Artenstein, 1969). The protective efficiency of this type of vaccine is about 90% (Lepow and Gold, 1981).

These vaccines are also immunogenic for children, but the antibody concentrations induced are only 50% that obtained in adults.

1.9.2 Cell-mediated Immune Response

Cell-mediated immunity is particularly important in host-defense against intracellular pathogens and a close temporal relationship has been demonstrated between development of delayed-type hypersensitivity and acquired resistance to intracellular pathogens: Mycobacterium tuberculosis, Brucella abortus and Listeria monocytogenes (Collins, 1971). Sensitized lymphocytes respond to antigen by elaborating a variety of factors, called lymphokines, one of the functions of which is to increase the bactericidal capacity of phagocytes. (Morley, Hanson and Rumjanek, 1984). In view of the intraphagocytic location of a large proportion of bacteria in gonorrhoea and meningococcal disease, lymphokine production may have a major role in host-defense against the pathogenic Neisseria species. In addition, K-lymphocytes (and mononuclear phagocytes) can act in concert with specific antibody to lyse cells. This is termed antibody-dependent cell-mediated cytotoxicity (ADCC).

1.9.2.1 Gonococcal Infection

Delayed-hypersensitivity to gonococcal antigens has been observed in patients with gonorrhoea. Corbus and Corbus (1941) reiterated this observations, which had been the subject of forty-six contributions since 1909. Lymphocytes isolated from patients with gonorrhoea have been shown to elicit a blastogenic response when stimulated with gonococcal antigens (Esquenazi and Streitfeld 1973).

Not all patients' lymphocytes respond to the same degree (Wyle, Rowlett and Blumenthal, 1977). Some individuals were no more sensitive to the stimulating antigen than uninfected controls. The incidence of non-responders was much greater among male patients than female patients.

Lymphocyte blastogenesis in cells taken from patients with gonorrhoea can also be stimulated by antigens from N.meningitidis (Wyle, Rowlett and Blumenthal, 1977), and, occasionally, from N.catarrhalis (Esquenazi and Streitfeld, 1973). This gives further support to observations of antigenic similarity among the Neisseria species. (Apicella et al., 1981; Gold et al., 1978).

1.9.2.2 Meningococcal Disease

Little effort has been made to investigate the cell-mediated immune response to meningococcal disease because of the evidence suggesting a dominant role for bactericidal antibody in host-defense against N.meningitidis. Lymphocytes from patients with acute meningococcal meningitis show a severely depressed response to meningococcal antigens and the plant lectin stimulant of lymphocyte blastogenesis, phytohaemagglutinin, (Greenwood, Onduloju and Ade-Serrano, 1979). Lymphocytes from vaccinees reacted in a similar fashion to those obtained from control subjects. Sparkes (1983) recently observed that meningococcal protein antigens in a $CaCl_2$ extract were strongly mitogenic for murine B-lymphocytes, but markedly impaired T-lymphocyte responses to mitogenic stimuli. This suppression of T-lymphocyte responsiveness might account for the anergy observed in acute meningococcal meningitis.

Antibody-dependent cell-mediated anti-meningococcal activity by K-lymphocytes and mononuclear phagocytes has been reported. (Lowell et al., 1979). IgG and IgA antibodies specific for N. meningitidis are equally effective in initiating this cell-mediated

killing of meningococci. Convalescent IgM induced by Group C meningococcal vaccine is ineffective. (Lowell et al., 1980). The immune specificity of this cell-mediated antibacterial system has been shown to depend on the antibodies to the group specific meningococcal capsular polysaccharides. (Smith and Lowell, 1980).

1.10 THE AIMS OF THIS STUDY

1.10.1 A comparison of sera of blood groups A and B in their capacity to kill *Neisseria gonorrhoea* and *Neisseria meningitidis*

The epidemiological data reviewed in section 1.3 *et seq* led to the investigation of the host-parasite interactions responsible for the observation that individuals of blood group B appear to be more susceptible to infection by *N.gonorrhoeae* than individuals of the other ABO blood groups (Foster and Labrum, 1976; Miler *et al.*, 1977; Kinane *et al.*, 1983). Gonococci have been found to absorb and stimulate production of human ABO isohaemagglutinins (Miler *et al.*, 1977). Anti-B isohaemagglutinins have also been shown to be capable of opsonising and killing *via* an antibody-complement mediated bactericidal reaction organisms, such as *Escherichia coli* 086, that have galactose as the immunodominant sugar of the LPS (Muschel and Osawa, 1959; Check *et al.*, 1972). Terminal galactose residues are also a feature of gonococcal LPS, and galactose-specific antibodies cross-react with purified gonococcal LPS (Bundle, 1979).

The suggestions of Foster and Labrum (1976) combined with this information about the biological activities of anti-B isohaemagglutinins and the chemical nature of gonococcal LPS led to the formulation of the following hypothesis :

> Gononocci, by virtue of the terminal galactose residues in the LPS molecule, react with human serum in a similar fashion to *E. coli* 086, and so are a) opsonised and b) killed much more effectively by anti-B antibodies than by anti-A antibodies.
>
> A comparison of normal sera from individuals of Groups A and B should show a greater opsonising and bactericidal activity against *N.gonorrhoeae* in group A sera (i.e. the sera which contain anti-B antibodies).

Studies by Blackwell et al (1983) have shown no differences in the opsonising capacity of A and B sera. Serum did not increase the degree of association between gonococci and neutrophils, Kinane et al (1983a) found that autologous serum enhanced gonococcal association with mononuclear phagocytes, especially to cells of blood group B. Moreover, this opsonic activity was absent in AB serum (which has no anti-A or anti-B antibodies) and in serum from which the ABO isohaemagglutinins had been removed (see section 1.7.2.1.2).

Consequently, the hypothesis proposing a greater opsonic activity for N.gonorrhoeae in group A serum in comparison with group B serum has been disproved for neutrophils, but still requires further investigation with mononucler phagocytes.

This study tests the second part of the hypothesis: normal serum from blood group A individuals has a greater antibody-complement mediated bactericidal activity against N.gonorrhoeae than serum from individuals of blood group B.

Since N.gonorrhoeae and N.meningitidis have similar outer membrane structures, particularly the LPS molecule (with which 'natural' bactericidal antibodies react (see Section 1.5.5 et seq)) comparisons will be made between the sensitivity of gonococcal and meningococcal strains to normal sera from individuals of blood groups A and B.

1.10.2 A comparison of the oxidative metabolic response of human peripheral blood monocytes of blood groups A, B, AB and O in response to stimulation with Neisseria gonorrhoeae

The work of Kinane et al, (1983a) demonstrated enhancement binding of N.gonorrhoeae to human peripheral blood monocytes by isohaemagglutinins. This enhanced binding was greatest for monocytes of group B in autologous serum. (See Section 1.7.2.1.2). The

second part of this investigation comprises a short pilot study to ascertain 1) whether there is any heterogeneity in response to stimulation by N.gonorrhoeae from monocytes of blood groups A,B,AB and O in the presence of autologous serum and 2) whether the response can be diminished by absorbtion of the 'natural' antibodies reactive with the stimulating gonococcal strain.

CHAPTER 2

MATERIALS AND METHODS

CONTENTS

2. MATERIALS

All chemicals and biochemicals were obtained commercially and were of a high technical grade or analytical grade of purity.

2.1 Buffers

2.1.1 Phosphate Buffered Saline (PBS)

50 mM Phosphate buffer pH 7.2 containing 0.15 M Sodium Chloride.

2.1.2 Dulbecco's Phosphate Buffered Saline (DPBS)

Dulbecco's PBS (pH 7.3) was prepared from Dubecco's A tablets (Oxoid Ltd., England) which give a solution containing NaCl (5g/l), KCl (0.2 g/ l), Na_2HPO_4 (1.15g/l) and KH_2PO_4 (0.2g/l), 0.2% Phenol Red indicator was added to give a final concentration of 0.0002%. Divalent cations were added by supplementing 100 ml DPBS with 0.5 ml Dulbecco B ($CaCl_2$ (20g/l), $MgCl_2$ (20g/l) (D.PBS ÷ 8).

2.1.3 Tris Buffer

This Tris buffer was used to store the partially purified pyocins. It contained 0.01M Tris buffer (pH 7.5) with 0.01 M $MgCl_2$ and 0.01M $MgSO_4$.

2.2 Bacterial Strains

Recent clinical isolates of *Neisseria gonorrhoeae* were obtained from Dr H. Young and colleagues, STD Diagnostic Laboratory, University of Edinburgh. Strains were also provided by Dr K. Shannon and Prof. I Phillips, St Thomas's Hospital Medical School, London and Dr Joan S. Knapp, Neisseria Reference Laboratory, United States Public Health Service, Seattle, Washington. Strains of *N.meningitidis* and *N. lactamica* were obtained from the culture collection of the Infection and Immunity Laboratory, Bacteriology Department, University of Edinburgh, as were the pyocin-producing strains of *Pseudomonas aeruginosa*. The Kageyama classification (Kageyama, 1975) for the pyocins produced by the strains of *Ps.aeruginosa* used in this study are shown in Table III.

Neisseria strains were cultured on MNYC medium at 37° C in a humidified incubator in an atmosphere of air enriched with 10% (V/V) CO_2. The *Pseudomonas* strains were cultivated on nutrient agar at 37°.

TABLE III

Kageyama classification of the pyocins

Kageyama Classification	Pyocin producing strains of *Pseudomonas aeruginosa*
R 1	ISD, 2285, IS4, IS6, IS8
R 2	ISB, R21, 430
R 3	ISE
R 4	Not available
R 5	ISA, ISC
Nongroupable	R205, 9579

2.3 Bacterial Culture Media

In addition to standard bacterial culture media, the following were used :

2.3.1 Modified New York City (MNYC) Medium

This was prepared as outlined by Young, (1978). The medium contained Difco GC Medium Base supplemented with 10% (W/V) Group O human blood lysed by Saponin (0.5% W/V), 2.5% (W/V) yeast dialysate, 0.1% (W/V) glucose, lincomycin (1µg/ml), colistin (6 µg/ml) amphotericin B (1µg/ml) and trimethoprim lactate (6.5 µg/ml).

2.3.2. Sodium Glutamate Broth (SGB)

This is a clear medium suitable for the production of pyocins (Kageyama and Egami, 1962). It contains sodium glutamate, 20g, glucose (20% (W/V solution) 20 ml, $MgSO_4 7H2O$ 0.1g, $NaH_2PO_4.12H_2O$ 5.63g, KH_2PO_4 0.25g, $Ca(NO_3)_2$ 10mg, yeast extract 1g; and distilled water to 1 litre. The glucose solution was sterilised by filtration and added to the autoclaved medium before use.

2.3.3 Defined Medium

This chemically defined liquid medium was used in the cultivation of N.gonorrhoeae for part of the study which was conducted in the Microbiology Department, University of Birmingham. It was prepared according to the directions given by Veale, Penn and Smith (1981).

2.4 Enumeration of Bacteria

2.4.1 Total Counts

Bacteria were counted microscopically in a Helber chamber. At least 200 bacteria were counted in each estimation.

2.4.2 Viable Counts

Viable counts of bacteria were performed by plating out tenfold dilutions of the bacterial suspension, either as six drops of 50 µl , as described by Miles, Misra and Irwin, (1938) or by spreading 0.3ml of each dilution over a plate. After overnight incubation the number of colonies was multiplied by the appropriate factor to give the viable count in the original suspension.

2.5 Pyocin Typing

2.5.1 Introduction

Pyocins are bacteriocins produced by Ps.aeruginosa that bind to and kill bacterial strains bearing the appropriate receptor molecules. Pyocin typing allows detection of bacteria possessing these receptor molecules. The basic procedure involves mixing the pyocin with the bacterial strain under test in conditions that allow pyocin-binding and killing to take place, then determination of the residual viable organisms. Bacteria expressing the pyocin receptor will be killed, whereas those which do not will remain viable. By using a range of pyocins which have different receptor molecules, a typing schema can be developed based on the sensitivity spectrum of pyocin-sensitive spectrum of pyocin-sensitive strains.

2.5.2 Induction and preparation

Pyocin-production and partial purification basically followed a procedure outlined by Blackwell, Young and Anderson (1979). An overnight nutrient-broth culture (2ml) of the pyocin-producing strain of Ps.aeruginosa was added to 25 ml of sodium glutamate broth (SGB). After 3 hours incubation at 30°C in an orbital incubator (Gallenkamp) running at 100 r.p.m., Mitomycin C (1.8 µg/ml) was added and the cultures were incubated in the dark until the cells lysed. This took approximately 3 hours. The lysate was centrifuged at 3000 xg for 30 mins. to remove the cells and debris. The supernates were shaken with chloroform (5% V/V) to kill any remaining bacteria and stored at 4° C overnight.

This crude pyocin lysate was ultracentrifuged (100,000xg/ 3 hours) and the supernatant, containing soluble pyocin activity and Mitomycin C, was discarded. The pellet, containing the rod-type (R-type) pyocins, was resuspended in Tris buffer.

2.5.3 Titration of pyocin activity - a plaque assay

Pyocins were titrated according to the protocol described by Blackwell, Winstanley and Telfer Brunton, (1982). Two fold dilutions of pyocin suspension were made in DPBS + B 15 µl drops of each dilution were placed on a dried nutrient agar plate. When the drops had been absorbed the plate was flooded with a suspension of Ps.aeruginosa strain ZD8/38 (10^7 cfu/ml), a strain which is susceptible to all the pyocins used in the study, and incubated at 37° C for 12-18 hours. The pyocin titre was the highest dilution that produced a clear area of inhibition of the bacterial lawn. The activity of each pyocin preparation was frequently checked against the indicator strain Ps.aeruginose ZD8/38. The pyocin preparations retained high activity over a period of several months.

2.5.4 Determination of the pyocin sensitivity spectrum of strains of N.gonorrhoeae and N.meningitidis by a plaque assay

Discrete 10 µl drops of each pyocin preparation (standardised to a titre of 64-128) were placed onto dried MNYC plates. After they had been absorbed the plate was flooded with an 18 hours culture of the bacteria suspended in DPBS + B (approx. 10^7 cfu/ml) and any excess fluid was removed by gentle aspiration. After overnight growth the results were recorded as + = inhibition, $\pm$ = inhibition with some overgrowth - = no inhibition.

2.5.5 Isolation of a mutant strain of N.gonorrhoeae which was resistant to Kageyama's class R1 pyocins

Colonies growing within a zone of inhibition produced by the R1 class pyocin, IS8, were subcultured and re-typed. One mutant strain P280µ, derived from a strain sensitive to pyocins of classes R5 and R1 (P280) was sensitive to Class R5 pyocins only. This mutant strain was used in this study.

2.5.6 Modified pyocin-typing procedure

A modification of the pyocin typing procedure was required for part of this study which was conducted in the Microbiology Department, University of Birmingham. The main modification involved the removal of unreacted pyocin prior to determination of the numbers of residual viable organisms. The procedure developed to meet these needs was as follows : 15 µl of the pyocin suspension was added to 100 µl of the test organisms suspended in Defined Medium (DM) (10^4 - 10^5 cfu/ml) in sterile plastic tubes (10 ml capacity). Control tubes received DM (15 µl) but no pyocin. After 30 min. incubation at 37°C 0.5 ml DM was added to each tube. The gonococci were deposited by centrifugation (1500 xg, 15 min) and the supernatant containing unreacted pyocin was removed. The gonococci were resuspended in 0.3 ml DM and plated onto 'chocolate' agar or MNYC medium. Results were recorded as +, ± and - when the viable counts were $< 5\%$, $< 20\%$ (but $> 5\%$) and $> 20\%$, respecitvely of the colony counts for the control tubes.

2.6 Bactericidal Tests

2.6.1 Introduction

Complement activation by specific antibody can result in killing and lysis of gram-negative bacteria. The aims of the bactericidal tests are to determine the presence of serum bactericidal antibodies reactive with test bacterial strains and to quantify these antibodies by titration. As with the pyocin sensitivity tests (Section 2.5 *et seq.*), the principle of the test is to mix serum, or a dilution of the serum, with a standard number of bacteria and a standard amount of complement in an environment conducive to the activation of complement by antigen-antibody complexes. After incubation at a standard temperature over a fixed period of time the number of residual viable organisms is determined. By comparison with a control experiment in which the antibody is absent, an estimation of the degree of bacterial killing can be made. The degree of killing which constitutes significant bactericidal activity is an arbitrary decision. In these experiments a significant bactericidal test results in a drop of at least 80% in the number of viable bacteria over a 30 min period at 37^{o} C.

The distinction between killing due to antibody - dependent activation of the classical complement pathway and that due to activation of the alternative pathway can be made by conducting bactericidal tests in medium containing 10 mM Mg - EGTA. This specifically chelates the calcium ions which are essential for activation of the classical complement pathway. Any bactericidal activity observed in this system is due to activation of the alternative complement pathway. The possibility that the alternative complement pathway might be activated by the test organisms can be greatly decreased by diluting the complement components by 1/16. This still allow activation of the classical (antibody-mediated) complement pathway (Schreiber *et al.* 1979).

2.6.2 Human Sera

Normal human sera from individuals of blood groups A and B were obtained in small quantities from women attending post-natal clinics. They were kindly supplied by Drs P.L. Yap and S. Urbaniak, Blood Transfusion Service, Royal Infirmary of Edinburgh. Sera from patients with gonorrhoea were kindly provided from Dr H Young and colleagues, STD Diagnostic Laboratories, University of Edinburgh. These sera were taken from the patents for syphilis serology, and I used the small amounts remaining after the diagnostic work was completed. All sera were heated to 56° C for 30 minutes to inactivate the complement. They were stored at -20° C prior to use.

2.6.3 Preparation of mouse immune serum against N.gonorrhoeae

2.6.3.1 Preparation of the immunising antigens

An eighteen hour growth of N.gonorrhoeae was harvested from MNYC plates, suspended and washed twice in PBS, then resuspended in 10 ml PBS to which an equal volume of PBS containing 2.4% formaldehyde was added. After overnight storage at 4° C the formaldehyde - treated organisms were pelleted and resuspended in PBS, containing 0.01% formaldehyde as a preservative, to the opacity of Brown's tube No. 3 as judged by eye (Brown, 1919-1920). This is equivalent to 1.1×10^9 gonococci/ml (Cunningham and Timothy, 1924).

2.6.3.2 Immunisation schedule

Groups of 10 female CF1 mice, 5-6 weeks old, each with a bactericidal titre of < 10 against the immunising gonococcal strain, were immunised via the lateral tail veins with 0.25 ml of the suspension of formaldehyde - treated bacteria. The mice were immunised on days 0, 7 and 10. On day 14 a small quantity of blood was taken from each mouse in the group via the retro-orbital plexus and pooled. The separated serum was heat-inactivated (56°C/30 mins), distributed

into small tubes and stored at -20°C until required. The mice thereafter received booster doses (0.25 ml) of gonococcal antigen at 14 day intervals and a small quantity of blood was taken from each mouse five days after each booster dose. This was continued for ten weeks with each group of mice.

2.6.4 Complement

Human AB serum from a donor with no history of gonococcal infection was absorbed twice over a period of 24 hours at 4° C with a 'cocktail' suspension of the bacterial strains to be used in the bactericidal test. This was done to remove any reactive antibodies. The bacteria were removed from the serum by centrifugation (1500 x g/30 mins) at 4° C. The absorbed serum was then passed through a sterilising membrane filter (pore diameter = 0.22 μm). Care was taken to avoid frothing since this might denature the complement proteins. This absorbtion procedure did not affect the haemolytic titre of the serum which remained at 32-64, but resulted in a serum which had no bactericidal activity at a dilution of 1:4 against any of the strains used in the absorbing 'cocktail'.

2.6.5 Assay of bactericidal activity in immune mouse serum

These tests were performed in round bottomed glass tubes (15 x 75 mm). An eighteen hour culture of the gonococcal strain to be tested was harvested from MNYC medium and suspended in D.PBS + B to a concentration of approximately 10^6/ml. 160 μl of this suspension was added to 20 μl of a dilution of the heat-inactivated mouse serum and 20 μl of recently thawed complement serum which had been diluted 1:2 with the buffer. Controls in which buffer replaced the mouse serum and/or complement were included for each test. After thirty minutes incubation at 37° C, 25 μl volumes of the test and control mixtures were removed and viable counts performed as described in section 2.4.2. A reduction in viable count of at least 80% compared with the complement - free control was considered to indicate a significant level of bactericidal antibody. The greatest dilution of immune mouse serum to give a significant result was the titre of the serum.

2.6.6 Titration of Bactericidal Antibody in Human serum

Each of the 96 wells of an 8 x 12 microtitre plate (either U - or V - well) was filled with 50 µl D.PBS + B and 50 µl of neat serum or, in some tests, a dilution of the serum, was added to the first well in each row. A series of six two-fold dilutions were then made along each row. By starting a series of rows at column 7 it was possible to titrate sixteen sera in the same plate. An 18 hour culture of the heat bacteria was harvested from MNYC medium and suspended in D.PBS + B to give approximately 10^4 organisms/ml. To each well was added 40 µl of the bacterial suspension and 10 µl of a 1:2 dilution of the absorbed complement source. Serum and/or complement-free controls were also included in the test. The plate was covered and incubated at 37°C for 30 minutes. Two discrete drops of 20µl from each well were plated onto dried MNYC plates and incubated at 37° overnight. Bactericidal activity of each serum against the bacterial strain under test was considered significant if there was a reduction in viable numbers of at least 80% that of the controls. Due to the small numbers of bacteria in the inoculum, a reduction of $\geqslant$ 80% in viable numbers resulted in less than 40 colonies growing from each 20 µl drop. The results of each experiment are expressed as the reciprocal titre of the highest serum dilution to have significantly reduced the viable count.

In some experiments D.PBS + B was replaced by D.PBS + 10 mM Mg - EGTA . The addition of 10mM Mg-EGTA results in sequestration of the calcium ions required for activation of the classical complement pathway yet permits activation of the alternative complement pathway. Tests conducted under these conditions will allow the detection of bacterial killing due to the alternative complement pathway.

2.6.7 Inhibition of serum killing of N. gonorrhoeae by isolated LPS

The normal bactericidal test procedure (section 2.6.6) was followed using serum at a final concentration of 1:10. Isolated LPS was added to the test mixture to give a final concentration of

either 20 µg/ml or 100 µg/ml. At times 0,10,20 and 30 minutes 20 µl aliquots were removed from the reaction mixture and viable counts performed as described in Section 2.4.2.

2.7 Titration of Isohaemagglutinins

50 µl of PBS was dispensed into each well of a U-well microtitre plate and a series of two-fold dilutions of the heat-inactivated test serum were made. A 0.5% (V/V) suspension of washed red blood cells of the appropriate ABO blood group was prepared in the same buffer and 50 µl volumes were added to each well. After mixing by gentle agitation the plate was left at room temperature for two hours. The titre of isohaemagglutinin was recorded as the reciprocal of the highest serum dilution to give visible agglutination of the red blood cells.

2.7.1 Absorbtion of Isohaemagglutinins or other antibodies from serum

Isohaemagglutinins were removed from serum by absorbtion with red blood cells of the appropriate ABO specificity and anti-bacterial antibodies were removed by absorbtion with a suspension of bacteria. The method used was as follows : A heavy suspension of the absorbant was mixed with heat inactivated serum and left at room temperature for one hour. The cells were deposited by centrifugation and the serum supernate was absorbed a second time for an eighteen hour period at 4^{o} C. After removal of the cells by centrifugation, the absorbed serum was passed through a sterilising membrane filter (pore diameter 0.22 µm). When red blood cells were used as the absorbant, this procedure reduced isohaemagglutinin titres to < 4.

2.8 Lipopolysaccharide isolation and Analysis

2.8.1 Lipopolysaccharide extraction

The aqueous-phenol procedure of Westphal and Lüderitz (1954) was used. Strains of N.gonorrhoeae were harvested from MNYC plates, washed twice in PBS and pelleted. The bacteria in the pellet were lysed by being suspended in distilled water to give approximately 10% (W/V). After 1 hour incubation at 37° C with ribonuclease and deoxyribonuclease, the mixture was heated to 65° C. An equal volume of 95% (W/V) phenol was added to the mixture and after 10 minutes stirring the mixture was put on ice to allow separation of the aqueous and phenolic phases. Centrifugation for 20 minutes at 2000xg assisted this phase separation. The upper aqueous layer was removed and dialysed against running tap water for 20 hours to remove the phenol. The volume was reduced by rotary evaporation and the solution was clarified by centrifugation (15,000 xg for 15 minutes). The LPS was concentrated by ultracentrifugation (100,000 xg for 3 hours). After being resuspended in distilled water and washed by centrifugation at 100,000 xg for 3 hours, the resulting clear pellet was freeze dried in a small plastic container.

2.8.2 Analysis of the isolated LPS

2.8.2.1 Introduction

Lipopolysaccharide, as the name indicates, is a molecule with lipid and polysaccharide moieties. The lipid portion is inserted into the membrane and the major antigenic determinant(s), the polysaccharide portion, is expressed on the surface of the bacterial outer membrane. Chemical differences between LPS molecules occur in the polysaccharide portion.

The monosaccharide constituents of the LPS can be liberated by hydrolysis and subsequently separated and identified by chromatography.

Gas-liquid chromatography can be used to separate and identify very small quantities (0.1μg/μl) of sugars after they have been converted to volatile alditol acetate derivatives. This procedure involves reduction of aldose sugars to alditols and subsequent acylation.

2.8.2.2 Hydrolysis of LPS and preparation of alditol acetates

In this experimental procedure analytical grade reagents and clean, acid-washed glassware were used throughout.

A small portion of LPS (approximately 2 mg) was dissolved in 0.25 ml, 2M HCl, sealed in a glass ampule and heated at 100° C for 3 hours to effect complete hydrolysis of the polysaccharide. The hydrolysate was dried in vacuo in a dessicator containing anhydrous P_2O5 and NaOH. The dried residue was washed with distilled water (0.2 ml) and dried in the evacuated dessicator three times. This removed any traces of HCl.

The residue was dissolved in 0.5 ml H_2O and transferred to a round-bottomed flask. The sugars were reduced by treating with approximately 10 mg potassium borohydride for at least 1 hour at room temperature. The excess borohydride was neutralised by addition of glacial acetic acid, sufficient to bring the pH down to < pH5 (a few drops was adequate). The borate was then removed by washing with methanol (3ml). The methyl borate was removed by rotary evaporation at 35°C. This washing procedure was repeated three times.

Acylation of the reduced sugars was carried out by addition of a mixture of acetic anhydride and pyridine (1:1), and heating the reaction mixture in a glass-stoppered vessel at 100° C for 1 hour.

The acetic anhydride and pyridine were removed by co-distillation in the rotary-evaporator (35°C) with successive volumes (5,7,9 and 10 ml) of toluene until the residue was dry. After one more co-distillation with 10 ml toluene, the residue was dissolved in

2 ml chloroform, and washed three times with 3 ml distilled water. The chloroform layer was then evaporated to dryness and the resulting alditol acetates were dissolved in 0.25 ml chloroform and stored at 4° C in a tightly stoppered small glass vessel.

This procedure is that used by Mr R Brown and Dr I Poxton, Microbial Pathogenicity Research Laboratory, Bacteriology Department, University of Edinburgh.

2.8.2.3 Separation and Identification of the Alditol Acetates by Gas Chromatography

This work was undertaken on equipment in the Department of Brewing and Biological Sciences. Heriot-Watt University, Edinburgh by kind permission of Mrs A Murray and Dr M Fleming.

The gas chromatography equipment consisted of a Packard Model 428 gas chromatograph containing a 50 m x 0.25 mm OV1 capillary column through which the carrier gas nitrogen flowed at a rate of 1.5 ml/min. The split ratio was set at 30:1. The injector and detector temperatures were set at 230° C and 275°C respectively. The detector was linked to a Carlo Erba MEGA series integrator which plotted the peaks, gave retention times and peak areas. The synchronisation of gas chromatograph and integrator was performed manually.

A 0.5 µl volume of the sample was injected into the instrument and the following temperature programme was followed : 175°C for 4 minutes followed by a rise of 4°C/min up to 260°C which was maintained for 5 minutes before ending the run.

The alditol acetates of the LPS hydrolysates were chromatgraphed, as were a series of alditol acetate derivatives of pure, standard monosaccharides, identification of the components in the LPS hydrolysate was made by comparing the retention times of the peaks with those obtained using known monosaccharide derivatives.

2.8.3 Extraction of outer membrane complexes

N.gonorrhoeae readily liberates outer membrane vesicles and the method used to extract these outer membrane complexes was simple. An overnight culture of N.gonorrhoeae was harvested and suspended in PBS + 10 mM EDTA to approximately 1% W/V. The suspension was heated to 45° C for 30 minutes after which it was vortexed for 3 minutes with 3mm glass beads. The supernate was decanted into a micro-centrifuge tube and the bacteria were deposited by centrifugation (approx. 10,000 xg for 5 minutes) in a Beckman Microfuge. The clear supernate containing the outer membrane complexes was separated and stored at -20°C until required for study.

2.9 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS - PAGE)

SDS-PAGE was conducted in slab gels (17 x 14 cm) using a Raven electrophoresis tank (Raven, Haverhill, Sussex, England). The gels were prepared using a stock solution of acrylaride - bisacrylamide (30:0.8) and a discontinuous buffer system containing 0.1% sodium dodecyl sulphate (SDS) as described by Laemmli (1970). The final concentrations of the buffers were as follows : stacking gel; 0.125 M Tris-HCl, pH 6.8, Resolving gel 0.375 M Tris-HCl, pH 8.8, Reservoir buffer; 0.025 M Tris, 0.192M glycine, pH 8.3. The final concentration of the acrylamide in the stacking gel was 2.5%, and in the resolving gel was 17.5%.

Samples were solublised in a buffer containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue in 0.0625 M Tris-HCl (pH 6.8) by heating to 100^{o} C for 5 minutes. This prolonged solublisation step was necessary to ensure disaggregation of the micelles of lipopolysaccharide. (Jann, Reske and Jann, 1975). After loading the samples onto the stacking gel, electrophoresis was carried out at constant voltage until the dye front was about 2 cm from the bottom of the gel.

On completion of electrophoresis the gel was carefully removed from the cassette and fixed overnight in a solution of 40% ethanol, 5% acetic acid in water prior to staining.

2.10 Silver Staining of Lipopolysaccharide

Lipopolysaccharide in polyacrylamide gels was stained using a slight modification of the sensitive silver stain designed by Tsai and Frasch(1982). As the authors remarked, it is essential that distilled water and very clean staining baths are used to prevent staining artefacts.

The staining procedure used was as follows : After overnight fixation in 40% ethanol - 5% acetic acid the fixative is replaced by 0.7% periodic acid in 40% ethanol - 5% acetic acid to oxidise the sugars in the LPS. After 45 minutes the oxidising solution was removed and the gel washed for 90 minutes in three volumes (500 - 1000 ml) of distilled water. The water was decanted and the gel immersed in freshly prepared staining reagent which was prepared as follows : 2ml NH_4OH (s.g. 0.880) was mixed with 28 ml of 0.1M NaOH. 5 ml of 20% (W/V) $AgNO3$ was added dropwise to this solution while it was being stirred. Water (115 ml) was then added to complete the staining reagent. After a staining period of 10 minutes, the stain was decanted and the gel was washed three times as described earlier. The waste ammoniacal silver stain is potentially explosive and so was discarded into a saturated solution of NaCl.

The stained and washed gel was developed with a formaldehyde developer : 50mg citric acid and 0.5 ml of 37% formaldehyde solution in 1 litre of water. Development was stopped by washing in water when the gel background showed slight signs of discolouration. The gel was washed and stored in water.

This procedure stained LPS strongly, whilst proteins showed much weak staining.

2.11 The Nitroblue Tetrazolium (NBT) Reduction Test

Professional phagocytes, both polymorphonuclear and mononuclear, respond to various particulate and soluble stimuli by an increase in oxidative metabolism. Phagocytosis of bacteria is associated with this phenomenon. The increase in oxidative metabolism does not provide the cell with extra energy; it is a means of producing various reduced forms of oxygen; superoxide radicals, hydroxyl radicals and hydrogen peroxide, which form one facet of the microbicidal machinery of the phagocyte. Determination of the degree to which bacteria stimulate the production of reactive oxygen species such as these is the purpose of a series of tests, including the NBT reduction test.

NBT is a redox indicator. In the oxidised state it is composed of yellow crystals which readily form aqueous solutions. When reduced, it becomes a blue formazan which is insoluble in water. NBT is reduced by professional phagocytes during phagocytosis. This results in insoluble blue formazan deposits within the cells. Baehner, Boxer and Davis (1976) suggested that NBT reduction by phagocytosing neutrophils was due exclusively to activity of a superoxide radical-generating oxidase.

These experiments on glass-adherent mononuclear phagocytes use NBT reduction as an index of the stimulation of the oxidative metabolism of individual cells by gonococci.

2.11.1 Isolation of monocytes from human peripheral blood

Human peripheral blood was obtained from healthy volunteers by venepuncture and was quickly transferred to plastic vessels containing heparin (10 Units per ml. of blood) as an anticoagulant. The mononuclear cell fraction, containing phagocytes and lymphocytes, was obtained from blood diluted 1:2 in DPBS + B by separation on Ficoll - Hypaque cushions (9% (W/V) Ficoll, 33.9% (W/V) Hypaque mixed in the ratio 2.4: 1 to give a specific gravity of 1.078) containing 0.1% (W/V) Na_2 EDTA, at 400 xg

for 30 min. (Böyum, 1968). The interface cells (monocytes and lymphocytes) were collected, washed twice in warmed D.PBS + B, and resuspended in Hank's solution. (Oxoid Ltd., England). 100 µl of the cell suspension was diluted 1:10 in white blood cell diluting fluid (0.01% gentian violet in 1% acetic acid) and loaded into a haemocytometer. On microscopic examination at x400 the monocytes, identified by their morphology, were counted. The cell suspension was then adjusted to give a monocyte concentration of 2×10^6 cells per ml.

2.11.2 Preparation of monocyte monolayers

Small monolayers (5mm diameter) of monocytes were prepared on clean glass microscope slides using an apparatus borrowed from Mr W Neill, Department of Bacteriology. This comprised a neoprene block, in which there were 10 evenly distributed 5 mm holes, which was opposed to the microscope slide and firmly held in place by sprung steel clips.

100 µl aliquots of the cell suspension (containing monocytes and lymphocytes) in Hank's solution containing 20% autologous fresh serum were distributed into the wells of two cleaned and sterilised micro-well units. After 90 minutes incubation at $37^{o}C$ in air enriched with 5% Co_2, each monolayer was gently washed several times with warmed Hank's solution to remove any non-adherent cells (which are predominantly lymphocytes). Microscopic examination of each monolayer using an inverted microscope showed the resultant monolayers to consist of well separated monocytes with very few contaminating lymphocytes.

2.11.3 Nitroblue Tetrazolium (NBT) reduction test)

An 18 hour culture of N.gonorrhoeae was harvested from MNYC medium, suspended and washed once in Hank's solution, and adjusted to a concentration of approximately 2×10^7 bacteria/ml. Another portion of the bacterial suspension was centrifuged (3000xg/15 mins) and the pellet was distributed into small volumes (about 100 µl) of fresh and heat-

inactivated (56° C/30 mins) serum autologous with the monocytes under test, to give a final concentration of about 10^9 bacteria/ml. These sera were kept in ice for 1 hour,after which the bacteria were quickly pelleted by centrifugation in a Beckman Microfuge (approx. 10,000 xg) for 1 minute. The supernatant sera were quickly removed and placed in chilled vessels in an ice bath. This absorbtion procedure did not reduce the activity of the complement in the fresh serum. In addition to the fresh and absorbed sera, the following reagents were also used in this test ; Nitroblue Tetrazolium (NBT); a sterile 1% solution of NBT in distilled water was stored frozen (-20°C) until required for use. Opsonised Zymosan; zymosan, opsonised with fresh AB serum using the method of Taffet and Russell (1981) and was standardised to 200 mg/ml and stored at - 20° C until required for use.

Pairs of monolayers were covered with the mixtures of reagents as shown in Table IV.

TABLE IV

Nitroblue Tetrazolium (NBT) reduction Test (volumes given in µl)

	Hank's Solution	1% NBT	Zymosan	FHS	HIS	Absorbed Sera FHS	Absorbed Sera HIS	Bacterial Suspension
A Stimulus - free Control	90	5	-	5	-	-	-	-
B Zymosan Control	85	5	5	5	-	-	-	-
C Test organism + FHS	-	5	-	5	-	-	-	90
D Test organism + HIS	-	5	-	-	5	-	-	90
E Test organism + Absorbed FHS	-	5	-	-	-	5	-	90
F Test organism + Absorbed HIS	-	5	-	-	-	-	5	90

After 50 minutes incubation at 37° C in the CO_2 incubator, the reaction mixtures were removed by gentle aspiration and the monolayers were fixed by filling each well with saline containing 3.7% formaldehyde and leaving at room temperature for 30 minutes. When the fixation procedure was completed the microscope slides were gently removed from the neoprene block and the monolayers were stained with 1% Neutral Red for 1 minute, washed and dried.

Microscopic examination was undertaken at a magnification of x1200 using a water-immersion objective. Each monolayer was examined by a series of S-shaped sweeps. 200 monocytes were counted and placed into one of three categories based on the amount of reduced NBT, a blue formazan deposit, within the cell. These categories, -, + and +++ are described as follows : -, monocytes in which there is no blue formazan; + monocytes which have slight blueing, especially around the periphery of the cell; +++ monocytes which have heavier blue formazan deposits.

2.12 Statistical Methods

Comparisons between the titration data were made using the Mann-Whitney U test. The results obtained from the NBT reduction experiments were compared by an analysis of variance. Comparison of the means obtained from this analysis were compared by the protected significant difference method described by Snedecor and Cochran (1980). The difference between a specific pair of means is significant if it exceeds the product of the standard error of the difference between two means and the 5% value of *t* for the appropriate number of degrees of freedom. This test is 'protected' since it should only be applied where the Variance Ratio (F) is significant.

RESULTS

CHAPTER 3

A comparison of the bactericidal activities of normal sera from individuals of blood groups A and B against strains of *N.gonorrhoeae*

CONTENTS

3.1 Hypothesis

This section tests the hypothesis stated earlier in Section 1.10.1 that sera from individuals of blood group A have a greater capacity to kill strains of N.gonorrhoeae in an antibody-complement mediated bactericidal reaction than sera of blood group B. This is due to the ability of the anti-B isohaemagglutinins, present in Group A sera, to bind to strains of N.gonorrhoeae and initiate a bactericidal reaction.

3.2 Experimental Design

Sera from individuals of blood groups A and B were obtained from women attending post-natal clinics. They were supplied by Dr. S. Urbaniak and his colleagues in the Blood Transfusion Service laboratories, Royal Infirmary of Edinburgh. This source of serum was chosen since this population has a very low incidence of recent gonococcal infection and, generally, high titres of isohaemagglutinins.

The target antigen for 'natural' bactericidal antibodies against *N.gonorrhoeae* is the LPS (Glynn and Ward, 1970; Tramont, Sadoff and Artenstein, 1974; Schneider *et al*, 1982; Section 1.8.3.1). Since differences in the pyocin type of gonococcal strains has been correlated with differences in the LPS molecule (Sadoff, Zollinger and Sidberry, 1978; Section 1.5.5.3), strains with different pyocin types were used in this study.

These strains had well defined, stable pyocin sensitivities: Group I strains were only sensitive to pyocins of Kageyama's class R5 and the unclassified pyocin 9579; Group II strains were sensitive to the same pyocins as strains of Group I and, in addition, to pyocins of Kageyama's class R1 and the unclassified pyocin, R205 (Kageyama, 1975, Section 1.5.5.3).

Since colony type has been shown not to influence the susceptibility of *N.gonorrhoeae* to bactericidal antibody (Tramont, Sadoff and Artenstein, 1974), non-pilate (T4) colony types were used in these experiments. This reduced the possibility of the bacteria clumping due to pili becoming entangled with each other, thereby decreasing the accuracy of the viable count estimations. (Kasper, Rice and McCormack, 1977). One strain, P 280μ, was a pyocin-resistant mutant derived from the parent strain, P280, as described in section 2.5.5. The pyocin sensitivities of the strains used in this section of the study are shown in Table V.

TABLE V

Pyocin Types of Strains of N.gonorrhoeae

		Pyocins with Kageyama group												
		R1					*Unclassified*		*R3*	*R2*			*R5*	
	Gonococcal strain	*ISD*	*2285*	*IS4*	*IS6*	*IS8*	*R205*	*9579*	*ISE*	*ISB*	*R21*	*430*	*ISA*	*ISC*
Group I	M9131													
	E757													
	*849209													
	*845650	−	−	−	−	−	−	+	−	−	−	−	+	+
	*934936													
	P280μ													
	P3309													
	†7425													
Group II	M8865	+	±	+	+	+	+	+	−	−	−	−	+	+
	P280													
	E2590													
	E728													
	E759	+	+	+	+	+	+	+	−	−	−	−	+	+
	M5287													
	P3290													
	†1560													

+ clear zone of inhibition.
± zone of partial inhibition.
− no inhibition.
* β-lactamase producing strains.
† strains from disseminated infection.

The bactericidal test described in Section 2.6 *et seq* was developed to allow optimal use of the small amounts (less than 1 ml) of serum available. A standard complement level was used throughout the tests. This was supplied by fresh human AB serum (which contains neither anti-A nor anti-B isohaemagglutinins) from a donor with no history of gonorrhoea. Before use the complement source was absorbed with a 'cocktail' of the gonococcal strains under test using the method described in Section 2.6.4 and was standardised in a haemolytic assay to a minimum haemolytic titre of 1/32 - 1/64. The absorbed complement source was added to the heat-inactivated A or B serum in the bactericidal tests to give a final concentration of 1/30 - 1/60. This concentration supplied sufficient complement to mediate antibody-dependent bacteriolysis whilst minimising any bactericidal activity due to activation of the alternative pathway. (Schreiber *et al*, 1979) Additional assays were also carried out in the presence of 10mM -Mg-EGTA to determine if any bactericidal activity due to activation of the alternative complement pathway occurred.

Small numbers of bacteria (approximately 10^4/ml) were used in this bactericidal test 1) to reduce the degree of bacterial clumping and 2) to allow easy determination of the end-points of titrations. Titre of bactericidal activity was arbitrarily assigned to the greatest dilution of serum that gave at least 80% decrease in viable count. All titrations were conducted in duplicate and the titres obtained were found to be comparable i.e. there was less than a fourfold difference in titre. On those occasions where a two-fold difference in titre between duplicate tests was observed, the lower value was taken as titre.

3.3 Results

The titres of bactericidal antibody in the sixteen group A and sixteen group B sera against the sixteen strains of N.gonorrhoeae are given in Table VI. The titres of isohaemagglutinin in some of the sera are also shown. The alternative complement pathway was not contributing to the bactericidal activity of the test system since all reactions with the serum-sensitive strains were inhibited by the presence of 10mM Mg-EGTA.

The geometric mean titres of the bactericidal antibody in the Group A and Group B sera are shown for each of the serum-sensitive strains in Table VII.

The distributions of the titres within sera of each blood group were compared using a robust, non-parametric statistical test, the Mann-Whitney U test. The one-tailed test was used to test the null hypothesis that the titres obtained with Group A sera were similar to and not significantly greater than those obtained with Group B sera. (Table VII). Some of the titres shown in Table VI have undetermined values i.e. < 15 or > 480. In making the statistical comparisons between the titres obtained with A and B sera the procedure outlined by Lutz (1978) was adopted. This involves substituting 'likely' values of the true titre for values where the titre is an undetermined value. I have considered a titre of < 15 to be equal to a titre of 10 for the purposes of this analysis. Any significant differences between the mean titres of the groups using this value would be even more significant if the true titre was, in fact, lower. In a similar manner, all titres of > 480 are taken to be equal to 480.

The titres of isohaemagglutinins (Table VI) tended to be greater for individuals of blood group A, but did not appear to reflect the titres of bactericidal antibody. For example; whereas the isohaemagglutinin titre of serum iii (Group B) was high (128), it had a low titre of bactericidal antibody. In contrast, serum v (Group B), which had a lower isohaemagglutinin titre (32), had high

TABLE VI

Titres of bactericidal antibody in Group A and Group B sera against strains of N.gonorrhoeae

Bactericidal titre of normal human serum from Group A individuals

	Test strain of N. gonorrhoeae	Sera [&] A [32]	B [128]	C [128]	D [64]	E [128]	F [128]	G [256]	H [128]	I	J	K	L	M	N	O	P
Group I	M9131	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
	E757																
	849209																
	P3309																
	934936																
	P280u																
	*7425																
Group II	M8865	15	240	60	60	60	30	120	120	30	120	120	60	30	30	15	60
	P280	>	>	>	>	>	>	240	240	240	60	120	30	120	120	30	120
	E2590	<	<	60	30	<	30	<	<	30	30	120	60	60	60	15	60
	P3290	15	15	30	30	30	30	15	60	120	120	15	15	15	60	30	120
	*1560	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<

Bactericidal titre of normal human serum from Group B individuals

	Test strain of N. gonorrhoeae	Sera [&] i [64]	ii [64]	iii [128]	iv [128]	v [32]	vi [64]	vii [32]	viii [32]	ix	x	xi	xii	xiii	xiv	xv	xvi
Group I	M9131	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
	E757																
	849209																
	P3309																
	934936																
	P280u																
	*7245																
Group II	M8865	15	30	30	30	>	15	120	30	15	15	<	30	<	15	30	15
	P280	<	15	<	15	240	15	15	30	120	60	60	60	240	60	120	60
	E2590	<	<	<	15	60	<	<	<	30	30	30	60	30	60	30	30
	P3290	<	<	<	30	60	<	120	60	120	30	30	15	15	120	30	60
	*1560	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<

< titre less than 15.
> titre greater than 480.
* strains from disseminated infection.
[&] isohaemagglutinin titre (where determined)

TABLE VII

Comparison of titres of bactericidal antibody against strains of *N.gonorrhoeae* in sera of blood groups A and B.

Strain	Geometric Mean Titre		U[1]	P[2]
	Group A sera	Group B sera		
P280	185.06	42.12	40.5	< 0.005
M8865	55.02	26.15	59	< 0.005
E2590	27.60	21.67	106	NS
E3290	32.72	30.87	107.5	NS

1 U = the Mann-Whitney U statistic

2 P = the probability (one-tailed) of the differences between the groups having arisen by chance

NS = Not significant

titres of bactericidal antibody.

A group A serum (K) and a group B serum (vii), each with high titres of bactericidal antibody, were absorbed with the appropriate red blood cells, using the method described in Section 2.7.1 to remove the isohaemagglutinins. This treatment did not result in a significant (i.e., greater than four-fold) reduction in the bactericidal titre of the sera against strains P280, M8865 or E2590 (Table VIII).

A group A serum was absorbed with strains of N.gonorrhoeae non-serogroupable N.meningitidis and N.lactamica. The bactericidal activity against the gonococcal strain P280 was removed by absorbtion with Group II strains of N.gonorrhoeae, but not by Group I strains. One of the two strains of N.meningitidis which had a Group II pyocin type (16797) also reduced the bactericidal titre of the serum, as did one of the two N.lactamica strains (Table IX).

TABLE VIII

Titres of bactericidal antibody before and after absorbtion of isohaemagglutinins

Serum	Gonococcal Strain	Titre	
		Unabsorbed serum	Absorbed serum
	P280	120	120
Group A	M8865	120	120
(k)	E2590	30	30-60
	M8865	120	120
Group B	P280	15	< 15
(vii)	E2590	15	15

TABLE IX

Removal of bactericidal antibody by absorbtion with various *Neisseria* species

	Bactericidal Titre of serum against *N.gonorrhoeae* strain P280
Unabsorbed serum	32
Serum absorbed with :	
N.gonorrhoeae strain E757 (Group I)	16
" " M9131 "	16
" " P280 (Group II)	< 4
" " M8865 "	< 4
N.lactamica " a	16
" " b	< 4
N.meningitidis " 15373 (pyocin resistant)	32
" 16797 (Group II)	4

3.4 Summary and discussion of the hypothesis

The comparison of the bactericidal activity of sera taken from women of blood groups A and B can only be made for the serum-sensitive strains of Group II. A cursory examination of the titration data shows that sera from both groups A and group B individuals exhibited bactericidal activity. Comparison of the geometric mean titres (Table VII) shows the greatest difference between A and B sera occurred in tests against strain P280. This strain was very sensitive to both A and B sera; group A sera gave a higher mean titre than group B sera. This difference is described as highly significant by the Mann-Whitney U-test ($P < 0.005$). Although group A sera also gave higher titres than group B sera against strain M8865, the difference is not so marked as that observed with strain P280, but the significance of the difference is still as great ($P < 0.005$). No significant differences between A and B sera were found in the analysis of the titres against strains E2590 and P3290.

Any difference between the titres of bactericidal antibody in NHS of groups A and B appears to depend on the gonococcal strain against which the sera are titrated. In the two situations in which significant differences were found, *viz*. titrations against strains P280 and M8865, sera from Group A individuals gave, on average, higher titres than sera from Group B individuals.

Absorption of isohaemagglutinins from a group A and a group B serum did not reduce the titre of bactericidal antibody when tested against strains P280, M8865 and E2590. These data suggest that isohaemagglutinins are not the antibodies in NHS which are bactericidal for these strains of *N.gonorrhoeae*.

The absorbtion of the bactericidal antibodies by a strain of *N.lactamica* and a non-serogroupable Group II strain of *N.meningitidis* reiterates the observations of cross-reactive antigens between *Neisseria*

species (Gold et al., 1978; Apicella et al; 1981). The inability of the Group I strains of N.gonorrhoeae to absorb the bactericidal antibody reinforces the proposal by Schneider and colleagues (1982) suggesting that gonococcal serum resistance was not due to the inability of the complement lytic complex, $\overline{C5b-9}$ to penetrate the outer membrane, but was due to absence of the antigenic determinants that react with natural bactericidal antibodies.

The most striking result of these experiments is the relationship between pyocin sensitivity and serum sensitivity of the strains of N.gonorrhoeae used in this study. LPS, the receptor for 'natural' bactericidal antibodies against N.gonorrhoeae (Glynn and Ward, 1970; section 1.8.3.1) is also the receptor for R-type pyocins in both Ps.aeruginosa (Kageyama, 1975; Meadow and Wells, 1978) and N.gonorrhoeae. (Sadoff, Zollinger and Sidberry, 1978; section 1.5.5.3). Investigations into the LPS structure of Ps.aeruginosa have shown that resistance to an R-type pyocin is due to absence of the appropriate sequence of sugar residues that make up the receptor site on the LPS molecule (Kageyama, 1975; Meadow and Wells, 1978). The molecular basis for pyocin sensitivity of the gonococcus has not yet been elucidated. However, it has been shown that pyocin resistant gonococci isolated from a pyocin-sensitive parent strain have altered LPS molecules compared with LPS isolated from the parents (Guymon, Esser and Schafer, 1982).

The pyocin-sensitivity patterns of the strains used in this study suggest that Group II strains have additional LPS determinants, absent (or cryptic) in strains of Group I, which are associated with sensitivity to the pyocins of Kageyama's class R1. These determinants might also be associated with the antigens to which 'natural' bactericidal antibodies react. One possible source of antigenic stimulation for the production of these antibodies may come from non-serogroupable strains of N.meningitidis with the Group II pyocin type which appear to cross-react with gonococcal antigens involved in the bactericidal reaction. All the Group II strains isolated from localised genital gonorrhoea

were sensitive to the majority of sera (both A and B) tested, whereas none of the Group I strains were sensitive to any of the sera tested in this system. Additional evidence for the involvement of the LPS portion associated with the R1 pyocin binding site in gonococcal sensitivity to NHS comes from the results obtained with the R1 pyocin-resistant mutant, P280 μ, which had been derived from a Group II strain, P280. Whereas the parent strain showed great sensitivity to the majority of sera tested, the mutant strain, which had the same pyocin-sensitivity pattern as the Group I strains (Table V), was resistant to all the sera tested (Table VI).

Although all Group II strains from localised genital infections were found to be generally serum sensitive, strain 1560, a Group II strain isolated from a disseminated infection, was resistant to all the sera tested. The availability of receptors for pyocins of class R1 does not appear to imply serum-sensitivity in all cases.

CHAPTER 4

Analysis of the lipopolysaccharide of *N.gonorrhoeae*, strains P280 and P280μ

CONTENTS

4.1 Introduction

The results presented in Chapter 3 indicated a correlation between pyocin type of N.gonorrhoeae and the sensitivity of the gonococcal strains to bactericidal antibodies in normal serum. These results suggested that structural differences may be found between the LPS molecules of serum sensitive (Group II) strains and serum-resistant (Group I) strains; serum-resistant strains lack LPS determinants associated with the R1 pyocin binding site present in the serum-sensitive strains. It was proposed that the LPS portion associated with the R1 pyocin binding site was the antigen to which the bactericidal antibody in normal human serum reacts.

4.2 Hypothesis

This section examines the hypothesis that the LPS in a Group I strain is chemically, physically and antigenically different from the LPS in a Group II strain.

4.3 Experimental Design

In order to minimise the differences between the Group I and Group II strains under examination, a comparison was made between the LPSs of strain P280 (Group II) and the R1-pyocin resistant, serum-resistant strain, P280μ, derived from strain P280 as described in section 2.5.5. Lipopolysaccharide was isolated from both strains by the method of Westphal and Lüderitz (1954), described in Section 2.8 *et seq.* The isolated LPSs were fully hydrolysed and the resulting monosaccharides were derivatised to form alditol acetates.

The alditol acetates were separated by gas chromatography as described in Section 2.8.2.3. By comparing the retention times of the derivatised components of the hydrolysate with alditol acetates of pure monosaccharides (Kindly provided by Dr I Poxton, Department of Bacteriology) putative identification of some of the LPS sugars was made.

Comparisons between LPSs were made by comparing the ratios of the main peaks of the chromatogram to a peak of substantial area which was present in both LPS from P280 and P280μ. In addition to the chemical studies on isolated LPS, the electrophoretic mobilities of the LPSs present in outer membrane vesicles, isolated from the two strains by the method described in Section 2.8.3, were compared. After electrophoresis in polyacrylamide gel, by the protocol described in Section 2.9, the LPS bands were visualised by means of a silver-stain (Tsai and Frasch, 1982) described in Section 2.10.

The ability of the purified LPS from both strains P280 and P280μ to inhibit the killing of strain P280 by normal serum was assessed. The bactericidal reaction (described in section 2.6.6) was performed in the presence of either 20μg/ml or 100μg/ml LPS. The number of residual viable organisms was assessed at 10,20 and 30 minutes after the start of the reaction as described in section 2.6.7.

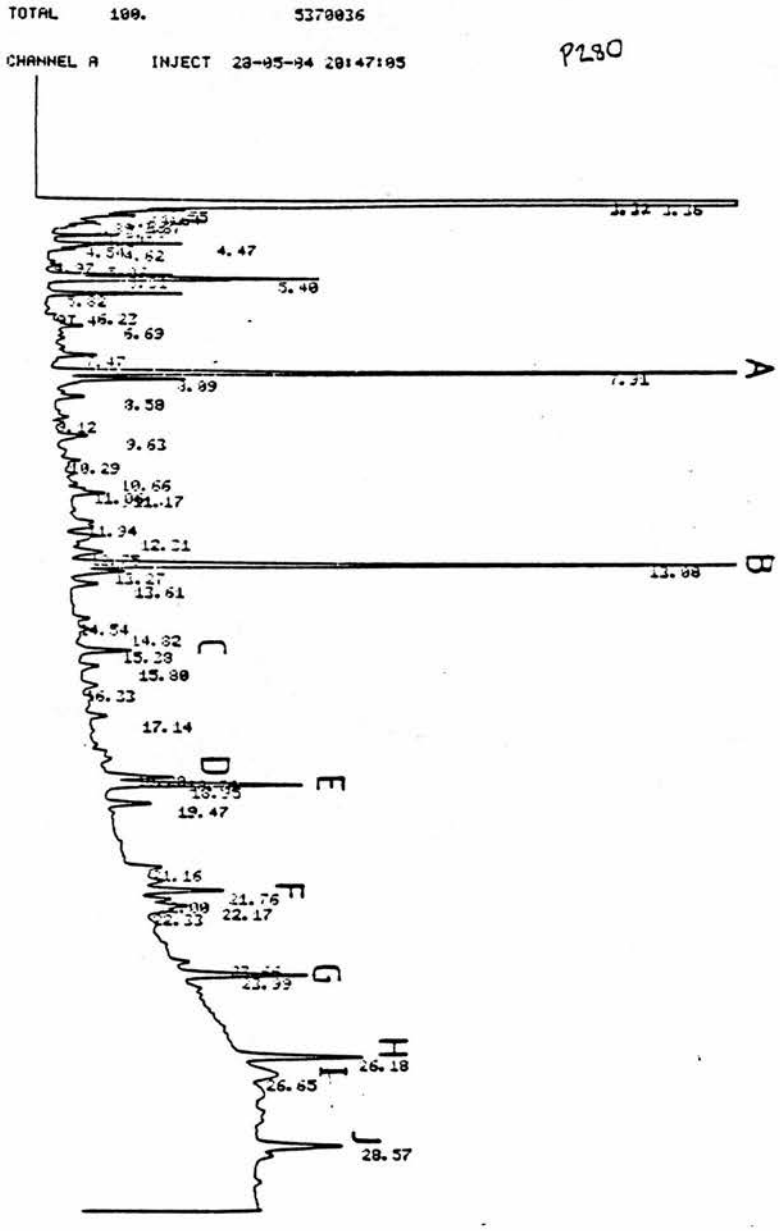


FIGURE 3, Chromatogram of Alditol Acetates from N.gonorrhoeae strain P280 LPS

FIGURE 4, Chromatogram of Alditol Acetates from N.gonorrhoeae strain P280μ LPS

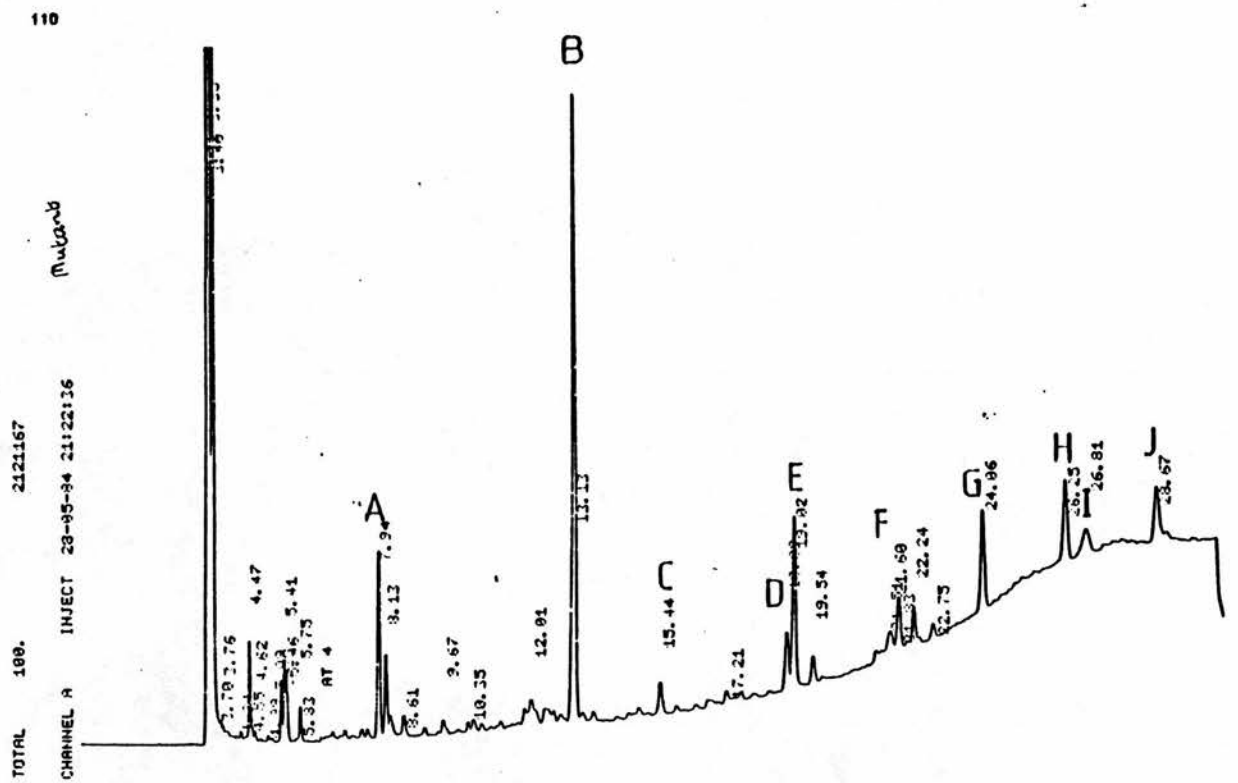


TABLE X

Normalised Peak Areas for Chromatograms of Alditol Acetates from LPSs of N.gonorrhoeae strains P280 and P280 μ

	P280		P280μ	
Ref. Peak =	18.95		Ref Peak =	19.02
Ref. Peak Area =	13308		Ref Peak Area =	11030
	Peak rt	Area/Ref. area	Peak rt	Area/Ref. area
A	7.91	2.92568	7.94	.71206
B	13.08	3.17583	13.13	3.40925
C	15.38	.20574	15.44	.16201
D	18.76	.34904	18.82	.31115
E	18.95	1.00000	19.02	1.00000
F	21.76	.45717	21.60	.18939
G	23.99	.66005	24.06	.62529
H	26.18	.65855	26.25	.51840
I	26.65	.27127	26.81	.24161
J	28.57	.48106	28.67	.39030

TABLE XI

Retention times of alditol acetate derivatives of reference sugars

Sugar	r.t. (in decimal minutes)
Deoxyribose	10.37
Ribose	13.13
Rhamnose	13.13
Fucose	13.59
Glucose	18.58
Galactose	18.76
Glucosamine	22.51
Galactosamine	23.92

4.4 Results

4.4.1 Analysis by Gas Chromatography

The chromatograms obtained from the alditol acetates of hydrolysed LPSs isolated from N.gonorrhoeae strains P280 and P280 μ are shown in Figures 3 and 4, respectively. The ratios of the areas of the major peaks A-J to the arbitrary reference peak, E, together with their retention times is given in Table X. The retention times of a few standard alditol acetates are given in Table XI.

Quantitative analysis of these chromatograms is not possible since no internal standard was used. Comparison between the chromatograms can only be made on a qualitative basis and by examination of the ratios of each component of the hydrolysate to an arbitrary reference peak which is found in both samples. The overall pattern of the chromatograms is the same. This suggests that the LPS from both strains is composed of the same collection of sugars. By comparing the ratios of the areas of each of the major peaks, A-J,with an arbitrary reference peak which, in this case, is peak E, the relative quantities of each sugar components can be determined for each LPS preparation. These ratios are presented in Table X Peak A represents a low molecular weight compound which is probably a breakdown product produced during hydrolysis or the derivatisation of the sugars. Comparison of the ratios of the peak areas to the area of peak E show great similarity for all the major peaks, except peak F. In this case the ratio in the P280 LPS is greater than twice that seen in the LPS from P280 μ; 0.46:1 compared with 0.19:1.

Comparison of the retention times of the peaks obtained from the LPSs with those obtained with the standard sugars gives an indication of the identities of the sugar in each peak. A temperature programme was used in this analysis and retention times obtained from two runs with the same sample varied slightly. Consequently, the true

identity of each peak in the LPS analyses can only be made by co-injection of a known standard sugar with the sample. The limited time available to use the gas chromatography machine did not permit this to be done. Nevertheless, comparison of the retention times of the standard sugars with those of the peaks obtained with the LPSs suggests the following sugars are present in the LPS isolated from both strains; glucose, galactose and galactosamine (Table XII).

TABLE XII

Putative Identification of the chromatogram peaks

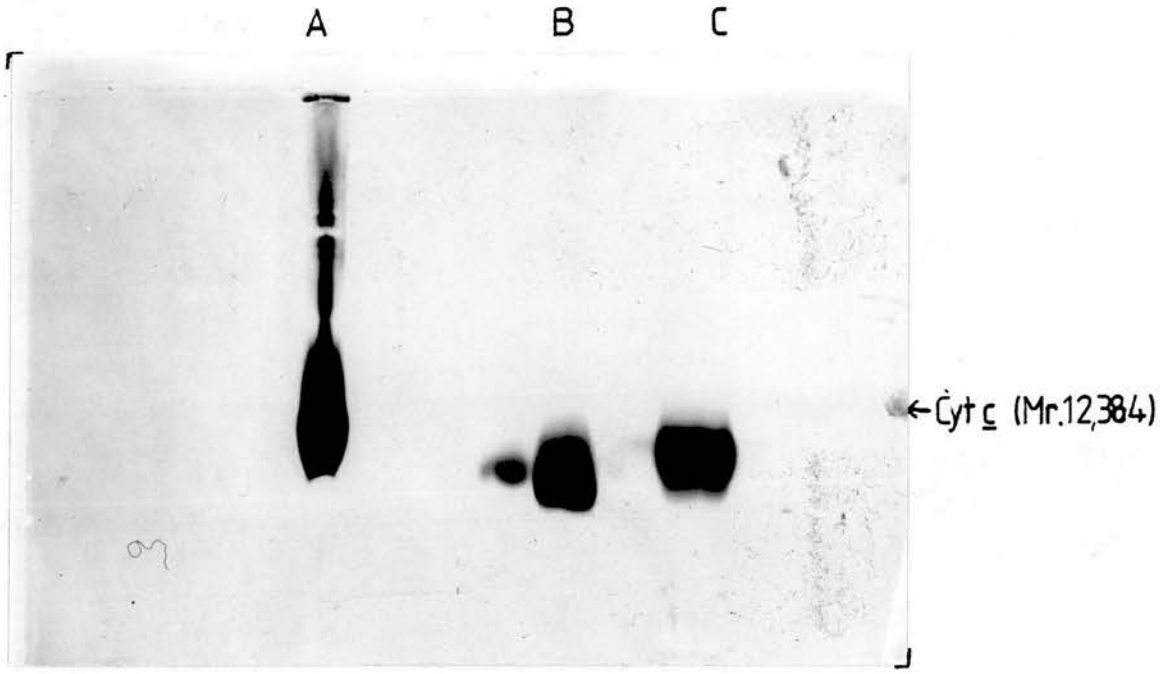
Peak	Sugar
B	Ribose or Rhamnose
C	-
D	Glucose
E	Galactose
F	-
G	Galactosamine
H	-
I	-
J	-

4.4.2 Analysis by Polyacrylamide Gel Electrophoresis

After electrophoresis of the outer membrane vesicles isolated from P280 and P280μ, the LPS components were visualised by silver staining. LPS from Escherichia coli and horse heart cytochrome c were included as molecular weight markers. (Figure 5). The two gonococcal LPSs differed in their electrophoretic mobility and, therefore, in molecular weight; the molecular weight of P280μ LPS was less than the P280 LPS. When compared with the molecular weight markers the molecular weight of P280 LPS was approximately that of the

FIGURE 5

Electrophoresis of outer membrane vesicles isolated from N.gonorrhoeae strains P280 and P280 μ



A=E.coli B = P 280μ C = P 280

FIGURE 6

Inhibition of Killing of N.gonorrhoeae strain P280 by LPS isolated from strain P280

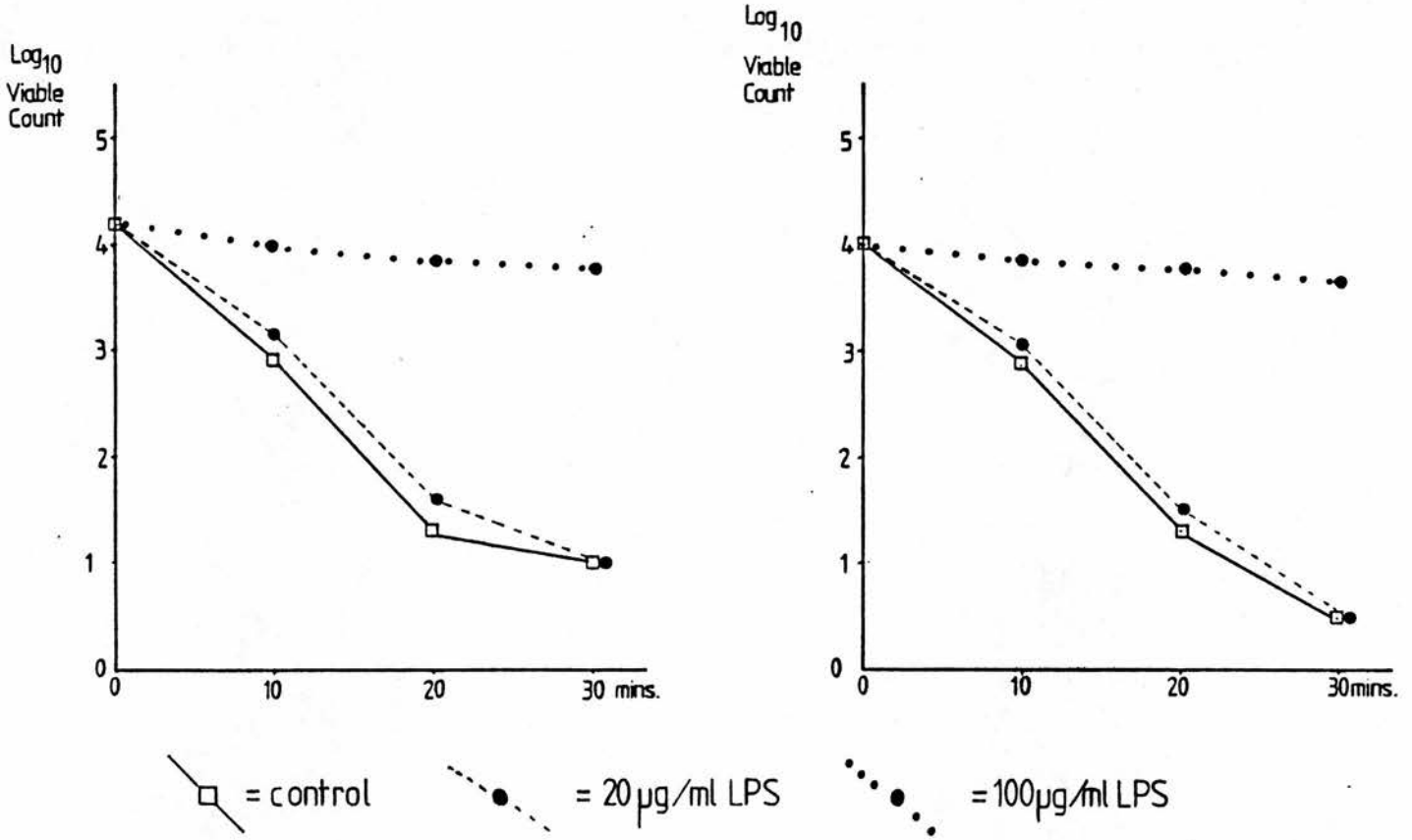
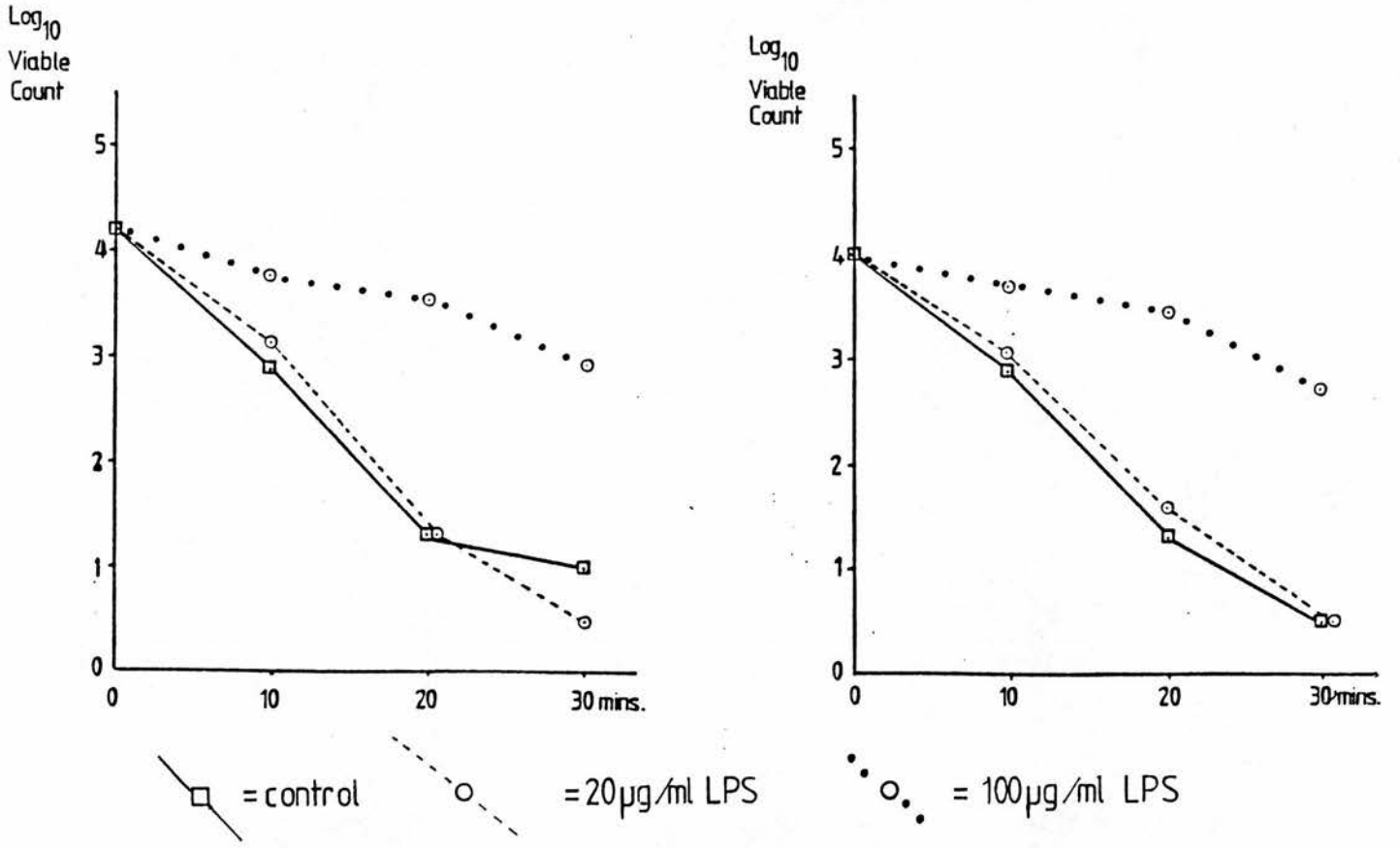


FIGURE 7

Inhibition of Killing of N.gonorrhoeae strain P280 by LPS isolated from strain P280 μ



LPS 'core' from *E.coli.* The LPS from P280 μ was a slightly smaller molecule (Figure 5).

4.4.3 Inhibition of serum killing of *N.gonorrhoeae* strain P280 by LPS isolated from strains P280 and P280 μ

The results are shown in Figures 6 and 7 Isolated LPS from both strains P280 and P280 μ showed no inhibition of serum killing at a concentration of 20 μg/ml. Both LPSs were found to inhibit serum killing of strain P280 at a concentration of 100 μg/ml, but the degree of inhibition was greater with the LPS isolated from strain P280.

4.5 Summary and Discussion of the Hypothesis

These results show that the LPS of strain P280μ has a lower molecular weight, determined by SDS-PAGE, than the LPS of strain P280. There does not appear to be any major difference in the qualitative compositional analyses of the polysaccharide moiety of the two LPSs, but there does appear to be a difference between the LPSs in the proportions of each sugar component in the LPS. This difference is in the sugar moiety responsible for peak F. In comparison with the other sugar types in each LPS molecule, there is less of the Peak F sugar in P280μ LPS than in P280 LPS.

Unequivocal identification of the sugars was not possible since there was insufficient time available on the gas chromatography machine to allow co-injection runs to be made. Comparison of the retention times of alditol acetates of known sugars with the retention times of the peaks obtained with the LPS hydrolysates suggested the identities shown in Table XII. Peak B has the same retention time as both Ribose and Rhamnose. Ribose could be present if the ribonuclease with which the cell lysate was treated prior to extraction of the LPS (Section 2.8.1) was inactive. Rhamnose, however, has been previously identified in the LPS of some gonococcal strains (Wiseman and Caird, 1977; Section 1.5.5.1). Clarification of this point would require a change of chromatographic conditions, or the use of mass spectroscopy linked to the present system. The other sugars for which provisional identification has been made, *viz.* glucose, galactose and galactosamine, have been described in compositional analyses of gonococcal lipopolysaccharides by other workers. (Stead *et al*, 1975; Perry *et al*; 1975; Wiseman and Caird, 1977; Section 1.5.5.1)

The ability of the LPS isolated from strain P280 to inhibit the killing of strain P280 by serum was expected. The same ability displayed by the LPS isolated from the serum-resistant strain P280 μ was surprising. However, the degree of inhibition was not as

great as with P280 LPS. After 30 minutes incubation there were at least 1 $\log_{10}$ fewer viable bacteria in the reaction mixture containing the P280 μ LPS (100 μg/ml) than in the mixture which contained the same quantity of P280 LPS. When compared with the initial inoculum, a significant degree of microbial killing ($\geqslant$ 80% drop is viable numbers) was observed in the presence of P280 μ LPS (100 μg/ml) but not in the presence of P280 LPS (100 μg/ml).

These results support the hypothesis (section 4.2) that the LPS of the serum sensitive gonococcal strain P280, is different in both chemical, physical and immunological characteristics to the LPS isolated from the serum-resistant variant, P280 μ. The lower molecular weight of P280 μ LPS suggests that its resistance to normal human serum and R1 class pyocins, compared with the parent strain, P280, is due to loss rather than masking of sugar residues.

CHAPTER 5

Changes in pyocin-sensitivity of *N.gonorrhoeae* associated with the induction of phenotypic serum resistance

CONTENTS

5.1 Introduction

'Natural' bactericidal antibodies in human serum react with LPS antigens of *N.gonorrhoeae*, (Glynn and Ward, 1970; Schneider *et al*, 1982; see Section 1.8.3.1) thought to be associated with a portion of the LPS molecule that serves as the receptor site (s) for Kageyama's class R1 pyocins. (Chapters 3 and 4). One reason for serum resistance among gonococcal strains is the absence of the LPS antigen to which these bactericidal antibodies bind (Schneider *et al*; 1982; Chapters 3 and 4).

The mechanism for the inducible phenotypic serum resistance in *N.gonorrhoeae* (Rittenberg *et al*., 1977; Veale, Penn and Smith, 1981; See Section 1.8.3.2) has not yet been elucidated. Development of phenotypic serum resistance requires metabolic activity on the part of the gonococcus (Veale, Penn and Smith, 1981) and is achieved by 77% (SE $\pm$ 27%) of a small inoculum (5 x 10^3/ml) within 3 hours of incubation with the inducting factor from human serum (Martin *et al*,1981).

In view of the excessive outer membrane production by the pathogenic *Neisseria* species during growth (DeVoe and Gilchrist, 1973; Stead *et al*, 1975) and the lag phase and requirement of bacterial metabolism observed in the induction of serum-resistance, inducible phenotypic serum resistance may be due to rapid expression of an LPS molecule lacking the determinants which react with the bactericidal antibodies in normal human sera.

5.2 Hypothesis

The hypothesis under examination in this Chapter is that the induction of phenotypic serum resistance in *N.gonorrhoeae* will be associated with a change in the LPS molecule resulting in resistance to pyocins of Kageyama's class R1.

5.3 Experimental Design

Pyocins were an ideal tool to probe the LPS structure of the gonococci induced to serum resistance, since only small numbers (10^4 - 10^5) of resistant organisms can be produced by each induction experiment. Other techniques lack the sensitivity required to analyse such a small number of bacteria.

Since the induced organisms revert to the serum sensitive state on culture in the absence of the inducer, it was necessary to ensure that any unreacted pyocins were removed from the mixture of pyocin and gonococcus prior to estimating the numbers of residual viable bacteria. The chosen technique, described in Section 2.5.6, involved mixing pyocin and gonococcus in liquid medium for a sufficient time to allow the pyocins to bind to and kill sensitive organisms. The bacteria were washed free from pyocin by making a dilution of the mixture, depositing the bacteria by centrifugation and decanting the supernqte containing unreacted pyocin. The bacteria were then resuspended and the number of remaining viable organisms was estimated. A comparison of the viable count with that obtained from a control experiment, in which the aliquot of pyocin was replaced by an aliquot of medium, allowed the degree of pyocin - sensitivity to be determined.

These experiments were conducted in the Department of Microbiology, University of Birmingham. The induction of N.gonorrhoeae strain BS4 (agar) to serum resistance by the method of Veale, Penn and Smith (1981) was performed by P.V. Patel. This technique involves incubation of the gonococcal strain with an ultrafiltrate from guinea pig serum in a chemically defined medium containing 0.1% (W/V) bovine serum albumin for 3 hours at 37° C. Serum sensitivity of strain BS4 (agar) prior to, and after induction of serum resistance was performed by Dr E L Tan. Concurrent examination of the sensitivity of strain BS 4 (agar) in the serum-sensitive and induced serum-resistant states to a panel of pyocins was performed with assistance from Dr E L Tan and Dr N J Parsons using the method described in Section 2.5.6.

TABLE XIII

Changes in pyocin sensitivity of N.gonorrhoeae strain BS4 (agar) induced to phenotypic serum resistance

Each result was obtained on at least four different tests with each pyocin and at least eight different tests with pyocins from strains IS6, IS8, R205, 9579 and ISC. The differences between the serum - susceptible and serum-resistant forms of BS4 (agar) are bracketed

			Inhibition* by pyocins from the Kageyama group									
	Strain of gonococci	Serum Resistance	Pyocin :	R1				Unclassified		R3	R2	R5	
			Strain :	ISD	2285	IS6	IS8	R205	9579	ISE	ISB	ISA	ISC
Before induction	BS4 (agar)	-		-	-	-	(±) (−)	(+) ( )	+	-	-	+	( ) (+)
After induction	BS4 (agar)	+		-	-	-	(-)	(-)	+	-	-	+	(±)

* Results were recorded as + ± and - when the viable counts were < 5% < 20% (but > 5%) and > 20% respectively, of the counts of the control tubes not containing pyocin.

5.4 Results

The results are summarised in Table XIII. During the 3 hour incubation with the inducer - containing fraction of guinea pig serum the serum susceptible form of strain BS4 (agar) had developed reduced sensitivity to the class R1 pyocin, IS8, and resistance to the unclassified pyocin, R205; also its full sensitivity to the class R5 pyocin, ISC, was decreased.

As a corollary to the findings of this experiment, a pilot study of the sequential changes in pyocin - sensitivity of gonococcal isolates during repeated subculture after primary isolation showed strains to generally follow this pattern : on primary isolation they show resistance to pyocins of classes R5 and R1 and to serum in a bactericidal test. In the course of daily subculture, strains become sensitive to R5 pyocins and, usually, some or all of the panel of R1 pyocins. The degree of serum-resistance was also decreased in many cases.

5.5 Summary and Discussion of the Hypothesis

The changes in pyocin sensitivity associated with the induction of serum resistance in N.gonorrhoeae strain BS4 (agar) suggest that a change in LPS results from 3 hours incubation at 37° C with a low molecular weight fraction (< 5000 daltons) of guinea pig serum. The mutant strain, P280 μ, selected for resistance to R1 pyocins, retained sensitivity to R5 pyocins, indicating that the receptor(s) for R5 pyocins is nearest the membrane, and the receptor(s) for the R1 pyocins more distal (Chapters 3 and 4). This is similar to the R5 and R1 pyocin receptor sites described for Ps.aeruginosa (Kageyama, 1975; Meadow and Wells, 1978). The loss of sensitivity to the R1 pyocin, IS8 and the R5 pyocin, ISC, suggest that changes to the polysaccharide moiety of the LPS may have taken place at sites both proximal and distal to the lipid portion.

The results of this study are in general agreement with the hypothesis that changes in expression of the polysaccharide portion of the LPS would occur during induction to serum resistance. Whether these changes in pyocin sensitivity are due directly to structural changes in the LPS or to masking of the pyocin binding sites by other components will require use of techniques of a more analytical nature.

The results obtained with the strains recently isolated from patients with gonorrhoea are in keeping with the general theme proposed by Ward, Watt and Glynn (1970) viz. that gonococci in urethral exudates are serum-resistant on primary isolation and, after subculture become sensitive. They also support the main result of this section, namely that the factor inducing serum-resistance in gonococci effects an alteration in gonococcal sensitivity to pyocins, presumably by stimulating alterations in the LPS structure.

CHAPTER 6

The immunogenicity of lipopolysaccharide in Group I strains of *N.gonorrhoeae* for mouse and man

CONTENTS

6.1 Hypothesis

Since sensitivity to class R5 pyocins is a feature common to both Group I and Group II strains, similar structural features may exist within the LPS molecules of both groups. If these common portions are antigenic and capable of stimulating bactericidal antibodies during infection, antibodies capable of killing Group I strains would be expected to appear during gonococcal infection.

Bactericidal antibodies reactive with Group I strains were not observed in a survey of 32 sera from normal individuals of blood groups A and B (Chapter 3). If the hypothesis is substantiated, the detection of these antibodies might form the basis for a serological screening test for gonococcal infection.

6.2 Experimental Design

Antibodies raised in mice against N.gonorrhoeae strains of Group I and Group II were used in an antibody-complement mediated bactericidal reaction to determine the cross-reactivity of gonococcal strains, both within and between the two groups. This tested the first section of the hypothesis :
similar antigenic determinants, putatively associated with the R5 pyocin binding site on the LPS, exist in strains of both groups.

Sera from patients with gonococcal infection were assayed in the antibody and complement - mediated bactericidal test to determine the activity against a strain of Group I and a strain of Group II. The infecting strain was also pyocin typed and used in the assay against the patient's serum wherever possible. The second section of the hypothesis was proved if bactericidal antibody against the Group I test organism was observed in the sera of infected patients.

TABLE XIV

Titration of Control and Absorbed murine sera raised against N.gonorrhoeae strains M9131 (Group I) and M8865 (Group II)

Antiserum	Strain	
	M9131	M8865
Anti-M9131	1024	1024
Anti-M9131 absorbed by M8865	64	< 4
Anti-M8865	256	1024
Anti-M8865 absorbed by M9131	64	64

6.3 Results

6.3.1 Cross reactivity of *N.gonorrhoeae* of Groups I and II demonstrated by murine antiserum

Antisera were raised in groups of mice against formalin-treated suspensions of *N.gonorrhoeae* strains M9131 (Group I) and M8865 (Group II) by the protocol described in Section 2.6.3 *et seq* Bactericidal tests employing the method described in Section 2.6.5 were conducted on the homologous immunising strains and a titre of 1024 for each antiserum was demonstrated. After cross-absorbing the sera with the heterologous strain (Section 2.7.1) the absorbed and control (unabsorbed) sera were titrated against the immunising strains. The titres obtained are given in Table XIV.

Bactericidal tests with each antiserum, at a final concentration of 1:100, were conducted on other gonococcal strains of Groups I and II. The results were variable between experiments with the same strain. The number of tests (out of four) giving at least 80% decrease in viable count in comparison with controls are given in Table XV.

6.3.2 Screening sera from patients with gonorrhoea for bactericidal antibody

Small quantities of heat-inactivated serum, taken for syphilis serology from patients with gonorrhoea, were kindly provided by Dr H Young and his colleagues, STD Diagnostic Laboratory, Bacteriology Department, University of Edinburgh. These sera were screened for bactericidal activity against strains E757 (Group I) and P280 (Group II). In many cases they were also tested for activity against the infecting strain. This was undertaken before the third *in vitro* sub-culture of the organism. Due to the small quantities of serum available, these tests could only be performed once with each serum. To ensure that

TABLE XV

Bactericidal action of immune mouse serum on strains of N.gonorrhoeae
(Number of tests out of 4 giving $\geqslant$80% decrease in viable count)

Strain	Antiserum	
	M9131	M8865
Group I	(Group I)	(Group II)
M9131	3	2
E757	1	2
849209	3	4
845650	4	4
934936	2	3
P280μ	0	3
Group II		
M8865	4	4
M5287	4	4
E728	3	2
E759	2	2
P280	1	4

TABLE XVI

Titres of bactericidal antibody in sera from patients with gonorrhoea against N.gonorrhoeae strains E757 (Gp I) and P280 (Gp II)

Patient No.	Sex	Pyocin Group	E757	P280	Homologous Strain
1	F	NT	< 4	> 128	16
2	F	NT	< 4	>128	>128
3	F	NT	<4	64-128	< 4
4	F	NT	4	64	NT
5	F	NT	< 4	64	< 4
6	F	NT	<4	32-64	4-8
7	F	NT	<4	64-128	4-8
8	F	NT	<4	32-64	< 4
9	M	NT	<4	32-64	<4
10	M	NT	<4	64-128	<4
11	M	NT	4-8	64-128	>128
12	F	NT	< 4	32-64	4-8
13	F	II	<4	> 128	< 4
14	F	II	<4	>128	>128
15	F	II	<4	64-128	8-16
16	F	I	< 4	16	8
17	F	II	<4	64	< 4
18	F	II	<4	> 128	8
19	F	II	<4	128	64
20	F	I	<4	> 128	< 4
21	F	II	4	> 128	<4
22	F	I	< 4	> 128	< 4

the system was working, a positive control serum with known activity against strain P280 was included in each set of experiments as a control. The results of these titrations are given in Table XVI.

The majority (19/22) of the sera were obtained from women; in addition to being the same gender as the sources of normal human serum, women generally have a longer period of infection prior to diagnosis and treatment than men. (Robertson, McMillan Young, 1980). This would give them potentially greater opportunity to produce an immune response to gonococcal antigens.

Only three of the twenty-two sera tested demonstrated any bactericidal antibody, at a titre of $\geqslant 4$, reactive with the Group I strain, E757. All the sera, however, showed activity at a titre of 16 against the Group II strain, P280. Reactions with the infecting strain were variable. The pyocin type was only determined for ten of the isolates; three were Group I strains and seven were Group II strains. These tests with the patient's serum against the infecting strain did not demonstrate the clear cut association between the Group II pyocin type and serum sensitivity observed earlier with the normal sera and laboratory strains. In some cases an infecting strain of Group II was resistant to the patient's serum, e.g. E4092, whereas the strain was typically serum-sensitive in other cases, e.g. E4080. In one instance, E4095, the patient's serum was bactericidal, at a titre of 8, for an infecting strain with the Group I pyocin type.

Similar results were obtained with a second set of 37 sera from the STD Diagnostic Laboratory. The diagnosis for the patient was not known at the time the sera were tested. They were screened for bactericidal activity against strain P280 and another Group I strain, M9131. It was later found that 12 sera were from patients with gonorrhoea, and 25 sera from patients from whom no Neisseria spp. were isolated. Although the titres against strain P280 were similar to those in Table XVI, for both patients with and without gonorrhoea, the titre against strain M9131 was < 4 for each serum.

6.4 Summary and Discussion of the Hypothesis

The anti-gonococcal bactericidal antibodies in the murine antisera raised against a strain of Group I (M9131) and a strain of Group II (P280) demonstrated cross-reactions both within each pyocin-defined group and between groups. This bactericidal antibody could be removed from the serum by absorption with cross-reacting strains of either group. (Table XIV). Inspection of Table XV shows that certain tests e.g. anti-M9131 serum reacting with strain P280, gave a positive result in only one out of the four occasions on which the test was conducted. However, the majority of tests demonstrated bactericidal activity on at least four occasions. The source of variability may be the incorporation of human complement in a system with mouse antibodies. This cross-species mixing of immunological reagents has shown variability in other systems. (Zollinger and Mandrell, 1983). The incorporation of normal mouse serum as the complement source might reduce the variability between tests. Another potential source of interference in the consistency of the tests could arise from the use of re-usable glass tubes as reaction vessels. The occasional tube may have been inadequately rinsed after washing, and any residual detergent would interfere with antigen-antibody interactions.

These experiments demonstrate the possession of shared antigenic determinants among the strains tested that are capable of stimulating bactericidal antibody in the mouse. The reactivity of the test strains with the panel of pyocins showed that shared LPS structures were associated with the R5 pyocin binding site(s). The LPS associated with this region may be, or be closely associated with the common antigen(s) suggested by these serological reactions.

The results obtained with human sera from patients with gonorrhoea were radically different to those observed with the immune mouse sera. During infection the human immune system does not appear to produce bactericidal antibodies reactive with the gonococcal antigen(s) associated with the Group I pyocin type. This contrasts with the ability of the murine immune system to produce bactericidal antibodies reactive with Group I strains when immunised with either a Group I

or a Group II strain. The antigen associated with the R5 pyocin binding site appears to be available to an immune system, but the human immune system either does not recognise it, or does not respond to the antigen in the same manner as the immune system of the mouse.

Differences between the immune responsiveness of mice and men have been observed for other antigens. Redhead (1984) described a similar situation in the immune responsiveness of mice and men to outer membrane proteins of Bordetella pertussis. Although an antibody response to the antigen was observed in mice, there was no similar response observed in children exposed to the antigens.

This apparent lack of a bactericidal antibody response might be due to unresponsiveness to certain antigens, such as has been observed with CBA/N mice (O'Brien,Scher and Metcalf, 1981). Alternatively, bactericidal antibody reactive with the Group I LPS determinants might be formed, but in conjunction with IgA specific for the same antigens. If the IgA was produced in sufficient quantity, it would block the bactericidal antibody and so result in an apparent lack of bactericidal antibody in the tests. This has been shown to occur in bactericidal tests against N.meningitidis using convalescent sera from patients with meningococcal disease. (Griffiss, 1975). Raised titres of antigonococcal antibodies have been observed in patients who have been infected with N.gonorrhoeae for up to 14 days. After this time, or after treatment, the titre of antigonococcal IgA was shown to decline rapidly (McMillan et al., 1979). If blocking IgA antibodies were present in the sera from patients with gonorrhoea, they are probably only reactive with those determinants associated with the LPS of Group I strains, since strain P280 and other Group I strains were killed by these sera. Screening sera from patients with gonorrhoea for IgA antibodies reactive with the LPS of Group I strains might clarify this point.

CHAPTER 7

The bactericidal effects of serum from normal individuals and patients with gonorrhoea on serogroupable and non-serogroupable strains of *N.meningitidis*

CONTENTS

7.1 Hypothesis

The results presented in Chapter 3 show that normal human sera from individuals of blood groups A and B contain bactericidal antibodies reactive with strains of N.gonorrhoeae which are sensitive to R-type pyocins of Kageyama's class R1. These antibodies could be absorbed by one meningococcal strain that was sensitive to R1 pyocins (strain 16797) but not by a meningococcal strain that was resistant to pyocins of classes R5 and R1.

Chapters 3 and 6 described the apparent absence of bactericidal antibodies reactive with Group I gonococcal strains - those reactive with class R5 pyocins only - in the sera of both uninfected normal individuals and patients with gonorrhoea. Meningococcal isolates with the Group I and Group II pyocin types have been described (Blackwell and Law, 1981).

The hypothesis under examination in this chapter proposes that meningococcal strains expressing the same pyocin sensitivities as Group I and Group II gonococcal strains will show the same patterns of sensitivity to the bactericidal antibodies in serum from normal individuals and patients with gonorrhoea as Group I and Group II strains of N.gonorrhoeae.

7.2 Experimental Design

A panel of non-serogroupable meningococcal strains of the same pyocin types as gonococcal strains of Group I and Group II, together with two pyocin resistant and three serogroupable strains were used as test organisms in the bactericidal test used in the previous study (Chapter 3). The sera of blood groups A and B were obtained from the same source as those used in Chapter 3, *viz.* women attending the post-natal clinic, and the sera from patients with gonorrhoea were obtained from the same source as those described in Chapter 6. Non-serogroupable meningococci accounted for most of the strains used in this study. They are more frequently observed to be pyocin sensitive than serogroupable strains. (Blackwell and Law, 1981).

TABLE XVII

Frequency Distribution of titres of Bactericidal Antibody against strains of *N.meningitidis* in sera of blood groups A and B

	Group A								Group B							
Log_{10} Titre	≤·6	·6	·9	1·2	1·5	1·8	2·1	2·4	≤·6	·6	·9	1·2	1·5	1·8	2·1	2·4
Strain																
GroupI																
PA1190	3	3	0	1	7	1	4	4	2	4	1	0	0	3	2	1
NA935113	0	7	0	0	2	3	3	0	2	8	0	0	0	1	1	4
42619-4	0	2	0	1	0	1	0	4	0	3	0	0	0	2	1	2
PA477	0	15	0	1	0	0	0	1	4	4	1	0	1	2	3	1
GroupII																
1419	0	2	1	0	3	1	6	3	3	3	0	0	1	2	4	3
PA469	2	2	0	0	0	1	4	4	3	3	0	0	0	0	5	6
0452	0	0	0	1	0	0	10	5	0	0	0	0	0	0	8	1
16795	1	2	4	6	4	0	1	0	3	0	0	5	1	3	0	0
Serogroupable																
Ref A	4	1	3	1	4	2	0	0	4	0	0	0	4	2	3	1
RefY	16	0	0	0	0	0	0	0	16	0	0	0	0	0	0	0
W135	2	0	3	1	2	0	0	0	4	1	1	2	0	0	0	0
Pyocin Resistant																
PA 846	4	1	3	3	0	5	0	0	5	2	1	0	4	4	0	0
PA 520	0	7	0	0	0	0	0	16	0	2	0	1	0	0	0	5

7.3 Results

The titres of bactericidal antibody found in a range of normal women of blood groups A and B against the panel of N.meningitidis strains are given in a frequency table (Table XVII). The geometric mean titres for sera of each blood group against each strain are compared in Table XVIII. The distributions of titres from sera of blood groups A and B against each serum-sensitive strain were compared by the Mann-Whitney U-test. No significant differences were observed in a one-tailed test at a probability level of 0.025.

Titres obtained using sera from patients with gonorrhoea are given in Table XIX. In these experiments the small quantity of serum allowed only one titration to be made against each test strain by the individual patients' serum. Control positive sera were included in each test to ensure that the experimental system was functioning correctly.

The results show that normal sera contain bactericidal antibodies which are reactive with both Group I and Group II strains of non-serogroupable N.meningitidis. (Table XVIII) They also react with the pyocin resistant strains, PA846 (also referred to as NG846) and PA520 and strains of serogroups A and W135. None of the sera have bactericidal antibody at a titre of $\geqslant 4$ against a strain of serogroup Y. There are no significant differences between titres obtained with group A and group B sera against each strain.

Sera from patients with gonorrhoea have similar titres of bactericidal antibody as those from normal individuals against two non-serogroupable strains, PA846 and Q452 (Table XIX). They contrast with the sera from normal individuals in having low titres or undetectable amounts of bactericidal antibody against the strain of serogroup A.

TABLE XVIII

Comparison of bactericidal titres obtained with sera of blood groups A and B against strains of N.meningitidis.

Strain	Geometric Mean Titres (Number of sera tested)		U	
	Group A sera	Group B sera		
Group I				
PA 1190	30.7 (23)	43.5 (13)	125.5	NS
NA 935113	18.2 (15)	15.2 (16)	112	"
42619-4	53.1 (8)	34.5 (8)	26	"
PA 477	5.5 (17)	14.0 (16)	114.5	"
Group II				
1419	57.9 (16)	29.0 (16)	104	NS
PA 469	45.9 (13)	42.0 (17)	108	"
0452	137.3 (16)	136.0 (9)	61.5	"
16795	13.6 (18)	14.1 (12)	98.5	"
Pyocin Resistant				
PA 846	11.7 (16)	11.2 (16)	123.5	NS
PA 520	71.2 (23)	63.1 (8)	89	"
Serogroupable				
Ref A	10.5 (15)	24.7 (14)	70	NS
Ref Y	2.0 (16)	2.0 (16)		"
Ref W135	8.7 (8)	4.3 (8)	20.5	"

U = value of U in Mann-Whitney U test

NS = not significant in a one-tailed test at a probability level of 0.025

TABLE XIX

Titres of bactericidal antibody against serogroupable and non-serogroupable strains of *N.meningitidis* in sera from patients with gonorrhoea

Patient No.	Sex	Serogroupable			Non-serogroupable	
		A	B	C	0452	PA(NG)846
1	F	< 4	< 4	4-8	⩾ 128	128
2	F	32-64	< 4	4	⩾ 128	⩾ 128
3	F	< 4	< 4	< 4	⩾ 128	8-16
4	F	⩽ 4	< 4	8-16	64-128	64-128
5	F	4	4	4-8	⩾ 128	32-64
6	F	⩾ 128	< 4	4-8	⩾ 128	⩾ 128
7	F	4-8	< 4	4-8	⩾ 128	⩾ 128
8	F	< 4	8-16	4-8	32-64	16-32
9	M	< 4	< 4	8-16	64-128	< 4
10	M	< 4	8-16	8-16	64-128	8-16
11	M	< 4	< 4	< 4	16-32	< 4
12	F	8-16	8-16	17-32	64-128	32-64
13	F	< 4	< 4	< 4	⩾ 128	64-128
14	F	< 4	< 4	< 4	⩾ 128	4-8
15	F	< 4	< 4	< 4	64-128	16-32
16	F	< 4	< 4	< 4	16-32	< 4
17	F	< 4	< 4	< 4	64-128	4-8
18	F	< 4	< 4	4-8	⩾ 128	< 4
Geometric Mean titres *		3.4	2.6	4.0	74.7	16.0

* Determined using < 4 = 2 and ⩾ 128 = 128

Tests using strains of the other two common serogroups, B and C, also show low titres of bactericidal antibody in sera from patients with gonorrhoea. The geometric mean titres obtained against meningococcal strains of serogroups A, B and C using sera from 18 patients are 3.4, 2.6 and 4.0, respectively (Table XIX).

7.4 Summary and Discussion of the Hypothesis

Normal sera reacted with N.meningitidis strains of the pyocin types of both Groups I and II. In addition, pyocin resistant and serogroupable strains were also susceptible to these sera. This contrasts with the results obtained using sera from patients with gonorrhoea. In these tests little or no bactericidal antibody was found to react with strains of serogroups A, B and C.

The spectrum of pyocin groups of meningococcal strains sensitive to the bactericidal antibodies of normal human sera is broader than that observed with strains of N.gonorrhoeae. This does not support the proposed hypothesis. Serum-sensitivity of the meningococcal strains of Group I could be due either to possession of other determinants reactive with normal serum or to subtle differences in the LPS associated with the binding site for the class R5 pyocins that allow reaction with the pyocins and also bactericidal antibodies. The pyocin-resistant strains may, in fact, be capsulate. Possession of a capsule may hinder the binding of pyocins to the LPS (Blackwell and Law, 1981) and also provide other polysaccharide antigens as receptors for bactericidal antibody (Goldschneider, Gotschlich and Artenstein, 1969).

CHAPTER 8

A comparison of the response of monocytes of blood groups A,B,AB and O to *N.gonorrhoeae* determined by Nitroblue Tetrazolium (NBT) reduction

CONTENTS

8.1 Introduction

This short series of experiments was a pilot study to develop a methodology for examining the oxidative metabolism of glass-adherent monolayers of human mononuclear phagocytes responding to stimulation by strains of N.gonorrhoeae. In addition, it provided an opportunity to determine whether the ABO blood group of the cells was a potential source of variation in the response of these cells to gonococci.

The need for a method of determining the burst of oxidative metabolism of monocytes stimulated with gonococci arises from the hypothesis of Novotny and colleagues (1977) that survival and replication of gonococci within mononuclear phagocytes is central to the pathogenesis of the disease. This hypothesis has received support from other workers, (Veale et al; 1976; section 1.7.2.1.1) who have demonstrated the ability of gonococci to survive and grow within human monocytes. In order to elucidate the mechanism by which gonococci survive the bactericidal compounds produced by the phagocyte, estimation of the production of bactericidal compounds is necessary. The phagocyte's bactericidal armoury can be classified into two main groups of compounds: those requiring oxygen for their formation - singlet oxygen, hydroxyl radicals and hydrogen peroxide - and those that are oxygen-independent - cationic proteins, lactoferrin and various proteolytic and saccharolytic enzymes.

The precursor of the oxygen-dependent bactericidal species is a one-electron reduction product of oxygen, the superoxide radical. Qualitative and quantitative determination of this component, or its dismutation product, hydrogen peroxide, forms the basis of tests to determine the oxidative burst associated with phagocytosis.

8.2 Experimental Design

Preliminary experiments employing ferricytochrome c reduction to quantify superoxide production were frustrated, presumably by the very high cytochrome oxidase activity in the gonococcal outer membrane reviewed by Morse, Cacciapuoti and Lysko, (1979). The determination of hydrogen peroxide was also subject to errors due to the exceedingly high catalose activity found in strains of N. gonorrhoeae (Norrod and Morse, 1979). The experiments reported here employed the redox indicator, nitroblue tetrazolium (NBT), to detect superoxide. Its properties have been described in Section 2.11. In the oxidised form it is soluble; when reduced it forms blue insoluble crystals. The soluble oxidised form of NBT incorporated into monolayers of phagocytes from the medium forms blue crystals in cells liberating superoxide. The proportion of stimulated (superoxide-producing) cells can be estimated by microscopic examination of the monolayers. Full experimental details are given in Section 2.11.1 et seq.

N.gonorrhoeae strains A and B (Both recent isolates of pyocin Group II with the double-highlight colonial morphology, a phenotype that is associated with gonococcal resistance to killing by phagocytes. (Penn et al, 1976; see Section 1.5.1.1) were incubated with monolayers of peripheral blood monocytes in the ratio of 10 bacteria per cell. Monocytes from one individual of each of the four ABO blood groups were used. Due to the toxic nature of NBT, it was incorporated in the test medium to give a low final concentration of 0.05%. Tests were conducted in Hank's medium supplemented with 5% autologous serum. In each experiment fresh and heat-inactivated serum was used to detect the role of complement in the stimulation of monocytes by gonococci.

In order to determine the role of 'natural' antibodies, tests incorporating either fresh or heat-inactivated autologous serum, which had been absorbed for one hour at 4^{o} C with the stimulating gonococcal strain,were also included. As a control procedure, opsonised zymosan was used to stimulate monocytes in a medium containing 5% FHS.

After the experiment the monolayers were fixed with a water-based fixative, formol saline, since the reduced NBT is soluble in organic solvents. The cells were counterstained with neutral red (1%) in order to give the greatest contrast with the blue formazan. Preliminary experiments with May-Grünwald and Giemsa stains did not allow any slight blueing of the cells to be detected.

200 monocytes were observed microscopically for each test, and cells were categorised -, + or +++, as described in Section 2.11.3. The counts were analysed by analysis of variance (Anova) using the Genstat computer programme.

TABLE XX

Numbers of formazan - containing monocytes in NBT test

		Blood Group A				Blood Group B				Blood Group AB				Blood Group O			
Stimulus	Response	+		+++		+		+++		+		+++		+		+++	
without zymosan	Expt. 1	25	6	17	1	7	2	2	1	14	8	6	0	18	28	4	7
	Expt. 2	3	6	8	9	23	18	6	7	8	12	1	3	17	5	2	4
with zymosan	Expt. 1	9	4	72	90	6	0	31	55	34	4	59	84	11	8	117	83
	Expt. 2	1	1	130	106	10	13	57	66	3	6	104	74	2	6	107	78
N.gonorrhoeae Strain A																	
+ FHS	Expt. 1	9	4	41	19	8	9	54	37	1	0	66	37	6	16	30	23
	Expt. 2	4	5	14	17	5	15	22	17	8	10	15	20	99	3	31	27
+ FHS Absorbed	Expt. 1	4	8	15	38	1	8	19	23	5	10	13	23	6	3	4	16
	Expt. 2	6	5	9	2	6	20	7	8	24	7	11	8	4	4	16	12
+ HIS	Expt. 1	9	17	10	3	5	5	15	16	8	5	12	21	13	7	19	4
	Expt. 2	14	11	4	8	13	26	13	8	6	17	7	5	12	4	19	20
+ HIS Absorbed	Expt. 1	8	19	16	10	8	7	13	7	14	6	17	4	13	14	14	17
	Expt. 2	8	8	6	6	16	11	8	7	10	32	12	8	12	21	17	16
N.gonorrhoeae Strain B																	
+ FHS	Expt. 1	9	8	10	20	10	7	21	5	12	9	6	13	16	9	57	29
	Expt. 2	3	3	34	17	8	14	20	23	2	4	41	45	1	3	49	51
+ FHS Absorbed	Expt. 1	1	3	6	5	4	7	8	1	6	10	7	15	6	3	23	17
	Expt. 2	5	2	24	20	15	13	4	10	4	7	30	36	7	11	18	17
+ HIS	Expt. 1	8	9	1	9	15	11	12	2	19	12	14	23	10	3	27	17
	Expt. 2	2	3	11	4	19	17	9	10	30	11	19	23	27	8	20	20
+ HIS Absorbed	Expt. 1	15	10	24	15	8	9	6	1	8	10	8	17	10	4	12	11
	Expt. 2	7	20	13	11	13	26	9	13	5	4	16	13	12	24	10	12

8.3 Results

The counts for each test are shown in Table XX. These values were square-root transformed prior to analysis. This transformation stabilises the variance. This manipulation ensures that the variance for data values such as counts that follow a Poisson distribution are independent of the mean. (Snedecor and Cochran, 1980). The results of duplicate experiments were pooled and subjected to an analysis of variance (Anova) to determine if the blood group of the monocytes, the serum treatment (heat-inactivation or absorption), or the stimulating strain (A or B) caused any significant heterogeneity in the NBT reduction test. The results are summarised by two Anova tables (Tables XXI and XXII).

The Anova table for the results obtained with the opsonised zymosan (Table XXI) shows that significant heterogeneity exists between; 1) cells giving a + response and those giving a +++ response; and 2) cells incubated with zymosan and those incubated in the absence of zymosan. No significant hetergeneity is observed between the responses of monocytes of the four ABO blood groups. Zymosan stimulation resulted in more cells reducing NBT than in monolayers without zymosan. Of those cells that reduced NBT, more cells gave a +++ response than gave a + response (Table XXII). The significant interactions were due to effects from the primary sources of variation. (Factors A and B in Table XXI).

A summary of the results obtained in the experiments with N.gonorrhoeae strains A and B as stimulants are shown in Anova Table 2 (Table XXIII). In these experiments no significant heterogeneity due to the stimulating strain viz, strain A or strain B was observed. The table of means for those factors that gave rise to significant heterogeneity is given in Table XXIV. As in the experiments with the control stimulus, opsonised zymosan, more cells gave a +++ response than a + response. The treatment of the serum was found to influence the numbers of cells reducing NBT. The greater number of cells

TABLE XXI

ANOVA TABLE 1

Stimulation of monocytes of Blood Groups A,B,AB and O by opsonised zymosan in FHS

Source of Variation		DF	Sum of Squares	Mean Square	Variance Ratio (F)	
A (Degree of response +, ++)		1	107.785	107.785	76.684	$p < 0.01$
B (With/without zymosan)		1	144.383	144.383	102.723	$p < 0.01$
C (Blood Group AB,A,O,B)		3	7.220	2.407	1.712	NS
Interactions	A, B	1	250.515	250.515	178.231	$p < 0.01$
	A, C	3	12.325	4.108	2.923	$p < 0.05$
	B, C	3	4.888	1.629	1.159	NS
	A,B,C	3	4.668	1.556	1,107	NS
Residual		48	67.467	1.406		
Total		63	599.248	9.512		

TABLE XXII

Table of Means for Anova Table 1

Source of Variation		Factors	Means	SED*
A (Degree of response)	(	+	2.87	0.296
	(	+++	5.46	
B(with/without zymosan)		without zymosan	2.66	0.296
		with zymosan	5.67	
Interactions				
A,B	without zymosan	+	3.34	0.419
		+++	1.98	
	with zymosan	+	2.39	
		+++	8.94	
A, C (Blood Group)				
	AB	+	3.11	0.593
		+++	5.10	
	A	+	2.33	
		+++	6.33	
	O	+	3.24	
		+++	5.90	
	B	+	2.79	
		+++	4.52	

Means are all means of square-root transformed counts

*SED = standard error of the differences between the means in each group

TABLE XXIII

ANOVA TABLE 2

Stimulation of monocytes of **Blood Groups** A,B,AB and O by N.gonorrhoeae **strains** A and B

Source of Variation	DF	Sum of Squares	Mean Square	Variance Ratio (F)	
A (Strain A, Strain B)	1	0.0114	0.0114	0.012	N S
B (Degree of Response + +++)	1	56.1268	56.1268	57.238	p < 0.01
C (Serum Treatment; FHS,HIS,FHS Abs,HIS abs)	3	19,647	6,549	6.679	p < 0.01
D (Blood Group AB, O, B, A)	3	13.045	4.3482	4.434	p < 0.01
Interactions					
A, B	1	0.0365	0.0365	0.037	NS
A,C	3	1.0976	0.3659	0.373	NS
B, C	3	73.0488	24.3496	24.832	p < 0.01
D, A	3	4.6644	1,5548	1,586	NS
D, B	3	16.0265	5.3422	5.448	p < 0.01
D, C	9	12.4871	1.3875	1.415	NS
No third and fourth order interactions were significant					
Residual	192	188.273	0.9806		
Total	255	426.8445	1.6739		

TABLE XXIV

Table of Means for Anova Table 2

Source of Variation		Factors	Means	SED*
B (response)		+	2.924	0.1238
		+++	3.861	
C (serum treatment)		FHS	3.828	0.1751
		FHS absorbed	3.058	
		HIS	3.334	
		HIS absorbed	3.350	
D (Blood Group)		O	3.664	0.1751
		AB	3.322	
		B	3.319	
		A	3.064	
Interactions				
B, C	FHS	+	2.520	0.2476
		+++	5.137	
	FHS Absorbed	+	2.509	
		+++	3.607	
	HIS	+	3.278	
		+++	3.389	
	HIS Absorbed	+	3.390	
		+++	3.309	
DB	O	+	2.900	0.2476
		+++	4.428	
	AB	+	2.924	
		+++	4.120	
	B	+	3.230	
		+++	3.409	
	A	+	2.643	
		+++	3.485	

Means are all means of square-root transformed counts

* SED = standard error of the differences between the means in each group

stimulated in medium containing FHS compared with medium containing absorbed FHS is the only significant difference of means in this variate ($p < 0.05$). Cells stimulated in medium containing HIS or absorbed HIS appear to give the same degree of response.

In contrast to the results obtained with the opsonised zymosan, the ABO blood group of the monocytes was a significant source of heterogeneity (Table XXIII). Comparison of the mean numbers of NBT reducing cells obtained for each blood group (Table XXIV) shows that Group A cells gave significantly fewer positive cells than Group O ($p < 0.05$) . No significant differences were noted between the responses observed with Group B cells and those of the other blood groups.

An examination of the interaction between B, the degree of response (+, +++) and C, the serum treatment, shows that the difference between the mean number of NBT reducing cells giving a +++ response to those giving a + response is significantly higher ($p < 0.05$) in these tests where a functional complement system is present i.e. tests containing FHS or absorbed FHS, but not in those tests containing heat-inactivated serum.

The interaction between the degree of response, B, and the ABO blood group, D (Table XXIV) demonstrates that ABO blood group influences the degree of response to the gonococcal stimulants used in these NBT tests. Whereas monocytes of blood groups AB, A and O give significantly greater numbers of cells giving a +++ response than a + response ($p < 0.05$), monocytes of group B do not.

8.4 Summary and Discussion of the results

These are preliminary experiments. Firm conclusions cannot be drawn from them. Nevertheless, they indicate potential areas for further investigation and provide the experimental technique to answer those questions. This NBT-reduction test provides a qualitative and semi-quantitative method for examining the responses of glass-adherent mononuclear phagocytes to standard stimulants, such as opsonised zymosan, and bacteria, such as N.gonorrhoeae. Despite the subjective and semi-quantitative aspects of this test, the results obtained using the control stimulus, opsonised zymosan, displayed no significant heterogeneity between four sets of duplicate experiments. The majority of NBT-reducing cells gave a strong, +++ , response to both the control stimulus and the test bacterial strains.

Both strains of N.gonorrhoeae tested, strains A and B, were pyocin Group II, double-highlight colonies. The monocyte responses to both these strains were very similar. The treatment of the serum (heat-inactivation or absorbtion) did not influence the numbers of monocytes that reduced NBT, but did have an effect on the degree of response (+ or +++). The presence of FHS in the medium resulted in a larger proportion of cells giving a +++ response than in those experiments that used HIS. This suggests a role for the complement proteins in stimulation of NBT reduction. Absorbtion of the serum was found to reduce the number of positive, NBT-reducing monocytes only for the experiments using FHS. (Table XXIV). No such reduction was seen between the results obtained with HIS and absorbed HIS. This, together with the results discussed above, suggests both reactive antibody and complement are required for a +++ response to stimulation by these strains of gonococcus.

Monocytes from the four individuals of blood groups AB, A,O and B gave a predominantly +++ response to the opsonised zymosan. (Table XXII). A similar pattern of response was observed when the

stimulant was N.gonorrhoeae for all tests except those using monocytes of blood group B. In these tests the ratio of cells giving a +++ response to those giving a + response was 1.114:1; this compares with the ratios 2.331:1, 1.985:1 and 1.740:1 observed with monocytes of blood groups O, AB and A, respectively.

In summary, the NBT-test described in this section, is a simple method to determine, in a semi-quantitative manner, the oxidative burst of individual glass-adherent peripheral blood monocytes stimulated by N.gonorrhoeae and zymosan. The response of these cells to N.gonorrhoeae is augmented by the presence of both complement and reactive antibody. Monocytes isolated from four individuals, each of a different ABO blood group, were stimulated with opsonised zymosan and two strains of N.gonorrhoeae and the responses were compared. Whereas the monocytes from each donor reacted to a similar degree in response to stimulation by opsonised zymosan, the degree of response to N.gonorrhoeae was heterogeneous among the four groups of monocytes; cells of blood group B gave a less intense response than cells of the other ABO blood groups.

CHAPTER 9

GENERAL DISCUSSION

CONTENTS

9.1 Serum sensitivity among strains of *N.gonorrhoeae* and *N. meningitidis*

Healthy uninfected people have been shown to possess bactericidal antibodies in their serum that react with certain strains of *N.gonorrhoeae* and initiate a complement-mediated bactericidal reaction. It was suggested in Chapter 3 that these antibodies were not isohaemagglutinins, thereby refuting the hypothesis described in the introductory Chapter (Section 1.10.1) : the increased susceptibility of individuals of blood group B was due to their lack of anti-B isohaemagglutinins, antibodies known to react with many species of gram-negative bacteria, including the gonococcus.

Greater bactericidal activity among group A sera, which possess these antibodies, was predicted, and removal of isohaemagglutinins should have reduced the bactericidal activity of group A sera. The results of these experiments in Chapter 3 support the former proposal for two of the four serum-sensitive strains studied; P280 and M8865. The latter proposal is, however, rejected.

If isohaemagglutinins are not a component of the 'natural' bactericidal antibodies reactive with *N.gonorrhoeae*, which other classes of 'natural' antibody might be involved in serum-killing of these organisms.? Recent investigations by Lalezari and colleagues (Lalezari *et al,* 1984; Lelezari and Jiang, 1984) have revealed that normal human sera contain a wide range of sugar-specific antibodies. Since gonococcal LPS is the 'target' antigen for bactericidal antibodies in normal human sera, one or some of these sugar-specific antibodies could be responsible for the 'natural' anti-gonococcal bactericidal antibody. One possible contender, found in 100% of 350 sera examined by Lalezari *et al,* (1984), is an antibody specific for melibiose. Melibiose has been shown to inhibit precipitation reactions between gonococcal LPS and immune hen serum (Perry *et al,* 1978). These authors suggested that a lactosyl unit might occupy a terminal position in the gonococcal LPS (Section 1.5.5.1). Lalezari *et al* (1984) found lactose-specific antibodies in 22.5% of 350 normal human sera. The reacting moiety of

the lactose residue was associated with the 1-4 linkage between the galactose and glucose residues (Lalezari and Jiang, 1984). This 1-4 linkage region between galactose and glucose is a structural feature common to the six LPS serogroups (GC1 to GC6) described by Apicella and Gagliardi (1979) (Apicella et al., 1981) . The possibility of these antibodies cross-reacting with the galactosyl residue,which is immunodominant in B blood group substance,may result in their formation being prevented in group B individuals. This may be one of the reasons for the bactericidal antibody against strains P280 and M8865 being observed at a higher titre in group A sera than in Group B sera (Table VII).

The antigenic stimulus responsible for 'natural' bactericidal antibodies reactive with N.gonorrhoeae is probably found in commensal bacteria. The observation that these antibodies can be removed from serum by absorbtion with a strain of N.lactamica and a Group II strain of meningococcus (Table IX) gives support to this idea.

The lack of similarity between the serum-sensitivity patterns observed for Group I and Group II strains of non-serogroupable meningococci (Chapter 7) is puzzling. Protein antigens might be acting in bactericidal reactions mediated by 'natural' antibody (Frasch and Gotschlich, 1974), thereby accounting for the serum-sensitivity of Group I strains of non-serogroupable meningococci. Natural antibodies to protein antigens of N.gonorrhoeae have been reported (Schiller, Friedman and Roberts, 1978; Sarafian, Tam and Morse, 1983),but opsonisation is the principal biological activity of these antibodies. Another possible explanation for the serum-sensitivity of Group I strains of meningococci could be that capsular antigens of a hitherto unknown serogroup may be reacting with bactericidal antibodies. If, however, the observed serum-sensitivity of Group I strains of meningococci is mediated via LPS antigens the possibility of differences in composition and/or structure between gonococcal and meningococcal LPS molecules must be considered.

9.2 The relationship between pyocin type and serum sensitivity in strains of *N.gonorrhoeae*

Gonococcal strains of pyocin Group I were uniformly resistant to NHS, whereas those of Group II were sensitive to the majority of the 32 sera tested at a titre of $\geqslant 15$. (Chapter 3). Examination of the LPS isolated from a Group II strain, P280, and a pyocin-resistant variant, P280μ that has the same pyocin type and serum-resistance as Group I strains, suggests that the LPS of Group II strains is a larger molecule than that found in Group I strains. This indicates that the portion of LPS associated with the binding site for the class R1 pyocins might be absent, rather than cryptic,in Group I strains. This provides circumstantial evidence that the LPS locus that binds bactericidal antibody is in the distal portion of the LPS and is associated with the binding site for class R1 pyocins.

In contrast,Morse and Apicella (1982) and Guymon, Esser and Shafer, (1982) observed that loss of pyocin-sensitivity in serum-resistant gonococcal strains was coincident with a shift to serum sensitivity. Only one of the pyocin classes used in these studies, pyocin 1 (used by Guymon, Esser and Shafer, 1982) has been classified by Kageyama. Pyocin 1 belongs to the R5 class of pyocins (Kageyama, 1975) that bind deep in the LPS molecule of *Ps.aeruginosa* (Kageyama, 1975; Section 1.5.5.3). If the gonococcal receptor for R5 pyocins has a similar location, and this report suggests that it does bind closer to the lipid moiety of the LPS than class R1 pyocins, then loss of its receptor by mutation would result in a LPS molecule possessing very little carbohydrate. These mutants may exhibit the high serum sensitivity observed in deep rough LPS mutants of *Salmonella* spp. (Taylor, 1983).

The serum-sensitive (Group II) strains in this study presumably have more carbohydrate residues, or different linkages between residues, in the LPS since they bind R1 pyocins. According to the *Pseudomonas* model and this report, these bind at a site on the LPS

distal to the binding site for class R5 pyocins. The Group I strains might, therefore, be similar to the serum-resistant strain FA19 described by Guymon, Esser and Shafer, (1982). Comparisons with the work of Morse and Apicella is hindered by the fact that their pyocin 611-131 has not been placed into one of Kageyama's classes (Kageyama, 1975).

The results of this report are supported by the findings of Schneider and colleagues (1982) who proposed that the basis for serum-resistance in strains of N.gonorrhoeae was due to absence of an LPS antigenic determinant rather than masking of an antigenic site.

9.3 The prevalence of Group I and Group II strains of *N.gonorrhoeae* in clinical isolates

The report by Blackwell, Young and Anderson (1979), determined the pyocin type for a range of gonococcal isolates from localised and disseminated infections. This was retrospectively analysed for the prevalence of Group I and Group II strains of *N.gonorrhoeae*. Strains that were sensitive to any of the R5 pyocins were allocated to Group I, and strains that were sensitive to any R5 and any R1 pyocins were allocated to Group II. The hypothesis under test was as follows : Strains isolated from DGI are all serum-resistant whereas those from localised infections tend to be serum-sensitive *in vitro*. Therefore, constitutively serum-resistant strains *i.e.* those of Group I, should be predominant among those isolated from DGI and serum-sensitive, Group II strains should form the majority in strains isolated from localised infection.

The analysis supported the hypothesis, 95 (86.4%) of 110 isolates from localised infections were of the Group II pyocin type, compared with only 15 (13.6%) of Group I. This contrasted with the isolates from DG1 in which only 14 (58.4%) out of 24 isolates were of Group II and a larger proportion 10/24 (41.6%), of Group I isolates were found.

These results complement the observations of Schoolnik, Buchanan and Holmes, (1976) who found 97% of strains isolated from DGI were serum-resistant, compared with 42% of strains isolated from localised infections. (See Section 1.8.3.5). The observation that a Group II strain isolated from DGI, strain 1560, has a similar resistance to serum as the Group I strains isolated from localised infection suggest that at least two types of constitutive serum resistance may occur. Lack of the LPS receptor for bactericidal antibody, such as that observed with Group I strains and that described by Schneider *et al* (1982) may present one form of serum-resistance. Another form of

serum-resistance, which may have been responsible for the results obtained with the Group II DGI isolate, strain 1560, may result from the possession of a low molecular weight (Mr 35K) protein I molecule that has been associated with resistance to serum. (James et al, 1982)

9.4 Inducible phenotypic serum-resistance in *N.gonorrhoeae* - a possible mechanism.

This report demonstrated that changes in pyocin - sensitivity are associated with the development of phenotypic serum resistance (Chapter 5). These changes suggest loss or masking of pyocin receptor sites, and are consistent with the ideas expressed in Chapters 3 and 4: loss of the LPS portion associated with the binding site for class R1 pyocins results in a shift from serum-sensitivity to resistance.

N.gonorrhoeae is capable of expressing two distinct LPS molecules simultaneously (Guymon *et al*, 1980; Rappuoli, 1981). Rappuoli (1981) reported that gonococcal strain R-10 simultaneously contained two antigenically distinct R-type lipopolysaccharides, only one of which contained the lactosyl determinant which Perry *et al* (1978) described as being an immunologically dominant feature. Both LPS types were shown to have similar compositions and molecular weights. The differences in antigenicity might be attributable to differences in the linkage between the constituent sugars of the LPS. Antigenically distinct gonococcal LPS with very similar molecular weights have also been described by Schneider *et al*, (1978).

If the LPS species containing the lactosyl moiety reacted with natural bactericidal antibodies and class R1 pyocins and the other LPS species, in which the lactosyl unit was absent, was resistant to these antibodies and pyocins, then a reduction in expression of the lactose-containing LPS would be associated with an increase in resistance to serum and R1 pyocins. Since the structure of the polysaccharide portion of LPS is dependent on the specificities of the glycosyl transferases, and the availability of the subunit sugars is dependent on membrane transport proteins and epimerases, interference with any one of these proteins could halt LPS synthesis. However, in the proposed mechanism for the development of phenotypic serum resistance, only one LPS species is proposed to be affected. Since the two LPS species described by Rappuoli (1981) had similar chemical compositions interference with a specific glycosyl transferase is the most likely event to result in inhibition of the production of one LPS species whilst permitting synthesis of the other. This interference

might be due to direct steric inhibition of a transferase, or to repression of the genes coding for the transferase by the serum-resistance inducing factor.

Another possible mechanism of inducing phenotypic serum resistance could involve the liberation of enzymes capable of degrading the polysaccharide moiety of the LPS molecule, thereby destroying the antigenic and pyocin-reactive properties of the molecule. Such enzymes have been observed in the supernates of sonic extracts of N.gonorrhoeae and N.meningitidis (Apicella, Breen and Gagliardi, 1978). Chemical analysis of culture supernatants before and after treatment with the inducer would test this hypothesis.

9.5 The absence of functional bactericidal antibody reactive with *N.meningitidis* strains of serogroups A,B, and C in the sera of patients with gonorrhoea

Sera from patients with gonorrhoea were screened for bactericidal antibody against meningococcal strains of serogroups A,B and C and two non-serogroupable strains, 0452 and PA(NG) 846. Normal titres against the non-serogroupable strains were found; the geometric mean titres were 74.7 and 16.0 for strains 0452 and PA(NG) 846, respectively. Antibodies reactive with the serogroupable strains were not detected in the majority of sera tested. The geometric mean titres against the strains of serogroups A,B and C were 3.4, 2.6 and 4.0, respectively. (Chapter 7). This lack of antibody against the serogroupable strains contrasts with the results obtained with serum from healthy people. (Chapter 7). Individuals of blood group B had a geometric mean titre of 24.7 against the serogroup A strain, and individuals of blood group A had a geometric mean titre of 10.5. Craven and colleagues (1982) report even higher titres against strains of serogroups A,B and C in the sera of seven normal individuals.

The absence of detectable bactericidal antibody against serogroupable meningococci but not against the non-serogroupable strains in the patients' sera could be due to lack of antibodies or to the presence of IgA blocking antibodies. The patients with gonorrhoea may never have had high titres of bactericidal antibody against the serogroupable meningococcal strains in this study. However, they were shown to have bactericidal antibody reactive with the non-serogroupable strains at titres similar to those observed in healthy uninfected people.

Anti-meningococcal antibodies may be absorbed by cross-reacting gonoccal antigens during infection. Antigenic similarities between gonococcal and meningococcal LPSs have been reported (Apicella *et al*; 1981; Section 1.5.5.2) and *Neisseria* species are

known to release free LPS and outer membrane vesicles during growth (De Voe and Gilchrist, 1973; Johnson, Perry and McDonald, 1975). These may act as absorbants of antibody, possibly leading to the observed reduction (transient?) in antibodies bactericidal for the serogroupable strains tested. A similar phenomenon occurs during pneumococcal infection (Downie, 1937).

Another explanation for these observations comes from the work of Griffiss (1975 and 1983) which shows that IgA specific for the same antigenic determinants as bactericidal IgM and IgG can block the binding, and, therefore, killing of meningococci by these antibodies. Moreover, Griffiss (1975) demonstrated the presence of these IgA blocking antibodies in the serum of patients convalescing after an episode of meningococcal disease. Infection with N.gonorrhoeae may result in the stimulation of IgA antibodies that cross-react with meningococcal antigens and block the binding of bactericidal antibodies to N.meningitidis, IgA antibodies reactive with serogroupable meningococcal strains have been described both in patients with gonorrhoea and in healthy controls, but no major differences between the two groups were observed (McMillan et al, 1979).

If the bactericidal activity was due to natural antibodies of the IgM class, as in the case of N.gonorrhoeae (Schoolnik, Ochs and Buchanan, 1979), then low titres of IgA blocking antibodies in patients' sera would produce a prozone (Griffiss, 1983). This was not observed in our experiments.

The role of bactericidal antibodies in preventing dissemination of N.meningitidis from the mucosa of the upper respiratory tract has been discussed in section 1.7.3. Absence of bactericidal activity against N.meningitidis, whether due to absence of bactericidal antibody, as seen in young children (Section 1.8.4) or due to the presence of IgA blocking antibodies inhibiting the action of the serum bactericidal antibodies, predisposes the human host to meningococcal disease. (Griffiss, 1983; Kähty et al, 1981).

Is there any link between infection with N.gonorrhoeae and susceptibility to meningococcal disease? An examination of some epidemiological data provides circumstantial evidence to suggest that there may be. Analysis of the age-distribution of patients with meningococcal disease shows the greatest morbidity to in the first four years of life. These children have not yet developed bactericidal antibodies against meningococci (Goldschneider, Gotschlich and Artenstein, 1969b). The group in which the next highest incidence occurs is the 15-19 year olds (Peltola, Kataja and Mäkelä, 1982; Section 1.3.2.3), an age range corresponding to the onset of sexual activity and in which there is a high incidence of gonorrhoea (Kallings and Moberg, 1977; Section 1.3.1)

Outbreaks of meningococcal disease in military institutes occur predominantly among recruits, 80% of infections developing within 5-6 weeks of their entry (Bristow, van Peenen and Volk, 1965; Varela and Gilmore, 1971). The majority of patients with meningococcal disease in one well documented outbreak in a military establishment fell into the age range of 18-24 years (Koppes, Ellenbogen and Gebhart, 1977) which is identical to that in which most military cases of gonorrhoea are to be found. (White and Blount, 1967).

The incidence of meningococcal colonisation of the pharynx is higher in patients with genital gonorrhoea than in people without the disease. (Young, Harris and Robertson, 1979; Ødegaard and Gedde-Dahl, 1979; Section 1.4.3). Analysis of the serogroup of the meningococcal strains isolated from the oropharynx of patients with gonorrhoea has revealed that women with gonorrhoea had a higher rate of colonisation with serogroupable meningococci than women without gonorrhoea. In contrast, no difference in carriage rate was observed among women for non-serogroupable meningococci. No differences in carriage of meningococci were observed between men with and without gonorrhoea.

The carriage rate of serogroupable meningococci has been shown to be reduced by the presence of serum bactericidal antibodies (Gotschlich, Goldschneider and Artenstein, 1969). It is tempting to speculate that the absence of bactericidal antibody reactive with serogroupable strains of meningococci among the sera from patients (Predominantly women) with gonorrhoea surveyed in this report (Chapter 7) may be one of the factors responsible for the increased carriage of N.meningitidis among women with genital gonorrhoea. Similar titres of bactericidal activity against non-serogroupable strains of N.meningitidis were present in sera from women with gonorrhoea and from healthy women.

These observations provide circumstantial evidence to support the suggestion that recent gonococcal infection may be one of the many factors contributing to susceptibility to meningococcal disease and could partially account for the higher incidence of this disease among young adults.

9.6 The oxidative metabolic response of human monocytes of blood groups A,B, AB and O to stimulation by strains of N.gonorrhoeae

This report describes a simple method for determining, in a qualitative and semi-quantitative manner, superoxide production of individual glass-adherent mononuclear phagocytes by reduction of the redox indicator, nitroblue tetrazolium (NBT). A standard stimulus, opsonised zymosan, gave similar responses in duplicate experiments using monocytes from four donors, one of each of the four ABO blood groups. This uniformity of response, as determined by analysis of variance (Tables XXI and XXII), provides a reference point to which other stimuli can be compared.

This was only a pilot experiment, designed to develop the technique and discover sources of hetergeneity in the oxidative metabolic responses of monocytes from donors of the four ABO blood groups to stimulation by strains of N.gonorrhoea. In view of the increased susceptibility of individuals of blood group B to gonorrhoea (Section 1.4.1.1) and the evidence suggesting that intracellular survival of gonococci within phagocytes is an important aspect of the pathogenesis of gonorrhoea (Section 1.7.2.1), the observation, in these experiments, that the monocytes from the blood group B donor produce superoxide less intensely than the monocytes from the donors of blood groups A,AB and O (Chapter 8) requires further investigation. If, after examining the monocytes from a larger sample of people, this observation was confirmed as a general phenomenon of monocytes from blood group B donors, it could be one of the factors causing the increased susceptibility of individuals of blood group B to gonorrhoea.

Blackwell et al (1984) conducted NBT reduction experiments stimulating monocytes in suspension with gonococci and other Neisseria species. Experiments conducted in the absence of serum gave more

formazan - containing monocytes than experiments in which serum was incorporated into the medium. These results were interpreted as indicating an inhibitory role for serum. This could, however, be a misinterpretation. Since the experiments were conducted on monocytes in suspension, a population that would contain potentially glass-adherent cells, the presence of serum may have assisted many of the cells, especially those stimulated by phagocytosis of the bacteria, to adhere to the reaction vessel and, therefore, not be present in the aliquot that was removed from the reaction mixture for mounting and microscopic examination.

The experiments in this report found that removal of reactive antibody and complement from the serum added to the reaction mixture resulted in a decrease in the number of superoxide - producing monocytes. (Tables XXIII and XXIV; Section 8.3), These differing responses might be due to mononuclear phagocytes being a heterogeneous population of cells with a variety of functions and surface components. (Dougherty and McBride, 1984). Examination of the distribution of Fc and complement receptors between the two populations, viz. glass-adherent monocytes and monocytes in suspension, might indicate the reason for these differences.

CHAPTER 10

FUTURE WORK

CONTENTS

10.1 Lipopolysaccharide type and serum-sensitivity of the pathogenic Neisseria species

Grouping by sensitivity to R-type pyocins has provided a simple and rapid means of discriminating between strains of the pathogenic Neisseria species on the basis of putative differences in the LPS molecule. In the case of the gonococcus, these differences have been associated with differences in the interaction of strains with the humoral and phagocytic components of the human immune system. The information provided by pyocin-sensitivity is primitive. Deeper understanding of the interactions between the LPS of Group I and Group II gonococcal strains with 'natural' bactericidal antibodies and the differences between serum-sensitivity of Group I strains of N.gonorrhoeae and Group I strains of non-serogroupable meningococci will depend on structural analysis of these molecules. LPS analyses are also an important step in determining the effects of the component of guinea pig and human blood that induces N.gonorrhoeae strains BS4 (agar) to serum-resistance.

At present, only small numbers of bacteria can be used in the induction experiments because of the small quantities of inducer available. If the inducer is actively taken up by the bacteria, isolation of the membrane receptor could provide a means for purifying and concentrating the inducer by affinity - chromatography.

10.2 Does gonorrhoea predispose to meningococcal disease?

This report shows that patients with gonorrhoea have lower titres of 'natural' bactericidal antibody against meningococci of serogroups A, B and C than normal healthy people. This may increase the susceptibility of patients with gonorrhoea to meningococcal disease. Anecdotal and circumstantial evidence suggests that gonorrhoea may be one of the factors that predispose the host to meningococcal disease. Validation of this hypothesis would require clinical observations to determine whether young adults with meningococcal disease had a recent history of gonorrhoea.

The possibility that gonococcal infection stimulates IgA blocking antibodies could be investigated by screening patients' sera for IgA antibodies reactive with capsule and LPS antigens.

10.3 The oxidative burst in monocytes stimulated by *N.gonorrhoeae*

The continuation of the preliminary experiments described in this report would involve an examination of the reactivities of monocytes taken from a larger number of donors of the four ABO blood groups. If the subdued response of blood group B monocytes to this stimulus was found to be a general phenomenon, two questions arise : 1) since complement was found to increase the intensity of superoxide production, do the stimulating strains of *N.gonorrhoeae* activate complement equally efficiently in sera of the four ABO blood groups? and 2) do monocytes from donors of blood group B have as many antibody and complement receptors per cell as monocytes of blood groups A, AB and O?.

Phagocyte responses to external stimuli, opsonised bacteria, complement fragments *etc.*, are triggered *via* a system that uses calcium as a secondary messenger (Romeo, 1982). The development of fluorescent indicators of cytosolic calcium, such as Quin-2, allow the ionic fluxes of cells, such as monocytes, to be monitored during stimulation. (Tsien, Pozzan and Rink, 1984). These techniques would allow the stimulation of monocytes by *N.gonorrhoeae* to be assessed at the level of an event that occurs after membrane stimulation but prior to and concurrent with the activation of the membrane oxidases responsible for the production of superoxide.

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PUBLICATIONS

Some of the data from this report have been published in the following papers:

Winstanley, F.P., Blackwell, C.C., Weir, D.M. and Kinane, D.F. (1983). ABO blood group and susceptibility to gonococcal infection. II. The relationship of lipopolysaccaride type to gonococcal sensitivity to the bactericidal activity of normal human serum. J Clin and Lab Immunol 11: 27-32.

Winstanley, F.P., Blackwell, C.C., Weir, D.M. and Kinane, D.F. (1983). Gonorrhoea: a predisposing factor for meningococcal disease? Lancet ii: 1135.

Winstanley, F.P., Blackwell, C.C., Tan, E.C., Patel, P.V., Parsons, N.J., Martin, P.M.V., and Smith, H. (1984). Alteration of pyocin-sensitivity pattern of Neisseria gonorrhoeae is associated with induced resistance to killing by human serum. J Gen Microbiol 130: 1303-1306.

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BLOOD GROUPS AND SUSCEPTIBILITY TO GONOCOCCAL INFECTION.
II. THE RELATIONSHIP OF LIPOPOLYSACCHARIDE TYPE TO GONOCOCCAL SENSITIVITY TO THE BACTERICIDAL ACTIVITY OF NORMAL HUMAN SERUM

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SUMMARY This study examines the bactericidal activity of normal human sera from individuals of blood groups A and B for gonococcal strains with simple and complex lipopolysaccharides (defined by pyocin-sensitivity) isolated from localised and disseminated infection. The bactericidal activity did not depend on A or B isohaemagglutinins. Resistance to normal human serum exhibited by strains from localised infections appeared to be due to lack of part of the lipopolysaccharide antigen, whereas resistance of strains from disseminated infection appeared to depend on a separate mechanism yet to be defined.

INTRODUCTION

INDIVIDUALS of blood group B are especially susceptible to genito-urinary infections with *Escherichia coli* (1, 2) and *Neisseria gonorrhoeae* (3, 4, 5). There is evidence that the anti-B isohaemagglutinin found in individuals of blood groups A and O may play a role in host-protection against infection by gram-negative bacteria (6).

In this study we investigated the sensitivity of a number of strains of *N. gonorrhoeae* with different lipopolysaccharide (LPS) to "normal human serum" (NHS) from individuals of blood groups A and B to determine whether anti-B isohaemagglutinin is involved in a non-specific host defence mechanism against gonorrhoea.

Previous work from this laboratory has indicated that attachment of gonococcal strains to polymorphonuclear leukocytes (PMN) varies according to differences in the structure of the LPS (7) as detected by differences in sensitivity to partially purified R-type pyocins of *Pseudomonas aeruginosa* (8). Since LPS is one of the major antigens involved in bactericidal killing (9, 10), strains of both of the pyocin-defined groups (7) were tested to determine whether differences in LPS structure could be correlated with differences in sensitivity to the bactericidal effects of NHS and conventional immune sera prepared in mice.

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MATERIALS AND METHODS

Bacterial Strains

Ten gonococcal strains from localised infections were supplied by the Department of Genito-urinary Medicine, Royal Infirmary of Edinburgh (R.I.E.). Three β-lactamase-producing strains were kindly supplied by Dr. K. Shannon and Professor I. Phillips, St. Thomas's Hospital Medical School, London, and two strains from disseminated infections were obtained from Dr. Joan S. Knapp, Neisseria Reference Laboratory, United States Public Health Service, Seattle, Washington. All strains, together with the grouping based on pyocin sensitivity (7) are listed in Table 1. They all conformed to the morphological and biochemical characteristics of *N. gonorrhoeae* and were of colony type 4 (11).

Culture

Strains were cultured on Modified New York City (MNYC) medium (12) at 37°C in a humidified incubator with 10% CO_2 in air.

Isolation of Pyocin-resistant Mutant

Colonies growing within a zone of inhibition produced by pyocin IS8 were isolated and re-typed. One mutant strain (P280μ), sensitive to Kageyama group R5 pyocins only, was used in the study.

Preparation of Immunising Antigens

An 18-hr growth of gonococci was harvested from MNYC plates with sterile cotton swabs and suspended in 0·01M phosphate-buffered saline (PBS) pH 7·2. An equal volume of PBS containing 2·4% formaldehyde was added to the suspension which was stored overnight at 4°C. The formaldehyde-treated organisms were centrifuged at 900 × g for 1 hr and resuspended in PBS containing 0·01% formaldehyde to the opacity of Brown's Tube No. 3 as judged by eye (13). This is equivalent to $1·1 \times 10^9$ gonococci/ml (14).

Table 1 Pyocin type of gonococcal strains

		Pyocins with Kageyama group												
		R1					*Unclassified*		*R3*	*R2*			*R5*	
	Gonococcal strain	*ISD*	*2285*	*IS4*	*IS6*	*IS8*	*R205*	*9579*	*ISE*	*ISB*	*R21*	*430*	*ISA*	*ISC*
Group I	M9131													
	E757													
	*849209													
	*845650	−	−	−	−	−	−	+	−	−	−	−	+	+
	*934936													
	P280μ													
	P3309													
	†7425													
Group II	M8865	+	±	+	+	+	+	+	−	−	−	−	+	+
	P280													
	E2590													
	E728													
	E759	+	+	+	+	+	+	+	−	−	−	−	+	+
	M5287													
	P3290													
	†1560													

+ clear zone of inhibition.
± zone of partial inhibition.
− no inhibition.
* β-lactamase producing strains.
† strains from disseminated infection.

Immunisation Schedule

Female CF1 mice, 5–6 weeks old, caged in groups of 10, were given 0·25 ml of the standard suspension of formaldehyde-treated gonococci (gonococcal antigen) intravenously, via the lateral tail veins. The mice were immunised at day 0 and again on days 7 and 10. On day 14 a small quantity of blood was taken from the retro-orbital plexus of each mouse of the group and pooled; the serum obtained was inactivated by heating to 56°C for 30 min and then distributed into small tubes and stored at −20°C until required. The mice thereafter received booster doses (0·25 ml) of the gonococcal antigen at 14-day intervals and blood was obtained 5 days after each immunisation.

Complement

Human AB serum from a donor with no history of gonococcal infection was adsorbed for 24 hr at 4°C with a live suspension of the gonococci used in the study, distributed in 200 μl aliquots and stored at −70°C before use. The minimum haemolytic titre of the serum was 32–64.

Normal Human Sera from Individuals of Blood Groups A and B

Kindly supplied by Drs. S. Urbaniak and P. L. Yap of the Blood Transfusion Service, R.I.E.

Screening Test for Bactericidal Activity of Normal Human Serum

An 18-hr culture of gonococci was suspended in Dulbecco's Phosphate Buffered Saline supplemented with Mg^{++} and Ca^{++} ions (0·5 mM and 0·9 mM respectively) (DPBS + B) to give approximately 10^4 cfu/ml.

A series of two-fold dilutions, from 15 to 480, were made in a microtitre plate for each serum to be screened to give a final volume of 50 μl per dilution, 40 μl of gonococcal suspension and 10 μl of a two-fold dilution of the complement source were added to each well and incubated at 37°C for 30 min. Two drops of 20 μl from each well were plated onto MNYC medium and incubated for 24 hr. The highest serum dilution to give a reduction in viable count of ≥ 80% was considered to be the bactericidal titre of the serum.

Bactericidal Assay with Immune Mouse Serum

These were performed in round-bottomed glass tubes (15 mm × 75 mm). An 18-hr culture of gonococci was suspended in D.PBS + B to give approximately 10^6 cfu/ml. The assay mixture contained 160 μl of bacterial suspension, 20 μl of a two-fold dilution of heat-inactivated mouse serum and 20 μl of freshly thawed AB serum as the complement source. Controls lacking serum and/or complement were included for each experiment. After static incubation at 37°C for 30 min in a humidified atmosphere with 10% CO_2 in air, 25 μl volumes were removed from each tube and viable counts were performed (15). A reduction in viable count of 80% or greater when compared with controls was considered to be significant.

Results

The gonococcal strains were all typed by pyocin sensitivity and classified into two broad groups: Group I strains were sensitive to Kageyama group R5 pyocine; Group II strains were sensitive to Kageyama groups R5 and R1 pyocins (table 1).

The AB serum used as the exogenous complement source for the bactericidal reactions was found to have slight microbicidal activity against strains of Group II. This activity was removed by absorption at 4°C with the sensitive strains and the procedure had no effect on the complement titres. Absorbed AB serum was used in all the bactericidal tests.

Table 2 Bactericidal titre of normal human serum from Group A individuals

	Test strain of N. gonorrhoeae	Sera A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
Group I	M9131, E757, 849209, P3309, 934936, P280μ, *7425	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Group II	M8865	15	240	60	60	60	30	120	120	30	120	120	60	30	30	15	60
	P280	>	>	>	>	>	>	240	240	240	60	120	30	120	120	30	120
	E2590	<	<	60	30	<	30	<	<	30	30	120	60	60	60	15	60
	P3290	15	15	30	30	30	30	15	60	120	120	15	15	15	60	30	120
	*1560	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<

< less than 15.
> greater than 480.
* strains from disseminated infection.

Normal human sera from individuals of blood groups A and B were found to be bactericidal to gonococcal strains of Group II to a titre of $\geqslant 15$, but no activity towards Group I strains from localised infections or a disseminated infection could be demonstrated at the lowest serum dilution of 15. The titre of bactericidal antibody against strains of Group II was slightly greater in individuals of blood group A (tables 2 and 3). No bactericidal activity could be demonstrated towards strain 1560 (Group II) from a disseminated infection (tables 2 and 3).

This bactericidal activity of NHS towards strains of Group II is inhibited by 10 mM Mg-EGTA which sequesters Ca^{++} ions necessary for the classical pathway of complement activation.

There was no discrimination between strains of Group I and Group II by the microbicidal mechanism in immune mouse serum. Serum raised against a strain from Group I (M9131) killed other strains in Group I and also strains in Group II. This same lack of discrimination was shown by antiserum raised against a strain from Group II (M8865) (table 4). Similar experiments with two gonococcal strains from disseminated infections (1560 and 7425) showed no detectable microbicidal activity.

One serum from each of the blood groups A and B was absorbed twice for 24 hr at 4°C with a heavy suspension of erythrocytes expressing A or B antigens to remove the iso-haemagglutinins. This absorption did not remove any bactericidal activity from the sera. Absorption with a heat-killed suspension of *P. aeruginosa* strain ZD8 slightly reduced the bactericidal activity of the sera (table 5).

Table 3 Bactericidal titre of normal human serum from Group B individuals

	Test strain of N. gonorrhoeae	Sera i	ii	iii	iv	v	vi	vii	viii	ix	x	xi	xii	xiii	xiv	xv	xvi
Group I	M9131, E757, 849209, P3309, 934936, P280μ, *7245	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<
Group II	M8865	15	30	30	30	>	15	120	30	15	15	<	30	<	15	30	15
	P280	<	15	<	15	240	15	15	30	120	60	60	60	240	60	120	60
	E2590	<	<	<	15	60	<	<	<	30	30	30	60	30	60	30	30
	P3290	<	<	<	30	60	<	120	60	120	30	30	15	15	120	30	60
	*1560	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<	<

< titre less than 15.
> titre greater than 480.
* strains from disseminated infection.

Table 4 Microbicidal action of immune mouse serum. Number of tests out of 4 giving ⩾ 80% kill

	Gonococcal strains	Antiserum	
		M9131 (group I)	*M8865 (group II)*
Group I	M9131	3	2
	E757	1	2
	849209	3	4
	845650	4	4
	934936	2	3
	P280μ	0	3
Group II	M8865	4	4
	M5287	4	4
	E728	3	2
	E759	2	2
	P280	1	4

Discussion

Our findings suggest that the increased susceptibility of individuals of blood group B to infection by *N. gonorrhoeae* is not related to differences in susceptibility of the organism to isohaemagglutinins. Although *N. gonorrhoeae* isolates have been shown to absorb anti-A and anti-B isohaemagglutinins (4) the bactericidal activity of normal human serum (NHS) could not be removed by absorbtion with red blood cells expressing A and B antigens.

Variations in the susceptibility of gonococci to the bactericidal effects of NHS have been reported (9, 16, 17). Although the LPS has been found to be the target antigen for "natural" antibody (16, 18), the particular components involved in the reaction had not been identified. Classification of gonococcal isolates by the sensitivity pattern to partially-purified R-type pyocins of *P. aeruginosa* has provided a method for detecting differences in LPS antigens. Clinical isolates of Group I which appear to have a simpler LPS structure are resistant to NHS while those with more complex LPS (Group II) are sensitive. The importance of LPS components in determining the reaction with NHS is illustrated by the change from sensitivity to resistance found in the mutant P280μ (Group I) derived from a NHS-sensitive clinical isolate, P280 (Group II). Alterations in the LPS associated with this mutation was shown by gel-electrophoresis and gas-liquid chromatography (unpublished data). These findings are similar to those of Morse and Apicella (19) for their gonococcal LPS mutant selected for resistance to pyocin 611–131.

Table 6 Relative positions of pyocin receptor sites on lipopolysaccharide (LPS) of *Pseudomonas aeruginosa*

*R3**	*R4**	*R2**	*R1**	*R5**
I	H	D	K	A
		F	L	B
		J	M	C
		U	N	E
			O	G
			P	
			Q	
(Sidberry and Sadoff 1977)			R	
			S	
			T	
ISE	—	ISB	ISD	ISC
		R21	IS4	ISA
		430	IS6	
			IS8	
(This study)			2285	

* Arranged in order of the receptor sites on the lipopolysaccharide (LPS) fraction of *P. aeruginosa* suggested by Kageyama (1975).
The receptor for R5 is nearest to, and that for R3 furthest from the cytoplasm.

A schematic representation of the relative positions of pyocin receptors on the LPS of *P. aeruginosa* proposed by (20) is shown for our range of pyocins and those used by Sidberry and Sadoff (21) in Table 6. The "natural" antibody of NHS appears to be directed against either: (1) the LPS structure(s) that form the receptor site(s) for pyocins of Kageyama's group R1; or (2) to one or more structures distal, but linked, to the receptor site for the R1 pyocins. Evidence for this second proposal comes from the work of Sadoff and co-workers (22). Gonococcal isolates were typed with pyocins from their collection and they found that the growth of serum-sensitive strains was inhibited by pyocins D, H and I. These three pyocins belong to Kageyama groups R2, R4 and R3 respectively which attach to LPS receptors distal to those for R1 (table 6). In contrast, our pyocins of these three groups

Table 5 Titres of bactericidal antibody before and after absorption

	N. gonorrhoeae strain	*Before absorbtion*	*Absorbed with erythrocytes*	*Absorbed with* P. aeruginosa
Group A serum (K)	P280	120	120	60
	M8865	120	120	60
	E2590	30	30–60	15
Group B serum (vii)	P280	120	120	15
	M8865	15	<	15
	E2590	15	15	<

had no activity against any of the gonococcal isolates tested (8).

The putative "natural" antibody responsible for the microbicidal activity of NHS differs in specificity compared with conventional immune sera raised in mice. While the "natural" antibody recognises determinants present only on organisms expressing the more complex LPS, the immune sera had a wider specificity and were bactericidal for strains of both Group I and Group II.

Previous work has suggested that resistance of gonococci to NHS is not due to the presence of masked antigens, blocking IgA antibodies, or deficiencies in complement function, but to the resistant organism's lack of an LPS determinant that binds the "natural" antibody (23); our findings support this view. The bactericidal activity of NHS towards strains of Group II could be absorbed in part by a heat-killed suspension of *P. aeruginosa* strain ZD8, suggesting that the activity may be due to "natural" antibody induced by commensal flora.

One implication of our findings is that the more NHS-sensitive Group II organisms should be restricted to localised infections and that Group I should predominate in disseminated infections. In an earlier study on pyocin types of gonococci from a number of geographic sources (8) 95 (86·4%) of 110 isolates from localised infections were of Group II compared with only 15 (13·6%) of Group I. In contrast, of the 24 isolates from disseminated infections, a higher percentage of Group I organisms (41·6%) were found. The proportion of isolates of Group II was 58·3%. Since organisms of Group II have been isolated from disseminated infections, simplicity of LPS does not appear to be the sole factor in determining the characteristics of a "disseminated" strain.

A Group II isolate from a disseminated infection was resistant to immune mouse serum in addition to NHS and this finding is the subject of further studies. This suggests that loss or masking of antigens can occur without interfering with the receptor sites for the R-type pyocins. This resistance to bactericidal antibody of a strain from disseminated infections was not found to be associated with anti-complementary activity of the organism.

We conclude that the bactericidal effect of NHS on strains of *N. gonorrhoeae* is mediated by antibodies that bind to a LPS portion associated with the binding site of R1 pyocins present in gonococci of Group II. Strains lacking this portion of LPS—(Group I)—are resistant to NHS. Thus "natural immunity" to gonorrhoea appears to be directed at organisms of Group II.

The basis for the increased susceptibility of blood group B individuals to gonorrhoea is still not established but seems not to be due to isohaemagglutinins. There is evidence in *E. coli* urinary tract infections that the anti-B titres are raised during infection with organisms expressing B-like antigens. This rise did not occur with anti-A isohaemagglutinins in response to A-like organisms (24). If this difference is reflected in other "natural antibody" responses of B individuals a possible basis for increased susceptibility to infection arises.

The sharing of antigens (galactose determinants) between the LPS of gonococci and blood group B antigen could also account for a diminished response in individuals of blood group B.

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of chloracne (72). The table shows the mortality figures of both subgroups stratified by year of birth. 25 men have died, 8 from myocardial infarction including sudden death and 8 from neoplasms. In contrast to the 8 who died from tumours, 7 of the 8 men who died from myocardial infarction/sudden death had had chloracne; 4 belonged to the group of 16 contract cleaners, who did the dirtiest work and had severe chloracne. These 16 men had many other exposures to chemicals before and after the period concerned, and alcohol consumption was relatively high among this group. The neoplasms do not show an organ related pattern, and are not considered to represent any excess mortality: expected number of deaths 21 (SMR 1·19, 95% confidence interval 0·78–1·70); expected number malignant diseases 6·94 (SMR 1·15, 95% confidence interval 0·49–2·09).

Morbidity data are being collected on 35 of the cohort, most of them being employees of the factory concerned. The data are being compared with those of a matched control group, and to date no significant differences have been found. The study continues.

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GONORRHOEA, A PREDISPOSING FACTOR FOR MENINGOCOCCAL DISEASE?

SIR,—Immunity to meningococcal disease correlates with the presence of bactericidal antibodies for *Neisseria meningitidis*. Development of protective antibodies in the absence of meningococcal disease is associated with carriage of non-serogroupable strains of the organism which are also usually non-pathogenic.[1,2] Children not yet colonised by these strains are at greater than usual risk of meningococcal disease. We have noted abnormally low levels of antibody bactericidal for *N meningitidis* serogroup A and two non-serogroupable strains among individuals with gonorrhoea,[3] and report here the bactericidal activity of serum from individuals with gonorrhoea against strains of *N meningitidis* serogroups A, B, and C, those most frequently associated with meningococcal disease, and against the two non-serogroupable strains (0452 and NG846).

Strains of *N meningitidis* were cultured on modified New York City medium at 37°C in a humidified atmosphere enriched with 10% CO_2. Titration of bactericidal antibody was done as described previously (see table).[4]

The results demonstrate very low or undetectable levels of antibodies bactericidal for the strains of meningococci serogroups A, B, or C in sera from patients with gonorrhoea. This contrasts with the titres against a group A strain found in serum of uninfected "normal" individuals from the same geographical area (only 5/16 sera having titres below 4)[3] and with titres against serogroups A, B, and C reported by Craven et al.[5]

Absence of bactericidal antibody against serogroupable meningococci is a common feature of individuals who contract meningococcal disease and, as Artenstein and Ellis[6] suggested, "since meningococcal disease is a rare event compared to the carrier state, it may well be that deficiencies in host defences may play a major role in determining whether systemic invasion occurs".

TITRE OF BACTERICIDAL ANTIBODY AGAINST N MENINGITIDIS STRAINS OF SEROGROUP A, B, AND C, AND TWO NON-GROUPABLE STRAINS 0452 AND NG846 IN SERA FROM PATIENTS WITH GONORRHOEA

Patient	A	B	C	0452	NG846
1	<4	8–16	4–8	32–64	16–32
2	<4	<4	8–16	64–128	<4
3	<4	8–16	8–16	64–128	8–16
4	<4	<4	<4	16–32	<4
5	8–16	8–16	16–32	64–128	32–64
6	<4	<4	8–16	⩾128	64–128
7	<4	<4	<4	⩾128	4–8
8	<4	<4	<4	64–128	16–32
9	<4	<4	<4	16–32	<4
10	<4	<4	<4	64–128	4–8
11	<4	<4	4–8	⩾128	<4
12	<4	<4	4–8	⩾128	128
13	32–64	<4	4	⩾128	⩾128
14	<4	<4	<4	⩾128	8–16
15	⩽4	<4	8–16	64–128	64–129
16	4	4	4–8	⩾128	32–64
17	⩾128	<4	4–8	⩾128	⩾128
18	4–8	<4	4–8	⩾128	⩾128

Meningococci and gonococci possess similar lipopolysaccharide structures,[7] and cross-reactions of monoclonal antibodies have been shown between lipopolysaccharide antigens of meningococci and gonococci.[8] Bactericidal antibodies against both these species appear to be directed against lipopolysaccharide antigens. The low titre of bactericidal antibody towards groups A, B, and C meningococci in sera from patients with gonorrhoea could be due to absorption of cross-reactive antibodies by the infecting strain of *N gonorrhoeae*.

We propose that gonorrhoea may compromise host defences against meningococcal disease and that this may explain in part the following observations:

(*a*) The age range most at risk of meningococcal disease is 0–4 years (ie, children lacking bactericidal antibodies[1]) but the group next most at risk is 15–19 years, the age when sexual activity begins and when the incidence of gonorrhoea is high.[9,10]

(*b*) During outbreaks of meningococcal disease in military establishments most patients are aged 18–24 years,[11,12] the range in which most cases of gonorrhoea in servicemen are found.

(*c*) The incidence of meningococcal colonisation of the pharynx is higher in patients with genital gonorrhoea (26%) than in those without (11%). The relative risk of harbouring meningococci when infected with gonococci is 2·44 (95% confidence limits, 1·4–4·2).[13,14]

A recent history of gonoccal infection or exposure to infection may be one of the contributing factors to the increased susceptibility of young adults to meningococcal disease.

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SHORT COMMUNICATION

Alteration of Pyocin-sensitivity Pattern of *Neisseria gonorrhoeae* is Associated with Induced Resistance to Killing by Human Serum

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A laboratory-grown strain of *Neisseria gonorrhoeae*, selected *in vivo*, BS4 (agar), is susceptible to complement-mediated killing by fresh human serum but is relatively resistant to killing by human phagocytes. It can be induced to serum resistance by incubation with a small molecular weight fraction of guinea pig serum. The serum-susceptible and induced-resistant forms show differences in pyocin sensitivity tests. This indicates either differences in the structure of their lipopolysaccharides or masking of some determinant(s). The pyocin sensitivity pattern of BS4 (agar) is only slightly different from that of a closely related strain, BSSH, which is more susceptible to killing by human phagocytes.

INTRODUCTION

A strain of *Neisseria gonorrhoeae* selected from a small colony forming, pilate laboratory strain (BS) by three or four passages through plastic chambers implanted subcutaneously in guinea pigs was resistant to killing by human serum and human phagocytes when examined without subculture (Penn *et al.*, 1976, 1977). When cultured in laboratory media, the *in vivo*-selected strain, BS4 (agar), lost its serum resistance but retained its resistance to killing by phagocytes (Penn *et al.*, 1976, 1977). The serum resistance could be restored phenotypically to BS4 (agar) by incubating it for 3 h at 37 °C in a defined medium (DM) containing 0·1% (w/v) bovine serum albumin (BSA) and small molecular weight fractions of either guinea pig or some human sera (Veale *et al.*, 1981; Martin *et al.*, 1981).

The determinant(s) of the induced resistance of BS4 (agar) to killing by human serum has not yet been investigated. The limited supply of host-derived inducer has prevented the production of sufficient organisms for investigation of outer membrane proteins (OMP) and lipopolysaccharides (LPS) comparable to those studies on the culture-stable type of serum resistance shown by strains from disseminated gonorrhoea (Brooks *et al.*, 1978; Lambden *et al.*, 1979; James *et al.*, 1982). An investigation of the LPS of susceptible and induced-resistant forms of BS4 (agar) seems particularly relevant because LPS appears to be the target antigen for the 'natural' antibody responsible for the bactericidal activity of normal human serum (Brooks *et al.*, 1978; Schneider *et al.*, 1982). A method for indicating changes in LPS of *N. gonorrhoeae* became available when it was shown that partially purified, Kageyama R-type pyocins from *Pseudomonas aeruginosa* (Kageyama, 1975), the receptor sites for which reside on the LPS (Meadow & Wells, 1978), reacted with gonococci and could form the basis of a typing system (Morse *et al.*, 1976; Sidberry & Sadoff, 1977; Blackwell *et al.*, 1979). Only small numbers of gonococci are required and the pyocin method has been used to indicate LPS differences in mutational and epidemiological studies on gonococcal sensitivity to serum killing and attachment to polymorphonuclear leucocytes (Guymon *et al.*, 1982; Blackwell *et al.*, 1983;

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Winstanley *et al.*, 1983). This paper reports tests with a range of R-type pyocins on strain BS4 (agar) before and after induction to serum resistance by the low molecular weight fraction from guinea pig serum.

The determinants of the resistance of strain BS4 (agar) to killing by phagocytes have been investigated by comparing it with a closely related phagocyte-susceptible strain, BSSH, selected *in vitro* by colonial appearance from the original parent strain BS (Penn *et al.*, 1977). In this case, sufficient organisms could be obtained for biochemical examination. The putative determinant of resistance was not pili and resided in outer membrane vesicles (OMV) (Parsons *et al.*, 1981). Examination of the proteins of purified OMV from BS4 (agar) and BSSH on sodium dodecyl sulphate polyacrylamide gel electrophoresis associated three OMP with the resistance of BS4 (agar) to killing by phagocytes (Parsons *et al.*, 1982). However, it was thought interesting to include in the pyocin tests a comparison of the phagocyte-resistant [BS4 (agar)] and phagocyte-susceptible (BSSH) strains.

METHODS

Neisseria gonorrhoeae. Strains BS4 (agar) and BSSH were derived, stored, cultured and counted as described previously (Penn *et al.*, 1976, 1977; Parsons *et al.*, 1981, 1982).

Induction of BS4 (agar) to serum resistance. The method used was described by Veale *et al.* (1981): 10^4–10^5 gonococci were incubated for 3 h at 37 °C in equal volumes of DM containing 0·1% (w/v) BSA and an ultrafiltrate of guinea pig serum containing molecules of less than 5000 daltons (Veale *et al.*, 1980; Martin *et al.*, 1981). The serum-susceptible BS4 (agar) used for comparison with the resistant organisms in the pyocin tests (Table 1) had been incubated in DM containing 0·1% BSA for 3 h at 37 °C without the inducer.

Pyocins, Representatives of four of the five groups (R1–R5) of R-type pyocins described by Kageyama (1975) were prepared from the following strains of *P. aeruginosa* and partially purified and stored as described by Blackwell *et al.* (1979). These included: R1 pyocins from strains ISD, 2285, IS6 and IS8; R2 pyocin from strain ISB; R3 pyocin from strain ISE; R5 pyocins from strains ISA and ISC; and two unclassified pyocins from strains R205 and 9579.

Tests for pyocin sensitivity. A suspension (15 µl) of partially purified pyocin was added to a suspension of gonococci (100 µl; 10^4–10^5 c.f.u. ml^{-1} in DM (Veale *et al.*, 1981) in a sterile plastic test tube (10 ml capacity). Control tubes received DM (15 µl) but no pyocin. After 30 min incubation at 37 °C, DM (0·5 ml) was added to each tube. The gonococci were deposited by centrifugation (1500 *g*, 15 min, 37 °C) and the supernatant was removed. The gonococci were resuspended in 0·3 ml DM, which was plated out on haemoglobin agar. After incubation at 37 °C in candle extinction cans the colonies were counted. Results of the experimental samples were recorded as +, ± and − when the viable counts were <5%, <20% (but >5%), and >20%, respectively, of the colony counts for the control tubes. All gonococcal strains were tested against the 10 different pyocins in many tests (see Table 1).

RESULTS

The results are summarized in Table 1. During the 3 h induction period to resistance the serum-susceptible form of BS4 (agar) had lost its sensitivity to the pyocin produced by strain R205 and its partial sensitivity to that produced by strain IS8; also its full sensitivity to the R5 pyocin produced by strain ISC became partial. The serum- and phagocyte-susceptible strain BSSH (Penn *et al.*, 1976, 1977) showed a partial sensitivity to the R1 pyocin from strain IS6 in contrast to the insensitivity of the two forms of BS4 (agar), both of which were relatively resistant to killing by phagocytes (Penn *et al.*, 1976, 1977; N. J. Parsons & H. Smith, unpublished observations). In reactions with the pyocins from strains IS8 and ISC, BSSH behaved similarly to the serum-susceptible form of BS4 (agar). With the pyocin from strain R205, BSSH was less sensitive than the serum-susceptible form of BS4 (agar), whereas the serum-resistant form of BS4 (agar) was fully resistant to this pyocin.

DISCUSSION

The pyocin sensitivity tests suggest that a change in LPS occurs when the serum-susceptible gonococcal strain BS4 (agar) is converted to serum resistance by the low molecular weight inducer from guinea pig serum in the short time of 3 h at 37 °C. Overall, a loss of sensitivity to three pyocins accompanied the increase in serum resistance and this indicates that the LPS

Table 1. *Activity of 10 different pyocins on strain BS4 (agar) before and after induction to serum resistance and on strain BSSH*

Each result was obtained on at least four different tests with each pyocin and at least eight different tests with pyocins from strains IS6, IS8, R205, 9579 and ISC. The differences between the serum-susceptible and serum-resistant forms of BS4 (agar) are bracketed.

Strain of gonococci	Serum resistance	Resistance to phagocyte killing	Pyocin:	Inhibition* by pyocins from the Kageyama group									
				R1				Unclassified		R3	R2	R5	
			Strain:	ISD	2285	IS6	IS8	R205	9579	ISE	ISB	ISA	ISC
BS4 (agar)	−	+		−	−	−	[±]	[+]	+	−	−	+	[+]
BS4 (agar)	+	+		−	−	−	[−]	[−]	+	−	−	+	[±]
BSSH	−	−		−	−	±	±	±	+	−	−	+	+

* Results were recorded as +, ± and − when the viable counts were <5%, <20% (but >5%), and >20%, respectively, of the counts of the control tubes not containing pyocin.

structure had been altered, since three receptor sites appear to have been removed or modified or masked by a new component. This result complements recent correlations made between the susceptibility of clinical isolates to serum killing and the presence of LPS receptors for the R1 pyocins (Winstanley *et al.*, 1983). Any speculation on the precise changes in LPS structure which accompanied the increase in serum resistance depends on the assumption that observations on pyocin reactions with the LPS of *P. aeruginosa* apply to the LPS of *N. gonorrhoeae*. Mutants of gonococci selected for resistance to R1 pyocins remain sensitive to R5 pyocins, indicating that the receptor for the R5 pyocins is in the portion of the LPS nearest to the cytoplasm and those for R1 pyocins are more distal (Winstanley *et al.*, 1983). These are similar to the results obtained for *P. aeruginosa* (Kageyama, 1975). Little can be drawn from the most marked change in pyocin sensitivity accompanying the increase in serum resistance, namely the complete loss of sensitivity to the unclassified pyocin from strain R205, since its receptor site is unknown even for the LPS of *P. aeruginosa*. It should be noted that in the majority of clinical isolates of gonococci, if the strain is sensitive to R1 pyocins it is also sensitive to the pyocin produced by strain R205 (Blackwell *et al.*, 1979). The smaller losses in sensitivity to the R1 pyocin from strain IS8 and the R5 pyocin from strain ISC suggest that receptor sites both in the polysaccharide side chain and in or near the core of the LPS may have been affected. The receptor sites may not have been produced, or they may have been modified, or even obscured by new structures such as proteins under the influence of the small molecular weight inducing factor whose mechanism of action is unknown. However, one possible masking agency, the formation of a capsule under the influence of the inducer, does not occur; in electron microscopy similar to that which demonstrated capsules on some gonococci (Demarco de Hormaeche *et al.*, 1978), capsules could not be seen on BS4 (agar) organisms either before or after being induced to resistance (P. M. V. Martin, unpublished observations).

It is tempting to suggest that changes in pyocin sensitivity which occur as the result of the action of the small molecular weight inducer indicate changes in LPS which are responsible for the increased resistance to serum killing. However, the evidence at present is only that of association (Smith, 1983) and can only be strengthened by deeper investigations on the connections with serum resistance of the LPS and any other bacterial components (for example, OMP) that may be formed under the influence of the inducer.

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SHORT ARTICLE

ABSENCE OF BACTERICIDAL ANTIBODIES AGAINST GROUP-I LIPOPOLYSACCHARIDE DETERMINANTS OF *NEISSERIA GONORRHOEAE* DURING HUMAN INFECTION

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SUMMARY. None of 34 sera from patients with gonorrhoea contained antibodies bactericidal for strains of *Neisseria gonorrhoeae* with Group-I lipopolysaccharide (LPS). All contained antibodies against a strain with Group-II LPS, as do sera from uninfected controls. The absence of Group I-LPS antibodies in infected humans contrasts with previous findings that mice immunised with strains from either of the LPS groups produced bactericidal antibody to Group I. Our hope that detection of antibodies to Group-I strains would provide a screening test for gonorrhoea was, therefore, not realised.

INTRODUCTION

Gonococcal isolates from uncomplicated genital infections and disseminated infections have been divided into two broad groups based upon their pyocin-sensitivity spectrum. This classification has been shown to correlate with interactions of strains from localised genital infections with both humoral and phagocytic components of the human immune system. Briefly, gonococcal strains sensitive to pyocins of Kageyama's group R5, lipopolysaccharide (LPS) Group I, are resistant to normal human serum and they bind in lower numbers to polymorphonuclear leukocytes than strains of Group II (Blackwell *et al.*, 1983). This second group is sensitive to pyocins of Kageyama's group R1 in addition to R5 and strains of this group are killed by the majority of normal human sera tested (Kageyama, 1975; Blackwell, Young and Anderson, 1979; Winstanley *et al.*, 1983). Strains of Group II are thought to have extra LPS components, absent or cryptic in strains of Group I, to which there are bactericidal antibodies in the majority of normal human sera (Schneider *et al.*, 1982; Winstanley *et al.*, 1983).

Earlier studies revealed that specific antisera raised in mice against strains of Group I or II were bactericidal for other strains of the homologous group. They were also active against strains of the heterologous group (Winstanley *et al.*, 1983). In that study we also screened sera from a control population of women attending a postnatal clinic for bactericidal antibodies to both groups of gonococci. Antibodies against Group II only were found. This is a population in which there is a very low incidence of gonorrhoea. We have now screened sera from individuals with gonorrhoea for bactericidal activity against strains of LPS Groups I and II. Our hypothesis was that individuals exposed to or infected with *N. gonorrhoeae* would, like the mouse model, have antibodies to both groups and that detection of bactericidal activity for Group I might be used as a serological screening test for gonococcal infection.

MATERIALS AND METHODS

Bacteria. Strains of LPS Group I (E757 and M9131) and Group II (P280) were isolated from patients attending the Department of Genitourinary Medicine, Royal Infirmary, Edinburgh. All were from localised genital infections (Winstanley *et al.*, 1983). They were

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cultivated on Modified New York City Medium (MNYC) (Young, 1978) at 37°C in a humidified atmosphere enriched with 10% CO_2.

Assay for bactericidal antibodies. Bactericidal tests were performed as described by Winstanley *et al.* (1983) and as outlined below.

Sera taken for syphilis serology from patients attending the Department of Genitourinary Medicine were obtained from Dr H. Young, STD Diagnostic Laboratory, Department of Bacteriology, University of Edinburgh. Human AB serum from a donor with no history of gonococcal infection was used as the source of complement. It was absorbed for 24 h at 4°C with a live suspension of the gonococcal strains used in the study, in order to remove cross-reacting antibodies, and was distributed in 200-μl portions and stored at −20°C before use. The haemolytic titre of this complement source was 32–64. An 18-h culture of each gonococcal strain was diluted in Dulbecco's phosphate buffered saline, supplemented with Mg^{++} and Ca^{++} ions (0·5mmol and 0·9mmol respectively), to give *c.* 10^4 cfu/ml. Twofold dilutions (50μl) of the sera to be tested were made in microtitre plates and to each was added 40μl of the gonococcal suspension and 10μl of the human complement source. After incubation at 37°C for 30 min, the numbers of viable organisms in each well were determined by culturing three 20-μl drops from each on MNYC. The bactericidal titre of a serum was the highest dilution that caused a reduction in viable count of 80%, compared with that of a control to which no complement was added.

Results

The titres of bactericidal antibody in sera from 22 patients with gonorrhoea against strains E757 (Group I) and P280 (Group II), are shown in the table. Similar results were obtained with a second set of 37 sera from the STD Diagnostic Laboratory; the patients' diagnoses were not known at the time the sera were tested. They were screened for bactericidal activity against strain P280 and Group-I strain M9131. Amongst these sera, 12 were from patients with gonococcal infection and 25 from patients from whom no neisseriae were isolated. There were no differences in titres of bactericidal antibodies to strain M9131 between sera from the infected

TABLE. *Titres of bactericidal antibody against gonococcal strains E757 (Group I) and P280 (Group II) in sera from patients with gonorrhoea.*

	Titre of antibody to strain	
Patient No.	E757	P280
1	<4	>128
2	<4	>128
3	<4	64–128
4	4	64
5	<4	64
6	<4	32–64
7	<4	64–128
8	<4	32–64
9	<4	32–64
10	<4	64–128
11	4–8	64–128
12	<4	32–64
13	<4	>128
14	<4	>128
15	<4	64–128
16	<4	16
17	<4	64
18	<4	>128
19	<4	128
20	<4	>128
21	4	>128
22	<4	>128

and uninfected patients; titres were $\leqslant 4$. The titres to strain P280 were similar to those recorded in the table.

Discussion

The absence or extremely low titres of bactericidal antibodies against strains M9131 and E757 in sera from patients with gonorrhoea refutes our hypothesis, based on results obtained with immune mouse sera (Winstanley *et al.*, 1983), that infection with *N. gonorrhoeae* in man would result in the production of antibodies bactericidal for strains of Group I. This failure may result from the stimulation by Group-I antigens of antibodies of classes that do not activate the classical complement pathway, e.g., classes IgA and IgG4, or by failure of the human immune system to respond to antigens of the gonococcal LPS associated with the binding site for R5 pyocins.

It has been suggested recently that susceptibility and resistance to gonorrhoea of a group of Chinese prostitutes was associated with differences in the major histocompatibility complex genes. The HLA "haplotype" A11, B15 appeared to be associated with resistance to the disease amongst these women who were at very high risk of exposure to infection (Chan and Rajan, 1982). Similarly, the ability to respond immunologically to LPS antigens associated with the binding site of R5 pyocins might depend upon the genetic constitution of the individual.

Bactericidal antibody is an important host defence that prevents the dissemination of gonococci from the mucosa (Brooks, Ingwer and Peterson, 1978). The majority (*c.* 85%) of gonococcal strains isolated from localised genital infection are of Group II (Winstanley *et al.*, 1983) and are thought to be prevented from dissemination by "natural" bactericidal antibodies and complement. Strains of Group I, however, being resistant to normal human serum, have a greater predisposition to cause bacteraemia; they are isolated from approximately 42% of disseminated infections (Winstanley *et al.*, 1983).

The apparent inability of man to produce antibodies bactericidal for gonococcal strains of Group I may be a host factor that retards recovery from disseminated gonococcal infection and it may also pose difficulties in the immunoprophylaxis of gonorrhoea. These results indicate that, like other attempts to identify antibodies against gonococcal LPS (Ward and Glynn, 1971 and 1972; Watt, Ward and Glynn, 1971; Maeland and Matre, 1975), screening of sera for bactericidal activity against LPS-group-I strains will not be of use as a diagnostic test.

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