AN INVESTIGATION OF SOME FACTORS INFLUENCING THE PATHOGENICITY OF *NEISSERIA MENINGITIDIS*

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C E R T I F I C A T I O N

This is to certify that the Thesis titled

"AN INVESTIGATION OF SOME FACTORS INFLUENCING THE PATHOGENICITY OF NEISSERIA MENINGITIDIS"

submitted to the School of Postgraduate Studies, University of Lagos for the award of the degree of Ph.D. in Medical Microbiology is a record of original research carried out by Mr. Taiwo O. Banjo in the Department of Medical Microbiology and Parasitology.

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Ph.D) in the department of Medical Microbiology and Parasitology, College of Medicine of the University of Lagos, Lagos, Nigeria.

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January, 1990

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DEDICATION

To the memory of my father

Okunola Olurotimi Banjo.

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ACKNOWLEDGEMENT

I wish to express my sincere gratitude to Professor Tolu Odugbemi for his able supervision, guidance, advice and support. I must also thank him for his patience and understanding. I am also grateful for the use of his personal research materials which I used for this work and the use of his personal library.

I am indeed indebted to Professor V.O. Rotimi, Head of Department of Medical Microbiology and Parasitology who first introduced me to the intriguing subject of Medical Microbiology some years ago. I am very grateful to him for sharing a part of his wealth of knowledge and experience with me and providing me with much inspiration. I must thank him for his unending support and continuous concern for my progress and access to his personal library and materials.

I must register my appreciation to Dr. A.O. Coker, Co-ordinator of Post-graduate studies and Prof. C.E. Anyiwo for their interest and support.

I also thank Dr. S.F. Lawal, Mrs. Macaulay and Mr. M.C. Obi for all their help and encouragement and the entire staff and students of the Department of Medical Microbiology for their various forms of assistance during these studies. I am also grateful to members of Professor Tolu Odugbemi's research laboratory : Mrs. Mowumi Ademidun, Mrs. Tolu Animashaun and Mrs. Anita Agbabiaka and Joe Ikea whom I enjoyed working with.

My thanks also go to Oby, School of Pharmacy for the dialysis tubes and Alhaji Isa, Unilag and Gbenga (LUTH) for taking the photographs in this thesis.

I am grateful to my dear friends and colleagues, Duro Akindutire, James Epoke, Promise Adewusi, Fred Esumeh, Dr. C. Obi, Dr. A. Alabi, Lampe, Niyi, Dele and Cyprian for their continuous support and encouragement throughout the period of this work. I must also thank Sola Solaru, Dr. S. Hassan and Dr. A. Adeyemo for their concern and encouragement.

My special thanks go to Dr. 'Kunbi Makanjuola for her love understanding and kindness at all times. I am extremely grateful to Uncle Bunmi for his fatherly advice and attention and to my Aunt Kofo, Tosin Funlola and Mowumi for their immense kindness and love, especially when spirits were low.

As always I am ever so grateful to my dear mother, Mrs. W.E. Banjo for her continuous support, encouragement and love and to my brother, Alaba and my sisters, Kemi and Remi for their love and kindness.

My thanks to Mr. Cyprian Tasie and Mrs. Kasali who worked tirelessly to type this thesis.

Finally, I am deeply grateful to the Almighty Father who made all this possible.

Taiwo Banjo

ABSTRACT

The nasopharyngeal carriage rate of Neisseria meningitidis among 639 school children i.. Ijede, Ikorodu, Lagos State was 6.2% (40/639). The proportion of male carriers 62.5% (25/40) was significantly higher than females (37.5%; 15/40 ($P < 0.05$). N. meningitidis groups C and A were the most frequently isolated serogroups accounting for 40% (16/40) and 22.5% (9/40) of the isolates respectively.

In-vitro assessment of the attachment of N. meningitidis to human nasopharyngeal epithelial cells (NEC) showed that meningococci of all serogroups attached in significantly higher numbers to NEC of infants (160/NEC) and children (164/NEC) than NEC from neonates, (115/NEC) adults (124/NEC) and elderly (109/NEC). Attachment of meningococci to NEC of males and females was not significantly different. N. meningitidis group C clinical strain attached significantly better to non-carrier NEC than to the NEC of Group C meningococcal carrier.

The cell free supernates of some strains of Escherichia coli, Staphylococcus aureus and viridans streptococci isolated from the upper respiratory tracts of meningococcal carriers and non-carriers were found to produce substances inhibitory to serogroups of meningococci in vitro.

Purified meningococcal endotoxin, and heat-killed meningococcal inocula from both clinical and carrier isolates were lethal for 12 day old chick

embryo on chorioallantoic vein and yolk sac inoculation. Experimental laboratory animals including day old chicks suckling mice and infant rats did not develop meningitis subsequent to inoculation with meningococci through various routes.

The collective results of these studies suggest that N. meningitidis possesses numerous pathogenicity factors which may act in synchrony depending on various host factors to enable this organism to overcome host defence and cause life threatening infections.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

General Characteristics and Historical perspective:

Neisseria meningitidis, commonly referred to as the meningococcus, is a gram negative spherical (coccus) shaped bacterium, usually found occurring in pairs (diplococcus) with the adjacent sides of each coccus flattened to give a characteristic bean-shaped appearance. The meningococcus possesses catalase and cytochrome oxidase activity, it is non-spore forming and non-motile. Some strains may possess a polysaccharide capsule. *Neisseria meningitidis* is the aetiological agent of epidemic cerebrospinal meningitis and a fulminating septicaemia (meningococcaemia), two important diseases which continue to be a health matter of great concern world wide.

The meningococcus and its diseases have been of profound interest to both scientists and physicians for many centuries. Although meningococcal diseases are believed to be ancient, the early history remains unclear because of the difficulty of differentiating them from other disease syndromes of a similar nature. This confusion is exemplified by some of the names used to describe the disease in the early 19th century such as sinking typhus, spotted fever and brain fever. Renown physicians of ancient times such as Abu-Bakr Ibn Zachariyya (c865-932) and the Renaissance doctors, Heromyrus (1530-1606) and David Sinnert (1572-1637)

although they did not know much concerning its aeitiology and course. Thomas Willis (1621-1675) gave the first detailed description of epidemic meningitis in his publication 'A description of an epidemical fever in London, 1661'. Many years later in 1805, Gaspard Vieusseux (1746-1814), in Geneva, Switzerland, wrote a report on epidemic meningitis and the course of the disease. He noted that the disease occurred chiefly in the poor districts, mostly among children and young adults and that it is often rapidly fatal.

Subsequently, Nathan Sling (1781-1837) and Elisha North (1771-1843) wrote a treatise on a malignant epidemic, commonly called spotted fever at that time, characterised by headache, vomiting, coma and delirium and appearance of spots, mainly on the neck and extremities, the latter being considered as an unfavourable prognostic sign.

The causative agent of epidemic meningitis was first described by Marchiafava and Celli in 1884 who found it in meningeal exudates. However, it was not until 1887 that the renown pathologist, Anton Weichselbaum (1945-1920) working in Vienna, Austria, was able to isolate the organism and named it Diplococcus intracellularis meningitidis. Councilman in 1898 found this organism, now known as Neisseria meningitidis, in 31 of 34 cases of meningitis and thus firmly established its aetiologic role in epidemic cerebrospinal meningitis

Kiefer in 1896 and Albrecht and Ghon, in 1901, found that healthy persons could become carriers of the meningococcus, while in 1909, Dopter first recognised different serogroups of Neisseria meningitidis.

In 1913, Flexner introduced serum therapy for the treatment of meningococcal infection.

Since 1880, the northern Savannah region of tropical Africa has been regularly afflicted by epidemics of meningococcal disease. Horn in 1908 described a severe outbreak that affected northern Ghana in 1906-1908. In 1948-1950 a severe epidemic affected northern Nigeria on a massive scale; 92,964 cases were reported with 14,273 deaths (Horn, 1951).

Meningococcal disease is still a major health problem in many parts of the world. According to an estimate by Feldman (1972) during 1939-1962, there were almost 600,000 cases in the world and more than 100,000 of them were fatal.

In 1981 there were 3,525 cases reported to the Centers for Disease Control (CDC) Atlanta, Georgia in the United States of America, compared with 1,478 cases reported in 1978. Infants and children accounted for over 50% of the 1981 patients (CDC, Morbidity and Mortality weekly report, 1982). Peltola, Kataja and Makela (1982) have pointed out that shifts in this age distribution of meningococcal disease in a population, can be used to forecast an epidemic situation, and that

relatively more cases arise in the 5-19 year old age group during epidemic than non-epidemic circumstances.

The nature of infection, prevalence of the disease and socio-economic conditions of affected populations appear to be important in determining the case fatality rate. In industrialised nations the case of fatality rate may be as low as 7%-19% (Andersen, 1978) while in some developing countries the fatality rate may reach 70% (De Morais et al., 1974).

Areas of the world which have experienced recent epidemic meningococcal disease include Finland, Mongolia, Alaska, sub-Saharan Africa, Canada and Brazil (Apicella, 1986).

Morphology and cultural characteristics of the meningococcus

Neisseria meningitidis is a gram negative diplococus approximately 0.6 - 0.8 microns in diameter. When in body fluids the cocci are often arranged in pairs with the adjacent sides flattened giving rise to a characteristic kidney or bean shape. The meningococci exhibit considerable variation in size and staining properties. They frequently possess metachromatic granules which may be seen under Loeffler's methylene blue or Neisser's stain. They tend to undergo autolysis readily, resulting in considerable size and shape variation in older cultures. The organisms also produce a polysaccharide capsule which is the basis of the serogroup classification system. They do not produce spores and are non-flagellated.

Meningococci are fastidious in their growth requirements and are sensitive to toxic substances which may be present in some growth media. Meningococcal colonies/about are 1-5mm in diameter. When isolated on transparent media the so-called lens effect may be observed; that is, when distant objects are viewed through the colony they become sharply focused. The colonies are usually convex and if large amounts of polysaccharide are present, will appear mucoid rather than smooth. Rake (1933) described both rough and smooth colony types and found that those colonies of strains more recently isolated were smoother than old stock cultures. He further associated the change from smooth colony types to rough with a partial loss of immunological specificity.

Due to their highly fastidious nature meningococci require accessory growth factors such as are present in blood, serum and certain vegetable extracts. These organisms are extremely sensitive to the toxic effects exerted by a variety of amino-acids, free fatty-acids and salts which may be present in various peptones or in agar. The toxic effects of free fatty-acids can be eliminated by adding a binding agent such as soluble starch or charcoal to the medium. The meningococci are also inhibited by low concentrations of gonadal steroids (Morse, 1978).

Blood agar base, trypticase soy agar, supplemented chocolate agar, Mueller Hinton agar and certain

selective media into which antibiotics have been incorporated (e.g. Thayer Martin's Medium and New York City medium) are usually adequate to support the growth of meningococci. The antimicrobial agents commonly incorporated into the media include nystatin, trimethroprim, vancomycin and colistin.

Optimal growth conditions are achieved in a moist environment at 35-37°C and an atmosphere of 5-10% carbon dioxide in air. Cheever (1965) observed that growth of meningococci in tryptose phosphate veal infusion broth in which starch casein hydrolysate is added is relatively poor, resulting in granular turbidity with little or no surface growth. The ability of meningococci to degrade carbohydrate is markedly restricted. N. meningitidis breaks down glucose and maltose to produce acid without gas production while it fails to degrade other sugars including sucrose, lactose and fructose. Variants of meningococci which degrade only glucose have been reported (Morse, 1978).

Physiology and Metabolism

The meningococci are aerobic and contain high levels of cytochrome-C-oxidase activity and carbohydrates are utilized by oxidative pathways resulting in the accummulation of acetic acid and under some conditions, lactic acid and acetylmethyl carbinol are produced (Morse, 1978). Indole and hydrogen sulphide are not formed. Morse (1978), using radiorespirometrical techniques defined the relative participation of glucose

catabolic pathways in meningococci and found 85% participation in the Entner-Douderoff pathway at pH 7.4 and temperature of 37°C. The Entner-Douderoff pathway is apparently the major route of glucose catabolism in Neisseria meningitidis. The meningococcus has a functional tricarboxylic acid (TCA) cycle (Morse, 1978). However, utilisation of this pathway is markedly influenced by growth conditions.

Purine metabolism has also been extensively studied among the meningococci. Adenosine monophosphate (AMP) and guanine monophosphate (GMP) are synthesized by this organism de novo (Catlin, 1973) and from exogeneous adenine (Morse, 1978). However, GMP cannot be converted into AMP by meningococci.

Studies on pyrimidine metabolism in the meningococci show that they do not require exogeneous pyrimidine base for growth (Catlin, 1973) and that this may account for the low uptake and incorporation of these compounds by meningococci. Pyrimidine nucleotides in N. meningitidis may be synthesised de novo or from exogenous uracil. The meningococcus lacks enzymes for uridine incorporation (Morse, 1978). These workers also reported that 5-fluoro-uracil inhibited meningococcal growth, a condition which could be reversed by addition of uracil but not uridine, thus suggesting the absence of phosphorylase and kinase.

Walstad et al. (1974) described the release of substances which inhibited many strains of Neisseria

gonorrhoeae (gonococcus) and Neisseria meningitidis during the growth of the gonococcus. These inhibitors were identified as free fatty acids and a minor phospholipid component known as lysophosphatidyl-ethanolamine. The mechanism by which these inhibitory free fatty-acids are produced was not determined but it is believed to be partly due to the presence of phospholipases which degraded phosphatidyl-ethanolamine to lysophosphatidyl-ethanolamine and glycerophosphatidyl-ethanolamine with the release of one or both fatty-acids.

Few organic ions are required for growth and maintenance of cells. Divalent cations, Mg^{2+} and Ca^{2+} markedly inhibit autolysis, presumably by stabilization of the cell membrane. Meningococci typically undergo autolysis in older cultures. The presence of divalent cations Mg^{2+} and Ca^{2+}, will prevent cellular lysis but will not inhibit peptidoglycan hydrolysis (Morse, 1978). Two enzymes have been described for Neisseria spp that are known to assimilate CO_2 to H^+ and HCO_3 (Cox and Baugh, 1977). Phosphenolypyruvate carboxylase, a cytoplasmic enzyme, has been reported for pathogenic Neisseria spp (Cox and Baugh, 1977; Morse, 1978).

The role of carbonic anehydrase in the metabolism of Neisseria meningitidis is still unknown. The inhibition of this enzyme by acetazolamide prevents growth of the organism, indicating that a vital cellular function has been interrupted(Morse, 1978). The usefulness of this membrane bound cytoplasmic

enzyme may lie in its involvement in the regulation of ion movement across the cytoplasmic membrane or its ability to concentrate either proteins or bicarbonate ions at the cell membrane.

De Voe (1982) reported that several organic (cysteine, glutamine, lanthione) and inorganic forms of sulphur (SO_3^{2-}, SO_4^{2-}, HSO_2^{2-}) can serve as sole sources of sulphur for the growth of meningococci in a defined medium. He also found thiosulphate reductase activity in cell free soluble extracts of meningococci. This activity, although not sensitive to oxygen, is present during the enzymatic reduction of thiosulphate. Meningococci are able to use various forms of iron as their sole source of iron, and utilization of this element is an important metabolic aspect of this organism. Free iron in the normal host is maintained at extremely low levels of about 10^{-18}M (Buddingh and Polk, 1939) which is far below 8×10^{-8}M which has been established as the minimum to sustain cell division in meningococci (Archibald and DeVoe, 1980). According to Archibald and De Voe, (1980) meningococci have a special mechanism for the acquisition of iron from a glycoprotein known as transferrin. This iron acquisition system is heat-sensitive being inactivated by heating at 60°C for 5 minutes, is sensitive to trypsin and to respiratory chain inhibitors.

Meningococci possess an active autolytic enzyme system which results in swelling, loss of staining

properties and the ultimate disappearance of the cells themselve in old cultures. Heating cultures at 56°C for 30 minutes result in inactivation of the autolytic enzymes. This inactivation may also be achieved by the addition of potassium cyanide or formalin to cultures (Morse, 1978).

It is conceivable that many aspects of the physiology and metabolism of meningococci may be related to its pathogenicity and as such it may be rewarding as a basis from which to direct future research.

Antigenic Classification of the Meningococci:

The history of the development of antigenic classification of the meningococci has been extensively reviewed by Cheever (1965): Soon after the meningococcus was identified as the aetiologic agent of epidemic meningitis and after the recognitio of healthy nasopharyngeal carriers, many workers carried out investigations into the applications of immunologic methods for the detection and differentiation of meningococci. This led to the discovery that antigenically distinct meningococci existed by Dopter in 1909. Kutscher in 1906, using absorption tests observed striking differences between strains of meningococci isolated from different sources but was unable to classify them by this method. In 1909, Elser and Hunton found that about 40% of their meningococcal isolates were agglutinable by monovalent serum. They termed these strains which showed reduced absorption capacity, Pseudomeningococci. Dopter in the same year noted the presence of organisms in nasopharyngeal mucus, which resembled meningococci in morphology,

cultural characteristics and fermentation reactions, but differed by their inability to agglutinate the available meningococcal serum in use at that time. He named these organisms, parameningococci. Arkwright in 1909, reported that strains isolated from sporadic cases of meningococcal meningitis tended to differ serologically from those isolated from epidemics. Dopter and Pawn in 1914 divided parameningococci into 3 types; alpha, beta and gamma. A year later, Ellis examined strains from six epidemic foci and found that they fell, by agglutination, into two types, I and II, of which type II appeared to be identical to Dopter's parameningococcus. Gorden and Murray (1915), later identified four antigenically distinct types of meningococci which they designated by Roman numerals I, II, III and IV. Interestingly none of the members of these groups showed any antigenic relationship with Dopter's parameningococcus (cited by Cheever, 1965).

A decade later Branham (1932) demonstrated that groups I and III were identical while she also described an epidemic strain which she considered to be atypical group II (group II alpha). It seems evident that the continued involvement of different laboratories led to the evolvement of a complex system of nomenclature which was not resolved until the mid 1950's when serogrouping nomeclature became standardised and was based on capital letters, thus unifying all existing systems and

simplifying bacterial taxonomy.

Rake and Scherp (1935) isolated the soluble specific polysaccharide responsible for the specificity of Group A meningococcal strains and Clapp et al. (1935) were able to establish the relationship between the polysaccharide and capsule using the Quellung's reaction on group A strains. Branham and Carlin (1942) went further to demonstrate that the meningococcal polysaccaride antigens could elicit antibody response which was able to confer protection in mice.

At first the introduction of antibiotics for the treatment and prophylaxis of meningococcal infection caused diminished interest in the development of group specific antigens for use as vaccines. However, the subsequent emergence of sulphonamide resistant meningococci created a clinical problem which provided renewed impetus for research on the immunology of the meningococcus.

Watson, Marinetti and Scherp (1958) identified the specific soluble substance from group C meningococci and showed its sialic acid nature. However, it is the capsular polysaccharide which provides the basis for grouping the meningococci. These organisms may be separated by sero-agglutination into at least 13 serogroups A, B, C, D, (Branham 1953; 1958); X,Y,Z, (Slaterus, 1961) 29E, W-135 (Evans et al. 1968) H, I, K, (Ding et al. 1981) and L (Ashton et al., 1983).

Gotschlich et al., (1969) and Robinson and Apicella

(1968) demonstrated the effectiveness of the group C vaccine in studies among United States of America army recruits achieving 87% reduction in the occurrence of the disease. Wyle (1972) reported that purified group A and C meningococcal capsular polysaccharide could induce bactericidal antibody when used for immunization.

A number of drawbacks are associated with meningococcal polysaccharide vaccines. For example :

(i) the polysaccharides are type -2 T-cell independent antigens and therefore do not give adequate immune response in the very young and do not provide long lasting immunity. However, Beuvery et al. (1983) circumvented these problems by linking the polysaccharide antigens to proteins

(ii) the group B capsular polysaccharide a homopolymer of alpha (2-8) sialic acid is poorly immunogenic (Wyle, 1972). The linear sugar epitopes of this polysaccharide do not seem to be immunogenic at all and the low-affinity antibodies when induced, appear to be directed against conformational epitopes (Jennings et al., 1985). Recently, (Jennings et al., 1987) showed that by replacing the N-acetyl by

N-propionyl in the group B polysaccharide it may be possible to induce group specific bactericidal antibodies that cannot be absorbed with unmodified polysaccharide. According to Poolman (1988) a possible explanation for this may be found in the observation that this epitope mimics the chemical bond between the polysaccharide and the lipids, sticking it into the outer membrane. Chemical definition of these newly discovered epitopes may eventually enable the induction of the alpha (2-8) sialic acid specific antibodies that might be potentially dangerous because of autoimmune effects.

(iii) antibodies directed against the meningococcal capsular polysaccharides may not be those with the greatest bactericidal potency, since for that, a close proximity to the outer membrane is required. Anticapsular immunity may depend more upon phagocytosis. Poolman (1988) suggests that in order to produce efficacious meningococcal vaccines protective antigens other than the capsular polysaccharides must be identified.

Recently Zollinger et al. (1979) and Poolman et al. (1986) have produced OMP vaccines. These vaccines comprise two OMPS (class 1 and 2/3 OMP) excepting the presence of some class-4 OMP and lipo-oligosaccharide (LOS) which cannot at the present time be eliminated. It is believed that the LOS may act as an adjuvant and the amount of LOS may leave a profound effect on the immune response. Either aluminium phosphate or aluminium hydroxide is usually included as adjuvant in the final vaccine preparation (Poolman et al. 1986).

Using murine monoclonal antibodies Poolman et al. (1987) have demonstrated that antibodies against class -1-OMP and LOS are bactericidal and that antibodies against the class -2/3 OMP are only weakly bactericidal especially when the test bacteria are grown on stationary phase or in conditions of glucose limitation. Saukonen et al. (1988) confirmed these findings by testing the protective capacity of these monoclonal antibodies in an infant-rat model.

However, the first generation OMP vaccines can only be used in situations in which most cases of a meningococcal disease are caused by a single strain or combination of strains sharing the same subtyping characteristics since the meningococci are quite heterogenous with respect to the class-1 and -2/3 OMPs (Poolman et al, 1986). A hybridoma bank is presently being built up to enable serotyping of most strains

(Poolman, 1988).

Immunity to meningococcal disease:

Goldschneider et al. (1969) and Reller, MacCregor and Beaty (1973) demonstrated that protective antibodies to meningococci may be acquired passively by transplacental passage of immunoglobulins or actively as the result of nasopharyngeal colonisation with both capsulated and non-capsulated meningococci. Furthermore, subclinical systemic infection may also provide a mechanism for active immunisation. The effect of passively acquired immunity is relatively short-lived but is effective in protecting new-born against meningococcal disease as evidenced by the low incidence of cases in the first six months of life (Smith, 1954). The prevalence of bactericidal antibody decreases after birth and reaches its nadir between the sixth and 24th months of life (Goldschneider et al,, 1969). Thereafter there is a linear increase in antibody until about 12 years of age.

Studies by Robbins and associates in 1972 indicate that serologic cross-reaction between the meningococcal group A polysaccharide occur with Bacillus pumilis and that the E. coli K1 antigen is immunologically and chemically identical to the group B capsular polysaccharide (Robbins et al., 1972).

These unrelated yet immunologically similar antigens may play a very important role in the development of natural immunity to the meningococcus and ultimately in protection against virulent meningococci (Apicella, 1986).

Griffis (1982) developed a hypothetical immuno-epidemiologic model for epidemic meningococcal disease and proposed that epidemic susceptibility is acquired by induction of meningococcal serum IgA by cross-reacting enteric bacteria. Simultaneous colonisation with the appropriate strain of meningococcus may result in disseminated disease.

Initial views that immunoglobulin deficiency may play a significant role in the occurrence and prognosis of meningococcal infection have not been substantiated; however, there seems to be no doubt that deficiencies of the late components of complement C5 - C9 predispose to frequent or recurrent attacks of meningococcaemia (Ross and Densen, 1984). It is believed that in the absence of antibody the main defence against bacteraemic invasion is lysis of the organisms by the complement pathway. Recent descriptions of individuals who lack any properdin illustrate this amply (Densen et al., 1987). Properdin stabilises the activated alternate pathway product C-3bBb, which in turn promotes further breakdown of C3 to

down and the alternative complement pathway cannot be sustained. These patients suffer very severe meningococcal disease with a high mortality. If they are immunised however, they can develop an antibody response and recruit the classical complement pathway thus diminishing their susceptibility to severe disease (Densen et al., 1987).

Treatment of meningococcal infections:

The introduction of antimicrobial therapy dramatically altered the prognosis of meningococcal disease for the better. Furthermore, the application of supportive care to treat complications of meningococcal infections such as shock, disseminated intravascular coagulation (DIC), heart-failure, pericarditis and prolonged mental obtrundation has also had an appreciable impact on prognosis.

Schwentker et al. (1937) demonstrated that sulphonamides could be successfully used to treat meningococcal meningitis and septicaemia. Feldman et al. (1942) later confirmed this observation, and as new antibiotics were introduced through the 1940's and 1950's, several reports on acceptable substitute for the treatment of this infection penicillin-allergic patients (Berkow, 1977). The duration of of antibiotic therapy may vary somewhat with the

presentation and manifestation of the disease and patient-response, but 10-14 days is usually sufficient (Apicella, 1986).

Vascular collapse and shock are common complications of meningococcal disease and effective treatment requires adequate preparation and close monitoring of the patient. Levin and Painter (1966) reported a high frequency of cardiac disease associated with meningococcal infection and they found the use of digitalis effective in raising arterial pressure, particularly when used in conjunction with isoproterenol.

The problem of disseminated intravascular coagulation is ominous and the use of heparin in the treatment of this complication of meningococcal disease is controversial (Corrigan and Jordan, 1970; Denmark and Knight, 1971). Administration of heparin should be based on a careful study of the clinical situation and laboratory data, particularly that relating to the clotting factors.

Other life threatening complications needing therapy include neurologic sequelae ranging from coma to diabetes insipidus and pericarditis (Apicella, 1986).

Chemoprophylaxis:

It was not long after the clinical use of

sulphonamides for the treatment of serious meningococcal disease, that it became apparent that short courses of sulfadiazine were also useful in the eradication of meningococcal carriage for long periods of time (Fairbrother, 1940). Feldman (1972) stated 'if there are no carriers, there are no cases' and it did appear that chemoprophylaxis of the meningococcal carrier commensurately decreased the number of cases.

The length of time for which the meningococcal carrier status was eradicated, was found to be a function of the initial dose of sulphonamide administered (Cheever , 1965) and after two doses of 3 and 2g of sulphadiazine, the carrier rate dropped from 79% to zero in 72 hours. On military bases and in closed environments such as boarding schools, institutions and family units in which cases arose, this form of chemoprophylaxis was effective in preventing the spread of meningococcal infection.

However, the recognition of widespread sulphonamide resistant-meningococci led to the abandonment of sulphadiazine for meningococcal prophylaxis (Brown and Condit, 1965) except in cases where the strains were known to be sulphur sensitive.

Since then, there has been an extensive search for new agents for chemoprophylaxis. Unfortunately penicillin has not proved to be useful - long acting

mixtures do not eradicate nasopharyngeal carriage and while massive doses cause individuals to become non-carriers, the carrier state promptly recurs after discontinuation of the drug (Feldman, 1972). Minocycline and rifampicin have been demonstrated to rapidly eradicate carrier status for up to 6-10 weeks after treatment (Guttler et al., 1971). However, problems are associated with both these antibiotics: Minocycline has been shown to cause vertigo (Jacobson and Daniel, 1975) while rifampicin treatment can result in the emergence of rifampicin-resistant-meningococci in up to 27% of patients. There are also reports documenting the efficacy of agents used alone or in combination (Blakebrough and Gillies 1980; Apicella, 1986).

Early studies of penicillin given in relatively low doses (120,000 units/day IM) indicated that it was not as effective as sulfonamides (Mead, Harris and Samper, 1944) except when used in higher doses of 360,000 units/day IM (Kinsman and D'Alonzo, 1946). McCrumb et al. (1951) demonstrated the efficacy of chloramphenicol as a therapeutic agent. The first generation cephalosporins were of doubtful efficacy when used in the 1960's since these agents produced variable results and indeed, their use is now contra-indicated in the treatment of meningococcal infection (Mangi et al., 1973). However, the third generation cephalosporins such as moxalactam, cefotaxime and cefaperazone demonstrate excellent in vitro activity

against meningococci and are believed to achieve adequate central nervous system concentrations of treatment of meningococcal meningitis (Apicella, 1986).

The emergence and spread of sulphonamide-resistant strains of meningococci (Gauld et al., 1965; Feldman, 1967), became a matter of great concern, and sulphonamides now have a limited role in the treatment of meningococcal infection. Fortunately, penicillin therapy for the treatment of meningococcal infection is still safe and effective and is usually administered intravenously or intramuscularly. The intrathecal route is contra-indicated because of the severe neurotoxicity of penicillin when in high concentrations in the central nervous system CNS (Apicella, 1986). Chloramphenicol has proved to be a useful alternative (Weidner, et al, 1971). According to Weidner, et al (1971) the major factor determining the efficacy of a meningococcal prophylactic agent is the capacity to achieve bactericidal levels in tears and saliva.

At the present time, rifampicin (600mg qid for 2 days for adults and 100mg/kg qid for 2 days for children) is the recommended therapy for meningococcal prophylaxis (Centers for Disease Control, 1976).

(1986) have demonstrated genotypic variability among meningococci, expressed in their ability to rapidly change their antigenic structure by quantitatively varying the class -5 OMP, pili and LPS. Putten *et al.* (1987) showed that meningococci adapt their outer-membrane composition to the growth rate and glucose limitation. Furthermore, meningococci can vary the rate of expression of class-1-OMP *in vivo* (Poolman *et al.*, 1986). This was reportedly done by examination of antibody responses and direct investigation on meningococci in CSF using immuno-electo-chemistry and monoclonal antibodies. This emphasizes the difficulty in discovering appropriate protective epitopes. The importance of field trials with outer-membrane component vaccines (i.e. poly-saccharides and lipo-oligo-saccharides and classes 1, 2, 3, 4 and 5 OMPs) is obvious. It is believed that the selection of the composition of the first of such vaccines to be tested will depend on the outer membrane characteristics of the major disease causing strains in the country where the vaccine trial will be carried out and also on the outer membrane components suspected to be protective antigens. Furthermore, LPS must be excluded or must be reduced to acceptable minimal levels because of their endotoxic activity.

Meningococcal vaccines and immunoprophylaxis of meningococcal infection:

The emergence of sulphonamide resistant meningococci led to intensified efforts towards the development of a vaccine for the prevention of meningococcal infections especially in high risk populations such as military recruits in stationary barrack situations (Apicella, 1986).

Goldschneider et al. (1969) demonstrated that individuals lacking bactericidal antibody against a circulating virulent strain had a risk of acquiring meningococcal meningitis. The protective bactericidal antibodies they detected in the base-line sera were directed against type specific antigens and at least two group C strains were circulating at that period only one of which was responsible for disease. The subsequent result was the development of two vaccine preparations derived from the polysaccharide of the group A and C meningococci. Artenstein et al. (1968) isolated the meningococcal polysaccharides from the supernatant of overnight cultures and from molecular sieve and ion-exchange chromatography. The chemical fractionisation method was employed by Rake and

Scherp (1935) for the separation of meningococcus into three fractions. These workers found a carbohydrate or 'C' substance common to all meningococci and a protein or 'P' substance similar to that found in gonococci and type III pneumococci, and a fraction comprising a sodium salt of polysaccharide acid. The chemical constituents of the capsular substances of meningococci have been extensively investigated, Neisseria meningitidis Group C polysaccharides can be biochemically divided into either neuraminidase-sensitive or resistant polysaccharides (Apicella, 1979). The former are designated C- and are the variant strains of group C. The role of O-acetylation in the antigenic specificity of groups B and C meningococci is still unclear, although recent evidence suggests this substitution is unimportant (Apicella, 1986). This assumption is based on the finding that both B and C variant strains do not possess O-acetylation groups and according to Bhattergee et al. (1975) while O-acetylation substitutions are restricted to carbon C-7 and C-8 atoms of the sialic acid residue, up to 24% of native C_1^+ polysaccharide is unacetylated.

The immunogenicity of groups A and C meningococci polysaccharide appears to be a function of molecular

size according to Brandt and Artenstein (1975) who showed a progressive decrease in the immune response to both groups A and C polysaccharides in persons immunized from batches of vaccines of decreasing molecular size.

Meningococci can be classified serologically into lipopolysaccharide (LPS) immunotypes and at least eleven LPS immunotypes of meningococci have been recognised (Apicella 1986). Such non-capsular cell wall antigens may be important for understanding the immunobiology of the meningococcus. There is no apparent correlation between the occurrence of LPS immunotype in meningococci except that immunotypes 10 and 11 have been observed only in serogroup A (De Voe, 1982).

Although the sugars released from LPS of various meningococci are identical, they can be separated into three groups on the basis of their galactose-glucose ratios. The cores of the groups A and C and 29E have galactose-glucose ratios of 1:2, 2:2 and 2:1 respectively. The majorfatty acids in the lipid-A portion of the LPS of serogroups A, B, X and Y are beta-hydroxylauric, beta-hydroxymyristic and lauric acids (Jennings et al., 1980).

Cultural conditions have been shown to influence the composition of meningococcal LPS (McDonald and Adams, 1971). Jennings et al. (1980) reported that LPS from representatives of all meningococcal serogroups lacked an 'O' antigenic polysaccharide chain as part

of the LPS despite their smooth colonial character and proven virulence. Jennings et al. (1980) further reported that the glycoses making up the meningococcal LPS were glucose, galactose, glycosamine, heptose and 2-keto-3 deoxyoctonate (KDO).

Interestingly, Davic, Ziegler and Arnold, (1978) found that antisera to endotoxin from Escherichia coli without O-antigen-side chain, was protective against localised and generalised Schwartzman reaction in rabbits treated with meningococcal LPS. This gives credence to the reports that Neisseria spp have similar core and lipid-A components to the enterobacteriaceae except that the former appear to lack the 'O' antigen side chain (McDonald and Adams, 1971; Stead et al., 1975 and Jennings et al., 1980).

Isolation and Laboratory identification of Neisseria meningitidis from clinical infections

The diagnosis of acute meningitis is a medical emergency and speed and accuracy are essential. The definitive diagnosis of serious meningococcal infections has as its pre-requisite the bacteriologic isolation of meningococci from normally sterile body fluid specimens of blood cerebrospinal fluid (CSF), synovial, pleural or pericardial fluids. Nasopharyngeal secretions, petechial aspirates and conjunctival swabs may also need to be examined for the presence of Neisseria meningitidis. In cases of suspected meningococcal

disease, specimens should be collected before treatment with antimicrobial agents because the latter reduce the rate of cultural isolation. Specimens as a rule should be rapidly transported to the laboratory with protection from drying and temperature extremes. The first sample of CSF collected is the best, and usually about 2-5ml is adequate, obtained by lumbar puncture procedure at the fourth and fifth lumbar interspace. For blood culture, three sets of blood specimens are preferably collected from the basillic vein, at separate intervals when the patient is experiencing chill or fever and should be inoculated directly into blood culture bottles (Shanson, 1981). Evacuated blood collection tubes containing anticoagulant have been found to be toxic for meningococci (Eng and Holton, 1977) usually, about 8-10ml of blood may be inoculated into 100ml of trypticase soy broth and approximately 0.1ml of the blood is spread over the surface of a blood-agar and a chocolate agar plate and then incubated at $37^{o}C$, in an atmosphere of 5-10% CO_2 with increased humidity.

The cerebrospinal fluid in bacterial meningitis usually shows a well marked increase in polymorphonuclear (PMN) cells, is turbid and contains 500-20,00 leucocytes/mm^3 of which 90% are PMN cells. Protein and globulin are moderately raised to about 80-500mg/ml while sugar concentration is usually reduced to less than 35mg/100ml (Welbourn (1964). However, the

sugar level estimation in CSF is of little value unless the blood sugar level is also known.

Direct examination of gram stain smear preparations is of diagnostic significance if gram negative diplococci are seen. Because only a few meningococci may be present during the early stages of infection, the CSF specimens may be concentrated by centrifugation or filtration. The organisms are usually found both extracellularly and intracellularly in PMN cells and the presence of PMN leucocytes is correlated with good host response and favourable prognosis.

It must be remembered that a number of other organisms possess similar gram stain appearance to meningococci: These include Branhamella catarrhalis, Moraxella sp, and Veillonella sp. It is imperative therefore, that full characterisation of isolates be carried out to ensure unequivocal result of identification.

Appropriate media for growing the meningococcus are Modified Thayer Martin (MTM) media using GC agar base (Oxoid) and New York City medium. MTM contains 3 µg/ml vancomycin to inhibit a wide range gram positive organisms, 7.5 µg/ml colistin for gram negative organisms and saprophytic Neisseria and 12.5 µg/ml nystatin to inhibit fungal growth and 5 µg/ml trimethroprim which prevents the swarming of Proteus sp. New York City medium has amphotericin B in place of nystatin. Selective media for isolation of Neisseria meningitidis are especially useful when screening per

nasopharyngeal swab for the presence of meningococci or for culture of sputum.

Meningococci can be presumptively recognised on conventional media by their charateristic colonial appearance. They produce glistening transparent colonies which are confirmed by gram stain, positive oxidase and catalase reactions and their ability to degrade only maltose and glucose among the fermentable sugars with acid but not gas production. Flynn and Waitkins (1978) described a serum free medium for testing the fermentation reactions of pathogenic Neisseria.

D'Amerto et al (1978) and Watson and Perrine (1978) employed chromogenic and fluorescent substrates respectively, for the identification of N. meningitidis. Odugbemi, MacEntegart and Hafiz (1978) while investigating the effects of various cations of gonococci noticed that meningococci can be differentiated from the gonococci by the resistance of the meningococcus to a concentration of 10,000uM of magnesium chloride and also its resistance to Cong red dye. Arko and Odugbemi (1984) also devised a rapid method for differentiating meningococci from gonococci based on the meningococci's slower reaction with superoxol (30% H_2O_2).

A number of serological methods have been used to group the meningococci. Agglutination is the most reliable and routinely used. The isolates may be matched against group specific antisera to polysaccharide capsule.

Zemenska et al. (1977) introduced the serum agar method which would be useful during an outbreak of meningococcal meningitis for grouping of the specific antigens involved in the outbreak. Indeed Zemenska et al. (1977) found there was no statistical difference between the serum agar method and slide agglutination with respect to their sensitivities and capabilities.

Errors due to non-specific agglutination may be guarded against by the use of a normal control serum diluted 1:50 and a saline suspension of organisms during the slide agglutination test. The counter-current immunoelectrophoresis (CIE) serological test system depends on the migration of soluble antigen and antibody through an agar block under the influence of an electric field and provides a rapid means for the detection of meningococcal antigens in the CSF of infected patients. However, it is not an unequivocal test and a significant amount of antigen must be present for intepretable results. Counter-current immunoelectrophoresis is not usually satisfactory for detecting group B antigens and furthermore, cross-reactions with Escherichia coli KI and group B meningococci may occur. Ogunbi and Odugbemi (1972) have used the system with some measure of success in Nigeria, but in a developing country there are attendant problems such as inadequate power supply maintenance of the equipment and cost effectiveness of the system. Latex agglutination and coagglutination tests for meningo-

coccal antigen in body fluids are commercially available (Verdos 1978). All kits contain polyvalent reagents for groups A,B and C meningococci and have Y and W-135 also. According to Ingram (1983) these systems are considerably more sensitive than CIE and positive results provide a rapid presumptive diagnosis which allows for prompt administration of appropriate therapy.

Epidemiology of Neisseria meningitidis and Meningococcal Disease:

Asymptomatic nasopharyngeal carriage of Neisseria meningitidis in healthy individuals was first recognised in 1909. However, early investigators were unable to reconcile the presence of these organisms and the absence of associated disease process. Glover in 1920 (cited by Frazer et al, 1973) postulated that when the carrier rate exceeded 20%, the community was in danger of an epidemic, usually due to the predominant serotype being carried. Although Glover's concept still prevails today there is little evidence in the literature to support him.

The carriage rate of meningococcus has been variously estimated to be between 5-36% between epidemics (Craven et al, 1980; Njoku-Obi and Agbo, 1976) and 20-60% during the epidemic periods (Goldschneider, Gotschlich and Artenstein, 1969).

According to Aycock and Mueller (1950) and Winzel et al (1978) serogrouping of meningococcal isolates gives significance to carriage data; and there has been little correlation between carriage rates and incidence of disease indeed the carriage rate itself may not be important since an individual who has a nasopharyngeal infection for a considerable period of time is probably not likely to succumb to

the strain. However, the rate of acquisition of pathogenic types in a population may be significant (Winzel et al., 1978).

Carriage stimulates bactericidal and other antibodies and such individuals are immune to the strains they carried.

The meningococcus is endemic in most populations and the disease is usually sporadic. In the USA, Devine, et al. (1971) studied the carriage of meningococci with time and the overall incidence of acquisition was 5.7% per year and modern generation of carriage was about 9½ months. According to Whittle et al. (1975) the endemic situation as seen in the USA is that adult males introduce the bacteria into the family and while other adult meningococcal carriers become immune in the process, children who are non-immune develop infection. This situation leads to a low but steady case-incidence, the great majority of cases being infants and young children.

Devine et al. (1972) have pointed out that the carrier to case ratio for group Y meningococci is approximately 10,000:1; B is 5,000:1 and C is about 500:1 and according to the data published by Hassan et al. (1979) this ratio for group A is 125:1. These differences may reflect the heterogenicity of the groups. Group A appears to be more homogenous since the serotype protein antigens extracted from all A

strains are identical (Sippel and Quan, 1971) and group A carriers are only found in populations associated with group A disease (Sippel and Girgis, 1978).

Studies of meningococcal carriage rates by Njoku-Obi and Agbo (1976) in Eastern Nigerian during an interepidemic period show that the nasopharyngeal carriage is about 10% and that the predominant serogroup is B and the highest percentage of isolates was from the age group 11-20 years, followed by 6-10 years.

Meningococcal diseases and clinical manifestations:

Neisseria meningitidis is primarily recognised characteristically for its role in causing epidemic cerebrospinal meningitis and fulminant septicaemia (meningococcaemia) two devastating diseases which have caused much untold suffering throughout the world. However, the clinical manifestations of meningococcal disease may be quite varied, ranging from transient fever and bacteraemia to fulminant disease culminating in death within a few hours of the onset of clinical symptoms. Wolfe and Birbara (1968) classify four clinical situations:

(1) Bacteraemia without sepsis - patients are usually admitted for upper respiratory tract infection or viral exathem and frequently recover without specific

antimicrobial therapy - however the results of the blood cultures are positive for N. meningitidis.

(2) Meningococcaemia without meningitis - the patient is septic with signs of leucocytosis, skin rashes generalised malaise, weakness, headache and hypotension.

(3) Meningitis with or without septicaemia - patients have headache, fever, cloudy CSF and meningeal signs and pathologic reflexes not present.

(4) Meningoencephalitic presentation - Patients are profoundly obtrund with meningeal signs and septic spinal fluid. The deep tendon reflexes and superficial reflexes are either absent or rarely hyperactive. Pathologic reflexes are frequently present. The patient may progress from one situation to another during the course of the disease.

Petechial lesions are a common sign of meningococcal infection and petechial rashes may commonly be seen in clusters under areas where pressure may be applied to the skin by elastic in underwear or stockings for example. The lesions may join together to form larger lesions which appear acclymotic. These lesions may actually be secondary to subcutaneous haemorrhage, may be vesicular and

frequently desquamate as the patient recovers. The petechiae correlate with the degree of thrombocytopaenia and are clinically important as an indicator in the evolution of bleeding complications secondary to disseminated intravascular coagulation (DIC) which usually follows. These features have been extensively reviewed by Apicella (1986).

Many authors have described a macropapular eruption of varying size also associated with meningococcal infection (Wolf and Birbara, 1968; Feldman, 1972). The rash is not purpuric and non-pruritic and is transient, generally lasting for less than 2 days and is usually gone hours after the first observation. Generalised musle tenderness may also be an important differential sign. There may be occasional pain from these myalgias causing considerable discomfort to the patient.

Evidence of meningeal irritation is common except in the very young and old. According to the findings of Feigin and Dodge (1976) focal neurologic signs and features are less common in meningococcal meningitis than in pneumococcal meningitis or that due to Haemophilus influenzae.

This correlates with the postmortem findings described by Thomas (1943), in which focal cerebral involvement in meningococcal meningitis was rare

and the cause of death was related to toxins produced by the organism or by cerebral oedema and to the secondary effects on vital centres in the mid-brain regions.

Interestingly, Ducker and Simmons (1976) supported these clinical observations when they found that meningococcal endotoxin produced no effect intravenously when introduced into the ventricular system of dogs, but produced massive haemorrhagic pulmonary oedema, visceral congestion and a renal haemorrhage. The lesions observed were similar to those seen outside the CNS in soldiers dying of meningococcal meningitis and septicaemia (Hardman, 1968). Levin and Painter (1968) and Kanter et al. (1956) have emphasized the myocardial problems associated with meningococcal infection. Evidence of myocardial failure as manifest by gallop rhythm, by congestive heart failure with pulmonary oedema and by high central venous pressures and simultaneous poor peripheral perfusion has been reported by Levin and Painter (1968).

Shock-state is a dominating feature of the infection. Usually the patient is poorly responsive and peripheral vasoconstrictionis maximal with cyanotic, poorly perfused extremities. Arterial blood-gas analysis demonstrates high acidosis and

anoxia with arterial PO below 70mm (Levin and Painter, 1968).

Further concommittant with the initial evaluation of the patient or later in the recovery phase of the illness, a number of unusual complications have been reported. These include arthritis, pericarditis, conus medullaris syndrome and cranial nerve dysfunctions particularly of the 6th, 7th and 8th cranial nerves (Feigin and Dodge, 1976). The pericarditis may cause massive tamponade.

Fulminant meningococcaemia is the most severe form of meingococcal disease because of the high mortality rate. This form, also called the Waterhouse-Friderichsen syndrome, occurs in approximately 5-15% of cases of meningococcal disease (Hoffman and Edwards, 1972). It begins with sudden high fever, chills, myalgias, weakness, nausea, vomiting and headache. Apprehension, restlessness and delirium occur within a matter of hours. The rash appears suddenly and is widespread purpuric and ecchymotic. Haemorrhages appear on the buccal mucosa and conjunctivae. Typically no signs of meningitis are present and cyanosis, hypotension and profound shock will eventually appear. Patients, with this form of meningococcaemia usually present with a high fever of about 40.6^{o}C and either a normal WBC count or leukopaenia. There may also

be a lowered prothrombin time, a lowered fibrinogen level, and circulating levels of fibrin split products. Serum complement may also be lowered. Pulmonary insufficiency develops within a few hours and many patients die despite appropriate antibiotic therapy, and intensive care (Apicella 1986).

Chronic meningococcaemia is characterised by intermittent bacteriaemic illness that lasts for at least one week anf as long as several months. The syndrome must be differentiated from the problem of recurrent episodes of meningococcal meningitis. Lion et al. (1976) demonstrated the absence of the sixth complement component in patients with chronic meningococcaemia while Alper (1970) and Petersen et al. (1979) reported deficiency of C-3 and C-8 respectively in their patients with this disease.

As chronic meningococcaemia progresses the temperature rises daily and fever may be more continuous. Eventually a skin eruption appears during the febrile episodes. However, chronic meningococcaemia is not a frequent form of meningococcal infection (Hoffman, 1986).

Meningococcal pneumonia has been a recognised clinical syndrome for many years, (Young, LaForce and Head 1972). However, due to the presence of asymptomatic nasopharyngeal

carriage of the meningococcus it would be hazardous to establish the diagnosis based on sputum culture alone. History of cough, chest pain, chills and previous upper respiratory tract infection occur in over 50% of patients (Koppes, Ellenbogan and Gebhart, 1977). Rales and fever and pharyngitis occur in about 80% of patients. In many instances the disease may involve more than one lobe, although the right lower and middle lobes are the ones most frequently affected (Koppes et al., 1977). There appears to be an association between meningococcal infection and preceeding viral respiratory infection (Young et al., 1972).

There are now increasing reports of the role of meningococci in causing urethritis (Miller et al. 1979; Faur et al., 1975) and an association between orogenital sex and acquisition of the organism has been suggested (Salit and Frasch, 1982). Localised disease such as conjunctivitis and otitis media and arthritis are also to be found in the literature (Bannister, 1988).

Pathogenesis of meningococcal infection

Among the early workers who investigated the pathogenesis of meningitis were the well known scientists, Councilman, Mallory and Wright

(1898) who found that removing small amounts of CSF during experimental bacteraemia led to the development of meningitis. They concluded that the occurrence of meningitis was facilitated in nature by transient lowering of CSF pressure. Although this hypothesis had no basis in experimental data, the role of a pressure differential between blood and CSF has been supported indirectly by other workers (DeVoe, 1982).

The CSF serves as an excellent culture medium for bacteria. Within hours of its inoculation with meningococci, an inflammatory reaction in the leptomeninges becomes apparent. Inflammation of the meninges in meningococcal disease is usually characterised by an exudate consisting primarily of PMN cells. The hallmark of meningococcal meningitis is a creamy pus in the subarachnoid space (DeVoe, 1982). There appears to be no direct invasion of cerebral tissue itself by the exudate, per se, but there are serious changes in the underlying brain. In the extreme, the brain becomes intensely oedematous and congested(DeVoe, 1982).

After entry of meningococci into the CSF, the outcome is almost invariably fatal in the absence of antimicrobial therapy, implying that the body cellular and humoral defences are practically helpless

against this organism.

It is generally accepted that nasopharyngeal colonisation is an essential step in the pathogenesis of infection due to meningococci. The organism may make its way from the nasopharynx to the CNS through two main routes. It may pass directly through the base of the skull to the inside of the cranium. This appears to be the more direct and obvious route. The second is less direct but more conventional i.e. by way of the bloodstream across the blood brain barrier. There is clinical and experimental evidence for both routes and there is also the possibility which combines the first two- that the organism passes directly to the CNS and that infection of the bloodstream is secondary to infection of the CSF.

Once the organisms are established in the nasopharynx they are separated, in terms of distance by only a few millimeters from the inside of the skull, but anatomically these are the nasopharyngeal mucosa, a plate of bone and the dura-mater between these organisms and the leptomeninges. The rarity of occurrence of meningitis indicates that these are adequate defences.

When the defences are breached, by injury for example, the meningococci may readily penetrate them and cause meningitis, but even when the

defences are intact there is experimental evidence to show that particles may still find a passage through this barrier (Christie, 1969).

The meningococcus has the capacity to produce fulminating illness culminating in death within a matter of a few hours and despite the availability of effective antimicrobial therapy and administration of appropriate vaccine the case fatality is still alarmingly high (Apicella, 1986), and a significant proportion of infants and children have been shown to develop severe neurologic sequelae (Sippel, 1981; Goldschneider, 1969).

Despite the wealth of knowledge available on the biology and epidemiology of Neisseria meningitidis, many aspects of its pathogenicity are still poorly understood.

The factors influencing the attachment of meningococci to nasopharyngeal epithelial cells, the effects of meningococcal endotoxin on ciliary activity of respiratory mucosa and the biologic interactions between N. meningitidis and the indigenous microbial flora of the upper respiratory tract may play an important role in the pathogenicity of N. meningitidis and the determination of the spectrum of susceptible hosts.

Objectives

The following were the set objectives of this study:

(1) To isolate and characterise meningococci from asymptomatic carriers and clinical specimens and to determine the carriership of meningococci among school children from a defined geographical locus.

(2) To investigate, *in vitro*, the factors influencing the attachment of *Neisseria meningitidis* to human naso-pharyngeal epithelial cells.

(3) To define using an *in vitro* model, the biologic antagonistic interactions between *Neisseria meningitidis* and upper respiratory tract microflora.

(4) To investigate the effect of meningococcal endotoxin on the activity of ciliated respiratory mucosa in adult rat trachea model.

(5) To investigate the role of endotoxin in the pathogenicity of carrier and clinical isolates of *Neisseria meningitidis* in a chick embryo model.

(6) To develop an animal model for meningococcal meningitis.

CHAPTER 2

NASOPHARYNGEAL CARRIAGE OF *NEISSERIA MENINGITIDIS* AMONG SCHOOL CHILDREN

INTRODUCTION

Numerous reports in the literature indicate that Neisseria meningitidis has its natural habitat in the mucous membranes of the nasopharynx of normal human hosts (Sippel, 1981; Apicella, 1986). Asymptomatic nasopharyngeal carriage of the meningococcus has been recognised since 1896 when the presence of these known pathogens and absence of associated disease process appeared somewhat paradoxical to the early investigators (Apicella, 1986).

In 1909, Bruns and Hohn recognised a linear relationship between carriership in a population and the course of meningococcal epidemic infection (Apicella, 1986). Glover in 1920 (cited by Fraser et al., 1973) from his studies conducted among military camps, postulated that when the carrier rate of meningococcus exceeded 20%, the community was in danger of an epidemic, usually due to the predominant serogroup. However, so far there have not been any reports in the literature to substantiate this hypothesis.

The meningococcus is endemic in most populations and the disease when it occurs is usually sporadic (Cheever, 1965). Despite the importance of meningococcal disease as a major cause of morbidity and mortality in this country, especially during the cyclically occurring epidemics (Greenwood et al., 1979; Hassan-King et al,, 1979) there have only been limited studies on the carrier rate of Neisseria meningitidis in Nigeria.

Whittle et al., (1975) undertook a study of the meningococcal carrier rate of a general population in northern Nigeria during an epidemic and reported a 34% carriage rate for group A meningococci. Later, Hassan-King et al. (1979) in the same environment, also investigated meningococcal carriage during an epidemic in Zaria, northern Nigeria and reported an overall carriage rate of 3.8% for the epidemic strain. Njoku-Obi and Agbo (1976) conducted a survey on the carriership of N. meningitidis in the eastern part of Nigeria, a region just outside the West African meningitis belt. They found the most prevalent serogroup isolated from asymptomatic carriers to be group B meningococci, and an overall carriage-rate of 10%.

Studies of carriership of meningococci among different populations are important in furthering our understanding of the epidemiology of Neisseria meningitidis. Such studies also provide information as to the prevalent serogroups within specific populations.

The meningococcal carriage rate among children in Lagos (in the south-western region of Nigeria) has not been previously reported. The present study was thus carried out to determine the carriage rate of meningococci amont school children in Ijede, Ikorodu in Lagos State and to determine the prevalent serogroups of Neisseria meningitidis in that area.

MATERIALS AND METHODS

Subjects:

The subjects used for this study were healthy school children between the ages of 5 and 15 years attending primary school in Ijede area of Ikorodu local government area in Lagos State, Nigeria. A total of 639 children were sampled (342 male and 297 female) after informed consent. The nasopharyngeal swabs were carefully taken by depressing the tongue with a wooden palate-depressor and swabbing the nasopharynx with plain cotton swabs (Sterilin, Middlesex) under direct vision. None of the children sampled was known to have received antibiotic therapy within two weeks of when swabs were taken.

Media:

The following media were used:- Chocolate agar comprising G.C. agar base (Oxoid) and 7.5% expired human blood) and Thayer-Martin agar (chocolate agar with 3.75 μg/ml of vancomycin, 7.5 μg/ml of colistin and 12.5 units/ml of nystatin (Thayer and Martin, 1966). TM is a selective medium for the isolation of pathogenic *Neisseria* and was the medium of choice for the isolation of *Neisseria meningitidis* in this study.

Processing of specimen:

After collection, each nasopharyngeal swab was immediately inoculated onto a plate of chocolate agar and TM agar and plated out according to the standard method previously described by Gillies and Dodds (1976).

The plates were incubated at 37°C in 5-10% CO_2 in a humid environment using a candle extinction jar, for 18-24 hours. Plates of TM not showing any growth after this initial period were further incubated for another 24 hours.

Identification of isolates:

Ater incubation,colonies resembling N. meningitidis on chocolate and TM agar were gram stained and observed for the presence of characteristic gram-negative diplococci. Isolates were further identified by their slow reaction with superoxol reagent (Arko and Odugbemi, 1984) against a known positive control, Neisseria gonorrhoeae and a known negative control, Neisseria meningitidis Group A, ATCC 1054. Using a sterile pipette a drop of superoxol reagent (30% H_2O_2) was allowed to fall onto suspicious colonies and observed for immediate effervescence (positive result) or slow effervescence (negative result). Oxidase activity was determined by emulsifying suspicious colonies onto a piece of Whatman's filter-paper freshly impregnated with oxidase reagent (BDH). A purple colour at the site of emulsification, obtained within thirty seconds, was taken as positive. Pseudomonas aeruginosa ATCC, 27853 and Staphylococcus aureus ATCC 25923-F49 were used as positive and negative controls respectively, for the oxidase test.

Colonies showing gram negative diplococci that were oxidase positive and superoxol negative were sub-

cultured onto chocolate agar and incubated for 18-24hr at 37°C in 5-10% CO_2 in preparation for the sugar utilisation tests. Sets of glucose maltose, lactose, fructose and sucrose sugars were prepared in bijoux bottles at a concentration of 1% using the method described by Flynn and Waitkins (1978) employing a serum-free agar slope comprising GC agar base and phenol red indicator (see appendix).

After inoculation of the bottles, they were incubated at 37°C for 18-24hr in 5-10% CO_2 and high humidity. Neisseria meningitidis isolates characteristically utilise only glucose and maltose with acid production as indicated by a change in the colour of the indicator in the medium from red to yellow.

The isolates were then serogrouped by slide agglutination using a complement of group specific antisera (CDC, Atlanta, Georgia) comprising serogroups A, B, C, X, Y, Z and W-135 which were kindly provided by Professor Tolu Odugbemi, Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos (CMUL). The results were analysed and compared statistically using student's t-test for significance at 95% (0.05) and 99% (0.01) confidence limits.

A total number of 639 school children were sampled for nasopharyngeal carriage of Neisseria meningitidis in Ijede Ikorodu, Lagos State. These comprised 342 (53.5%) male and 297 (46.5%) female.

Their ages ranged between 5 and 15 years and the overall mean age was 7.6 years and a modal age of 7 years. The mean age among males sampled was 7 years, while for females the mean age was 8.2 years.

Of the 639 children sampled for nasopharyngeal carriage of meningococci, 40 were found to be carriers thus giving a carriage rate of 6.2%. Twenty-five (3.9%) were males while 15 (2.3%) were female (table 2.1).

Table 2.2 shows the proportion of meningococcal carriers among the Ijede School Children by sex. Males accounted for 62.5% of the 40 nasopharyngeal carriers of meningococcal while females accounted for 37.5%. Statistical analysis by student's t-test showed that the proportion of male carriers was significantly higher than that of female carriers ($P < 0.05$).

Table 2.3 shows the meningococcal serogroup distribution among the 40 isolates from the school children. Serogroup C Neisseria meningitidis accounted for 16 of the 40 isolates representing a percentage occurrence of 40%. This was the most frequently isolated serogroup from this study.

Serogroups A and B accounted for 9/40 (22.5%) and 7/40 (17.5%) of the positive isolates respectively. Group W-135 was isolated from 3/40 (7.5%) positive isolates while 5/40 (12.5%) were found to auto-agglutinate with the meningococcal group specific antisera. Serogroups X, Y and Z were not isolated in this study.

The isolation rate of group C meningococcus was significantly higher than the rates obtained from groups B and W-135 ($P < 0.05$).

Table 2:1

Summary of statistical characteristics of Population studied at Ijede, Ikorodu Lagos State

Total Number of children studied	639
Male	342 (53%)
Female	297 (47%)
Age Range	5-15years
Mean age (Male)	7 years
Mean age (Female)	8.2years
Overall mean age	7.6years
Modal age	8years

Table 2:2

Nasopharyngeal Carrier Rate of *Neisseria meningitidis* among School Children at Ijede, Ikorodu Lagos State (N = 639)

Sex of Child	No. of children colonised by *N. meningitidis* at nasopharynx	Carrier Rate of *N. meningitidis* (%)
Male	25	3.9
Female	15	2.3
Total	40	6.2

N = Total number of School Children sampled in study.

Table 2:3

Proportion of nasopharyngeal carriers of meningococci at Ijede, Ikorodu, Lagos State by sex

Sex of Child	No. of nasopharyngeal carriers of N. meningitidis	Proportion of carriers (%)
Male	25	62.5
Female	15	37.5
Total	40	100

N = Total number of children colonised at nasopharynx by N. meningitidis = 40

Table 2:4

Distribution of serogroups of *N. meningitidis* among 40 positive isolates from school children at Ijede, Ikorodu, Lagos State.

Serogroup	Male (N=25)	Female (N=15)	Total	Percentage occurrence (%)
A	6	3	9	22.5
B	4	3	7	17.5
C	10	6	16	40
W-135	2	1	3	7.5
Autoagglutinated with available antisera	3	2	5	12.5

N = Total number of children in group.

Serogroups X, Y and Z were not isolated.

DISCUSSION

In the present study the nasopharyngeal carriage rate of N. meningitidis among 639 primary school children between the ages of 5 and 15 years was found to be 6.2%. The region from which the sample population was taken is Ijede in Ikorodu, a local government area of Lagos State, a coastal region situated about 900km south of the Nigerian meningitis belt. Epidemic meningococcal infection has not been reported at Ijede, but sporadic incidents of the disease do occur. This is in contrast to the seasonal cyclic epidemics which have been reported in the northern part of Nigeria (Archibald, 1962; Greenwood et al, 1979; and Hassan-King et al, 1979).

This study was directed at school children because this age group comprises those reported to be most susceptible to meningococcal diseases (Greenwood et al., 1979; Sippel, 1981; Apicella, 1986).

The carriage rate of 6.2% is relatively low compared with those reported by other workers. For example, 21% was reported by Fraser et al. (1973) at a naval school for boys in England, 34% by Sandborn et al. (1972) and 10% by Njoku-Obi and Agbo (1976). However, this current figure is higher than the rate of 3.8% earlier reported by Hassan-King (1979) for a general population in Zaria in northern Nigeria. In epidemiologic investigations, the recovery of organisms such as meningococci which are relatively fastidious in their isolation requirements,

depends largely upon the isolation techniques employed The use of chocolate agar and selective Thayer Martin agar ensured the adequate isolation of meningococci from the potentially mixed flora of the nasopharynx. Furthermore, the use of serum-free agar slope for sugar utilization tests prevented equivocal results which may otherwise have been the case if phenol-red peptone water were used for identification (Flynn and Waitkins, 1978).

Meningococcal carriage rates may fluctuate with season of the year; Hassan-King et al. (1979) observed that the carriage rate of meningococci was 34% during the dry season and 7% towards the end of that period. The present study was carried out during January to April, the dry season, and the carriage rate appears to be quite low compared to 34% reported by Hassan-King et al. (1979).

In the present study it was observed that the carriage rate was significantly higher among males than females. The reason for the significant difference in carriage rate between the sexes is not immediately clear. However, this may not be unrelated to behavioural differences between the sexes which may find males of this age-group indulging in more physical group activities (e.g. wrestling) thus allowing for closer contact and facilitate the spread of meningococci. Hassan-King et al. (1979) also reported a higher meningococcal carrier rate among males below the age of 20 years and females of the same age group. However, Holten et al. (1978) studied meningococcal carriage in a military population and found that sex made little

difference to serogroup acquisition rates and duration of carriage. It is possible that in military establishments both sexes are exposed to similar physical conditions and boarding. However, it is also feasible that physiological differences between the two sexes may influence attachment of meningococci at the epithelial cell surfaces. This is yet to be investigated.

In this present study, serogroups of N. meningitidis, A, B, C and W-135 were isolated, while X, Y and Z were not. In recent years epidemics of meningitis in northern Nigeria have been due to meningococcal groups A and C (Greenwood et al., 1979; Hassan-King et al., 1979) while group B strains have been largely responsible for sporadic cases of meningococcal meningitis observed in other parts. Serogrouping the isolates of meningococcal from the school children studies in Ijede gives more significance to the carrier data. For instance, it is interesting to note that group C was the most frequently isolated serogroup of meningococcus from this study followed by group A. This is quite unlike the observation of Njoku-Obi and Agbo (1976) who found that group B meningococcus was the most predominant in the area studied in the eastern part of Nigeria, just below the meningitis belt. However, it must be noted that their study was done over a decade ago and there are reports in the literature that serogroup distribution of Neisseria meningitidis often changes (Gold et al., 1975; Apicella, 1986). If this is so, this emphasizes the importance of continuous monitoring of serogroups of meningococci in different populations worldwide.

The reason for the changing serogroup pattern is still unclear, but may not be unrelated to the development of immunity to a prevalent strain by communities over a period of time.

Whether the strains of groups A and C isolated among school children in Lagos are identical to the epidemic strains encountered in the northern part of Nigeria, cannot be ascertained without employing finer epidemiological tools for typing.

It is believed that the present study has provided baseline information of epidemiological significance regarding the carriership and serogroup distribution of meningococci in Lagos.

CHAPTER 3

IN-VITRO ASSESSMENT OF FACTORS INFLUENCING ATTACHMENT OF *NEISSERIA MENINGITIDIS* TO HUMAN NASOPHARYNGEAL EPITHELIAL CELLS

Neisseria meningitidis colonises the nasopharynx of about 5-30% of the normal population during inter epidemic periods (Griffis and Artenstein, 1976; Njoku-Obi and Agbo, 1976; Craven et al., 1980) and 20.60% during epidemics (Goldschneider et al. 1969; Whittle et al., 1979).

According to Cheever (1965) and Sippel (1981), N. meningitidis gains access to the human body via the nasopharynx and subsequent to becoming implanted there, they may remain completely quiescent, giving rise to a carrier status, or may set up localised inflammatory reactions which in turn may progress to purulent rhinitis and other severe infections. Moreover, it is from the nasopharynx that meningococci are disseminated into the environment through aerosols produced by spasms of coughs and sneezes (Apicella, 1986).

It is apparent therefore, that the capacity for meningococci to colonise and establish themselves in the nasopharynx cannot be over-emphasized as a contribution to the pathogenesis and spread of meningococcal infection. Craven et al. (1980) reported that isolates of groups B and C meningococci from patients adhered better to buccal epithelial cells (BEC) than strains isolated from meningococcal carriers. However, they could not correlate their observations of attachment to BEC with a specific capsular polysaccharide serogroup, outer membrane protein (OMP) serotype or quantitative

and Morton (1981) demonstrated that meningococci exhibited some degree of epithelial-cell specificity in that they attached significantly better to posterior pharyngeal cells than to buccal epithelial cells. This specific recognition-reaction between the cell-membrane of pharyngeal cells and bacterial adhesins, may help to explain the localisation of meningococci in the posterior pharynx and may influence the characterististic colonisation pattern observed. Salit and Morton (1981) further reported that, whereas meningococcal pili mediate attachment to oropharngeal cells, the presence of meningococcal capsular polysaccharide impedes attachment.

Despite the importance of Neisseria meningitidis as a human pathogen and its known portal of entry the factors influencing its adherence to human nasopharyngeal epithelial cells have not been adequately investigated. There are for example, no reports in the literature on the attempts to determine the possible relationship between ages of human donors of nasopharyngeal epithelial cells (NEC) and the specificity of attachment of meningococci of different serogroups to NEC of such donors. This may prove to be of some significance, since according to several reports in the literature, the susceptible age groups of those who develop meningococcal disease are usually children and adolescents (Sippel, 1981; Apicella, 1986).

Furthermore, the presence of indigenous microflora in the nasopharynx may exert competitive pressure for

binding to available receptor sites on the NEC, or produce toxic or other substances which may interfere with meningococcal attachment to NEC.

The purpose of this study was to evaluate and compare, using an in-vitro system, the attachment of different meningococcal serogroups comprising standard meningococcal reference strains (ATCC) and local isolates from carriers and clinical cases, to human nasopharyngeal epithelial cells.

MATERIALS AND METHODS

Bacterial strains:

The following standard bacterial strains were used in this study:

(a) *Neisseria meningitidis* Group A, ATCC (American Type Culture Collection) 1894; Group B, ATCC 2091; Group C, ATCC 1054 and a strain of *N. meningitidis* group W-135. These were kindly provided by Prof. Tolu Odugbemi, Department of Medical Microbiology, College of Medicine (CMUL). A strain of group C, *N. meningitidis* isolated from a clinical case of cerebro-spinal fever in Lagos University Teaching Hospital (LUTH) designated LUTH-405, and a strain of *N. meningitidis* group C isolated from the nasopharynx of an asymptomatic carrier, designated M-625, were used as representatives of clinical and carrier strains respectively.

(b) *Staphylococcus aureus*, ATCC 25923-F49.

(c) *Escherichia coli*, ATCC 25922-F50.

(d) *Psuedomonas aeruginosa* ATCC 27853; all provided by Prof. Tolu Odugbemi.

(e) Strains of group A streptococci (*Streptococcus pyogenes*) and Group C

streptococci, kindly provided by Mr. S.F. Lawal of the same department.

(f) Strains of Branhamella catarrhalis and Neisseria lactamica, N. sicca obtained from Mr. M.C. Obi of the same department.

(g) Strains of strept viridans were obtained from Miss G. Ngbenebor.

Nasopharyngeal epithelial cell:

Donor nasopharyngeal epithelial cells (NEC) were collected from healthy neonates, infants (1-3 years) and children (4-15 years) after informed consent from gaurdians; and from volunteers comprising adolescents (16-25years) adults (26-59 years) and elderly (60 years and above). The donors comprised Lagos University Teaching Hospital (LUTH) and College of Medicine University of Lagos (CMUL) staff and their children, and neonates and infants in the LUTH Paediatric Unit. None of the subjects from whom NEC were obtained was known to be on antibiotic therapy peior to the time of collection of the samples. Donors were screened to determine whether or not they were nasopharyngeal carriers of meningococci by plating their nasopharyngeal swabs onto selective modified Thayer Martin (MTM) medium. The

swabs were also plated onto chocolate agar, blood agar and MacConkey agar to determine the resident micro flora of the nasopharynx of the donors.

Preparation of nasopharyngeal epithelial cells:

For the experiment on attachment as related to age group, nasopharyngeal cells were obtained from five individuals in each age-group, who where confirmed non-nasopharyngeal carriers of meningococci. The NEC were obtained by swabbing the nasopharynx vigorously with sterile cotton swabs (Sterilin, Middlesex) under direct vision after depressing the tongue with a sterile wooden applicator. The swabs from each of the donors were swirled into phosphate buffered saline PBS (Oxoid) of pH 7.2 previously distributed into sterile universal b bottles. The NEC were washed in five changes of PBS and centrifuged at 500 rpm after each wash to detach bacteria already adherent to the cells according to the method of Salit and Morton (1981). The NEC were passed over sterile filter paper (Whatman No. 1) and resuspended in 10ml of sterile PBS. In this way, two sets of NEC per donor were prepared separately into sterile universal bottles. The NEC were then counted using a Nauber chamber and

diluted to give a final concentration of 10^4 NEC/ml in PBS.

Media:

All strains of meningococci were maintained by subculture on chocolate agar (GC agar base, Oxoid) and incubated in 5-10% Co_2 in air using a candle extinction jar, while the environment was humidified to about 50% by enclosing some moist cotton wool in the jar. Mannitol salt agar (MSA)(Oxoid) was used as the selective medium for culture of S. aureus and S. epidermidis while E. coli and Ps. aeruginosa were cultured onto MacConkey agar (Oxoid), Branhamella catarrhalis and Neisseria lactamica were cultured on chocolate agar. Liquid medium and suspension medium used for growth and/or suspension of the organisms include: Mueller Hinton broth (Oxoid) and phosphate buffered saline (PBS). All organisms isolated during this study were identified according to standard methods previously described by Cowan and Steel (1974).

Attachment studies:

Standardisation of tmperature and pH was first performed in order to obtain the conditions required for optimum attachment meningococci to NEC. The

attachment assays were done using a modification of the methods reported by Craven et al. (1980) and Salit and Morton (1981). Briefly, representative meningococcal strains from overnight growth on chocolate agar were suspended in PBS prepared with sterile distilled water, to a concentration of 3×10^8 bacterial/ml corresponding to No. 1 McFarland's barium sulphate standards (appendix). Equal volumes (1ml each) of NEC in PBS previously counted and diluted to give 10^4 NEC/ml, and meningococcal suspensions, were dispensed into McCartney bottles by graduated pipette and the pH adjusted at sets of pH3, 5, 7.2 and 8.5 using drops of 0.02N HCl and 0.05N NaOH as appropriate. The pH was determined with a Corning model-7 type pH meter.

Each set of 10^4 NEC/ml and 10^8 cfu/ml N. meningitidis was made in duplicate and the reaction-mixture incubated at 37°C. A drop of trypan blue was added to each reaction mixture to determine the viability of the epithelial cells. (Non-viable cells stained blue). Clumped and damaged NEC were excluded from the count. Each set was done in duplicate and the mean of the average number of meningococci attaching to 20 NEC per donor per group was taken as the mean number

of gram negative diplococci per NEC. This number was multiplied by two to obtain the mean number of meningococci per NEC.

The above procedure was repeated at a pH 7.2 but with varied temperatures; 4°C (refrigerator), 25°C and 37°C (incubator), 42°C and 60°C (water bath). After incubation of the reaction mixtures, a drop was put on a clean microscope slide with a pasteur pipette. This was allowed to dry and fixed with 100% methanol (BDH) and gram-stained. The number of gram-negative cocci was determined by counting them directly, under oil immersion objective of a light microscope.Nasopharyngeal epithelial cells, from the reaction mixtures were examined for attached meningococci at intervals of 5 min for up to sixty minutes. The rate of attachment of N. meningitidis to NEC at pH 7.2 and temperature 37°C was calculated as the ratio between the mean number of meningococcal cells per NEC and the time interval taken for these cells to attach and was expressed as number of N. meningitidis per minute.

All subsequent attachment assays were standardised and done at 37°C and pH of the reaction-mixture was pH 7.2. Incubation time was thirty-minutes. These were the optimum values obtained from the pilot standardisation experiments described above.

Nasopharyngeal epithelial cells were obtained from five individuals from each age group (except the elderly age group which comprised 3 individuals) and these were separately examined for the mean number of meningococci attaching to the NEC of each individual by counting the average for 20 NEC per person.

Investigation of the effect of cell free supernates of different bacterial species on attachment of Neisseria meningitidis to NEC:

Mueller Hinton broth was inoculated with the respective organisms (see bacterial strains used) and incubated at 37°C for 18h. The overnight growth was centrifuged at 1000 rpm for 30 minutes and the supernate decanted into sterile containers, through Millipore membrane filters (pore size 45 μm.) The NEC were then added to the supernate and enumerated in a Nauber chamber then diluted with supernate to give a concentration of 10^4 NEC/ml. One ml of NEC in supernate was inoculated with 1ml of 10^8 bacteria per ml and incubated at 37°C for 30 minutes after which a drop was gram-stained and the number of meningococcal cells attaching to NEC was counted.

RESULTS

N. meningitidis of serogroups A, B. C and W-135 were found to attach optimally to donor nasopharyngeal epithelial cells (NEC) at a temperature of 37°C (Table 3.1) and pH 7.2 (Table 3.2). Attachment at this temperature and pH was significantly greater than any other combination ($P < 0.01$) with a total average of 152 meningococcal cells/NEC. The next highest attachment ratio was still at pH 7.2 but with temperature of 42°C; a ratio of a 100 meningococcal cells/NEC was observed. Individually among the serogroups, Group C, *N. meningitidis* clinical strain LUTH 405 had the highest mean number of meningococcal cells attaching to NEC:; tne count was an average of 164/NEC. However, this mean count was not significantly different from those recorded for serogroups A and B but serogroup W-135 significantly had the lowest mean number of meningococci attaching to NEC with a count of 128 meningococci/NEC ($P < 0.01$)

The rate of attachment of meningococci to NEC was found to be highest between the 15th and 30th minutes of incubation (Table 3.3) and was determined to be about 8 meningococci attaching per minute. The mean rate of attachment did not alter significantly after the first 30 minutes of incubation of the reaction-mixtures.

Table 3.4 shows the attachment of meningococcal serogroups to the NEC of male and female non-meningococcal-carrier donors. The highest number of meningococci attaching to NEC of female-donor was 170/NEC recorded for Group A, ATCC 1894. However, this number was not significantly different from the values obtained for meningococcal attachment to male-donor NEC (162/NEC).

The attachment of meningococci to the donor NEC of different age groups is shown in Table 3.5. The overall mean number of meningococci attaching to the NEC was significantly higher in infant (160/NEC), children (164/NEC) and adolescents (163/NEC) than for neonate (115/NEC), adult (124/NEC) and aged (109/NEC) ($P < 0.01$).

However, there was no statistically significant difference in the mean number of meningococci/NEC between the infant, children and adolescent age groups.

The lowest overall mean number of meningococci/NEC was observed when NEC of aged donors were used. Table 3.8 shows the effect of meningococcal carrier status of the NEC donors on the attachment of clinical and carrier isolates of meningococci. The attachment ratio for group C clinical strain (LUTH-405)

of 158/NEC was significantly higher ($P < 0.01$) for non-carrier NEC than NEC of group C meningococcal carrier. However, this count was not significantly different from the count obtained for the attachment to NEC of group A carrier (160/NEC).

Pre-treatment of NEC with supernates of *E. coli* significantly reduced the attachment of serogroups A, B, C and W-135 to the NEC of the two categories of donors compared with untreated control NEC ($P < 0.01$). The supernate of *B. catarrhalis* reduced the attachment of only group B meningococci to NEC significantly compared to the control (see table 3.6). At the other end of the whole spectrum of organisms used, the supernates of *N. sicca* and *N. lactamica* did not have significant influence on the number of meningococcal cells that got attached to the donor NEC.

The cell-free supernates of *S. aureus* and *S. epidermidis* reduced the numbers of meningococcal cell/NEC for serogroups A, B, W-135 clinical strain LJTH 405 and group C, M625 compared with the control. Viridans streptococci cell free supernates reduced the attachment of groups A and C and W-135 to NEC of donors (see table 3.8).

Table 3.7 shows the cell-free supernates of different meningococcal serogroups tested which

demonstrated no significant effect on the attachment of N. meningitidis to the NEC; the supernate of group B N. meningitidis however, significantly reduced the attachment of Group B and W-135 meningococci to donor NEC ($P < 0.01$).

Table 3.1

The *in-vitro* effect of varying the temperature of environment on the attachment of serogroups of *Neisseria meningitidis* to nasopharyngeal epithelial cells (NEC) at pH 7.2 for 30 minutes

N. meningitidis strains	Mean number of bacterial cell NEC at varying temperature (°C):				
	4	25	37	42	60
Group A, ATCC 1894	24	68	152	106	40
Group B, ATCC 2091	16	38	150	98	56
Group C, ATCC 1054	30	38	162	96	60
Group W-135	28	52	128	110	94
Group C, LUTH 405 (clinical strain)	36	70	164	106	74
Group C, M625 (carrier strain)	48	68	158	98	78
Total overall mean for all strains	30	57	152	102	67

Table 3.2

The effect of varying the pH of environment on the attachment of serogroups of *N. meningitidis* to NEC at 37°C for 30 minutes

N. meningitidis strain	Mean number of *N. meningitidis* NEC at varying pH:			
	3	5	7.2	8.5
Group A, ATCC 1894	54	74	160	92
Group B, ATCC 2091	26	84	150	86
Group C, ATCC 1054	38	106	158	84
Group W-135	46	110	136	68
Group C, LUTH 405 (clinical strain)	22	94	160	120
Group C, M625 (carrier strain)	40	64	158	88
Total overall mean for all strains	38	88	154	90

Table 3.3 Rate of attachment of *N. meningitidis* (Group A, ATCC 1894) to NEC at pH 7.2 and 37°C

Length of incubation in minutes	mean number of meningococci/NEC	Rate of attachment meningo/min
0	0	0
0 - 5	16	3.2
5 - 10	28	2.4
10 - 15	40	2.4
15 - 20	110	14
20 - 25	125	3
25 - 30	158	6
30 - 35	162	1
35 - 40	160	-
40 - 45	154	-
45 - 50	160	-
50 - 55	158	-
55 - 60	164	-

- = negligible.

Table 3.4

Sex of NEC donor and attachment of *N. meningitidis* of NEC of non-meningococcal carriers (Temp 37°C, pH 7.2)

N. meningitidis strain	Mean No. of *N. meningitidis* NEC by sex of donor	
	Male	Female
Group A, ATCC 1894	162	154
Group C, LUTH 405 (clinical)	156	148
Group C, M625 (carrier)	160	158

Table 3.5

Attachment of *N. meningitidis* to the NEC of donors representing different age groups

(Temp 37°C, pH 7.2)

N. meningitidis strain	Mean No. of *N. meningitidis*/NEC/per different groups					
	Neonate	Infant	Children	Adolescent	Adult	Aged
Group A, ATCC 1894	98	160	166	140	130	102
Group B, ATCC 2091	114	172	178	174	110	98
Group C, ATCC 1054	102	148	156	160	120	98
Group W-135	120	162	164	164	128	120
Group C, LUTH 504 (clinical)	120	158	162	168	136	110
Group C, M625 (carrier)	132	164	158	168	120	128
Overall mean for all serogroups	115	160	164	163	124	109

Table 3.6

Effect of pre-treatment of nasopharyngeal epithelial cells (NEC) of donors with supernates of some Gram-negative organisms on attachment of *Neisseria meningitidis*

N. meningitidis strain	Mean No. of *N. meningitidis*/ NEC treated with supernates of:					
	BC	NL	EC	NS	PA	Control
Group A, ATCC 1894	132	162	58	154	110	168
Group B, ATCC 2091	110	160	68	160	124	154
Group C, ATCC 1054	122	168	86	158	148	162
Group C, LUTH 405 (clinical	116	148	128	170	164	168
Group C, M625 (carrier)	122	154	110	162	114	160

BC = *Branhamella catarrhalis*

EC = *Escherichia coli*

NL = *Neisseria lactamica*

NS = *Neisseria sicca*

PA = *Pseudomonas aeruginosa*

Control = Untreated donor NEC

Table 3.7 Effect of pre-treatment of donor nasopharyngeal epithelial cells with supernates of selected serogroups of *Neisseria meningitidis* on attachment of meningococci to NEC (37°C, pH 7.2)

Meningococcus strain	Mean No. of meningococci/NEC pre-treat with supernates of :					
	A	B	C	C/LUTH	CM625	Control
Group A	152	160	146	152	140	148
Group B	158	110	158	160	168	162
Group C	158	152	166	148	156	154
Group W-135	168	116	160	146	158	160
Group C, LUTH 405 (Clinical)	160	168	154	150	164	162
Group C, M625 (Carrier)	150	162	158	156	162	154

A = Group A *N. meningitidis* ATCC 1894

B = Group B *N. meningitidis* ATCC 2091

C = Group C *N. meningitidis* ATCC 1054

C, LUTH = Group C *N. meningitidis* LUTH 405 (clinical isolate)

C, M625 = Group C *N. meningitidis* M625 (carrier isolate)

TABLE 3-8

Effect of pre-treatment of Nasopharyngeal Epithelial cells of donors with supernates of some gram positive organisms on attachment of N.menignitidis

Strain of N.Meningitidis	Mean No of Meningococci/NEC Treated with Supernates of				
	SA	SE	VS	GAS	Control
Group A ATCC I894	II8	I34	I30	I52	I68
Group B ATCC 209I	I24	I20	I48	I60	I62
Group C ATCC I054	II2	I32	I28	I62	I68
Group W-I35	I06	I22	II2	I48	I54
Group C Clinical -LUTH 405	I28	I08	I24	I56	I66
Group C Carrier M625	I24	II8	I20	I50	I58

SA = Staph. aureus

SE = Staph. epidermidis

VS = Viridans streptococcus

GAS = Group A streptococcus

Control = Untreated donor NEC

Table 3.9 Effect of meningococcal carrier status of NEC donors on the attachment of clinical and carrier isolates of meningococci to NEC

Strain of *N. meningitidis*	Mean No. of meningococci/donor NEC	
	Group C carrier donor	Group C non-carrier donor
Group A, ATCC 1894	160	156
Group C LUTH 405 (clinical)	132	156
Group C M625 (carrier)	156	154

Table 3.10 Comparison of attachment of *N. meningitidis* with non-pathogenic *Neisseria* and *Branhamella catarrhalis* to donor NEC (Temp 37°C, pH 7.2)

Bacterial strain	Mean number of bacteria/NEC
Neisseria meningitidis Group A, ATCC 1894	158
Branhamella catarrhalis	120
Neisseria lactamica	58
Neisseria sicca	84

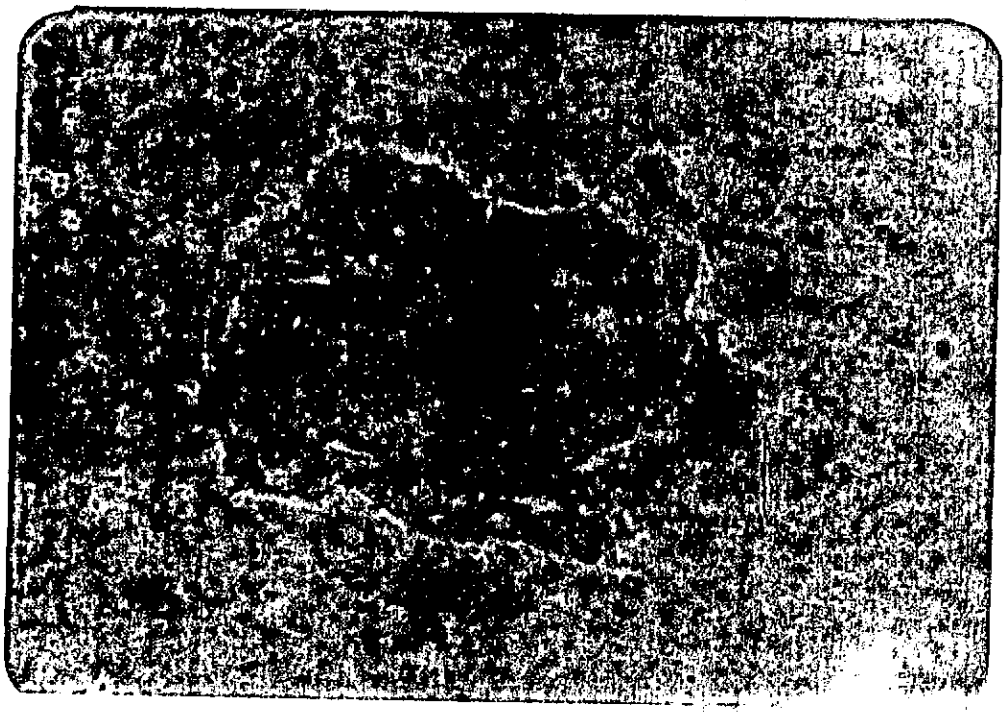

Fig. 3.1 Human nasopharyngeal epithelial cell control.

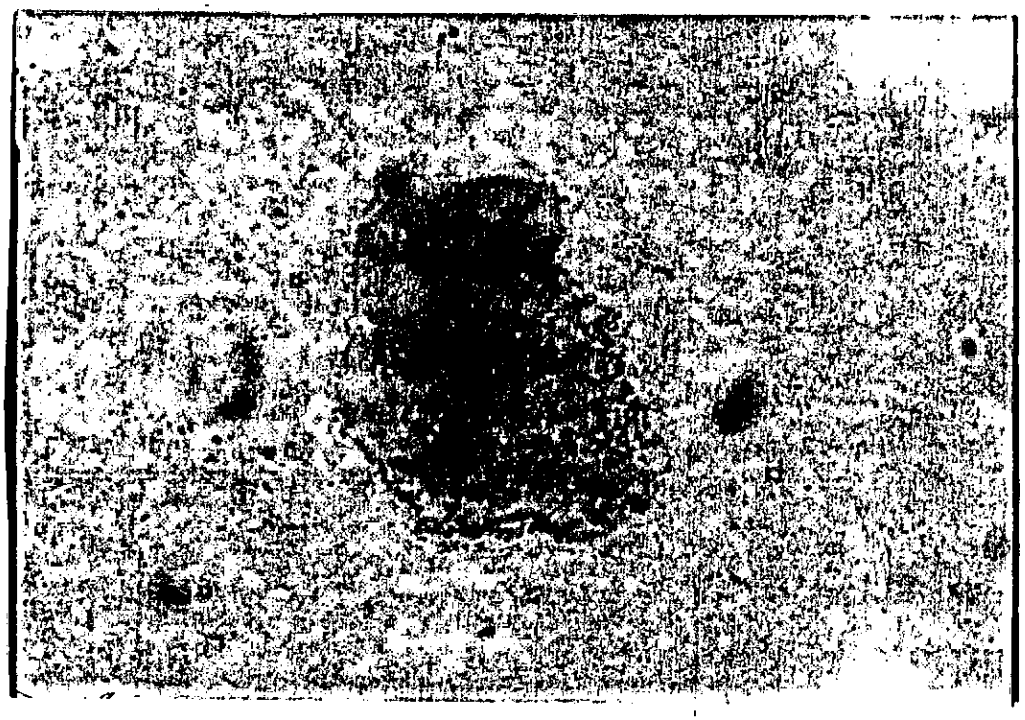

Fig. 3.2 Attachment of Neisseria meningitidis group C LUTH 405 to human nasopharyngeal epithelial cells.

DISCUSSION

The present study was carried out to investigate the factors which may influence the attachment of meningococci to nasopharyngeal epithelial cells (NEC) using an *in-vitro* system. The results of this work indicate that environmental temperature and pH are important parameters affecting the attachment of meingococci to NEC. Meningococci were found to attach optimally at pH 7.2 and temperature of 37°C. This is in agreement with the report by Craven *et al.* (1980) and Stephens, Hoffman and McGee (1983). These findings are not surprising. Similar conditions of temperature and pH are encountered in the host environment where the organism may be found under natural circumstances. Further to these observations on the physiological requirements of the meningococci, the work of (Catlin, 1973) also supports this finding.

The meningococci are fastidious organisms and exacting in their growth requirements and are susceptible to even subtle changes in the environment. The significant variations in the results for attachment at different temperatures and pH confirmed the need for a baseline standardization at which subsequent in-vitro assessments of attachment should be performed. On this basis, the values of pH 7.2

and temperature, 37°C were chosen. Furthermore, a pilot investigation indicated that an optimum number of meningococci is attached to NEC by the end of 35 minutes incubation and any further incubation of the reaction mixture was not useful. This finding is contrary to those of Aly et al. (1977) who reported maximum attachment between 90 minutes and 2 hours but agrees with that of Craven et al. (1980) and Salit and Morton (1981) who reported that maximum attachment usually took place at 30 minutes incubation.

It is also interesting to note that the number of meningococci which became attached to NEC increased with time in a positive correlation (r = 0.98 on Pearson's scale) for the first thirty-five minutes of incubation. However, the highest rate of attachment occurred between 15-20 minutes after incubation. This may be due to the orientation of organisms for specific binding sites on NEC and may reflect the mechanisms involved in binding and factors influencing it such as hydrophobic interactions at respective surfaces. That the number of meningococci attaching to donor NEC does not significantly increase after thirty minutes suggest the presence of perhaps a limited number of binding or receptor

sites on the NEC or that only a certain percentage of the total organisms possess the necessary mechanisms to recognise receptor sites. This problem has not been properly addressed by earlier investigators who have worked on the attachment of bacteria to epithelial cells. Indeed, the specific receptor sites on human epithelial cells need to be identified and quantitated, moresor since bacterial attachment to epithelial cells plays an important role in mucous membrane colonisation (Aly et al., 1980; Lampe et al., 1982) and correlates closely with disease pathogenesis (Salit and Morton 1981).

In the present study, not only were the strain characteristics (i.e. meningococcal isolates from clinical cases and asymptomatic carriers) considered, but also the status of the NEC donors (i.e. meningococcal carrier or non-carrier status) taken into consideration in determining the affinity for attachment. Although there was no significant difference between the general attachment of carrier strains and clinical strains of meningococci to donor carrier and non-carrier NEC it is interesting to note that clinical isolates of group C meningococcal attached significantly better to NEC of non-carrier donors than carrier donors ($P < 0.01$). These

observations may suggest that hosts who have not been previously colonised by meningococci may be at higher risk to colonisation by clinical isolates when such strains are prevalent in the environment. This inference is correlated well by the report of Goldschneider *et al.* (1969) which claimed that asymptomatic colonisation by meningococci is an immunising process resulting in the production of protective antibody. A clinical strain of group C (LUTH 405), the clinical isolate of meningococcus used in this study, bound in significantly higher numbers to the nasopharyngeal cells obtained from a group A meningococcus carrier than to those of a group C carrier ($P < 0.01$). This indicates further that there may exist a specificity in the protection afforded colonised persons to clinical isolates of meningococci. During the course of this study, local clinical isolates of group A *N. meningitidis* could not be obtained, thus it could not be determined if the same would be true of clinical group A meningococci isolates from Nigerian patients.

The specificity of attachment of meningococci of various serogroups and of clinical and carrier strains of Group C to NEC of donors from different age groups was also investigated in this study. The results obtained showed that meningococci

adhered in significantly greater number to the NEC of infants, children and adolescents than to the NEC obtained from neonates, adults and elderly persons ($P < 0.01$). Such preferential attachment may be important in determining the susceptible group which may subsequently develop meningococcal disease and correlates well with the finding that meningococcal infections are more commonly described in children and adolescents (Aycock et al., 1980; Apicella, 1986). The reason for favoured attachment to NEC of a particular age-group is still unclear but may not be unrelated to the apparent absence of corresponding humoral antibody in the groups which attachment is highest (Goldschneider et al., 1969, Apicella, 1986). Fothergill and Wright (1933) and Goldschneider et al. (1969) pointed out a possible inverse relationship between the age of maximum disease incidence and age-related prevalence of bactericidal antibody by using in-vitro tests. Furthermore, new born up to the age of about six months have been shown to possess maternal-acquired passive immunity to meningococcal infection (Goldschneider 1969; Sippel 1981). It is interesting to note that there were no significant differences in the pattern of attachment of selected serogroups to the NEC of different age group. This implies that each serogroup possesses

a similar capacity to colonise individuals of each age group.

According to Craven et al. (1980) and Beachey (1980), the ability to attach to mucosal surfaces may be regarded as a measure of the pathogenic potential of an organism, and the attachment process represents the establishment of a base from which tissue penetration or cell invasion can be initiated.

In this study, the adherence capacity of two species of non-pathogenic Neisseria (N. lactamica and N. sicca) and Branhamella catarrhalis was compared with that of Neisseria meningitidis. Meningococci were observed to attach in significantly greater numbers to donor NEC than the non-pathogenic Neisseria sp. and B. catarrhalis which were isolates from the upper respiratory tract of patients in Lagos University Teaching Hospital (LUTH). The enhanced capacity for attachment by meningococci to NEC may to some extent account for the meningococcus more significant role in disease causation and reflect its greater potential for pathogenicity than the classically non-pathogenic Neisseria sp.

The mechanisms involved in the binding of some species of Streptococcus, Staphylococcus, and Escherichia coli to epithelial cells from different sources have been extensively investigated (Beachey,

1975; Beachey, 1980).

Teichoic acid (TA) and lipoteichoic acid (LTA) in the cell wall of S. aureus and Strept pyogenes respectively, have been shown to mediate attachment of these organisms. An interesting report by Aly et al. (1980) described the inhibition of binding of Staphylococci to nasal epithelial cells by pre-treatment of the cells with streptococcal lipoteichoic acid. However, pre-treatment of the cells with staphylococcal teichoic acid surprisingly did not inhibit the binding of Streptococci to the nasal epithelial cells. Craven et al. (1980) observed that meningococcal strains demonstrated affinity for buccal epithelial cells and postulated that this was possibly due to interactions between hydrophobic subunits of pili and buccal epithelial cell surfaces. As human beings are the only natural host for the meningococcus it seems reasonable to speculate that there is some unique property in the human epithelial cells e.g. a specific epithelial cell receptor, that permits attachment of meningococci and subsequent nasopharyngeal colonisation.

In the present work, the effect of cell free supernates of some strains of S. aureus, E. coli and S. epidermidis were found to inhibit attachment of

these organisms to NEC in-vitro as evidenced by the significantly lower number of meningococci attaching to NEC which had been pre-treated with the supernate of these organisms. The mechanisms by which inhibition occurs is still not clear. However, there are a number of speculations which appear feasible:

(1) Competition for binding sites by some bacterial cell constituents present in the supernates, and

(2) Direct bactericidal effects of cellular and metabolic products of the interfering organisms (see chapter 4).

Pre-treatment of donor NEC with cell-free filtrates of group A meningococci also significantly reduced the numbers of groups A and C meningococci binding to NEC.

That some organisms such as S. aureus and S. epidermidis which are normally part of resident microflora of the nasopharynx of a high percentage of the population, are able to inhibit attachment of meningococci to NEC in-vitro, is of interest and may sugggest that some individuals colonised with such organisms may be less susceptible to colonisation with meningococci. However, the mechanisms of attachment are not yet fully understood and it is

possible that the specific members or populations of inhibitory organisms may also have to be taken into consideration in this regard.

The results of these investigations have demonstrated the complexity of meningococcal attachment to NEC in the sense that many factors may be operative in synchrony to determine if such process should indeed take place. These include the meningococcal carrier status of the host, characteristic of the strain (i.e clinical or carrier isolates) and the presence of other micro-organisms in the nasopharynx.

This study also includes the first report in the literature of the preferential attachment of meningococci to NEC of infants and adolescents. It is conceivable that all these factors and others yet to be determined and presently outside the scope of this study, may interplay to determine the spectrum of host susceptible to meningococcal nasopharyngeal colonisation and possibly the sub-sequent development of meningococcal infection.

CHAPTER 4

AN IN-VITRO INVESTIGATION OF THE BIOLOGIC ANTAGONISTIC INTERACTIONS BETWEEN *NEISSERIA MENINGITIDIS* AND UPPER RESPIRATORY TRACT MICROFLORA

INTRODUCTION

The interactions between man's indigenous microflora and potentially pathogenic organisms have been the subject of sporadic investigations and continuous speculations for several decades (Sanders, 1969; Crowe et al., 1973). There is evidence in the literature which suggest; that bacterial interference by the mucosal normal flora may enhance resistance to the acquisition of some pathogenic bacteria or to their associated disease (Kraus et al., 1976). Sanders (1969) demonstrated that viridans streptococci isolated from the normal flora of the throat were highly inhibitory to the growth of group A streptococci, while Crowe et al. (1973) reported that some species of Neisseria possessed bactericidal activity against group A streptococci. Hentges (1967) reported the inhibition of Shigella flexnerii by Klebsiella spp. present in the normal intestinal microflora. Kraus et al. (1976) and Movin et al. (1980) observed that gonococcal immunity may be mediated by bacterial interference from urethral organisms.

In an earlier report, Sanders (1969) showed that strains of viridans streptococci isolated from the throat and S. epidermidis, were able to inhibit

the growth of meningococci *in vitro*. Furthermore, Jyssum and Allunans (1984) observed that meningococci themselves were capable of producing substances inhibitory to other meningococci. Recently, Filice *et al*. (1985) using an agar overlay technique demonstrated the inhibitory effect of *Strept faecalis* and *Bacillus pumilis* against group A meningococci. Despite the importance of *N. meningitidis* as the causative agent of cerebrospinal fever and meningococcaemia, and its paradoxical asymptomatic presence in the nasopharynx of colonised hosts, the interaction between meningococci and the normal nasopharyngeal microflora has not been adequately investigated.

Furthermore, the results of the study in chapter 3 of this thesis have demonstrated that the cell free supernates of some bacteria such as *S. aureus* and *E. coli*, were capable of inhibiting the attachment of meningococci to human nasopharyngeal epithelial cells. This also indicated the need to further investigate the mechanisms of interactions between these organisms and *Neisseria meningitidis*.

This study was undertaken to investigate and compare the antimeningococcal activities of bacteria isolated from the upper-respiratory tracts of meningococcal carriers and non-carriers using an *in-vitro* assay system.

MATERIALS AND METHODS

Bacterial strains:

The following standard bacterial strains were used in this study: *N. meningitidis* serogroup A, ATCC 1894, group B, ATCC 2091, group C ATCC 1054 and a strain of group W-135; *Ps. aeruginosa*, ATCC 27853, *S. aureus* ATCC, 25923 - F49 and *E. coli*, ATCC 25922 - F50. These organisms were kindly provided by Prof. Tolu Odugbemi, Department of Medical Microbiology, College of Medicine, University of Lagos. Strains of group A Streptococcus and Group C Streptococcus were provided by Mr. S.F. Lawal, Department of Medical Microbiology, College of Medicine, University of Lagos and viridans streptococci were obtained from Miss. G. Negbenebor of the same department. Other bacterial strains used during the course of this study were obtained by swabbing volunteers at the nasopharyngeal site and culturing these specimen onto appropriate media.

Media

The media used for this study were as follows: Chocolate agar (Oxoid), Thayer-Martin agar, Blood agar, crystal violet blood agar (CVBA), MaConkey agar and Mannitol salt agar. Mueller-Hinton broth, nutrient broth and brain heart infusion broth were the liquid media used. Details of the formulation

and preparation of these media are given in the appendix.

Isolation and identification of bacterial organisms:

Nasopharyngeal swabs were plated out onto the media mentioned above and plates were either incubated in 5-10% Co_2 in air in a candle extinction jar (chocolate agar, Thayer-Martin agar and crystal violet blood agar) or in air (Blood agar, MacConkey agar and Mannitol salt agar (MSA) for 18-24h. The isolates obtained were Gram stained and then identified following the standard methods of Cowan (1974). The organisms were maintained by subculture onto media as follows: E. coli, Ps. aeruginosa onto MacConkey agar; Neisseria spp. onto chocolate agar, Staphylococcus spp. and Streptococcus spp. onto blood agar.

Preparation of sterile cell-free-filtrates: A modification of the filtrate technique described by Crowe et al, (1978) was used to prepare the cell free filtrate of the organisms used for this work.

Overnight growth of the organisms on solid media were inoculated into 9ml of brain heart infusion broth which had already been dispensed into sterile universal bottles. The bottle were then

incubated for 24 hours in 5-10% Co_2 in candle-extinction jars. All the bacteria present in the broth cultures were removed by passage through membrane-filters (pore size 0.45 µm; Millipore Co) and the filtrate was collected and stored at 4°C inside sterile universal bottles (Sterilin).

Determination of inhibitory activity of filtrates by filter paper disc: Filter paper discs were prepared from Whatman's No. 1 filter paper with a standard perforator and sterilised by autoclave and impregnated with 0.02ml of cell-free-filtrate and placed on a plate of chocolate agar flooded with 10^6 cfu/ml of meningococci of respective serogroups. The plates were incubated at 37°C for 18hrs in 5-10% CO_2. Inhibition was observed as a zone of no growth immediately around the disc.

Agar overlay technique:

This method has previously been described by Crowe et al. (1978) and Jyssum and Allunans (1984). The test organism was emulsified in saline to 10^8 cfu/ml corresponding to McFarland's No. 1 opacity and 2mls spread on a plate of chocolate agar, this was then incubated at 37°C overnight. The plates were then replicate-plated onto a fresh chocolate agar plate and then overlayed with

molten chocolate agar which was allowed to solidify.

Standard suspensions of 10^6 cfu/ml were spread on the plates and then incubated overnight at 37°C in 5-10% Co_2 in air. The plates were then observed for zones of inhibition on the overlay.

Inhibition of growth in Mueller Hinton broth containing cell-free-filtrate:

Five mls of Mueller Hinton broth and five mls of cell-free-filtrate were inoculated with 0.1 ml of 10^8 cfu/ml of meningococci and incubated at 37°C for 18 hours while controls comprising Mueller Hinton broth inoculated with meningococci were also set up under the same conditions. The bacteria were enumerated using modified Miles and Misra (1938) surface viable count using a 0.02ml dropping pipette. Counts lower than 10^5 cfu/ml for cell-free-filtrate treated Mueller Hinton broth were regarded as having inhibitory activity.

RESULTS

The antagonistic interactions between the bacteria from the upper respiratory tracts of meningococcal carriers and non-carriers was investigated *in-vitro* by agar overlay technique, inhibition of growth in broth and filtrate impregnated filter-paper disc methods. Table 4.1 shows the inhibitory effects of the cell-free filtrates of some standard bacterial strains on the growth of different serogroups of *N. meningitidis*. All the strains of meningococci tested were inhibited by *S. aureus* ATCC 25923-F49 as demonstrated by a zone of inhibition around the filter paper disc. The cell free filtrates of *E. coli* ATCC 25922-F50 inhibited the growth of Groups A, B, W-135 and group C M625 (carrier strain) meningococci. However *Ps. aeruginosa* ATCC 27853 showed inhibitory activity only against Group B meningococcus. Gram positive organisms isolated from the upper respiratory tract of non-meningococcal carriers (*S. aureus*, *S. epidermidis*, Group A, beta-haemolytic streptococci and viridans streptococci) had inhibitory activity against serogroups A, B, C, W-135 meningococci. Six out of 10 (60%) of isolates of *S. aureus* from the upper-respiratory tract of non-meningococcal carriers inhibited the growth of groups A, B and C

meningococci while 5/10 (50%) inhibited the growth of Group W-135. (Table 4.2).

S. epidermidis strains were also found to inhibit meningococci 4/10(40%) against groups A, W-135 and group C clinical and carrier isolates and 3/10 (30%) against Group B and C meningococci. Two out of 8 of the isolates of S. pyogenes and 4/8 (50%) of viridans streptococci were able to inhibit the growth of meningococci.

Table 4.3 shows the inhibition of N. meningitidis by gram negative bacteria (B. catarrhalis, E. coli, N. lactamica and P. aeruginosa). E. coli was able to inhibit the growth of all the serogroups of meningococci tested while N. lactamica inhibited the growth of serogroups B and C, N. meningitidis.

Some strains of gram psotive bacteria from the URT of meningococcal carriers were also able to inhibit the growth of meningococcal serogroups A, B, C and W-135 (see table 4.4). Five out of ten (50%) of the strains of S. aureus and 2/10 (20%) of S. epidermidis from this group inhibited the growth of meningococci of serogroups A, B, C and W-135. However, Group C beta-haemolytic streptococci did not exhibit any inhibition of growth against the meningococci (Table 4.4).

None of the strains of B. catarrhalis, N. lactamica and Ps. aeruginosa inhibited the growth

meningococci (see Table 4.5).

Table 4.6 shows that dialysis of the filtrate of inhibitory strains of S. aureus, S. epidermidis, E. coli and viridans streptococci had no effect on their inhibitory activity. However, after heat treatment, strains of S. aureus, S. epidermidis and viridans streptococci lost all inhibitory activity (see also table 4.7). On the other hand, heat treatment of 60°C of E. coli cell free filtrates did not lead to loss of its inhibitory activity.

The inhibitory activity of Staph. aureus ATCC 25923 - F49 against N. meningitidis Group C, ATCC 1054 by cell free filtrate impregnanted disc is demonstrated in Fig. 4.1.

Table 4.1

Inhibitory effect of cell free filtrates of some standard bacterial strains on the growth of *N. meningitidis* in vitro

Bacterial strain	Effect of cell-free filtrates of:			
	SA	PA	EC	Control
Group A, ATCC 1894	+	–	+	–
Group B, ATCC 2091	+	+	+	–
Group C, ATCC 1054	+	–	–	–
Group W-135	+	–	–	–
Group C, LUTH 405	+	–	–	–
Group C, M625	+	–	+	–

SA = *Staph. aureus* ATCC 25923 - F49

EC = *Escherichia coli* ATCC 25922-F50

PA = *Pseudomonas aeruginosa* ATCC 27853

Control = Filter paper disc impregnated with Mueller-Hinton broth.

Table 4.2

The inhibitory effects of gram-positive organisms isolated from the upper respiratory tract of non-meningococcal carriers on different serogroups of *N. meningitidis*

Strains of meningococcus	Number (%) of strains of test organism inhibiting growth of meningococci					
	SA (N=10)	SE (N=10)	GAS (N=8)	GCS (N=4)	VS (N=8)	Control
Group A	6 (60)	4 (40)	3(38)	0 (0)	4(50)	0
Group B	6 (60)	3 (30)	2(25)	0 (0)	4(50	0
Group C	6 (60)	3 (30)	2(25	0 (0)	4(50)	0
Group W-135	5 (50)	4 (40)	2(25)	0 (0)	4(50)	0
Group C, LUTH405	6 (60)	4 (40)	2(25)	0 (0)	4(50)	0
Group M625	6 (60)	4 (40)	2(25)	0 (0)	4(50)	0

% = in parenthesis

SA = *S. aureus*

SE = *S. epidermidis*

GAS = Strept pyogenes or Group A B-haemolytic streptococci

GCS = Group C, B-haemolytic streptococci

VS = Viridans streptococci

Table 4.3

Inhibition of *N. meningitidis* by gram negative bacteria isolated from the URT of non-meningococcal carriers

Serogroups of *N. meningitidis*	Number (%) of strains of test organisms inhibiting growth of meningococci				
	BC (N=8)	EC (N=5)	NL (N=3)	PA (N=1)	Control
A, ATCC 1894	-	3 (60)	-	-	-
B, ATCC 2091	-	3 (60)	1 (33)	-	-
C, ATCC 1054	-	2 (40)	1 (33)	-	-
W-135	-	3 (60)	-	-	-
C, LUTH 405 (Clinical strain)	-	2 (40)	-	-	-
C, M625 (Carrier strain)	-	3 (60)	-	-	-

- = inhibition not observed

BC = *Branhamella catarrhalis*

EC = *Escherichia coli*

NL = *Neisseria lactamica*

PA = *Pseudomonas aeruginosa*

URT = Upper Respiratory Tract

Table 4.4

Inhibition of *N. meningitidis* by gram-positive bacteria isolated from the URT of meningococcal carriers

Strain of meningococcus	Number (%) of strains of test organisms inhibiting growth of meningococci				
	SA (N=10)	SE (N=10)	GAS (N=4)	VS (N=6)	Control
Group A	5 (50)	2 (20)	1 (25)	2 (33)	0
Group B	5 (50)	2 (20)	1 (25)	2 (33)	0
Group C	5 (50)	2 (20)	0 (0)	3 (50)	0
Group W-135	5 (50)	2 (20)	0 (0)	3 (50)	0
Group C, LUTH	5 (50)	2 (20)	1 (25)	2 (33)	0
Group C, M625	5 (50)	2 (20)	1 (25)	2 (33)	0

% = in parentheses

SA = *S. aureus*

SE = *S. epidermidis*

GAS = Streptococcus Group A

VS = Viridans streptococcus

Table 4.5

Inhibition of *N. meningitidis* by gram-negative bacteria isolated from the URT of meningococcal carriers

Serogroups of meningococcus	Number (%) of strains of test organism inhibiting growth of meningococci				
	BC (N=6)	EC (N=4)	NL (N=2)	PA (N=1)	Control
A, ATCC 1894	-	2 (50	-	-	-
B, ATCC 2091	-	2 (50)	-	+	-
C, ATCC 1054	-	2 (50)	-	-	-
W-135	-	2 (50)	-	-	-
C, LUTH 405	-	2 (50)	-	-	-
C, M625	-	2 (50)	-	-	-

BC = *Branhamella catarrhalis*

NL = *Neisseria lactamica*

EC = *Escherichia coli*

PA = *Pseudomonas aeruginosa*

\- = No inhibition

Table 4.6

Effect of Dialysed filtrate of bacterial strains on their inhibitory activities against *N. meningitidis*

Strain of meningococcus	Test organism inhibiting growth of meningococci after dialysis of filtrate				
	SA	SE	EC	VS	Control
Group A	+	+	+	+	-
Group B	+	+	+	+	-
Group C	+	+	+	+	-

\+ = inhibition

\- = no inhibition

SA = *S. aureus*

SE = *S. epidermidis*

EC = *E. coli*

VS = Viridans streptococcus

Table 4.7

Effect of heat-treated filtrate of bacterial strains on their inhibitory activities against *N. meningitidis*

Strain of meningococcus	Test organism inhibiting growth of meningococci after heat treatment of filtrate				
	SA	SE	EC	VS	Control
Group A	-	-	+	-	-
Group B	-	-	+	-	-
Group C	-	-	+	-	-

\+ = inhibition

\- = no inhibition

SA = *S. aureus*

SE = *S. epidermidis*

EC = *E. coli*

VS = Viridans streptococcus

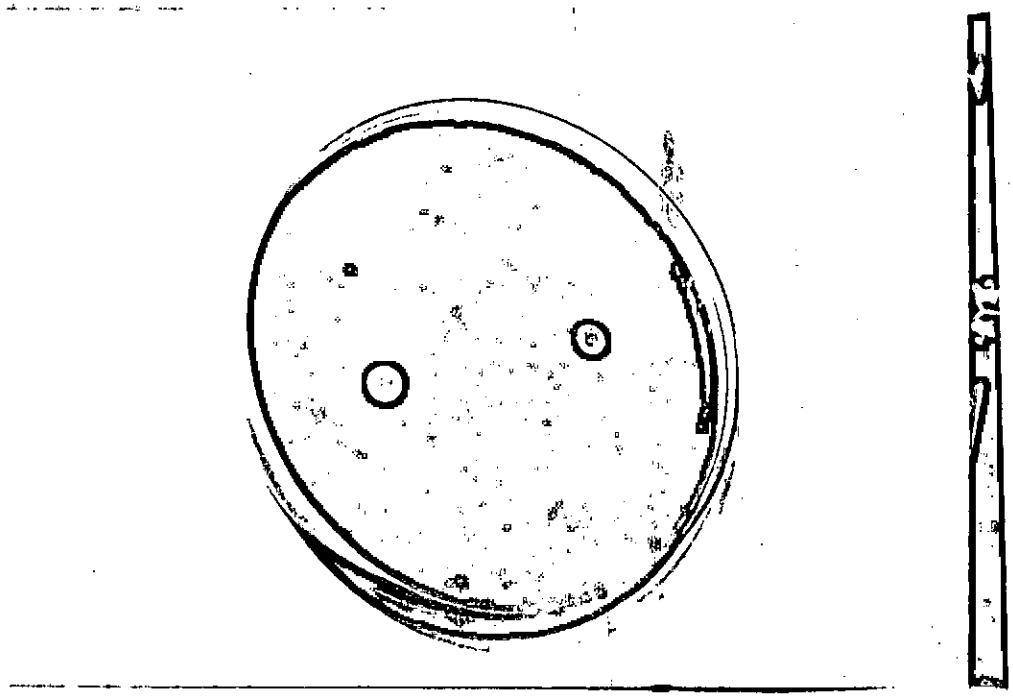

Fig. 4.1 Inhibitory activity of Staph. aureus ATCC 25923 - F49 against N. meningitidis Group C ATCC 1054 by cell free filtrate impregnanted disc.

SA = Staph. aureus

C = Control disc.

DISCUSSION

The present study was prospectively designed to investigate and compare the antagonistic interactions between the different serogroups of N. meningitidis and other bacterial organisms which are part of the normal microflora of the upper respiratory tract of humans. The results obtained from this work demonstrate that cell free filtrates of some strains of S. aureus, E. coli, viridans streptococcus and S. epidermidis were able to inhibit the growth of meningococci group A, B, C and W-135 in-vitro.

This is not the first report of antagonostic activity of organisms from the nasopharynx inhibiting the growth of meningococci. Filice et al. (1985) reported that some strains of Bacillus pumilus and viridans streptococci as well as Strept. faecalis have inhibitory effect on the growth of group A meningococci isolated from children in a skid-row community in Seattle, USA. However, there is no information about the meningococcal carrier status of the children investigated. Some results of part of this study confirm and extend the report of Filice et al. (1985).

The present work was also able to demonstrate that strains of bacteria with inhibitory activity

could be isolated from both meningococcal carriers and non-carriers. This particular finding is interesting. This may imply that the actual numbers or concentrations of inhibitory organisms found in the nasopharynx may be a more important factor in determining the presence or absence or meningococci at this site in an individual than its mere presence. Thus the more heavily colonised an individual is with inhibitory strains, the less likely it is that such a person will be successfully colonised by meningococci which are sensitive to those strains.

Determination of antagonistic bacterial activity using filter paper discs impregnated with cell free supernates of overnight growth of the test organism, proved to be a simpler and better method than the agar-overlay technique in determining antagonistic acitivity, as demonstrated by the fact that none of the strains shown to be inhibitory by the disc method was so for the agar-overlay technique in this study.

The mechanism whereby inhibitory organisms produced their effects in this study is largely unknown. According to Sanders (1969) antagonism of one bacterium by another may result from any three possible mechanisms (1) elaboration of an antibiotic susbstance (2) depletion of essential substrate from growth medium and (3) creation of a restrictive

physiologic environment. Since in the present study, the inhibitory effects against meningococci were also investigated by the effects of cell-free filtrate impregnated into discs, it is highly improbable that inhibition was due to depletion of substrate or restrictive physiologic environment. This suggests that inhibitory strains were operative by means of producing antimeningococcal factors, the nature of which is yet to be elucidated. However, the results of this study have shown that these factors vary among different species or organisms producing them. For example, whereas the anti-meningococcal factors produced by S. aureus, S. epidermidis and Viridans streptococci are heat-sensitive and non-dialysable against distilled water, that of E. coli is heat-stable. This proposition becomes even more attractive when spatial arrangements of interfering organisms are considered and space for attachment to binding sites becomes depleted. However, this concept is still speculative and yet to be experimentally substantiated.

The phenomenon of bacterial antagonism or interference has already been exploited in the control of staphylococcal disease in newborn (Sanders, 1969).

It is quite possible that bacterial interference by mucosal microflora might increase resistance

to the acquisition of meningococcus or its associated diseases at the nasopharyngeal site. Some studies have already provided evidence that mucosal flora which inhibit the growth of S. aureus (Shinefield et al, 1963), and Group A B-haemolytic streptococci (Crowe et al, 1973) have been associated with resistance to diseases caused by these pathogens.

Jyssum and Allun.ans (1984) recently reported that meningococci were capable of producing substances (meningococcocin) inhibitory to other strains of meningococci. In the present work, this phenomenon was not observed among the strains of meningococci used, but does not preclude the existence of such strains in this environment and may only reflect low incidence of these strains.

An interesting aspect of the present work is the novel method designed to investigate bacterial antagonism If the precise nature and mode of action of the anti-meningococcal factors can be further elucidated, it may prove to be useful in preventing meningococcal colonisation of the upper-respiratory tract and other easily accessible mucosal surfaces.

The present work has demonstrated that some bacteria normally resident in the human nasopharynx may produce substances inhibitory to meningococci. It is conceivable that such a phenomenon may play a significant role in determining, depending on the

concentrations of such bacteria, those individuals who will eventually become nasopharyngeal carriers of N. meningitidis and subsequently develop meningococcal disease. It has also described a novel and simple method for investigating antagonistic interactions between organisms using filtrate impregnated filter paper discs.

CHAPTER 5

THE ROLE OF ENDOTOXIN IN THE PATHOGENICITY OF CARRIER AND CLINICAL ISOLATES OF *NEISSERIA MENINGITIDIS* IN A CHICK EMBRYO MODEL

INTRODUCTION

Neisseria meningitidis is associated with epidemic outbreaks of leptomeningeal inflammation and fulminating septicaemia which continue to constitute a major health problem worldwide (Greenwood *et al.*, 1979; Whittle *et al.*, 1975; Sippel, 1981; Apicella, 1986).

Meningococcal meningitis may be rapidly progressive and approximately half of those hospitalized within the first 24 hours of the onset of the illness, subsequently die (DeVoe, 1982). Therefore meningococcal meningitis is regarded as a medical emergency. Meningococcaemia may progress to septic shock with intravascular clotting, irreversible circulatory failure and shock, a condition known as Waterhouse-Friedrichsen Syndrome. It is usually associated with a characteristically high mortality rate (Finklestein, *et al.*, 1969).

There are several antigenic or toxic components of *N. meningitidis* believed to be important in the pathogenesis of meningococcal disease. These include the group specific capsular polysaccharide, the lipopolysaccharide (LPS) endotoxin-cell-wall complex and a genus specific protein (Paterson, 1980; DeVoe, 1982; Apicella, 1986).

A significant proportion of the meningococcal

cell wall comprises the macromolecular LPS - endotoxin complex (DeVoe, 1982). These are released into the extracellular environment during the log-phase of growth or upon autolysis and death of the organism. Injection of small doses of endotoxins derived from a variety of gram-negative organism into experimental animals, have been shown to elicit dramatic changes in blood, body temperature, metabolism, humoral and cellular immunity and resistance to infection (DeVoe, 1982; Braude, 1986). In most animals, injection of LPS in doses ranging from 0.1 µg to 10 µg will cause rapid onset of fever neutropaenia and hypotension Berry, 1975; DeVoe et al, 1979; Braude, 1986).

Endotoxins derived from strains of Neisseria meningitidis have been used extensively in studies on mammalian tissue-injury and have been demonstrated to elicit generalised Schwartzman reaction in rabbits (Gaskin and Dalldorf, 1976; Ducker and Simmons, 1978; Apicella, 1986).

Many of the physiologic and immunohisto-pathologic events subsequent to the injection of endotoxin into experimental animals, are commonly observed in patients developing septic shock during the course of meningococcal disease (Berry, 1980). Chick embryos have been used for the study of neisserial

virulence, endotoxic activity and passive immunity by several investigators (Buddingh and Polk, 1939; Finklestein, 1967; Ueda et al., 1971; Odugbemi, 1978; Hafiz, 1986). However, there are no reports in the literature comparing the effects of endotoxin from meningococcal strains isolated from clinical cases and those isolated from carriers using a chick embyro model.

The present study was carried out with the aim of comparing the activities of endotoxin of sero-groups A, B, C, W-135 and also those of clinical and carrier strains of meningococci. In addition, this work also envisaged to investigate the possibility that clinical strains may be more lethal in their effects than carrier strains. A chicken embryo model (Hafiz, 1986) was used for this study.

MATERIALS AND METHODS

Bacterial strains

The following standard bacterial strains were used for this study. *N. meningitidis* serogroup A, ATCC, 1894; *N. meningitidis* Group B, ATCC 2091; Group C, ATCC 1054; and a strain of group W-135 which were kindly provided by Prof. Tolu Odugbemi, Department of Medical Microbiology, College of Medicine, University of Lagos. A strain of *N. meningitidis* group C isolated from a clinical case of meningitis in Lagos University Teaching Hospital and designated LUTH 405 and a strain of Group C meningococcus isolated from an asymptomatic carrier in Ijede, Lagos and designated M625, were also used.

Experimental animals

The embryonated chicks used in this study were obtained from a well-known poultry farm, Samrose Agricultural Industries Ltd., Agege, Lagos. The eggs were maintained in an humidified incubator at $37^{o}C$ and rotated every 6h until required at 12 days of age.

Media

The solid media used during this study were chocolate agar and Thayer-Martin agar comprising GC agar base (Oxoid).

Brain heart infusion broth (Oxoid) and Mueller Hinton broth (Oxoid) were also used.

Preparation of endotoxin

Endotoxin was extracted and prepared from the isolates of the different serogroup strains of N. meningitidis using a modification of the method previously described by Hafiz (1986) used for the extraction of endotoxin from Neisseria gonorrhoeae. The strains of meningococci were resucitated from lyophylised ampoules and cultured onto chocolate agar. Isolates were confirmed by gram-stain (gram-negative diplococci), slow reaction with superoxol reagent (Arko and Odugbemi, 1984) presence of catalase and oxidase activity and their ability to utilise only glucose and maltose incorporated as 1% in a serum-free agar slope (Flynn and Waitkins, 1978). They were then serogrouped against a complement of meningococcal antisera (CDC, Atlanta, Georgia) by the slide agglutination procedure.

The overnight growth of respective serogroups on chocolate agar, were harvested into sterile universal bottles (about 20-25 plates were needed to yield about 1g wet weight of organisms). To this was added 20ml of 45% aqueous phenol following the

procedure of Maeland (1968). The aqueous phenol suspensions of meningococci were incubated at 37°C for 30 minutes and centrifuged at 2,500 rpm for 15 minutes. This resulted in three distinct layers. The centrifuged contents were kept at 4°C for 24 hours after which the free endotoxin could be recovered in the aqueous phase (represented by the uppermost layer). This was done by pipetting the top layer into sterile universal bottles (Sterilin, Middlesex) using sterile pasteur pipettes.

The volatile phenol was further separated from the aqueous layer by heating in an open container in a water-bath at 55°C for 1 hour. Four milligrams of RNase A (BDH) were then added to the cooled endotoxin solution which was incubated further at 37°C for 18 - 24 hours (Perry et al., 1975). The crude endotoxin extract which resulted, was dialysed against cold distilled water for 2 days in a Visking cellophane dialysis tubing. The product was lyophylised in a conical flask in a freeze-drying machine.

Endotoxin was recovered as a powder, weighed and diluted with sterile distilled water to give a stock solution of 0.1mg/ml. This was kept in sterile universal bottles at 4°C until required.

Preparation of live meningococci

Live suspensions of N. meningitidis were also prepared for inoculation into the chick-embryo. The meningococci of the respective serogroups were grown separately on chocolate agar (GC agar base) for 18 hours and suspended in sterile PBS maintained at pH 7.2, in MacCartney bottles and diluted to give a concentration of 10^6 organisms/ml determined by diluting a suspension made to the equivalent of MacFarland's barium sulphate opacity tube No. 1. The viability of the suspensions was checked by the observation of meningococcal growth upon subculture onto chocolate agar.

Preparation of heat-killed N. meningitidis

A suspension of 10^6 organisms/ml of meningococci of each representative strain in sterile PBS, was heated in a water-bath maintained at $60^{\circ}C$ for 30 inutes. The sterility of the suspensions was checked by observing the absence of growth upon subculture on chocolate agar after 18 - 24h incubation in 5 - 10% CO_2 in air.

Preparation of Formal-saline-treated N. meningitidis

The overnight growth of representative strains of N. meningitidis on chocolate agar was harvested in

PBS pH 7.2 and centrifuged at 600 revolutions per minute (rpm) for 30 minutes. The supernatant was decanted leaving the deposited cells in the universal bottles used. The deposit was heated in 10% formal saline (prepared as formaldehyde 10% in normal saline) overnight. The cells were then washed three times in PBS at pH 7.2 and centrifuged at 600 rpm for 30 minutes. The cells were resuspended in PBS to give a concentration of 10^6 cfu/ml using the MacFarland's barium sulphate opacity tube No. 1 and making the relevant dilutions.

The embryonated eggs were examined over a candler in a dark room and a rectangle of 4mm x 6mm was drawn over a prominent chorioallantoic vein observed under candling. A dental drill was used to carefully cut the egg-shell over the markings and the shell flap was lifted gently to expose the intact chorioallantoic membrane. The eggs were then placed in a humidified incubator at $37^{o}C$ for 2h to allow fixation of the veins before inoculation and to allow the embryo to settle.

Effect of endotoxin

A set of 6 eggs per concentration of endotoxin ranging from 1 µg/ml, 10 µg/ml, 20 µg/ml and 50 µg/ml was inoculated with 0.1ml of each concentration of endotoxin for each serogroup

into the chorioallantoic vein of the embryo using a sterile 27 gauge needle (stenstet). The inoculated eggs were placed along with the controls, inoculated with 0.1mlPBS, in a humidified incubator at 37°C.

Effect of formalin treated meningococci

A set of 6 eggs was inoculated with 0.1ml 10^9, 10^8, 10^7 and 10^6 formalin-killed meningococci per serogroup through the chorioallantoic vein as described above. The chick embryo were also inoculated with 0.1ml of 10^9, 10^8, 10^7 and 10^6 heat-treated meningococci per millilitre through the chorioallantoic vein. The same procedure was repeated for live-organisms using the same concentration of organisms.

All the inoculations were performed aseptically under sterile conditions under an inoculating hood which had been sterilized by exposure to ultra-violet radiation for some hours. Sterility was confirmed by absence of growth of organisms on an exposed plate of blood agar placed in the hood and later incubated at 37°C for 18 - 24h.

The eggs were replaced in the humidified incubation at 37°C after inoculation and examined every six hours over a period of 72 hours. Suspected

deaths were investigated by observing lack of movement of the embryo under the candler. The deaths were confirmed by opening up the eggs.

For each set of eggs inoculated with endotoxin, heat-or formal-saline treated meningococci a control was se up comprising 12 day old chick embryo inoculated with 0.1ml of PBS (pH 7.2) and also observing over a period of 72 hours along with the other experimental eggs. The 50 percent lethal dose (LD_{50}) of the different inocula was calculated using the Reed and Muench (1938) method which is based on the assumption that animals dying at a stated dose would also have been killed by greater amounts of agent and conversely that those surviving would also have survived smaller doses (Cruickshank et al, 1975).

After opening the embryo, parts of the body (brain, heart, lungs and other tissues were chopped separately and homogenised into PBS (pH 7.2) and 0.1ml was spread onto Thayer Martin medium and checked for the recovery of meningococci after incubation in 5 - 10% CO_2 in air for 18 - 24h. Specimens were also plated onto blood agar plates to cross-check if other organisms could be isolated.

RESULTS

The results show that injection of 0.1 µg/ml and 1µg/ml of endotoxin obtained from all the strains used did not have any lethal effect on the chick embryo., However, at a concentration of 10 µg/ml, 6/6(100%) of chick embryo inoculated with whole-cell of group A and group C clinical isolates, 83% inoculated with Group B and C standard strains and 67% inoculated with Group C carrier isolate died within 36h inoculation of the endotoxin via the chorioallantoic vein (Table 5.1).

Observing the inoculated eggs over time intervals of 6h each showed that all those inoculated with 10 µg/ml endotoxin from the clinical strains died within 12hs, while those inoculated with endotoxin from other groups died 18 hours after inoculation.

Inoculation of 50 µg/ml of endotoxin from all strains resulted in death of all the chick embryo by the end of 36 hours (Table 5.1). Eighty-three percent (5/6) of those inoculated with this concentration of clinical isolate died within 6 hours of inoculation while other strains took up to 12 hours for lethal effects to manifest.

Table 5.2 shows the effect of formalin-killed organisms inoculated at concentration of 10^5, 10^6, 10^7 and 10^8 bacterial cell/ml into chick embryo model. A concentration of 10^7 formalin treated cells/ml of Group A and Group C clinical isolates caused death of two out of six (34%) chick embryos inoculated for these organisms. However, at a

higher concentration of formalin-treated organisms for these two groups, the lethality dropped to 17% for group A and 0% for group C clinical isolates. No serogroup caused greater than 17% lethality at 10^8 cells per ml (Table 5.2).

Inoculation of 10^7 heat killed meningococci resulted in the death of 83% (Group C standard strain and Group C LUTH 405 strain) 50% (Croup A and group W-135 and Group C carrier strain. All the serogroups caused death of 100% of chick embryo upon inoculation of 10^8 heat-killed meningococci except Group W-135 where mortality was 83%. (See table 5.3).

In the control groups which were inoculated with PBS there were no deaths within the time period.

Table 5.4 summarises the effect of inoculating chick embryo with different concentrations of live meningococci from the various serogroups and clinical and carrier strains. 10^6 live organisms of clinical isolate caused death of 50% of chick embryo inoculated. At 10^8 organisms/ml all serogroup strains caused death in all chick-embryo inoculated except group W-135 in which 67% of chicks were killed at this concentration of live meningococci. The LD50 for meningococcal endotoxin ranged between $10^{-3.5}$ and 10^{-2} negative log-titre for group A and W-135 meningococci respectively (Table 5.5). The LD50 for live-meningococcal inocula is shown in table 5.7, the lowest being 10^6 organisms/ml for group C, clinical isolate. Table 5.6

shows the LD50 for heat-killed meningococcal inocula injected into the chick-embryo; Group C, M625, carrier isolate had the lowest LD50 of 10^7 while the highest was for Group C LUTH 405 clinical isolate ($10^{7.39}$).

Table 5.1 Effect of inoculation of 0.1µg 1µg, 10µg and50µg/ml of meningococcal endotoxin of different serogroups into chick embryo 36 hours after inoculation

Group and strain of Meningococcus	Number (%) of chick embryo death after 36h inoculation of endotoxin of concentration				
	0.1µg/ml	1µg/ml	10µg/ml	50µg/ml	Control
Group A	0/6(0%)	0/6(0%)	6/6(100%)	6/6(100%)	0/6(0%)
Group B	1/6(17%)	0/6(0%)	5/6(83%)	6/6(100%)	0/6(0)
Group C	1/6(17%)	0/6(0%)	5/6(83%)	5/6(83%)	0/6(0)
Group W-135	0/6(0%)	0/6(0%)	3/6(50%)	6/6(100%)	0/6(0)
Clinical isolate	0/6(0%)	0/6(0%)	6/6(100%)	6/6(100%)	0/6(0)
Carrier isolate	1/5(17%)	0/6(0%)	4/6(67%)	6/6(100%)	0/6(0)

Table 5.2 Effect of Formalin-treated meningococcal inocula chick embryo 36 hours after inoculation

Strains of *N. meningitidis*	Number (%) of chick embryo dead after 36h inoculation of formalin killed meningococci at concentration				
	10^5/ml	10^6/ml	10^7/ml	10^8/ml	Control
Group A	0/6(0)	0/6(0)	2/6(34)	1/6(17)	0/6(0)
Group B	0/6(0)	1/6(17)	0/6(0)	1/6(17)	1/6(17)
Group C	1/6(17)	0/6(0)	0/6(0)	0/6(0)	2/6(34)
Group W-135	0/6(0)	0/6(0)	0/6(0)	0/6(0)	0/6(0)
Clinical isolate Group C LUTH 405	1/6(17)	0/6(0)	2/6(0)	2/6(34)	0/6(0)
Carrier isolate Group C M625	1/6(17)	0/6(0)	0/6(0)	0/6(0)	0/6(0)

Table 5.3 Effect of heat-killed meningococcal inocula on chick embryo 35 hours after inoculation

Group/strain of meningococcus Strains of *N. meningitidis*	Number (%) of dead chick embryo at concentration of				
	10^5/ml	10^6/ml	10^7/ml	10^8/ml	PBS Control
Group A	0/6(0%)	1/6(17%)	3/6(50%)	6/6(100	0/6(0)
Group B	0/6(0%)	0/6(0%)	4/6(67%)	6/6(100)	0/6(0)
Group C	0/6(0%)	1/6(17%)	5/6(83%)	5/6(83%)	1/6(0)
Group W-135	0/6(0%)	2/6(33%)	3/6(50%)	6/6(100%)	0/6(0)
Group C LUTH 405	0/6(0%)	0/6(0%)	5/6(83%)	6/6(100%)	0/6(0)
Group C M625	0/6(0%)	0/6(0%)	3/6(50%)	6/6(100%)	0/6(0)

Control = PBS

Table 5.4 Effect of live meningococcal inocula on chick embryo 36 hrs post inoculation

Group/strain of meningococcus Strains of *N. meningitidis*	Number (%) of dead chick embryo at concentration of				
	10^5	10^6	10^7	10^8	PBS Control
Group A	0/6(0)	0/6(0)	4/6(67)	6/6(100)	0/6(0)
Group B	0/6(0)	2/6(34)	4/6(67)	6/6(100)	0/6(0)
Group C	1/6(17)	1/6(17)	3/6(50)	6/6(100)	0/6(0)
Group W-135	0/6(0)	1/6(17)	2/6(34)	4/6(67)	0/6(0)
Clinical isolate	0/6(0)	3/6(50)	6/6(100)	6/6(100)	0/6(0)
Carrier isolate	0/6(0)	1/6(17)	4/6(67)	6/6(100)	0/6(0)

Table 5.5 Fifty-percent lethal dose of endotoxin from respective serogroups of *Neisseria meningitidis*

Group of *N. meningitidis*	LD50 (Negative log) of LD50 titre
Group A ATCC 1894	$10^{-3.5}$
" B ATCC 2091	$10^{-2.49}$
" C ATCC 1054	$10^{-2.5}$
" W-135	10^{-2}
" C, LUTH 405	$10^{-3.5}$
" C M625	$10^{-2.35}$

Table 5.6 Fifty-percent lethal dose (LD50) of heat killed meningococcal inocula for different serogroups of *N. meningitidis*

Group of *N. meningitidis*	LD50 (organisms/ml)
Group A, ATCC, 1894	$10^{7.15}$
Group B, ATCC 2091	$10^{7.24}$
Group C, ATCC 1054	$10^{7.4}$
Group W-135	$10^{7.3}$
Group C LUTH 405	$10^{7.39}$
Group C M625	$10^{7.0}$

Table 5.7 Fifty-percent lethal dose LD50 of live meningococcal inocula for different serogroups

Group and Strain of *N. meningitidis*	LD50 organisms/ml
Group A 1894	$10^{7.24}$
" B 2091	$10^{7.15}$
" C 1054	$10^{7.29}$
Group W-135	$10^{8.6}$
" C LUTH 405	10^{6}
" C,M625	$10^{7.2}$

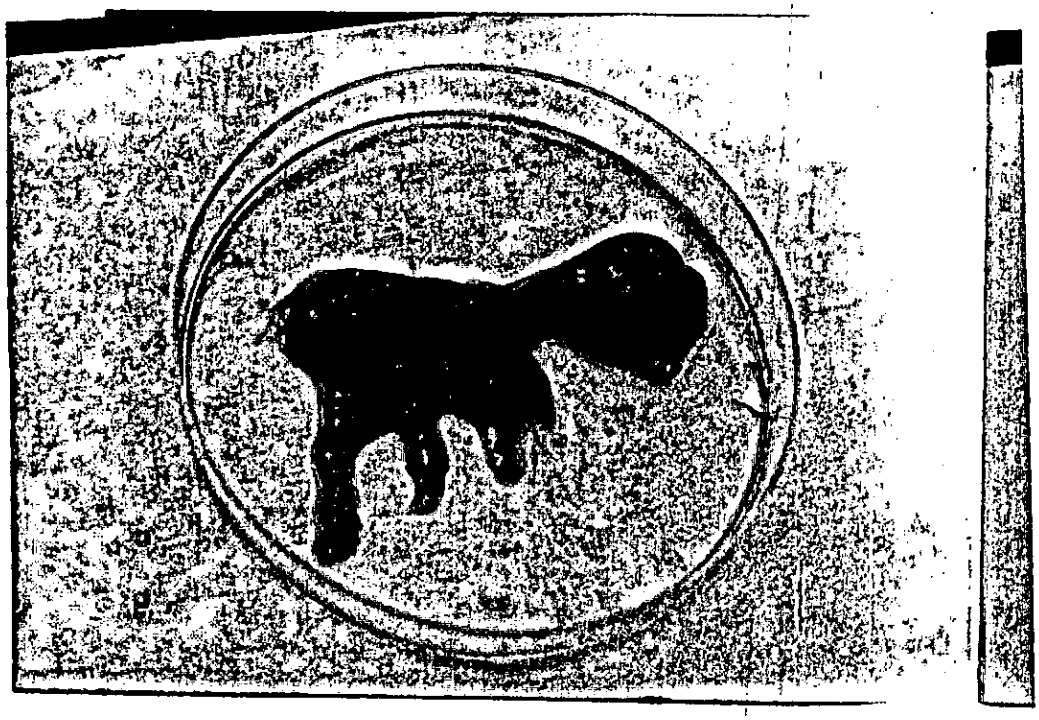

Fig. 5.1 Death of 12-day old chick embryo six hours after inoculation with 10µg endotoxin of *N. meningitidis* Group C LUTH 405.

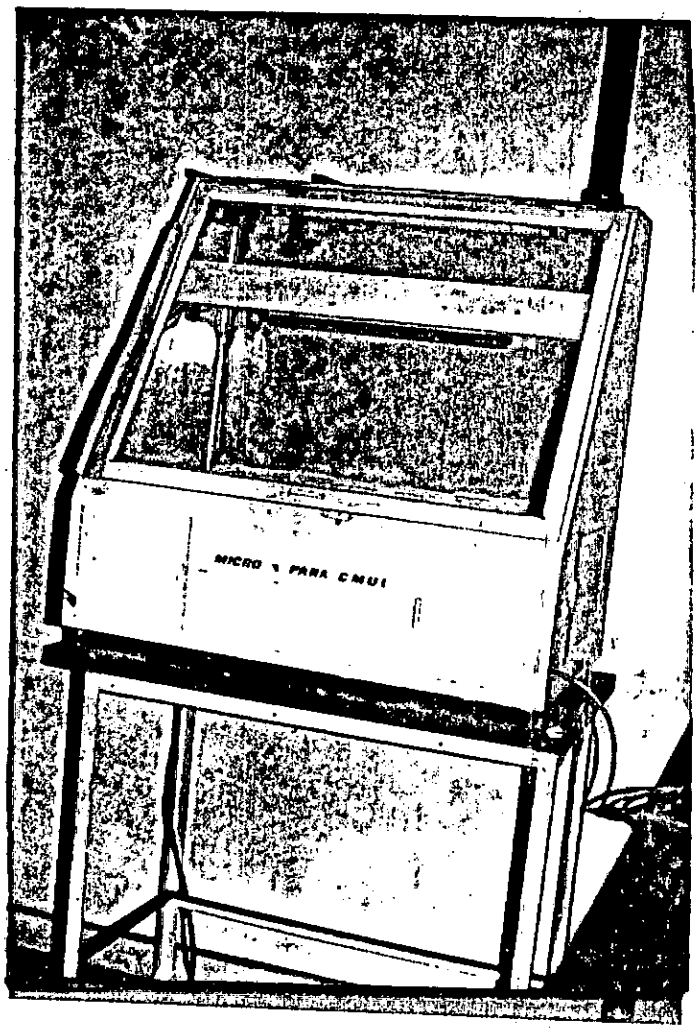

Fig. 5.2 Ultra-violet light sterilized inoculating hood, Dept. of Medical Microbiology and Parasitology, College of Medicine, University of Lagos.

DISCUSSION

The results of the present study have demonstrated that meningococcal endotoxins extracted and purified from Neisseria meningitidis of serogroups A, B, C and W-135 are lethal for 12 day old chick embryo subsequent to chorioallantoic vein inoculation of the endotoxin. This is similar to the finding of Kaino (1982) and the report of DeVoe (1982) who demonstrated that embryonated chicks are susceptible to meningococcal endotoxin subsequent to yolk sac inoculation.

Although endotoxin from each serogroup was lethal to chick embryo, it is interesting to note from the results of the present study that group W-135 appeared to be less potent (LD50 = 10^{-2}) than those of groups A, B and C, and the effects being manifest only after 24 hours of chorioallantoic inoculation of 10 µg of endotoxin. Group W-135 is not commonly reported as a cause of meningococcal epidemics. The lesser capacity of this group to cause disease in humans may be reflected in the lower potency of endotoxin from this serogroup compared to A, B and C which have been classically associated with epidemics (Whittle et al., 1975; Sippel, 1981; Apicella, 1986).

Comparing the lethalality of clinical and carrier isolates of group C meningococci for the chick embryo, it was observed during the course of this work, that the endotoxin from the clinical isolate caused death of the

embryo within 12 hours of chorio-allantoic vein inoculation of 10 µg/ml of the endotoxin whereas with endotoxin from the carrier. This result further strengthens the popular thinking that the endotoxin is the primary pathogenicity factor of the meningococci. The deaths of two chick-embryo inoculated with formalin killed organisms may be attributed to contamination with formalin which may not have been totally removed during washing of the cells and as thus appears to be incidental.

It is interesting to note that heat-killed organisms at concentrations of 10^8 and 10^7 organisms/ml had similar lethal effects to the inoculation of endotoxin. Heating, has been shown to liberate endotoxin from gram-negative organisms (Apicella, 1986). However the inoculation of 10^8 live meningococci caused death of the chick embryo only after 48 hours of chorioallantoic vein inoculation. Although the reason for this is not immediately clear it may not be unrelated to the slower rate of endotoxin release via blebs in the cell-membrane and eventual autolysis of meningococcal cells. This result seems to de-emphasize the invasive capacity of the meningococcal organisms and stresses the role of endotoxin in pathogenicity. However, recovery of the meningococci in chick embryo tissues in similar numbers to the inoculating dose, on solid media, indicates their ability to establish and survive within this host.

In summary, the results of this study have demonstrated that meningococcal endotoxin may play an

important role in the pathogenicity of both clinical and carrier isolates of N. meningitidis.

It is feasible to speculate that the clinical strain (LD50 = $10^{-3.5}$) than carrier strain (LD50 = $10^{-2.35}$) produced a more potent endotoxin thus suggesting the quantitative and qualitative differences between the endotoxins produced by these strains from the same serogroup. If this is so, the implications for the role of clinical isolates in disease causation are important, especially if endotoxin is to be regarded as a principal pathogenicity factor of the meningococcus. Other reports in the literature also provide evidence that clinical strains appear to be more virulent than carrier strains (Craven, et al., 1980, Andersen, 1978).

Holbein (1981) had earlier reported that clinical strains of N. meningitidis are more virulent for mice than carrier strains. A useful comparism of the pathogenic effects of different preparations of meningococcal organismx and their endotoxins may be readily drawn from this study. For instance it is interesting to note that injection of formalin-killed organisms via chorioallantoic vein route in concentrations of up to 10^8 organisms/ml was unable to cause death in the 12 day old chick embryo model used. This is not surprising and is similar to the finding of Hafiz (1986) who reported that formalin-killed Neisseria gonorrhoease could not elicit death of chick embryo. Formalin treatment of gram-negative organisms

such as meningococci, kills the bacterial cells while simultaneously maintaining the integrity of the cell-wall and preventing the release of endotoxin. It would be interesting to determine the factors which dictate whether a strain should be a carrier strain or a clinical strain and whether such statuses are interchangeable.

CHAPTER 6

EFFECT OF TREATED AND UNTREATED MENINGOCOCCAL CELLS AND ENDOTOXINS ON CILIARY ACTIVITY OF RESPIRATORY MUCOSA OF RAT TRACHEA

INTRODUCTION

Although *Neisseria meningitidis* continues to be an important cause of disease in both industrialised and developing countries (Sippel, 1981; Binkin and Band, 1982) our knowledge of the mechanisms of its pathogenicity is still incomplete. Meningococci gain entrance to the human body via the nasopharynx and after becoming implanted at this site, they may set up local inflammatory reactions such as rhinitis, or they may remain completely quiescent without giving rise to any symptoms (DeVoe, 1982; Apicella, 1986). It is clear that to establish within the host, the meningococcus must necessarily circumvent host defence mechanisms.

The possession of pili for attachment to mucosal epthelial cell surfaces and capsule to resist phagocytosis, have been postulated as some of the factors which help meningococci to survive in the adverse host environment (Craven *et al.*, 1980; Sippel, 1981). The mucociliary epithelial linings of the nasopharynx and respiratory tract, by their rapid movement create steady currents which transport mucus and foreign particles embedded in it to the pharyngeal mucociliary escalator (Wilson and Miles, 1975). This suggests that the ciliated

epithelium acts as a non-specific host defence or mechanised barrier preventing the colonisation and establishment of bacteria within the host. It is conceivable that organisms such as N. meningitidis reported as nasopharyngeal colonists (Craven, 1980; DeVoe, 1982) may have means of overcoming these defences.

Denny (1974) and Johnson and Inzana (1986) reported that Haemophilus influenzae could elicit loss of ciliary activity in organ cultures of rat trachea treated with lipo-oligosaccharide (LOS). H. influenzae was also demonstrated to cause sloughing of the epithelial cells of the respiratory mucosa (Johnson and Inzana (1986); the LOS of H. influenzae being the endotoxin. The endotoxin of the outer membrane of the meningococcus has been studied for its toxic properties (Davies and Arnold, 1974), antigenic properties (Zollinger and Mandrill, 1980) and biochemistry (Jennings et al., 1980). However, the effects of meningococcal endotoxin on ciliated respiratory epithelium has not been reported in the literature.

In order to further our understanding of the pathogenesis of meningococcal infection the present work was carried out to investigate the effects of meningococcal endotoxin on the ciliary activity of rat respiratory epithelium.

MATERIALS AND METHODS

Bacterial Strains:

The following bacterial strains previously described in Chapter 5 were used during the course of this work: N. meningitidis serogroup A, (ATCC 1894), serogroup B, (ATCC 2091), Serogroup ,(ATCC 1054) and serogroup W-135 which were kindly provided by Prof. Tolu Odugbemi, Department of Medical Microbiologu, College of Medicine, University of Lagos. A strain of group C, N. meningitidis isolated from a clinical case of meningitis at the Lagos University Teaching Hospital (LUTH) designated (LUTH CS 405) and a strain of group C, N. meningitidis isolated from an asymptomatic carrier in Ijede designated M625, were also used. All the strains were resuscitated from freeze-dried ampoules prior to use.

Experimental Animals:

The experimental animals used for this study were white albino rats obtained from the Laboratory Animal Centre, College of Medicine, University of Lagos (CMUL). The rats weighed an average of 20g. They were fed ad-libitum on standard rat chow and water.

Media:

The following media were used for the present study: Chocolate agar comprising GC agar base (Oxoid) and Thayer-Martin agar, (see appendix).

Preparation of Endotoxin from serogroups of Neisseria Meningitidis:

The preparation of endotoxin was as described in detail in chapter 5 of this thesis. Overnight culture of meningococci from respective serogroups on chocolate agar were harvested into sterile universal bottles and 20ml of 45% aqueous phenol added (Maeland, 1968). The resulting aqueous suspensions were incubated at 37^{o}C for 30 minutes then centrifuged at 2,500rpm for 15 minutes and kept at 4^{o}C for 24 hours after which the free endotoxin was recovered in the aqueous phase. Volatile pehnol was separated from the aqueous layer and 4mg of RNase A (BDH) was added. The crude endotoxin was dialysed against cold distilled water for 2 days. The dialysate was lyophylised in a high vacuum freeze drying machine (model and manufacturer's address)

The endotoxin was recovered as a powder and weighed and diluted with PBS, HEPES and MEMS; pH 7.2 to give a stock solution of 0.1 mg/ml.

Preparation of live suspension of meningococci:

The meningococci of respective serogroups were grown separately on chocolate agar for 18 hours and suspended in PBS, pH 7.2 in MaCartney bottles and diluted to give concentrations of 10^9, 10^8, 10^7, 10^6 cfu/ml using McFarland's barium sulphate opacity standards.

Preparation of heat-killed N. meningitidis:

The live organisms were prepared as described above but were heated in a water bath for 30 minutes at 60°C. The sterility of the suspensions was checked by absence of growth upon subculture on chocolate agar.

Preparation of formal saline treated suspension of N. meningitidis:

The bacterial cells of representative strains were harvested in PBS (pH 7.2) in universal bottles and centrifuged at 600xg for 30 minutes. The resulting deposit was treated with 10% formal saline overnight. The cells were washed three time in PBS (pH 7.2) by centrifugation at 600 x g after which they were resuspended in PBS to a concentration of 10^9 cfu/ml and diluted down to 10^6 cfu/ml. The

suspensions were also checked for sterility absence of growth on chocolate agar, 18 - 24h at 37°C.

Preparation of ciliated respiratory epithelium of rats:

The rats were dissected and the trachea removed asceptically by the method of Johnson et al. (1983) and placed in sterile petri-dishes (sterilin) containing Eagle's minimum essential medium (MEM) buffered at pH 7.2 with 50MM HEPES (BDH Chemicals, Ltd.) supplemented with 3.75 µg/ml of vancomycin prepared with sterile distilled water. Each trachea was cut into a series of rings one cartilage (about 0.2mm thick, with a sterile blade. They were then placed in sterile tissue culture tubes containing 1ml of MEM and incubated at 37°C in 5-10% CO_2 in air. This was a modification of the method described by Johnson and Inzana (1986).

Assessment of ciliary activity:

Motility of cilia was ascertained by direct microscopic observation using an inverted microscope (Zeiss) with dark-background illumination (Cherry and Taylor-Robinson, 1974). If motile, cilia could be observed beating from the inner circumference of the tracheal lumen. If ciliary activity was

less than 50% compared to the control, the environment was considered to be ciliostatic (+) while if the activity was greater than 50% after treatment with the different materials it was regarded as non-ciliostatic (-).

Assessment of the ciliostatic effect of endotoxin:

Purified endotoxin powder was reconstituted in MEMS + PBS + HEPES and appropriate antibiotics to give a stock solution of 100 µg/ml and diluted to give concentrations of 1, 10, 20, 50 µg/ml of endotoxin which were placed in 5ml amounts into sterile petridishes containing the CRE of the rat tracheas.

RESULTS

The effect of endotoxin:

The ciliostatic activities of the different concentration of the endotoxin are demonstrated in table 6.1 - 6.3. There was no ciliostatic effect by any of the serogroups after 6 hours and 12 hours of treatment with 10 µg/ml of endotoxin. Only reference standard strain of group C, ATCC 1054; local clinical strain of group C (LUTH 405) and a carrier strain of Group C (M625) exposure of the ciliated epithelium to the endotoxin for 18 hours. However, 10 µg/ml of the endotoxin from all the meningococcal strains at 24 hours of exposure: these observations are aptly demonstrated in table 6.1.

Table 6.2 shows the ciliostatic effect of 20 µg/ml of meningococcal endotoxin extracted from different serogroups on rat ciliated epithelium. At this concentration the endotoxin from all the strains of meningococci caused ciliostasis after 18 hours of exposure of ciliated epithelium. Only the local clinical strain of Group C (LUTH 405) induced ciliostasis after 12 hours at this concentration. Control of PBS plus HEPES and MEMS had no effect on the ciliated epithelium up to 24 hours.

At a concentration of 50 µg/ml the endotoxin of all the meningococcal serogroups tested caused ciliostasis much more rapidly than at lower concentrations; ciliostasis was achieved in all but one case by 12 hours. Group W-135 elicited ciliostasis after 18 hours being the only exception (see table 6.3). Notably, local clinical strain of Group C LUTH 405 induced ciliostatic effect at this concentration at a faster contact time of 6 hours, thus being the only strain to demonstrate this effect at an earlier time. The lowest concentration of edotoxin at which ciliostatic effect could be observed on the ciliated epithelium by any of the serogroups was 10 µg/ml. There was no ciliostatic activity observed over a period of 30h after treating with 1 µg.

The effect of whole live cells on ciliary activity:

Table 6.4 shows the ciliostatic effect of treatment of rat ciliated epithelium with 10^9 cfu/ml of live meningococci on ciliated respiratory epithelium. Notably as was with the effect of the endotoxin group W-135 had the least effect on the CRE; ciliostasis only occurred at 30 hours contact-time with the organism. Groups A, B and C standard strains elicited ciliostasis after 18 hours of exposure. Control suspension made up of PBS, HEPES

and MEMS, set up in parallel did not effect the motility of the cilia for up to 30 hours distinctly however, clinical strain of Group C (LUTH 405, caused ciliostasis within 6 hours.

The effect of formalin-treated bacterial cells on ciliary activity:

The ciliostatic effect of 10^9 cfu/ml of formalin treated serogroups of meningococci on rat-ciliated epithelium is shown in table 6.5. Up to 24 hours none of the strains had effect on the ciliary mobility of the ciliated respiratory epithelium. However, after 30 hours exposure to the groups A and B of the standard strains and group C M625 carrier strain, there was seizure of ciliary activity.

The effect of heat killed bacterial cell on ciliary activity:

The ciliated epithelial cells of rat trachea were exposed to 10^8 bacterial cells/ml of heat killed meningococci over the same periods of time as before. The effect of the heat-killed meningococci on the CRE is demonstrated in table 6.6. Clinical strain of Group C, (LUTH405) caused ciliostasis after 12 hours while groups B and C

of the standard strains and group C, M625 carrier isolate caused ciliostasis after 18 hours. Group A, ATCC 1894 induced ciliostasis only after 24 hours. The cilia of respiratory epithelium in the control remained motile for up to 24 hours.

Table 6.1

Ciliostatic effect of 10 µg/ml of endotoxin from different meningococcal serogroups on Rat ciliated respiratory epithelium (CRE)

Endotoxin from different strains of *N. meningitidis*	Ciliostatic on CRE effect after treatment with endotoxin:			
	6h	12h	18h	24h
Group A, ATCC 1894	-	-	-	+
Group B, ATCC 2091	-	-	-	+
Group C, ATCC 1054	-	-	+	+
Group W-135	-	-	-	+
Group C, LUTH 405	-	+	+	+
Group C, M625	-	-	+	+
Control (PBS+HEPES+MEMS)	-	-	-	-

Notes:

\+ = Ciliostasis

\- = No ciliostasis

CRE = Ciliated respiratory epithelium.

Table 6.2

Ciliostatic effect of 20 µg/ml of endotoxin from different meningococcal serogroups on Rat ciliated respiratory epithelium

Endotoxin from different strains of *N. meningitidis*	Ciliostatic effect on CRE after treatment with edotoxin:			
	6h	12h	18h	24h
Group A, ATCC 1894	-	+	+	+
Group B, ATCC 2091	-	-	+	+
Group C, ATCC 1054	-	-	+	+
Group W-135	-	-	+	+
Group C, LUTH 405	-	-	+	+
Group C, M625	-	-	+	+
Control (PBS+HEPES+MEMS)	-	-	+	+

\+ = Ciliostatis

\- = No ciliostasis

Table 6.3

Ciliostatic effect of 50 µg/ml of endotoxin from different meningococcal serogroups on Rat ciliated respiratory epithelium.

Endotoxin from different strains of *N. meningitidis*	Ciliostatic effect on CRE after treatment with endotoxin:			
	6h	12h	18h	14h
Group A, ATCC 1894	–	+	+	+
Group B, ATCC 2091	–	+	+	+
Group C, ATCC 1054	–	+	+	+
Group W-135	–	–	+	+
Group C, LUTH 405	+	+	+	+
Group C, M625	–	+	+	+
Control (PBS+HEPES+MEMS)	–	–	–	–

\+ = Ciliostasis

– = No ciliostasis

Table 6.4

Ciliostatic effect of 10^8 live bacteria ml on Rat ciliated Respiratory epithelium

Meningococcal strains	Ciliostatic effect on CRE after treatment with 10^8 live bacteria/ml on ciliated respiratory epithelium:				
	6h	12h	18h	24h	30h
Group A, ATCC	-	-	+	+	+
Group B, ATCC	-	-	+	+	+
Group C, ATCC	-	-	+	+	+
Group W-135	-	-	-	-	+
Group C, LUTH 405	+	+	+	+	+
Group C, M625	-	-	+	+	+
Control (PBS+HEPES+MEMS)	-	-	-	-	-

\+ = Ciliostasis

\- = No ciliostatic effect

Table 6.5

Ciliostatic effect of 10^9 cfu/ml of formalin treated serogroups of meningococci on rat ciliated respiratory epithelium

Meningococcal strains	Ciliostatic effect on CRE after treatment with formal-saline:				
	6h	12h	18h	24h	30h
Group A, ATCC 1984	-	-	-	-	+
Group B, ATCC 2091	-	-	-	-	+
Group C, ATCC 1054	-	-	-	-	-
Group W-135	-	-	-	-	-
Group C, LUTH 405	-	-	-	-	-
Group C, M625	-	-	-	-	+
Control (PBS+HEPES+MEMS)	-	-	-	-	-

\+ = ciliostasis

\- = No. ciliostasis

Table 6.6

Ciliostatic effect of 10^8 cfu/ml of meningococci after heat-treatment on Rat respiratory epithelium

Meningococcal strain	Ciliostatic effect on CRE after treatment with heat:			
	6h	12h	18h	24h
Group A, ATCC 1894	-	-	-	+
Group B, ATCC 2091	-	-	+	+
Group C, ATCC 1054	-	-	+	+
Group W-135	-	-	-	+
Group C, 405 LUTH	-	+	+	+
Group C, M625	-	-	+	+
Control (PBS+HEPES+MEMS)	-	-	-	-

\+ = Ciliostasis

\- = No ciliostasis

DISCUSSION

In order to become well established within the host system, bacteria must be capable of circumventing host defence mechanisms. The present study was carried out to determine the effect of meningococcal endotoxin and live organisms on the ciliated respiratory epithelium of rat trachea.

In this study it was observed that meningococcal endotoxin extracted from serogroups A, B, C and W-135 were all capable of causing ciliostasis in rat trachea ciliated epithelium within 18 hours of exposure to a concentration of 20 µg/ml, and 12 hours treatment with 50 µg/ml of endotoxin. This is the first report in the literature of the ciliostatic effect of meningococcal endotoxin in the ciliated respiratory epithelium in a rat model. A comparable finding similar to this observation was noted with Haemophilus influenzae type B lipo-oligosaccharide which was reported by Johnson and Inzana (1986) and Denny (1974). In these reports they found that their organism caused ciliostasis of the ciliated respiratory epithelium of rats.

The ciliated epithelial cells of the naso-pharynx and respiratory tracts acts as a non-specific host defence or mechanised barrier preventing the

establishment of bacteria within the host at these sites. If the ability of N. meningitidis to interfere with the ciliary motion of mucosal epithelial cells in vitro is a reflection of what happens in vivo, it is conceivable that this phenomenon constitutes an important virulence factor for the meningococcus. This is a novel approach to the pathogenesis of meningococcal infection which has not yet been explored. However, caution must be exercised in directly extrapolating these findings with animal tissue to similar circumstances in man. Regarding the capacity of meningococcal endotoxin to induce ciliostasis as a pathogenicity factor is at present speculative until these effects are produced in an in vivo experiment. However, it is interesting to note that Wilson et al. (1985) recently reported that lipo-oligosaccharide from H. influenzae could induce sloughing of epithelial cells of the ciliated nasal epithelium of humans. It is conceivable that this may also be the case with meningococcal endotoxin. It would be rewarding to conduct similar experiments with endotoxin from N. meningitidis. Furthermore, it is possible that endotoxin may cause even more extensive damage to the epithelial cell and facilitate penetration of meningococci into host cells.

The finding that rat trachea cilia is susceptible to the toxic effects of meningococcal endotoxin is interesting, moreso since these animals are generally normally resistant to infection with meningococci (Holbein, et al., 1982), as would be demonstrated in chapter 7. The mode of action of action of endotoxin in causing ciliostasis is not yet clear. However,

(1) interference with cellular metabolism

(2) recognition of cell-receptor sites on epithelium

(3) direct cellular toxicity are feasible obstructive mechanisms which may be explored further.

The ciliostatic potential of endotoxin from clinical isolate LUTH 405 was significantly greater than that of the carrier isolate M625 as observed by the ability of 10 µg/ml of the former to cause ciliostasis within 12 hours compared to 10 µg/ml in 18 hours for the latter. This is an important finding which has support from evidence produced by many other investigators who showed that there is greater virulence in clinical strains when compared with carrier strains (DeVoe 1982; Apicella, 1986).

The present work has demonstrated that meningococcal endotoxin from different meningococcal serogroups can produce ciliostatic effect on rat trachea ciliated epithelium *in-vitro* and indicates the possible role of endotoxin in maintaining meningococcal at mucosal surfaces by interfering with the function of ciliated epithelial cells. It provides further evidence for a novel role of endotoxin in the pathogenesis of meningococcal infection.

CHAPTER 7

STUDIES ON THE DEVELOPMENT OF EXPERIMENTAL ANIMALS MODELS FOR MENINGOCOCCAL MENINGITIS

INTRODUCTION

Niesseria meningitidis has the capacity to produce fulminating illness and death within a matter of a few hours from the onset of infection (Sippel, 1981; DeVoe, 1982; Apicella,1986) and outbreaks of meningococcal disease have frequently posed serious medical and epidemiological problems (Binkin and Band, 1983; Goldschneider et al., 1969). Despite the availability of effective antimicrobial therapy, the case fatality rate still remains alarmingly high and a significant proportion of infants and children may develop neurologic sequelae (Goldschneider. et al 1969; Cheever, 1965).

In spite of the wealth of knowledge available on the biology of N. meningitidis its pathogenicity is till poorly understood. This is not unrelated to the absence of a suitable animal model in which simulated disease may be observed. Man is the only known natural host of the meningococcus (Cheever, 1965; Bell and Silber, 1981) and laboratory animals are known to resist natural infection and only become susceptible to inoculation of meningococci when the infective dose is so large that it approaches, the lethal dose of the heat-killed organisms.

Several animal models have been designed for the study of meningococcal disease, each providing

certain advantages over the other. Harter and Petersdorf (1960) have suggested that for an ideal animal infection model:

1. the portal of entry and route of dissemination of the organism must be similar to those in humans
2. the bacterium must be virulent for humans as well as the experimental animals
3. the cause of disease must be relatively predictable
4. the disease must be reproducible within the limits of biological variation
5. the lesions in the experimental infection must be morphologically similar to those seen in humans
6. the techniques should be relatively simple
7. the pathophysiological events must be similar to those in humans especially if the animals are to be used for detailed studies on pathogenic mechanisms.

One of the earliest animal models for meningococcal infection was the monkey, reported by Flexner in 1907 (cited by DeVoe, 1982) and the mouse model by Miller (1933). When meningococci are suspended in 2.5% hog gastric mucin a smaller number of organisms is required to initiate fatal infection

than when phosphate buffered saline is used.

Brener, DeVoe and Holbein (1981) demonstrated that N. meningitidis increased in virulence after in vitro iron limited growth at low pH and Latendre and Holbein (1983) showed that iron availability enhanced experimental meningococcal infection in mice. Holbein (1981) found many of the forms of iron used to be highly toxic for mice and reported that iron in form of dextran was more useful. Embryonated eggs are also susceptible to infection following inoculation of organisms via the yolk sac route.

However, none of the animal models mentioned so far produce disease that bears much resemblance of the natural disease as it occurs in man and hence their usefullness is limited.

The present study describes attempts to develop an animal model for meningococcal meningitis.

MATERIALS AND METHODS

Experimental animals:

The following animals were used in an attempt to develop a suitable animal model for meningococcal meningitis.

1. Adult albino mice and suckling albino mice which were obtained from the experimental animal laboratory centre of the College of Medicine, University of Lagos. They were specially bred pathogen free animals with an average weight of 20g (adult), and 3.2g (suckling).
2. Infant and adult rats: These were obtained from the same place as the mice as above. The rats and mice were kept in standard cages and fed daily on standard rat chow supplied by the animal laboratory centre and tap-water ad-libitum.
3. Embryonated eggs :
   These were obtained from Samrose Agricultural Industries Ltd., a well-known farm in Agege, Lagos and kept at 37^{o}C in a humidified incubator until ready for use at 12-days of age.
4. Day-old chicks: These were also obtained from the Samrose Agricultural Industries directly. Alternatively, embryonated eggs were allowed to hatch and develop for about

for about 24 hours.

Bacterial strains

The following bacterial organisms were used for this study: N. meningitidis, Group A (ATCC 1894), Group B (ATCC 2091), Group C (ATCC 1054), and Group W-135. These strains were kindly provided by Prof. Tolu Odugbemi, Department of Medical Microbiology, College of Medicine, University of Lagos. A strain of Meningococcus Group C isolated from a clinical case of meningitis in Lagos University Teaching hospital (LUTH) and designated LUTH 405 and a strain of Meningococcus Group C isolated from an asymptomatic carrier and designated M625 were also used.

Media

The media used in this study were chocolate agar (GC agar base, Oxoid), Thayer-Martin agar, charcoal agar, Mueller Hinton agar (Oxoid), Nutrient broth, Mueller Hinton broth and brain heart infusion broth.

The following inoculating media were also prepared for experimental animal inoculation during the course of this study:

1. Semi-solid chocolate agar (0.3%) w/v of normal constituent.

2. Starch-solution in PBS with ranges of 5-10% starch (BDH chemical Ltd.)
3. Semi-solid charcoal agar 0.3% w/v of normal constituent
4. Defined gonococcal supplement (see appendix)
5. Trypan blue solution 0.01% - 0.1%.

Bacterial inoculation

All the bacterial strains used were resuscitated from freeze-dried ampoules and subcultured onto chocolate agar and Thayer-Martin agar and inoculated for 18-24h at 37°C. Suspensions of live organisms were made in PBS pH 7.2 and pH 6.5 corresponding to Brown's No. 5 opacity tube giving a concentration of 10^{10} organisms/ml. This was diluted in appropriate inoculating media, thus giving concentrations of 10^6, 10^7, 10^8, 19^9, 10^{10} cfu/ml.

Anaesthesia

The rats, mice and day old chicks were anaesthetised with chloroform on cotton wool. Animals were carefully shaved with sharp scalpel, if necessary, at relevant site before inoculation. Controls comprising animals inoculated with similar quantities of inoculating media without meningococci were set up in parallel.

Routes of Inoculation

The experimental animals were inoculated with No. 20 gauage needles through the following routes:

1. Intraperitoneal
2. Intracerebral
3. Intra-aural
4. Intravenous
5. Intranasal
6. Intraocular

Chick-embryo were inoculated through the yolk sac and chorioallantoic vein. All experimental animals were inoculated with concentration of meningococci ranging from 10^6 to 10^{10} organisms/ml.through the various routes.

Assessment of Infection:

The body organs (liver, kidney, heart and brain tissue) and viscera of experimental animals were chopped up and emulsified in PBS. Specimens were plated onto chocolate agar and Thayer-Martin agar.

Isolates on TM were gram-stained and those showing gram-negative diplococci were further identified and confirmed as N. meningitidis by their ability to utilise only glucose and maltose in serum-free fermentation medium (Flynn and Waitkins, 1978) and serogrouped using slide agglutination technique against battery of N. meningitidis group specific antisera (CDC, Atlanta, Georgia, USA). The organisms were quantitated using modified Miles and Misera (1938) semi-quantitative count method.

RESULTS

The chick embryo model was inoculated with 10^8 live meningococci/ml. All died within 36 hours of inoculation via the chorioallantoic vein and the yolk sac routes.

Meningococci of the various serogroups inoculated were recovered in greater numbers per/ml than in the inoculum of 10^8 cfu/ml (see table 7.1), ranging from 1.5×10^8 to 1.4×10^9 meningococci/ml. Colloidal starch solution, semi-solid charcoal, agar defined gonococcal supplement and semi-solid chocolate agar which were used as suspending media for meningococci, did not increase the infectivity of meningococci by lowering number of meningococci required to cause death in chick embryo. Trypan blue was toxic for chick embryo at 0.01% concentration; all chick embryo inoculated died within 24h and meningococci could not be recovered from the animal.

The route of inoculation of meningococci did not have any effect on the mortality of mice and rats inoculated (tables 7.2 and 7.3). However, 1/10 adult mice inoculated via intravenous route, 3/10 inoculated by intraperitoneal route and 1/10 inoculated by intercerebral route died within 24 hours upon intravenous, intraperitoneal and intracerebral inoculation. Meningococci could not however, be recovered from the brain or meninges of any of the inoculated experimental animals.

Table 7.1 Average count of meningococci recovered at death from chick embryo inoculated with 10^8 organisms/ml of meningococci through chorio-allantoic vein and yolk sac

Group of *N. meningitidis*	Inoculum size (cfu/ml)	No. of organisms (cfu/ml) Recovered after inoculation through	
		Chorioallantoic	Yolk sac
A	10^8	12×10^8	1.5×10^8
B	10^8	$2 \times 3 \times 10^8$	1.6×10^8
C	10^8	1.4×10^9	1.9×10^8
Clinical strain Group C	10^8	1×10^8	2.1×10^9
Carrier strain Group C	10^8	2.2×10^8	2.1×10^8
Control (PBS)	0	0	0

Table 7.2 Effect of route of inoculation of Group C (Strain LUTH 405) *N. meningitidis* on mortality of adult mice

Route of inoculation	meningococcal inoculum (cfu/ml)					
	10^6	10^7	10^8	10^9	10^{10}	Control (PBS)
IV	-	-	-	-	1/10	-
IP	-	-	-	-	3/10	-
IC	-	-	-	-	1/10	-
IN	-	-	-	-	-	-
IO	-	-	-	-	-	-

IV = intravenous

IP = intraperitoneal

IC = intracerebral

IN = intranasal

IO = Intra-ocular

- = no death recorded

Table 3. Effect of route of inoculation of Group C (Strain LUTH 405) *N. meningitidis* on mortality of infant rats

Route of inoculation	meningococcal inoculum (cfu/ml)					
	10^6	10^7	10^8	10^9	10^{10}	Control (PBS)
IV	-	-	-	-	3/10	-
IP	-	-	-	-	2/10	-
IC	-	-	-	-	1/10	-
IN	-	-	-	-	-	-
IA	-	-	-	-	-	-
IO	-	-	-	-	-	-

IV = intravenous

IP = intraperitoneal

IC = intracerebral

IN = intranasal

IA = intra aural

\- = no death recorded

IO = Intra-ocular

DISCUSSION

In the present work attempts were made to develop a suitable animal model in which meningococcal infection could be observed and the factors involved in its pathogenesis could be followed.

Of the animals used, the results appear to be most favourable for the chick embryo model. Inoculation of 10^8 live meningococci/ml was able to cause death of the inoculated embryo within 36h via both the chorioallantoic vein or yolk sac routes. Furthermore, meningococci could be recovered from chick embryo tissue in numbers equivalent to or greater than the inoculated dose, suggesting the capacity for meningococci to survive in this animal. However, there was no evidence of meningeal invasion and neither could the organisms be isolated from the brain of the chick embryo. This differs from the finding of Buddingh and Polk (1939) who reported that virulent meningococci could be recovered from the brain after injection into a 12 day old chick embryo. This difference may point to the fact that strain differences could be of significance in determining the cause of meningococcal infection. However, even the clinical isolate of group C meningococcus (Strain LUTH 405), used in this work could not be isolated from the brain or meninges. Payne and Finklestein (1978) have reported various levels of virulence among meningococci, based on their ability to acquire iron in chick embryo.

The various suspending media used for the inoculation of meningococci were employed with the hope of enhancing the infectivity of the organisms; Colloidal-starch solution and semi-solid charcoal have been reported to enhance the survival of meningococci *in vitro* (Morse, 1978). However, this did not appear to be the case *in-vivo* in the rat and mouse models employed. None of the suspending media were capable of enhancing infectivity or allowing the meningococci to invade the CNS of these animals. The pH of inoculating media was also not found to significantly affect the infectivity of the meningococci although Masson and Holbein (1985) reported that a low pH of 6.6 could increase meningococcal virulence.

It is interesting to note that intranasal instillation of up to 10^{10} cfu/ml of meningococci did not result in infection of the rats, mice and day old chick. It has been well established that the nasal route is the major portal of entry of meningococcci into human beings (Goldschneider *et al*, 1969; DeVoe, 1982: Apicella, 1986). It is quite possible that the meningococci are unable to colonise this site in these experimental animals.

Saukkonen *et al*. (1988) recently reported an infant rat model for group B meningococcal infection by intraperitoneal challenge of 10^6 cfu/ml. However, the results of this study could not support their finding. Intra-

peritoneal inoculation of 10^{10} cfu/ml resulted in the death of only 3/10 infant rats and furthermore viable meningococci could not be cultured from the CSF brain or meninges of infected rats. This difference may be attributed to the different strains of rats and/or meningococci used in the two studies. However, the results of the present study are consistent with Sippel (1981) who reported the resistance of rats and mice to meningococcal infection.

The LD_{50} of 10^{10}cfu/ml is not apparently useful for an animal model, since such a number is greater than that of heat-killed organisms required to cause mortality, suggesting that death may be due to meningococcal endotoxin. More importantly, meningococci could not be isolated from the brain and meninges of the inoculated experimental animals.

The present study supports the general observation that experimental laboratory animals are not readily susceptible to infection with N. meningitidis.

CHAPTER 8

CONCLUDING DISCUSSION

CONCULDING DISCUSSION

The purpose of the experiments carried out in this report, was to further investigate some of the factors influencing the pathogenicity of *N. meningitidis* and attempt to develop an animal model in which meningococcal infection could be simulated as it occurs in humans and also provide further information on the pathogenesis of the disease.

An important baseline data for the investigation of factors influencing the pathogenicity of meningococci was obtained from the study of the attachment of meningococci to human nasopharyngeal epithelial cells (NEC). This appears to be a logical point from which to analyse the first step of host-bacteria interaction. Bacterial colonisation of mucosal surfaces has always been regarded as an important prelude to the infectious process (Beachey, 1975; McGee *et al.*, 1983). Earlier studies on this aspect had focused principally upon the role of pili and adhesins on attachment and host factors were not adequately accounted for (Craven *et al.*, 1980; Salit and Morton, 1981).

The results obtained from the study on factors influencing the attachment of meningococci to NEC in this report, clearly illustrate the importance

of host status as it is demonstrated by the finding that meningococci attach significantly better to the NEC of children, infants and adolescents compared with adults, neonates and aged donors of NEC. Furthermore, the carrier status of the host appears to be equally important in determining the specificity of attachment. As mentioned earlier in chapter 3, this preferential attachment may be important in determining the susceptible group which may subsequently develop meningococcal disease and correlates well with the finding that meningococcal infections are more common among children and adolescents possibly due to absence of humoral antibody (Aycock et al., 1950; Goldschneider et al., 1969; Apicella, 1986).

There appears to be the need among workers on attachment of bacteria to mucosal surfaces to universally standardise their experimental procedures to allow for more critical analysis and comparison of the capacity for attachment in different environments and among different bacterial strains. At the present time, there appear to be too many different techniques to make any comparison useful. Thus efforts in this vein may prove more rewarding for future documentation.

The nasopharynx serves as the reservoir for the

spread of meningococci and meningococcal infection within a population and the organism is disseminated by means of aerosols (Artenstein et al., 1968) coupled with the finding that organisms which are part of the indigenous microflora of the nasopharynx of humans, may also exert an influence on attachment. The complexity of the factors influencing the attachment of meningococci to NEC comes into perspective and the permutations of the probability of an individual becoming a nasopharyngeal carrier appears difficult to compute. This is even more so when the factors innate to the meningococcus itself such as piliation, encapsulation or whether the organism is a carrier isolate or clinial strain are considered. The study also revealed that the ability for attachment did not differ significantly among serogroups A, B, C and W-135 thus suggesting that the carriage of particular serogroup may depend more upon the prevalence of the serogroup in that area. However, epidemics of meningococcal meningitis are often seasonal occurrences (Goldschenider et al., 1969; Whittle et al., 1975; Binkin and Band 1983) and it seems possible that environmental conditions may further influence host characteristics by inducing physiological changes especially at the mucous membrane interface and this may also influcence

colonisation patterns. This is an area which requires further investigation.

The relationship between meningococcal carrier rate and occurrence of endemic or epidemic meningococcal disease is still uncertain. Glover (1920) reported that when the carrier rate exceeded 20% the community was in danger of an epidemic usually due to the predominant serogroup. He proposed "spacing out" to prevent epidemics in military establishments and achieved some degree of success. However, Hassan-King et al. (1979) found that during an epidemic in Zaria, northern Nigeria, the carriage rate was only 3.8%. Thus the relevance of carriage data to the occurrence of meningococcal epidemic disease is still enigmatic.

In the present study an epidemiological investigation of carriership among school children in Lagos, Nigeria was carried out. The carriage rate was found to be 6.2%, and serogroups A and C, were the most commonly isolated serogroups, while groups X, Y and Z were not isolated. Furthermore, male carriership was found to be significantly higher than in females. These results are interesting. Firstly, groups A and C meningococci have been reported as the cause of epidemics in the northern part of Nigeria over the past few decades (Greenwood

and Wali, 1980; Whittle et al., 1978) and furthermore, group B meningococci have characteristically been the predominant groups in regions which are outside the zones where epidemics have been experienced in Africa (Njoku-Obi and Agbo 1976). The present result has provided evidence to support the changing patterns of serogroups over a period of time in some regions (Sippel, 1981; Sandborn and Toure, 1984). The reason for changing serogroup patterns are still obscure however.

This work also describes the first report in the literature of the toxic effects of meningococcal endotoxins on ciliated respiratory epithelium using trachea from adult rats. Meningococcal endotoxin caused cilostasis of cilia. Denny (1974) and Johnson and Inzana (1986) have reported a similar phenomenon for Haemophilus influenzae. Interference of mucociliary apparatus of the epithelial cells of the respiratory tract seems to be an intriguing concept which may well be an important aspect in the steps involved in the pathogenesis of meningococcal infection and the progression of meningococcal disease. It is worthy of note and further investigations on this line of thinking using human tissue may prove to be rewarding. The nasopharyngeal epithelium is, afterall, the first port of call at which this organism impinges after dissemination and

inhalation of aerosols. It is also interesting to note that the endotoxin from the clinical strain of N. meningitidis was significantly more effective in eliciting ciliostatic effect than that from the carrier isolate, thus further supporting the several reports in the literature that clinical strains are more virulent than carrier strains (DeVoe, 1982; Apicella, 1986).

It was demonstrated in this work that meningococcal endotoxin is lethal for 12 day old chick embryo via choriollantoic vein inoculation. Injection of small doses of endotoxin into experimental animals causes dramatic changes in blood-pressure, breathing capacity, body temperature circulating enythrocytes, metabolism and humoral and cellular immunity and resistance to infection (DeVoe, 1982). Sets of chick embryo inoculated with formal-saline treated cells of N. meningitidis to prevent release of endotoxic components survived as long as the controls inoculated with PBS thus providing further evidence that endotoxin was responsible for lethality. As with the effect of endotoxin on ciliated respiratory epithelium it was also observed that the clinical strains endotoxin was more potent than the carrier strain of the same serogroup. One of the most important questions which still remains unanswered concerning the pathogenicity

of N. meningitidis why different strains possess varying degrees of virulence.

One of the major problems encountered in studying the pathogenicity of N. meningitidis is the absence of a suitable animal model with which to study the progression of the disease. Most animals used in the laboratory are resistant to infection with meningococci.

During the course of these studies it was observed that the chick-embryo method appeared to be the most suitable to investigate meningococcal disease. A lower number of infective meningococci were needed when compared to rat and suckling mouse models. However, it appears the search for a suitable animal model will entail the use of some factors which will make such an animal susceptible to infection.

Many aspects regarding the interaction of the meningococcus with its host still remain unaswered. Specific adherence of meningococci to host tissues, properties of invasiveness, paracrine and endocrine hormone interactions with host in response to endotoxin and pathogenesis of meningitis are areas requiring intense research in the near future.

The physiology of the meningococcus must be explored in further detail to determine pathogen

related properties. Indeed, according to DeVoe (1982) it is feasible that the cytoplasmic membrane, cell wall and surface components will prove to be rewarding areas in which most physiology related to pathogenic mechanisms will be found. In a general analysis it appears that the pathogenicity of the meningococcus depends on its behaviour in the host and no doubt other factors included in the external ecology of the organism.

We are still ignorant of the factors responsible for dictating the regular appearance of major epidemics of meningococcal disease within the African meningitis belt.

The result of experiments reported here have no doubt shed some light on the factors influencing the attachment of meningococci to epithelial cells, interactions between nasopharyngeal microflora and meningococci, ciliostatic mechanisms of meningococcal endotoxins on mucociliary epithelium and also its lethality for chick embryo. This work has also contributed, in some measure to our knowledge of the factors influencing the pathogenicity of N. meningitidis which at present is still a very vast and complex subject.

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APPENDIX

MEDIA AND REAGENTS USED

Blood Agar Base No. 2

Code CM271

Formula

Proteose Peptone	grams per litre	15.0
Liver Digest		2.5
Yeast Extract		5.0
Sodium chloride		5.0
Agar		12.0
pH 7.4 ± 0.2		

Direction

Suspend 40g in 1 litre of distilled water, bring to the boil to dissolve completely, Mix and sterilize by autoclaving at 121°C for 15 minutes. Cool to 45 - 50°C and add 7% blood.

Brain Heart Infusion:

Code CM225

Formula

Calf brain infusion solids	grams per litre	12.5
Beef heart infusion solids		5.0
Proteose Peptone		10.0
Dextrose		2.0
Sodium chloride		5.0
Disodium phosphate		2.5
pH 7.4 ± 0.2		

Directions

Add 37g to 1 litre of distilled water. Mix well and distribute into final co tainers. Sterilize by autoclaving at 121°C for 15 minutes.

Brain Heart Infusion Agar

Code CM375

Formula

Calf brain infusion solids	grams per litre	12.5
Beef heart infusion solids		5.0
Proteose Peptone		10.0
Sodium chloride		5.0
Dextrose		2.0
Disodium phosphate		2.5
Agar No. 1		10.0
pH 7.4 ± 0.2		

Directions

Suspend 47 grams in 1 litre of distilled water. Boil to dissolve the medium completely. Distribute into tubes or flasks and sterilize by autoclaving at 121°C for 15 minutes.

Charcoal Agar

Code CM 119

Formula

'Lab-Lemco' Powder	gramsper litre	10.0
Peptone		10.0
Starch		10.0
Charcoal-Bacteriological		4.0
Sodium chloride		5.0
Nicotinic acid		0.001
Agar		12.0
pH 7.4 ± 0.2		

Directions

Suspend 51g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes.

Chocolate Agar (Heated blood agar) using GC agar base

Formula

Special Peptone	grams per litre	15.0
Corn starch		1.0
Sodium chloride		5.0
Dipotassium hydrogen phosphate		4.0
Potassium dihydrogen phosphate		1.0
Agar No. 1		10.0
pH 7.2 ± 0.2		

Directions

Soak for 15 minutes, mix and autoclave at 121°C for 15 minutes. Allow to cool to 75°C in a water bath, add 5 percent sterile human blood.

Allow to remain at 75°C and mix gently from time to time until the blood turns Chocolate brown colour. Plates are then poured.

MacConkey Agar

Code CM7

Formula

Peptone	grams per litre	20.0
Lactose		10.0
Bile Salts		5.0
Sodium chloride		5.0
Neutral red		0.075
Agar		12.0
pH 7.4 ± 0.2		

Directions

Suspend 52g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. Dry the surface of the gel before inoculation.

Mannitol Salt Agar

Code CM85

Formula

'Lab-Lemco' Powder	grams per litre	1.0
Peptone		10.0
Mannitol		10.0
Sodium chloride		75.0
Phenol red		0.025
Agar		15.0
pH 7.5 ± 0.2		

Directions

Suspend 111g in 1 litre of distilled water.and bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

Mueller Hinton Broth

Code CM405

Formula

Beef, infusion from	grams per litre	300.0
Casein hydrolsate		17.5
Starch		1.5
pH 7.4 ± 0.2		

Directions

Dissolve 25 grams in 1 litre of distilled water. Sterilize by autoclaving at 121°C for 15 minutes.

Mueller Hinton Agar

Code CM337

Formula

Meat infusion	grams per litre	6.0
Casein Hydrolysate		17.5
Starch		1.5
Agar No. 1		10.0
pH 7.4 ± 0.2		

Directions

Suspend 35 grams in 1 litre of distilled water. Bring to the boil to dissolve the medium completely. Sterilize autoclaving at 121°C for 15 minutes.

Nutrient Broth

Code Powder CM1

Formula

'Lab-Lemco' Powder	grams per litre	1
Yeast Extract		2
Peptone		5
Sodium chloride		5
pH 7.4 ± 0.2		

Directions

Add 13g to 1 litre of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes.

Nutrient Agar

Code Powder CM3

Formula

'Lab-Lemco' Powder	grams per litre	1
Yeast Extract		2
Peptone		5
Sodium chloride		5
Agar		15

pH 7.4 ± 0.2

Directions

Powder : Suspend 28g in 1 litre of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

Thayer Martin Medium

GC Agar base (Oxoid) - 18 grams

Distilled Water - 485 ml.

Soak for 15 minutes, mix and autoclave at 121°C for 15 minutes. Allow to cool to 75°C in a water bath, add 5 percent sterile human blood.

Allow to remain at 75°C and mix gently from time to time until the blood turns a Chocolate brown colour. Cool to 56°C, mixing at intervals. Add 15 ml of sterile distilled water to VCNT Antibiotic Supplement in vials. Shake vigorously till it dissolves, add to Chocolate agar. Pour plates.

Formula (per vial)

Yeast Autolysate	-	5.0 gram
Dextrose	-	0.75 gram
Sodium bicarbonate	-	0.075 gram
Colistin methane sulphate	-	3.75 grams
Nystatin	-	6,250 units
Trimetoprim	-	2.5 mg

Fermentation Media (Flynn and Waitkins, 1972)

Serum Agar Sugars :

Glucose, maltose, lactose and sucrose were used and these were obtained from BDH Chemicals Ltd, England.

Serum Free Fermentation Medium

Oxoid GC Agar base - containing

Special peptone	-	15 grams
Corn starch	-	1.0 grams
Sodium chloride	-	4.0 grams
Di-potassium hydrogen phosphate	-	4.0 grams
Potassium di-hydrogen phosphate	-	1.0 grams
Agar	-	10.0 grams

pH 7.2 $\pm$ 0.2

Gonococcal Supplement

Prepared by additing 90 ml of A to 10 ml of B.

(A) Glutamine Solution:

L-glutamine (Koch-light Lab. Ltd)	-	1.0 gram
Distilled Water	-	90 ml

Mix and dissolve.

(B) Ferric Nitrate Solution

Ferric Nitrate (Analar) - 0.05 gram

Distilled water - 10 ml

Mix and dissolve.

Phenol Red - 0.2% stock solution.

Sugars - 10% solutions of either glucose, maltose sucrose and lactose sterilized by Seitz filter.

Preparation of Fermentation Medium:

GC Medium Base (Oxoid) - 36 grams

Distilled water - 970 ml

Mix by heating and when clear added

Supplement - 20 ml

Phenol Red Solution (0.2%) - 10 ml

After further mixing, the pH of the medium was adjusted to 7.6 with N. NaOH. This was then distributed in 90 ml volumes into 100 ml screw capped bottles, which were then autoclaved at 121°C for 10 minutes. After cooling to 50°C, 10 ml of the appropriate sugar was added aseptically to give a final concentration of 1%. Three millilitre amounts were dispersed into sterile bijoux bottles and sloped. The sugars were identified by colour code on the caps.

Phosphate Buffered Saline:

Using Dulbecco 'A' Tablets

Formula:

Sodium chloride	-	8.0 g/L
Potassium chloride	-	0.2 g/L
Disodium hydrogen phosphate	-	1.15 g/L
Potassium dihydrogen phosphate	-	0.2 g/L

pH 7.3

One tablet was dissolved in 100ml of distilled water and autoclaved for 10 minutes at 115^{o}C before dispensing into sterile bijoux bottles.