EFFECTS OF INTERACTION OF *NEISSERIA GONORRHOEAE* WITH HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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By

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ABSTRACT

Human neutrophils were shown to undergo several metabolic changes coincident with ingestion of Neisseria gonorrhoeae, including an increase in oxygen consumption, changes in the electrophysiology of neutrophil membranes, and increased transport of chloride ions (Cl^-) across the membrane. Mean oxygen consumption and chloride uptake by neutrophils were increased by challenge with both piliated (P^+) and nonpiliated (P^-) gonococci, although levels were lower in the presence of P^+ organisms. P^+ gonococci were also found to initiate only hyperpolarization of cell membranes, in contrast with P^- organisms which caused a period of hyperpolarization followed by a prolonged period of depolarization.

The strains of organisms examined in this study could be divided into two groups. The first group consisted of P^+ organisms which stimulate lower levels of response than the corresponding P^- isotype. The second group consisted of strains which initiated variable responses by the leukocytes. High performance liquid chromatography analysis of neutrophil extracts, both unstimulated and challenged with gonococci confirmed production of hyperchlorous acid (HOCl) in the leukocytes. Furthermore, the addition of radiolabelled Cl^- to the neutrophils revealed that some of the Cl^- taken up by the cells in response to gonococcal challenge was utilized in the production of HOCl, suggesting a direct relationship between stimulation of Cl^- uptake and production of active chlorinating compounds in neutrophils.

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INTRODUCTION

Gonorrhea is a sexually transmitted disease which only affects humans. Its cause is a gram-negative diplococcus, Neisseria gonorrhoeae, first identified by Neisser in 1879 (cited by Davis et al, 1973). It has been shown that colonial morphology is directly related to virulence (Kellogg, 1963).

At the time of interaction with polymorphonuclear leukocytes (PMN), gonococci induce a number of metabolic changes in the PMNs including increased oxygen consumption, a drop in intravacuolar pH and other oxidative and oxygen-independent changes. It is generally agreed that virulent (pilated) gonococci affect PMN metabolism to a lesser extent than avirulent (non-pilated) counterparts.

Wiseman et al (1984) and Wiseman and Martin (1986) have shown that gonococci also alter chloride transport across PMN membranes; pilated cells again having a reduced effect. The current study endeavours to reassess all these changes in PMN metabolism and to relate them to visual estimates of phagocytosis and to changes in membrane electrical properties.

LITERATURE REVIEW

A. BIOLOGY OF N.GONORRHOEAE

1. Colonial Morphology of N.gonorrhoeae

Leistikow and Loeffler initially cultivated N.gonorrhoeae on artificial media in 1882 (Scherp, 1955). In 1904, Lipshultz reported differences in the colonial morphology of the organism (cited by Brown and Kraus, 1974). Since then, others have recorded observations regarding the appearance of colonies (Hill, 1948).

A correlation between colonial morphology and virulence of N.gonorrhoeae was reported by Kellogg et al (1963). Colony types 1 and 2, found in primary cultures from clinical material, produced infections in human volunteers after 69 selective in vitro passages. Types 3 and 4 colonies predominated if the growth was transferred without selection, and lacked ability to infect volunteers. As shown in Table 1, colonial types are morphologically distinct.

Jephcott et al (1971) discovered that types 1 and 2 gonococci possessed filamentous surface appendages described as fimbriae or pili. Type 3 and 4 organisms were nonpiliated. Within a short period of time, it was demonstrated that piliated gonococci were more virulent than nonpiliated organisms for the chimpanzee (Brown et al, 1972) and the chick embryo (Buchanan and Gotschlich, 1973; Bumgarner and Finkelstein, 1973), and interest in pili as a virulence factor increased.

Variations in the color/opacity of gonococcal colonies were described by Swanson (1978). Gonococci forming opaque colonies were more highly aggregated than organisms in transparent (nonopaque) colonies and possessed one or more cell wall proteins in addition to those found on gonococci from transparent colonies. Opaque colony forming organisms were also more susceptible to trypsin killing.

TABLE 1. Colonial morphology of N.gonorrhoeae.

Type	Size (mm)	Edge	Color	Pili	Virulence
1	0.5	Entire	Dark Gold	+	+
2	0.5	Crenated	Dark Gold	+	+
3	1.0	Entire	Light Brown	-	-
4	1.0	Entire	Colorless	-	-
5	1.4	Crenated	Dark Brown	-	-

References: Kellogg (1963)

Swanson (1978, 1980)

2. Gonococcal Outer Membrane Components

The envelope of the gonococcus is typical of gram-negative bacteria (Swanson, 1972; Novotny et al, 1975). An outer membrane containing protein, lipopolysaccharides (LPS) and pili is the outer-most component of the cell wall. There is, however, evidence that gonococci may possess a capsule (Hendley et al, 1977; James and Swanson, 1977; Richardson and Sadoff, 1977). The outer membrane encloses a periplasmic space which contains a thin layer of peptidoglycan. This in turn surrounds an inner cytoplasmic membrane which is directly in contact with the cell cytosol. Immunologically, the outer membrane and its associated components (protein, LPS and pili) are most important with regard to pathogenicity and virulence of the organism.

a) Outer Membrane Proteins. The outer membrane of N.gonorrhoeae contains a limited number of proteins and is less complex than that of E.coli (Wolf-Watz et al, 1976; Johnston and Gotschlich, 1974). Protein-1 (P1) is the predominant polypeptide of the outer membrane of the gonococcus and accounts for over 50% of the total protein (Johnston and Gotschlich, 1974). Initial studies reported that this protein had a molecular weight of 34.5 K, but further investigations have revealed that this species can range from 32-39 K (Johnston et al, 1976; Hildebrandt et al, 1978; Heckels, 1977, 1978; Swanson, 1978). P1 is the basis of a serotyping system (Wang et al, 1977; Sandstrom et al, 1982) and it has been found in all strains examined. The protein spans the outer membrane and may exist as a trimeric complex (Blake, 1985). The main function of P1 may be to serve as a porin for the gonococcus (Blake and Gotschlich, 1982; Lynch et al, 1983). It can insert into both artificial as well as erythrocyte membranes, and this may be responsible for changes in the ion transport

of host cells when gonococci adhere to them. Certain variants of P1 have been implicated in the resistance of strains to the bactericidal effects of serum (James et al, 1982). The same variants have also been associated with disseminated gonococcal infections (Cannon et al, 1983; Hildebrandt et al, 1978; Hildebrandt and Buchanan, 1978).

Protein-2 (P2) is a heat-labile trypsin sensitive protein whose presence has been linked with the opacity of gonococcal colonies (Swanson, 1977, 1978; James and Swanson, 1978). Its molecular weight ranges from 24-32 K and immunological studies have shown that it is surface exposed (Swanson and Barrera, 1983; Judd, 1985; Robinson et al, 1988). The antigenicity of P2 varies both between strains as well as within individual strains. Isolates of a single strain are able to express different forms of P2, and the degree of expression of the protein changes at a high frequency (Diaz and Heckels, 1982; Swanson, 1982). P2 has been implicated in attachment of gonococci to both human epithelial cells and human leukocytes (Lambden, 1979; Sugasawara, 1983). This protein has a high isoelectric point and it could be that electrostatic attraction facilitates attachment of $P2^+$ organisms to both negatively charged epithelial cells, as well as to themselves in order to form infectious units of more than one diplococcus (Schoolnik, 1985).

Protein 3 (P3) appears to be complexed with P1 to form porins (Wilfert and Gutman, 1988). It has a molecular weight of approximately 35 K and is thought to be located on the cytoplasmic side of the outer membrane (Heckels, 1978). The protein has been found in all strains examined.

Recently, a new gonococcal outer membrane protein, referred to as the H.8 antigen, was identified and cloned (Cannon et al, 1984; Black

and Cannon, 1985). Although the protein has been found in all strains of N.gonorrhoeae and N.meningitidis tested, its role in the pathogenesis of the organisms is unknown. H.8 appears to be homogeneous within a given strain, and although there is variation of the antigen among strains, all strains have been found to have one or more common surface epitopes (Hitchcock et al, 1985).

Swanson and King (1978) identified a gonococcal surface protein with a molecular weight of 28-29 K. The protein was found to be involved in the adherence of gonococci to human leukocytes, and was therefore named leukocyte association factor (LAF).

Analysis of gonococcal outer membrane proteins by 2-dimensional (2-D) gel electrophoresis has shown that the expression of certain proteins is repressed by anaerobic growth, while expression of other proteins is induced by the same growth conditions (Clark et al, 1987). This same technique has also revealed that there is a group of proteins other than pili which is involved in the transition of gonococci from type 1 to type 4 colony forming organisms (Klimpel and Clark, 1988).

b) Lipopolysaccharide (LPS). The LPS of N.gonorrhoeae consists of a collection of heterologous glycolipids which range in molecular weight from 3.2-7.2 K. They do not contain repeating oligosaccharides and strains differ in the number and molecular weight of LPS components (Griffis et al, 1987). It has been shown that individual strains produce a number of different LPS's, but the types produced by each strain are limited and are characteristic for a given strain (Mandrell et al, 1986; Schneider et al, 1986; Apicella et al, 1987). Chemical studies of gonococcal LPS have shown that they commonly contain glucose, galactose, glucosamine, heptose, 3-deoxy-octulosonic acid (KDO),

phosphate and lipid A (Perry et al, 1975; Stead et al, 1975; Wiseman and Caird, 1977). Rhamnose, xylose, fucose, galactosamine, mannose and N-acetylneuraminic acid are less frequently present. LPS is involved in the attachment of piliated and nonpiliated gonococci to host cells (Watt et al, 1978) and has also been shown to play a role in serum resistance (Schneider et al, 1982).

c) Pili. Gonococci from colony types 1 and 2, but not 3 or 4, have pili (Jephcott et al, 1971; Swanson et al, 1971). The pili are composed of a large number of subunits, termed pilin, which are polypeptides of varying molecular weight (16-24 K) (Buchanan et al, 1977; Pearce and Buchanan, 1978; Hermodson et al, 1978). The subunits are primarily of one antigenic type in the pili of a given strain. Some gonococcal pili have hexose residues and phosphate groups associated with their subunits (Robertson et al, 1977). Pili from different strains are antigenically heterogeneous and antibodies to one serotype react minimally with different serotypes (Buchanan, 1975). It has more recently been shown that individual strains produce a number of antigenically different pili which show differing specificity of adhesion to various epithelial cells (Lambden, 1982; Virji et al, 1982; Heckels and Virji, 1985). The pili of gonococci are important in attachment of the bacteria to human epithelial cells, as well as to erythrocytes and leukocytes (Pusalang and Sawyer, 1973; Blake and Swanson, 1975; Buchanan and Pearce, 1976). Pusalang and Sawyer found that piliated (P^+) gonococci adhere to human epithelial cells to a greater extent than nonpiliated (P^-) organisms, but if the pili were removed, their adherence mimicked that of P^- gonococci. There is evidence suggesting that P^- gonococci are more resistant than P^+ organisms to killing by normal serum (McCutchan et al, 1976; James, 1985).

Swanson (1985) discovered that phenotypically P^- gonococci may possess unassembled pilin units on their cell surface.

d) Capsule. A number of groups have reported that N.gonorrhoeae may possess an antiphagocytic capsule composed of polysaccharide (Hendley et al, 1977; James and Swanson, 1977; Richardson and Sadoff, 1977; DeHormaeche et al, 1978). The capsule can be visualized with a light microscope by the technique of India ink staining. It can also be seen with an electron microscope using a stain specific for the polysaccharide in the capsule, or anticapsular antibodies. The width of the capsule is 1-2 times the diameter of the organism, and is present in all colony types. Presence of the capsule appears to correlate with inhibition of phagocytosis by human leukocytes (Richardson and Sadoff, 1977), and there is evidence that encapsulated variants adhere less well to human buccal cells and Hep-2 cells than non-encapsulated forms. The quality and quantity of gonococcal capsules seems to be strain-dependent, and its presence seems to depend on the culture media.

3. Virulence and Pathogenicity of N.gonorrhoeae

a) Relation of Colonial Morphology to Virulence of N.gonorrhoeae. Cultures of N.gonorrhoeae typically display five morphologically distinct clonal types. Types 1 and 2 (T1, T2) colonies predominate in initial cultures but they rapidly undergo a transition to types 3, 4 and 5 (T3, T4, T5) colonies. This transition was demonstrated to be associated with a loss of virulence (Kellogg et al, 1963, 1968). Sparling (1966) found that a loss of competence of gonococci for DNA-mediated transformation also appeared to accompany this shift to T3 and T4. T1 and T2 have transformation frequencies of about 10^{-3},

while T3 and T4 transform at rates of 10^{-7} or less. Pili were found only on the surface of T1 and T2 organisms.

Because competence of gonococci for DNA-mediated transfer is associated with piliation, it was thought that pili or pilin was involved in transformation. However, Mathis and Scocca (1984) showed that pilin did not bind DNA and that antibodies to purified pilin had no effect on transformation frequencies. Other proteins must therefore be present to account for differences in ability for transformation. Analysis of whole-cell lysates and outer membrane preparations of gonococci by 2-D electrophoresis revealed that there are differences in the expression of a number of proteins between T1 and T4 cells (Klimpel and Clark, 1988). A minimum of 8 T1-specific and 4 T4-specific outer membrane proteins were identified.

Pili play an important role in the virulence of gonococci. They are involved in adherance of organisms to human epithelial cells (Pusalang and Sawyer, 1973) and inhibition of phagocytosis by leukocytes, but it is unlikely that they are the only proteins which are responsible for differences in virulence between P^+ and P^- gonococci. Colonial type was found to have no bearing on LPS chemical structure (Stead et al, 1975), and so this surface component is not suspected of being involved in the difference in virulence between types. This is in contrast with the findings of Wiseman and Caird (1977), who found slight differences in the amount and type of certain sugars between colonial types.

Research on N.gonorrhoeae has been hampered by the lack of a suitable animal model which accurately mimics the disease in humans. Early work with animals in the study of gonococci has been reviewed by

Hill (1943). Most of these efforts to establish disease in animals were unsuccessful.

Miller (1948) succeeded in producing gonococcal infections in the eye of rabbits by inoculating N.gonorrhoeae into the anterior chamber. However, the lack of similarity of this model to the human disease, as well as the discomfort this technique induced in the animals made this system inadequate in many respects.

In the early seventies, researchers at the Center for Disease Control in Atlanta used chimpanzees as subjects for the study of gonococcal infections (Lucas et al, 1971; Brown et al, 1972). They were able to induce gonococcal urethritis in male chimpanzees, and showed that the animals could pass the disease sexually to female cage mates. Studies using these animals have shown that chimpanzees could gain strain-specific immunity to gonococci by being vaccinated with a suspension of formalin-treated bacteria. Chimpanzees seem to be the animals which can most accurately reproduce the symptoms of human gonorrhea, but the expense and unavailability of these animals makes them beyond the reach of most laboratories.

A procedure which made it possible to use rodents to study gonococci was published by Arko (1972). Hollow plastic practice golf balls were surgically implanted in the subcutaneous tissue of rabbits and guinea pigs. After the wounds had healed, the subcutaneous balls or chambers were inoculated with N.gonorrhoeae. Fluid was drawn out of the chambers at various times and cultured for gonococci. This technique has been very useful in studying various host-bacteria interactions, and has also been helpful in identifying certain virulence factors of the gonococcus (Penn et al, 1977; Turner and Novotny, 1976; Veale et al, 1975).

Chick embryos have also been used to study gonococci. Bang (1941, 1943) was able to inoculate 9-15 day embryos with gonococci by placing organisms on the chorioallantoic membrane. Buchanan and Gotschlich (1973) reproduced this technique and showed that P^+ organisms were much more lethal for the embryos than P^- bacteria. Intravenous inoculation of the embryos was found by Bumgarner and Finkelstein (1973) to be the preferred method of inoculation and has often been subsequently used to study the organism.

The difficulties in finding a suitable animal host for gonococcal infections led to the use of human organ cultures. Carney and Taylor-Robinson (1973) used fallopian tube organ cultures to study interactions between mucosal epithelium and N.gonorrhoeae. They showed that the organisms damaged the tissue and inhibited ciliary action. Ward et al (1974) modified this technique to create circumstances which more nearly duplicated the natural situation by perfusing fallopian tubes with medium containing gonococci. Zell McGee and co-workers designed a technique to quantitatively measure virulence of organisms by assessing the damage done to infected fallopian tube organ cultures (McGee et al, 1976). Studies using these techniques showed that virulent gonococci are able to adhere to and damage human fallopian tissue, but are unable to cross the species barrier and damage fallopian tissue of various other species (Johnson et al, 1977).

b) Interactions Between N.gonorrhoeae and Human Leukocytes. Gonococci can be isolated from the urethral exudates of infected human males. Microscopic examinations of these exudates have shown that the organisms were closely associated with leukocytes. These cells were usually granulocytes, but occasionally monocytes were present. The bacteria were attached to the surface of the phagocytes and could

often be found within the cells (Novotny et al, 1975; Ouchinnihov and Delehtorskij, 1977).

In vitro studies have shown that, in the presence of opsonic serum, avirulent gonococci are rapidly ingested and killed by human neutrophils (Dilworth et al, 1975; Gibbs and Roberts, 1975) but the fate of virulent gonococci is somewhat controversial. Many researchers have reported that virulent gonococci adhere to the surface of human neutrophils but fail to be ingested (Dilworth et al, 1975; Gibbs and Roberts, 1975; Thongthai and Sawyer, 1973). They therefore claimed that virulent gonococci are resistant to killing by human neutrophils and that this resistance is due to a decrease in ingestion of the organisms.

Other laboratories have demonstrated that virulent gonococci are ingested by human neutrophils but that the bacteria are able to survive intracellularly and occasionally have been observed to multiply within phagocytes (Ward et al, 1972; Witt et al, 1976). This implies that the virulent organisms can withstand the bactericidal effects of the leukocytes while within the cells. Shafer et al (1986) have shown that Cathepsin G, an enzyme which is stored in the granules of neutrophils, is quite efficient in killing P^- gonococci, but its effects on the viability of P^+ organisms was not measured.

However it is shown, virulent gonococci seem to be more resistant than avirulent gonococci to the bactericidal effects of human granulocytes, and this is most likely important in the ability of the organisms to initiate an infection.

c) Immunogenicity of Components of Principal Importance. The antigenic composition of N.gonorrhoeae is complex. Antigens include LPS, pili (pilin) and outer membrane proteins. LPS varies greatly among

strains of gonococci, but the number and antigenic types of LPS structures produced by a single strain is considered to be stable (Schneider et al, 1986; Griffis et al, 1987). Gonococcal LPS's contain three categories of antigenic determinants; the serotype, the variable, and the common determinants. Apicella and Gagliardi (1979) have shown that the common determinants from several different gonococcal LPS are recognized by a single monoclonal antibody. It may be useful to incorporate this LPS determinant in a vaccine; however, recent work suggests that the expression of LPS epitopes by N.gonorrhoeae is less stable than previously thought (Apicella et al, 1987; Schneider et al, 1988), and this may limit the effectiveness of this component as an immunogen.

Gonococcal pilin is composed of a constant region in which the amino acid sequence is conserved, a semivariable region, and a highly variable region. The highly variable region is immunodominant over the conserved sequence (Wilfert and Gutman, 1988). The specificity of antibodies directed against pili are therefore typically heterogeneous. Certain investigators have found that the administration of a vaccine containing pili to humans conferred significant protection against infection with gonococci possessing pili identical to that used for immunization (Briton et al, 1978). However, since antibodies to one pilus serotype react minimally with different serotypes (Buchanan, 1975), and individual strains are able to readily change their pili, it seems unlikely that this component could be used to produce an efficient vaccine.

Protein-1 (P1) is found in all strains of gonococci, and is identical in all clonal types of a given strain. This protein has been used to serotype gonococcal strains, and it has been demonstrated that

the immunological variation between strains is in excess of 90 serotypes (Sandstrom et al, 1985). Therefore, a polyvalent P1 vaccine could elicit antibodies which might prevent severe gonococcal infections. Robinson et al (1987) determined that the surface accessibility of P1 varies within populations of gonococci belonging to a single strain. This diversity in the immunological accessibility of P1 may affect the efficiency of a vaccine composed of this antigen.

The H.8 antigen is the most promising candidate for inclusion in a gonococcal vaccine. This surface-exposed protein is found in both gonococci and meningococci, and antibodies to H.8 are elicited in human systemic gonococcal and meningococcal infections (Black et al, 1985). The protein exhibits variations in molecular weight among strains, but all strains have one or more common surface-exposed epitopes (Hitchcock et al, 1985). Initial studies of this antigen using slide agglutination, immune electron microscopy, and an indirect fluorescent antibody assay showed that H.8 is abundantly and uniformly distributed on the gonococcal outer membrane. The distribution of H.8 was later examined using H.8 specific antibodies linked to gold spheres (Robinson et al, 1987). The results of this study reveal that like P1, the accessibility of H.8 on the surface of the organisms varied among gonococci of a given strain. These findings suggest that an effective vaccine must contain more than a single component of the gonococcal outer membrane.

B. BIOCHEMISTRY OF PHAGOCYTOSIS

Neutrophils are professional phagocytes of the immune system. They circulate in the blood until they receive a signal which comes in the form of a gradient of chemotactic molecules. Factors which are chemotactic for

neutrophils include fragments of complement factors (C5a), compounds released from damaged tissue, and molecules released from the invading organism (Ward, 1968). The neutrophils migrate along the gradient until they reach the chemotactic source (Zigmond, 1978).

Once the phagocytes reach the organisms, they attempt to adhere to and ingest the microbe. Adherance is facilitated by the presence of opsonin. Opsonin consists of fragments of complement component C3 and immunoglobulin G. These proteins bind to the invading bacteria and are then bound to the neutrophils by means of specific surface-bound receptors (Spitznagel, 1983). The cell is then able to extend pseudopodia and engulf the organism into a phagolysosome. This process is known as opsonophagocytosis. Another mechanism neutrophils use to adhere to bacteria involves the interactions of carbohydrate binding proteins on one cell with sugars on the surface of the other cell. This process is termed lectinophagocytosis and has recently been reviewed by Ofek and Sharon (1988).

When neutrophils make contact with the invading organisms, they undergo a metabolic burst. This consists of a number of changes in the metabolism of cells including an increase in the production of cAMP (Simchowitz, 1980), changes in intracellular Ca^{++} ion concentrations (Lazzari *et al*, 1986), changes in membrane potential, an increase in oxygen consumption and the production of oxidative compounds (Spitznagel, 1983). A change in transmembrane potential has been found to be one of the earliest measurable responses of neutrophils to stimulation (Korchak and Weismann, 1978; Cohen *et al*, 1981). Depolarization was thought to be necessary for activation of neutrophils, but Lyman *et al* (1987) have shown that unopsonized *Candida albicans* hyphae are able to stimulate a metabolic burst without causing membrane depolarization.

1. Oxygen-Independent Metabolism

Once organisms are ingested by neutrophils, they are subjected to both oxygen-independent and oxygen-dependent antimicrobial systems. Oxygen-independent mechanisms include a drop in the phagosomal pH and the release of lysozyme, lactoferrin, and granular cationic proteins into the phagosome. Studies have shown that the pH of phagocytic vacuoles is more acidic than the cytoplasmic pH, with estimates of pH values ranging from 3.0-6.5 (Jessen and Baiton, 1973; Klebanoff, 1975). The source of the acid is unknown, however, increased lactic acid formation may be partially responsible for the fall in pH (Kakinuma, 1970; Mandell, 1970). Carbonic anhydrase is also thought to play a role in lowering the pH of the phagosome (Cline, 1973). Pneumococci have been found to be highly acid-sensitive and may be killed in the vacuole by acid alone (Avery and Cullen, 1919).

Lysozyme, a basic protein with a molecular weight of approximately 14.5 K, is stored in neutrophil granules (Spitznagel, 1983; Cohn and Hirsh, 1960). It is released into the phagocytic vacuole following phagocytosis, where it degrades cell wall components of ingested bacteria. The enzyme hydrolyzes the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine acid residues in bacterial peptidoglycan. M.lysodeikticus is susceptible to the actions of lysozyme (Blumfitt and Glynn, 1961), while S.aureus is resistant to attack by this enzyme (Ayoub and McCarty, 1968; Gallis et al, 1976).

Lactoferrin is an iron-binding protein which is released into the phagosome after ingestion. It is able to inhibit the growth of microorganisms by binding iron which is required as an essential nutrient by the organisms. This protein is microbiostatic rather than microbicidal. It is unlikely that lactoferrin by itself could significantly affect the viabil-

ity of ingested organisms, but it has been found to have a lytic effect on gram-negative bacteria in the presence of certain chelating agents (Masson et al, 1969).

Granular cationic proteins (GCP) are heat-stable acid resistant antimicrobial enzymes. They bind to acidic groups on the organism and interfere with growth and viability of the cells (Odeberg and Olsson, 1975; Lehrer et al, 1975; Weiss et al, 1978). The microbicidal action of these proteins is dependent on LPS structure with smooth LPS inhibiting function of the enzymes to a greater extent than rough LPS (Modrzakowski and Spitznagel, 1979; Weiss et al, 1980). Daley et al (1982) have shown that differences in the susceptibility of gonococci to the bactericidal action of GCP's is due to variations in the cross-linking of cell wall peptidoglycan. Shafer et al (1986) have demonstrated that a particular GCP, Cathepsin G, is at least 100-fold more active against gonococci compared with other bacteria such as S.aureus and E.coli.

2. Oxygen-Dependent Metabolism

Phagocytosis by neutrophils is associated with a burst of oxidative metabolism (Karnovsky, 1962). The oxygen is converted, in part, to hydrogen peroxide (H_2O_2) by the action of a NADPH-dependent oxidase. H_2O_2 in high concentrations is antimicrobial (Miller, 1969). Superoxide anion (O_2^-) is considered to be an intermediate in the production of H_2O_2 from O_2, and can also be toxic to certain microorganisms (McCord et al, 1971). Hydroxyradical (.OH) and singlet oxygen (O.) have also been found to be present within actively phagocytizing neutrophils, and these molecules may also play a microbicidal role in the leukocytes (Gregory and Fridovich, 1974; Klebanoff, 1975).

3. The Myeloperoxidase-Hypohalide System

Myeloperoxidase (MPO), H_2O_2 and an oxidizable cofactor such as halide combine to form a potent antimicrobial system (Klebanoff, 1967, 1968; McRipley and Sbarra, 1967). H_2O_2 and chloride ion (Cl^-) are combined by the action of MPO to form hypochlorous acid (HOCl). Iodide ion (I^-) is less frequently used as a cofactor in this reaction. HOCl is proposed to kill microorganisms by oxidizing essential sulfhydryl groups (Albrich et al, 1981; Barrette et al, 1987). Virulent types of H.ducreyi have been found to be more resistant to the action of HOCl than avirulent types (Odumeru et al, unpublished observations). HOCl may also react with nitrogenous compounds to form chloramines, such as taurine, which are also microbicidal in neutrophils (Zgliczynski et al, 1971). Foote et al (1983) has calculated that at least 28% of oxygen consumed by neutrophils during the metabolic burst is converted to active chlorinating agents, such as HOCl and chloramines.

MATERIALS AND METHODS

A. ORGANISMS

Six strains of N.gonorrhoeae were used in this study. Three strains (191, 44269 and F-62) were stock laboratory strains. The other three were recent isolates supplied by the Health Sciences Centre Microbiology Laboratory. All were originally isolated from urethral exudates of infected individuals. The organisms were maintained in skim milk cultures and stored at -70°C. When required, cultures were thawed and plated on gonococcus agar base (Baltimore Biological Laboratories) supplemented with 2% isovitalex (Appendix B). Plates were incubated overnight (18-22 hours) in a hotpak CO_2 incubator at 36°C, 95% relative humidity. CO_2 concentration was set at 4%.

Colonial morphology and opacity were determined with a Bausch and Lomb stereomicroscope. Type 1 and 2 strains were designated pilus-positive (P^+) while type 3 and 4 colonies were referred to as pilus-negative (P^-). Opacity-positive and opacity-negative strains were designated O^+ and O^-, respectively (Swanson, 1978, 1980). Concentrations of organisms were determined by monitoring absorbance of bacterial suspensions at 650 nm using a Unicam SP800B spectrophotometer (Unicam Instruments Ltd., Cambridge, England). Optical density (O.D.) of cell suspensions was plotted against a concentration gradient to obtain a standard curve.

Assessment of bacteria for pili was done with the assistance of L. Cressman and P. Hazelton. Bacterial preparations were negatively stained with uranyl acetate on grids and examined under the transmission electron microscope at a magnification of 30,000x.

B. PREPARATION OF LEUKOCYTES

Blood was drawn from a human volunteer no more than six hours before the conclusion of the day's experiment. Neutrophils were obtained using the procedure of Odumeru et al (1984). Heparinized blood from a donor with no history of gonorrhea was sedimented for 60 minutes by adding 2 ml of 6% dextran in saline to 8 ml of blood. The leukocyte-rich supernatant was collected and the cells were pelleted by centrifugation at 200x g for 10 minutes. The cells were washed twice with heparinized saline, then suspended in a solution of 0.84% NH_4Cl in water and incubated at 37°C for 10 minutes. The cells were pelleted then washed and resuspended in the appropriate suspending fluid (Hank's balanced salt solution [HBSS] or proteose peptone saline [PPS]; Appendix A). Leukocytes were tested for viability by trypan blue exclusion and counted with a Helber counting chamber. Serum used as opsonin in assays was obtained by allowing blood to clot at room temperature for approximately 30 minutes and then centrifuged at 2000 xg for 10 minutes at 4°C.

Blood from one individual was used for the majority of experiments, but occasionally other persons were used as donors. Comparison of $^{36}Cl^-$ uptake of leukocytes from several people was made (data not shown). Although there was minor variation from person to person, differences were not deemed large enough to warrant strict control.

C. IMMUNOBLOTTING

Serum used as opsonin was tested for the presence of anti-gonococcal antibodies. Western blots of gonococcal outer membrane proteins (OMP) were blocked with 4% bovin serum albumin (BSA) then incubated with either test serum or serum positive for gonococcus antibodies for 60 minutes at 37°C. The blots were washed with Tris-saline (pH 7.2) then incubated with horse

radish peroxidase (HRP)-conjugated anti-human light and heavy chain antibody. Bands were visualized by rinsing blots in 100 ml phosphate buffered saline containing 100 ul H_2O_2 and 50 mg diamino-benzidine diamine.

D. ANALYSIS OF OUTER MEMBRANE PROTEINS (OMP)

Sarcosinate-insoluble OMP was prepared from 18-22 hour cultures of N.gonorrhoeae by the method of Barenkamp et al (1981). Proteins were dissolved at 100°C for 5 minutes in 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol and 0.0625 M Tris-hydrochloride (pH 6.8). Samples containing 10 ug of protein from each strain were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 1.5 mm thick, 12% gels, as described by Studier (1973). Bands were visualized by staining gels with 0.05% Coomasie blue.

E. UPTAKE OF $^{36}Cl^-$ BY NEUTROPHILS

Uptake of $^{36}Cl^-$ by neutrophils was measured using a modification of the rapid sampling technique of Hawkins and Berlin (1969). Neutrophils suspended in PPS (10^{-5} ml^{-1}) were pipetted onto circular (10 mm) glass coverslips and incubated in a moisture-containing petri dish at 37°C for 60 minutes. Excess fluid was removed from the coverslips and was replaced with 100 uL of a suspension of gonococci in PPS. Bacteria were at a concentration of 10^7 ml^{-1} and 2% normal human serum (NHS) was included in the suspension as opsonin. $Na^{36}Cl$ solution was added to each coverslip at a concentration of 0.015 mBq. The coverslips were incubated at 37°C and at appropriate times (5, 10, 20 and 30 minutes) were removed and washed four times with PPS. The coverslips were then placed in scintillation vials and the leukocytes were lysed with 0.5 M NaOH for 45 minutes at 37°C. The contents of the vial were neutralized with 0.5 M HCl to pH 7.0 and 5 ml of

scintillation cocktail (Appendix E) were added to each vial. The activity was read in an LKB liquid scintillation counter (Model 1215 Rackbeta II).

F. PHAGOCYTOSIS ASSAY

Ingestion of gonococci by human neutrophils was determined with the acridine orange method described by Odumeru et al (1984). Acridine orange-labelled bacteria fluoresce green when viable and red when non-viable. Crystal violet serves to quench fluorescence of noningested organisms. Leukocyte suspensions were pipetted onto No. 1 coverslips (22 x 22 mm) and incubated at 37°C in a humidified 4% CO_2 incubator for 60 minutes. The coverslips were rinsed with HBSS (pH 7.2) at 37°C and bacterial cells (10^8 CFU ml^{-1}) in HBSS preopsonized with 2% NHS were added to each coverslip. The coverslips were reincubated then removed at various times (15, 30, 45 and 60 minutes). The slips were washed with HBSS then stained with acridine orange (14.4 mg/L) in Gey's balanced salt solution (pH 7.2) (Appendix C) at 37°C for 60 seconds. Coverslips were washed with HBSS then counter-stained with crystal violet (1 mg/ml) in 0.15 M NaCl at 37°C for 60 seconds. The coverslips were washed once more with HBSS and were then placed cell side down onto glass slides. The slides were examined under a Leitz ultra-violet epiluminescence microscope using a Leitz x100 oil immersion objective. One hundred neutrophils were counted and the percentage of leukocytes containing ingested gonococci was referred to as percent phagocytosis.

G. OXYGEN CONSUMPTION BY NEUTROPHILS

Oxygen consumption in leukocytes was measured with the YSI model 5300 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH, USA) equipped with the standard bath assembly and two Clark oxygen

probes. Leukocyte suspensions were maintained at 37°C by connecting the bath assembly to a Haake model FE2 circulating water bath. Suspensions were continuously stirred by magnetic stirring bars. Prior to each experiment, the probes were calibrated with air-saturated double distilled water.

Three ml of PPS containing 0.5-1 x 10^6 neutrophils ml^{-1} and NHS (20 ul ml^{-1}) were added to each chamber. One chamber (control) contained leukocytes and serum alone while the other chamber (test) also contained gonococci at a concentration of 10^7 ml^{-1}. Probes were inserted into the chambers and oxygen levels were monitored every few minutes until one of the readings reached zero. Data was converted to read as uL oxygen consumed (Appendix D).

H. TRANSMEMBRANE POTENTIAL IN NEUTROPHILS

Changes in the transmembrane potential of neutrophils were monitored using the method of Lyman et al (1987). The procedure utilized the cationic lipophilic fluorescent dye 3,3'-dipropylthiodicarbocyanine iodide (Di-S-C_3-[5]). This dye absorbs electromagnetic radiation at a wavelength of 620 nm and re-emits it at a wavelength of 670 nm. The degree of fluorescence of a suspension of Di-S-C_3-[5] and neutrophils depends on the amount of dye that enters the cells, and this in turn is dependent on the membrane potential of the cells. An increase in the negativity of the membrane, which would constitute hyperpolarization, would cause less of the cationic dye to enter the cell, and fluorescence would decrease. Depolarization, or a decrease in the negativity of the membrane, would cause more of the positively charged dye molecules to enter the cell, and this would cause an increase in the fluorescence emitted by the suspensions. Leukocytes at a concentration of 106 ml-1 were combined with dye at a concentra-

tion of 10^{-6} M in 1 ml of HEPES-buffered HBSS (Seligmann et al, 1980; Appendix A) and the suspension was placed in a quartz cuvette. The cuvette was placed in an Aminco Bowman spectrophotofluorometer with excitation and emission wavelengths set at 620 and 670 nm, respectively. Fluorescence was allowed to stabilize for 6-8 minutes. Ten ml of solution were removed and replaced with 10 ml of a gonococcus suspension (180 ul gonococci [10^9 CFU ml^{-1}] and 20 ul NHS). Readings were taken at two minute intervals and data was plotted as fluorescence units (% transmission) against time. Samples were maintained at 37°C and were constantly stirred to prevent settling of cells.

I. HPLC (High Performance Liquid Chromatogrphy) Methods

In order to monitor hypochlorous acid (HOCl) production in neutrophils, we employed the method of Foote et al (1983) which involved the trapping of active Cl^- ion with an aromatic compound. The trapping compound was 1,3,5-trimethoxybenzene (TMB). Molecules of this compound can be chlorinated by active Cl which is in a formal +1 oxidation state, such as the Cl found in HOCl. Both the chlorinated compound, 1,3,5-trimethoxylbenzene chloride (TMBCl), as well as the nonchlorinated precursor, absorb electromagnetic radiation at a wavelength of 216 nm, and both molecules can be separated from each other by HPLC.

Leukocytes were suspended in HBSS at a concentration of 0.5-5 x 10^6 ml^{-1} as appropriate. The cells were rotated on a Labquake tube rotator (Lab Industries, Berkley, CA) at 37°C for 30 or 90 minutes. Test suspensions of leukocytes were stimulated with gonococci (10^7 CFU ml^{-1}) preopsonized with 2% NHS. TMB at a concentration of 100 ug ml^{-1} was also included in the suspensions. After rotary mixing, the cells were lysed by sonication and allowed to stand at room temperature for 10 minutes. Excess

sodium thiosulfate (about 2 g) was added to the lysates, and the mixture was then passed through a Waters C_{15} Sep-Pak cartridge (Waters Associates, Milford, MA). The cartridge was extracted with 6 ml of distilled water and then extracted again with 6 ml of methanol. The methanol extract was evaporated and resuspended in 100-200 ul methanol as required. The resuspended extract was then filtered through a Millipore HU (0.45 um) syringe filter and 25-50 ul of sample were injected into the HPLC system.

The HPLC pump (Waters M-45) was operated at a constant flow of 0.7 ml min^{-1}. The mobile phase was a solution of equal parts of acitonitrile and double-distilled water. The stationary phase was an Alltech Econosil C18 5 um column (Alltech Associates Inc., Deerfield, IL). The sample was detected with a Shimadzu SPD-2A UV detector (Shimadzu Corp., Tokyo, Japan). Sensitivity of the detector was set at either 0.02 or 0.32 at 216 nm. After the sample was injected into the system, 2 minute (1.4 ml volume) fractions were collected for 30 minutes. Scintillation cocktail, when required, was added to each fraction in 5 ml volumes and analyzed for radiolabel in the LKB Rackbeta II liquid scintillation counter in some experiments.

RESULTS

A. SDS-PAGE PROFILES OF GONOCOCCUS STRAINS

In Figure 1, SDS-PAGE Coomasic blue stained profiles of gonococcus strains used in this study are shown. The patterns are characteristic of gonococcus outer membranes with the most prominent feature being a band at about 36 K attributed to protein-1. There are slight variations in both the amount and the molecular weight of the protein, but it was found in every strain examined. Protein-3, which has an apparent molecular weight of approximately 34 K, was also found in all preparations. All strains, except F-62 P^+ and P^- and DC-1 P^- (lanes B, C and E) appear to contain protein-2 (30-32 K) in varying amounts. Since the H.8 antigen is said to stain only faintly, it is difficult to say whether this protein appears in any of the lanes of the profile, but its position, if present, would be just below protein-2.

B. IMMUNOBLOTS OF SERA EMPLOYED IN TESTS AS A COMPLEMENT SOURCE

Immunoblots of gonococcal outer membrane preparations with serum used as opsonin in assays yielded reactions which were at background levels (Figure 2). It is therefore assumed that the only source of opsonin in the serum is complement factors C_3b and iC_3b. The neutrophil receptors for these molecules are likely the main receptors utilized for adherence by these organisms. The weak reactions obtained with the test serum and the negative control could be due to the presence of antibody-Fc fragment binding ligands on the surface of gonococcal membranes. These receptors would non-specifically bind the second peroxidase labelled anti-human antibody, and this would give a false positive reaction.

FIGURE 1. SDS-PAGE profiles of Coomasic-blue stained outer membrane preparations of gonococcus strains. Strains F-62 and DC-1 were tested at a different time from strains represented in lanes F-N, but are shown in the same scale. Molecular weight standards are given in lane A. Strains F-62 P^+, P^-, DC-1 P^+ and P^- are shown in lanes B-E, respectively. Strains 191 P^+, P^- and U6 P^- are shown in lanes F-H, and strains DC-5 P^+, P^-, 44269 P^+, P^- and DC-2 P^+, P^- are given in lanes I-N. All strains are O^+ except F-62 and 191, which are O^-. Protein-1, 2 and 3 are marked on the right margin.

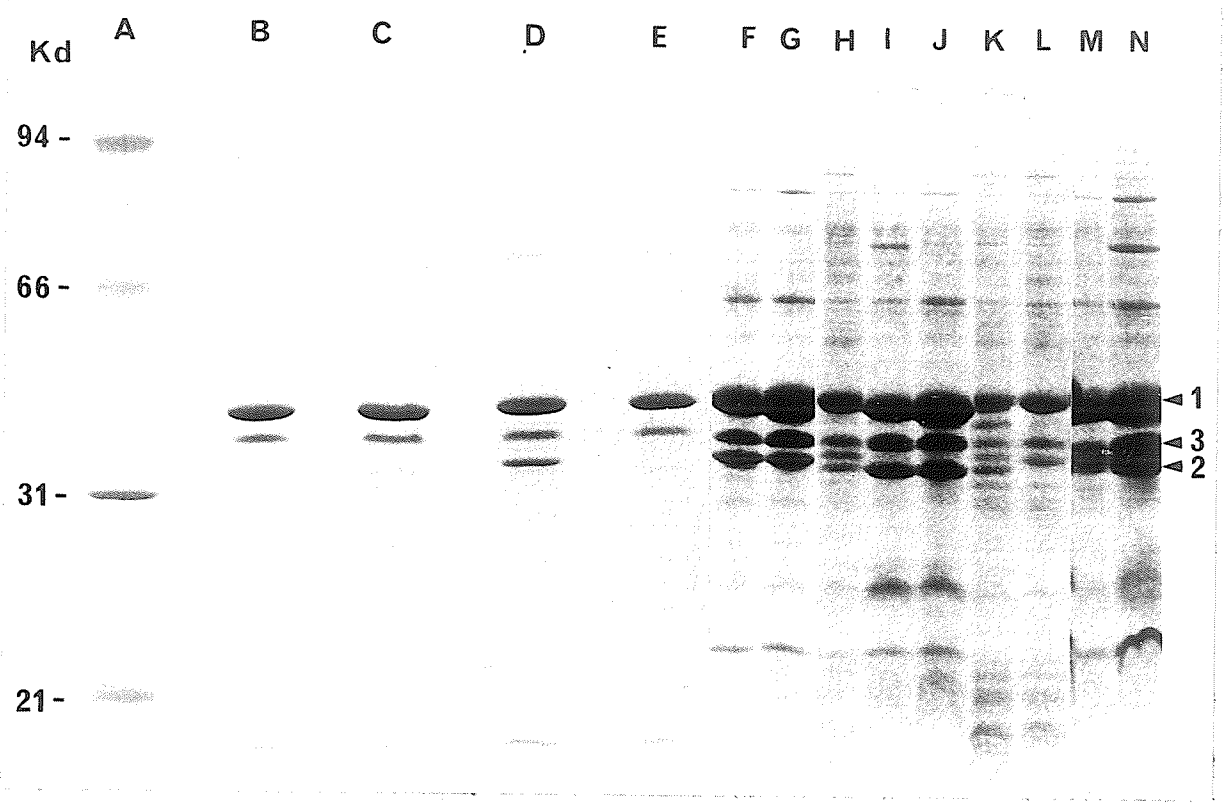
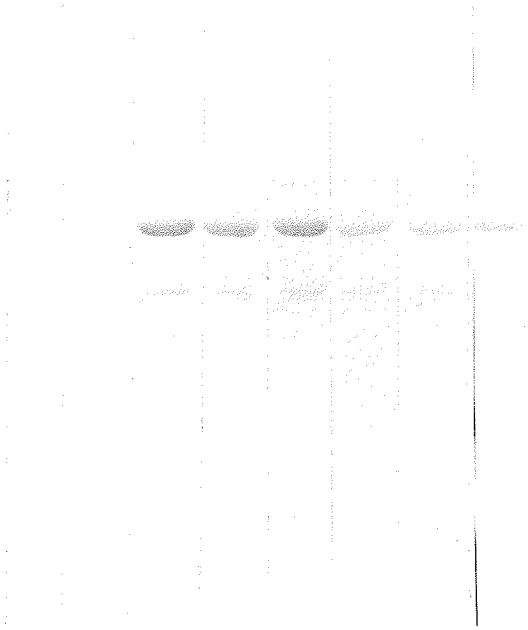

Kd
A
B
C
D
E
F
G
H
I
J
K
L
M
N
94 -
66 -
31 -
21 -
1
3
2

FIGURE 2. Immunoblots of gonococcal outer membrane proteins with test serum. Lane A represents negative controls, reacted with no first antibody. Lanes B-D represent positive controls, reacted with serum positive for gonococci (B 1:1000; C 1:1500; D 1:2000). Lanes E-G contain gonococcal proteins reacted with the serum used as opsonin in the assays (E 1:1000; F 1:1500; G 1:2000).

A B C D E F G



C. CALCULATION OF THE PHAGOCYTIC INDEX

It has been reported by Thongthai and Sawyer (1973) and Ward et al (1972) that nonpilated N.gonorrhoeae are ingested with greater frequency than pilated organisms. In order to determine if the strains used in this study behaved in accordance with these findings, the acridine orange assay was used to calculate a phagocytic index. These data also provided a baseline for comparison with other properties of phagocytosis.

As shown in Table 2, P^+ gonococci are not ingested by neutrophils (index = 29.5%) as well as P^- organisms (index = 44.7%). A T-value calculated for the two groups was -2.753, significant at $P<0.025>0.0125$. The results for individual strains show that, in each case, P^- types were ingested to a greater extent than P^+ types, but there is considerable variation from strain to strain. These observations generally confirm data reported in the literature over the years.

D. OXYGEN CONSUMPTION BY NEUTROPHILS

Oxygen consumption by human neutrophils in the presence and absence of P^+ and P^- gonococci was measured using a Clark oxygen probe. As shown in Figure 3 A-L, with the exception of strain DC-1 P^- (Figure 3 L), uptake is linear when ul oxygen is plotted against time. There is, however, wide variation in the rate of uptake, as evidenced by slope. The largest difference between uptake stimulated by P^+ and P^- organisms is attributed to strains F-62 and DC-1 (Figure 3 I-L). P^- bacteria stimulate greater uptake than controls, with the exception of strain 44269 P^- (Figure 3 F). For P^+ organisms, two of the six strains (F-62 and DC-1, Figure 3 I and K) fail to enhance oxygen uptake in neutrophils.

Table 3 summarizes differences in consumption between test and control neutrophils for all strains at a 30 minute time interval (as taken from the

TABLE 2. Ingestion of gonococci by neutrophils.

Strain	Opacity	% Phagocytosis	
		P^+	P^-
DC-2	+	24	37
191	-	30	60
44269	+	20	32
DC-5	+	30	34
F-62	-	36	52
DC-1	+	37	53
X ± SE		29.5 ± 2.7	44.7 ± 4.8

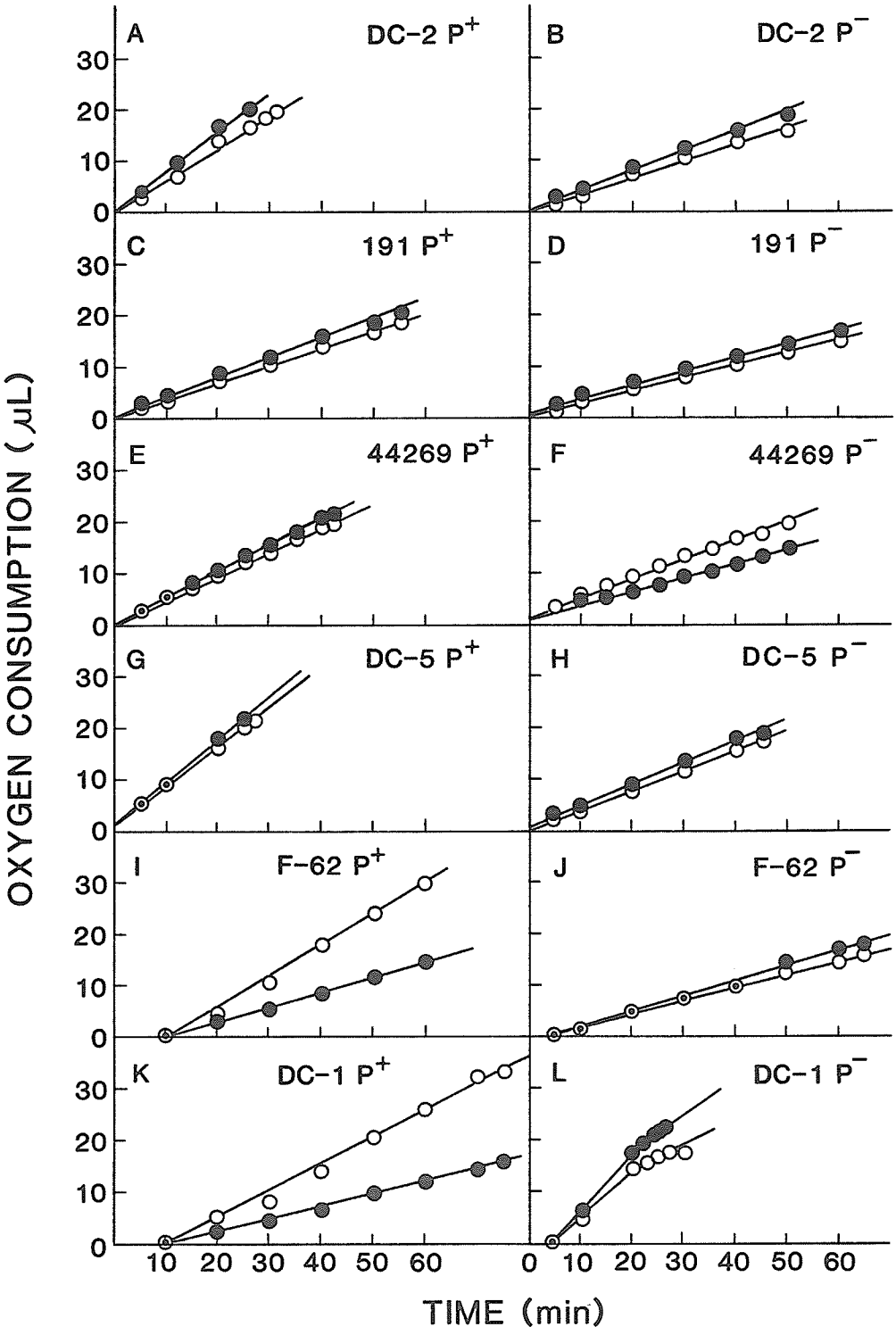
TABLE 3. Oxygen uptake by stimulated neutrophils.

Strain	Opacity	Uptake of O_2 by Neutrophils*	
		P^+	P^-
DC-2	+	4.0	1.9
191	-	1.3	1.5
44269	+	1.1	-3.5
DC-5	+	1.0	1.5
F-62	-	-1.2	1.2
DC-1	+	-5.0	5.0
X ± SE		0.2 ± 1.2	1.3 ± 1.1

* Differences determined at T=30 min.

(-) denotes test value < control.

FIGURE 3. Oxygen consumption in human neutrophils challenged with P^+ and P^- gonococci (A, C, E, G, I, K-P^+; B, D, F, H, J, L-P^-). Solid dots represent test values and open circles represent control values.


A
DC-2 P+
B
DC-2 P−
C
191 P+
D
191 P−
E
44269 P+
F
44269 P−
G
DC-5 P+
H
DC-5 P−
I
F-62 P+
J
F-62 P−
K
DC-1 P+
L
DC-1 P−
OXYGEN CONSUMPTION (μL)
TIME (min)
0
10
20
30
40
50
60

plots). These results confirm that both P^+ and P^- organisms stimulate oxygen consumption by neutrophils and that uptake is greater with P^- bacteria than with P^+ cells (1.3 vs 0.2 ul). A T-value of T=-0.64, where P>0.20<0.30, was calculated for P^+ and P^- organisms.

E. UPTAKE OF Cl^- ION BY NEUTROPHILS

It was previously shown (Wiseman and Martin, 1986) that Cl^- ion exchange in human neutrophils was altered in the presence of gonococci. The current study confirmed this with the use of radiolabelled $^{36}Cl^-$ which permitted Cl^- uptake to be determined.

As shown in Figure 4, P^- organisms of strains DC-2 stimulate greater uptake of radiolabel than P^+ gonococci. Strain DC-2 P^+ actually depressed uptake of the label. For strain 44269, P^+ gonococci stimulate slightly higher uptake than P^- cells. Results shown in Table 4 are expressed as the ratio of counts min^{-1} obtained from lysates of leukocytes stimulated with gonococci over those obtained from neutrophil controls. Mean values for P^+ and P^- groups of strains indicate that both stimulate uptake over controls to some extent, but the mean value for P^+ cells was lower. A T-value for the two groups was calculated (T = -1.002) and was significant at P<0.20>0.10.

F. NEUTROPHIL TRANSMEMBRANE POTENTIAL IN THE PRESENCE OF GONOCOCCI

Determinations of transmembrane potential (TP) of neutrophils in the presence of gonococci are shown in Figure 5. In the presence of the dye Di-S-C_3(5), leukocytes showed little or no depolarization when interacting with P^+ gonococci. The phenomenon induced by these organisms is technically termed hyperpolarization. The only exception is strain 44269 P^+ which induced depolarization of cell membranes. By contrast, P^- strains may

TABLE 4. Radiolabelled chloride uptake by neutrophils.

Strain	Opacity	Uptake of $^{36}Cl^-$ by Neutrophils*	
		P^+/Control	P^-/Control
DC-2	+	0.87	1.16
191	-	0.96	1.35
44269	+	1.06	1.03
DC-5	+	2.08	7.17
F-62	-	1.04	1.29
DC-1	+	1.38	1.43
X ± SE		1.23 ± 0.18	2.24 ± 0.99

* Ratios determined at T=20 min.

FIGURE 4. $^{36}Cl^-$ uptake by human neutrophils in the presence of gonococci. A) strain DC-2; B) 44269.

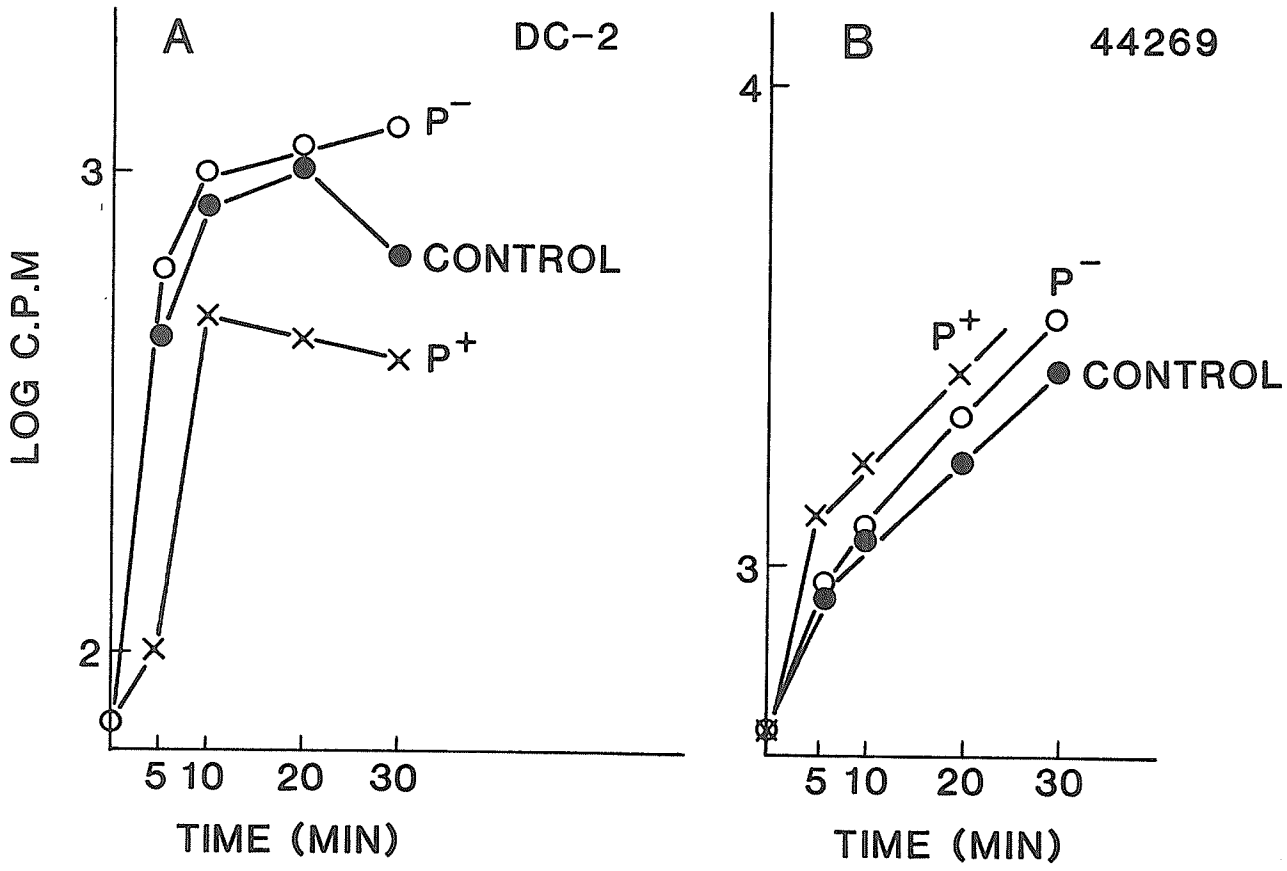
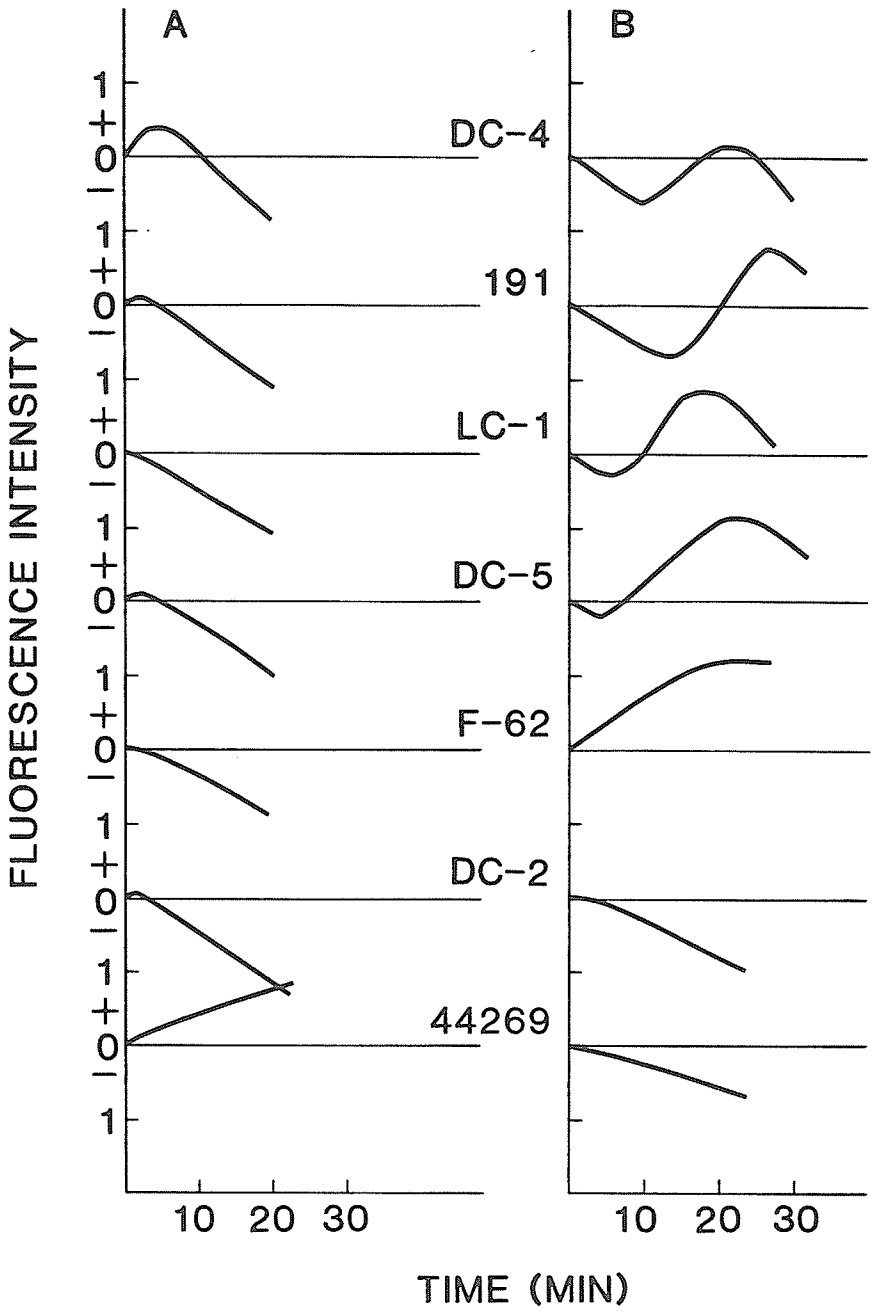

A
DC-2
B
44269
LOG C.P.M
3
2
4
3
P⁻
CONTROL
P⁺
5 10 20 30
TIME (MIN)

FIGURE 5. Leukocyte transmembrane potential in the presence of gonococci. A) P^+; B) P^-.


A
B
DC-4
191
LC-1
DC-5
F-62
DC-2
44269
1
+
0
−
1
10
20
30
FLUORESCENCE INTENSITY
TIME (MIN)

cause a brief period of hyperpolarization, but it is rapidly followed by depolarization. Exceptions to this are strains DC-1 and 44269, responses to which resembled those associated with P^+ strains. Strains DC-4 and LC-1 were only used in the TP assays.

Table 5 summarizes TP values taken at T=20 min. for neutrophils challenged with five strains of gonococci. The mean value for P^+ strains is more negative (-2.27), and indicates hyperpolarization. A positive value of 1.04 for P^- gonococci indicates that these organisms tend to stimulate a depolarization response. A T-value of -1.668 was calculated for the two groups of gonococci and was significant at $P<0.10>0.05$.

G. COMPARISON OF NEUTROPHIL BEHAVIOUR IN THE PRESENCE OF ISOGENIC PAIRS OF GONOCOCCI

A summary of changes in neutrophil properties in response to gonococcal challenge is shown in Table 6. P^+ bacteria are predictably ingested by neutrophils at a lower frequency than P^- gonococci. This serves as a reference point for the other data. Mean values for the $^{36}Cl^-$ uptake, oxygen consumption and TP assays indicate that neutrophils ingesting P^+ organisms behave differently from cells ingesting P^- organisms. P^- gonococci stimulate greater uptake of both Cl^- and O_2 (mean values 1.23 vs 2.24 and 0.2 vs 1.3). There are, of course, some variations among strains, such as negative oxygen uptake values for strain 44269 P^-. TP values are more negative (hyperpolarization) for neutrophils ingesting P^+ organisms and more positive (indicating depolarization) for leukocytes ingesting P^- strains.

There was overall good agreement between the acridine orange ingestion assay and the other properties, quite apart from the T test analyses.

TABLE 5. Leukocyte transmembrane potential in the presence of gonococci.

Strain	Opacity	Transmembrane Potential*	
		P^+/Control	P^-/Control
DC-2	+	-3.8	-1.5
191	-	-3.0	3.0
44269	+	3.8	-2.2
DC-5	+	-5.5	2.75
F-62	-	-2.85	3.17
DC-1	+	-	-
X ± SE		-2.27 ± 1.59	1.04 ± 1.19

* Fluorescence units determined at T-20 min.

(-) denotes test value < control (polarization).

TABLE 6. Comparison of human neutrophil biological properties in response to challenge with gonococci.

		% Phagocytosis		Uptake of $^{36}Cl^-$ by PMN[a]		Uptake of O_2 by PMN[b]		Transmembrane Potential[c]	
Strain	Opacity	P^+	P^-	P^+/Control	P^-/Control	P^+	P^-	P^+	P^-
DC-2	+	24	37	0.87	1.16	4.0	1.9	-3.8	-1.5
191	-	30	60	0.96	1.35	1.3	1.5	-3.0	3.0
44269	+	20	32	1.06	1.03	1.1	-3.5	3.8	-2.2
DC-5	+	30	34	2.08	7.17	1.0	1.5	-5.5	2.75
F-62	-	36	52	1.04	1.29	-1.2	1.2	-2.85	3.17
DC-1	+	37	53	1.38	1.43	-5.0	5.0	-	-
X±S.E.		29.5±2.7	44.7±4.8	1.23±0.18	2.24±0.99	0.2±1.2	1.3±1.1	-2.27±1.59	1.04±1.19

a Ratios determined at T=20 min.

b Differences from control determined at T=30 min. (-) denotes test value < control.

c Fluorescence units determined at T=20 min. (-) denotes test value < control (polarization).

H. PRODUCTION OF HYPOCHLOROUS ACID (HOCl) IN NEUTROPHILS

We wished to determine if Cl^- anions which had crossed the leukocyte membrane in response to gonococcal challenge were used by the myeloperoxidase system in the production of HOCl. Neutrophils were stimulated with gonococci in the presence of both TMB and $^{36}Cl^-$ (see Materials and Methods). It was thought that if any $^{36}Cl^-$ which entered the stimulated leukocytes was used to produce $HO^{36}Cl$, some of the radiolabelled acid might chlorinate the TMB to form $TMB^{36}Cl$. The newly chlorinated compound could be detected and isolated by HPLC and activity of fractions could be measured by liquid scintillation.

Figure 6A shows results with unchallenged control leukocytes. This preparation did not contain TMB. Results of TMB and TMBCl standards dissolved in methanol and passed through the HPLC system are shown in Figure 6B. In Figure 6C, neutrophils stimulated with gonococcal strain U6 P^- in the presence of TMB showed peaks identified as TMB and TMBCl. Naturally produced TMBCl is absent in control leukocytes (Figure 6A). Unstimulated neutrophils also converted TMB to TMBCl, but to a lesser extent than stimulated cells (data not shown).

In another experiment, neutrophils stimulated with gonococci (U6 P^-) were incubated with both TMB and $Na^{36}Cl$. Extracts were analyzed by HPLC. In three separate experiments, large amounts of radioactivity were detected in the 1-4 ml volume of initial effluent which came off the HPLC column (Figure 7). These fractions correspond to the void volume of the column and may represent nonspecific labelling. Three other peaks appear and are reproduced in each experiment. They came off the column in 9.8, 14 and 21 ml volumes. The peak of activity at 14 ml corresponds with the emergence of TMBCl standard. This indicates that some of the $^{36}Cl^-$ anion taken in by

FIGURE 6. HPLC profiles of human neutrophil extracts. A) leukocyte control; B) methanol solution of TMB and TMBCl; C) leukocyte extract from cells challenged with gonococcus strain U-6 P^-. TMB is uncombined compound originally added to neutrophils. TMBCl peak is produced by the leukocytes.

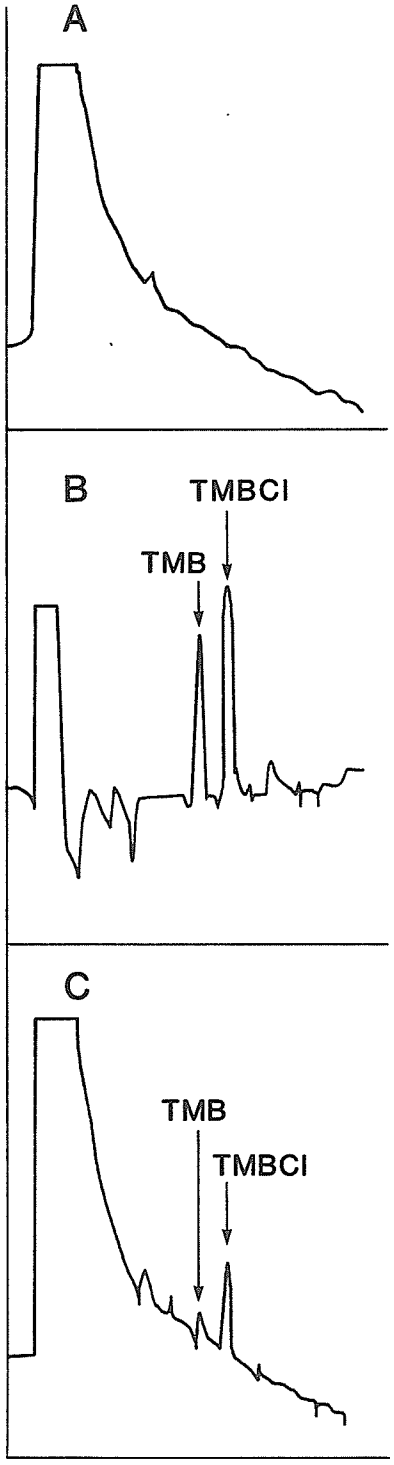
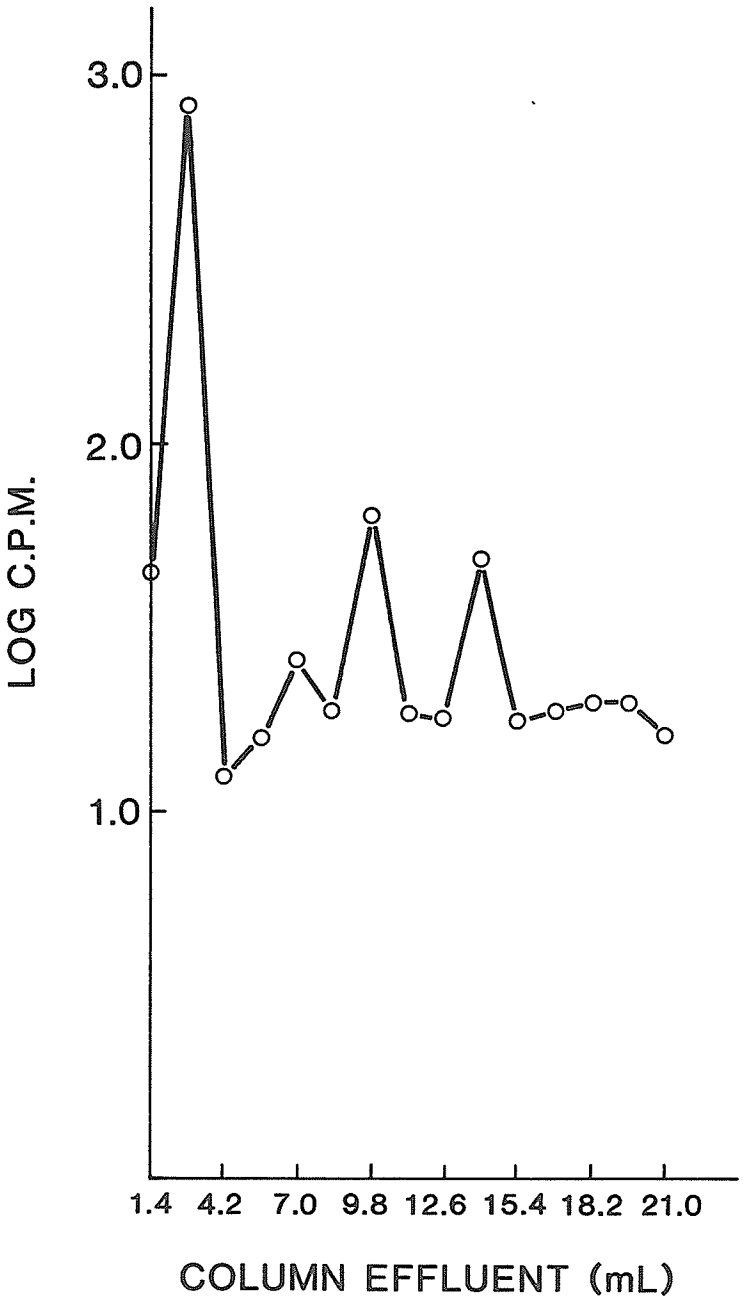

A
B
TMBCl
TMB
C
TMB
TMBCl

FIGURE 7. Formation of $^{36}Cl^-$ labelled compounds in leukocytes challenged with gonococcus strain U-6 P^-. Radioactivity peak at 14 mL corresponds to location of TMBCl standard. Average of three experiments.


3.0
2.0
1.0
LOG C.P.M.
1.4 4.2 7.0 9.8 12.6 15.4 18.2 21.0
COLUMN EFFLUENT (mL)

the neutrophils in response to gonococcal challenge is used in the manufacture of HOCl.

DISCUSSION

In the past, it has been observed that when N.gonorrhoeae adhere to human erythrocytes and neutrophils, the organisms initiate several changes in the metabolism of these host cells (Wiseman et al, 1984; Wiseman and Martin, 1986). These changes include alterations of K^+ and Cl^- transport across the host membrane, depression of Na-K-ATPase activity and stimulation of pyruvate kinase activity. The magnitude of the effects depended on whether the organisms were piliated. Piliation in gonococci is correlated with virulence.

In the present study, two related questions of gonococcus-neutrophil interactions were examined. The first question centred on the different effects P^+ and P^- gonococci had on leukocyte metabolism. The second question focussed on stimulation or inhibition of Cl^- uptake by neutrophils in response to gonococcal challenge. We wished to determine if Cl^- taken up by stimulated leukocytes was used by the myeloperoxidase-hypohalide system to generate microbicidal HOCl.

The results of experiments on Cl^- uptake by neutrophils show that, on the average, both P^+ and P^- gonococci stimulate transport of Cl^- across leukocyte membranes, and the P^- organisms tend to induce greater uptake. There are, of course, variations. The six strains can be divided into two groups. Group 1 consists of strains 191, DC-5, DC-1, and F-62, the non-piliated organisms of which stimulate greater responses than piliated types. The second group consists of strains DC-2 and 44269 which give variable results. For these strains, P^+ gonococci stimulate equivalent or greater Cl^- uptake than P^- gonococci, in spite of the fact that the phagocytic indexes were similar to those obtained for group 1 strains. These differences could likely be due to differences in outer membrane surface components; however, SDS-PAGE profiles of outer membrane protein prepara-

tions showed no noteworthy dissimilarities between group 1 and group 2 organisms.

Previous work done in this laboratory revealed that gonococci altered uptake of Cl^- in human erythrocytes. There is evidence that Band 3, the anion transport protein in erythrocyte membranes is the receptor for gonococci. The evidence for this was the following: purified Band 3 inhibits erythrocyte hemagglutination titres induced by gonococci; Cl^- transport across erythrocyte membranes was altered when gonococci adhere to them; and known inhibitors of anion transport, 4,4'-disiothiocyano-2,2'-disulphonic acid stilbene (DIDS) and α-cyano-4-hydroxycinnamic acid (α-CHC), reduced hemagglutination titres and uptake of Cl^- by erythrocytes.

The gonococcus receptor in leukocytes is unknown, although in the presence of normal serum, it is likely C_3b and/or iC_3b receptors. Gordon et al (1986) were able to inhibit phagocytosis of serotypes of pneumococci which, after being incubated with normal human serum, bore exclusively iC_3b ligands on their cell surface. Inhibition was attained by blocking iC_3b receptors on neutrophils with monoclonal antibodies. They further showed that the iC_3b ligand is the primary trigger for stimulation of intracellular bactericidal processes in the absence of immunoglobulin.

Since gonococci alter Cl^- transport in neutrophils, it is possible that the receptors for the organisms serve as anion channels in the leukocyte membranes. Chloride uptake by neutrophils can be inhibited by DIDS and α-CHC (Wiseman, unpublished data) but it is unknown if these compounds also inhibit interaction of gonococci with neutrophils. Jessen et al (1986) found that DIDS inhibited chloride exchange in Ehlich ascites tumour cells, and that DIDS acts on the outside of the cell membrane, as it does in human erythrocytes. They also discovered that ^3H-DIDS bound to a 30 K

membrane protein which has less than one-third of the molecular weight of Band 3.

There is increasing evidence suggesting that membrane electrophysiological processes play an important role in the function of both mononuclear phagocytes and granulocytes. Ince et al (1987) have shown, using the patch-clamp technique, that the hyperpolarization response that occurs in macrophages is associated with the action of ionic channels. The results of the current study reveal that depolarization of neutrophil membranes may also be influenced by changes in ion transport. Piliated organisms of group 1 strains evoke significantly different changes in membrane potential than do nonpiliated bacteria. The avirulent gonococci stimulate in neutrophils a brief period of hyperpolarization followed by prolonged membrane depolarization. This is the typical response of these cells to stimuli. The continuous hyperpolarization induced by virulent gonococci is not consistent with neutrophil stimulation. However, altered membrane electrophysiology correlates with altered effects on chloride transport caused by these organisms. The variable strains of group 2 induce effects on membrane potential which are anomalous. Both P^+ and P^- gonococci of strain DC-2 stimulate membrane hyperpolarization while 44269 P^- stimulates hyperpolarization in contrast with P^+ organisms which induce depolarization.

Miller et al (1972) stated that the ability of a bacterial strain to induce elevated oxygen consumption in neutrophils may be related to its in vivo virulence. They found that avirulent strains of Salmonella typhi or Staphylococcus aureus induced higher levels of oxygen consumption than virulent strains of S.typhi. Kossack et al (1981) essentially confirmed these findings. Virulent S.typhi stimulated much smaller increases in post-phagocytic oxidative metabolism in neutrophils than avirulent organ-

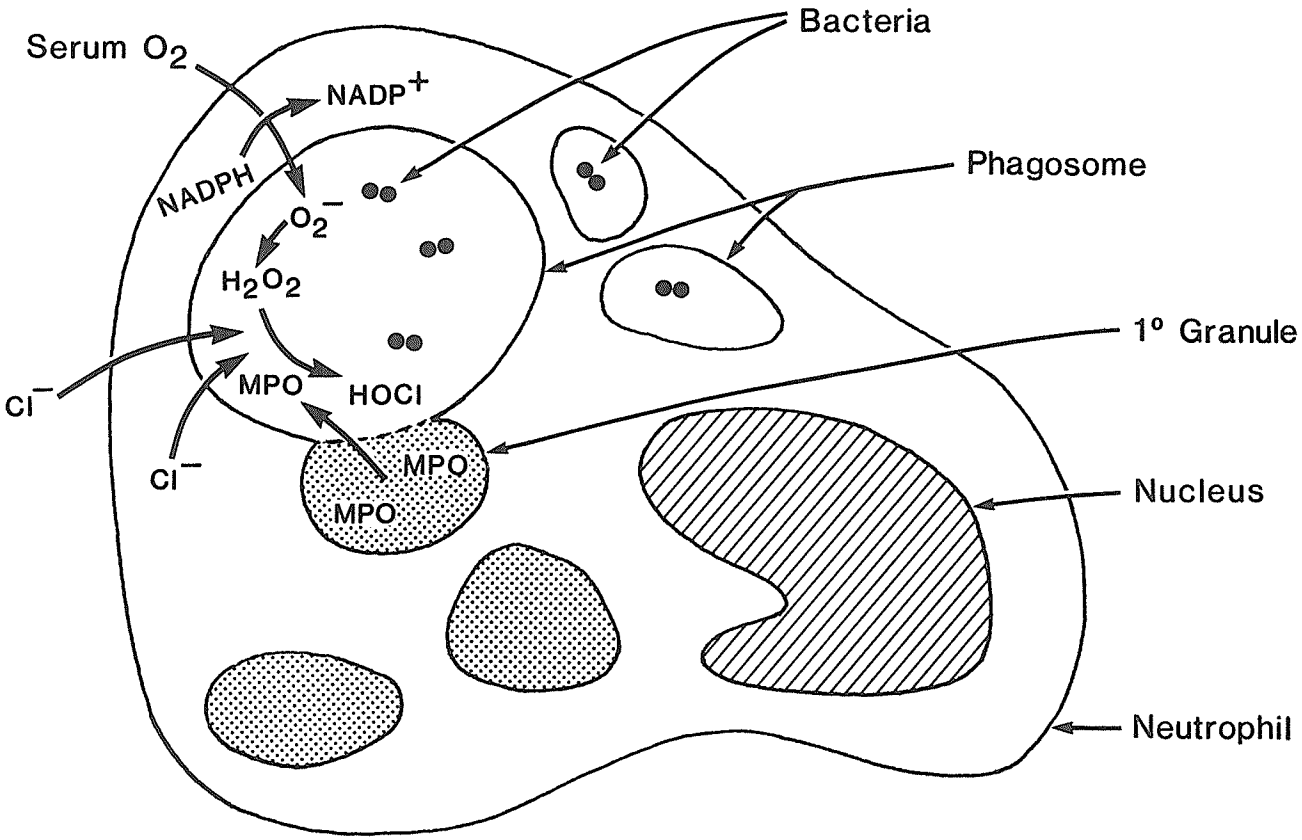
isms. Ingestion of virulent S.typhi also resulted in significantly less protein iodination than did ingestion of avirulent strains. The authors concluded that these results confirmed their hypothesis that virulent S.typhi fail to stimulate receptors which trigger neutrophil oxidative metabolism. While investigating the effects of gonococci on human neutrophil metabolism, Densen and Mandell (1978) observed that in the presence of normal serum, P^+ gonococci stimulated a slightly lower rate of oxygen consumption than P^- cells. They further reported that P^+ gonococci stimulated significantly lower levels of myeloperoxidase-mediated protein iodination than P^- organisms.

Of the six isogenic pairs examined in this study, only one P^- strain failed to stimulate increased oxygen consumption over control leukocytes. Piliated organisms of group 1 strains stimulated lower levels of oxygen consumption than their P^- counterparts. The results agree with the findings of Densen and Mandell (1978). There is, however, no indication that low oxygen consumption is associated with increased virulence for group 2 strains. Nonpiliated organisms of this group induce lower oxygen consumption than P^+ gonococci. Variable results in oxygen consumption, Cl^- ion uptake, and changes in membrane potential are all common to group 2 strains. This suggests that these three characteristics of neutrophil metabolism are associated with each other.

Foote et al (1983) demonstrated that the formation of chlorinating agents (HOCl and chloramines) by stimulated neutrophils coincides with elevated oxygen consumption, and that at least 28% of the oxygen consumed was used in the production of these chlorinating agents. The source of Cl^- is not known. However, as Klebanoff (1975) observed, rabbit neutrophils contain a large store of intracellular Cl^-, and this reserve of ion may be mobilized by the myeloperoxidase system to form HOCl and chloramines.

The results of this and another study (Wiseman and Martin, 1986) indicate that gonococci stimulate transport of Cl^- across neutrophil membranes and that avirulent organisms tend to elicit a greater response. Our view is that Cl^- taken up by neutrophils in response to stimulation is converted to HOCl by the myeloperoxidase-hypohalide system. Experiments performed on neutrophils stimulated with gonococci in the presence of the chloride trapping compound TMB and $^{36}Cl^-$ basically provided evidence for this view. Radiolabelled Cl^- appeared in fractions which also contained TMBCl. It seems that some of the $^{36}Cl^-$ taken up by the neutrophils was used to produce chlorinating agents which in turn chlorinated TMB to form radiolabelled TMBCl. These findings suggest that there is a direct relationship between the stimulation of Cl^- uptake and the production of active chlorinating agents in human neutrophils (Figure 8).

FIGURE 8. Metabolism of stimulated neutrophils. Serum oxygen is reduced in the leukocyte to form O_2^-. This molecule then undergoes dismutation to form H_2O_2. Myeloperoxidase (MPO), made available to the phagosome by fusion with primary (1°) granules, combines H_2O_2 with Cl^- to form bactericidal HOCl. The source of Cl^- may be both from within leukocytes as well as from Cl^- recently taken into the cell from the extracellular environment in response to stimulation.


Serum O_2
NADP$^+$
NADPH
O_2^-
H_2O_2
Cl^-
MPO
HOCl
Cl^-
MPO
MPO
Bacteria
Phagosome
1° Granule
Nucleus
Neutrophil

SUMMARY

1. Stimulation of Cl^- uptake by neutrophils in response to gonococcal challenge was demonstrated, with P^- organisms effecting a greater response.

2. The increased susceptibility of P^- gonococci over P^+ organisms to neutrophils was confirmed using the acridine orange assay.

3. P^- gonococci were generally shown to stimulate greater oxygen consumption by neutrophils than P^+ bacteria.

4. Depolarization of neutrophil membranes was stimulated only by P^- gonococci, with P^+ organisms initiating only prolonged hyperpolarization.

5. Production of HOCl by stimulated neutrophils was dependant, in part, on increased uptake of Cl^- by the leukocytes.

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APPENDIX A

SUSPENDING FLUIDS

Hank's Balanced Salt Solution (HBSS) (pH 7.2)

(Formula per litre of distilled water)

NaCl	8.0 g
KCl	0.4 g
$MgSO_4$ $7H_2O$	0.2 g
Na_2HPO_4	0.048 g
KH_2PO_4	0.06 g
$CaCl_2$	0.14 g

HEPES Buffered HBSS (pH 7.3)

(Formula per litre of distilled water)

NaCl	7.54 g
KCl	.313 g
Na_2HPO_4	.128 g
KH_2PO_4	.054 g
$MgSO_4$	.024 g
$MgCl_2$	.020 g
Glucose	1.98 g
HEPES*	2.38 g

*N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid.

Proteose Peptone Saline (pH 7.2)

10 g Proteose peptone (Difco) in 1 litre of 0.15 M (8.77 g) NaCl

APPENDIX B

ISOVITALEX

Formula per litre of distilled water	
Vitamin B_{12}	0.01 g
Diphosphopyridine nucleotide, oxidized (NAD^+)	0.25 g
Cocarboxylase	0.1 g
L glutamine	10.0 g
Adenine	1.0 g
Guanine HCl	0.03 g
P-aminobenzoic acid	0.013 g
Thiamine HCl	0.003 g
L cysteine HCl	25.9 g
Dextrose	100.0 g
Ferric nitrate	0.02 g

APPENDIX C

GEY'S BALANCED SALT SOLUTION

Formula per litre of distilled water (pH 7.2)

NaCl	7.00 g
KCl	0.37 g
$CaCl_2$	0.17 g
$MgSO_4$ $7H_2O$	0.07 g
$MgCl_2$ $6H_2O$	0.21 g
$NaHPO_4$ $2H_2O$	0.15 g
KH_2PO_4	0.03 g
Glucose	1.00 g
$NaHCO_3$	2.27 g

APPENDIX D

CONVERSION OF OXYGEN UNITS TO uL

H_2O equilibrated with air at 37°C -----> 5.02 uL O_2/mL* at 1 atmosphere.

Probe gives reading of 20.8 units with air saturated H_2O at 37°C:

$$20.8 \text{ units} = 5.02 \text{ uL/mL}$$

$$1 \text{ unit} = X \text{ uL/mL}$$

$$20.8\,X = 5.02$$

$$X = \frac{5.02}{20.8}$$

$$= 0.24134$$

$$X = 0.241 \text{ uL } O_2\text{/mL}$$

$$\therefore \text{ 1 unit} = 0.241 \text{ uL } O_2\text{/mL}$$

* YSI model 5300 instruction manual.

APPENDIX E

SCINTILLATION COCKTAIL

Scintiprep.	20.4 mL
Toluene	510.0 mL
Triton X-100	170.0 mL
H_2O	35.0 mL